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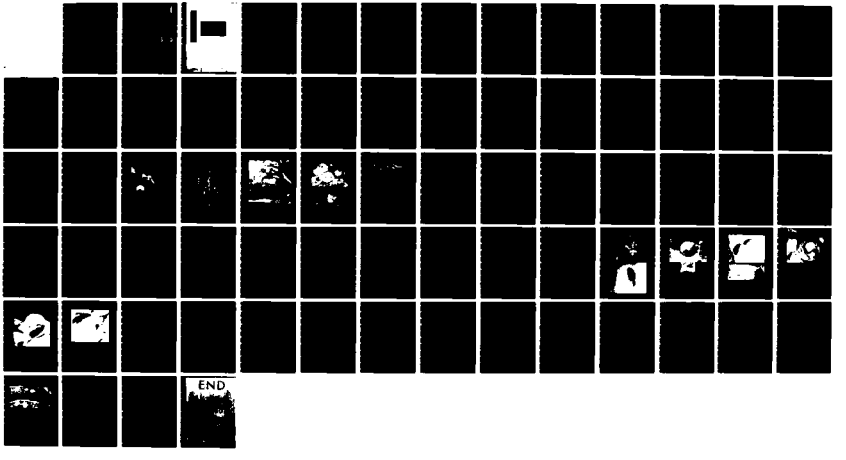

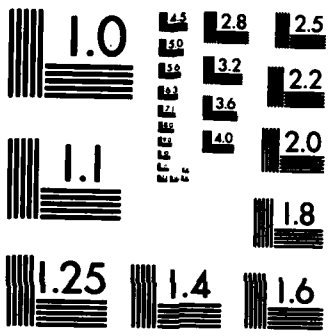

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ANNUAL REPORT #2

Electron Microscopy of Intracellular Protozoa

August, 1981

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Annual Report #2

ELECTRON MICROSCOPY OF INTRACELLULAR PROTOZOA

Masamichi Aikawa, M.D.
August, 1981

Supported by

U. S. Army Medical Research and Development
Command, Fort Detrick, Frederick, Maryland, 21701
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The Institute of Pathology, Case Western Reserve
University, Cleveland, Ohio, 44106

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Host-parasite interaction, electron microscopy, *Leishmania mexicana*, malaria, freeze-fracture, erythrocyte entry, macrophage entry

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

During this fiscal year, in collaboration with Dr. Hendricks and his associates of WRAIR, we studied the interactions between macrophages and *Leishmania mexicana in vitro* in the absence or presence of cytochalasin B, since there has not been a general agreement as to whether or not the promastigotes of *Leishmania* can actively penetrate the host macrophages. Our study showed that almost 90% of the promastigotes attached to the macrophages with their flagellum within 2 minute incubation periods of the parasite and macrophages. On the other hand, the macrophages showed many pseudopods and the promastigotes attached to the

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macrophages without any particular orientation after a 5 minute incubation period. No reduction of host cell entry by the parasites treated with cytochalasin B was noted within the 2 minute incubation periods, while a significant reduction of macrophage entry by cytochalasin-treated promastigotes was observed after a 5 minute incubation period. These observations indicate that the promastigotes of *L. mexicana* enter into the macrophages actively with the flagellum end first during the first 2 minute interaction period. However, when the macrophages become actively phagocytic after a 5 minute interaction period, many of the parasites are phagocytosed by the macrophages. Therefore, we conclude that the promastigotes of *Leishmania mexicana* initially invade the macrophages actively, but they are also phagocytosed by the macrophages to gain entry when the macrophages become actively phagocytic in the later stage of the interaction.

Studies on the invasion of the erythrocytes by malarial merozoites demonstrated that the formation of a junction between the merozoite and the erythrocyte is essential for the successful erythrocyte entry by a merozoite. However, the exact nature of this junction was difficult to analyze by the thin-sectioning technique alone. Therefore, freeze-fracture was performed on the erythrocytes invaded by malarial merozoites. This study showed, for the first time, a narrow circumferential band of rhomboidally arrayed particles on the P face of the erythrocyte at the neck of the erythrocyte invagination and matching rhomboidally arrayed pits on the E face. This band corresponds to the junction between the erythrocyte and the merozoite membranes observed in thin sections and may represent the anchorage sites of the contractile proteins within the erythrocyte. Intramembranous particles (IMP) on the P face of the erythrocyte membrane disappear beyond this junction. When the erythrocyte and cytochalasin B-treated merozoites are incubated together, the merozoite attaches to the erythrocyte membrane and a junction is formed between the two, but the invasion process does not advance further and no movement of the junction occurs. Despite the fact that there is no entry of the parasite the erythrocyte membrane still invaginates. Freeze-fracture shows that the P face of the invaginated erythrocyte membrane is almost devoid of the IMP that is found elsewhere on the membrane, suggesting that the attachment process in and of itself is sufficient to create a relatively IMP-free bilayer.

ELECTRON MICROSCOPY OF INTRACELLULAR PROTOZOA

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Summary

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Studies on the invasion of erythrocytes by malarial merozoites demonstrated that the formation of a junction between the merozoite and the erythrocyte is essential for successful erythrocyte entry by the merozoite. However, the exact nature of this junction was difficult to analyze by the thin sectioning technique alone. Therefore, freeze-fracture was performed on the erythrocytes invaded by

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Forward

In conducting the research described in this report, the investigators adhered to the Guide for Laboratory Animal Facilities and Care, as promulgated by The Committee on The Guide for Laboratory Animal Resources, National Academy of Science - National Research Council.

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b) Freeze-Fracture Study on the Erythrocyte Membrane During Malarial Parasite Invasion.

c) The Protective Antigen of Malarial Sporozoites (*Plasmodium berghei*) is a Differentiation Antigen.

Interactions Between Macrophages and *Leishmania mexicana In Vitro*

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Introduction

Leishmanias are protozoa which infect cells in the reticuloendothelial system of the mammalian hosts. Because macrophages of mammalian hosts are believed to be the cells which first interact with the promastigotes of *Leishmania*, many investigators (1,2,3) studied the interaction between the promastigotes of *Leishmania* and macrophages using light and electron microscopy. However, there has not been a general agreement as to whether or not the promastigote can actively penetrate macrophages. Pulvertaft and Hoyle (12), and Miller and Twohy (10) reported that *L. donovani* enters into the macrophages by its flagellar end first and suggested active entry of the parasite into the macrophages. Similarly Merino *et al.* (9) suggested that macrophage entry by *L. brasiliensis* involved in active participation of the parasite membrane through receptor sites. The other evidence came from an experiment by Lewis (7) who was unable to block *L. mexicana* entry into the macrophages with cytochalasin. On the other hand, Chang (5) reported no preferable orientation of *L. donovani* during macrophage entry. Also, he (4) showed *in vitro* that the presence of cytochalasin B prevented the entry of the parasite into the macrophages and also prevented the formation of macrophage psuedopodia which were usually observed during the *L. donovani* entry into macrophages. A similar observation has been made by Alexander (2) who used cytochalasin B to inhibit the entry of *L. mexicana* promastigotes into macrophages.

Recently, Zenian *et al.* (14) studied, by scanning electron microscopy, the interaction between the promastigotes of *L. tropica* and mouse macrophages and reported that the parasites attached to the host cells by their flagellar tips which were enveloped by the macrophage lamellar sheaths. The attachment and engulfment of the parasites by macrophages was inhibited by cytochalasin B.

Therefore, they concluded that macrophage invasion is through phagocytosis rather than penetration.

In order to clarify whether or not the promastigotes of *Leishmania* actively penetrate the macrophages, we studied, by light and electron microscopy, macrophage entry by *L. mexicana* promastigotes and amastigotes *in vitro* either in the presence or absence of cytochalasin B. Since the amastigotes show no active penetration of the macrophages, comparison between the promastigote and the amastigote entry into the macrophages, in the presence of cytochalasin B, will clarify the active participation of promastigotes into the macrophage. Furthermore, transmission electron microscopy on the interaction between the parasites and macrophages in the earlier stages of penetration at 2 and 5 minutes, would elucidate the interaction since the initial interaction will most likely occur earlier as indicated by Zenian *et al.* (14). Therefore, we have attempted to investigate the early stages of interaction between the macrophages and parasites in particular those stages occurring within the first 5 minutes and also a comparison of macrophage entry by the promastigotes and amastigotes of *L. mexicana* in the presence or absence of cytochalasin B. This experimental emphasis would elucidate the mechanism of macrophage entry by *L. mexicana.*

Materials and Methods

WR127 strains (Texas) of *Leishmania mexicana* (Texas) have been cultivated on 199 medium for several months at Walter Reed Army Institute of Research. Macrophages (P388) were obtained from Dr. J. Marr of the University of Georgia and have been cultivated for several months on 9.5 x 35 mm coverslips in Leighton tubules containing 2.0µl tissue culture medium 199 containing 5 U heparin/ml and 10% (v/v) inactivated calf serum. The number of macrophages/coverslip was 368,000.

1. Macrophage - *L. mexicana* interaction experiment.

Promastigotes were added to the Leighton tubules in which a monolayer of macrophages was growing. The final concentration of promastigotes/tubule was $4 \times 10^7/\mu l$. The tubules containing the parasites and macrophages were gently rocked as soon as the promastigotes were added, and incubated at 37°C. Samples were taken at 2, 5 and 60 minute intervals and fixed with 2.5% glutaraldehyde containing 4% sucrose and 0.1M cacodylate buffer (pH 7.4) after several washes with Hank's balanced salt solution. Amastigotes of *L. mexicana* were also added to the Leighton tubules which contain macrophages as described above. The concentration of the amastigotes/tube was $4 \times 10^6/\mu l$. The cell suspension was incubated at 37°C and fixed with 2.5% glutaraldehyde solution at 2, 5 and 60 minutes.

2) Cytochalasin B experiment.

Cytochalasin B was dissolved in a DMSO (dimethyl sulphoxide) and was added to the solution containing promastigotes to make the final concentration of cytochalasin B 10μg in 0.1% DMSO. The mixture was incubated at room temperature for 3 minutes. As a control, 0.1% DMSO was added to the vials of promastigotes. After room temperature incubation, the vial was warmed briefly at 37°C and washed with cell suspension medium to eliminate the excess cytochalasin B in the solution. Cytochalasin B-treated promastigotes were immediately added to the tubules containing macrophages and were incubated at 37°C. At 2 and 5 minute intervals the samples were fixed with 2.5% glutaraldehyde solution containing 4% sucrose and buffered with 0.1M cacodylate buffer (pH 7.4). In the other experiment, macrophages were treated with cytochalasin B (10μg/ml) for 3 minutes. After treating the macrophages with cytochalasin B, they were washed briefly with culture medium and promastigotes were added in a manner similar to the experiment described above. Samples were fixed again at 2 minutes and 5 minutes after incubation

for electron microscopy.

3) Electron microscopy.

Samples fixed in 2.5% glutaraldehyde solution were washed with 0.1M cacodylate buffer several times and then post-fixed in 1% osmium tetroxide for one hour. After dehydrating in an ascending concentration of alcohol, the samples were embedded in Spurr. The resulting blocks were cut with a Porter-Blum MT-2 ultramicrotome. Several thick sections (1μ thick) were obtained form each experiment, stained with 1% toluidene blue solution and were used for quantitative analysis of macrophage-parasite attachment observation. At least 200 macrophages were counted for the quantitative analysis of the attachment. Differences between the experiments were evaluated by the Student's *t* test.

The thin sections were placed on copper grids and stained with 1% uranyl acetate and lead citrate. The sections were then examined under a JEOL 100CX electron microscope.

Results

1) The interaction between macrophages and untreated promastigotes.

Two minute incubation of the macrophages and promastigotes showed the number of attached promastigotes per macrophage as 0.46 ± 0.14. Five minute incubation of the macrophages with promastigotes resulted in 0.73 ± 0.23 promastigotes attached to a macrophage. The number of parasites attached to the macrophage increased significantly ($P<0.001$) after 60 minute incubation and was 1.42 ± 0.26 (Table 1).

When examined by electron microscopy after 2 minute incubation, 90% of the attached promastigotes were attached to the macrophages with the tip of the

flagellum (Fig. 1), while 10% of the promastigotes were attached to the macrophages without any particular orientation. After 5 minute incubation, the attachment of the promastigotes to the macrophages did not show a preferred orientation. Fifty percent of the promastigotes were attached to the macrophages with their flagellar tips (Fig. 2), while the other 50% showed no particular orientation in relation to the macrophages. A similar observation was also made between the promastigotes and macrophages after 60 minute incubation (Fig. 3).

Even though insertion of the flagellar tips into the macrophages is observed during the 2 minute incubation, the macrophages did not form pseudopods around the inserted flagellum. Also, no aggregation of microfilaments in the macrophages was seen around the parasite flagellum during this period. To the contrary, many macrophage's pseudopods were seen around the inserting flagellum after 5 minute and 60 minute incubation. In these instances, aggregates of microfilaments in the macrophage cytoplasm were observed adjacent to the inserted flagellum (fig. 4). Several areas of close contact between the flagellar membrane and the invaginated macrophage plasma membrane was observed. Also noted are aggregations of small vesicles in the macrophage cytoplasm adjacent to the invaginated plasma membrane.

2) <u>The interaction between macrophages and untreated amastigotes</u>.

When amastigotes were incbuated with macrophages for 2 or 5 minutes, no attachment was observed between them. Similarly, the macrophages did not contain any amastigotes. In spite of the absence of attachment between the macrophages and amastigotes, pseudopods are present in the macrophages 5 minutes after incubation. On the other hand, 5% of the amastigotes were attached to the macrophages after 60 minute incubation (Fig. 5).

3) The interaction between cytochalasin B-treated promastigotes and macrophages.

The number of attached promastigotes per macrophage was 0.30 ± 0.11 after two minute incubation of the macrophage and cytochalasin B-treated promastigotes. After 5 minute incubation of the macrophages and cytochalasin B-treated promastigotes, the number of attached promastigotes per macrophage was 1.28 ± 0.32 (Table 2). When cytochalasin B-treated promastigotes and macrophages were incubated together for 2 minutes, no attachment was observed by electron microscopy between the flagellum of the promastigotes and macrophages, although a few promastigotes were attached to the macrophages without any particular orientation. After 5 minute incubation, about 50% of the attached promastigotes adhered to the macrophages without any particular orientation while the remainder were attached to the macrophages with the flagellum (Fig. 6). There were pseudopod formation, aggregation of the microfilaments and vesicles in the macrophage cytoplasm around the parasite attachment site. The results of the interaction between DMSO-treated promastigotes and macrophages were similar to those found between untreated promastigotes and macrophages.

4) The interaction between promastigotes and cytochalasin B-treated macrophages.

The number of attached promastigotes per macrophage was 0.82 ± 0.32 after two minute incubation of cytochalasin B-treated macrophages and promastigotes. After 5 minute incubation the number of attached promastigotes was 0.21 ± 0.15 (Table 3). Cytochalasin B-treated macrophages showed fewer pseudopods during the interaction with promastigotes than untreated macrophages. After 2 minute incubation, about 80% of the promastigotes attached to the macrophages showed insertion of the flagellum into the macrophage cytoplasm. The remainder of the promastigotes were attached to the macrophage membrane without any particular

orientation. Few macrophage pseudopods were present around the parasites. After 5 minute incubation, about 50% of the attached promastigotes showed their flagellum inserting into the macrophage cytoplasm and the remainder laying around the macrophage membrane (Fig. 7). Again, pseudopod formation was not prominent. The interaction between DMSO-treated macrophages and promastigotes showed that about 90% of the promastigotes inserted their flagellum into the cytoplasm.

Discussion

Interaction between macrophages and leishmanial promastigotes has been studied extensively to elucidate the mechanism of macrophage entry by the promastigotes (1-5). However, whether macrophages engulf the leishmanial promastigotes by phagocytosis or the parasite actively penetrates the cell is still controversial. Purvertaft and Hoyle (12) demonstrated that the promastigotes of *L. donovani* enter hamster macrophages by inserting their flagellar tip first, indicating that the parasites enter into the macrophages actively. A similar observation was also mde by Miller and Twohy (10). In order to prove that leishmanial promastigotes can enter into the cells without their phagocytotic activity, Lewis (7) studied the interaction between the promastigotes of *L. mexicana* and non-phagocytic sarcoma cells in the presence of cytochalasin B which is known to inhibit phagocytic activity. He found that the parasites can infect the sarcoma cells in spite of the presence of cytochalasin B, suggesting that the parasites make an active contribution to host cell infection.

The evidence supporting leishmanial promastigote entry into host cells by phagocytosis is the presence of abundant pseudopods, no preferable orientation during entry, and inhibition of the parasite entry by cytochalasin B. Akiyama and Haight (1) reported, by light microscopy, that most of the promastigotes of

L. donovani were engulfed at the posterior end first by the macrophage and a few by the flagellum. *L. mexicana* entry into the macrophages was inhibited by the use of cytochalasin B. Chang (3) using scanning and transmission electron microscopy reported that promastigotes of *L. donovani* depend on phagocytic activity of macrophages to gain intracellular entrance, but he also suggested that parasite-specific activities and/or properties might also play a role in the parasite entry. Macrophages are known to become phagocytic within 10 minutes after stimulation (6). Therefore, it is likely that the macrophages are already actively phagocytic within 10 minutes after the interaction between the parasites and the macrophages. Our data indicates that the macrophages showed many pseudopodia and the promastigotes were attached to the macrophages without any particular orientation after 5 minute incubation. On the other hand, in the observation made 2 minutes after the interaction, almost 90% of the attached promastigotes interacted with the macrophages with the flagellum and only a few pseudopodia were seen on the macrophages. This finding suggests that the macrophages are not actively phagocytic during the first 2 minutes of stimulation by the parasite, but become actively phagocytic after a 5 minute interaction. Our experiment with cytochalasin B-treated macrophages also showed similar results. The significant reduction of macrophage entry by the parasites in the presence of cytochalasin B after 5 minute incubation and no reduction after 2 minute incubation also indicates that the macrophages are only capable of phagocytizing the parasite after a 5 minute interaction but not within a 2 minute interaction. A similar observation was made by Zenian *et al.* (14) using *L. tropica*. They reported that the attachment of promastigotes of *L. tropica* to the macrophages occurred by the flagellar tips and there was no visible response of the macrophages in the early stage of initial contact. However, by 4 minutes, the protrusion of a lamellar sheath extended from the macrophage fitting closely around the attached flagellum. After 10 minute exposure, the parasites were engulfed by the macrophages. This data is in agreement with our transmission electron microscopy.

Therefore, we suggest that the promastigotes of *L. mexicana* enter into the macrophages actively with the flagellum end first during the first 2 minute interaction period. But, when the macrophage starts to have phagocytic activity at 5 minute incubation periods, the parasites are phagocytized by the macrophages to gain entry. This suggestion is supported further by the fact that amastigotes of *L. mexicana* known to enter the macrophages by phagocytosis were only found in the macrophage after 60 minute incubation periods (6). Furthermore, cytochalasin B blocked the entry of the amastigotes into the macrophages.

Of particular interest are the changes in the macrophage cytoplasm adjacent to the inserting flagellum. Aggregation of microfilaments in the macrophage cytoplasm around the inserting flagellum is similar to that found in macrophages phagocytizing other organisms. These filaments are supposed to be contractile proteins and aid phagocytosis (8,13). In the case of leishmanial promastigote entry into macrophages, aggregation of the microfilaments become prominent at 5 minute interaction, but not before a 2 minute interaction. This finding further supports our contention that leishmanial promastigotes enter into the macrophages within a 2 minute interaction without being phagocytized but the promastigotes enter macrophages either actively or passively after 5 minute interaction. The nature of vesicle aggregation around the inserting flagellum is unknown. However, it appears that the macrophage cytoplasm reacts to the invaginating plasma membrane by forming many vesicles. Several close contacts between the flagellar membrane and the invaginating macrophage plasma membrane suggest a close relationship between them. When *Plasmodium* enters into the erythrocytes, the two membranes come in close contact and a prominent junction is formed between the plasmodial and erythrocyte membrane. This close contact between the flagellar membrane and macrophage membrane may be a similar phenomenon as seen in *Plasmodium*-erythrocyte interaction although the formation of a junction does

not occur during the interaction between leishmanial promastigotes and macrophages. Recently, Change (4) indicated that *L. donovani* promastigote-macrophage binding is a ligand-receptor interaction involving their surface antigenic membrane proteins. Therefore, it is possible that point of close contact between the parasite and macrophage membrane may be the site of ligand-receptor interaction.

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Figure Legends

Fig. 1 Electron micrographs showing a promastigote's flagellum contacting a macrophage after 2 minute incubation. X 18,000.

Fig. 2 Electron micrograph showing that a promastigote's flagellum inserts into a macrophage cytoplasm after a 5 minute incubation period. X 13,000.

Fig. 3 Electron micrograph showing intracellular promastigotes of *L. mexicana* after a 60 minute incubation period. X 25,000.

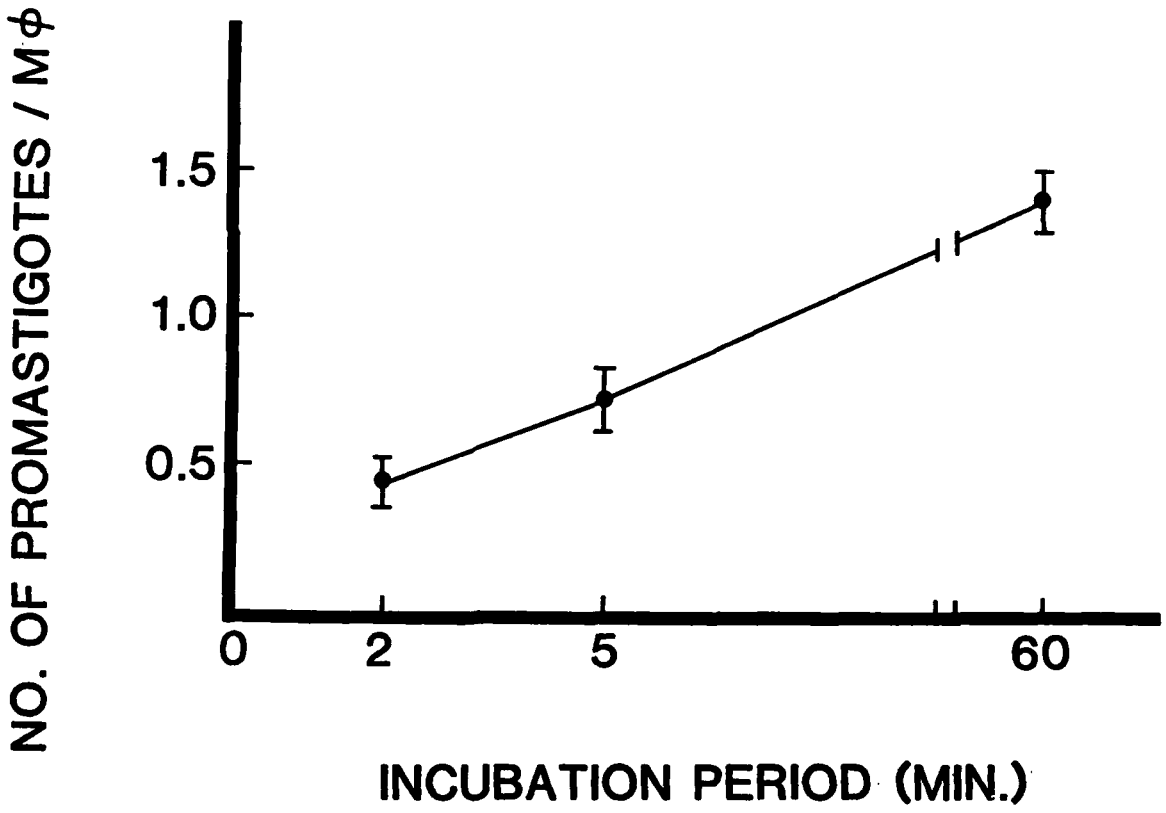
Fig. 4 Electron micrograph showing aggregates of microfilaments in the macrophage cytoplasm adjacent to the inserted flagellum. X 60,000.

Fig. 5 Electron micrograph showing many amastigotes engulfed by the macrophage after a 60 minute incubation period. X 14,000.

Fig. 6 Electron micrograph showing a cytochalasin-treated promastigote being phagocytosed by a macrophage after a 5 minute incubation. X 27,000.

Fig. 7 Electron micrograph showing a promastigote inside a cytochalasin-treated macrophage. X 30,000.

TABLE 1



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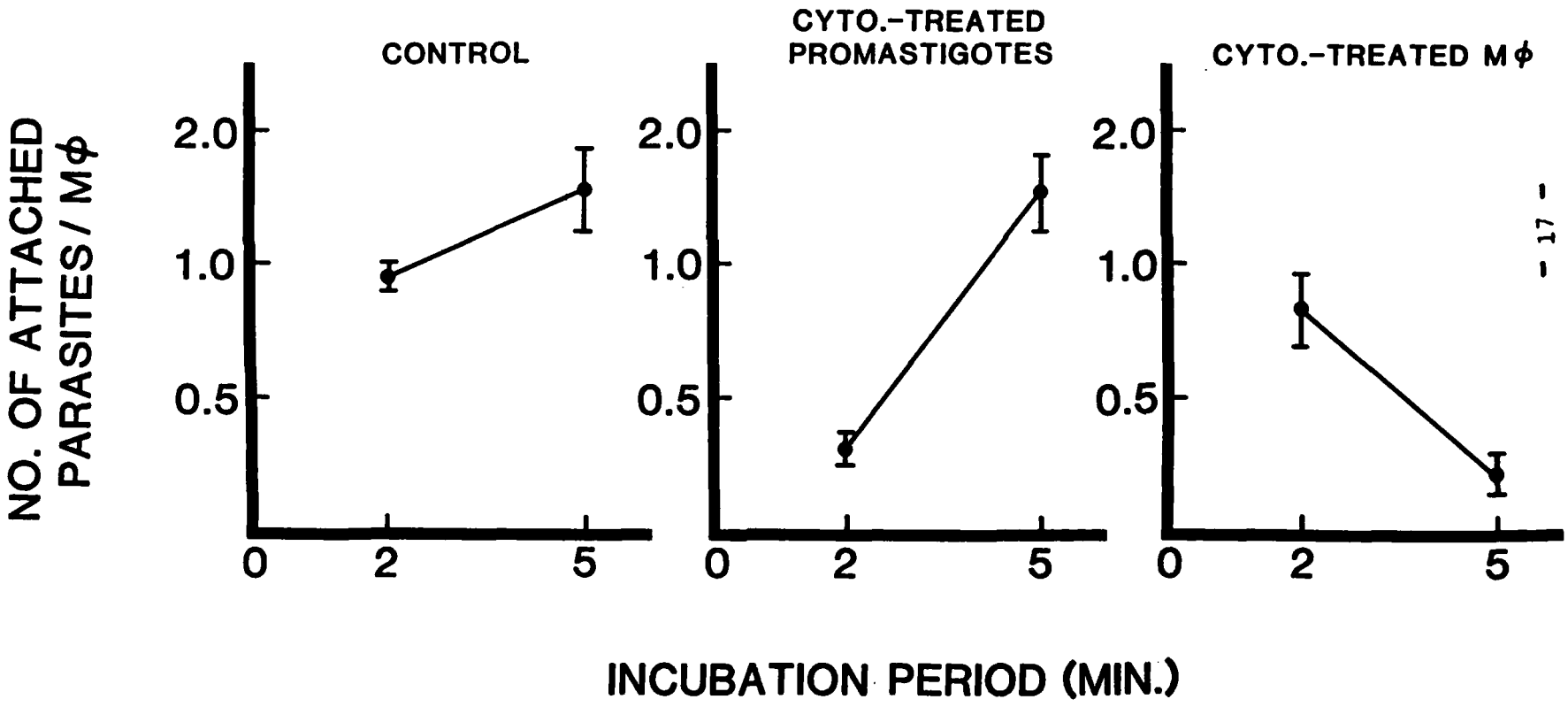
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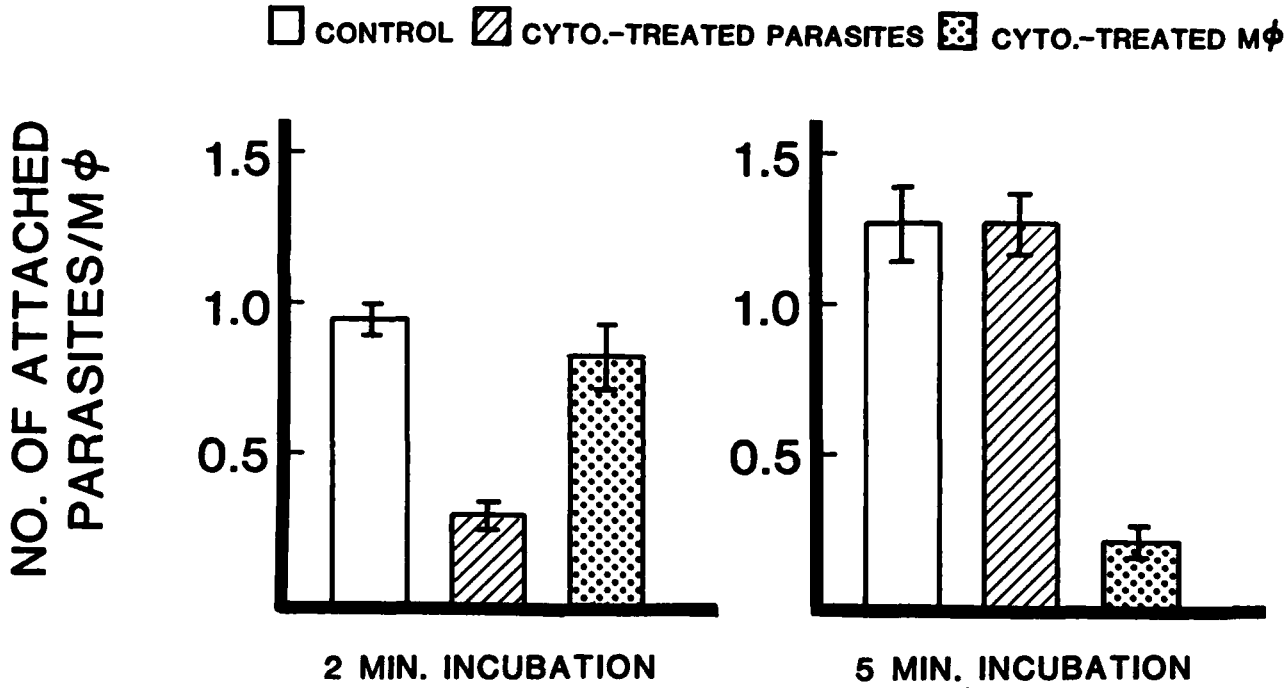
1.0
0.5
0
2
5
INCUBATION PERIOD (MIN.)

- 17 -

TABLE 3


CONTROL
CYTO.-TREATED PARASITES
CYTO.-TREATED Mϕ
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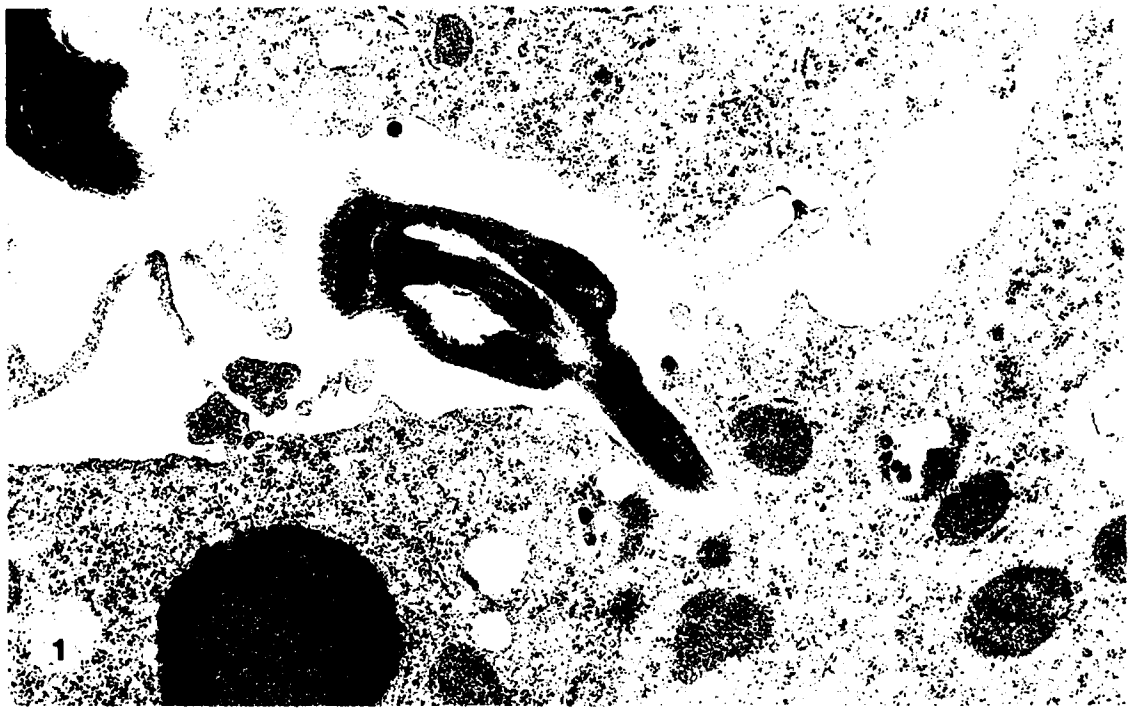
1.0
0.5
2 MIN. INCUBATION
5 MIN. INCUBATION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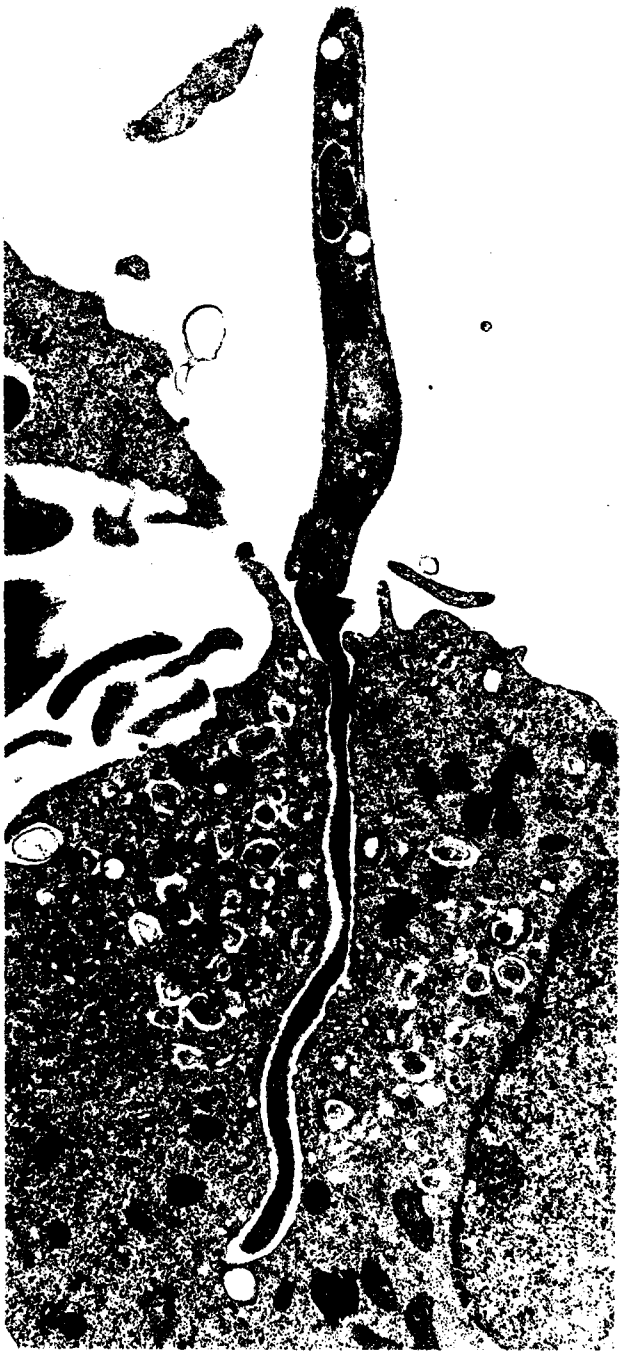

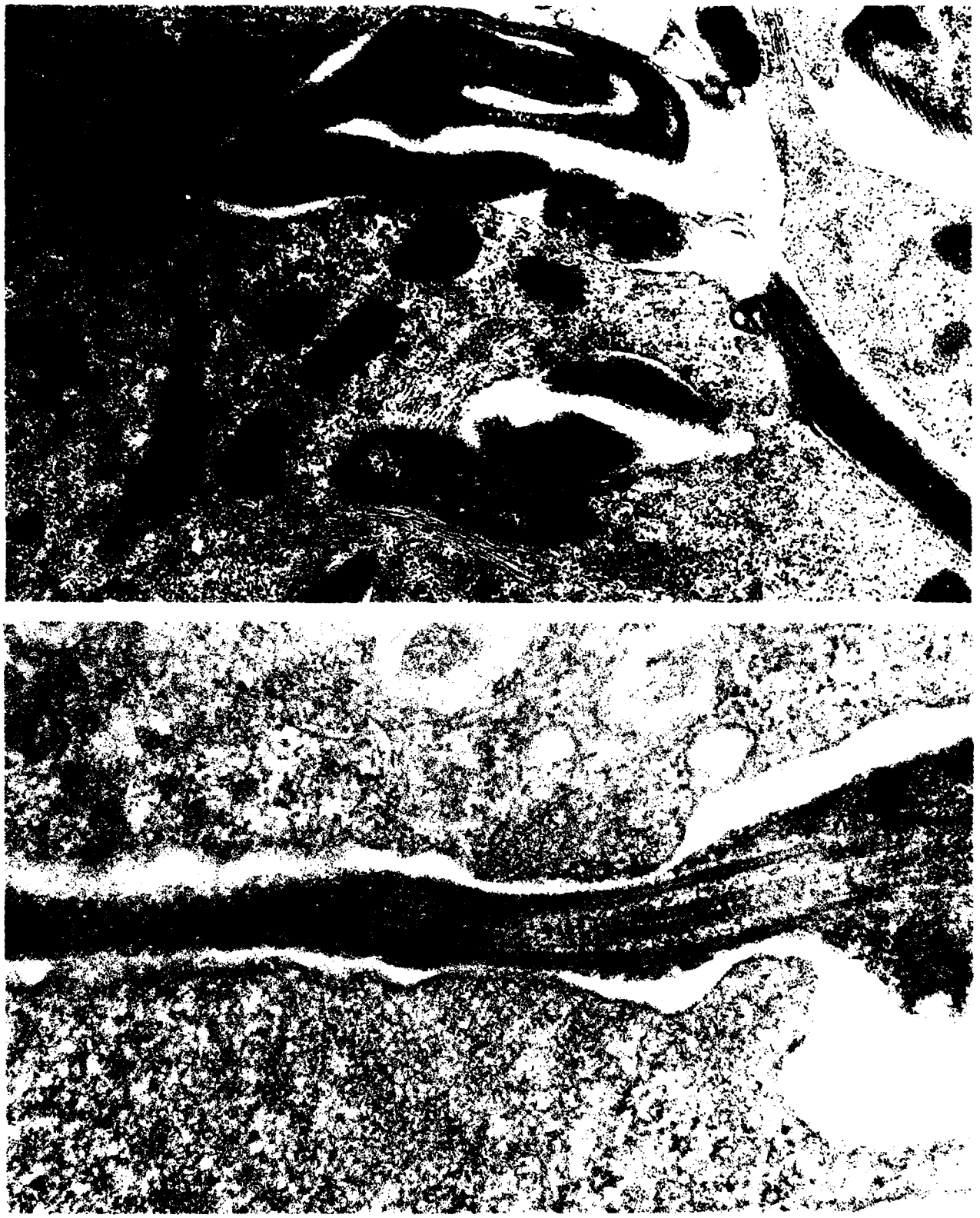

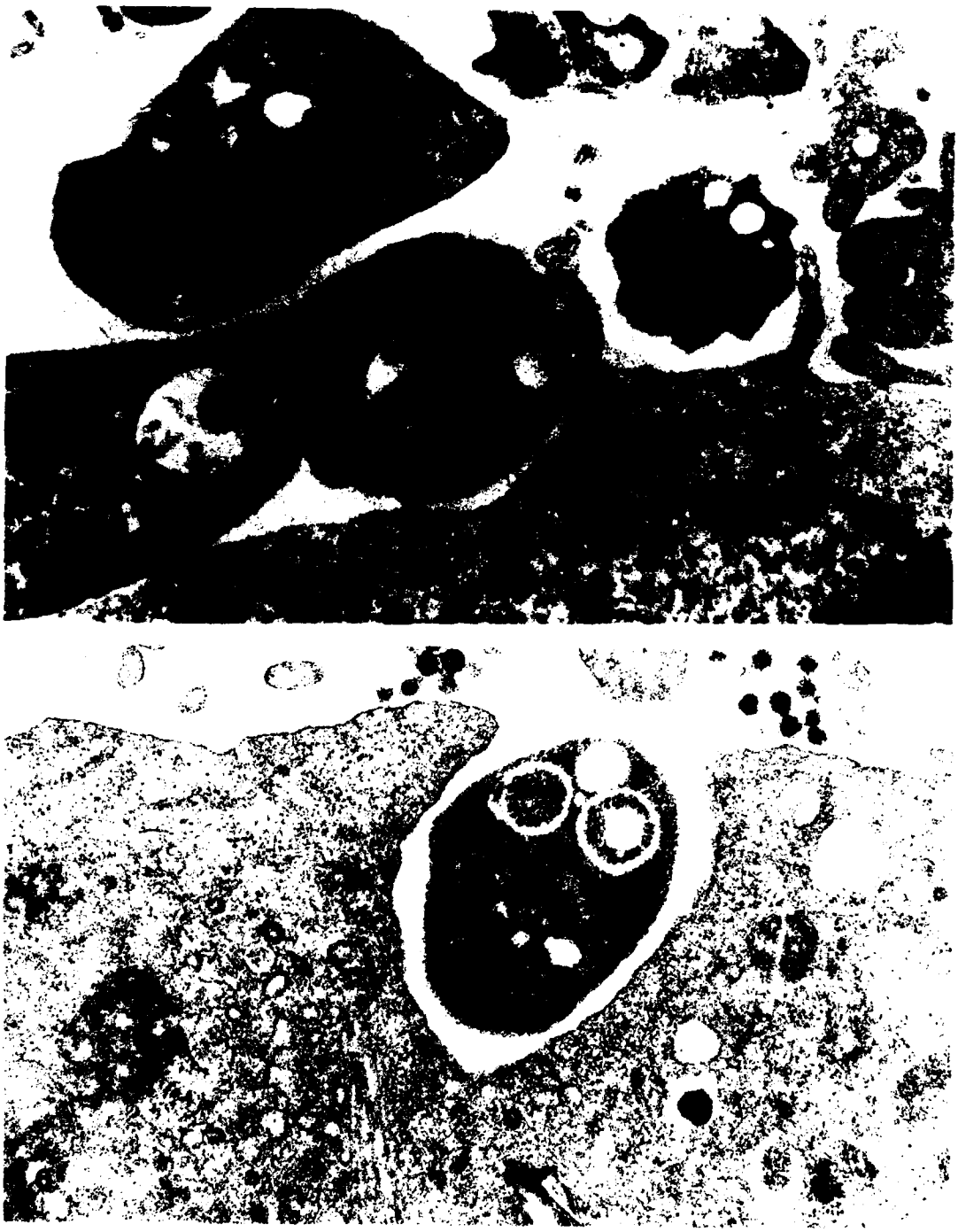

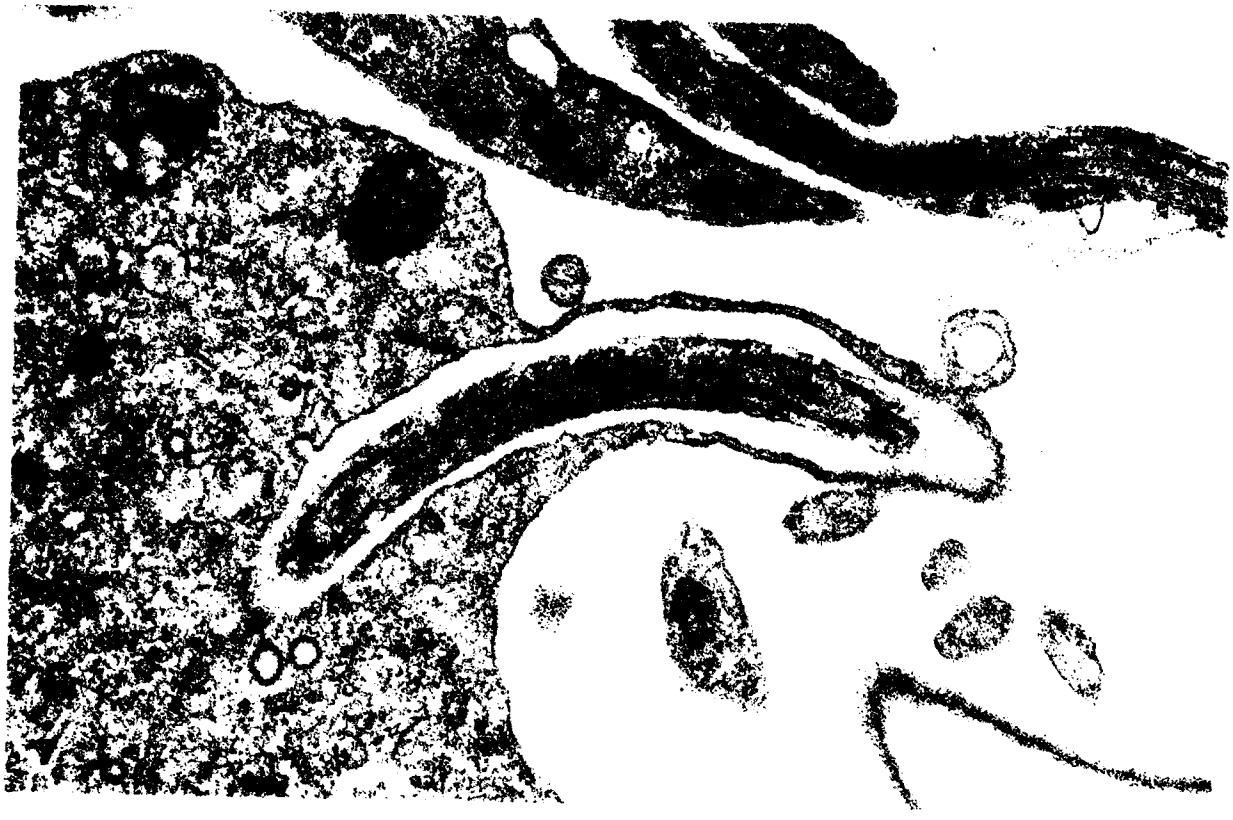

Freeze-Fracture Study on the Erythrocyte Membrane During Malarial Parasite Invasion.

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Running Title: Freeze-fracture on erythrocyte entry by *Plasmodium*.

Abstract

Invasion of erythrocytes by malarial merozoites requires the formation of a junction between the merozoite and the erythrocyte. Migration of the junction parallel to the long axis of the merozoite occurs during the entry of the merozoite into an invagination of the erythrocyte. Freeze-fracture shows a narrow circumferential band of rhomboidally arrayed particles on the P face of the erythrocyte membrane at the neck of the erythrocyte invagination and matching rhomboidally arrayed pits on the E face. The band corresponds to the junction between the erythrocyte and merozoite membranes observed in thin sections and may represent the anchorage sites of the contractile proteins within the erythrocyte. Intramembrane particles (IMP) on the P face of the erythrocyte membrane disappear beyond this junction. When the erythrocytes and cytochalasin B-treated merozoites are incubated together, the merozoite attaches to the erythrocyte membrane and a junction is formed between the two, but the invasion process does not advance further and no movement of the junction occurs. Despite the fact that there is no entry of the parasite the erythrocyte membrane still invaginates. Freeze-fracture shows that the P face of the invaginated erythrocyte membrane is almost devoid of the IMP that is found elsewhere on the membrane, suggesting that the attachment process in and of itself is sufficient to create a relatively IMP-free bilayer.

Introduction

Invasion of the erythrocytes by malarial merozoites requires the formation of a junction between the merozoite and the erythrocyte (1). Migration of the junction parallel to the long axis of the merozoite occurs during the entry of the merozoite into an invagination of the erythrocyte. As observed by thin-section electron microscopy, this junction is a region of close apposition between the merozoite and the erythrocyte, where the inner leaflet of the erythrocyte membrane appears thickened (1). The invaginated erythrocyte membrane beyond the junction has been shown by freeze-fracture to be devoid of intramembrane particles (IMP) (11). Cytochalasin-treated merozoites that can attach to but not enter into the erythrocyte also form a junction between the apical end of the merozoite and the erythrocyte (12). Even though the cytochalasin-treated merozoite never enters the erythrocyte, membrane invaginations occur within the erythrocyte in the region of the attached parasite. To further characterize the junctions and the erythrocyte membrane invaginations, we have analyzed them by freeze-fracture during normal invasion and after attachment of cytochalasin-treated merozoites to the erythrocytes.

Materials and Methods

Viable, invasive merozoites of a Malaysian strain of *Plasmodium knowlesi* were isolated according to the method of Dennis *et al.* (2) as modified by Johnson *et al.* (5). The merozoites were released into a protein-free medium containing medium RPMI 1640/15mM Hepes/34.5mM HCO_3 gassed with 5% CO_2 in air. One ml of the merozoite suspension contained ~5 x 10^7 merozoites. For invasion studies, 200µl of heat-inactivated fetal calf serum and 100µl of rhesus monkey erythrocytes (5 x 10^8/ml) in medium 199/10% fetal calf serum were added

to 2 ml of the merozoite suspension. The cell suspension was mixed in 37°C water bath for two minutes and then added to 13 ml of glutaraldehyde fixative (2% glutaraldehyde/116mM sucrose/50mM phosphate buffer, pH 7.4). For attachment studies, cytochalasin B in dimethyl sulfoxide (DMSO) was added to 1 ml of merozoite suspension so that the final concentration was 10µg/ml of cytochalasin B in 0.1% DMSO. After 1 minute incubation at room temperature, 100µl of rhesus erythrocytes in medium 199/10% fetal calf serum (10^8/ml) were added, and the suspension was mixed for 4 minutes at 37°C. The suspension was centrifuged for 2 minutes at 1000 x g, the supernatant was removed, and the pellet was resuspended in the final drop of medium. The cells were then fixed in 2 ml of glutaraldehyde fixative, dehydrated in an ascending alcohol series and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 100CX electron microscope.

For freeze-fracturing, the samples were fixed in glutaraldehyde fixative for one hour, washed several times in 50mM phosphate buffer and soaked for one hour in the same buffer containing 20% (V/V) glycerol as the cryoprotective agent. Freeze-fracturing was performed in a Balzers freeze-fracture etching unit. Fracturing was carried out at -120°C under a vacuum of 2×10^{-7} Torr. The surface obtained was replicated with platinum and carbon. Replicas were cleaned in 2% sodium hypochlorite to remove adherent organic material and then rinsed in distilled water. Particle counts were made by counting the number of IMP which fell in a 1/16µm^2 scribed square placed over each membrane face (11). At least 20 such counts were made to give values of density/square micron. Differences between particle counts were evaluated by Student's *t* test.

Results

a) Studies on erythrocytes during invasion by merozoites.

Since thin-section transmission electron microscopy on the invasion of the erythrocyte of *P. knowlesi* merozoites has been reported in detail (1), only a brief description of this process is presented here. Initially, the apical end of the merozoite makes contact with the erythrocyte and creates a depression in the erythrocyte membrane (Fig. 1). The zone of the erythrocyte membrane to which the merozoite is attached becomes thickened (~15nm), probably by the accumulation of electron-dense material along its inner face, and forms a junction (intercellular space measures ~10nm) with the plasma membrane of the merozoite (Fig. 1). Fine fibrils appear to span the intrajunctional space. As the merozoite enters the invagination of the erythrocyte surface, the junction moves along the confronted membranes to maintain its position at the neck of the invagination (Fig. 1). When entry is completed, the neck closes behind the merozoite in the fashion of an iris diaphragm and the junction becomes a part of the parasitophorous vacuole. The merozoite is now situated within a parasitophorous vacuole, the membrane of which originated from the erythrocyte plasmalemma.

Freeze-fracture shows that the merozoite enters an invagination of the erythrocyte membrane (Fig. 2). As the merozoite invasion progresses, the invagination of the erythrocyte membrane is shaped like a florence-flask (Fig. 3). The P face of the erythrocyte at the neck of the invagination is covered with IMP (Fig. 3). At the point just before the neck of the invagination abruptly expands into the parasitophorous vacuole, a narrow band (120nm in width) of rhomboidally arrayed particles is seen on the P face of the erythrocyte membrane (Fig. 3, inset) and matching rhomboidally arrayed pits (Figs.

4 & 5) are seen on the E face. Arrayed pits on the E face are in a corresponding pattern to the arrayed particles on the P face. This circumferential band disappears at the expansion point of the parasitophorous vacuole. This band corresponds to the junction region between the erythrocyte and merozoite membranes observed by thin-section electron microscopy. Arrayed particles and pits were not observed on the fracture face of the erythrocyte membrane away from the point of invasion or within the parasitophorous vacuole (Fig. 5). The number of IMP on the E and P faces of the erythrocyte membrane outside the invagination and on the E and P faces of the vacuole membrane created by the invaginated erythrocyte membrane is shown in Table 1. A significant difference was noted in the number of IMP present on the P face of the erythrocyte membrane and the P face of the vacuole membrane. P value calculated by the Student's *t* test was less than 0.01. On the other hand, no obvious difference in the number of IMP was noted between the E face of the erythrocyte membrane and the E face of the vacuole membrane.

A small dimple measuring ~50nm in diameter is noted on the P face of the parasite plasma membrane at the tip of the apical end (Figs. 2 & 8). This may correspond to the opening of the rhoptry duct present at the apical end of the merozoite.

b) Studies on erythrocyte attachment by cytochalasin-treated merozoites.

When rhesus erythrocytes and cytochalsin B-treated merozoites are incubated together, the apical end of the merozoite attaches to the erythrocyte membrane in a manner identical to that of the untreated merozoites. The erythrocyte membrane to which the cytochalasin B-treated merozoite is attached becomes thickened, forms a junction with the plasma membrane of the merozoite,

and invaginates slightly to cover the apical end (Fig. 6). However, the invasion process does not advance further and no movement of the junction occurs. Several membrane-bound vacuoles with an electron-translucent matrix appear in the erythrocyte cytoplasm near the attachment site (Fig. 6).

When the cytochalasin B-treated merozoites contact the erythrocyte membrane, freeze-fracture shows changes on the erythrocyte membrane similar to those seen during normal invasion with untreated merozoites (Figs. 7 & 8). At the end of the invagination neck, IMP on the P face of the erythrocyte membrane abruptly disappear so that the P face of the parasitophorous vacuole membrane possesses few IMP (Fig. 7).

Although the cytochalasin B-treated merozoites only attach to the erythrocyte membrane, the invagination of the erythrocyte extends far beyond the apical end of the merozoite (Figs. 7 & 8); this was not evident when thin sections were studied. In some instances, several secondary vacuoles are seen budding from the initial vacuole into the erythrocyte cytoplasm. The P face of the membranes in these secondary vacuoles also possess few IMP; the E face of these vacuoles is similar to that of the E face of the erythrocyte membrane.

Discussion

It is apparent that the translocation of the junction between the merozoites and the erythrocytes is an important component of the mechanism by which the merozoites enter the erythrocyte. Aikawa *et al.* (1) hypothesized that the movement of this junction is related to lateral displacement of the junction by membrane flow. However, the precise nature of this junction was difficult to analyze by thin-section transmission electron microscopy. Although the freeze-

fracture technique has been used to study erythrocyte invasion by merozoites, the junction remained unrecognized (11). Using freeze-fracture, our study demonstrates that a band of rhomboidally arrayed particles on the P face and pits on the E face correspond to the junction observed in thin sections. The presence of rhomboidally arrayed particles and pits at the junction site indicates that IMP on the erythrocyte membrane rearrange themselves at the site of *Plasmodium* entry for local membrane specialization (15).

Studies on membrane-membrane interactions such as membrane fusions between cells (10), vesicle-vesicle fusion (13), exocytosis (8) and myoblast fusion (6) have demonstrated that IMP are displaced laterally into adjacent membrane regions before the fusion process and that fusion occurs between protein-depleted lipid bilayers. In contrast to this lateral displacement of IMP, our study showed reorganized IMP at the site of the erythrocyte-*Plasmodium* interaction. This difference appears to be due to the face that the interaction between the erythrocyte and parasite membranes at the junction site is a transient phenomenon and is not a fusion process.

About 30% of the protein of the erythrocyte membrane consists of spectrin and actin, which form a lattice-like contractile system located on the inner aspect of the erythrocyte membrane (4,14,16). The arrangement of the contractile protein into a network is thought to facilitate changes in the shape of the erythrocyte. Therefore, it is possible that the presence of the dense zone at the site of the junction may represent aggregates of contractile proteins. Furthermore, rhomboidally arrayed pits on the E face seen by freeze-fracture may be the points where the contractile proteins anchor to the transmembrane proteins in the erythrocyte membrane (7).

Both during normal invasion and after the attachment of cytochalasin-treated merozoites, the P face of the vacuolar membrane is almost devoid of IMP. Since cytochalasin-treated merozoites remain outside the erythrocyte, the formation of a vacuole membrane does not require entry by the parasite. Our freeze-fracture observations show that the secondary vacuoles form an irregular chain that is in direct continuity with the original invagination of the erythrocyte membrane. Because this chain follows a tortuous path, thin sections give the spurious impression of independent small vacuoles subjacent to the site of the attachment. The vacuoles that are continuous with the invagination also possess few IMP. The contents of rhoptries, which are located in the apical region of the parasite, have been suggested to flow and diffuse into the erythrocyte membrane (1,12) and may be involved in the formation of the relatively protein-free vacuole membrane. Possible mechanisms might include cross-linking of spectrin (3), phospholipase activity (9), or actual creation of phospholipid bilayers by the rhoptries (11).

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Acknowledgements

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Figure Legends - (An arrow at the lower right hand corner indicates the direction of shadowing).

Abbreviations - (Pe): P face of the erythrocyte membrane; (Pv): P face of the vacuole membrane; (Pm): P face of the merozoite membrane; (Ee): E face of the erythrocyte membrane; (Ev): E face of the vacuole membrane; (Em): E face of the merozoite membrane.

Fig. 1 Thin-section electron micrograph of erythrocyte (rbc) entry by a merozoite (m). The merozoite is located within an invagination of the erythrocyte membrane. A junction (j) formed between the merozoite and the erythrocyte membranes is always located at the orifice of the invagination. X 54,000. Inset: High magnification of the junction shows thickening of the erythrocyte membrane where the merozoite is attached (arrow) and fine fibrils which appear to span the intrajunctional space. X 160,000.

Fig. 2 Freeze-fracture electron micrograph showing that the merozoite (m) is creating a slight invagination of the erythrocyte membrane during the initial stage of invasion. A small indentation (arrow) is present at the apical end. (Pm) is the P face of the merozoite plasma membrane. X 63,000.

Fig. 3 Freeze-fracture electron micrograph showing the P face (Pv) of a florence flask-shaped parasitophorous vacuole which is created by the invagination of the erythrocyte membrane during a merozoite invasion. The P face (Pe) of the ery-

throcyte membrane at the neck of the invagination is covered with IMP, but they disappear beyond the point where the neck of the invagination abruptly expands. Arrows outline the fracture edge of the erythrocyte membrane. X 52,000. Inset: High magnification electron micrograph of the P face (Pe) of the erythrocyte membrane at the neck of the invagination. A band of rhomboidally arrayed particles (arrow) is seen at a point just before the neck of the erythrocyte membrane invagination abruptly expands into the parasitophorous vacuole. X 85,000.

Fig. 4 Freeze-fracture electron micrograph of erythrocyte (rbc) entry by a merozoite (m). The E face of the erythrocyte membrane at the neck of the invagination consists of a narrow circumferential band of rhomboidally arrayed pits (arrow). (Ev) is the E face of the vacuole membrane. X 56,000.

Fig. 5 High power electron micrograph showing a band of rhomboidally arrayed pits (arrow) at the neck of the invagination. The E face (Ev) above this band shows few IMP. (Pm) is the P face of the merozoite membrane. X 104,000.

Fig. 6 Thin-section electron micrograph showing attachment of a cytochalasin B-treated merozoite (m) to an erythrocyte (rbc). The erythrocyte membrane becomes thickened (arrow) and forms a junction with the plasma membrane of the merozoite at the point of attachment. Several vacuoles (v) appear in the erythrocyte cytoplasm near the attachment site. X 48,000.

Inset: High magnification electron micrograph showing the thickened erythrocyte membrane (arrow) at the point of merozoite (m) attachment. X 80,000.

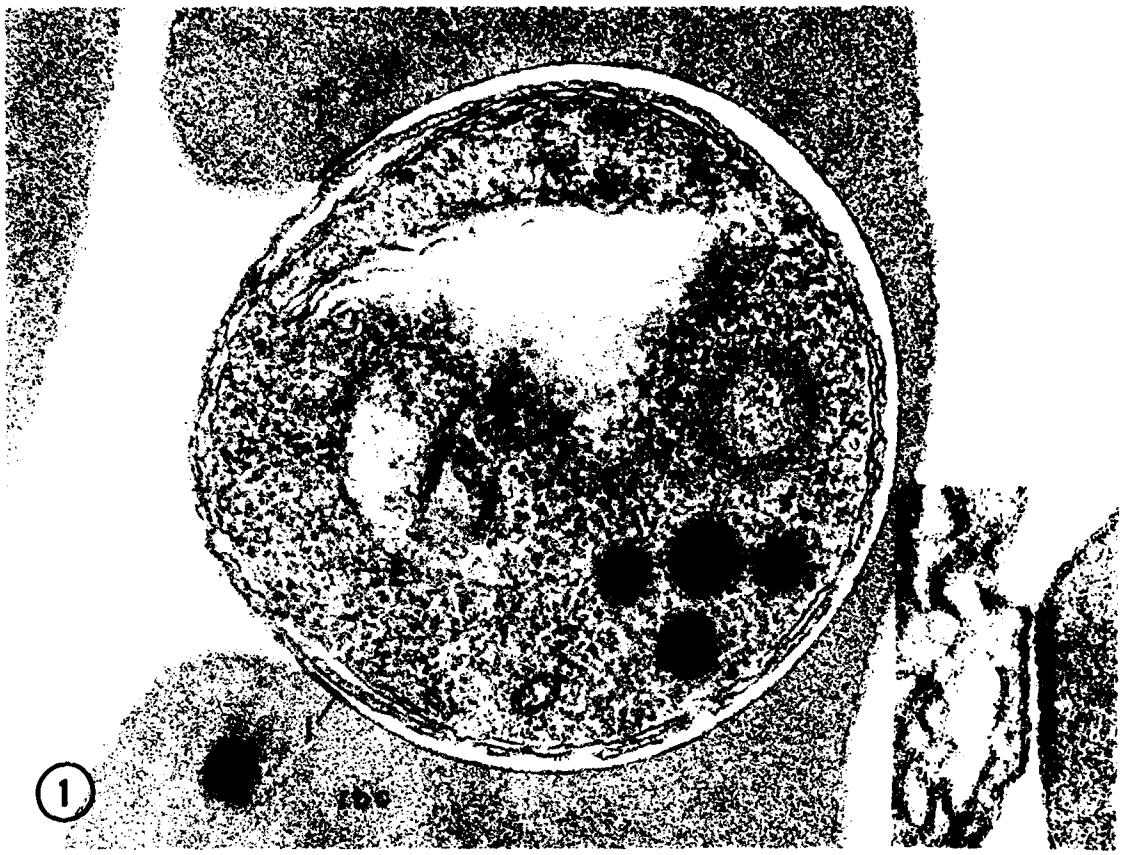
Fig. 7 Freeze-fracture electron micrograph showing attachment of a cytochalasin B-treated merozoite to the erythrocyte membrane. The invagination of the erythrocyte membrane extends beyond the apical end (ap) of the cytochalasin B-treated merozoite. IMP on the P face (Pe) of the erythrocyte membrane at the end of the invagination neck (white arrow) abruptly disappear and the P face (Pv) of the vacuole membrane possesses few IMP. Also present are secondary vacuoles (sv) near the erythrocyte invagination. Arrows outline the fracture edge of the erythrocyte membrane. X 63,000.

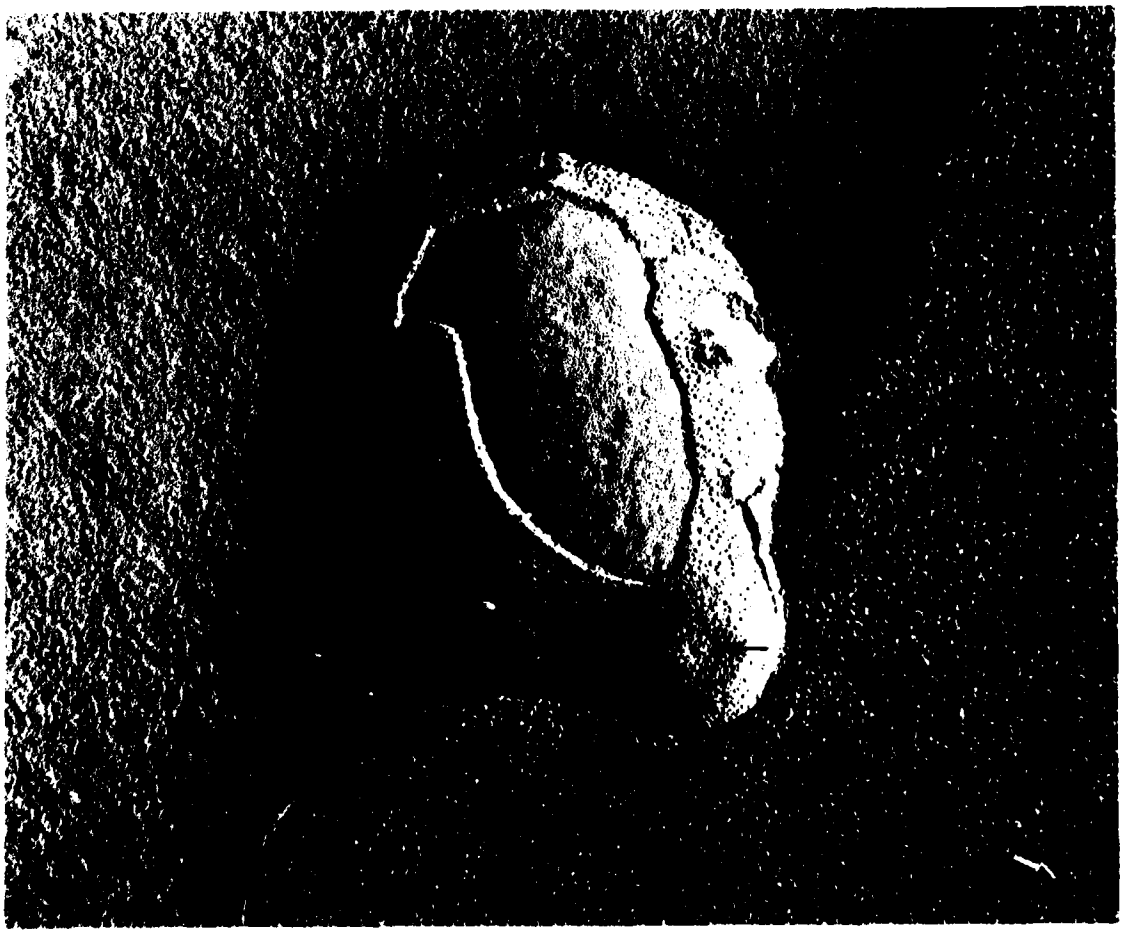
Fig. 8 Freeze-fracture electron micrograph showing an attachment between the cytochalasin B-treated merozoite and erythrocyte (rbc). Although the merozoite only attaches to the erythrocyte membrane, the invagination extends far beyond the apical end of the merozoite. A small dimple (white arrow) is present at the apical end of the merozoite. (Pm): The P face of the merozoite plasma membrane; (Ev): The E face of the vacuole membrane. Arrows outline the fracture edge of the erythrocyte. X 64,000.

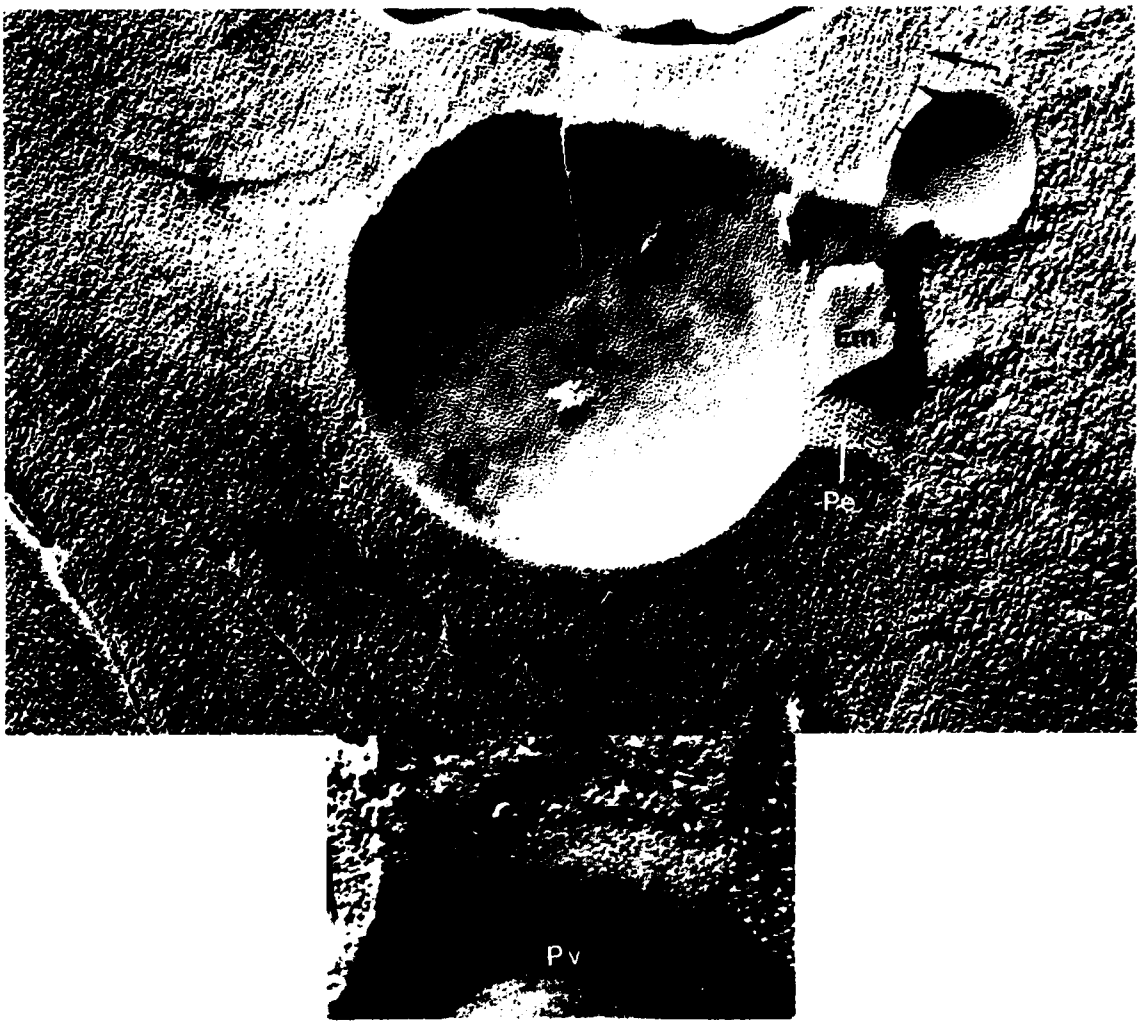
TABLE 1

Mean IMP Densities/μm^2 on the Erythrocyte and Vacuole Membranes

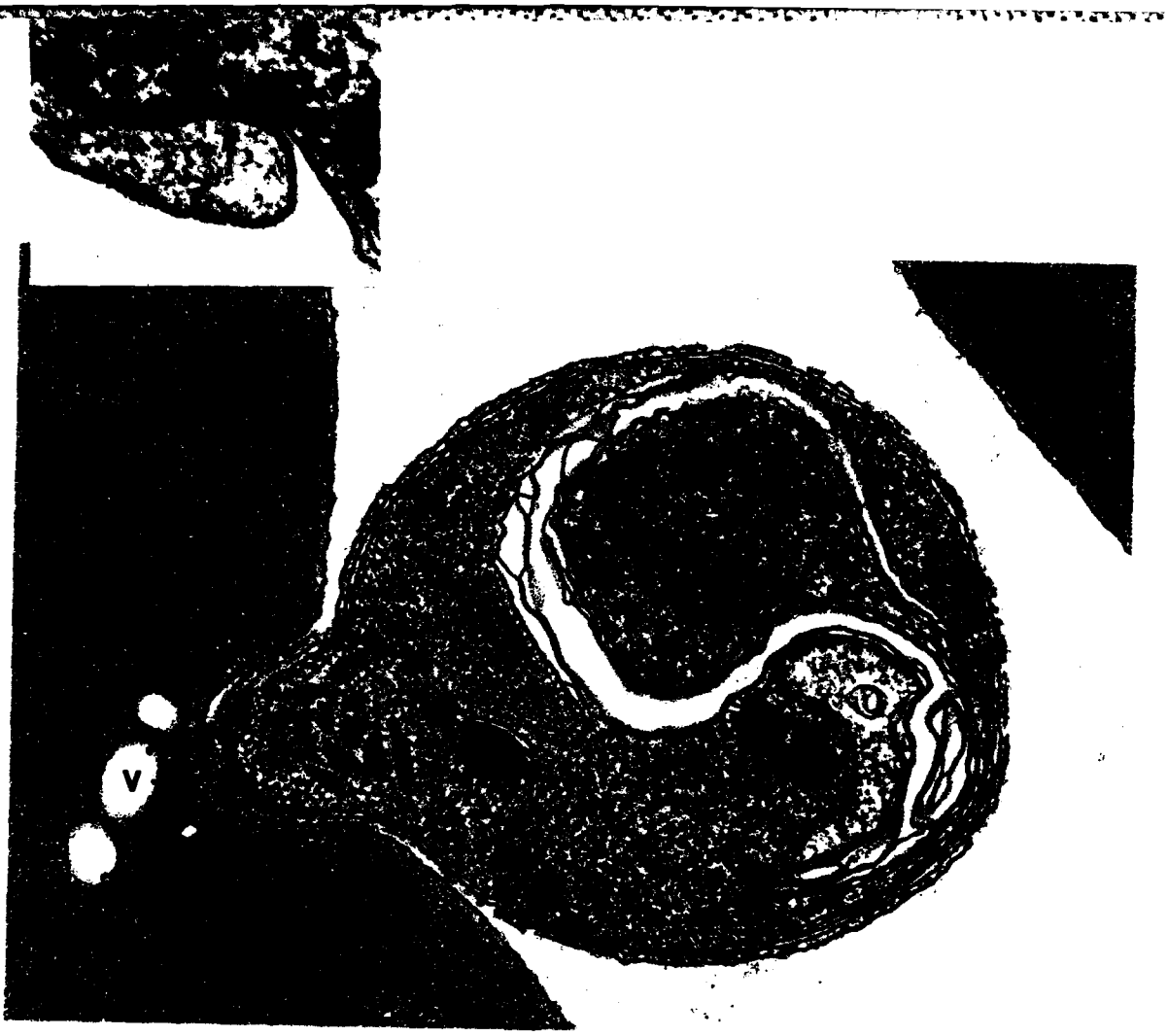
	P Face	E Face
Erythrocyte Membrane	2109 ± 431	389 ± 100
Vacuole Membrane	564 ± 148	334 ± 65






Em
Pe
Pv


5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The protective antigen of malarial sporozoites *(Plasmodium berghei)* is a differentiation antigen.*

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Running Title: Protective Antigen of Malarial Sporozoites.

Abbreviations,

Pb44: A protein with a molecular weight of 44,000 found on the surface of sporozoites of *P. berghei*.

Summary

Pb44, the protective antigen of rodent malaria sporozoites (*Plasmodium berghei*) covers the entire surface of mature, salivary gland sporozoites. This antigen is undetectable in approximately 50% of the immature, i.e. oocyst sporozoites. On the surface of the remaining oocyst sporozoites, Pb44 is found in patches. Pb44 is present in early exoerythrocytic liver stages of *P. berghei*, but becomes undetectable after 30 hours of intrahepatocytic development. It seems likely that Pb44 is a differentiation antigen associated with an unique function of the sporozoites, perhaps penetration in the target host cells.

Sporozoites are the infective stage of malaria parasites, introduced into the host through a mosquito bite. They develop within the oocysts, in the midgut of *Anopheles* mosquitoes, and subsequently migrate to the mosquito's salivary glands. Although sporozoites from oocysts and salivary glands are morphologically very similar, they differ in that only the salivary gland forms are infective, and can be used to vaccinate mammalian hosts (1,2).

Recently, it has been reported that a hybridoma, formed by fusion of a plasmacytoma cell line with spleen cells of mice immunized with γ-irradiated *P. berghei* sporozoites, produced monoclonal antibodies against a surface antigen of this parasite (3). *In vitro* incubation of salivary gland sporozoites with Fab fragments of the monoclonal antibody abolishes their infectivity. Passive transfer of relatively small doses of the purified antibody fully protects against a sporozoite-induced infection (4). The antibody reacts with a protein (Pb44) with an apparent molecular weight of 44,000, found on the surface of the salivary gland sporozoites.

Here we used this monoclonal antibody to search for the presence of Pb44 on the membrane of several developmental stages of *Plasmodium berghei*. We have found that Pb44 has the characteristics of a differentiation antigen, and is associated with the infective stage of the parasite.

Salivary gland and oocyst sporozoites of *P. berghei* (NK65 strain) were obtained by dissection of laboratory-bred, infected *Anopheles stephensi*, and purified as described in detail (3). Salivary gland sporozoites of *P. knowlesi*, kindly provided by Dr. R. Gwadz (Laboratory of Parasitic Diseases, NIAID, NIH) were obtained from *A. balabacensis*, fed on rhesus monkeys infected with the H strain of the parasite. Free forms of erythrocytic stages of *P. berghei* were obtained by lysing with 0.01% saponin the red blood cells of mice infected with the NK65 strain of the parasite.

Monoclonal antibodies against Pb44 were purified from ascitic fluid of mice bearing the hybridoma tumor 3D11 (4). The antibodies were conjugated with 4X crystalized ferritin (5,6) and filtered through a Sepharose 6B column, previously standardized for molecular weight determinations. Fractions containing conjugates consisting mainly of one molecule of ferritin and one molecule of antibody were pooled and kept at 4°C until used. In all the experiments the parasites were incubated at 4°C with the conjugate to avoid the redistribution of antigen on its surface membrane. After 30 minutes of incubation, the parasites were washed with TC 199, by repeated centrifugation, and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) containing 4% sucrose. The parasites were then processed for electron microscopy (7), and the thin sections were examined with a JEOL 100CX electron microscope.

Liver sections, containing exoerythrocytic stages of development of *P. berghei* were obtained from three week old rats, injected intravenously with 3-5 x 10^5 non-irradiated salivary gland sporozoites. The rats were sacrificed 12,18, 24, 32 and 42 hours after parasite inoculation, and their livers fixed in 10% buffered formaldehyde. Sections were prepared for light microscopy, incubated with the monoclonal antibodies to Pb44 and then stained with a preparation of fluorescein-labeled goat antibodies to mouse immunoglobulins. The sections were examined for fluorescence with a Zeiss microscope (630X), and then counterstained with hematoxylin-eosin confirming the diagnosis of exoerythrocytic stages, also by light microscopy.

Salivary gland sporozoites of *P. berghei*, incubated at 0°C with the ferritin conjugate, showed a uniform distribution of ferritin particles over the entire parasite membrane (Fig. 1a & b). In contrast, 50% of the sporozoites obtained from the oocyst localized in the mosquito's midgut, exhibited a complete absence of ferritin label. On the surface of the remaining oocyst sporozoite ferritin

particles were distributed irregularly and in patches (Figs. 2a & b). Using the same ferritin conjugated preparations of monoclonal antibody no label was found on the erythrocytic stages of *P. berghei*, nor on the salivary gland sporozoites of *P. knowlesi*.

Exoerythrocytic forms of the parasites were examined to determine, more precisely, the stage at which the antigenic determinants which were recognized by the monoclonal antibodies to Pb44 disappeared from the subsequent developmental stages of *P. berghei*. Twelve, eighteen and twenty-four hours after the inoculation of the sporozoites, the exoerythrocytic forms were detected by both light microscopy and by indirect immunofluorescence, using monoclonal 3D11 antibodies. After 30 hours the exoerythrocytic forms were only seen by light microscopy. This corroborates our earlier observations, using polyvalent hyperimmune sera, which established that early exoerythrocytic stages react with antisporozoite antibodies and late exoerythrocytic forms can only be detected by antiblood form antisera (8). It should be pointed out that the antigens present both inside and on the surface membrane of the parasites might be detected by immunofluorescence performed on fixed tissue sections. Considering that salivary gland sporozoites synthesize a precursor of Pb44 (Yoshida *et al.*, in preparation) the antigens detected in 12, 18 and 24 hours old exoerythrocytic forms could be located in the parasite's cytoplasm. That is, the membrane-associated Pb44 could have been lost even earlier, i.e. during or soon after sporozoite penetration into the hepatocytes.

In short, these findings support the idea that Pb44 is a differentiation antigen, involved in an unique, presumably essential function associated with mature sporozoites. One intriguing possibility is that Pb44 participates

in the process of the recognition and/or penetration of the parasite into the target (hepatocyte) host cell. If this is indeed the case, the search of homologues of Pb44 in sporozoites of human malaria would be of considerable interest.

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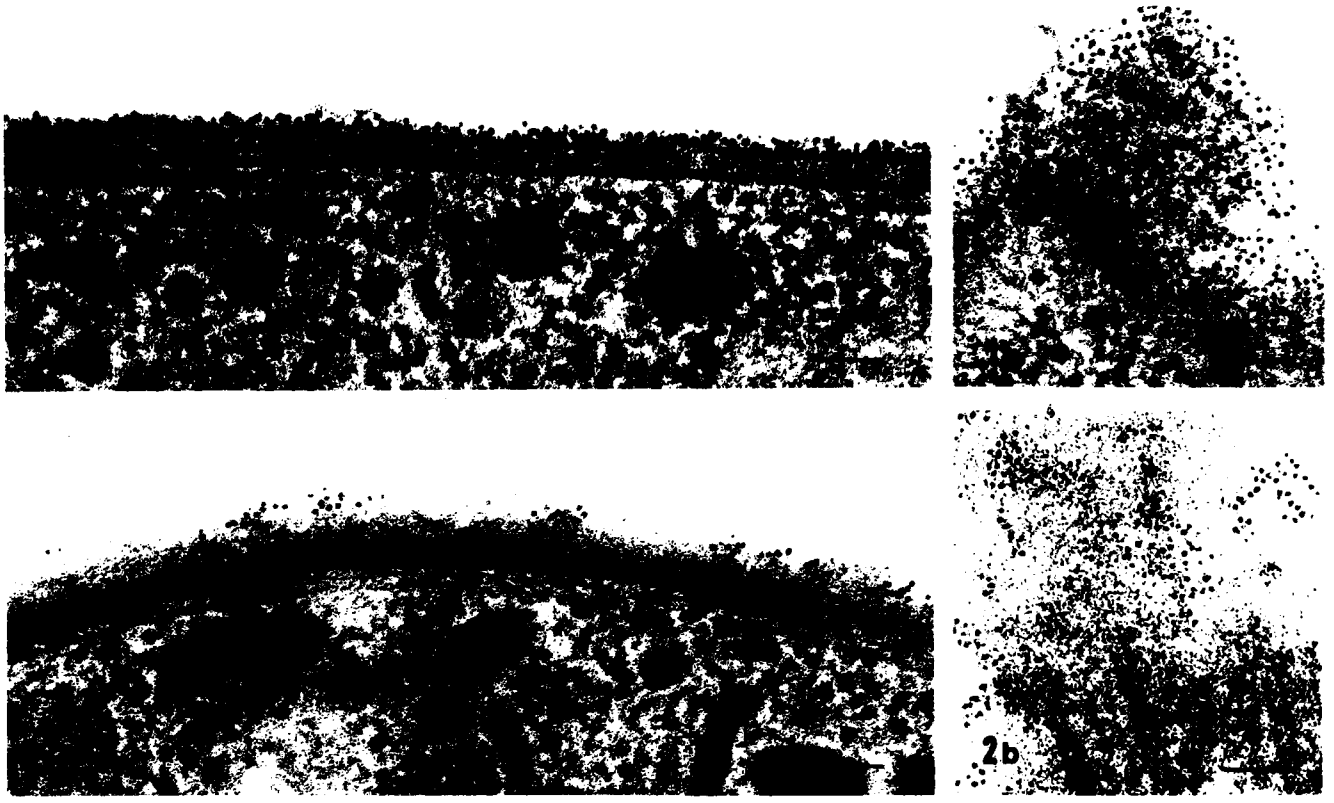
Figure Legends

Fig. 1 Electron micrographs of longitudinal (a) and tangiential sections (b) of salivary gland sporozoites of P. berghei incubated with ferritin-conjugated monoclonal antibody showing a uniform distribution of ferritin particles over the entire parasite surface membrane. Scale bar: 0.1μ.

Fig. 2 Electron micrographs of longitudinal (a) and tangiential sections (b) of oocyst-sporozoites of P. berghei incubated with ferritin-conjugated monoclonal antibody showing a patchy distribution of ferritin particles on their surface. Scale bar: 0.1μ.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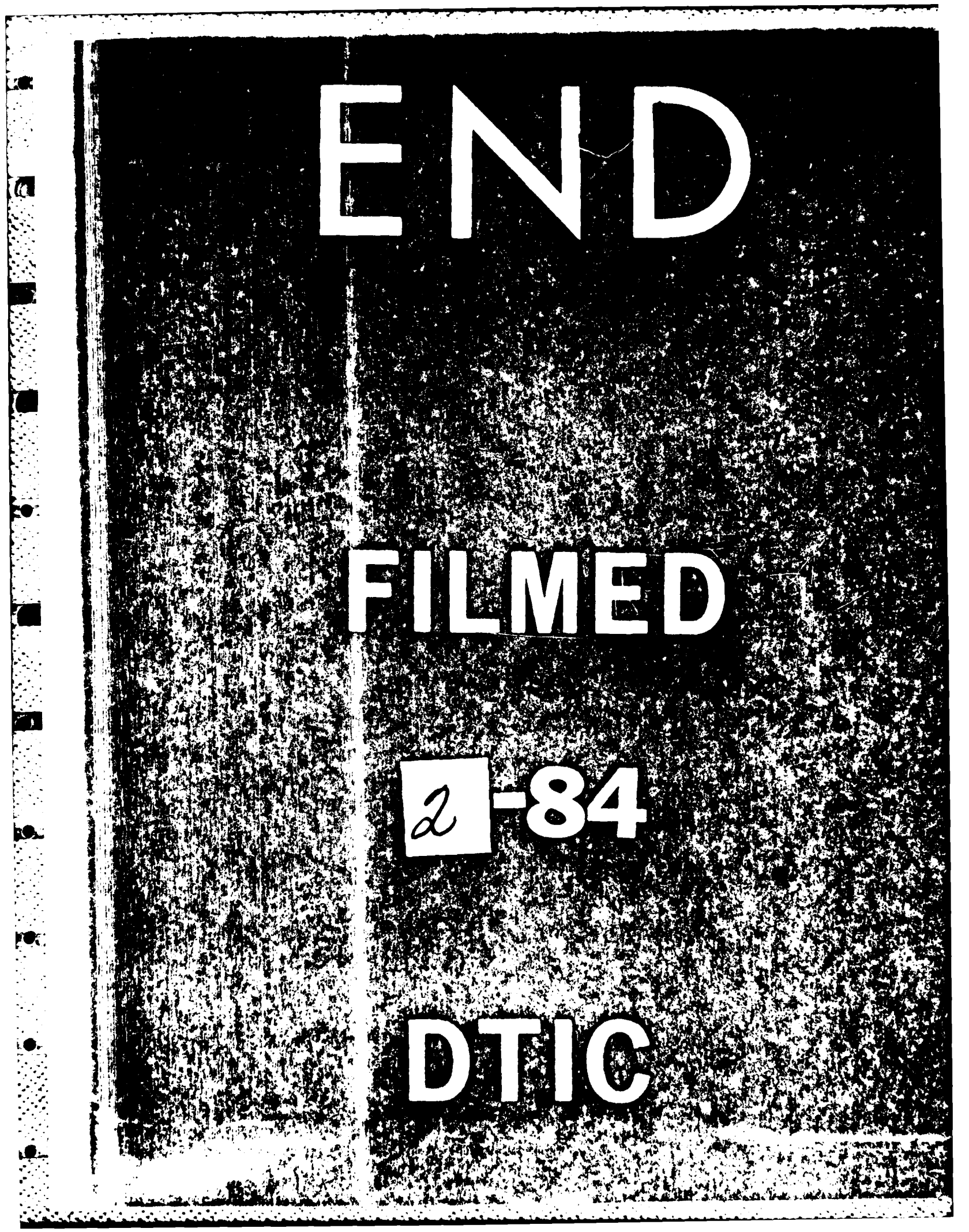
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