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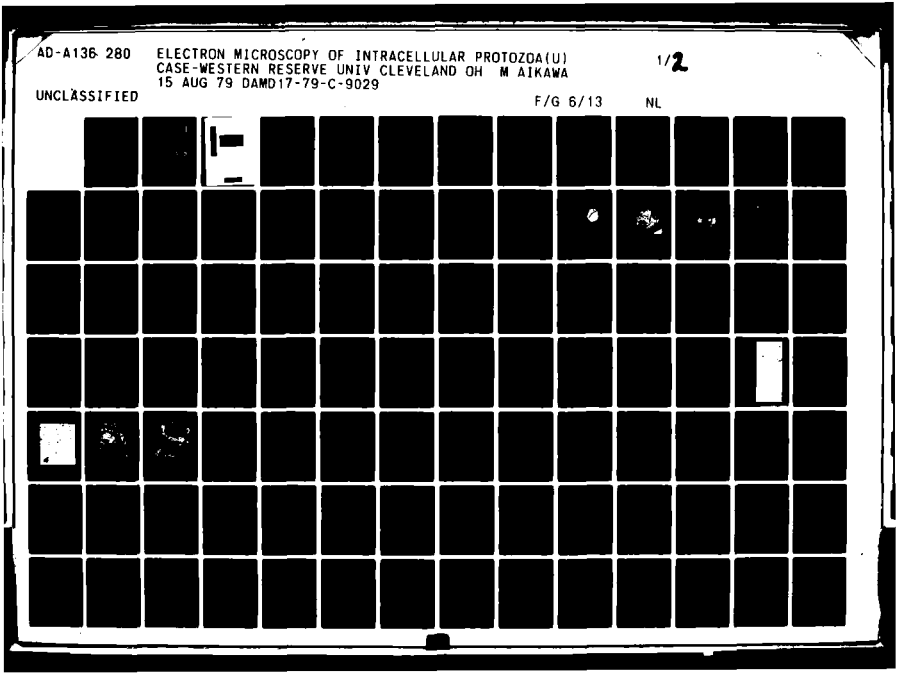

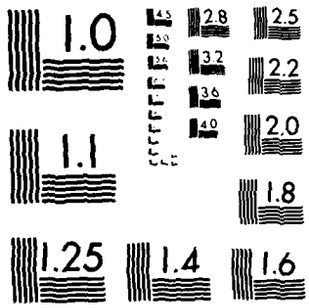

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Aikawa, Masamichi
Contract DAMD17-79-C-9029

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| REPORT DOCUMENTATION PAGE | | READ INSTRUCTIONS BEFORE COMPLETING FORM |
|---|---|---|
| 1. REPORT NUMBER | 2. GOVT ACCESSION NO. | 3. RECIPIENT'S CATALOG NUMBER |
| 4. TITLE (and Subtitle)<br>Electron Microscopy of Intracellular Protozoa | | 5. TYPE OF REPORT & PERIOD COVERED<br>Annual report, January 1, 1979 to August 15, 1979 |
| | | 6. PERFORMING ORG. REPORT NUMBER |
| 7. AUTHOR(s)<br>Masamichi Aikawa, M.D. | | 8. CONTRACT OR GRANT NUMBER(s)<br>DAMD 17-79-C-9029 |
| 9. PERFORMING ORGANIZATION NAME AND ADDRESS<br>Case Western Reserve University<br>Cleveland, Ohio 44106 | | 10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS<br>61102A.3M161102BS10.AF.050 |
| 11. CONTROLLING OFFICE NAME AND ADDRESS<br>U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, Maryland 21701 | | 12. REPORT DATE<br>August 15, 1979 |
| | | 13. NUMBER OF PAGES<br>147 |
| 14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office) | | 15. SECURITY CLASS. (of this report)<br>unclassified |
| | | 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE |

16. DISTRIBUTION STATEMENT (of this Report)
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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)
Malarial parasites, Electron microscopy, Leishmania, Host interaction

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
The effects of immune serum on P. falciparum-infected erythrocytes grown in vitro were investigated by electron microscopy. When immune serum was added to partially synchronized cultures of P. falciparum, parasites matured normally during the first day from ring forms to trophozoites and schizonts. In contrast, the number of new rings detected two days following reinvasion was reduced by 90%, compared with cultures containing normal serum.

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Clusters of merozoites were seen in cultures grown with immune serum, but rarely in cultures with normal serum. Electron microscopy showed many extracellular merozoites in the culture with immune serum. All of the extracellular merozoites were covered with a thick surface coat. Agglutination of these merozoites occurred as adherence between the surface coat of adjacent parasites. Also noted was the presence of many lysed erythrocytes infected with P. falciparum. The erythrocytes infected with P. falciparum showed electron-dense excrescences on the erythrocyte membrane. When incubated with immune serum, a distinct electron-dense surface coat was seen over the excrescences, indicating the presence of malaria antigen on the excrescences.

The effects of immune serum on sporozoites of P. berghei and P. knowlesi were also studied. Sporozoites incubated in noraml serum interact with macrophage and actively penetrate into the macrophages. On the other hand, sporozoites incubated in immune serum showed a distinct surface coat and were phagositized by the macrophages. They degenerated within vacuoles of the macrophages, while sporozoites incubated in normal serum did not show degenerative changes within the macrophages. Rat Kupffer cells appear to display the same interaction with sporozoites as that seen with the peritoneal macrophages. Based on these results, macrophages might serve to localize the sporozoites with normal serum in the immediate vicinity of the hepatocytes, while in the immunized animals, macrophages would remove and destroy these antibody-coated parasites, thus contributing to the mechanism of sporozoite-induced resistance.

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Annual Report No. 1

# ELECTRON MICROSCOPY OF INTRACELLULAR PROTOZOA

Masamichi Aikawa, M.D.

August, 1979

Supported by

U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701
(Contract No. DAMD 17-79-C 9029)

The Institute of Pathology, Case Western Reserve University
Cleveland, Ohio 44106

# Electron Microscopy of Intracellular Protozoa

Masamichi Aikawa, M.D.

September, 1979

Supported by

U.S. Army Medical Research and Development Command

Washington, D.C. 20314

Contract No. DAMD 17-79-C-9029

The Institute of Pathology

Case Western Reserve University

Cleveland, Ohio 44106

Summary

Host-parasite interaction is of major importance to intracellular parasites, since parasite survival depends on the host. Studies on the interaction are essential for better understanding of intracellular parasites. During the last fiscal year, we have concentrated our investigation on several aspects of the interaction between host cells and P. falciparum and L. brasiliense. In addition, we reviewed Pathology of Malaria in order to update our knowledge on malaria pathology.

In collaboration with Dr. Diggs and his associates at WRAIR, we studied the effects of immune serum on P. falciparum-infected erythrocytes grown in vitro. When immune serum was added to partially synchronized cultures of P. falciparum, parasites matured normally during the first day from ring forms to trophozoites and schizonts. In contrast, the number of new rings detected two days following reinvasion was reduced by 90%, compared with cultures containing normal serum. Clusters of merozoites, which were often present around clumps of malarial pigment were seen in cultures grown with immune serum, but rarely in cultures with normal serum.

Electron microscopy showed many extracellular merozoites in the culture with immune monkey serum. Many of them were partially lysed and showed the disrupted plasma membrane. All of the extracellular merozoites were covered with a thick surface coat. Agglutination of these merozoites occurred as adherence between the surface coat of adjacent parasites. Also noted was the presence of many partially lysed erythrocytes infected with P. falciparum. These parasites within the lysed erythrocytes were covered

with a surface coat. The erythrocytes infected with P. falciparum showed electron-dense excrescences on the erythrocyte membrane. When incubated with immune serum, a distinct electron-dense surface coat was seen over the excrescences. This finding suggests the presence of malaria antigen on the excrescences. Lysis of P. falciparum-infected erythrocytes is probably initiated at the site of the excrescences by the formation of immune complexes as observed by electron microscopy.

The effects of immune serum on sporozoites of P. berghei and P. knowlesi were also studied in collaboration with Dr. Nussenzweig and her associates. Sporozoites of P. berghei and P. knowlesi, incubated in normal serum interact with peritoneal macrophages. Interiorization of the sporozoite required that both serum and macrophages be obtained from an animal susceptible to infection by the malarial parasites. Phagocytosis by the macrophages appears not to be essential for the parasites to become intracellular. Our findings indicate that active penetration of the sporozoites into the macrophage occur. Antibodies present in the serum of sporozoite-immunized mice are important in establishing the fate of both the intracellular sporozoites and the macrophages containing the parasite. Sporozoites coated with antibodies degenerate within vacuoles of the macrophages, whereas sporozoites incubated in normal serum do not degenerate within macrophages. Rat Kupffer cells appear to display the same kind of interaction with sporozoites as that seen with the peritoneal macrophages. It is posturated that Kupffer cells play a dual role in sporozoite-host cell interaction. In normal animals these cells might serve to localize the sporozoites in the immediate vicinity

of the hepatocytes. In the immunized animals, macrophages would remove and destroy these antibody coated parasites, thus contributing to the mechanism of sporozoite induced resistance.

In collaboration with Drs. Hendricks and Alving of WRAIR, macrophage entry by L. brasiliense was studied in vitro. The flagellum initiates macrophage entry. Of particular interest was the aggregation of multi-laminated bodies in the cytoplasm in which the flagellum is inserted. Since these multi-laminated bodies resemble liposomes in morphology, we studied interaction between liposomes and Leishmania. Liposomes coated with anti-lipid A antibody were taken up by macrophages in one hour. These liposomes were seen in close proximity to intracellular Leishmania, indicating a close interaction between Leishmania and liposomes within the macrophages. We are planning to study the interaction between Leishmania and liposomes tagged with anti-leishmania drugs in order to study effects of the drugs in this system.

In recent years, new knowledge of malaria pathology has accumulated because of the use of modern techniques such as electron microscopy and immunofluorescence microscopy. This new knowledge has increased the understanding of pathological processes of malaria. Therefore, the review was made to update our current knowledge on malaria pathology with a particular emphasis on the prominent organ changes associated with Plasmodium infection and some of the physiopathological mechanisms involving malaria infection.

Foreward

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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Detailed Report

During the last fiscal year, we have investigated three major problems in relation to host-parasite interaction. They include: 1) interaction between P. falciparum infected erythrocytes and immune serum against P. falciparum, 2) interaction between malaria sporozoites and macrophages, and 3) interaction between Leishmania brasiliense and macrophages.

1) Inhibition of in vitro growth of Plasmodium falciparum by immune serum: A light and electron microscopic study.

There has been general agreement that humoral factors are important in protective immunity against malaria. The mechanism whereby serum exerts protection is unclear. One hypothesis suggests that the mechanism of humoral immunity depends upon antibodies directed against merozoites which prevent their attachment and/or penetration into new erythrocytes and interrupt the parasite life cycle (Miller, Aikawa and Dvorak, J. Immunol., 114:1237, 1975). However, there is also evidence that animals may demonstrate in vivo immunity in the absence of detectable anti-merozoite antibodies (Miller, Power, and Shiroishi, Exp. Parasitol., 41:105, 1977). Clark et al. (Lancet, 2:1128, 1975) demonstrated intracellular death of rodent malarial parasites just prior to the reduction in parasitemia and they felt that these abnormal intracellular parasites were analogous to the crisis forms described by Taliaferro and Taliaferro (J. Infect. Dis., 75:1, 1944). However, similar crisis forms have not been described for P. falciparum perhaps because the mature parasites are rarely seen in peripheral blood. In order to elucidate the effects of humoral antibody on intracellular P. falciparum, in collaboration with Drs. Chulay, Diggs and Haynes of WRAIR,

we undertook light and electron microscopic evaluation on the effects of antibody against P. falciparum growth in vitro.

Owl monkeys, previously infected with P. falciparum and treated with chloroquine are resistant to subsequent challenge with the same organism. Serum from such immune animals protects against initial infection, when passively transferred to normal owl monkeys. When immune serum was added to partially synchronized cultures of P. falciparum, parasites matured normally during the first day, from ring forms to trophozoites and schizonts. In contrast, the number of new rings detected two days following reinvasion was reduced by 90%, compared with cultures containing normal serum. Clusters of merozoites, which were often present around clumps of malarial pigment were seen frequently in cultures grown with immune serum but rarely in cultures with normal serum.

Electron microscopy showed many extracellular merozoites in the culture with immune monkey serum. Many of them were partially lysed and showed the disrupted plasma membrane. All of these extracellular merozoites were covered with a thick, loosely-packed surface coat measuring 60 nm in thickness. Agglutination of these merozoites occurred as adherences between the surface coat of adjacent parasites.

Intracellular uninucleate trophozoites and schizonts from the culture with immune monkey serum showed enlarged food vacuoles filled with electron-dense granular materials together with a few scattered pigment particles. However, no surface coat was observed in the intracellular parasites. Also noted was the presence of many partially lysed erythrocytes infected with the parasites. These parasites within the lysed ery-

throcytes were covered with a coat of about 60nm. The parasites incubated in normal serum did not show detectable morphological changes.

The erythrocytes infected with P. falciparu show electron-dense excrescences on the erythrocyte membrane. There are more excrescences on the erythrocytes infected with schizonts than those with infected uninucleate trophozoites. When incubated with immune serum, a distinct electron-opaque surface coat was seen over the excrescences. This finding indicates the presence of malaria antigens in the excrescences as previously suggested by Kilejian (Kilejian, Abati and Trager, Exp. Parasitol., 42:157, 1977). These data indicate that immune serum effects not only the free merozoites, but also intracellular parasites by lysing P. falciparum-infected erythrocytes. Lysis of P. falciparum-infected erythrocytes is probably initiated at the site of the excrescences by the formation of immune complexes as observed by electron microscopy.

These data will be presented at the meeting of the American Society of Microbiology in 1979 and are in preparation for publication.

2) Attachment, interiorization and intracellular fate of P. berghei and P. knowlesi.

The factors involved in the resistance or susceptibility of mammalian hosts to infection by the sporozoite remain obscure. It is also uncertain whether sporozoites, once injected by mosquito vector into the mammalian's host circulation, attach and penetrate directly into hepatocytes. The penetration into hepatocytes might be preceded by interaction with another cell type, such as Kupffer cells, the macrophages which line the hepatic sinusoids. Therefore, we, in collaboration with Dr. R. Nussenzweig and

her associates, first attempted to characterize sporozoite attachment, interiorization and intracellular fate within macrophages. Sporozoites of P. berghei and P. knowlesi were observed to interact with macrophages obtained from mice and rhesus monkeys. Attachment of the sporozoites onto the macrophages was found to be less specific than that seen for interiorization of the parasite. It was necessary to have both the macrophages and serum derived from a species of animals susceptible to infection by either species of malarial sporozoites studied, in order for the parasites to become intracellular in vitro.

The intracellular fate of P. berghei sporozoite was dependent upon exposure of the parasite to normal serum from a sporozoite immunized host. The sporozoites which demonstrated a surface coat deposition associated with immune serum incubation were seen degenerating within vacuoles of the macrophages. On the other hand, sporozoites preincubated in normal serum appeared unaltered within the macrophage after 60 minutes of incubation. In order to elucidate whether or not these parasites were phagocytosed by the macrophages, mouse macrophages were treated with cytochalasin B which blocks both specific and non-specific phagocytosis. Cytochalasin B-treated macrophages were found to contain approximately the same number of intracellular sporozoites as control cells. This indicates that interiorization of the sporozoites is not entirely due to phagocytosis by the macrophages. Hence active penetration of these sporozoites into the macrophage must occur. This study, therefore, suggests that the sporozoites may first enter into the macrophage (Kupffer cells) of the liver before they proceed to extra-erythrocytic stages in the hepatocytes. Also, it indicates the role

of macrophages as an effector mechanism of sporozoite-induced immunity. This data has been submitted for publication.

3) Penetration of macrophages by Leishmania brasiliense.

In collaboration with Drs. L. Hendricks and C. Alving of WRAIR, macrophage entry by Leishmania brasiliense was studied in vitro. As other investigators demonstrated in Leishmania donovani, the flagellum initiates macrophage entry. Of particular interest was the aggregation of multi-laminated bodies in the cytoplasm in which the flagellum is inserted. Since these multi-laminated bodies resemble the liposomes in morphology, we have attempted to study interaction between liposomes and Leishmania. Liposomes coated with anti-lipid A antibody were taken up by macrophages in one hour. When the promastigate form of Leishmania and liposomes coated with anti-lipid A antibody suspended in water containing ferritin, many liposomes labeled with ferritin were seen in close proximity to intracellular Leishmania. However, so far we were not able to observe a close contact between them. Currently we are further investigating the interaction between Leishmania and liposomes.

# Inhibition of *in vitro* growth of *Plasmodium falciparum* by immune serum: A light and electron microscopic study.

Jeffrey D. Chulay, Masamichi Aikawa,
J. David Haynes and Carter L. Diggs

Department of Immunology, Walter Reed Army Institute of Research, Washington, D.C. 20012 and Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106

There has been general agreement that humoral factors are important in protective immunity against malaria. The mechanism whereby serum exerts protection is unclear. One hypothesis suggests that the mechanism of humoral immunity depends upon antibodies directed against merozoites which prevent their attachment and/or penetration into new erythrocytes and interrupt the parasite life cycle (Miller, Aikawa and Dvorak, J. Immunol., 114:1237, 1975). However, there is also evidence that animals may demonstrate in vivo immunity in the absence of detectable anti-merozoite antibodies (Miller, Power and Shiroishi, Exp. Parasitol., 41:105, 1977). Clark et al. (Lancet, 2:1128, 1975) demonstrated intracellular death of rodent malarial parasites just prior to the reduction in parasitemia and they felt that these abnormal intracellular parasites were analogous to the crisis forms described by Taliaferro and Taliaferro (J. Infect. Dis., 75:1, 1944). However, similar crisis forms have not been described for P. falciparum perhaps because the mature parasites are rarely seen in peripheral blood. In order to elucidate the effects of humoral antibody on intracellular P. falciparum, in collaboration with Dr. Chulay, Diggs and Haynes of WRAIR, we undertook light and electron microscopic evaluation on the effects of antibody against P. falciparum growth in vitro.

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Intracellular uninucleate trophozoites and schizonts from the culture with immune monkey serum showed enlarged food vacuoles (Fig. 2) filled with electron-dense granular materials together with a few scattered pigment particles. However, no surface coat was observed in the intracellular parasites. Also noted was the presence of many partially lysed erythrocytes infected with the parasites. These parasites within the lysed erythrocytes were covered with a coat of about 60nm (Fig. 3). The parasites incubated in normal serum did not show detectable morphological changes.

The erythrocytes infected with P. falciparum show electron-dense excrescences on the erythrocyte membrane.( Fig. 2) There are more excrescences on the erythrocytes infected with schizonts than those with infected uninucleate trophozoites. When incubated with immune serum, a

distinct electron-opaque surface coat was seen over the excrescences. (Fig. 5) This finding indicates the presence of malaria antigens in the excrescences as previously suggested by Kilejian (Kilejian, Abati and Trager, Exp. Parasitol., 42:157, 1977). These data indicate that immune serum effects not only the free merozoites, but also intracellular parasites by lysing P. falciparum-infected erythrocytes. Lysis of P. falciparum-infected erythrocytes is probably initiated at the site of the excrescences by the formation of immune complexes as observed by electron microscopy.

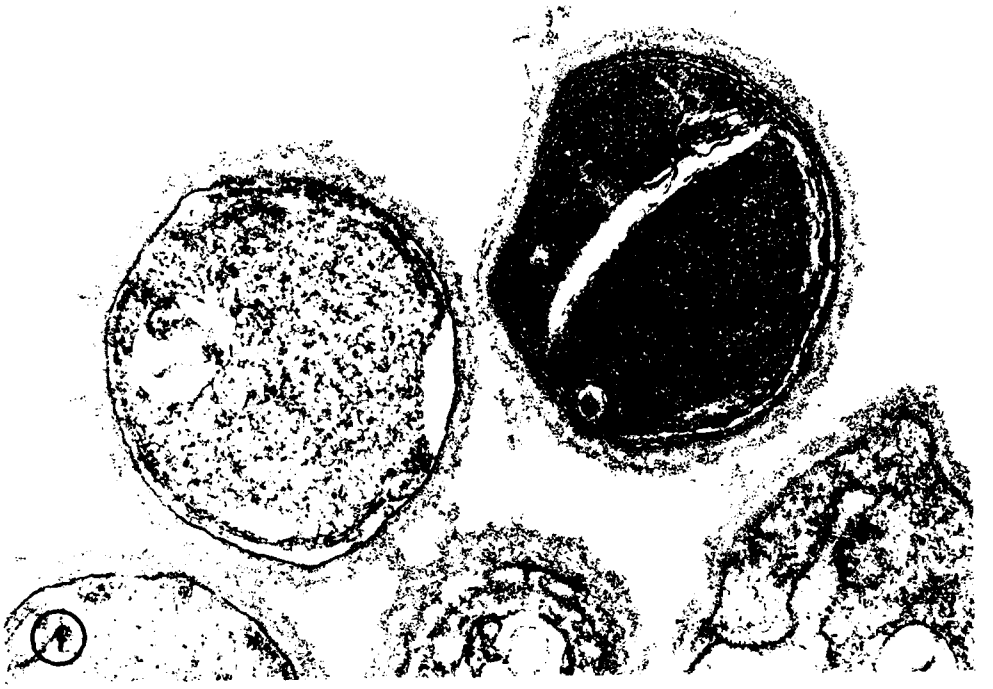
Figure Legends

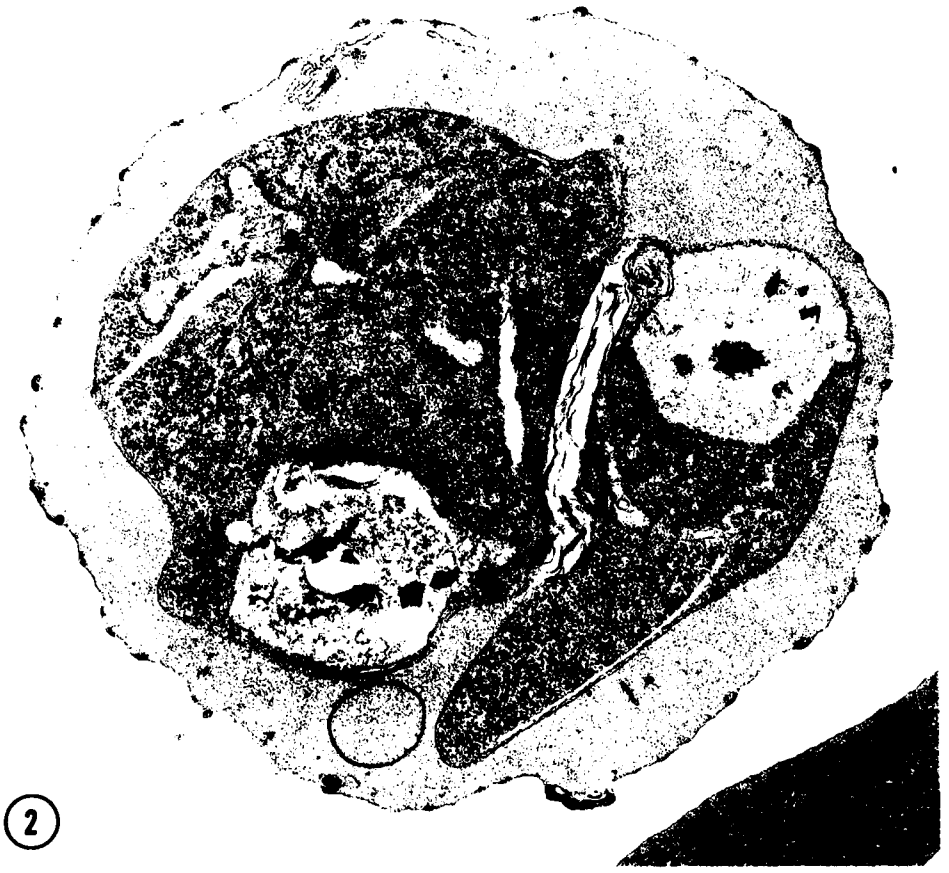
Fig. 1 Electron micrograph of free merozoites aggregated in immune monkey serum. Note adherence between the surface coats of adjacent merozoites. X 42,000.

Fig. 2 Electron micrograph of an erythrocyte infected with P. falciparum in immune monkey serum. Note enlarged food vacuoles and excrescences on the erythrocyte membrane. X 24,000.

Fig. 3 Electron micrograph of an extracellular schizont in immune monkey serum. The surface is covered with an electron-dense surface coat. X 60,000.

Fig. 4 Electron micrograph of a partially lysed erythrocyte infected with P. falciparum in the presence of immune monkey serum. Electron-dense surface coats are seen over excrescences of the erythrocyte membrane. X 30,000. Inset: High magnification of excrescences covered with a surface coat. X 55,000.





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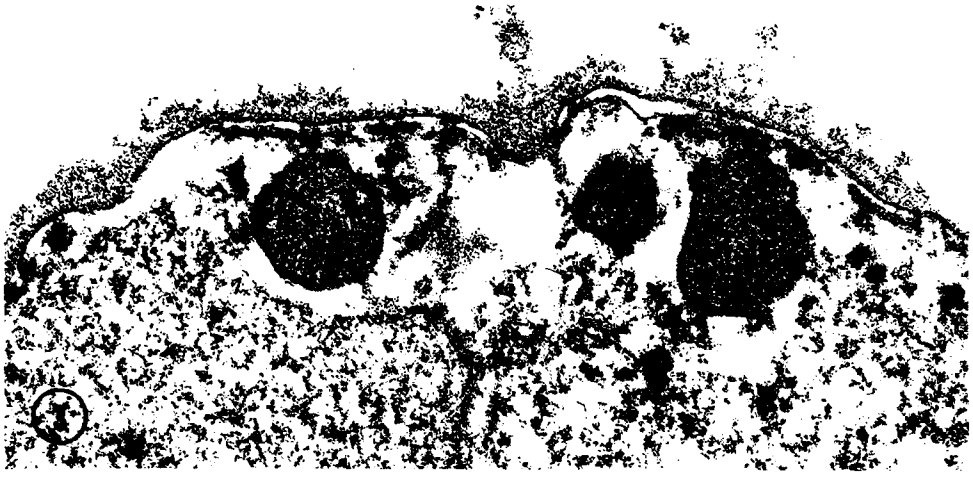

# Sporozoites of Mammalian Malaria: Attachment, Interiorization and Intracellular Fate Within Macrophages

Harry D. Danforth, Masamichi Aikawa, Alan H. Cochrane, and R. S. Nussenzweig

From the Division of Parasitology, Department of Microbiology
New York University Medical Center and the
Institute of Pathology, Case Western Reserve University

Running Title: Malarial Sporozoite-Macrophage Interaction

## SYNOPSIS

Sporozoites of Plasmodium berghei and Plasmodium knowlesi, incubated in normal serum readily interact with peritoneal macrophages of mice or rhesus monkeys respectively. Interiorization of the sporozoite requires that both serum and macrophages be obtained from an animal susceptible to infection by the malaria parasite. Serum requirements for sporozoite attachment to the macrophage is less specific.

Phagocytosis is not essential for the parasites to become intracellular. Our findings indicate that active penetration of the sporozoites into the macrophages does occur.

Antibodies present in the serum of sporozoite-immunized mice are important in establishing the fate of both the intracellular sporozoites and the macrophages containing the parasite. Sporozoites coated with antibodies degenerate within vacuoles of the macrophages, which show no morphological alteration. Sporozoites incubated in normal serum do not degenerate within macrophages, but the parasitized macrophages become morphologically altered and are destroyed.

Rat Kupffer cells appear to display the same kind of interaction with sporozoites as that seen with the peritoneal mouse macrophages. It is postulated that Kupffer cells play a dual role in sporozoite-host cell interaction. In normal animals these cells might serve to localize the sporozoites in the immediate vicinity of the hepatocytes. In the immunized animals, macrophages would remove and destroy these antibody coated parasites, thus contributing to the mechanism of sporozoite induced resistance.

Index Key Words: Plasmodium berghei; Plasmodium knowlesi; sporozoites; cytochalasin B; attachment to, interiorization and intracellular fate within peritoneal macrophages.

# INTRODUCTION

The factors involved in the resistance or susceptibility of mammalian hosts to infection by the mosquito stage of the malaria parasite, the sporozoite, remain obscure. This is due to lack of precise knowledge of the initial pre-erythrocytic phase of development of malaria parasites.

Hepatocytes are the only cells in which pre-erythrocytic parasite multiplication and development has been observed in sporozoite-inoculated mammalian hosts. However, in vitro experiments have so far consistently failed to detect any evidence of sporozoite-hepatocyte interaction. It is therefore uncertain whether sporozoites injected by the mosquito vector into the circulation of a mammalian host attach to and directly enter hepatocytes.

Alternatively, development within hepatocytes might be preceded by interaction with another cell type. Kupffer cells, the fixed macrophages which line the hepatic sinuses of the liver, could be important as a site of recognition and attachment for sporozoites as they enter the liver via the blood circulation. It is also possible that an initial phase of sporozoite differentiation or development might occur within these cells, preceding exoerythrocytic development within hepatocytes. Since Kupffer cells are phagocytic, they could function in certain circumstances to form a barrier to prevent sporozoite invasion of the liver cells, especially in sporozoite immunized animals.

We investigated sporozoite interaction with a phagocytic cell by characterizing the parasite's attachment, interiorization and intracellular fate within peritoneal macrophages in vitro. Sporozoites incubated in normal serum became attached to and were also found within macrophages, provided that

both the macrophages and the serum were derived from animals susceptible to infection by the malaria parasite. The presence or absence of antisporozoite antibodies in the serum was found to be quite important in determining the fate of both sporozoites and macrophages. Sporozoites incubated in immune serum were destroyed within the macrophages, while parasites incubated in normal serum appeared intact within the macrophages and caused a morphological degeneration of these cells.

## MATERIALS AND METHODS

Sporozoites. Sporozoites were obtained from the salivary glands of either Anopheles stephensi mosquitoes (Liston) infected with the NK-65 strain of Plasmodium berghei (Vincke and Lips) or Anopheles balabacensis balabacensis mosquitoes (Baisas) heavily infected with Plasmodium knowlesi (Stinton and Mulligan). The A. b. balabacensis mosquitoes were provided through the courtesy of Dr. Robert Gwadz, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland. Mosquito salivary glands were triturated in tissue culture medium (TC 199, Grand Island Biological Co) and the sporozoites were purified by passage through a DEAE-cellulose column, according to previously described technique (13). After concentration by centrifugation at 1000 g for 30 min., the sporozoites were resuspended in minimal essential medium (MEM, Grand Island Biological Co.) counted in a hemocytometer, and held on ice until further use. A temperature of 4°C was maintained during the processing of the sporozoites.

Macrophages. Peritoneal macrophages were obtained from A/J mice (Jackson Memorial Laboratories) 4 to 8 weeks of age, using the technique of Cohn and Benson (7). Rhesus monkey macrophages were obtained from the peritoneal cavity of an animal undergoing splenectomy. The peritoneal cavity of this animal was washed with warm MEM (37°C) using a large syringe fitted with a flexible canula (Intramedic polyethylene tubing, Fisher Scientific Co.). This medium was allowed to settle in the peritoneal cavity for 5 min. and was

then removed. Following centrifugation of the collected medium at 140 g for 10 min., the pelleted cells were resuspended in MEM with 10%(v/v)fetal calf serum (FCS, Flow Laboratories) and counted. Both rhesus and mouse macrophages were cultivated on 15 mm round glass or plastic coverslips (Thermox, Lux Scientific Corp.) in MEM with 10% (v/v) FCS at a concentration of 2 to 3 x $10^5$ cells per coverslip. These coverslips were incubated at 37°C in 10% $CO_2$ for 45 min. to allow for cell attachment. The coverslips were then washed in MEM, and fresh medium containing 10% FCS, 100 µ/ml penicillin and 100 µg/ml streptomycin was placed on the cells. Macrophages for all experiments were used within 4 hours after plating. Approximately 40% of the cells obtained from the monkey peritoneal cavity attached to the coverslips. The majority of these cells ingested sheep erythrocytes sensitized with IgG and were therefore considered to be macrophages.

Antisporozoite Antisera. A/J mice were immunized with irradiated sporozoites of P. berghei as described previously (16). After the immunized animals were found to be resistant to sporozoite challenge, they were bled for serum.

Incubation of Sporozoites with Serum. Sporozoites of P. berghei or P. knowlesi were incubated at room temperature in MEM containing normal serum in concentrations of 10-20% (v/v) obtained from the following animals: A/J mice (NMS) (Jackson Memorial Laboratories), Sprague Dawley rats (NRS) (Charles River Supply), rhesus monkeys (NRhS) and rabbits (NRaS). In some experiments, sporozoites of P. berghei were also incubated in 20% (v/v) heat inactivated normal mouse serum (NMS), 20% (v/v) immune mouse serum (IMS), or 3.5% (v/v) bovine serum albumin (BSA). Following the incubation period of 30 min. at

room temperature, the sporozoites were placed on the macrophages.

Sporozoite Inactivation. Sporozoites of P. berghei in MEM were held at 45°C for 45 min. in water bath (18) or treated with $10^{-2}$M colchicine (Sigma Chemical Co.) in a 37°C incubator for 60 min. Either treatment caused a loss of viability of the sporozoites as determined by their inability to cause infections in mice.

Sporozoite Incubation with Macrophages. Mouse or monkey macrophages plated on coverslips were washed free of the serum-containing medium with MEM and placed in plastic petri dishes (Falcon, 60 x 15 mm). The dishes were placed on ice and the cells were overlaid with 0.1 to 0.2 ml of suspension of sporozoites in 10 or 20% serum or 3.5% BSA, at a concentration of 1 to 2 x $10^6$ sporozoites/ml. The sporozoites were allowed to settle on the macrophages for 30 min. in a 10% $CO_2$ atmosphere. The macrophages with the sporozoites were then placed in a 37°C incubator and incubated according to experimental design for 10 to 240 min. The cells were then washed in MEM, and fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4).

Light Microscopy Observations of Sporozoite-Macrophage Interaction. The fixed specimens on the glass coverslips were inverted on to a microscopic slide and examined by phase contrast microscopy. Sporozoite-macrophage interaction was evaluated by counting 200 macrophages on duplicate coverslips to determine (a) the number of sporozoites attached to the macrophages, (b) the total number of intracellular sporozoites, (c) the percentage of macrophages which had intracellular sporozoites, (d) the percentage of macrophages which had sporozoite attachment, and (e) the percentage of degenerating macrophages.

Electron Microscopy Observations of Sporozoite-Macrophage Interaction. After fixation with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer, the specimens on the plastic coverslips were washed in phosphate buffer, post-fixed in 1% (w/v) phosphate buffered $O_sO_4$, prestained in 2% (w/v) aqueous uranyl acetate, and dehydrated in a gradated series of alcohols. The cells were embedded *in situ* according to a method previously described (5). The embedded macrophages were then viewed under phase microscopy to select the cells to be mounted on Epon studs (4). The specimens were thin sectioned with a Porter-Blum MT-2 ultramicrotome and examined in a Seimens Elmiskop 101 electron microscope. For scanning microscopy, specimens on the glass coverslips were critical point dried after fixation and dehydration, coated with palladium-gold, and examined with an ARM scanning electron microscope.

Preparation of Sensitized Erythrocytes and Phagocytic Assay. Sheep erythrocytes (E), used less than two weeks after storage in Alsever's solution, were sensitized with IgG (EIgG) (3). Then, 0.1 ml of the 0.5% EIgG suspension in MEM with or without 20% NMS was added to washed macrophages, and after remaining on ice for 30 min. , the cells were incubated for 45 min. at 37°C. The non-ingested EIgG were lysed by rinsing the coverslips in phosphate buffered saline (PBS) diluted 1:5 in distilled water, and the macrophages were fixed in buffered 2.5% glutaraldehyde. Erythrocytes ingested by 200 macrophages were counted by phase microscopy on duplicate coverslips.

Phagocytic Assay with Zymosan Particles. Zymosan (Sigma Chemical Co.), suspended in PBS, was washed twice in MEM and resuspended in the same medium with or without 20% NMS at a concentration of 0.2 mg/ml. A volume of 0.1 ml

of this suspension was placed on the macrophages. Preparations were first incubated for 30 min. on ice and then for 60 min. at 37°C. After fixation, phagocytosis was evaluated by counting ingested particles within 200 macrophages using phase microscopy.

Effect of Cytochalasin B on Macrophage-Sporozoite Interaction. Cytochalasin B (Sigma Chemical Co., lot #96C-0165) was dissolved in 0.1 ml dimethyl sulfoxide and diluted in MEM to the desired concentration. The macrophages on plastic or glass coverslips were washed with MEM and preincubated at 37°C for 15 min. with various concentrations of cytochalasin B ranging from 2.5 to 12.5 μg/ml. A suspension of either EIgG, zymosan, or P. berghei sporozoites in 20% NMS and the same concentration of cytochalasin B that was used to treat the macrophages was then added to the coverslips. After 1 hr. of incubation at 37°C both the phagocytosis of EIgG and zymosan, and sporozoite interaction was determined by light and/or electron microscopy. In a single experiment, we also preincubated sporozoites of P. berghei with cytochalasin B at a concentration of 10 μg/ml in MEM with 20% NMS at 37°C for 30 min. These parasites were then placed on the macrophages, and their interaction with these cells in the presence of cytochalasin B was evaluated as described above.

## RESULTS

The overall purpose of these experiments was to determine the mode of interaction of sporozoites of rodent and simian malaria with peritoneal macrophages, and the intracellular fate of these sporozoites within cells of the mononuclear phagocytic system.

A. Requirement of Normal Mouse Serum for P. berghei Sporozoite Attachment to and Interiorization within Mouse Macrophages.

In the initial series of experiments, it was verified that little or no interiorization occurred when P. berghei sporozoites in serum free medium were placed on mouse macrophages (Table I). In contrast, sporozoites of P. berghei incubated in MEM with 20% NMS, were observed by phase microscopy to be attached to and within mouse peritoneal macrophages by 10 min. after incubation at 37°C (Fig. 1a and Table I). With an increase of the incubation time to 1 hr., a greater number of intracellular sporozoites were seen in the mouse macrophages (Table I). At this time, some of the macrophages with attached or interiorized sporozoites demonstrated substantial morphological alterations as indicated by cytoplasmic blebbing and decreased spreading of the cells (Fig. 1b), when compared to the controls (Fig. 1c). As is shown in Fig. 2, the percentage of degenerating macrophages increased proportionally with the number of sporozoites incubated with the cells.

Scanning and transmission electron microscopy showed that sporozoites, in the presence of NMS, entered mouse macrophages by 10 to 30 min. of incubation at 37°C (Figs. 3a, b, c and 4). In some specimens, partially intracellular sporozoites were covered with macrophages' pseudopodia (Figs. 3b and 4). Numerous sporozites were seen both attached to and entering some of the

macrophages (Fig. 3c). These cells appeared to be morphologically altered as indicated by a rounding up of the macrophages(Fig. 3c). By 60 min. of incubation, most of the macrophages with interiorized sporozoites were completely fragmented.

B. Effect of Immune Mouse Serum on the Interaction between P. berghei Sporozoites and Mouse Macrophages.

Sporozoites incubated in IMS were more rapidly interiorized than those incubated in NMS (Table II). A relatively small proportion of the immune serum coated sporozoites remained attached to the macrophage membrane. This differed from the results obtained with sporozoites which had been incubated in normal serum, where attachment to macrophages was considerably more frequent than intracellular localization (Table II).

Transmission electron microscopy demonstrated that sporozoites incubated in immune serum, which were attached to the macrophages, were surrounded by a surface deposition (Fig. 5). Earlier work (8) had shown that this deposition was due to the interaction of antisporozoite antibodies with sporozoite surface antigen(s). This deposition was also observed within membrane bound vacuoles within the macrophage (Fig. 5), and surrounding the intracellular sporozoites.

C. Intracellular Fate of Sporozoites of P. berghei

Transmission electron microscopy showed that intracellular sporozoites of P. berghei incubated in either NMS or IMS were handled differently within mouse macrophages. Sporozoites of P. berghei in NMS were seen within membrane bound macrophage vacuoles by 30 min. of incubation (Fig. 6). These sporozoites appeared to be completely intact and showed no ultrastructural

degeneration. By 1 hr. of incubation, these intracellular sporozoites were still intact, but the macrophages containing these interiorized parasites were undergoing extensive morphological alteration. Observations of sporozoite-macrophage interaction at later time points were not possibly due to death and detachment of the macrophages.

Sporozoites preincubated in IMS, surrounded by a thick surface deposition, were found within membrane bound vacuoles within macrophages (Fig. 7). Many of these parasites appeared to be degenerating as early as 30 min. after incubation of the sporozoites with macrophages. These intracellular parasites showed a loss of ultrastructural organization, and a breakdown of their pellicular membranes. No ultrastructural alterations of the macrophages were observed upon their interaction with immune serum incubated sporozoites.

D. Serum Requirement for Sporozoite-Macrophage Interaction

These experiments were performed to determine whether sporozoite-macrophage interaction would be altered by the presence of serum obtained from various animal species.

The percentage of mouse macrophages with either attached or intracellular sporozoites of P. berghei, a rodent malaria parasite, was similar when the parasites were incubated in either NMS, NRS, or NMS-INACT (Figs. 8a and b). However, when these sporozoites were incubated in NRhS or FCS, there was a considerable decrease in the number of macrophages containing intracellular parasites (Fig. 8a). Attachment of the sporozoites incubated in FCS was similar to that seen with the various normal rodent sera, while sporozoites in NRhS showed decreased attachment (Fig. 8b). Sporozoites incubated in NRaS or 3.5% BSA did not interact with the macrophages as defined by the two parameters of attachment and interiorization (Figs. 8a and b). It is apparent that serum from animals susceptible to infection by P. berghei (i.e. small rodents) must

be present for maximal interiorization of the sporozoites to occur.

E. Macrophage Specificity for Sporozoite Attachment and Interiorization

Mouse peritoneal macrophages interacted preferentially with sporozoites of P. berghei in NMS. By 1 hr. of incubation, 68% of these macrophages had intracellular P. berghei sporozoites (Fig. 9a). At the same time point, only 10% of these macrophages contained intracellular P. berghei sporozoites incubated in NRhS. When mouse macrophages of the same preparation were overlaid with sporozoites of the simian malaria parasite P. knowlesi, incubated in either NMS or NRhS, interiorization occurred in only a small percentage of the macrophages (16% and 4%, respectively, Fig. 9a). Attachment of the P. knowlesi sporozoites to mouse macrophages was, however, similar to that seen with P. berghei sporozoites when both parasites were incubated with NMS (data not shown). The interiorization of P. knowlesi sporozoites was considerably greater (34% of the macrophages) when these parasites, incubated in NRhS, were placed on peritoneal macrophages of a rhesus monkey (Fig. 9b). Less interiorization occurred when the P. knowlesi sporozoites were incubated in NMS (13% of the macrophages, Fig. 9b). It is therefore evident, that greater parasite interiorization is seen when both serum and macrophages are obtained from animals which are susceptible to infection with P. berghei and P. knowlesi sporozoites respectively.

F. Effect of Cytochalasin B and Sporozoite Inactivation on Macrophage-Sporozoite Interaction

The next series of experiments was designed to determine whether sporozoite interiorization was due to phagocytosis and/or active penetration of sporozoites into macrophages. This was first examined by adding various concentrations of cytochalasin B, a known inhibitor of phagocytosis (9), to the macrophages prior to and during their incubation with sporozoites. In other experiments, the role of active penetration of sporozoites was investigated by inactivation

of the parasites by heat treatment or colchicine exposure before they were placed on untreated macrophages.

Treatment of the macrophages with cytochalasin B at a concentration of 10 µg/ml, completely inhibited phagocytosis, as measured by uptake of EIgG and zymosan granules. In contrast, the same concentration of cytochalasin B had no effect on the penetration of sporozoites, as compared with that observed with untreated macrophages. Additional experiments using cytochalasin B concentrations varying from 2.5 µg/ml to 12.5 µg/ml did not interfere with sporozoite penetration, but did effectively block phagocytosis of EIgG (Fig. 10).

Cytochalasin B at a concentration of 10 µg/ml was added to the sporozoites during their 30 min. preincubation in NMS. When these parasites were placed on cytochalasin B-treated macrophages, the penetration of the macrophages by these sporozoites was found to be similar to that seen with the non-treated controls. These cytochalasin B pretreated sporozoites were found to retain their infectivity for mice.

Heat treatment of sporozoites or colchicine exposure caused a loss of sporozoite infectivity for mice and resulted in a decrease in sporozoite-macrophage interaction. Thus, the number of these inactivated sporozoites which were interiorized was substantially reduced when compared with their respective controls (Table III). There was also a decrease in the number of inactivated sporozoites attached to the mouse macrophages. The macrophages which were incubated with these inactivated sporozoites, did not undergo the morphological changes that had been observed when viable sporozoites were placed on the cells.

## DISCUSSION

The results of the experiments presented in this report indicate that malaria sporozoites both attach to and are rapidly interiorized within macrophages. This interaction, particularly the intracellular fate of the parasites, is primarily dependent upon the presence of normal or immune serum in the incubation medium.

It is interesting that normal serum enhances the attachment of sporozoites to macrophages. In nature, these parasites are introduced by mosquito bite into the host's bloodstream, which transports them to the liver where they initiate the intracellular exoerythrocytic phase of development. Previous work, done in vitro, has shown that normal serum added to the incubation medium enhances sporozoite motility and infectivity (17, 19), and also increases immunogenicity of irradiated sporozoites of P. berghei (Orjih and Nussenzweig, in preparation). It is therefore possible, that normal serum increases the frequency of contact of the sporozoites with the macrophage surface by promoting parasite motility. However, sporozoite motility does not appear to be the only factor which enhances parasite attachment to macrophages. In fact, we only observed minimal attachment of P. berghei sporozoites maintained in a medium containing BSA, which has also been shown to promote motility (19).

It is possible that the contact with serum triggers some changes in the sporozoite's surface membrane, which might be essential for the parasites subsequent interaction and development within the mammalian host cells. Alternatively, the contact with serum might be essential just to maintain and prolong sporozoite viability in vivo and in vitro.

The serum component(s) essential for promoting sporozoite attachment to macrophages has not yet been characterized. However, it is certain that

parasite interiorization is greatly enhanced by the presence of serum from an animal susceptible to infection by a particular species of malaria parasite.

Sporozoites of P. knowlesi were observed to display a marked selectivity in their interaction with macrophages of different mammalian species. Thus, sporozoites of the simian malaria parasite were interiorized much more efficiently in monkey macrophages than by mouse peritoneal macrophages. This interaction was favored by the presence of normal rhesus serum.

Previous investigations have shown that the uptake by macrophages of certain developmental stages of protozoan parasites, including Leishmania species (2, 6), Trypanosoma cruzi (2, 14) and Toxoplasma gondii (11) is due to the phagocytosis of these parasites. In order to clarify how malaria sporozoites become interiorized these parasites were incubated with macrophages treated with cytochalasin B, a known inhibitor of phagocytosis (9). The fact that numerous intracellular sporozoites were detected under these conditions, which abolished uptake of EIgG and zymosan particles, indicated that sporozoites actively contribute to their interiorization and that phagocytosis of sporozoites by the macrophages is not essential. The active role of sporozoites in their interaction with macrophages, was further documented by the finding that heat-treated or colchicine-inhibited sporozoites failed to become interiorized.

It is interesting that the effect of cytochalasin B incubation of protozoan parasites varies with the stage of the life-cycle and the species of parasite. Although we observed no inhibition of penetration of macrophages by P. berghei sporozoites, merozoites of P. knowlesi preincubated in cytochalasin B failed to invade red blood cells (12). This was interpreted to be due to cytochalasin B interference with the junction formation between the merozoite and the red blood cell membrane, which inhibited the movement of the parasite into the cell. Cytochalasin B pretreatment of sporozoites of Eimeria magna also

inhibited in vitro penetration of host cells, which was attributed to a lose of motility of these sporozoites (10).

Earlier work, reviewed by Nussenzweig (15), has shown that sporozoite-immunized animals produce antibodies primarily directed against the parasite's surface antigens. Upon incubation with sporozoites, these antibodies coat the parasites, resulting in a thick surface deposition (8). These antisporozoite antibodies are important in determining the fate of both the intracellular P. berghei sporozoite and the macrophages in which the parasites are interiorized. Sporozoites coated with these antibodies are destroyed within the macrophages which show no subsequent degeneration. In the absences of immune serum, intracellular sporozoites fail to undergo ultrastructural degeneration within the macrophages. However, the macrophages containing these intracellular parasites become morphologically altered and are destroyed.

Preliminary experiments, with rat Kupffer cells obtained by liver perfusion, have shown the same pattern of interaction of these cells with P. berghei sporozoites as is seen with mouse peritoneal macrophages (H.D. Danforth, unpublished results). Thus, it is possible that in vivo Kupffer cell-sporozoite interaction might play a role in concentrating sporozoites in the immediate vicinity of the hepatocytes in which the parasite's final development takes place. However, it is not yet known whether the intracellular sporozoites remain viable within the macrophages, and then destroy these cells to invade the hepatocytes.

The role of Kupffer cells may be very different within sporozoite immunized animals. Under these conditions it is possible that the removal and destruction of antibody coated sporozoites by macrophages plays a significant part in preventing these parasites from invading the hepatocytes to continue their development. Macrophages, especially Kupffer cells, may therefore, have an essential role in the effector mechanisms of sporozoite-induced immunity against malaria.

ACKNOWLEDGEMENTS

The authors wish to thank Mmes. Rita Altszuler, Marilyn Maracic and Gail Topper, and Mr. John Rabbege for their technical assistance; Mmes. Sharon Hecht-Ponger and Beatrice Robles for the typing of the manuscript; and Dr. Celso Bianco and Dr. Hannah Lustig Shear for discussion and review of the manuscript.

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Fig. 1 Phase-contrast micrographs of the in vitro interaction of P. berghei sporozoites in NMS with mouse peritoneal macrophages. (a) Sporozoite attachment to and interiorization (arrows) within macrophages at 10 min. incubation at 37°C. X 1125. (b) Sporozoite attachment to and interiorization (short arrow) within macrophages at 60 min. incubation at 37°C. Note blebbing of cytoplasm (long arrow) at the periphery of some cells, and the rounded up appearance of the macrophages. X 1125. (c) Control macrophages without sporozoites at 60 min. incubation at 37°C. Note characteristic spread appearance of the cells. X 1125.

Fig. 2 The effect of the size of the sporozoite inoculum on the percentage of degenerating macrophages (MØ) seen at different time periods of incubation at 37°C. Data represent the per cent of degenerating macrophages per 200 macrophages counted on duplicate coverslips ●—●, $1 \times 10^5$ sporozoites per coverslip; ○—○, $2 \times 10^5$ sporozoites per coverslips; △—△, $3 \times 10^5$ sporozoites per coverslip.

Fig. 3 Scanning electron micrographs of the in vitro interaction of P. berghei sporozoites incubated in NMS with mouse peritoneal macrophages. (a) Attachment and partial interiorization of sporozoites (arrows) after 10 min. incubation at 37°C with macrophages. X 900. (b) Two sporozoites (Sp) partially interiorized at 10 min. incubation at 37°C. Note macrophage pseudopodia (arrow) extending over part of each sporozoite. X 3200. (c) Macrophage with numerous sporozoites attached and others partially intracellular after 30 min. incubation at 37°C. Note apparent degeneration of the macrophage indicated by rounded up appearance of the cell. X 4500.

Fig. 4 Sporozoite (Sp) of P. berghei in NMS entering a macrophage at 10 min. incubation at 37°C. Note electron lucent space surrounding the intracellular portion of the parasite (arrow) and a pseudopod extending over the extracellular portion of the sporozoite (double arrow). X 18,000.

Fig. 5 Sporozoite of P. berghei, with surface coat (double arrow) due to incubation with IMS, attached to mouse macrophage after 30 min incubation at 37°C. Note presence of material similar in appearance to the surface coat in a macrophage vacuole (arrow). X 15,000.

Fig. 6 Intracellular sporozoite (Sp) of P. berghei in NMS after 30 min. incubation at 37°C. Note that the parasite shows no ultrastructural degeneration and is enclosed in a membrane-bound parasitophorous vacuole within the macrophage. X 25,000.

Fig. 7 Sporozoite of P. berghei with antibody surface coat deposition (arrow) within a macrophage vacuole after 30 min. incubation at 37°C. Note that there is an apparent degeneration of the sporozoite (double arrow) as indicated by the loss of ultrastructural organization. X 34,000.

Fig. 8 Interaction of P. berghei sporozoites with mouse peritoneal macrophages (MØ) in the presence of normal serum from different animal species or BSA. Data based on 200 macrophages counted on duplicate coverslips after 60 min. incubation at 37°C. A, Percentage of macrophages with intracellular sporozoites; B, Percentage of macrophages with attached sporozoites. ▭, normal mouse serum (NMS); ▨, Heat inactiviated normal mouse serum (NMS-inact.); ☰, normal rat serum (NRS); ▨, normal rhesus serum (NRhS); ▩, normal rabbit serum (NRaS); ▭, fetal calf serum (FCS); ▭, bovine serum albumin (BSA).

Fig. 9 Comparison of the percentage of mouse or rhesus peritoneal macrophages (MØ) with intracellular P. berghei or P. knowlesi sporozoites in either normal mouse or rhesus serum after 60 min. incubation at 37°C. Data based on 200 macrophages counted on duplicate coverslips. A, Mouse macrophages; B, Rhesus macrophages. ☐, normal mouse serum (NMS) ▨, normal rhesus serum (NRhS).

Fig. 10 The effect of treatment with various concentrations of cytochalasin B on the percentage of mouse macrophages (MØ) with intracellular cells (EIgG or P. berghei sporozoites), after 60 min. incubation at 37°C. The data represent the per cent of macrophages with intracellular sporozoites or ingested EIgG per 200 macrophages counted on duplicate coverslips. O----O, ingested EIgG; O—O, interiorized sporozoites.

TABLE I

Effect of Normal Mouse Serum Incubation on the Number of Intracellular Sporozoites of P. berghei

| Sporozoites Incubated with | | Number of Intracellular Sporozoites[3] 10 min. 37°C Incubation | 60 min. 37°C Incubation |
|---|---|---|---|
| 20% NMS[1] | Exp. 1 | 42 | 64 |
| | Exp. 2 | 104 | 210 |
| MEM[2] | Exp. 1 | 0 | 0 |
| | Exp. 2 | 4 | 13 |

1 NMS = normal mouse serum

2 MEM = minimal essential medium

3 Data represent mean number of intracellular sporozoites per 200 MØ counted on duplicate coverslips - Approximately 200,000 sporozoites placed on coverslips plated with 200,000 MØ.

TABLE II

Attachment and Interiorization of P. berghei Sporozoites

in Normal and Immune Mouse Serum

| | 10 min. Incubation at 37°C | | 30 min. Incubation at 37°C | |
|---|---|---|---|---|
| | No. of Sporozoites Attached to MØ[3] | % MØ with Intracellular Sporozoites | No. of Sporozoites Attached to MØ[3] | % MØ with Intracellular Sporozoites |
| NMS[1] | 25 | 6 | 76 | 29 |
| IMS[2] | 7 | 13 | 28 | 24 |

1 NMS = 20% normal mouse serum

2 IMS = 20% immune mouse serum

3 Data represent mean number of sporozoites attached to MØ or % of MØ with intracellular sporozoites per 200 MØ counted on duplicate coverslips. Approximately 200,000 sporozoites placed on coverslips plated with 200,000 MØ.

TABLE III

Interaction of Mouse Peritoneal Macrophages with Heat-Inactivated and Colchicine-Treated *P. berghei* Sporozoites

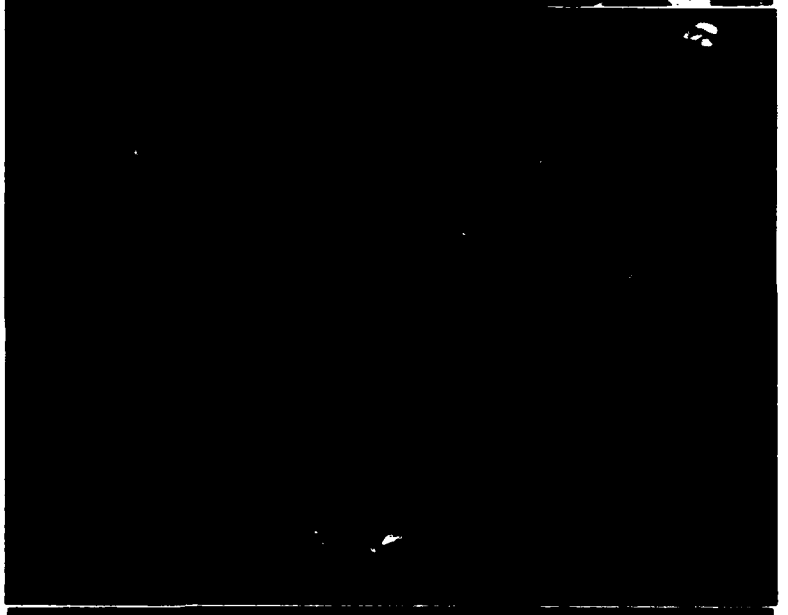
| Sporozoite Pre-Treatment | No. of Intracellular Sporozoites[3] (per 200 MØ) | No. of Sporozoites attached[3] (per 200 MØ) |
|---|---|---|
| Heat Inactivation[1] | 2 | 20 |
| None | 61 | 72 |
| Colchicine Incubation[2] | 2 | 6 |
| None | 115 | 180 |

1 Sporozoites pre-incubated at 45°C for 45 min.

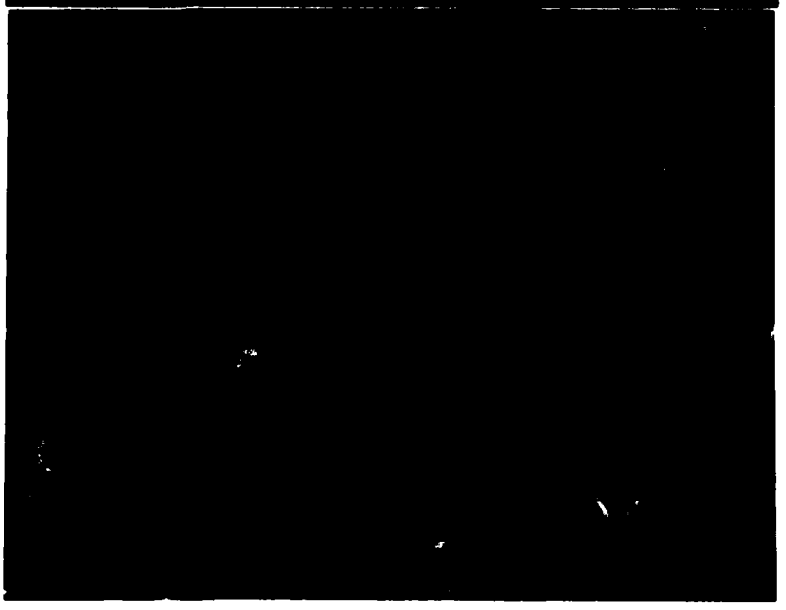
2 Sporozoites preincubated with $10^{-2}$M colchicine at 37°C for 30 min.

3 Data represents either mean number of intracellular sporozoites or number of sporozoites attached to MØ per 200 MØ counted on duplicate coverslips at 60 min. incubation at 37°C. Approximately 200,000 sporozoites incubated in NMS placed on coverslips plated with 200,000 MØ.

1a



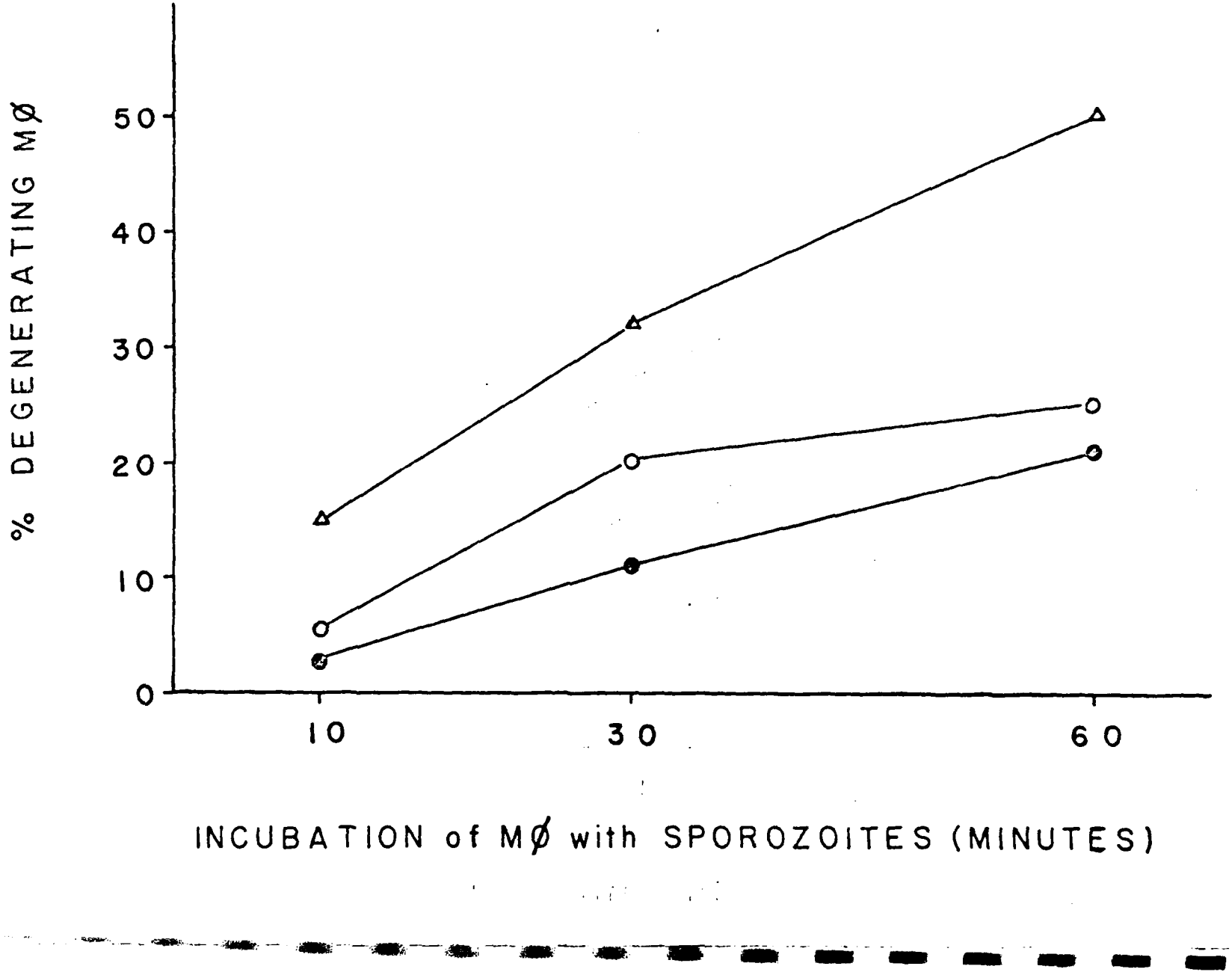
1b

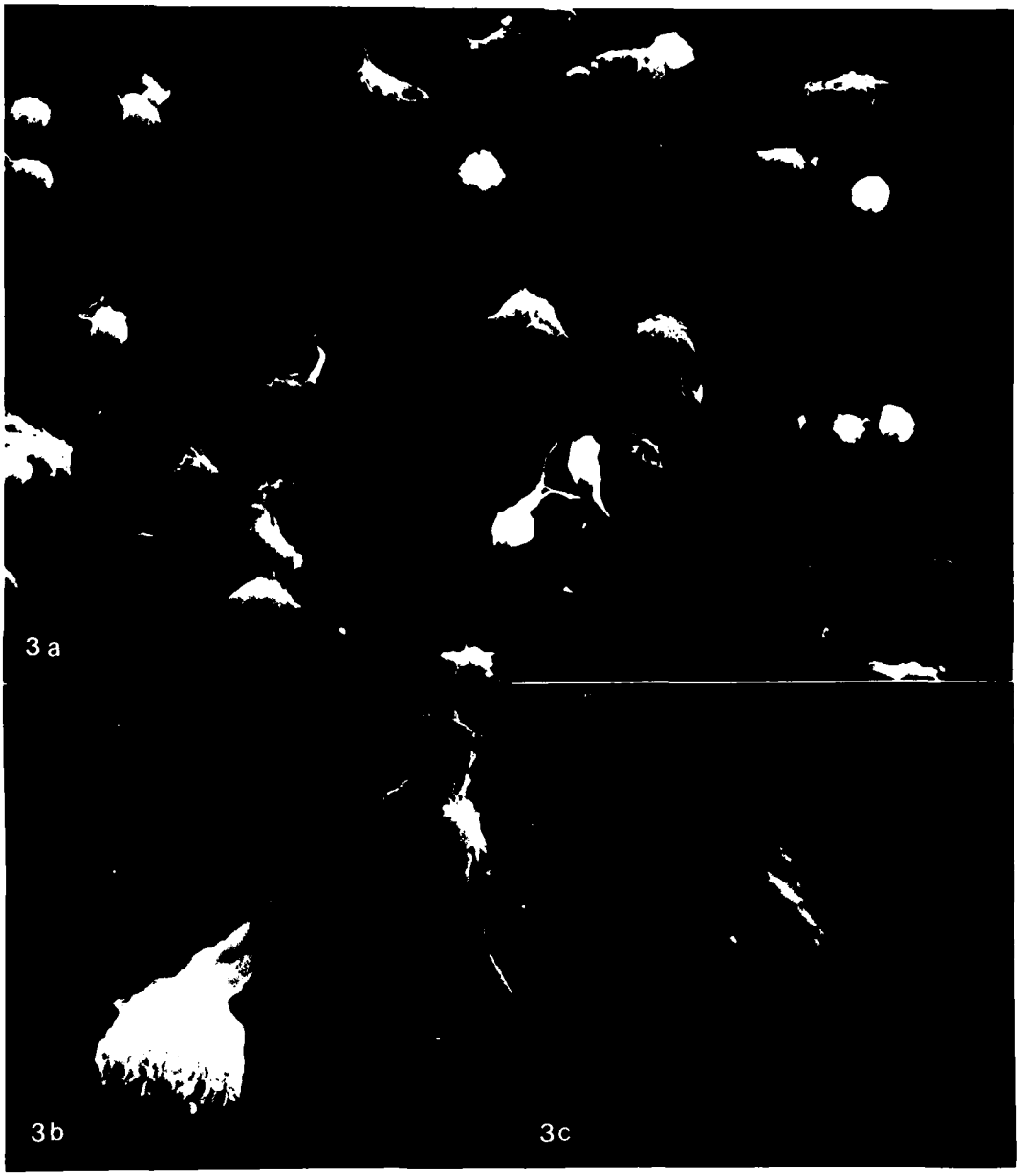


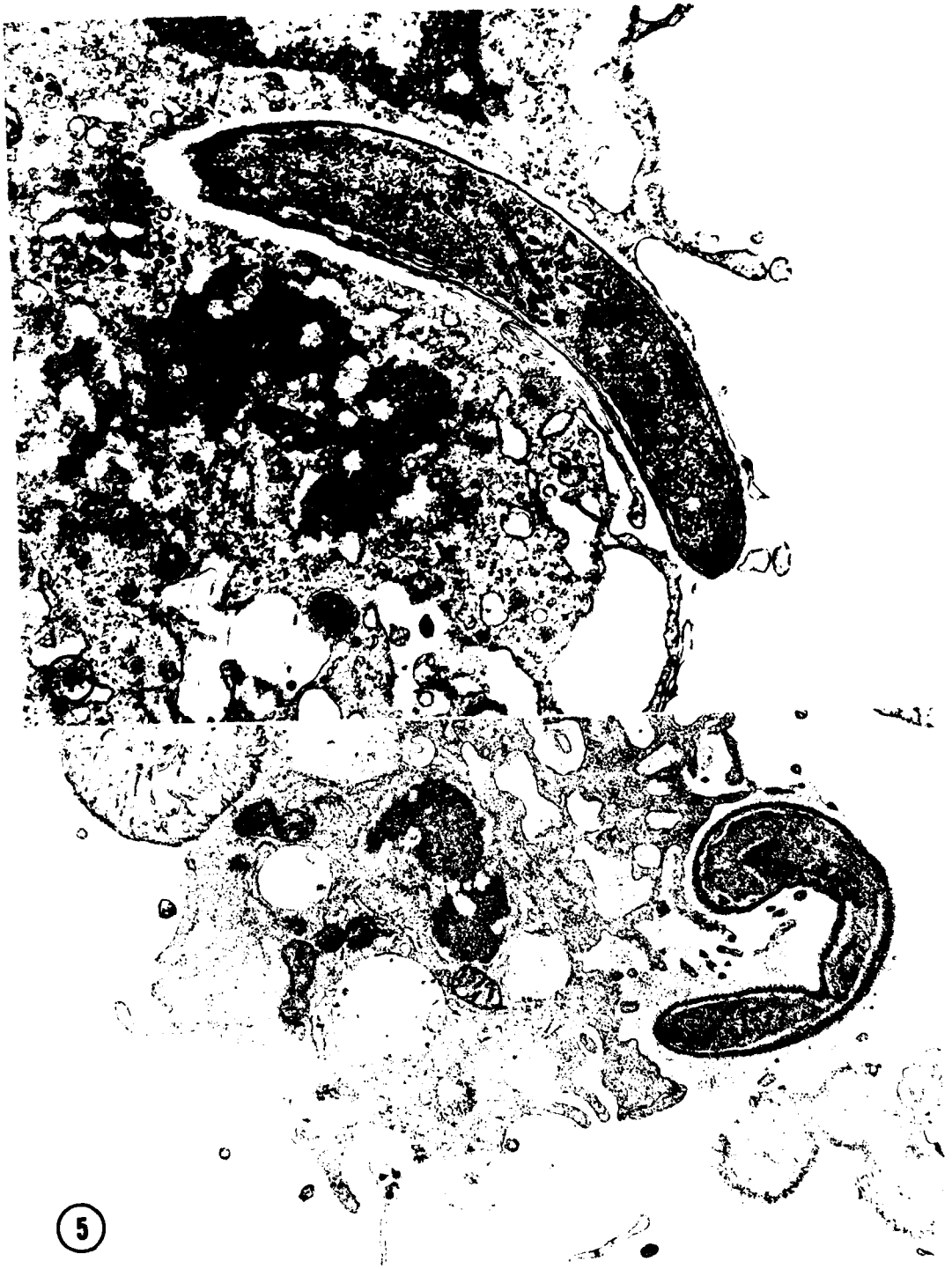
1c

Fig 1.

FIG. 2


% DEGENERATING MØ
50
40
30
20
10
0
10
30
60
INCUBATION of MØ with SPOROZOITES (MINUTES)


3a
3b
3c



(5)

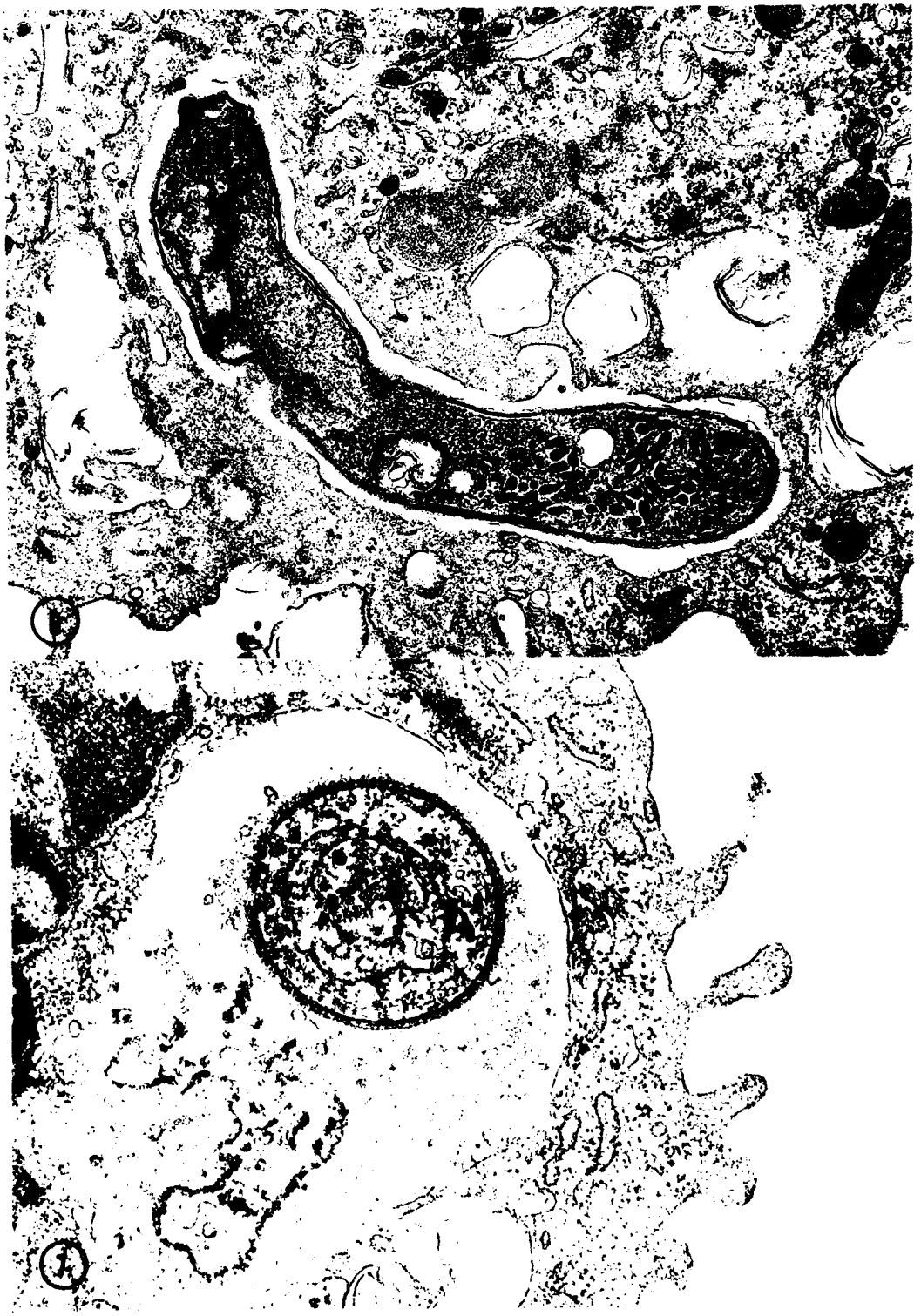


FIG. 8

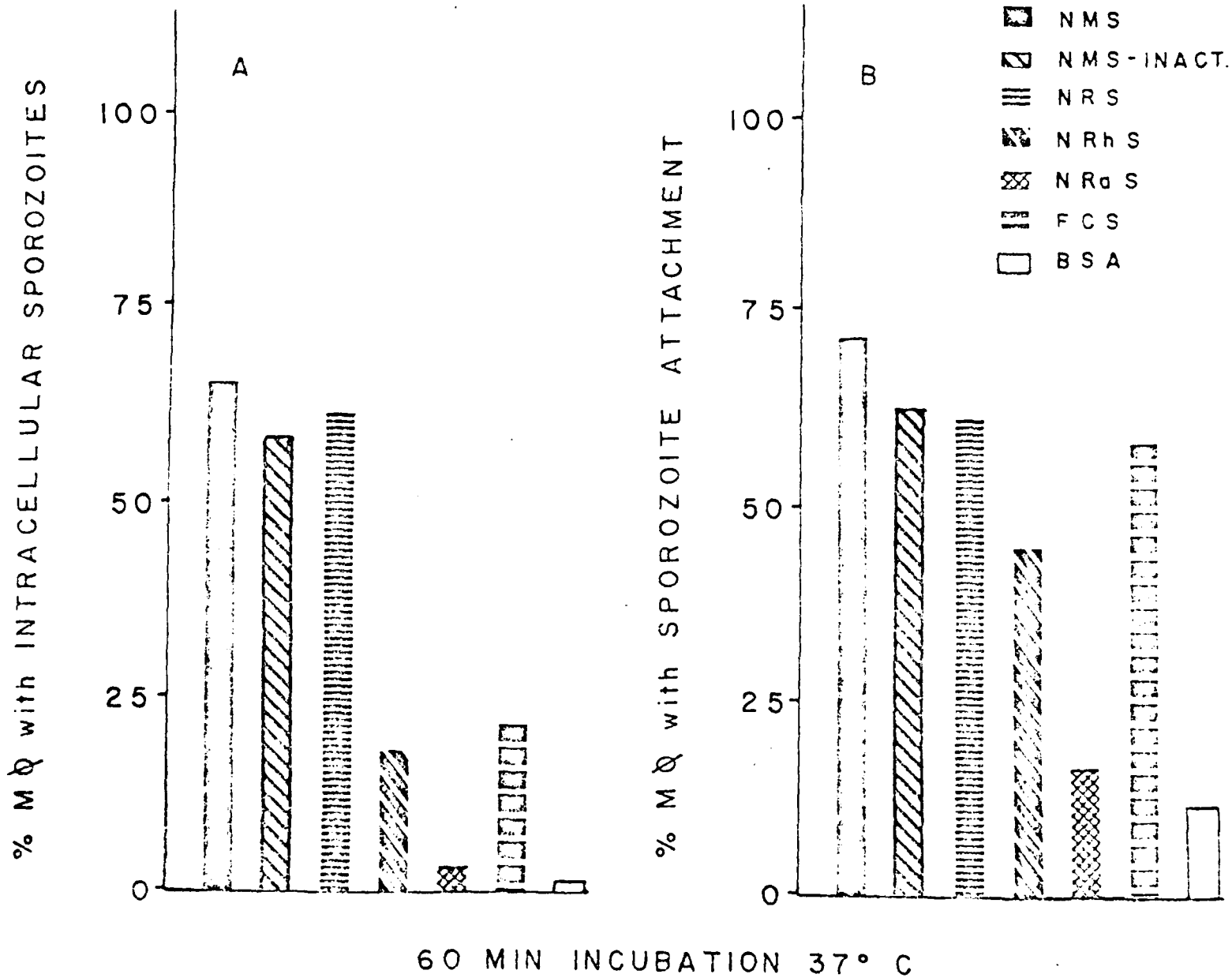
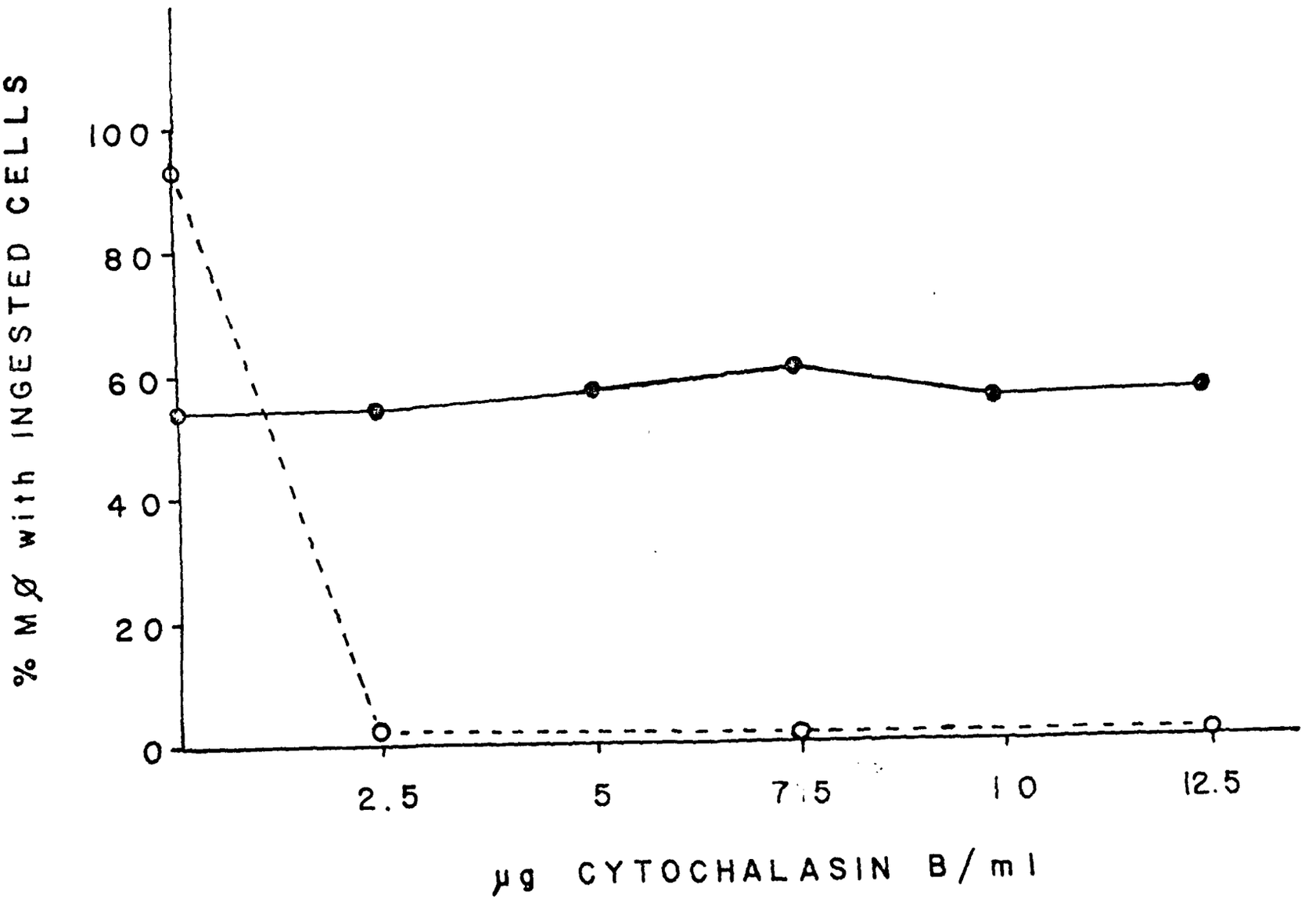

A
B
% MΦ with INTRACELLULAR SPOROZOITES
% MΦ with SPOROZOITE ATTACHMENT
100
75
50
25
0
NMS
NMS-INACT.
NRS
NRhS
NRaS
FCS
BSA
60 MIN INCUBATION 37° C

FIG. 9


% MOUSE MΦ with INTRACELLULAR SPOROZOITES
100
75
50
25
0
A
NMS
NRhS
P. berghei
P. knowlesi
% RHESUS MΦ with INTRACELLULAR SPOROZOITES
100
75
50
25
0
B
P. knowlesi


% MØ with INGESTED CELLS
100
80
60
40
20
0
2.5
5
7.5
10
12.5
µg CYTOCHALASIN B/ml

# Pathology of Malaria

Masamichi Aikawa, Mamoru Suzuki,
and Yezid Gutierrez

Institute of Pathology
Case Western Reserve University
Cleveland, Ohio 44106 U.S.A.

and

Department of Parasitology, School of
Medicine, Gunma University,
Maebashi, JAPAN

Acknowledgements:

This work was supported in part by Research Grant (AI-10645 and AI-13366) from the U.S. Public Health Service, the U.S. Army R & D. Command (DAMD17-79C-9029) and the WHO M2/181/145.

The authors wish to thank Drs. D. Connor, H. Jervis, D. MacCallum and B. Schnitzer for supplying micrographs and Dr. V. Boonpucknavig for supplying autopsy material used in this chapter.

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# I. INTRODUCTION

Even before the discovery of the causative agent, Plasmodium, the presence of a characteristic brownish pigment in the spleen, liver and brain was noted by most pathologists who had conducted necropsies on people who died of malaria. In 1847, Meckel pointed out that the brownish condition of the organs was dependent upon the accumulation of pigment removed from the blood, and later, Frerichs and Virchow confirmed this observation. This finding was of importance because it was the starting point of Laveran's research leading to the discovery of the plasmodial parasites.

There have been many reviews of malaria pathology in the past such as those by Marchiafava and Bignami (1900), Thayer (1900), Mannaberg (1905), Craig (1909), Marchoux (1926), Taliaferro and Mulligan (1937), and Maegraith (1966). These reviews contain detailed gross and microscopic descriptions of the changes produced by malarial parasites in man. In recent years, new knowledge has accumulated in many fields of Medicine because of the use of modern techniques such as electron microscopy and immunofluorescence microscopy. This new knowledge has increased the understanding of many disease processes including some aspects of malaria. Moreover, the successful infection of non-human primates by human malarial parasites has recently been accomplished allowing studies of morphological or immunological changes which may occur at any stage of human infection.

Pathological changes in malaria result primarily from the infection of erythrocytes by Plasmodium and from the host's response. To understand these changes three factors contributing to the development of the pathological lesions in various organs must be considered. They are: 1) parasitemia, 2) destruction of damaged erythrocytes and, 3) defense response of the host against the infection, including phagocytosis and the development of immunity. Malarial parasites invading erythrocytes initiate the pathological process and the consequences of this infection influence other host's tissues and organs. Destruction of host red blood cells occur not only when plasmodia rupture the erythrocytes at the end of schizogony, but also through the phagocytosis of infected and non-infected erythrocytes. The destruction of erythrocytes can result in some degree of anemia and contribute to anoxia. However, profound tissue anoxia resulting in shock and death occurs in infections such as P. falciparum due to sequestration of infected erythrocytes in the capillary bed.

The mechanism of host's defense against plasmodial parasites is not well understood, but there is a natural resistance to infection in endemic populations which can exhibit high parasitemias without apparent ill effects. The development of immunity affects both parasites and host tissues. The parasites are killed more rapidly and in the host there is hyperplasia of the reticulo-endothelial system with stimulation of its phagocytic properties especially in the spleen, liver and bone marrow. The difference between pathological and physiological changes, such as those associated with the

immune responses are difficult to recognize. Conventionally, the morphological changes occurring during the malarial infection have been described by pathologists, but attempts to correlate these changes with modern pathophysiological concepts of disease have not been made. In this chapter the prominent organ changes associated with Plasmodium infection will be described and some of the physiopathological mechanisms involved will be presented.

## II. Spleen Pathology

The spleen is the organ which shows the earliest changes in malaria infections. Spleen enlargement is a well-known physical sign of infection (Fig. 1) and its increase rate in human populations was used for the evaluation of malaria prevalence within a region (Boyd, 1949). Changes in the spleen size in experimental non-human primate malaria were evaluated by Coggeshall (1937) who demonstrated that M. rhesus infected with different doses of P. knowlesi and dying from three to seven days after infection, showed an average 57% organ size increase. If infections were to last longer because of treatment, the increase was 91%. In P. inui, a low-grade pathogen monkey malaria, the chronic infection in the rhesus produced an average spleen size increase of 171%.

Recently, Jervis et al., (1972) working with the P. falciparum-Aotus model found the spleen to be larger and heavier than those of control groups. Animals killed two weeks after the infection had larger spleens than the monkeys killed earlier, but there was no correlation between the infection time and the spleen size. Variations were interpreted as being the result

of several factors s[illegible]s cellular responses associated with acquired immunity, degree of [illegible]ular destruction and hyperplasia.

Post-mortem [illegible]amination of the spleen in acute malaria deaths reveals a dark red to chocolate color due to congestion and accumulation of malarial pigment (Fig. 2). The pulp's consistency is soft, friable and easily ruptured. Microscopically, the white and red pulps show hyperplasia and hyperemia (Fig. 3). The sinusoids are dilated with numerous red blood cells, many of which have parasites in different stages of development. White pulp hyperplasia is due to proliferation of endothelial cells, macrophages, and lymphoid elements which show many mitoses and large immature cells. Neutrophil infiltration is abundant especially in areas of necrosis (Taliaferro and Mulligan, 1937). The hyperemia is severe and it occurs in animals with high parasitemias during the first week of infection. Pigment is seen in macrophages, polymorphonuclear leukocytes and parasitized red blood cells and its presence is the histopathological whole mark of the infection (Fig. 4).

Following the acute changes there is a decrease of hyperemia and as the infection becomes established the white pulp may be markedly depleted in the chronic phase. Proliferative or hyperplastic changes take place in animals with malaria, although degenerative changes have been more commonly described. In chronic malaria the color of the organ becomes darker in proportion with the duration of the infection due to increase of pigment in the tissue. The spleen becomes firm because hyperemia is less evident; the fibrous tissue of the trabeculae, follicles

and capsule are increased and the reticulo-endothelial system shows marked proliferation. In long-lasting infections, these changes are more pronounced with diminution of the follicles and the pulp loses its character. Proliferative changes are particularly prominent in man, rodents and some non-human primates, but in Aotus experimentally infected with P. falciparum, the reticulo-endothelial system has only moderate changes (Jervis et al., 1972). The spleen neutrophil infiltration decrease and those present are diffusely scattered throughout the spleen.

The degree of accumulation of malarial pigment in the spleen phagocytic cells is directly proportional to both parasitemia and the duration of the infection. The phagocytized pigment can be found in the monocyte-macrophage series, neutrophils, and parasitized red blood cells, with pigment particles varying considerably in size and shape. The pigment occurs in small discrete masses during the early acute stages, while in animals with long-standing infections it is seen in clumps and large masses. If the infection is cured, the pigment will be processed by the host and it will disappear ultimately, but it can be seen for at least one year. The malarial pigment, the altered and parasitized red blood cells, and the parasites, stimulate the reticulo-endothelial system throughout the body with resultant marked hyperplasia and increased activity which in turn speeds up the rate of erythrocyte destruction by the spleen, leading to profound hemolysis. It is well-known that after a splenectomy of animals with experimentally induced malaria there is an increase in the peripheral blood parasitemia.

Hemorrhagic areas in the splenic pulp have been attributed to partial or complete circulatory obstruction by thrombosis. The thrombosis is observed in the arterioles and capillaries of the spleen and can be due to both the destruction of the erythrocytes and sequestration of infected red blood cells in P. falciparum infections. These thrombi cause decreased pressure with hemorrhage, necroses, and infarctions. Schnitzer et al. (1972) reported that in the spleen of Macaca-mulatta infected with P. knowlesi there was electron microscopic evidence of pitting of the portion of erythrocytes containing malarial parasites (Fig. 5). Cordal macrophages "pit" the parasite from the infected red blood cell and this phenomena may explain the presence of non-parasitized spherocytes in peripheral blood as well as the discrepancy between the degree of hemolysis and the number of parasitized erythrocytes. Quinn and Wyler (1979) studied the clearance of $^{51}$Cr-labeled P. berghei-infected erythrocytes in rats, in order to study the removal of parasitized erythrocytes by the spleen. Infected erythrocytes were removed more rapidly from the circulation than uninfected erythrocytes. The accelerated clearance appeared to result from greater splenic uptake in immune rats and correlate with spleen size. They suggested that rheologic alterations of parasitized erythrocytes might be an important determinant of clearance than antibody-dependent process.

The anemia seen in malarial infections of man and animals can sometimes be profound and extramedullary erythropoiesis is often a response seen in the spleen. Singer (1954) found that after the 7th day of P. berghei infection the chief activity of mice spleen was erythropoiesis.

The tropical splenomegaly syndrome (Pitney, 1968) is, perhaps, the most important consideration today. It occurs when malaria is endemic and several lines of work have suggested a causal relationship between the malarial infection and the syndrome. Its incidence has decreased in those endemic areas where massive chemoprophylaxis has been carried out. Moreover, it is suggested that in Uganda, 45% of patients with tropical splenomegaly syndrome have infection with P. malariae, a species capable of producing long-lasting infections in man (Marsden et al., 1965). However, similar studies in New Guinea failed to reveal such association (Marsden et al., 1967).

III. Liver Pathology

Hepatomegaly is a common sign of malaria infection in man, though variable in experimental animals (Fig. 1 ). Jervis et al. (1972) found that in the P. falciparum-Aotus model the wet and dry weights of the liver were not significantly altered. However, the wet weight in experimental animals with P. berghei remained unchanged, while the dry weight decreased, indicating that the increase is due to edema (Jervis et al., 1968). P. cathemerium also produces a marked liver enlargement in canaries especially at the crisis and shortly thereafter (Taliaferro and Mulligan, 1937). Another significant liver abnormality is a progressive change in color from pink tan to dark brown. When the infection is prolonged, it becomes almost black and this color is attributed to deposition of the malarial pigment in the reticulo-endothelial cells. In individuals with acute malaria, the organ becomes extremely friable and easily torn. In

chronic infections its consistency is definitely increased, the color is dark and the hepatic lobules are accentuated by the presence of pigment concentrated in large clumps in the portal areas (Fig. 6).

Light microscopy shows changes mainly involving the reticulo-endothelial system. During the early infection, the perilobular Kupffer cells start to hypertrophy and show active phagocytosis of pigment and infected erythrocytes (Figs. 7 & 8). As the infection progresses, mid-zonal and central lobular Kupffer cells hypertrophy. This distribution has been correlated in a man with the blood flow. MacCallum (1969a) reported that the order of response of intra-hepatic macrophages in guinea pigs infected with P. berghei is also the function of their spatial relationship to the portal blood flow. However, Maegraith (1954) working with the same parasite in rats reported an initial midzonal activation of Kupffer cells. It is generally accepted that the endothelial cells lining the liver sinusoids are also phagocytic in nature, and that they can transform into Kupffer cells (Aikawa et al., 1968). These transformed endothelial cells divide rapidly to increase the number of macrophages. Moreover, the macrophages containing pigment enter the sinusoids with the portal blood especially due to influx of splenic macrophages via the splenic vein. All of these factors increase the numbers of phagocytic cells in the liver and their distribution within the lobule.

The sinusoidal spaces also contain parasitized red blood cells varying in amount in accordance with the parasite species. In P. falciparum of man and experimental monkeys (Jervis et al., 1972; Gutierrez

et al., 1976), large numbers of parasitized red cells are present, "attached" to the endothelial cell. The same phenomena is true of P. knowlesi of non-human primates. The increased number of cells in the sinusoids account for the sluggish circulation in the organ with resultant congestion and central necrosis due to portal hypertension. Added to this slow blood flow there are other factors which could affect the circulation through the liver. Skirrow et al. (1964) found that terminal P. knowlesi malaria in monkeys leads to marked constriction of the portal vein and its branches. This constriction is relieved by sympatholytic drugs, and liver necrosis can be definitely prevented, if sympathectomy is performed before infection (Ray and Sharma, 1958). Finally, ultrastructural changes have been described in the sinusoidal endothelial cell lining in the P. falciparum-Aotus model and these changes may alter permeability as well as circulation (Gutierrez, et al., 1976).

In the acute phase, the malarial pigment is dispersed as small globules and diving macrophages are frequently observed. Later in the infection, the amount of ingested malarial pigment is increased, in clumps, and phagocytes become rounded instead of elongated or stellate and are lying free within the sinusoids. The nuclei of the phagocytes becomes irregular and prominent nucleoli develop. As the disease progresses, the amount of pigment within the phagocytic Kupffer cells increases and clumps into even larger masses (Taliaferro and Mulligan, 1937).

Malarial pigment is the end product of red blood cell hemoglobin digested by the parasite. The pigment is a ferric ion containing porphyrin conjugated with a protein moiety derived from the partial proteolysis of

the globin portion of hemoglobin. The amino acid composition of this moiety varies in different batches of pigment. Although earlier investigators thought that pigment was toxic to the host and could produce cell changes, it is now regarded as inert. Clumping of malarial pigment can be produced by treatment with various drugs including sulfadiazine, pyrimethamine, chloroquine and piperazine. Pigment laden macrophages aggregate within the sinusoids and eventually the macrophage cell membranes fuse resulting in the formation of giant cells.

There is another pigment in malaria infection, hemosiderin due to red blood cell lysis. This is a yellow pigment seen in many other conditions where red blood cell destruction takes place. It is found in small granules in the central portion of the lobule and dimishing towards the periphery, contained both in macrophages and hepatocytes. It is differentiated from malarial pigment because it does not give the Prussian blue reaction.

Electron microscopy has shown that the Kupffer cells are vacuolated and contain cytoplasmic electron dense bodies, large lysosomes with infected erythrocytes (Figs. 9 & 10) and malarial pigment particles (Aikawa and Antonovych, 1964). The pigment shows no apparent digestion but ingested parasitized red blood cells and acid phosphatase activity has been demonstrated in the phagolysosome (Aikawa et al., 1968). The ultrastructural appearance of the pigment differs with the species. In mammals, it has a characteristic rectangular crystaloid shape, while in avian and reptilian malariae it appears as a uniformly electron dense material (Aikawa, 1971).

Electron microscopic studies of the P. falciparum-Aotus model show a direct relationship between the number of parasitized erythrocytes and liver changes. In heavily infected animals, there are structural alterations and marked diminuation of hepatocyte mitochondria. The mitochondria become swollen, the cristae disappear, and its matrix is replaced by an amorphous electron dense granular material (Fig. 9) (Gutierrez et al., 1976). Liver mitochondria of mice infected with P. berghei have been correlated with biochemical changes such as abnormal respiration and oxidative-phosphorylation (Riley and Deegan, 1960; Riley and Maegraith, 1962). However, mitochondria from monkeys infected with P. knowlesi appear biochemically normal (Maegraith et al., 1962), suggesting that rodent and monkey malaria have different pathophysiologic mechanisms. Biochemical alterations of mitochondria are difficult to explain pathophysiologically. Animals with P. knowlesi and P. berghei infections have in their sera substances capable of producing biochemical disturbances on liver function in vitro (Riley and Maegraith, 1961; Maegraith et al., 1963). Both the ultrastructural and the biochemical changes of the liver mitochondria in experimental malaria are thought to be initiated by the parasite's erythrocytic phase directly or through mediators released into the animals' serum (Maegraith, 1966). Tissue anoxia produced either by an inadequate oxygen supply or by cell inability to use oxygen, is the principal cause of shock and eventual death in animals and man with malaria. The mitochondrial ultrastructural changes are non-specific response to various insults resulting in shock

and eventually in death; however, it is not known which is the mechanism that initiates these changes in animals with experimental malaria.

The hepatocyte shows changes in the malaria infections, also. The amount of fat augments and as the infection progresses, there is a slight increase in the number of iron containing granules, and glycogen loss. Bile canaliculi of monkey livers infected with P. falciparum show increase of alkaline phosphatase activity but this increase is non-specific since it is observed in several animal species subjected to various types of stress. Ultrastructurally, the hepatocytes in infected Aotus and man with P. falciparum are swollen with loss of microvilli both in the space of Disse and in the bile canaliculi. Cytoplasmic glycogen is first depleted and disappears later beginning in the central portion of the lobule, but the significance of these changes is not well understood (Jervis et al., 1968). In human infections Brito et al., (1969) speculated that alterations of bile canaliculi microvilli could be responsible for the hyperbilirubinaemic state of some of his patients. Interestingly, in the six cases reported by Brito et al., (1969) one had P. falciparum for 13 days and the rest had P. vivax from days to months. Yet, the ultrastructural hepatocyte lesions were very similar. Bhamarapravati et al. (1973) attributed jaundice in malaria patients to impairment in bilirubin transport either because of reticulo-endothelial cell blockage or disturbance of the hepatocyte microvilli.

Finally, the liver shows cellular infiltration by lymphocytes and granulocytes of the portal triads (Fig. 11). Small nests of erythropoietic, granulocytopoetic and megakaryopoetic cells can be seen within the liver

sinusoids when there is severe anemia.

IV. Heart and Vascular Pathology

There are no striking gross changes in the heart of patients dying of malaria. Microscopically (Fig. 12) there is fatty degeneration, focal fragmentation and necrosis of the cardiac muscle due to capillary blockage by parasitized erythrocytes especially in P. falciparum infection.

Ultrastructural changes of the heart have been studied recently in the experimental P. falciparum-Aotus model (Fig. 13). There is lipid droplet infiltration of muscle cells usually next to the mitochondria. Mitochondria are swollen with loss of cristae detail and the cardiac muscle is disorganized with fragmentation of the sarcomeres and A and I bands, around the intercalated disc (Gutierrez et al., 1976). These ultrastructural changes are non-specific because similar morphology has been described in animals dying of a variety of conditions such as hemorrhagic shock (Martin et al., 1964; Hiott, 1969). However, Aotus dying of hemorrhagic shock have other cardiac lesions such as marked myofibrillar derangement with widening and fragmentation of muscle Z bands.

The vascular pathology associated with malaria consists mainly of capillary occlusions due to masses of agglutinated infected erythrocytes (Fig. 14). These capillary occlusions cause hemorrhage and necrosis in the perivascular areas of the brain, myocardium, intestinal mucosa, skin (seen as purpura), and other organs especially in patients and animals dying of P. falciparum (Spitz, 1946).

There may be several causes for erythrocyte clumping in the small

arterioles and capillaries. Erythrocytes infected with P. falciparum have membrane changes described as excrescences (Fig. 15). These excrescences play an important role in the "attachment" of infected red blood cells on the vascular endothelium (Fig. 16) resulting in the occurrence of the parasite's schizogony deep in the vasculature (Luse and Miller, 1971; Aikawa et al., 1972). On the other hand, similar excrescences have been described in erythrocytes infected with both asexual forms and gametocytes of P. coatneyi, P. malariae, and P. brasilianum, but they must have a different function because they do not play an apparent role in deep vascular schizogony; however, they may contribute to erythrocyte agglutination (Aikawa et al., 1975). The manner in which infected erythrocytes join together or to the endothelial membrane is not known. Kilejian et al. (1977) has demonstrated the presence of malarial parasite antigens in the excrescences. When erythrocytes infected with P. falciparum are incubated with homologous antibody a prominent coat is formed on the excrescence surface (Fig. 17) supporting the idea that erythro-agglutination is due to an antigen-antibody reaction (Chulay et al., 1979). Other causes contributing to agglutination appear to be the escape of abnormal amounts of protein and fluid through the endothelial cell membrane which is rendered abnormally permeable by multiple factors of which anoxia may be the most important one.

The end result of erythrocyte agglutination and especially of attachment to the endothelial wall is the formation of thrombi in the capillaries as described in the classical literature. Similarly P. berghei 17x strain in rodents have been found to be capable of crossing the blood-brain

barrier, cause intravascular sequestration and produce multiple thrombi composed of infected erythrocytes and pigment (Yoeli and Hargreaves, 1974). The important consequence of thrombi formation in the host is vascular occlusion with resultant hemorrhage and tissue necrosis. Local tissue anoxia, plus anoxia associated with anemia due to erythrocyte destruction, if widespread, could result in shock.

There are other hemodynamic changes which can follow the above events. Animals infected with P. falciparum show terminally a typical syndrome of disseminated intravascular coagulation (DIC) with the characteristic thrombocytopenia, decrease of blood coagulation factors and increase of fibrin degradation products (Wellde et al., 1972). The occurrence of hemorrhages and thrombosis in patients with malaria suggests that DIC plays an important role in the pathophysiology of death in this infection. Jervis et al. (1972) rarely observed thrombi in autopsies performed in the P. falciparum-Aotus model, but fibrin strands were seen commonly in dilated capillaries and veins, suggesting that fibrinolysis was taking place. These findings are consistent with DIC syndrome with other etiologies in animals.

Goodwin and Richards (1960) detected pharmacologically active peptides in the blood and urine of mice infected with P. berghei. One of these peptides, bradykinin, present in rhesus monkeys sera with P. inui and P. coatneyi infections increases vascular permeability. Desowitz and Pavanand (1967) working with monkeys infected with P. coatneyi suggested that increased vascular permeability may be induced by antigen-antibody

complexes with a rapid decrease in plasma volume, shock, and changes in the liver, such as centrilobular necrosis. Similarly, Maegraith and Onabanjo (1970) demonstrated an increase in blood histamine of monkeys infected with P. knowlesi which they believe contributes to increased vascular permeability and capillary damage.

V. Hematopathology

Malarial parasites produce morphological changes in circulating infected erythrocytes, some of which are helpful in the specific diagnosis (Kreier et al., 1972). P. vivax enlarges parasitized red blood cells greatly, and changes their shape to angular when it presses neighboring erythrocytes. In P. falciparum, parasitized cells tend to be smaller than normal and there is some degree of crenation of infected and non-infected erythrocytes. In P. ovale a moderate enlargement with a fimbriated end especially in quickly dried blood films is common, and P. malariae produces no changes in parasitized red blood cells. The morphological red blood cell alterations seen with malaria parasites can be somewhat modified in chronic infections, when there is some degree of anemia (Field and Shute, 1956).

Malarial parasites are also known for having preferences for certain red blood cell types. P. falciparum invades both young and old erythrocytes; P. vivax, the young ones; and P. malariae, old erythrocytes. A similar phenomenon is seen in P. berghei which is considered to be reticulotropic. The availability of these cells in peripheral blood is considered

as a limiting factor in the infection. In infected rodents reticulocytosis follows peak parasitemia, but in human malaria it is observed as a response to the anemia produced by the parasite.

Changes in the number of circulating red blood cells in malaria can be followed by hematocrit and erythrocyte counts (Wellde et al., 1971a; 1971b; 1972). The anemia in animals with natural or experimental malaria appears to be due to red blood cell destruction by several mechanisms: 1) erythrocyte rupture at the end of the parasite's erythrocytic phase; 2) increased erythrophagocytosis of parasitized and altered red blood cells; (Fig.18) and 3)direct hemolysis due to an immune reaction against red blood cell membrane. In relation to the third postulate, the presence of antigen on the membrane of infected erythrocytes has been described in P. falciparum (Kilejian et al., 1977) and P. vivax (Aikawa et al., 1975). Other autoimmune hemolytic mechanisms may also be operating, but they are not fully understood at present.

P. brasilianum experimental infections have been studied (Taliaferro and Kluver, 1940) from the hematological point of view. A mild anemia is observed especially after the crisis at the end of fatal infections, or in the chronic phase. Changes in erythrocytes consisting of polychromatophilia and anisocytosis were also observed as well as reticulocytosis. Similarly, in P. coatneyi infections, Desowitz et al. (1967) noted anemia, bone marrow erythroid hyperplasia, and increased normoblasts, and reticulocytes in the peripheral blood. The hemogram returned to normal as soon as the animals enter the chronic phase of the disease.

Schnitzer et al. (1973) demonstrated pitting of infected erythrocytes in the spleen resulting in production of small spherocytes which are more susceptible to hemolysis. Increased osmotic fragility has also been demonstrated in infected red blood cells. Red blood cell lysis has been studied in P. knowlesi experimental monkey infections where the acute infection usually produces two types of lytic patterns. One is manifested by profound hemolysis following schizogony, with a marked drop in red blood cell mass, hemoglobinuria and death. The second pattern belongs to those infections with a moderate decrease of red blood cell count at the end of schizogony without hemoglobinuria (Devakul and Maegraith, 1959). The red blood cells in animals belonging to the first group are more susceptible to lysis. The explanation of this phenomenon is not clear, but it is attributed to changes brought about by the parasite (Devakul and Maegraith, 1959). Similar studies (Seed et al., 1976) have shown that erythrocytes from P. berghei infected rodents have an increased osmotic fragility.

In P. gallinaceum infections the erythrocytes have shown a lysis propensity even during the prepatent period, returning to normal when the animal is placed under therapy and the parasites clear from the circulation (Swann and Kreier, 1973).

There are other phenomena produced by the red blood cell-parasite association. Under P. falciparum endemic conditions, there is an apparent protection to infection in people with sickle cell disease (Allison, 1954). Under laboratory conditions such protection has been documented with

the use of P. falciparum in vitro cultures in sickle cell hemoglobin (HbS), homozygous (SS) and heterozygous (SA). With the electron microscopy, parasites in SS cells appeared disrupted by deoxy-HbS aggregates in needle-like form (Friedman, 1979). It is speculated that these ultrastructural alterations detected in vitro are the basis for the HbS gene carrier resistance (Friedman, 1979). Recently Miller et al. (1975) reported that initial recognition and attachment between P. knowlesi merozoites and erythrocytes probably involves specific determinants associated with Duffy blood group related antigens. Duffy-negative erythrocytes were refractory to invasion by P. knowlesi and P. vivax (Miller et al., 1976). Electron microscopic study (Miller et al., 1979) demonstrated that there was no junction formation between Duffy-negative erythrocytes and P. knowlesi merozoites (see chapter "Morphology of Plasmodia").

Of some importance is the stippling found in malaria infected red blood cells. This stippling has different characteristics varying with the species and is sometimes useful for the diagnosis. For example, in the vivax- and ovale-type malariae, the red blood cell stippling is referred to as Schuffner's dots; in the falciparum-type usually as Maurer's clefts and in the malariae-type as Ziemann's stippling. The electron microscopy of these structures and its relationship to the excrescences, caveolae, and caveola-vesicle complex (Aikawa et al., 1975) has been described in the chapter "Morphology of Plasmodia" in this book.

The effects of malaria infections on the other blood cell elements have been studied also. A leukocytosis has been found in P. berghei infections, mainly due to an increase of peripheral monocytes (Singer,

1954). Wellde et al. (1971a; 1971b; 1972) in the P. falciparum-Aotus model found leukopenia at the beginning with a moderate to marked leukocytosis, if the infection lasted longer than 12 days. This increase was always accompanied by a rise in mononuclear cells. Monocytosis seen in malaria infections correlates with the stimulation of the reticulo-endothelial system to handle malarial pigment and the accelerated destruction of infected and non-infected red blood cells. In P. brasilianum infections Taliaferro and Kluver (1940) found that granulocytes decreased a few days after the malarial crisis and monocytes increased slightly to 3-8% one to two weeks after the infection became patent. When the infection is prolonged and intense, the monocytes will maintain this level, but will return to normal if it is a chronic one with low parasitemia. In the same model, lymphocytes vary erratically in the acute phase, but could reach 50% during the chronic phase; eosinophils and basophils decrease gradually and almost disappear in chronic infections. Desowitz et al. (1967) found persistent leukocytosis beginning during the acute phase and continuing for a 5 month period in P. coatneyi. A close relationship between malaria infection and Burkitt's lymphoma has been suggested on the basis of sero-epidemiological data. Nkrumah et al. (1979) reported a link between P. falciparum infection and Burkitt's lymphoma in African children by studying immunoglobulin levels.

There are a few studies of platelets during malaria infections. In the patent period there is thrombocytopenia in all animals with P. falciparum-Aotus model following an inverse relationship with the parasitemia(Voller et al., 1969). This thrombocytopenia has been studied in conjunction with other coagulation factors. At least in one experimental animal it has been found that cephaline time was prolonged and the factor VIII reduced, with no change in fibrinogen or factor V (Voller et al., 1969). The thrombo-

cytopenia, cephaline time, and red[illegible]n of factor VIII, have been interpreted as consistent with disseminated intravascular coagulation. The normal fibrinogen values in the animals have been explained due to unusual high levels of antithrombin present in the Aotus species (Voller et al., 1969).

Dennis et al. (1966a; 1966b; 1977) found that in humans dying of acute falciparum there is thrombocytopenia, a prolonged prothrombin time, a decrease of multiple coagulation factors, decrease of plasminogen and increase of fibrinogen degradation products. Devakul et al. (1966) have also demonstrated a precipitous decrease with the use of $^{131}$I-labeled fibrinogen in patients with acute P. falciparum infections. Both groups of investigators have concluded that in man disseminated intravascular coagulation is present.

Serum chemistry in malarial infections has been studied on a limited basis. Blood urea nitrogen levels are increased after the 4th day of the infection in P. berghei, probably due to kidney changes (Sadun et al., 1965). The same authors also found an increase in SGPT and SGOT as early as 2 days after infection, probably related to erythrocyte destruction and liver damage.

Lower glucose levels have been seen in heavily infected animals starting with the 4th day of infection returning to normal two days later. However, both in man and animals glucose levels in malaria infections seem to depend on the stage of the disease and the severity of the infection. In P. vivax and P. falciparum a rise in glucose levels has been observed especially during the parxysm due to the increased metabolism during the fever or to hepatic cell damage. In P. knowlesi infection in monkeys,

hypoglycemia has been detected especially in animals with low liver storages (Fulton, 1939) or in terminal infections (Devakul and Maegraith, 1958). Similarly, hypoglycemia has been observed terminally in P. lophurae infections (Marvin and Rigdon, 1945). Davakul (1960) described a case of fatal P. falciparum in man with 7.4 mg% of glucose, but other patients did not show such drastic changes. The serum proteins are reduced in most malaria infections mainly due to a decrease in the albumin because of liver damage (Sadun et al., 1965).

## VI. Pulmonary Pathology

The main pathological changes occurring in the lungs during malaria infection include pulmonary edema, congestion, and the accumulation of pigment-laden macrophages in the capillaries (Figs. 19 and 20). Taliaferro and Mulligan (1937) found no specific changes in lungs of animals dying of acute malaria, but acute congestion and hemorrhagic infarcts have been described. Animals with chronic malaria have pale and anaemic lungs. MacCallum (1969b) reported large numbers of macrophages with abundant malarial pigment accumulated in the pulmonary vascular bed of hamsters with P. berghei as early as 6 days after the infection. These cells adhere to the capillary wall giving the appearance of total occlusion (Fig. 21), but on the 8th and 9th days of the infection, endothelial cells grew over to exclude them from the circulation.

MacCallum (1969b) also reported that hamsters with advanced stages of P. berghei infection have dilated lymphatic vessels with fibrin clots (Fig. 22) and that the alveolar spaces contained a protein-rich granular exudate (Fig. 24). Based on these findings he postulated that alveolar capillary congestion and filling of pulmonary veins by macrophages (Fig. 23) resulted in increased blood pressure altering fluid exchange across capillary membranes. He considers that pulmonary edema is caused by this blood pressure increase and by the obstruction and dilation of the lymphatic system. Godard et al. (1971) demonstrated, by x-ray examination of one patient with acute malaria, interstitial pulmonary edema without alveolar edema similar to that thought to occur in allergic hypersensitivity reactions. Suzuki (1974) studied ultrastructural lung changes in mice infected with P. yoelii and found many polymorphonuclear leukocytes present in the pulmonary capillary lumens and alveolar spaces (Figs. 25 & 26). In the later stages of infection, polymorphonuclear leukocytes were attached to the capillary basement membrane and cytoplasmic extensions of the endothelial cell were piled one upon the other. Singer (1954) working with P. berghei in mice, found also an increase in granulocytes and lymphocytes early in the infection. Macrophages are seen starting on the 5th day and phagocytosis is most active during the 8th day.

Patients with P. falciparum malaria especially in Southeast Asia can present with a syndrome of acute pulmonary insufficiency. Marks et al. (1977) studied a similar case in Rhodesia. The syndrome appears abruptly

in people with high parasitemia, usually associated with cerebral or renal complications but not heart failure. Hall (1976) suggested that impairment of the alveolar capillary microcirculation and excessive fluid therapy are the main causes for the acute pulmonary insufficiency in malaria. Grossly, the lungs of these patients show a marked increase in weight, congestion, and edema, as well as hemorrhagic consolidation (Stone et al., 1970). Microscopically, there is thickened alveolar septi (Fig. 27), diffuse alveolar edema, focal or widespread hyaline membrane formation, pulmonary congestion and focal intra-alveolar hemorrhages (Brooks et al., 1968; Stone et al., 1970; and Deaton, 1970; and Punyagupta, 1974). Sheehy et al. (1967) suggested that aggregated parasitized erythrocytes or microthrombi in the hypothalamus might cause release of anti-diuretic hormones and retention of fluid. Many investigators have noted that microcirculation dysfunction is followed by engorgement and accumulation of edema fluid and alveolar hyaline membrane formation.

Immunological reactions may be involved in the production of pulmonary injury in malaria. The pulmonary lesions in falciparum infection are often seen in individuals with immune-complex deposits in the kidneys. The special immunological relationship which exists between the basement membranes of lungs and kidneys is demonstrated by diseases such as Goodpasture's syndrome and provide a model for pulmonary lesions related to host immune reactions.

## VII. Thymus Pathology

Although T-lymphocyte functions and behavior have been extensively

studied, information on thymus histopathology is very limited. Al-Dabag (1966) studied birds infected with P. junstanucleare, P. gallinaceum, P. rouxi, P. cathemerium and P. elongatum and observed that during the patent period the thymus is highly active with a large number of mitotic figures present. In the chronic stage lymphocytes are depleted, and increased lymphorrhexis is present; he also observed that Hassel's corpuscles were plentiful in infected animals as compared with controls. Taliaferro and Taliaferro (1955) also observed lymphoid depletion in the thymuses of chickens chronically infected with P. gallinaceum.

Eling et al. (1977) studied the relationship between thymus and body weight during P. berghei infection in mice and found a weight increase during the first week, but in general the thymus-body weight ratio decreased as the infection progressed. Gravely et al. (1976) observed that the thymus of healthy young rats atrophied during P. berghei infection. Tanabe et al. (1977) examined the thymuses from Balb/c mice infected with P. berghei and also reported a decrease in the size and weight (Fig. 28). Histopathological examination showed a depletion of thymocytes and loss of distinction between the cortex and medulla (Fig. 29). P. chabaudi infections in mice result in thymus changes similar to those in P. berghei and they reverse to normal around the 94th day after recovering from the infection.

Several investigators have demonstrated that thymocytes may work adversely in malaria. Wright et al. (1971) implicated T-lymphocytes in the development of cerebral lesions in golden hamsters infected with P. berghei, because antithymocyte serum prevented cerebral hemorrhages.

Waki and Suzuki (1977) noted that athymic nude mice survived three times longer than did phenotypically normal mice (Fig. 30); this finding was confirmed with thymocyte replacement. Immunity to P. berghei blood forms is thymus-dependent and it is transferred by cells more efficiently than by serum.

VIII. Renal Pathology

a) P. malariae Associated Renal Pathology

Since Watson in 1905 noted the presence of edema in a patient with malaria in Malaysia, the relationship between P. malariae infection and nephrotic syndrome has been well documented. In 1940, Boyd observed albuminuria in 43 patients with quartan malaria, providing evidence for an etiological role between P. malariae and nephrotic syndrome. Gilles and Hendrickse (1960; 1963) also demonstrated a relationship between P. malariae and nephrotic syndrome in Nigerian patients. They found that in children the peak incidence of nephrotic syndrome and P. malariae infection coincides. Kibukamusoke (1973) observed a relationship between rainfall, mosquito density and frequency of hospital admissions for nephrotic syndrome at Lagos University Hospital in Uganda.

Histologically, there is a proliferative glomerulonephropathy with increased endothelial and mesangial cells. Kibukamusoke and Hutt (1967) classified these changes into five subgroups based on 77 biopsies from nephrotic patients in Uganda: 1) diffuse type with diffuse changes throughout the glomerular tufts; 2) lobular type with lesions in a lobular pattern; 3) focal type with abnormalities in less than 50% of the glomeruli; 4)

chronic type with glomerular changes plus capsular adhesions, secondary membrane thickening and sclerosis; and 5) minimal type with minor abnormalities in focal or sequential lesions. In 31 kidney biopsies from children, 28 corresponded to a type of proliferative glomerulonephropathy and the rest were only minimally changed. In 46 biopsies from adults, 27 had proliferative glomerulonephropathy and 11 corresponded to the diffuse type. Nine biopsies had diffuse uniform thickening of the basement membrane with no evidence of proliferative changes and the remaining 10 had other changes not related to malaria.

On the other hand, Gilles and Hendrickse (1963) described membranous glomerulonephritis in patients with quartan malaria and nephrotic syndrome. Biopsies revealed subendothelial basement membrane lesions characterized either by a double contoured or by a plexiform arrangement of PAS positive material and argyrophilic fibrils (Hendrickse et al., 1972; White, 1973; Edington and Gilles, 1976)(Fig. 31 and 32). In biopsies from patients with early lesions, the glomerular changes were found only in occasional capillary loops, but in the more advanced cases there was diffuse capillary wall thickening and almost absent mesangial cell proliferation. They proposed the name "quartan malaria nephropathy" for this condition because of its unique characteristics (Hendrickse et al., 1972).

Allison et al. (1969) reported moderate basement membrane thickening and circumscribed subepithelial electron-dense deposits in kidney biopsies from Nigerian children. They concluded that early stages of nephrotic syndrome have lesions similar to those of immune complex glomerulonephritis of experimental animals (Feldman, 1963). Dixon

(1966) and Allison et al. (1969) studied renal biopsies of Nigerian children with immunofluorescence and found irregular beaded deposits of host gamma and $B_1C$ globulins along the glomerular basement membrane suggesting deposition of antigen-antibody complexes in this site. Using a similar technique, Ward and Kibukamusoke (1969) found IgM, IgG, IgA, complement, fibrin, and malarial anitgens in the glomeruli of East African patients who developed renal disease and nephrotic syndrome following quartan malaria. They noted that IgM was the predominant immunoglobulin and that there were P. malariae antigens in the glomerular deposits in 3 of the 13 cases. Ward and Conran (1969) examined 44 renal biopsies from patients with nephrotic syndrome and found that IgM immunoglobulin in the glomerular deposits was most abundant.

There is little information available onthe tubular changes which occur in quartan malaria nephropathy, but their extent appears to reflect the degree of glomerular damage. The epithelial cells of the proximal convoluted tubules may contain hyaline droplets (Kibukamusoke and Hutt, 1967; White, 1973) and fatty vacuolation (Allen, 1962). Marked glomerular sclerosis, tubular atrophy, interstitial fibrosis, and, in severely affected kidneys, degenerative changes, including eosinophilic granular degeneration of the proximal tubular epithelium were present (Gilles and Hendrickse, 1963).

Allison et al. (1969) detected well-defined fluorescent deposits between the lumen and the nuclei of tubular cells after staining with an anti-complement conjugate. Hoube et al. (1971) reported that deposits of IgM and IgG were present in 17 out of 50 kidneys examined. One fifth of the kidneys

had complement deposits in the tubules and P. malariae antigen in 11 out of the 36. In serial biopsies of patients with malaria, IgG and IgM staining was consistently positive in the tubules. Only three patients showed tubular staining with the anti-complement conjugate initially, but all became positive as the disease progressed.

Morel-Maroger et al. (1975) studied renal biopsies in Senegal where the prevalence of quartan malaria is low, but nephrotic syndrome in children is common. Two distinct nephrotic syndrome types were seen. One was hypocomplementaemic with extra-membranous glomerulonephritis, termed "tropical extramembranous glomerulonephritis". The other showed features of progressive and segmental glomerulosclerosis similar to the Ivory Coast and Nigerian quartan malaria nephropathy and termed "tropical nephropathy". Morphologically the tropical extramembranous glomerulonephritis was characterized by proliferation of endothelial and mesangial cells with occasional subepithelial electron dense deposits. Immunofluorescence studies showed diffuse granular IgG deposits along the peripheral capillary loops. IgA, properdin, Clq and $C_4$ were also detected in these kidneys. The etiology of these two nephropathies is unknown, although malaria may be involved. Viral, bacterial and helminthic infection is prevalent among the Sengalese children and may also be involved in this unique nephropathy.

Soothil and Hendrickse (1967) collected random sera from children at the nephrosis clinic in Ibadan and found complement component $B_1C$ in a complex macromolecular form indicating that it can be incorporated into circulating soluble antigen-antibody complexes. Epidemiological

evidence has indicated that the nephrotic syndrome of Nigerian children is related to P. malariae infection. Therefore, it is probable that complement is incorporated in circulating immune complexes of people with quartan malaria nephrotic syndrome and that these complexes are similar to those deposited in the kidney.

b) P. falciparum Associated Renal Pathology

It appears that the pathological changes in kidneys of patients with falciparum malaria vary from case to case depending on the stage of the disease and its severity.

Spitz (1940) observed glomerulonephritis with proliferative changes and basement membrane thickening in patients with falciparum malaria. Berger et al. (1967) reported nephrotic syndrome associated with falciparum malaria in three patients. They described hypercellularity, adhesion of Bowman's capsule, and infiltration of polymorphonuclear leukocytes, but splitting and mild thickening of the glomerular basement membrane were also present in scattered glomeruli. Glomerular mesangial expansion, hypercellularity and basement membrane thickening have been reported by Hartenbower et al. (1972) and Bhamarapravatti et al. (1973) (Fig. 33).

Electron microscopy (Hartenbower et al., 1972) showed irregular basement membrane thickening with alterations of its electron density, abnormal deposits of material in the subendothelial regions, large amounts of mesangial matrix, and focal endothelial cell proliferation. These changes were consistent with focal membranoproliferative glomerulonephritis. The same authors also showed by immunofluorescence that the kidneys of people with falciparum malaria contained small focal

deposits of IgG, IgM and $B_1C$-globulin within the mesangium and along the basement membrane. Bhamarapravatti et al. (1973) using immunofluorescence, studied 10 biopsies from Thai patients with acute falciparum infection and found fine granular deposits in the mesangial areas and occasionally along the luminal sides of the glomerular capillaries. The granules consisted primarily of IgM.(9/10 cases), $B_1C$ (8/10 cases) and IgG (3/10 cases). IgA was identified only in one specimen from a patient who developed acute renal failure. P. falciparum antigen was detected in one patient.

Glomerular immune complexes disappear after patients have been treated and recover from malaria. Immunoglobulin deposits disappeared from kidneys of patients who responded to prednisone, cyclophosphoamind and azathioprine treatment (Adeniyi et al., 1970). On the other hand, IgG and $B_1C$ remained in patients refractory to treatment with those drugs. One micrograph in Adeniyi's et al. paper shows linear immunoglobulin deposits in the kidney, a pattern which is characteristic of autoimmune nephritis. The patients with these linear deposits had a poor response to asatioprine. Moreover, in these individuals the distribution pattern appeared to change from granular to linear after treatment, implying that at some point in the course of the disease, the pathogenic mechanism may have switched from antigen-antibody complex deposition to autoimmune damage. Hendrickse and Gilles (1963) suggested that untreated attacks of malaria in some patients may evoke an abnormal immunological response where the glomerular basement membrane is damaged

by the antigen-antibody complexes and leads to an autoimmune response. A glomerular pattern changing from granular to linear may be a sign that damage mediated by immune complexes has become an autoimmune deterioration. Based on these reports it appears that immune complexes are involved in the genesis of glomerular lesions associated with falciparum and malariae infections in man.

Pronounced renal tubular alterations have been reported in patients with falciparum malaria. There are hyaline droplets with cloudy swelling of the tubular epithelium and, in severly affected kidneys, fatty degeneration and necrosis. Changes are more pronounced in the distal convoluted tubules than the proximal ones (Boonpuckavig and Sitprija, 1978). Biopsies just taken after recovery showed abnormally large amounts of interstitial connective tissue and infiltration of lymphocytes, histiocytes and eosinophils and later focal interstitial scarring occurred (Berger et al., 1967). Hemoglobin casts may be seen in the distal convoluted and collecting tubules (Winslow et al., 1975).

Blackwater fever is an acute hemolytic condition associated with fever, anemia, jaundice, and hemoglobinuria and it is generally considered to be a complication of P. falciparum infection (Edington and Gilles, 1976). There is usually a history of irregular chemosuppression or inadequate chemotherapy, especially with quinine. The diagnosis of blackwater fever can be made only in patients without other drug-induced hemolysis (Gilles and Ikeme, 1960). For example, patients with erythrocytic glucose-6-phosphage dehydrogenase deficiency who manifest hemolytic anemia and "black urine" when treated with certain antimalarial drugs have a different

disorder from classical blackwater fever (Edington and Gilles, 1976).

The incidence of blackwater fever has decreased; however, because of immunity reduction in people who live in endemic areas, it may be presently in a surge. For example, Dukes et al. (1968) reported the syndrome in six Rhodesians (5 of them Africans) mostly town dwellers after 10 years without a case seen. In addition to the danger of blackwater fever in individuals whose immunity has waned, the increased use of quinine, as a result of the appearance of chloroquine resistant strains of P. falciparum, may also augment its incidence (WHO, 1973).

Grossly, the kidneys of patients dying of blackwater fever are dark in color, enlarged, and edematous. The cut surface has a pale color with evidence of cortical swelling and small hemorrhages, and congestion of the medulla (Fig. 34).

Microscopically, there are slightly abnormal glomeruli (Macgraith, 1948). Deposits of amorphous hyaline material are present in the capsular spaces (Edington and Gilles, 1976). Rosen et al. (1968a) reported hyalinization and segmental fibrosis of glomeruli in a biopsy specimen. The principal histologic changes are different degrees of degenerative changes up to necrosis, mainly in the loops of Henle, and in the distal convoluted tubules (Edington and Gilles, 1976). Epithelial, hyaline, or granular casts are often seen in the lumen. The number of hemoglobin casts in the kidneys of patients dying with falciparum acute renal failure is less than in patients with clinical evidence of "blackwater syndrome" (Winslow et al., 1975). Moreover, patients with blackwater fever have

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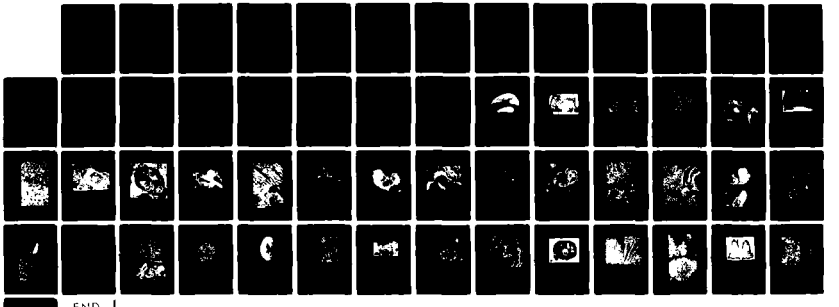

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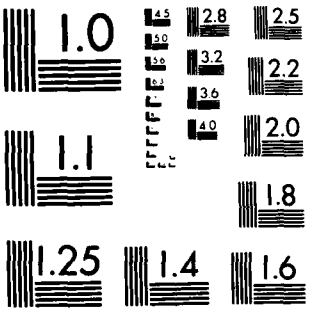

diffuse or focal stromal increase with interstitial lymphocytic infiltration (Fig. 35) (Rosen et al., 1968a). Abnormal amounts of collagen and scattered fibroblasts were also found. Osmiophilic rectangularly-shaped inclusion bodies which appeared to be malarial pigment particles were seen within fibroblasts.

c) Renal Pathology in Experimental Animals.

There has been work done in experimental animals to clarify the mechanisms of renal disease associated with malaria infection. Ward and Conran (1966) demonstrated malarial antigen, $\gamma$-globulin and $B_1C$ in the glomeruli of splenectomized monkeys infected with P. cynomolgi. If monkeys were splenectomized and unilaterally nephrectomized to increase the amount of circulating immune-complexes, there were no histologic alterations in 31 animals though granular deposits of IgG, $C_2$ and low amounts of malarial antigen were present over a 3 to 4 day period just after the primary peak of parasitemia (Ward and Conran, 1969). The deposits reappeared during a more sustained secondary parasitemia peak and changes in their concentration paralleled changes in parasitemia. Although there were circulating immune-complexes, Ward and Conran (1966, 1969) did not succeed in producing a picture of nephrotic syndrome in monkeys. Geiman and Siddiqui (1969) infected Aotus with P. malariae to study quartan malaria nephropathy experimentally. Voller et al. (1971) used the same model and observed facial edema and proteinuria in these animals during recrudescences. In spite of anti-malarial therapy, clinical deterioration occurred. There was generalized diffuse glomerulo-

nephritis with proliferative, and membranous changes. Prominent granular IgM deposits were seen in all glomeruli, but IgG, complements and malarial antigen were not present. Degeneration of the proximal tubules and focal collection of lymphocytes, plasma cells and occasional eosinophils in the interstitial tissue were also observed. Voller et al. (1973) also studied the kidneys of Aotus infected with P. malariae and P. brasilianium. In the acute phase there was an increase of mesangial matrix and proliferation of endothelial and mesangial cells in some glomeruli. IgM deposits were found in the mesangial regions with immunofluorescence but not with electron microscopy. In chronic infections the animals showed segmental or diffuse changes in the glomeruli characterized by swelling and proliferation of mesangial cells with abnormally large amounts of mesangial matrix, sometimes extending to the peripheral capillary zone. IgM and $B_1C/B_1A$ were identified by immunofluorescence. Electron microscopy showed variable thickening of the basement membrane with areas of altered electron density and occasional small inclusions. No deposits were present in the basement membrane. The general features of Aotus nephrosis following P. malariae infection are similar to those described in patients with quartan malaria nephrotic syndrome (Voller et al., 1971).

Histopathological kidney studies of rodents with malaria are scanty (Miller et al., 1968). Greenwood and Voller (1970a, 1970b) were the first workers to address the problem in New Zealand mice infected with P. berghei. Although there were several interesting findings reported by

these authors, unfortunately because of spontaneous development of nephropathy and the possibility of a latent virus infection in this mice strain, they are not suitable for study. Ehrich and Voller (1972) inoculated Swiss albino TO mice with P. berghei yoelii strain 17x and observed that glomerular immunoglobulin deposits paralleled parasitemia development. One month after the disappearance of parasitemia, the glomerular gamma-globulin deposits were no longer detected. Serum proteins did not appear in the urine until the 6th day of inoculation (Weise et al., 1972), but on the 8th day there was a tenfold increase and immune-complexes were detected. Suzuki (1972) also reported IgM glomerular deposits in mice infected with virulent P. berghei (NK65 strain) and treated. Boonpucknavig et al. (1972) found in infected mice with P. berghei, antigen deposited along the glomerular capillary walls in a granular pattern and extending into the mesangial areas. Plasmodial antigen was detected on the 3rd day after inoculation and antibody and complement on the 7th day. Histologically, the glomerular lesion consisted of mesangial cell proliferation, endothelial cell hypertrophy, polymorphonuclear cell infiltrate, and thickening of glomerular basement membrane. There was hemosiderin pigment in the proximal tubular cells.

Rhesus monkeys (Macaca mulatta), infected with P. knowlesi (Rosen et al., 1968b) excreted large quantities of hemoglobin, became oliguric and developed acute renal failure resembling human blackwater fever. The rapid intravascular hemolysis was reflected by progressive hematocrit, decrease and fall of total serum proteins. Light microscopy revealed hyaline droplet degeneration and hemoglobin granules in the proximal

tubules (Rosen et al., 1968b; Boonpuckanavig, V., 1973). Suzuki (1974) used P. berghei in mice in conjunction with sulfamonomethoxine (DJ1550) treatment to produce immune-complex disease. There was recrudescence during a 60 day period and in 15 mice studied there was generalized or localized diffuse or disseminated glomerular IgM deposits, but none had IgG. In 7 out of 15 mice, focal antigen deposits were found in the glomeruli (Fig. 36). Histologically there was a massive mesangial matrix increase accompanied by glomerular hypercellularity (Figs. 37 & 38). George et al. (1976) studied mouse malaria nephropathy using several strains of mice infected with P. berghei yoelii 17x. They detected glomerular deposits of IgG, IgM, $C_3$ and IgA; $C_3$ persisted longer than the immunoglobulins and malarial antigen was detected by the indirect fluorescent antibody technique.

All mice studied so far developed acute glomerulonephritis during plasmodial infection, similar to the acute falciparum glomerulonephritis, but none have developed lesions resembling P. malariae nephrotic syndrome. The plasmodia antigen, or antigens, responsible for malarial nephropathy observed in rodents, have not been isolated yet. Waki (unpublished observation) inoculated nude mice with P. berghei (NK65) and detected IgG and IgM in low titers during the primary attack. The IgG response would indicate that T cell-independent antigens occur in Plasmodium parasites. Suzuki (unpublished data) observed typical glomerular injuries in nude mice, suggesting that malarial glomerulonephritis is at least partly induced by T cell-independent antigen.

Kibukamusoke and Voller (1970) in Uganda, compared IgG, IgM

and malarial antibody levels in people with active nephrotic syndrome, with nephrotic syndrome in remission, and without nephrotic syndrome. The first two groups had high levels of IgM, antiplasmodial antibody and immune complex deposits in renal biopsies, suggesting that IgM is related to malarial nephropathy. IgM immunoglobulin has also been observed in human glomeruli (Ward and Kibukamusoke, 1969; Ward and Conran, 1969; Bhamarapravatti et al., 1973), Aotus (Voller et al., 1971; Voller et al., 1973), and mice (Suzuki, 1974). If IgM dominates the malarial nephropathy, then, it is a peculiar type as most nephropathies are associated with IgG. Dressman (1972) reported that some rabbits injected with BSA produced large-sized immune complexes whose major antibody class is weakly IgM binding. In those animals the immune response is associated with a unique glomerular lesion consisting of a focal glomerular necrosis without diffuse proliferative changes. The nature of this IgM induced lesion is reminiscent of glomerular lesions seen in P. malariae where IgM is predominant and, moreover, it resembles non-proliferative glomerulosclerosis type described by White (1973).

Lambert and Houba (1974) showed that $^{125}$I-labeled anti-P. malariae IgG persisted as a complex in Aotus monkeys infected with P. malariae but not in control animals. Anti-malarial antibodies were also demonstrated with labeled IgG fractions in the infected monkeys. IgG specific for P. malariae is found in greater amounts in glomeruli of monkeys infected with P. malariae than in P. falciparum infections. Houba et al. (1976) examined the fate of $^{125}$I-labeled anti-P. malariae IgG in Nigerian

nephrotics after i. v. administration and found a significant rapid loss of anti-malariae IgG from circulation than normal IgG. Subsequent biopsies from the same patients injected with anti-P. malariae IgG showed greater amounts of IgG in the eluates than did the controls.

Lambert and Houba (1974) measured levels of complement components $C_3$, $C_4$ and $C_3$ proactivator (C3PA) in plasma of infected monkeys with P. brasilianum and found an increase associated with parasitemia peaks. This pattern of complement components levels during parasitemia peaks may reflect promoted host synthesis and absorption by circulating or deposited antigen-antibody complexes, when the parasites are removed by the immune system.

## IX. Neuropathology

There are good reviews of cerebral malaria (Spitz, 1940; Thomas, 1971; Marsden and Bruce Chwatt, 1975) especially dealing with P. falciparum. Marked CNS changes occur with this parasite in approximately 2% of the patients with acute disease (Daroff et al., 1967). Postmortem examination of patients dying of cerebral malaria reveals an edematous brain with broadening and flattening of gyri (Fig. 39). The arachnoid blood vessels are congested giving the classical "pink brain" appearance (Fig. 39). The cortex is usually grayish-blue, because of deposition of pigment, and pigment, and petechial hemorrhages are common.

Microscopically, capillaries are occluded by erythrocyte masses (Fig. 40) (Conner et al., 1976). Many red blood cells contain parasites and in medium sized vessels they are seen marginated against the endothelial cells which are often swollen, necrotic or desquamated (Fig. 41).

Malaria pigment is present and in good preparations schizonts are easily recognized. Medium-sized arteries are usually not occluded, and chronic inflammatory cells are common especially around the vessels.

After 10 days of infection, concomitant with thrombi formation, ring hemorrhages appear around the blood vessels (Figs. 42 & 43). The brain stroma particularly around the neurons and the blood vessels is vacuolated as a result of severe edema, with necrosis and nerve fibers demyelinization. Later, these lesions will result in the formation of microgranulomata known as Durck's granulomas (Durck, 1917; Dhayagude and Purandare, 1943).

The final stages of the pathophysiology of cerebral falciparum malaria is not well-known. Anoxia plays an important role in the acute phase of the disease (Rigdon, 1944; Maegraith, 1965) and brain lesions are reversible, because treated patients recover and show no apparent sequelae later in life. There are studies in animals (Maegraith and Omabanyo, 1970; Onabanyo and Maegraith, 1970; 1971) suggesting that the clogging of brain capillaries produce an irreversible impedance of local blood flow. Kallikrein, an active blood peptide isolated from monkeys infected with P. knowlesi has been found to produce increased brain capillary permeability. If the peptide plus protamine blue dye is injected into normal guinea pigs brain, there is staining of brain substance and appearance of the dye in CSF. Migasera and Maegraith (1965) reported that a blood-brain barrier breakdown occurs in animals with acute P. knowlesi and P. berghei infections, suggesting that pharmacologically active substances are released

when antibody and antigen react. The action of these substances on endothelial permeability could be one mechanism for the production of lesions in animal brains, where parasitized erythrocytes are clumped in brain capillaries. Their experiments suggest that in malaria the endothelial lesion is a combination of both mechanical and chemical mediated damage.

Yoeli and Hargreave (1974) described a virulent (17x) strain of P. berghei (P. b. yoelii) which produced infection in mice with prominent intravascular sequestration of parasitized and non-parasitized erythrocytes in the brain. The capillaries are blocked by sequestred erythrocytes, and ballooning of endothelial cells and fine petechial hemorrhages on the brain surface and in the stroma are seen. They suggested that the strain virulence is based on its ability to induce intravascular sequestration of the infected erythrocytes with blockage of small and large brain capillaries. On the other hand, other virulent strains of P. berghei such as NK65 and KS61 do not produce brain vascular blockage, although they result in fatal infections. Mice infected with P. vinckei vinckei may also die, but only rarely are infected erythrocytes found in cerebral capillaries. Therefore, the 17x strain of P. berghei appears to be the only Plasmodium strain useful for studies of cerebral malaria.

Mercado (1965) described paralysis of rats infected with P. berghei. Their brains contained extensive focal cerebral hemorrhages. This paralysis can be prevented apparently, by splenectomy prior to infection suggesting an immunological mechanism in its development (Mercado, 1973).

## X Placenta Pathology

The placenta is an important organ in malaria infections. Grossly, there is an enlargement and the color has a slate-gray hue (Fig. 44). Microscopically, abundant parasites are seen in the maternal circulation in the intervillous spaces (Fig. 45). Pigment deposits are seen in macrophages located in the intervillous spaces and rarely in the parenchyme (Fig. 46). Lymphocytic infiltration may be focally present with a picture of lymphocytic and histiocytic villitis. Placental infection by any organism serves as a focus for fetal infections and cases of intrauterine acquired malaria are common in endemic areas. The effect of malaria placental infection of the fetus has been studied in Africa by Archibald (1956) who found that at least 2% of all births are premature due to malari infections. Tchakamakow (1954) reported postmortem findings in 20 stillborn infants from mothers with malaria in Macedonia and suggested that asphyxia is the cause of death.

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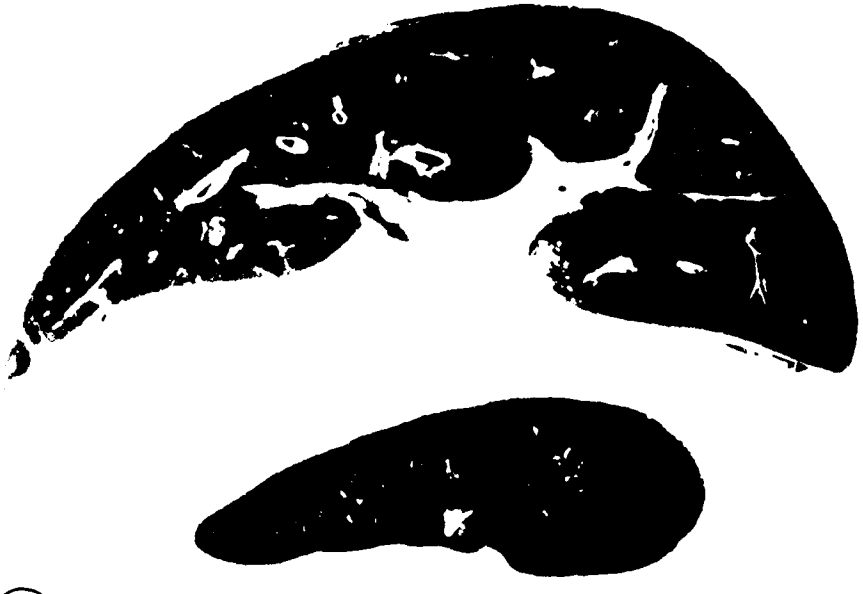

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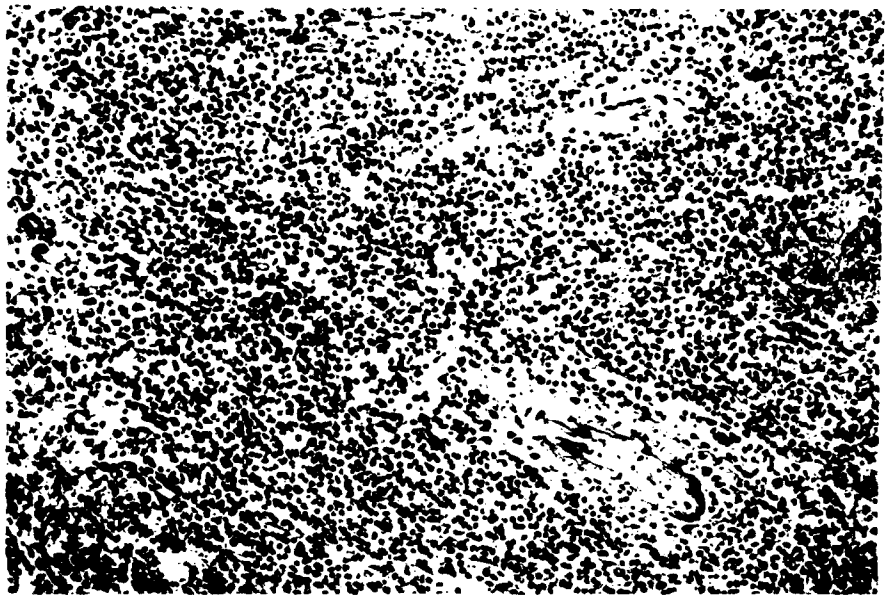

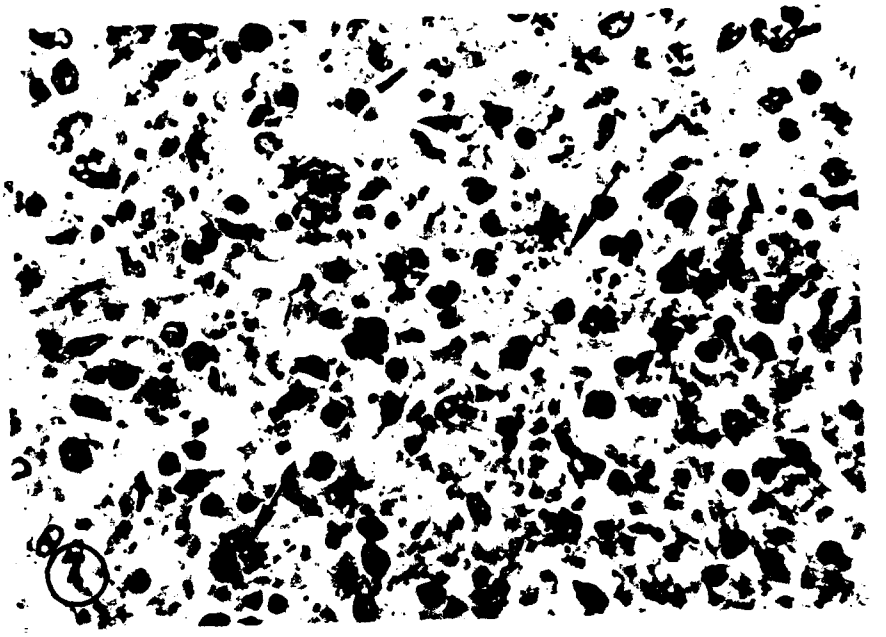

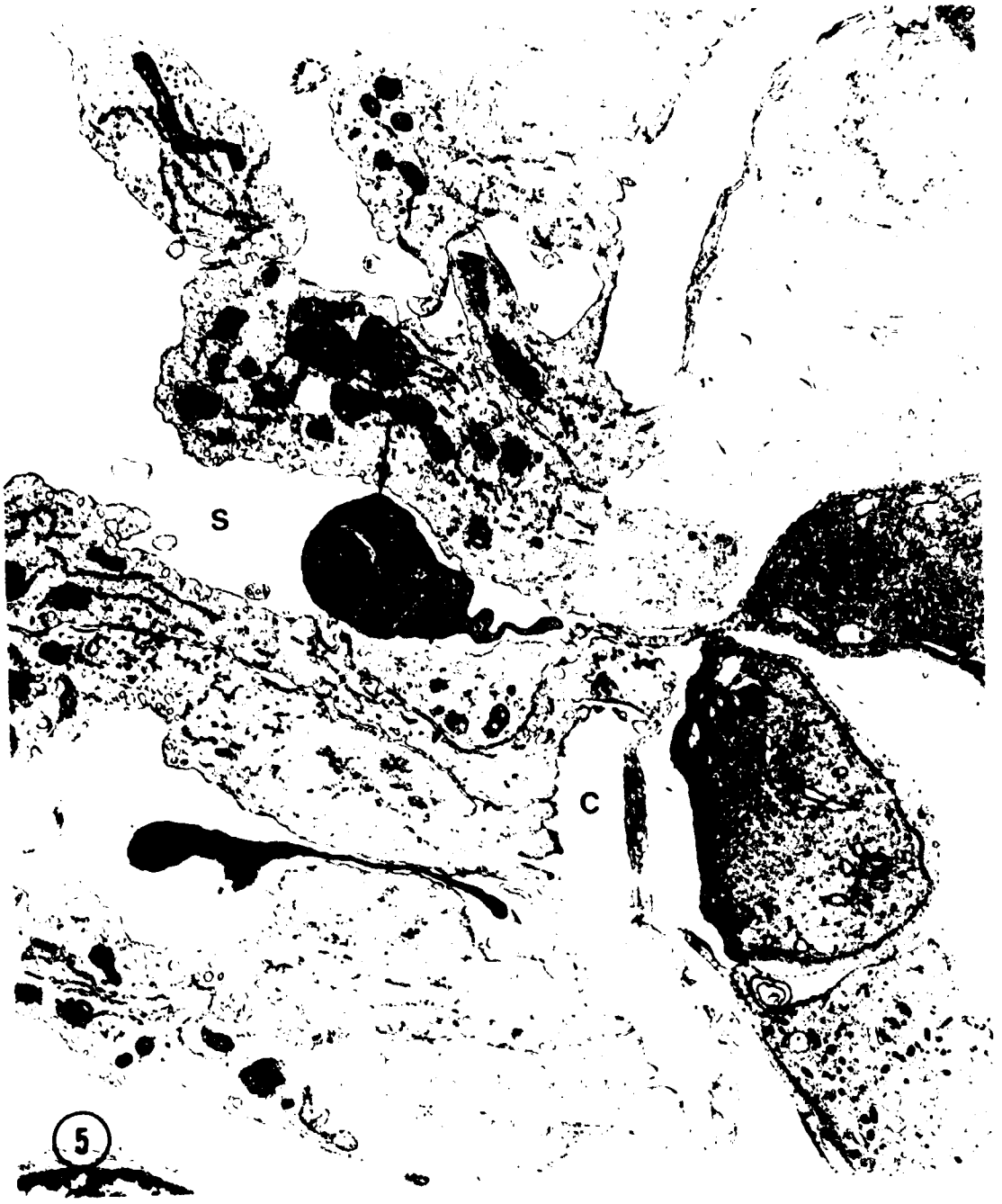
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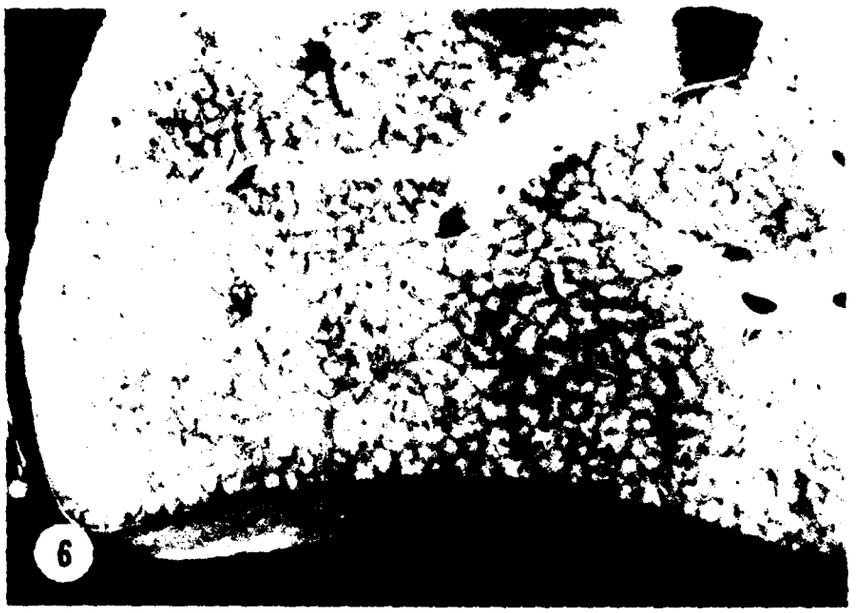
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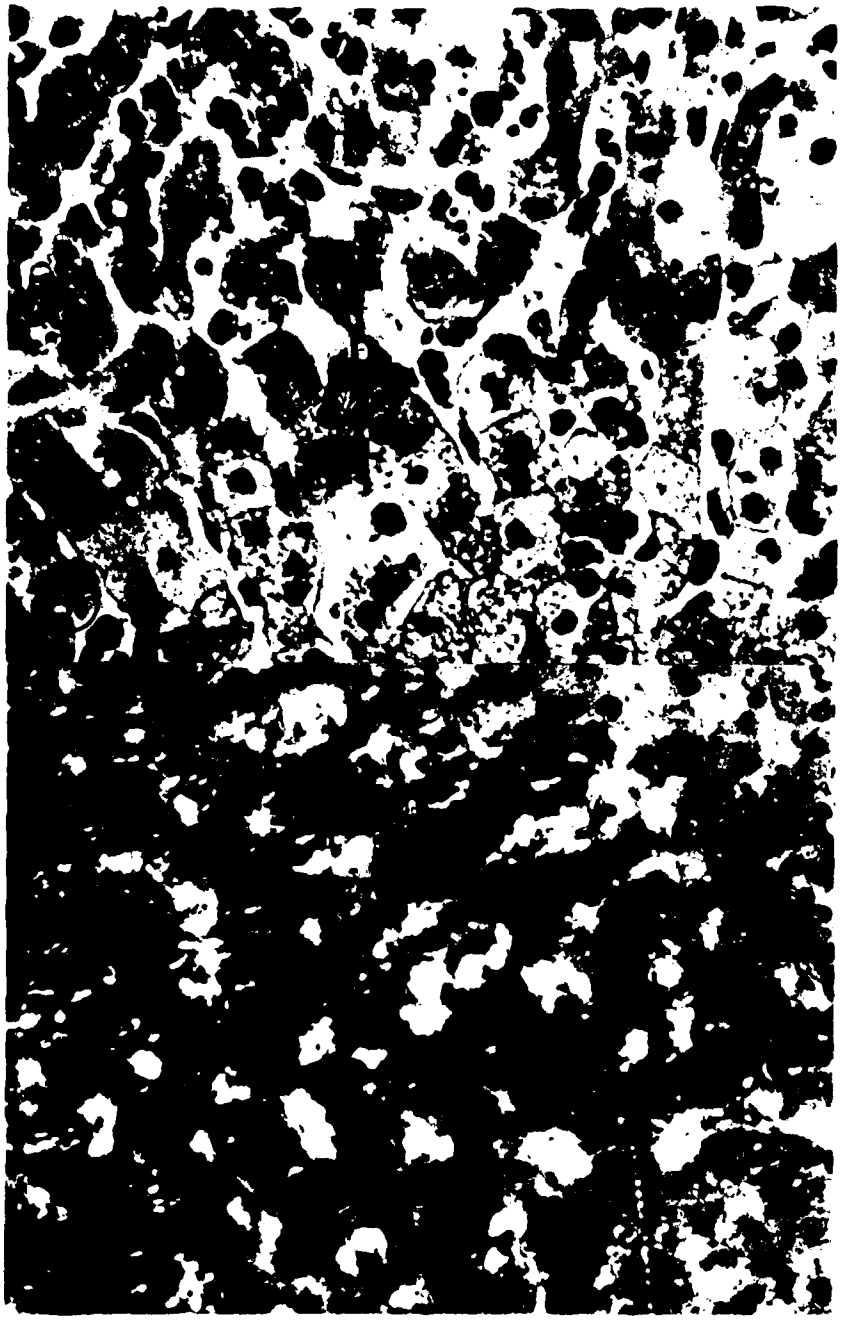

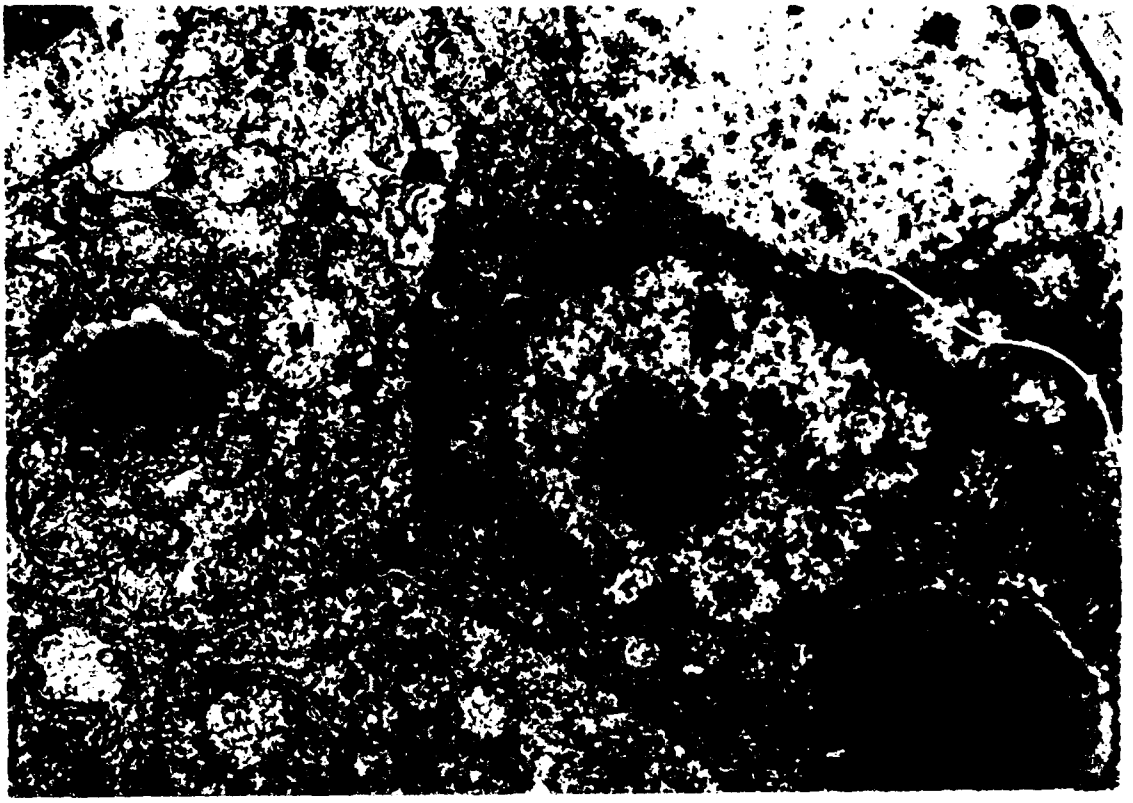

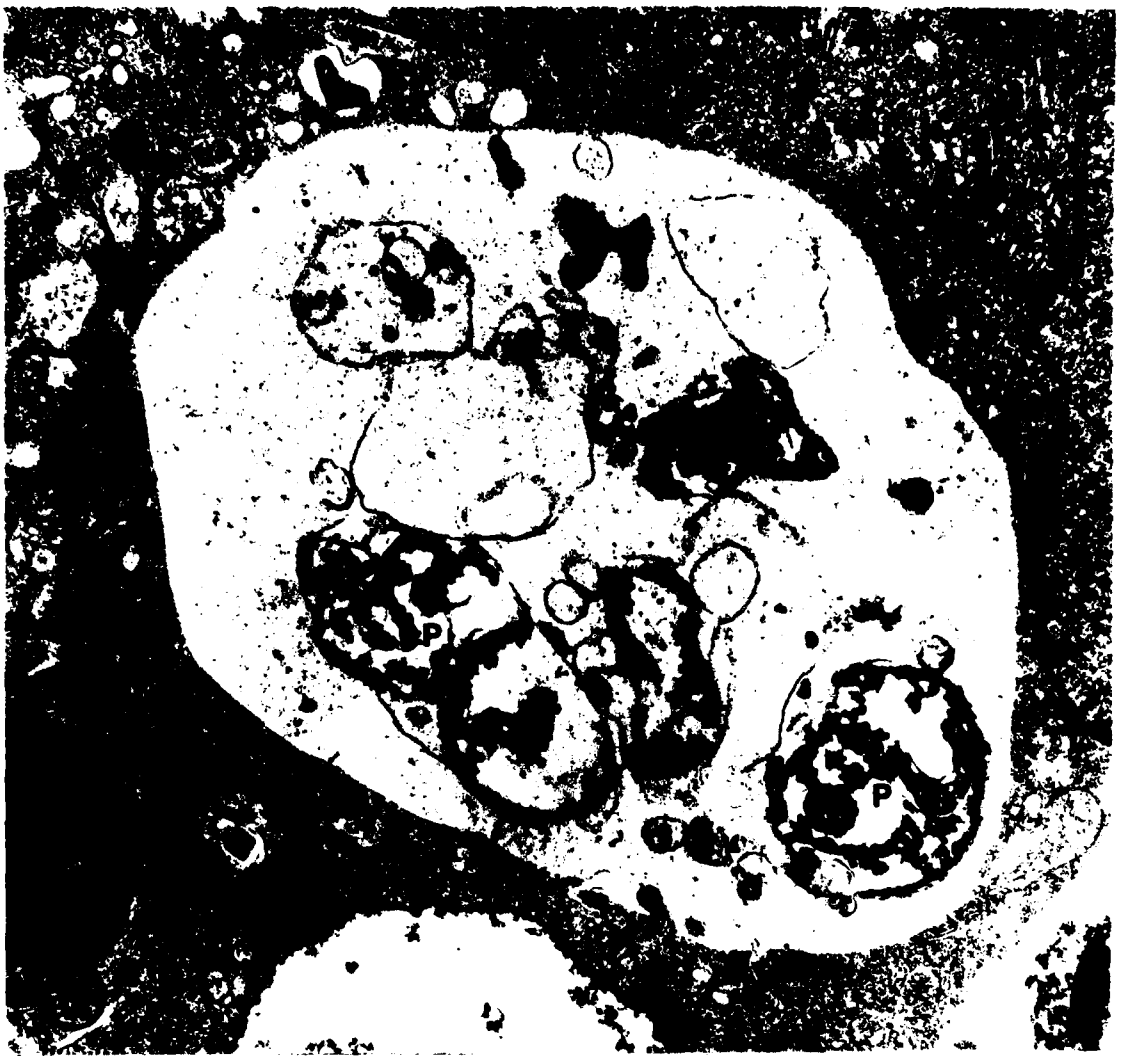
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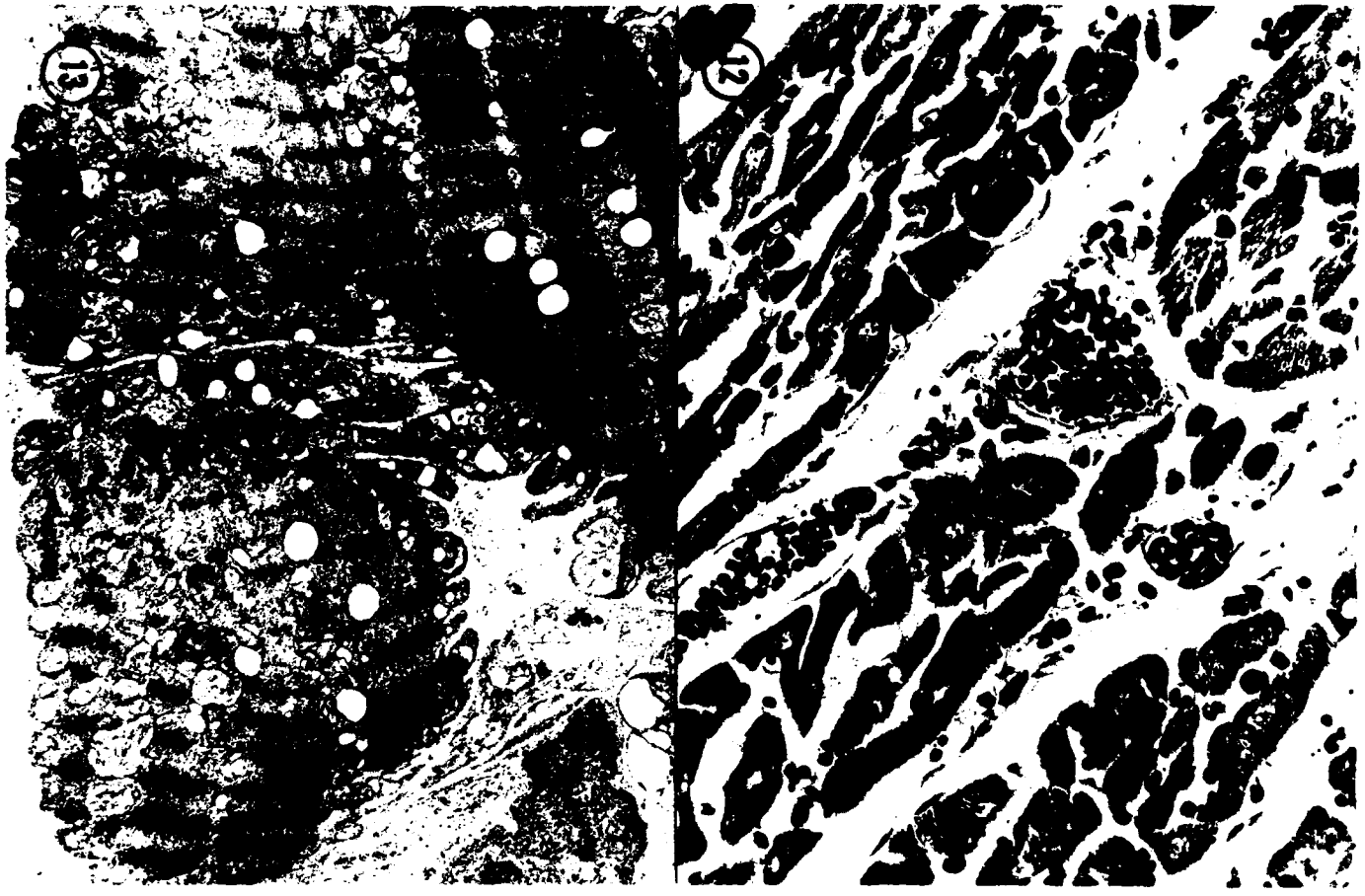
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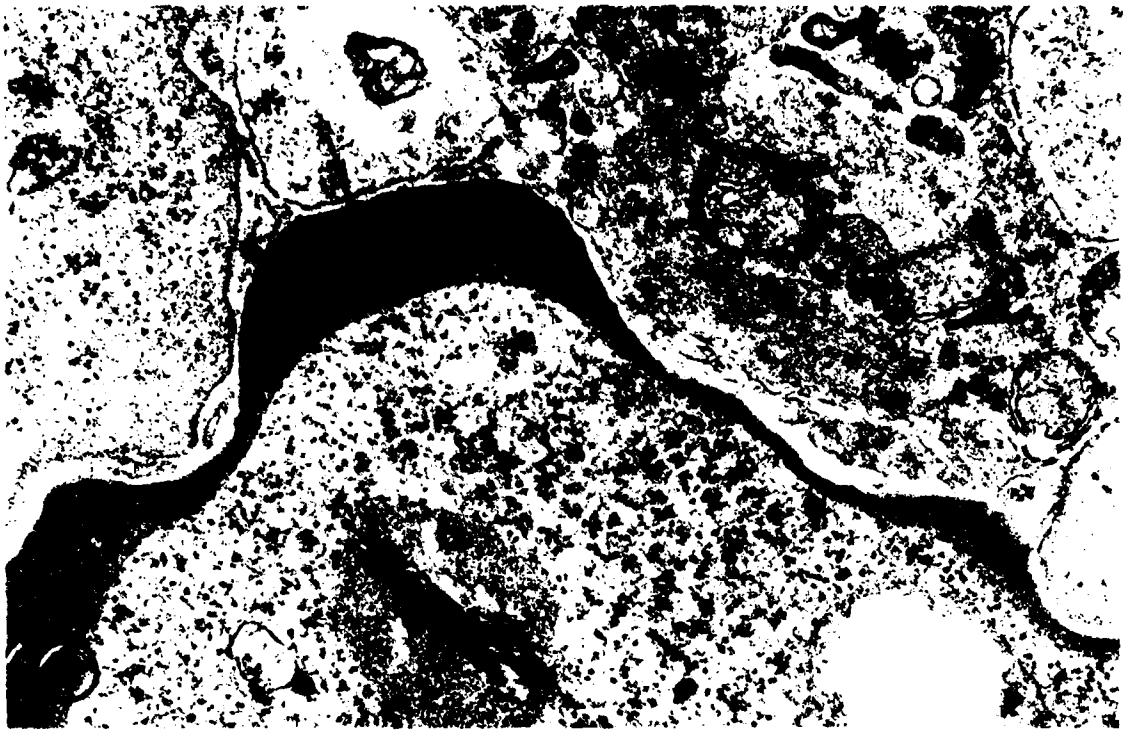

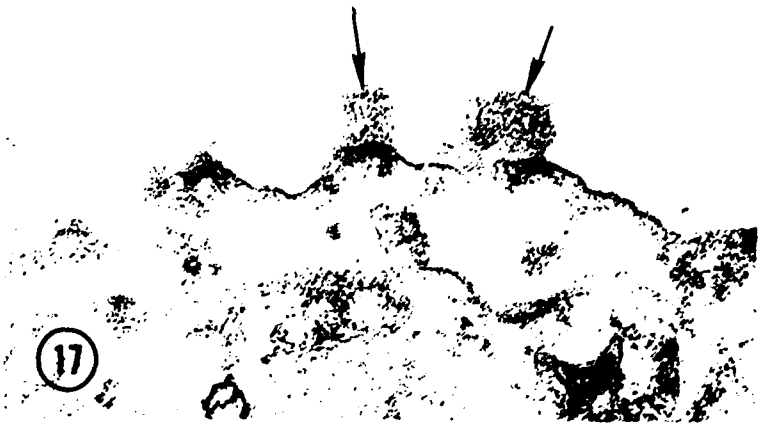
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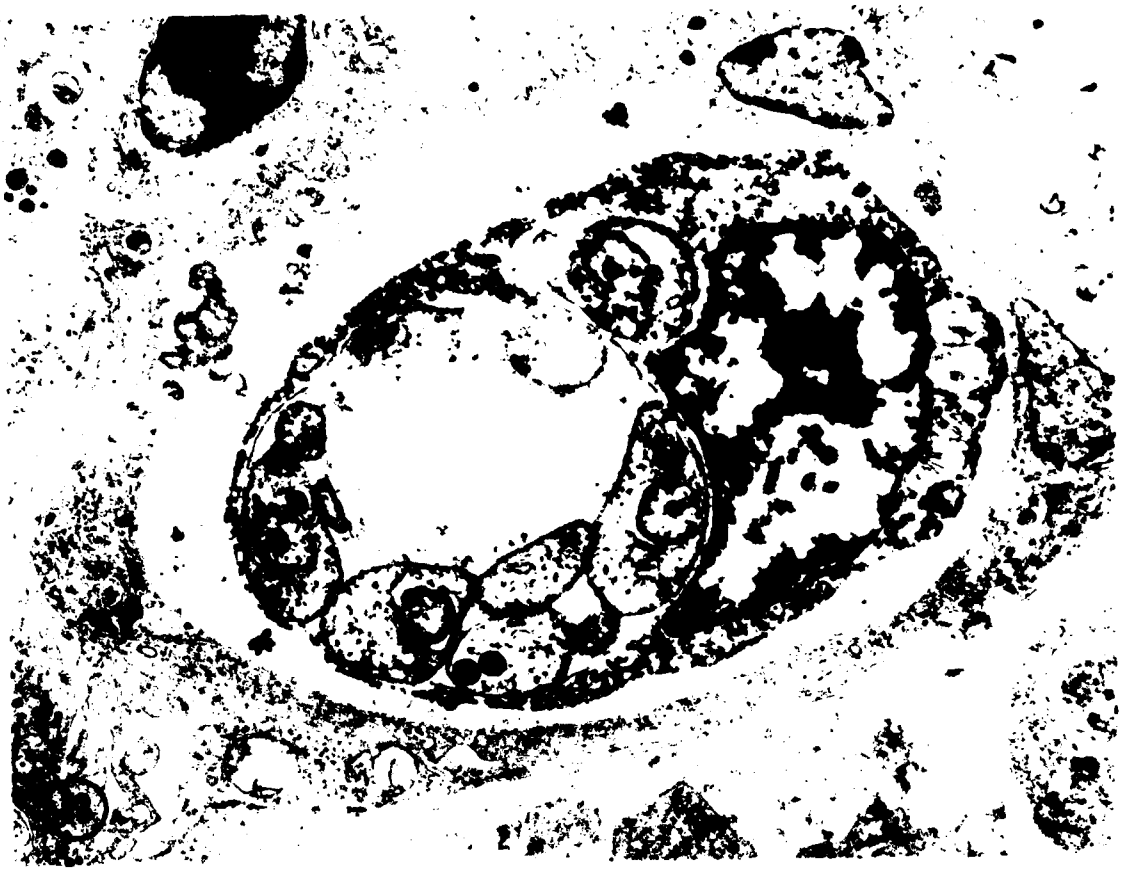


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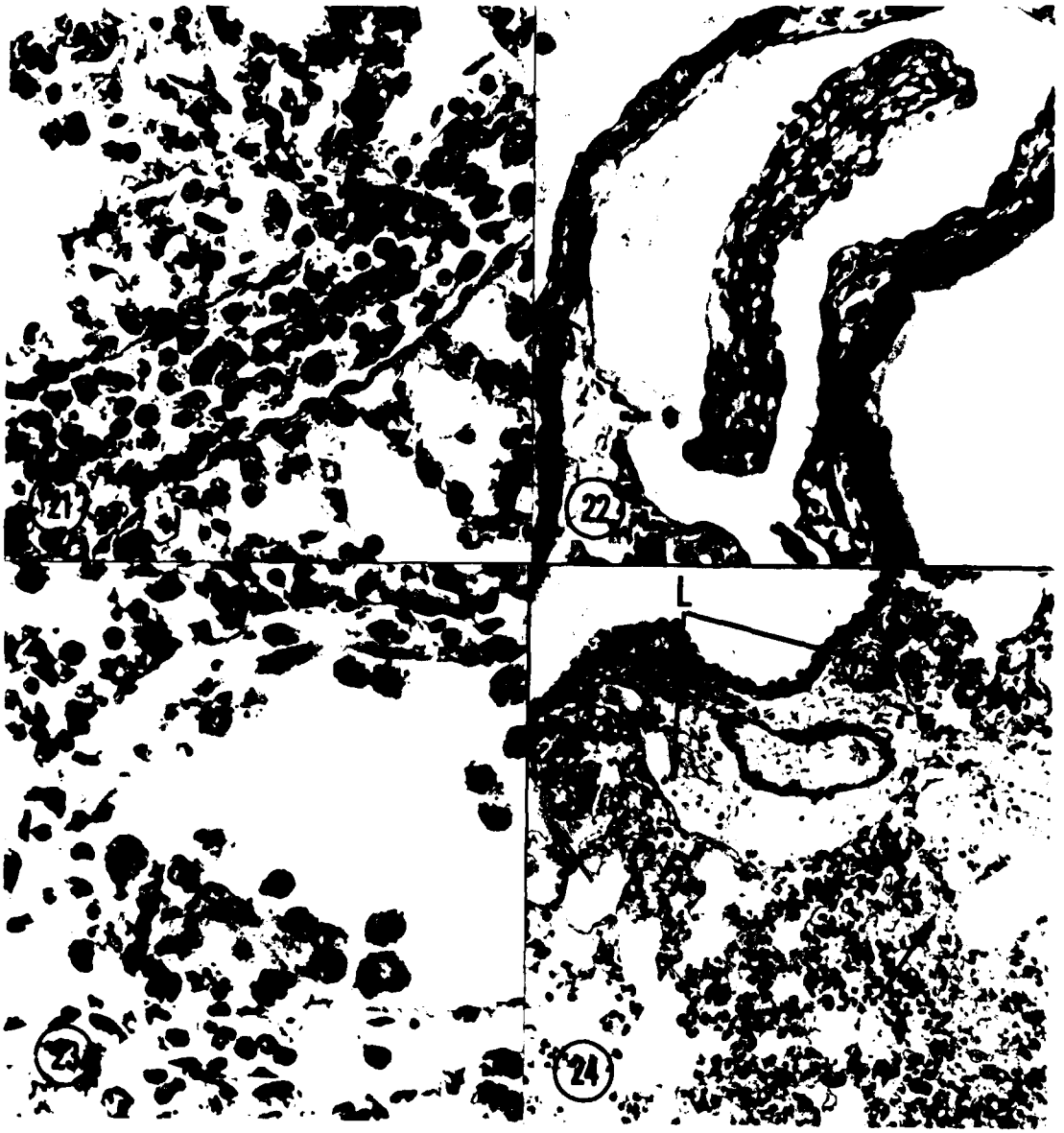
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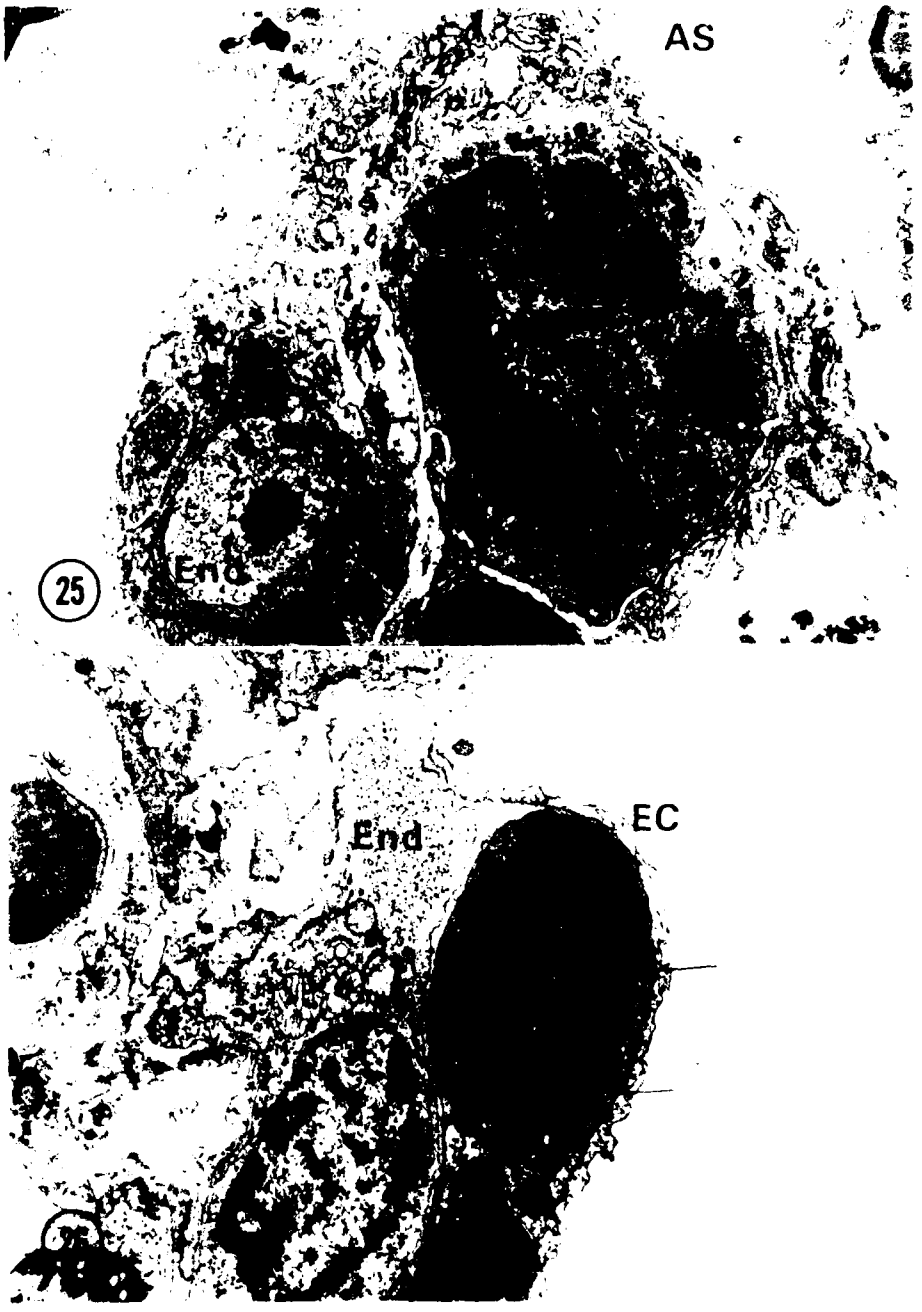
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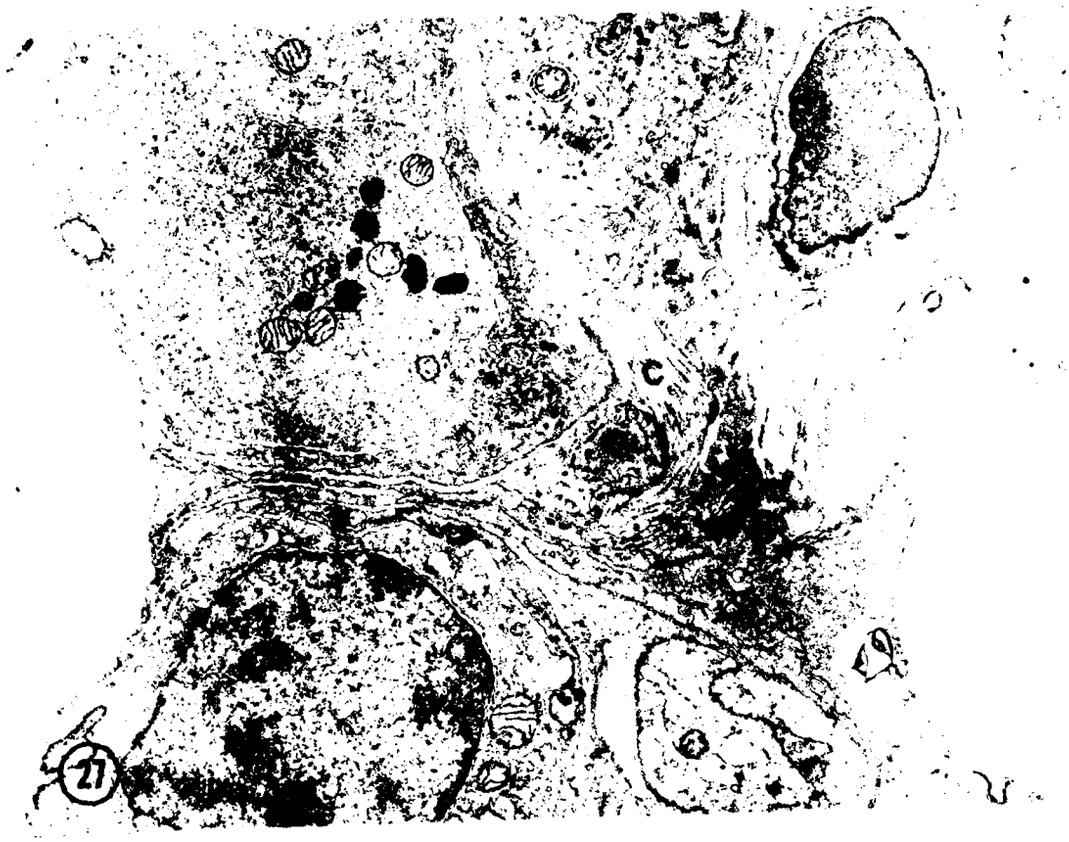
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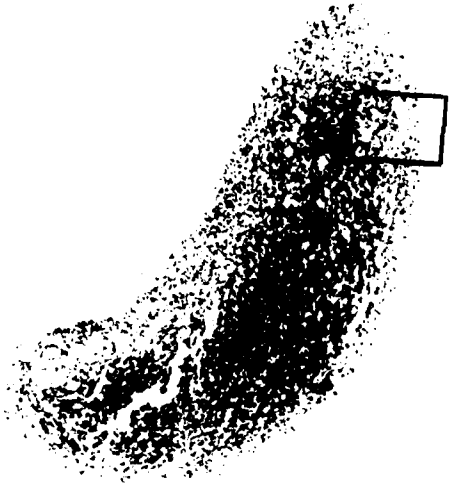

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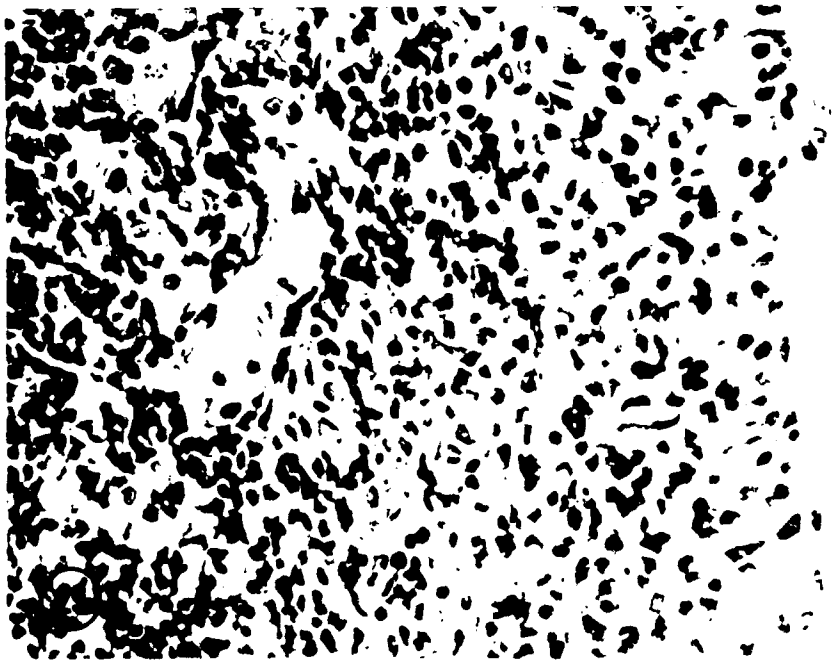
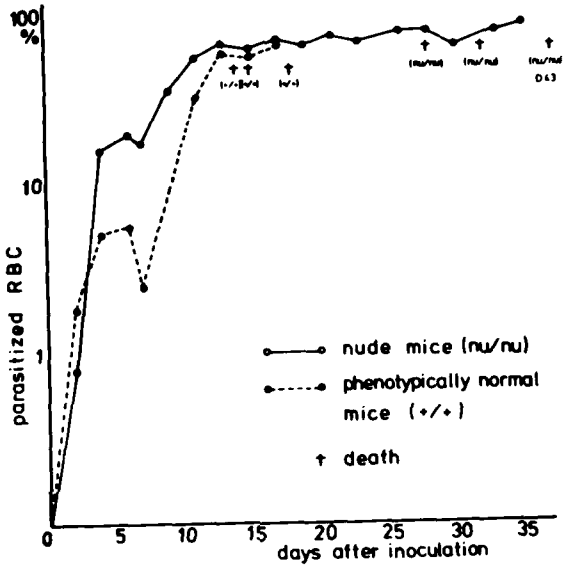


Plasmodium berghei (NK65) infection in nude mice

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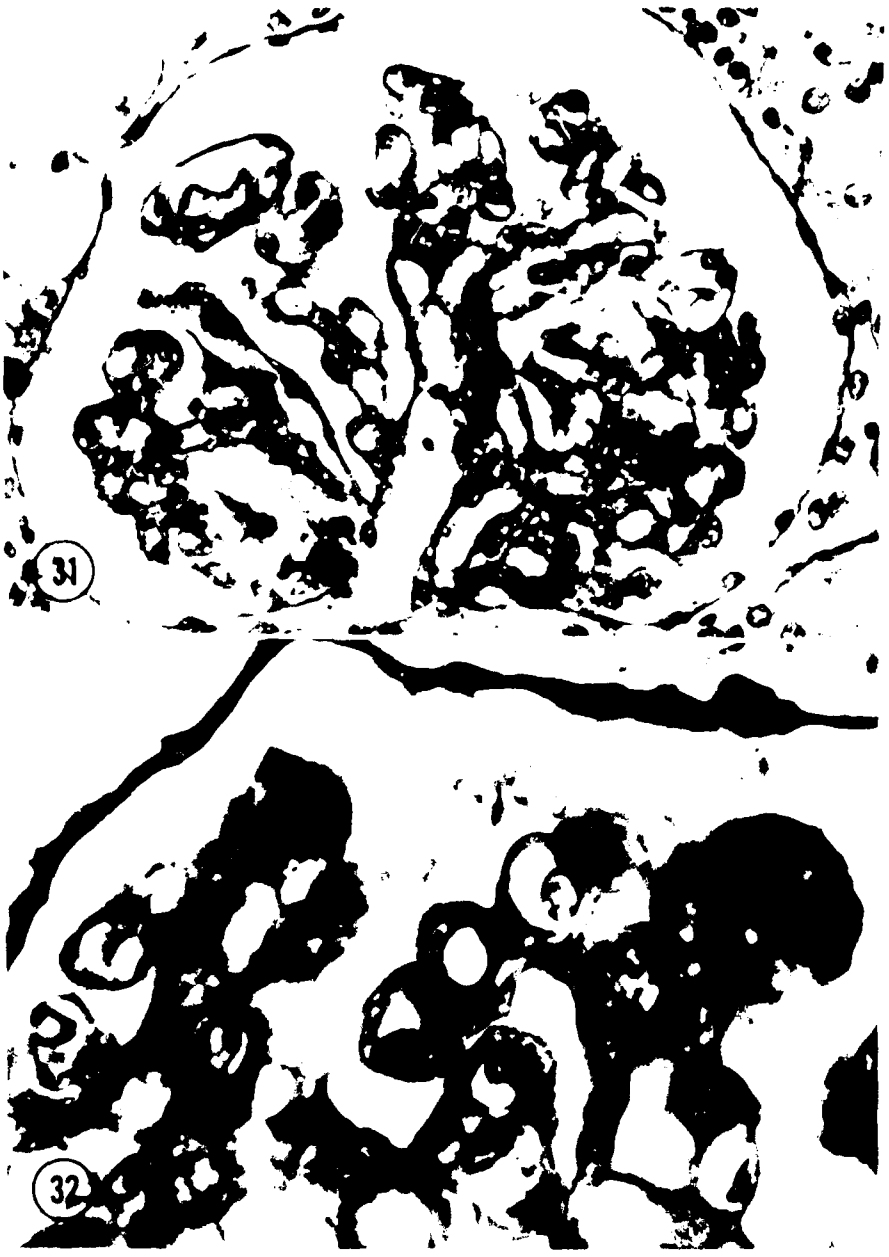
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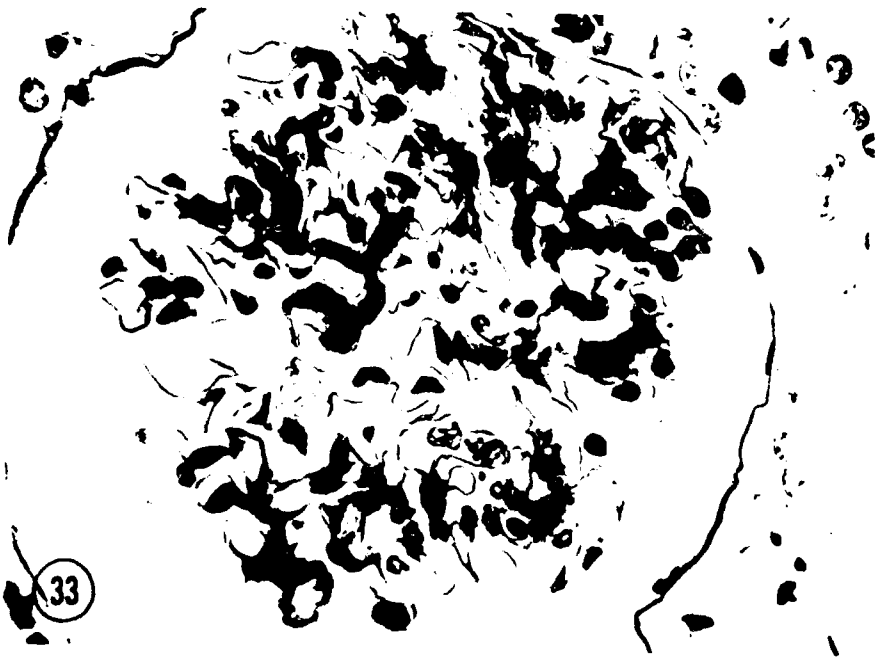
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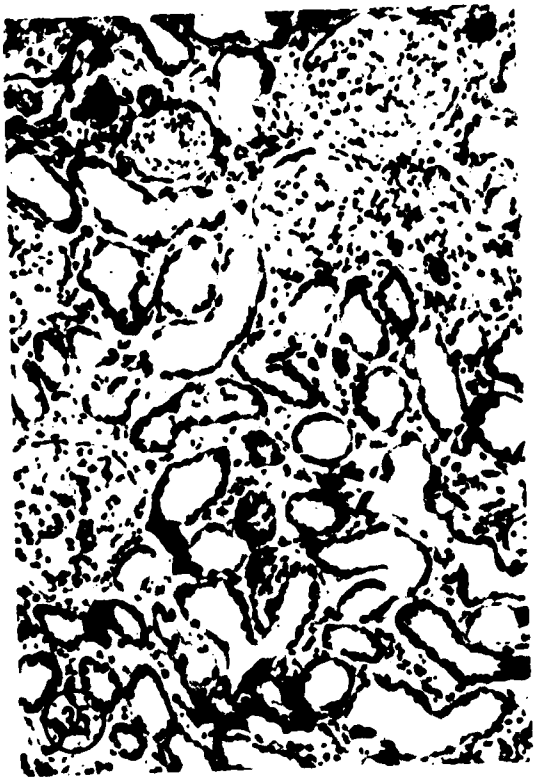


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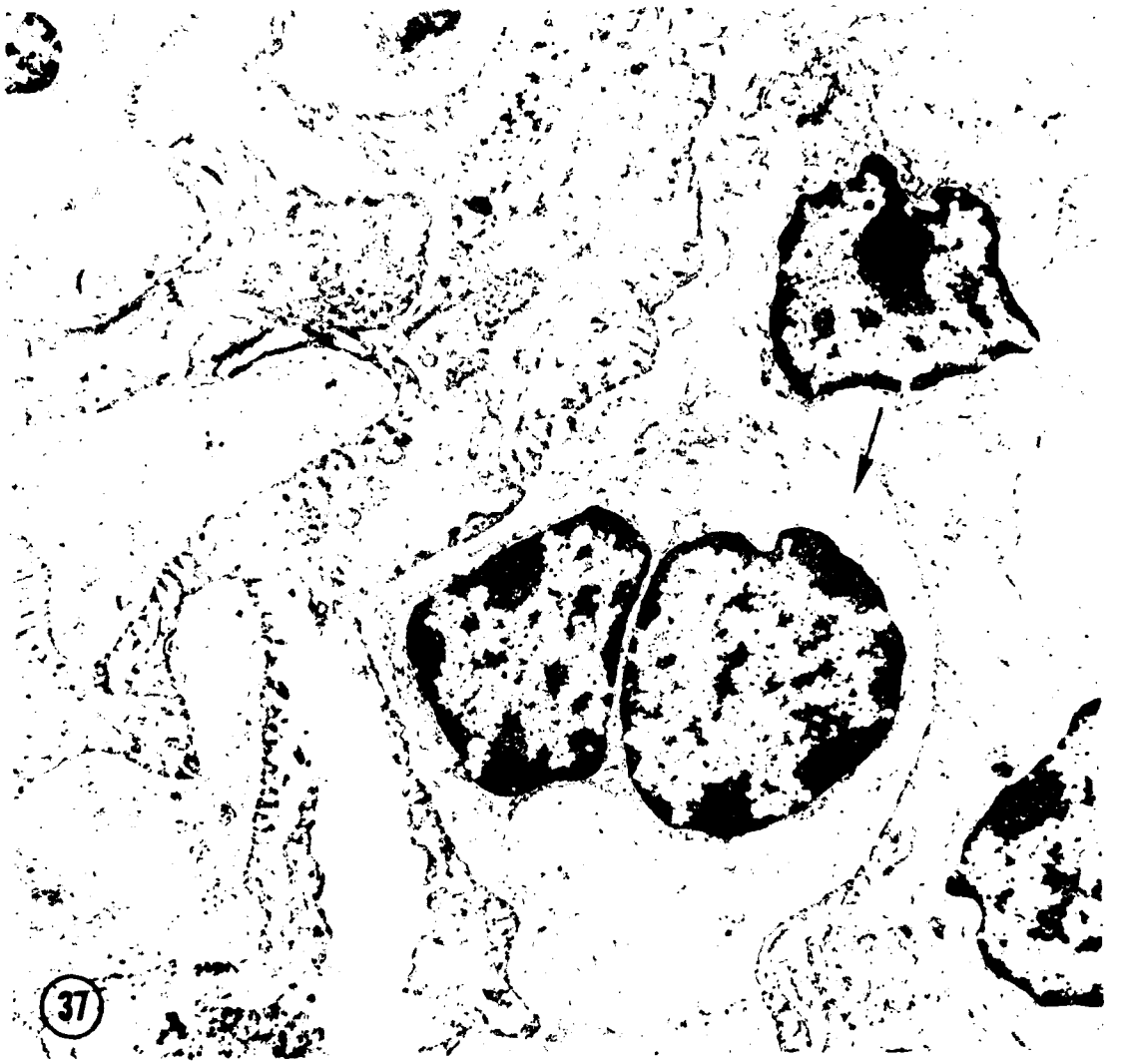
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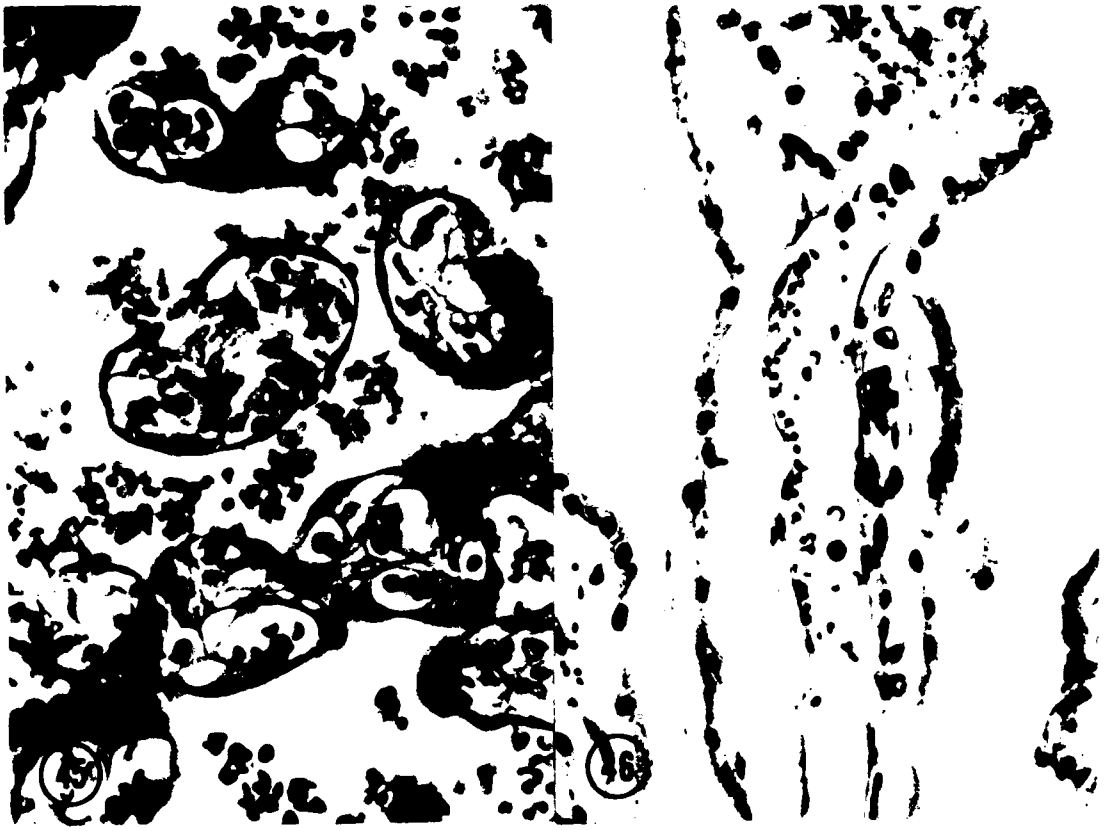

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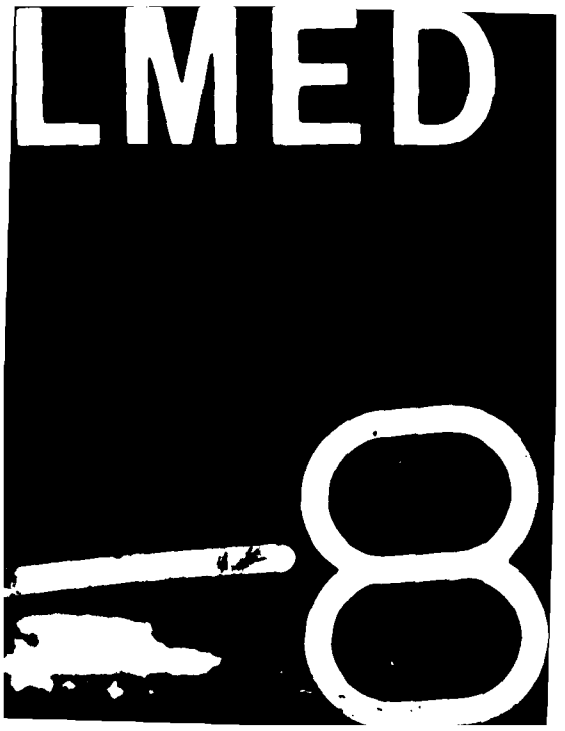
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