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ELECTRON MICROSCOPY TO CORRELATE CELL STRUCTURE
AND BIOCHEMICAL ACTIVITY

FINAL REPORT

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MASAMICHI AIKAWA

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13. ABSTRACT (Maximum 200 words) We performed immuno-electron microscopy to study intra-cellular localization and fate of liposome-encapsulated malarial antigen after phagocytosis by macrophages. Liposome-encapsulate protein that is phagocytosed by macrophages can enter an intracellular compartment in which at least some of the antigenic epitopes are not degraded by lysosomal enzymes. We also localized P. falciparum antigen having molecular weight 175 kDa (EBA) within the parasite by immunoelectron microscopy. EBA is specifically localized in micronemes of P. falciparum merozoites. The CS protein, SSP2, the Duffy receptor and now EBA-175 have been identified in the micronemes, suggesting that these organelles play a critical role in the storage and release of parasite proteins that act as receptors for invasion into host cells. P. coatneyl produces knobs on the membrane of PRBC and these PRBC appear to sequester in the vasculature of infected rhesus monkeys. We studied the pathology of CNS of rhesus monkeys infected with P. coatneyl and found that cytoadherence of PRBC to endothelial cells is a consistent feature of infections with this primate parasite. Cerebral microvessels with sequestered PRBC were shown by immunohistochemistry to possess CD36, TSP and ICAM-1. Our study indicates, for the first time, that rhesus monkeys infected with P. coatneyl can be used as a primate model to study human cerebral malaria.

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INTRODUCTION:

The main objective of our work was to function as a basic core electron microscopy facility in collaboration with scientists at WRAIR. In particular we proposed to carry out studies on morphological effects of drugs on various microorganisms by electron microscopy and autoradiography in order to understand the mode of actions of these drugs.

In the past, electron microscopy has been used to investigate the mode of action of chemotherapeutic agents on a variety of microorganisms. Studies of chloroquine, primaquine, pyrimethamine, qinghaosu, pentamidine and WR-163577 have indicated that each compound produces specific changes in particular organelles (1-7). The results obtained by electron microscopy have correlated well with biochemical observations on the action of drugs. Therefore, studies of the morphological effects of drugs on various microorganisms can contribute greatly to our understanding of their mode of action.

Secondly, immuno-electron microscopy has been used to characterize protective antigens of malarial parasites. Techniques of immunology and molecular biology have been used to identify and isolate specific proteins that may be capable of producing protective immunity against malarial parasites. Immuno-electron microscopy has played an important role in identifying specific immune targets and characterizing the precise location and expression of these targets (8,9). Such work will help to develop successful vaccines against microorganisms.

BODY:

During this period, we have performed electron microscopy (standard and immuno-electron microscopy) to investigate the mode of action of antimalarials and localization and characterization of protective malarial antigens in host and parasites. Important findings are described as follows:

a)   Phagocytosis of liposomes by macrophages: intracellular fate of liposomal malaria antigen.

Liposomes continue to be useful as models for understanding the disposition patterns of materials ingested by macrophages. The fate of liposome-encapsulated protein in macrophages is of particular interest in immunology. Liposomes are highly effective in the stimulation of immune responses to encapsulated protein antigens (10). It is widely believed that the process of immunological presentation of protein antigen to cells in the immune system involves initial processing of the antigen by specialized antigen presenting cells such as macrophages and B lymphocytes. It is presumed that the ability of liposomes to enhance immune responses is due to natural targeting of liposomes to macrophages. In collaboration with Col. Carl R. Alving and his associates of the Department of Membrane biochemistry, WRAIR, we studied intracellular localization and fate of liposomes and liposome-encapsulated malarial antigen after phagocytosis by macrophages.

In the present study, we performed immunoelectron microscopy to detect the presence of all surface-associated and intracellular liposome-associated malarial antigens. After incubation of macrophages with liposomes containing malarial antigen, vacuoles appeared in the cytoplasm and the vacuoles contained numerous liposomes that were densely labelled by antibody to malarial antigen. Many of the protein-containing liposomes apparently remained intact with a considerable amount of liposome-associated antigen for at least as long as 6 hours after incubation. Liposomes were mainly located at the peripheries of the vacuoles and often seemed to adhere to old vacuolar membranes (Fig.1). Antigenic epitopes detected by the antibody were often observed in the cytoplasm next to the vacuoles. The original antigen consisted largely of repeats of ASN-ALA-ASN-PRO, and it is possible that fragments containing ASN-ALA-ASN-PRO were recognized by the antibody. However, liposomes were invariably present only within the vacuoles and liposome-like structures were rarely, if ever, present in the cytosol of associated with any other subcellular organelies.

We concluded that liposome-encapsulated protein that is phagocytosed by macrophages can enter an intracellular compartment in which at least some of the antigenic epitopes are not degraded by lysosomal enzymes. Furthermore, although liposomes do enter large vacuoles, at least some of the antigenic epitopes, as detected by a monoclonal antibody, can escape into the cytoplasm. These experiments therefore provide evidence to support the

possible existence of a pathway in which lipsomal contents can bypass lysosomal degradation. The occurrence of an intracellular pathway for endocytosed or phagocytosed materials that avoids lysosomes has been previously hypothesized and such a pathway therefore might be used as a basis for delivery of non-degraded liposomal antigenic protein epitopes to the cytosol and to the cell surface.

b) A study on a primate model for human cerebral malaria

Human cerebral malaria is a pernicious manifestation of infection with P. *falciparum*. Possible factors contributing to the development of cerebral malaria include the blockage of cerebral microvessels by parasitized erythrocytes (PRBC), deposition of immune complexes in cerebral microvessels, reduced humoral or cell-mediated immune responses and action of tumor necrosis factor (TNF) (11).

Electron microscopy revealed multiple electron dense knobs protruding from the membrane of PRBC seen in the cerebral microvessels of cerebral malaria patients (12-13). These knobs attach via a parasite ligand to receptors on endothelial cells, resulting in the blockage of cerebral microvessels (Fig.2,3). Recently several investigators reported that host cell molecules such as CD36, thrombospondin (TSP) and intercellular adhesion molecule-1 (ICAM-1) may function as the endothelial cell surface receptors for PRBC. However, there is little information as to

whether these molecules actually play a role in cytoadherence of PRBC in vivo. We have demonstrated by immunohistochemistry the presence of CD36, TSP and ICAM-1 on the endothelial cells of cerebral microvessels of fresh-frozen, non-malarious human brain tissues (12). However, formalin-fixed and paraffin-embedded brain tissues do not stain well with antibodies against these molecules by immunohistochemical methods. Thus, the use of fresh brain tissue from cerebral malarial patients appears to be essential for studies of the potential role of these molecules in the development of cerebral malaria. However, it is difficult to obtain fresh brain tissue from cerebral malaria patients, since they often die in malaria endemic areas where no freezing facilities are available.

P. coatneyi produces knobs on the membrane of PRBC and these PRBC appear to sequester in the vasculature of infected rhesus monkeys (14). There have been no reports, however, indicating whether cytoadherence of PRBC to endothelial cells of cerebral microvessels occurs in vivo. Therefore, in collaboration with Col. Webster, Maj. Brown and Maj. Smith of AFRIMS, Bangkok, Thailand, we studied the pathology of the central nervous system (CNS) of rhesus monkeys infected with P. coatneyi in order to determine whether cytoadherence of PRBC to endothelial cells is a consistent feature of infections with this primate parasite and, if so, whether putative receptors for cytoadherence in human cerebral microvessels such as CD36, TSP and ICAM-1 are also present.

Our study demonstrated PRBC sequestration (Fig.4) and cytoadherence of knobs on PRBC to endothelial cells in cerebral microvessels of these monkeys (Fig.5). Cerebral microvessels with sequestered PRBC were shown by immunohistochemistry to possess CD36, TSP and ICAM-1. These proteins were not evident in cerebral microvessels of uninfected control monkeys. Therefore, our study indicates, for the first time, that rhesus monkeys infected with P. coatneyi can be used as a primate model to study human cerebral malaria. By using this animal model, we may be able to make strategies to develop vaccines to prevent human cerebral malaria (15).

In addition, we are planning to study morphological effects of antimalarial drugs on sequestered parasitized erythrocytes in order to understand how sequestered PRBC become detached from endothelial cells of cerebral microvessels by various antimalarials. Since this aspect of human cerebral malaria has never been studied in the past, our study will help to understand the mode of action of various antimalarial drugs on human cerebral malaria.

c)   A primate model for Human Cerebral Malaria: Effects of
      Artesunate (Qinghaosu derivative) on <u>Plasmodium</u> <u>coatneyi</u>-
      infected rhesus monkeys

In collaboration with Drs. Kyle Webster, Dennis E. Kyle,
Arthur Brown, C. Dahlem Smith, C. Ockenhouse, and Kevin Corcoran of
WRAIR and AFRIMS, we studied the effects of artesunate on rhesus
monkeys infected with ¦Plasmodium coatneyi¦ (15).   Sixteen rhesus
monkeys were placed in four groups; group I consisted of three
monkeys which were splenectomized and were treated with 3 doses
(loading dose: 3.3 mg/kg; maintenance doses: 1.7 mg/kg) of
artesunate; group II consisted of three monkeys which were treated
with 3 doses of artesunate; group III consisted of two monkeys
which were treated with one dose (3.3 mg/kg) of artesunate; group
IV consisted of five untreated monkeys.   Parasitemia of these
groups ranged from 13.3 to 19.5% before treatment.   Twenty-four
hours after administration, the parasitemia was reduced to 2.2% in
group I and <0.1% in group II; parasitemia was lowered to 10.6% in
group III only three hours after drug administration.   The rate of
sequestration in the cerebral microvessels, which was 29.4% in
untreated animals, was <0.1% in groups I and II (24 hours after
treatment), and 2.0% in group III (3 hours after treatment).   These
data clearly indicate that artesunate not only reduced parasitemia,
but also reduced the rate of parasitized red blood cell (PRBC)
sequestration in cerebral microvessels.   In an immunohistological
study, endothelial-leukocyte adhesion molecule-1 (ELAM-1) became
negative in group I after treatment with artesunate, although the

presence of CD36, thrombospondin (TSP), intercellular adhesion molecule-1 (ICAM-1), IgG, and C3 in the cerebral microvessels was not altered. This is the first in vivo study to show that artesunate interferes with continued PRBC sequestration in the cerebral microvessels in cerebral malaria. By using this animal model, we may be able to evaluate strategies for management of severe malaria as well as for the development of antimalarials and vaccines to prevent and/or cure cerebral malaria.

d) Morphological effects of artemisinin in Plasmodium falciparum

In collaboration with Dr. Milhous of WRAR, ultrastructural changes induced in ¦Plasmodium falciparum¦ by artemisinin were studied in vitro. Two hours after administration, changes were observed in parasite mitochondria, rough endoplasmic reticulum, and nuclear envelope. At four hours, in addition to the earlier changes, nuclear membranes and, to a lesser extent, plasma membranes may form myelin figures. In addition, there may be disappearance of ribosomes, and destruction of food vacuole membranes. These changes may lead to the total disorganization of the parasites. About 30% of the parasites manifested these alterations. Electron microscope autoradiography was performed after [^{3}H]-dihydroartemisinin and [^{14}C]-artemisinin were administered to infected erythrocytes in vitro. These drugs consistently were located in mitochondria (Fig.5) and food vacuoles (Fig.6).

e) Human vascular endothelial cell adhesion receptors

The clinical complications associated with severe and cerebral malaria occur as a result of the intravascular mechanical obstruction of erythrocytes infected with the asexual stages of Plasmodium falciparum. In collaboration with Dr. Ockenhouse of WRAIR, we report that a primary P. falciparum-infected erythrocyte (parasitized red blood cell [PRBC] isolate from a patient with severe complicated malaria binds to cytokine-induced human vascular endothelial cells, and that this adhesion is in part mediated by endothelial leukocyte adhesion molecule 1 (ELAM-1) and vascular cell adhesion molecule 1 (VCAM-1). PRBC binding to tumor necrosis factor α (TNF-α)-activated human vascular endothelial cells is partially inhibited by antibodies to ELAM-1 and ICAM-1 and the inhibitory effects of these antibodies is additive, PRBCs selected in vitro by sequential panning on purified adhesion molecules bind concurrently to recombinant soluble ELAM-1 and VCAM-1, and to two previously identified endothelial cell receptors for PRBCs, ICAM-1, and CD36. Post-mortem brain tissue from patients who died from cerebral malaria expressed multiple cell adhesion molecules including ELAM-1 and VCAM-1 on cerebral microvascular endothelium not expressed in brains of individuals who died from other causes. These results ascribe novel pathological functions for both ELAM-1 and VCAM-1 and may help delineate alternative adhesion pathways PRBCs use to modify malaria pathology.

CONCLUSIONS: 14

As indicated in the section "Introduction" we have collaborated with scientists from WRAIR and AFRIMS in order to investigate morphological effects of drugs, particularly antimalarials on malarial parasites and hosts.

We performed immuno-electron microscopy to study intracellular localization and fate of liposomes and liposome-encapsulated malarial antigen after phagocytosis by macrophages, since the ability of liposomes to enhance immune responses is due to natural targeting of liposomes to macrophages. Our study indicated that liposome-encapsulated protein that is phagocytosed by macrophages can enter an intracellular compartment in which at least some of the antigenic epitopes are not degraded by lysosomal enzymes.

Our attempt to make a primate model for human cerebral malaria has been successful. PRBC sequestration and cytoadherence of knobs on PRBC to endothelial cells in cerebral microvessels were demonstrated in rhesus monkeys infected with P. coatneyi. These cerebral microvessels with sequestered PRBC were shown to possess CD36, TSP and ICAM-1. By using this animal model, we studied morphological effects of artemisinin on sequestered PRBC within cerebral microvessels. In addition, by using this model, we may be able to develop vaccine candidates to prevent human cerebral malaria.

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APPENDIX:

Figure Legend

Fig. 1   Immunoelectron micrograph of macrophages. The
          macrophages were fixed 6 hours after incubation with
          liposomes containing malaria antigen and the malaria
          antigen was detected by specific monoclonal antibody
          (pf 1B2.2) to the antigen followed by the treatment
          with gold-labeled second antibody. Densely labeled
          liposomes are present within a membrane bound vacuole.
          X 50,000

Fig. 2   Electron micrograph showing cytoadherence (arrow) of
          PRBC knobs to endothelial cells (E) of rhesus cerebral
          microvessels. X 56,000

Fig. 3   Electron micrograph showing PRBC sequestration in a
          cerebral microvessel of rhesus monkey. X 18,000

Fig. 4   Immunoelectron micrograph showing the presence of EBA-
          peptide 4 in micronemes (M) of P. falciparum merozoites.
          No gold particles are seen associated with rhoptries
          (R). X 100,000

Fig. 5&6 Autoradiography of P. falciparum treated with ^{3}H-
          dihydroartemisinin. Silver grains are associated with
          mitochondria (M) (Fig.5) x 64,000 and food vacuoles (F)
          (Fig.6). X 39,000
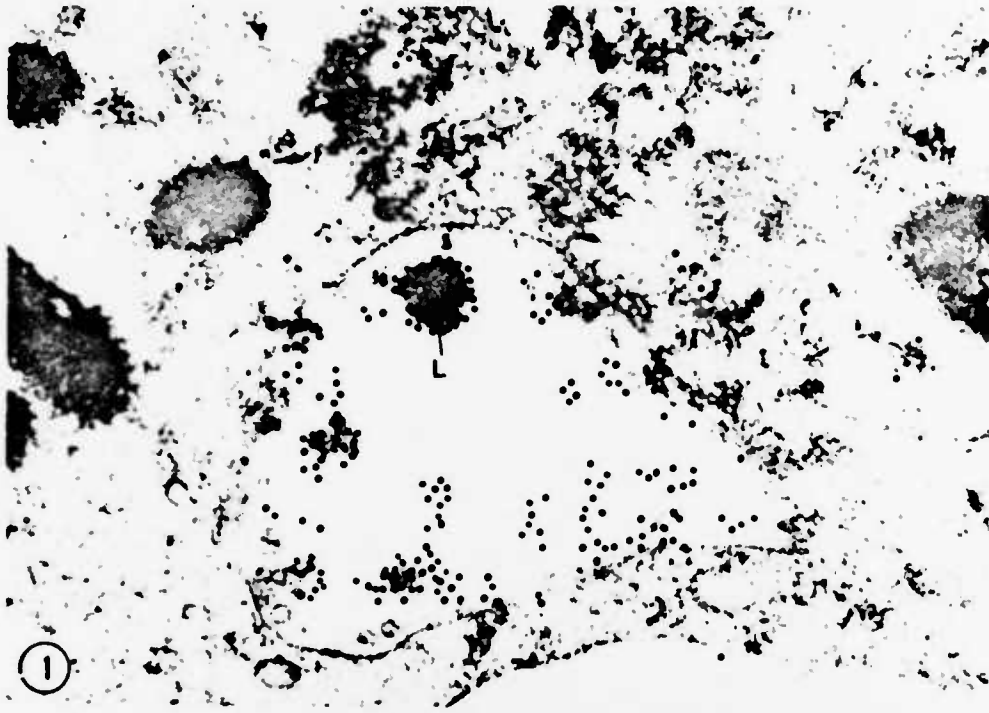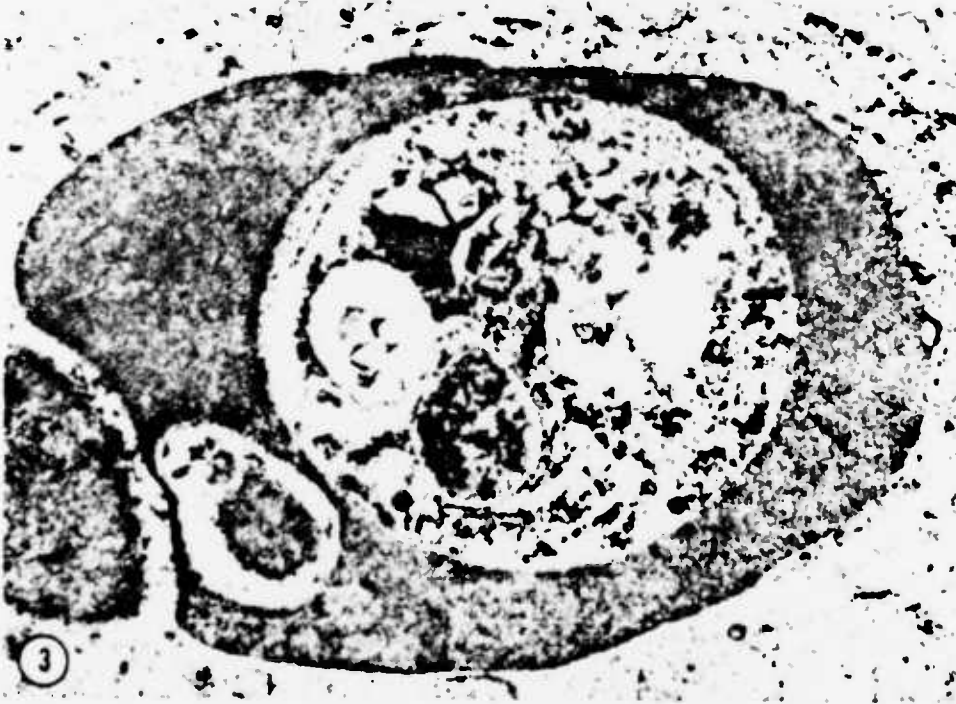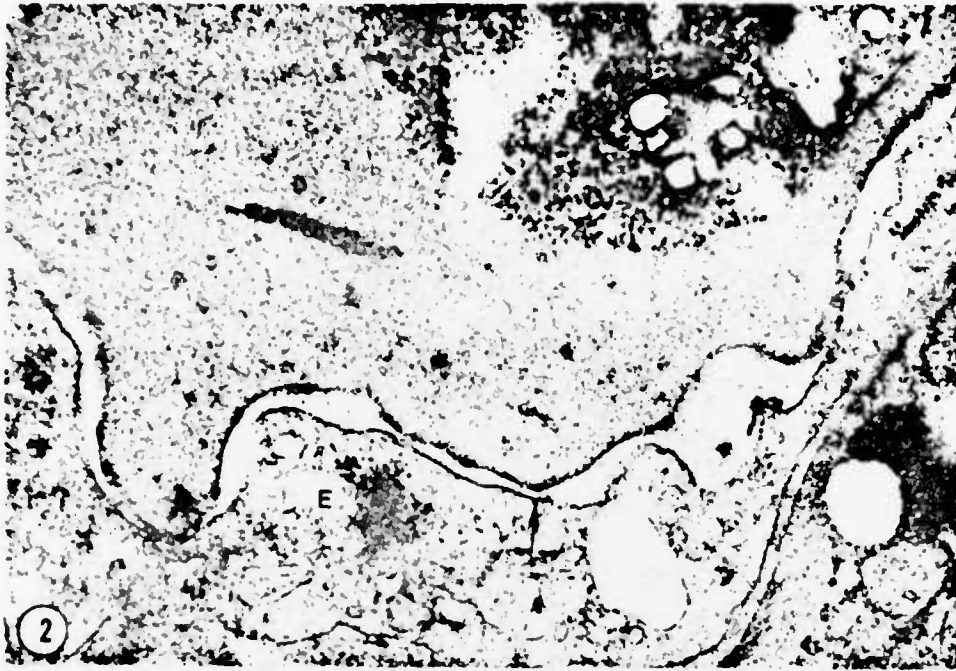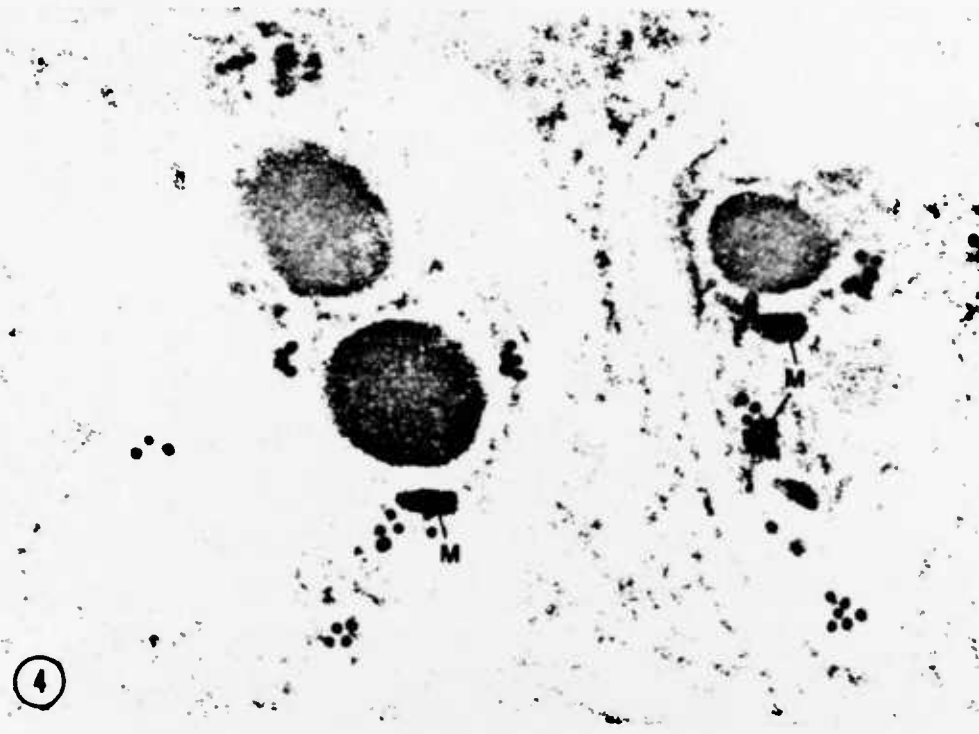
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