

IMMUNOPATHOLOGY AND HISTOPATHOLOGY
OF
MURINE CEREBRAL MALARIA

BY

BANCHOB SRIPA



A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
(PATHOBIOLOGY)

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OF
MURINE CEREBRAL MALARIA

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March 27, 1984.

Signature

B. Sripa

Banchob Sripa, B.Sc.

Candidate

Sommate Boonpucknavig

Sommate Boonpucknavig, M.D.

Preceptor

Pairote Prempre

Pairote Prempre, Ph.D.

Dean,

Faculty of Science

Mahidol University

Montree Chulasamaya

Montree Chulasamaya, M.D., Ph.D.

Dean,

Faculty of Graduate Studies,

Mahidol University.

EVALUATION OF THE FINAL EXAMINATION
THE DEFENSE OF THESIS

We, the members of the Supervisory Graduate Committee
for

BANCHOB SRIPA

unanimously approve the thesis entitled

IMMUNOPATHOLOGY AND HISTOPATHOLOGY

OF

MURINE CEREBRAL MALARIA

We further agree that he has satisfactorily defended
his thesis at the examination given by the Supervisory
committee

on

April 20th, 1984

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We recommend therefore that

BANCHOB SRIPA

be awarded the degree of MASTER OF SCIENCE in
Basic Medical Sciences (Pathobiology)
from Mahidol University

Signature

Sommate Boonpucknavig

Sommate Boonpucknavig, M.D.

Vijitr Boonpucknavig

Vijitr Boonpucknavig, M.D.

Panya Temcharoen

Panya Temcharoen, D.V.M., M.S.

Subhkiy A.

Subhkiy Angsubhakorn, D.V.M., M.

Galayanee Ekapanyakul

Galayanee Ekapanyakul, B.Sc., M.

M. Chulasamaya

Montree Chulasamaya, M.D., Ph.D.

Dean,

Faculty of Graduate Studies,

Mahidol University.

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BIOGRAPHY

NAME: BANCHOB SRIPA

DATE OF BIRTH: December 4, 1958

PLACE OF BIRTH: Ubonrajchathani, Thailand

INSTITUTIONS ATTENDED:

Benjamamaharaj School, Ubonrajchathani
March, 1975 : Certificate of M.S. III

Benjamamaharaj School, Ubonrajchathani
March, 1977 : Certificate of M.S. V

Khon Kaen University, Khon Kaen
March, 1981 : Bachelor of Science (Biology)
B.Sc.



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ABBREVIATIONS

BB	=	Borate buffer
BUN	=	Blood urea nitrogen
B1C	=	Beta one region of complement
°C	=	Degree centigrade or Celsius
CIC	=	Circulating immune complexes
FITC	=	Fluorescein isothiocyanate
FA	=	Fluorescent-antibody technique
g	=	Gram
γ	=	Gamma
H&E	=	Hematoxylin and Eosin
hr	=	Hour
i.p.	=	Intraperitoneal
i.v.	=	Intravenous
IEP	=	Immunoelectrophoresis
IFA	=	Indirect immunofluorescent-antibody technique
Ig	=	Immunoglobulin
Kg	=	Kilogram
LM	=	Light microscope
U	=	Micron
ul	=	Microlitre
M	=	Molar
mg	=	Milligram
ml	=	Mililiter

mm	=	Millimeter
min	=	Minute
mA	=	Milliampere
M.W.	=	Molecular Weight
nm	=	Nanometer
NSS	=	Normal saline solution
OD	=	Optical density
PB	=	Phosphate buffer
PBS	=	Phosphate-buffered saline
PEG-CC	=	Polyethylene glycol precipitation complement consumption
PMN	=	Polymorphonuclear cell
PRBC	=	Parasitized red blood cell
RBC	=	Red blood cell
rpm	=	Revolution per minute
SRBC	=	Sheep red blood blood cell
VB	=	Veronal buffer

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DEPARTMENT OF PATHOBIOLOGY

FACULTY OF SCIENCE, MAHIDOL UNIVERSITY

BANGKOK, THAILAND

ABSTRACT

Cerebral malaria was studied in three species of malaria parasite infections; P. berghei, P. chabaudi and P. yoelii. Mice were intravenously inoculated with 10^5 parasitized erythrocytes of each malaria parasite. For P. berghei infected group mice were sacrificed daily from day 2 to day 6 and thereafter sacrificed alternative day. While for P. chabaudi and P. yoelii infected groups, mice were sacrificed daily for 5 days and then sacrificed 3 days interval until day 20. Circulating immune complexes (CIC), antimalaria antibody, hematocrit and parasitemia were studied in relation to the immunopathological and histopathological findings. Immunofluorescent study, corpuscular

form of malarial antigen was first detected in the choroid plexus of P.yoelii infected mice on day 5. From day 8 onwards in all groups, both corpuscular and granular forms of malarial antigens were detected in all parts of the brain and were absent in mice infected with P.chabaudi and P.yoelii examined on day 20. High mortality and acute deaths occurred in P.berghei and P.chabaudi infections and, histopathologically, marked cerebral lesions were found in these groups by day 9, including congestion of meningeal and cerebral veins and capillaries, plugging of these vessels by heavy parasitized or "agglutinated" red cells and hemorrhage. Such lesions were minimal in P.yoelii infection and those sacrificed during the course of infection of all groups. The presence of cerebral lesions were not directly related to the level of CIC or antimalaria antibody or anemia. Most mice died with cerebral involvement showed no presence of antimalaria antibody. Markedly increase of parasitemia with a high proportion of irregular or spur-shaped red cells was observed in all acutely dead mice. Histopathological studies on tissue sections of liver, kidney, lung and spleen and immunopathological study of kidney sections were also described. The results suggest that less immune status of host and the alteration of rheologic properties of parasitized and non-parasitized erythrocytes may play in

important role in the pathogenesis of cerebral malaria in this study.



INTRODUCTION

Malaria still remains a major cause of morbidity and mortality in tropical and subtropical areas^(24,194), especially in Southeast Asia⁽⁷⁰⁾. One of the most serious and frequently fatal complication associated with human Plasmodium falciparum infection is cerebral malaria. Many investigators^(45,46,154,181,183) have recognized the brain lesions in patients who died of malaria disease. The typical pathological findings were petechial hemorrhages and capillary blockage with parasitized erythrocytes and/or malarial pigment. Various hypothesis have been raised up^(89,113,151,152,201) to explain the pathogenesis of the lesions but none is fully proven. Since histopathologically of human cerebral malaria studied were done in autopsy tissue which showed fully developed pathological appearances. Understanding the pathogenesis of cerebral malaria will lead to a proper treatments and may decrease the incidence of cerebral complicated malarial cases which is frequent cause of death in children and non-immune adults⁽¹¹⁵⁾.

The causes and effects of cerebral malaria were investigated in laboratory animal models either by histopathology or electron microscopy^(116,150,151,152). Wright⁽¹⁹⁷⁾ and Wright et.al.⁽¹⁹⁸⁾ had attempted to study

cerebral lesions in normal and neonatal thymectomized golden hamsters and found that non-thymectomized animals showed multiple small hemorrhages in the brains which were not seen in thymectomized hamsters. Yoeli and Hargreaves⁽²⁰²⁾ published a study of cerebral malaria in mice infected with a mutated strain of P.yoelii and severe brain hemorrhages were observed⁽²⁰⁴⁾. Susceptibility of different strain of mice were the one factor that can influence the severity of malaria infection⁽¹¹²⁾.

The pathogenesis of cerebral malaria is not entirely elucidated and the immunologic interaction between host and parasites appears to play an important role^(52,53, 197,198). The purpose of this experiment is to study the immunopathology and histopathology of mice infected with three different species of malaria parasites : Plasmodium berghei (P. berghei), P.chabaudi, and P.yoelii. Cerebral malaria was of particular interest in this study. Other pathological findings were also investigated in relation to parasitemia, circulating immune complexes (CIC), antimalaria antibody, blood urea nitrogen (BUN), and proteinuria.

LITERATURE REVIEWS

Murine rodent malaria has been intensively studied for over 30 years after the first discovery of malarial parasite of a rodent by Prof. Ignace Vincke in 1948. The rodent malaria parasite provides as an experimental model for a wide range studying of the disease since human malaria can be studied within limited conditions.

I. GENERAL HISTORY

Plasmodium berghei was discovered in thicket rat (Grammomys surdaster) by Vinke during his surveys in Katanga in 1948. The natural vector of this malaria parasite is Anopheles durenii millescampsii. P.berghei was named after Vinke's friend, Dr. Louis Van den Berghe who made a formal announcement of the new discovery at the Fourth International Congress of Malaria and Tropical Medicine in Washington in September 1948. From the early days of Vincke's discovery several strains of P.berghei were widely studied in different hosts and vectors. A complete cyclical transmission of several strains of P.berghei from Anopheline mosquitoes to various animals; including thicket rat (Thammomys), white mouse, rat and hamster were done by Yoelii and his colleagues⁽²⁰²⁾. During that period new species of rodent malaria parasites were also discovered. In 1965, Landau and Chabaud found a P.berghei-like Plasmodium in Thammomys

rutilans in Central African Republic and the new species was named P.chabaudi. After many discoveries of new species and subspecies of P.berghei of rodents, a taxonomic pattern of murine rodent plasmodia became increasingly complex. Killick-Kendrick⁽⁹³⁾ had gathered three subspecies of P.berghei-like parasite from the Lower Guinea Forest as a polytypic species of P.yoelii. At present, the taxonomy of rodent malaria parasites by Killick-Kenchick is now generally accepted. This classifies murine rodent malaria into four distinct species : P.berghei, P.yoelii, P.vinckei and P.chabaudi. This taxonomy is based on morphology of blood stage parasites, characteristics of sporogony, growth rate and size of preerythrocytic stage and the electrophoretic mobility of enzymes.

II. LIFE CYCLES

The life cycle of each murine Plasmodium is different in details of each stage, generally it is similar. This can be divided into 3 stages:

1. Preerythrocytic stage
2. Erythrocytic stage
3. Sporogonic stage

Preerythrocytic stage : This is the first stage after inoculation of sporozoites into host blood stream or by mosquitoes biting. Some sporozoites can enter the hepatocytes via blood stream and then differentiate into preerythrocytic schizonts. These schizonts will mature

within 42 to 72 hr after injection and release as merozoites into the circulation.

Erythrocytic stage : Merozoites enter red blood cells and undergo several cell divisions to trophozoites, schizonts and merozoites. These merozoites in turn can invade new red cells and again undergo schizogony. A certain number of schizonts will develop into gametocytes. Killick-Kendrick and Waren⁽⁹⁵⁾ have also shown that merozoites released from hepatocyte can develop directly into gametocytes.

Sporogonic stage : Gametocytes will be ingested by Anopheles mosquito via a blood meal. In the midgut of the infected mosquito, the gametocyte will transform into macrogametocyte and microgametocyte. Microgametocytes will undergo exflagellation, liberating male gametes. After fertilization take place, the motile ookinete will develop and pass through the midgut wall. At outer side of the midgut an ookinete develops into a mature oocyst within 8 to 15 days depending upon the environmental temperature. After maturation, the oocyst ruptures and liberates sporozoites moving toward the salivary glands. The sporozoites will move to the liver of the infected murine host and start the preerythrocytic stage.

III. BIOCHEMISTRY

A. Carbohydrate Metabolism

Glycolysis : The intraerythrocytic stage of rodent malarial parasites do not store glycogen or other polysaccharide⁽¹⁶³⁾. Glucose consumption per hour of 10^9 parasitized erythrocytes in vitro varies from 145 ug to over 18,000 ug while 10^9 free parasites consume about 60 ug to 1,200 ug. The glucose used by parasites is converted to lactate up to about 85%⁽³⁷⁾. Except lactate, no significant amounts of other metabolites of glucose metabolism by P.berghei have been identified. The enzymes involved in glycolysis have not been well studied because of some interferences of leukocytes and immature red cells in experiment.

Pentose phosphate pathway : This pathway in malarial parasites alone is not fully understood because of the interference from reticulocyte and mature red cells^(82,159). In P.berghei infected erythrocytes about 2% of glucoses can be used by this pathway⁽¹⁶⁾.

Citric acid cycle : Only 0.3-0.4% of the metabolized 6-¹⁴C-glucose are converted to labelled carbon dioxide in the presence of parasitized erythrocytes or free parasites⁽¹⁶⁾.

Carbon dioxide fixation : Nagarajan⁽¹²⁸⁾ reported that P.berghei, either free form or within the rat reticulocyte, can fix labelled carbon dioxide into organic acids, mainly malate. The results could be explained by the operation of the citric acid cycle, although the enzymes of this cycle could not be detected⁽¹²⁷⁾.

B. Amino Acid and Protein Metabolism

Protein digestion : P.berghei can degrade the hemoglobin of mouse erythrocytes, incorporate amino acids into its own protein and leave the unused haem as pigment⁽¹⁸⁰⁾. Cook et al., had investigated the parasite proteolytic enzymes in extracts of P.berghei released from mouse red cells. The extracts had two pH optimum for the digestion of hemoglobin, one at a pH of about 4, other at pH of about 8. The end-product of the digestion of hemoglobin by malaria parasites is malarial pigment. The composition of this pigment was studied in several species. Deegan and Maegraith⁽⁴¹⁾ suggested that the pigment of primate malaria contained haematin combined with other nitrogenous substance. Sherman et al.⁽¹⁶⁵⁾ reported that the nitrogenous part of the pigment of P.berghei was protein which produced by the partial digestion of hemoglobin. Homewood et al.,⁽⁷⁵⁾ pointed out that about 10% of the pigment appeared to be haemin.

Amino Acid Metabolism : The amino acid metabolism of rodent malarial parasites has not been well studied. Enzyme glutamate dehydrogenase which take part in amino acid metabolism were studied in P.chabaudi⁽¹⁸⁹⁾. The activity of glutamate dehydrogenase enzyme increases during the intraerythrocytic cycle of parasite, suggesting that it should originate in the parasite itself. P.berghei can

incorporate exogenous methionine and leucine into its protein^(179,180).

C. Lipid Metabolism

The study of synthesis of lipids by rodent malaria parasites is difficult to separate from that of reticulocyte and of white blood cells. Cenedella⁽²⁸⁾ found that cells from mice infected with P.berghei incorporated more glucose carbon into lipid than cells from normal mice. Evidence of oleic acid incorporation may suggest that P.berghei could obtain some part of its fatty acids from the plasma⁽²⁹⁾. It has been reported that P.berghei also contributes fatty acids to the plasma by breakdown of host lipids^(28,30).

D. Nucleic Acid Metabolism

The DNA of rodent malaria parasite is associated with its own histone and non-histone protein. DNA synthesis continues throughout the growth of the blood stage parasite. RNA is not well recognized and possesses fairly typical eukaryotic ribosomes consisting of ribonucleoprotein as 2 subunits, 60S and 40S. In higher organisms these subunits are derived from a precursor synthesized in the nucleolus, the structure is not so far recognized in murine plasmodia. It has been suggested that the host itself may synthesize one of the RNA subunit of parasite⁽¹⁸²⁾. Rodent malaria parasites can use exogenous purines for nucleic acid

synthesis. It is unable to utilize exogenous pyrimidines, but it can be synthesized by the parasite de novo.

Whereas the parasites recycle purines extensively by salvage pathways they appear to lack the necessary pathways for recycling purimidines⁽¹³¹⁾.

IV. IMMUNOLOGY OF RODENT MALARIA

Immune responses in rodent malaria have been investigated extensively both in humoral and cell mediated immune responses^(133,209). The information from the studies can be applied for human malaria in many types of research such as vaccine production, chemotherapy, etc.

A. Immunity to Malarial Infection

Host resistance to malaria infection had evidenced from the innate resistance to infection, actively acquired immunity and passive transferred immunity.

Innate immunity : Host factors should play a significant role in resistance to infection since a particular host has been found to be susceptible to certain species of Plasmodium but resistant to others^(71,72). Greenberg and Kendrick⁽⁶⁵⁾ had studied the susceptibility of rodents to infection and found considerable differences in various strain of rodents. Some species were susceptible to infection, others developed low-grade infections. However, the differences in the course of infection were unclear in rodents. In primate, there is evidence indicating

that susceptibility to plasmodial species depend on receptors on surface of erythrocyte membrane⁽²⁶⁾. These specific receptors are responsible for invasion of merozoites⁽¹¹⁸⁾. Age is also an importance factor of the innate immunity in some rodents⁽²⁰⁸⁾. Young rats are found more susceptible to P.berghei infection than adult rats whereas no this evidence in mice.

Actively acquired immunity : Mice recovered from blood-induced P.berghei infection by administration of antimalarial drugs can develop resistance to challenge^(17,39). The use of antimalarial drug during infection allows the host to acquire a certain degree of immunity. Diet or food is also a factor that can influence host immunity. Mice maintained on para-aminobenzoic acid-deficient milk diet during infection developed a strong and long-lasting resistance to subsequent challenge of P.berghei infection⁽⁸⁸⁾. Immunization with parasite antigen can also induce protective immunity to malarial infection. Partial protection to P.berghei infection was obtained by immunization with formalin-inactivated blood-stage parasites^(123,124,126), irradiated blood-stage parasites^(78,177). Mice infected with P.yoelii have a protection to rechallenge with blood infection^(58,125). Many adjuvants have been employed to potentiate the protective immunity to different malarial parasite in experimental animals^(44,106,107). Recently,

mice immunized with purified protective antigen on merozoite or schizont of P.yoelii recognized by monoclonal antibody have shown to have protection against challenge infection^(55,59). Immunity to sporozoite infection have been studied in various ways^(1,5,6,69,136). The irradiate-inactivated sporozoite seem to have the most effective protection. The protective antigen on sporozoite surface was found to be the protein of M.W. of 44,000 (Pb 44) by using hybridoma technique^(206,207).

Passively acquired immunity : Humoral factors play an important role in protection either by congenital or serum transfer. Immunity to P.berghei infection can be transmitted through the milk of immune mother rats to their offsprings⁽²³⁾. Passive transfer of immune serum can induce some degree of protection against plasmodial infection in rat^(25,42). But in mice treated with rat hyperimmune serum expressed only significant delay in patent infection and all eventually died with high parasitemias^(19,61). Protective activity to challenge infection of P.yoelii has been observed in mice received hyperimmune serum^(58,87,125). Whereas the passive transfer of immune serum of P.chabaudi induced a delay in the onset of parasitemia⁽¹¹⁰⁾.

B. Humoral Immune Response

Antibodies to blood stage of Plasmodia : Generally,

humoral factors represent, in part, an important role in acquired immunity to plasmodial infection^(34,43,62,144,155). It is evident from the polyclonal B-cell activation and the infection^(56,66). A consequence of polyclonal B-cell activation is a marked increase of immunoglobulin (Ig) levels. These antibodies are specifically directed against plasmodial antigens. Only a small part of these antibodies display a protective activity against malaria infection⁽⁶⁶⁾. The mechanisms which may be involved in antibody-mediated protection against blood stage malarial parasites are 1) inhibition of merozoite invasion by binding of merozoite ligands^(117,140,142,195), 2) inhibition of merozoites release from schizonts⁽⁶⁴⁾, 3) agglutination or opsonization or phagocytosis of merozoites⁽¹⁸⁴⁾, schizonts, and trophozoites^(81,100), 4) antibody-dependent cellular cytotoxicity of schizonts and trophozoites⁽²⁰⁾ 5) inhibition of PRBC binding to endothelial cells⁽¹⁸⁶⁾, and 6) blocking erythrocyte receptors for merozoite⁽¹¹⁷⁾. A number of experiments have been done to clarify the mechanisms of action of protective antibodies^(35,59,124,148). Recently, experiment using monoclonal antibodies raised against P.yoelii, upon passive transfer, can react with merozoites or schizonts and protected mice from lethal P.yoelii infection⁽⁵⁹⁾. The P.yoelii antigens recognized by monoclonal antibodies have been identified and purified.

Antibodies to sporozoite : Anti-sporozoite

antibodies can be detected after the sporozoite entering the host by many investigations^(130,134,174). The protection against sporozoite infected rodents can be achieved for months by intravenous injection of irradiated sporozoite or repeated bites of infected mosquitoes^(153,172). Studies using IFA technique have indicated that sera from mice immunized with sporozoites recognized four classes of antigens: species-specific antigens, stage-specific antigens, antigens shared with other developmental stages (e.g. blood stage), and mosquito antigens contaminating during the sporozoite preparation used for immunization⁽⁵⁾. On hybridoma technique the monoclonal antibody producing was found to react with a sporozoite surface component of 44 K (Pb 44)^(146,206). This component, Pb 44, is stage specific antigen and rodent-specific antigen. This antigen is densely distributed in sporozoites located at the salivary glands but sparsely distributed or absent in sporozoites located at the mid gut of the mosquitoes⁽²¹⁾. This antigen (Pb 44) was also found on the sporozoite of P.yoelii nigerinensis but not in either simian or human malaria parasites. Monoclonal antibodies directed against Pb 44 antigen also conferred protection against lethal infection of P.berghei in mice by passive transfer^(146,206) or in vitro⁽⁷³⁾.

C. CELL-MEDIATED IMMUNE RESPONSE

The immune response to malaria parasite infection is a complex process involving both humoral and cell-mediated responses. Initially, the adoptive transfer of spleen cells from immune animals was shown to confer protection^(141,156). Many investigators have also demonstrated the involvement of T cell in immunity^(84,86,155). This was evident by Roberts and Weidanz⁽⁵⁰⁾ who reported that B-cell deficient animals recovered from infection with P.yoelii were resistant to reinfection. It was suggested that antibody independent cell-mediated response played a role in protection against malaria infection. The specific delayed type hypersensitivity (DTH) detected in P.chabaudi and P.yoelii infected mice implied the cell-mediated immunity^(51,144). Passive transfer of P.yoelii immune serum to the recipient mice was found to have a protection against P.yoelii infection. This serum transfer produced only transient inhibitory effect in T cell-deprived recipients, whereas immune spleen cell transfer conferred a significant degree of protection⁽⁸⁶⁾.

Thymectomized rats are more susceptible to P.berghei infection than intact controls⁽²⁰⁹⁾. Congenitally athymic, (nu/nu) mice developed a lethal infection upon inoculation with a mild strain of P.yoelii⁽³³⁾. The same was found in the mice administered with antithymocyte serum

(ATS)⁽¹⁷¹⁾. Cerebral hemorrhage development was found in intact hamster infected with P.berghei while neonatal thymectomized or ATS treated hamster did not^(197,198). In addition, it has been observed recently that T cell-deficient nu/nu mice did not exhibit cerebral malaria⁽⁵³⁾.

D. IMMUNOSUPPRESSION

The immunosuppression have been found during the course of malaria parasite infection^(190,209). Humoral response is depressed upon administration of unrelated antigen, such as tetanus toxoid⁽⁴⁾, BCG⁽¹⁷⁰⁾, human transferin⁽¹⁷⁵⁾. Eventually, mice infected with blood stages of P.berghei show an impaired host resistance to immunization with irradiated sporozoites of the same malarial parasite⁽¹³⁵⁾. The immune response, measured by anti-sporozoite antibody, was severely depressed after the blood-stage immunized mice were infected with irradiated sporozoites. Alteration of cell-mediated response was also found during malaria infection⁽⁸⁵⁾. In P.yoelii-infected euthymic mice both splenic plaque-forming cells and serum antibody to pneumococcal polysaccharide (SSSIII) were severely depressed whereas in athymic nude mice and antithymocyte-treated mice were normal⁽¹²²⁾. Suppression of immune response of mice infected with P.chabaudi⁽³⁸⁾ or P.yoelii⁽¹⁹²⁾ to mitogen was also investigated.

Non-specific suppression to malaria infection may be, in

part, due to macrophage⁽¹⁰¹⁾ and suppressor T cells⁽¹⁰²⁾. Recently, Khansari et.al.⁽⁹¹⁾ had extracted a soluble suppressive factor derived from P.berghei infected blood and found to have suppressive activity. However, the mechanism of immunosuppression have not yet been elucidated.

V. PATHOLOGY OF MALARIA INFECTION

The pathological changes in rodent malaria infection have been studied in various aspects^(112,27). Most of the pathological changes are related to the intensity of parasitemia^(98,99). P.berghei parasite has been studied more than P.yoelii and P.chabaudi parasites.

Brain : The pathological changes seen in the brain of mice^(53,112,150,202) and hamster^(152,198) infected with murine malaria parasites have been studied in both of histopathological and electronmicroscopic levels. Typical changes were cerebral hemorrhage and congestion of blood vessels, which is similar to those found in human cerebral malaria⁽²⁷⁾. Grossly, the brains show petechial hemorrhage and congestion of cerebral capillaries. Microscopically, congestion affected meningeal and many intracerebral veins and capillaries and choroid plexus. Central chromatolysis of Purkinje cells and neurone was associated with the area of hemorrhage and occlusion⁽¹⁵⁰⁾.

Extravasated blood contained both parasitized and non-parasitized erythrocyte at the site of hemorrhage.

Ballooning and aneurism formation in blocked capillaries were observed in mutant P.yoelii infection⁽²⁰²⁾.

Electronmicroscopic study revealed that numerous monocytes attached to the endothelial wall of blood vessel⁽¹⁵²⁾.

But there have not been demonstrated concurrent thrombosis in murine cerebral malaria. Immunological study show granular deposits of IgG in the basement membrane of the choroid plexus but these immune complexes seem not to play an important role in the cerebral pathology^(89,112).

Kidney : Abnormality of kidney functions have been described in mice⁽¹³³⁾. The principle histological changes are congestion of veins and capillaries, interstitial hemorrhage, infiltration of mononuclear cells and accumulation of pigment-laden macrophages in vessels and glomerular tufts^(8,167). Slightly proliferation of mesangial cells was also observed^(9,167). Focal glomerulonephritis was demonstrated in hyperimmune state⁽¹³⁾. Mouse Ig, B1C globulin and malarial antigen have been detected in complexes localized in the glomerular capillary wall^(9,10,11,90) mostly in mesangial area⁽¹⁷⁶⁾. These immune complexes deposits may be the cause of proteinuria in rodent malaria⁽¹²⁾.

Liver : Liver is grossly enlarged, dark brown in color due to malarial pigments. The histopathological findings found in the liver include congestion of hepatic and portal veins, distension of sinusoids by swollen Kupffer cells filled with malarial pigment, portal infiltration of monuclear cells and extramedullary haemopoiesis (111,168,187).

Lung : P.berghei infection results in a striking accumulation of pigment-laden phagocytic cells within the pulmonary alveolar capillaries. During parasitemia develop, the increase of vascular permeability was observed with lung edema (193). In some cases, there have been shown that the immune complexes deposit are IgG and IgM in the alveoli and adherent to the vascular walls (192,193).

Spleen : Splenomegaly has been observed in malarial infection. The spleen is dark brown in color. Striking histological changes affected both red and white pulps. Congestion of red pulp and hyperplasia of white pulps have been observed as infection progress. The sinusoids are greatly expanded as a result of a marked increase in the number of reticuloendothelial cells which show erythrophagocytosis (112). Accumulation of pigment-laden phagocytic cells in the red pulp and extramedullary haemopoiesis increase during the development of infection.

Anemia : The association of anemia with malaria has been known for a long time. The obvious cause of anemia in malaria was the destruction of erythrocytes⁽⁹⁸⁾. A number of mechanisms have been postulated to explain the cause of anemia. These include a possible concomitant infection^(104,109), alterations of the erythropoietic mechanism^(54,108), erythrocyte membrane alterations and/or hemolysis by the action of parasite products^(49,74,96), autoimmunity^(54,157), and reticuloendothelial hyperphagocytosis directed against host erythrocytes^(158,164,166,210). All of these mechanisms seem to be responsible for the cause of anemia⁽²⁰⁹⁾. Recently, malaria-associated anemia has been attracted on non-parasitized erythrocytes. The clearance of ⁵¹Cr-labelled nonparasitized erythrocytes separated from a P.berghei infected mouse were more rapid than normal red cells after intravenous injection into normal mice⁽⁷⁹⁾. These may be explained by the alteration of surface membrane of non-parasitized erythrocytes⁽⁶⁸⁾. This leads to rapid clearance from the circulation by the spleen⁽¹⁶⁶⁾ and subsequently cause anemia. The study of survival time of erythrocytes is reduced for 4-5 weeks in recovered patient in P.falciparum malaria⁽¹⁹⁶⁾.

EXPERIMENTAL DESIGN

One hundred and twenty mice were divided into three groups of 40 mice. The malaria parasites used were Plasmodium berghei (P.berghei), P.chabaudi, and P.yoelii. Mice were inoculated with 1×10^5 parasitized erythrocytes intravenously (i.v.) by lateral tail vein of each strain of malaria parasites. Three experimental mice and one normal mice were killed as schedule and summarized in fig 1.

On the day of killing, mice were decapitated under ether anesthesia and blood was taken by heart puncture. Brain, kidney, liver, spleen, and lung, were quickly removed for immunopathological and histopathological studies. Immunofluorescent staining of frozen tissue sections was done in the same day. For histopathological studies, kidney, and spleen, were fixed in Zenker-formol solution, the other organs were fixed in 10% neutral buffered formalin.

Blood : Blood smears were prepared to determine the parasitemia by Giemsa staining. Hematocrit were also done by using the method of Benjamin⁽⁷⁾.

Serum : Serum was collected for determination of malarial antibody titre by indirect immunofluorescent technique (IFA)⁽¹²⁹⁾, for circulating immune complexes by polyethylene glycol precipitation

complement consumption test (PEG-CC)⁽¹⁸⁾ and for blood urea nitrogen (B.U.N.) by the method of Croker⁽⁴⁰⁾.

Urine : Urine was collected for determination of protein. All fluorescein-isothiocyanate (FITC)-labelled antibodies were prepared for use during the experiment.

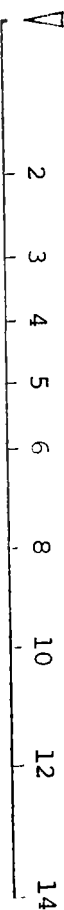


FIG. 1 EXPERIMENTAL DESIGN

Number of mice : 40 mice/group

10^5 PRBC (i.v.)

P. berghei



10^5 PRBC (i.v.)

P. yoelii



10^5 PRBC (i.v.)

P. chabaudi



day of sacrifice

Control : kill simultaneously with the experimental group

Sample size : Experiment group = 3 mice killed
control = 1 mouse killed

Collections : Brain Malarial antigen (all organs)

kidney FA Mouse γ -globulin and FLC (kidney)

Spleen 10% Neutral buffer fromalin (all organs)

Liver LM Fixed in

Lung Zenker-formal solution (kidney and spleen)

Blood $\triangleright$ Parasitemia, Hematocrit

Serum $\triangleright$ Antimalarial antibody, CTC, BUN, Urine Proteinuria

PRBC = Parasitized red blood cells, FA = Fluorescent antibody study, LM = Light microscopic study

CTC = Circulating immune complexes, BUN = Blood urea nitrogen, i.v. = intravenous injection

MATERIALS AND METHODS

1. ANIMALS

Female Swiss Albino mice of random breeding, ICR strain, aged 8-12 weeks were used throughout the experiment. These mice were bred at the Laboratory Animal Center of the Faculty of Science, Mahidol University and maintained in an animal room of the Department of Pathobiology. Mice were housed in metal cages and fed pellet (Gold Coin Ltd. Singapore) and water ad libitum.

2. MALARIA PARASITES

Plasmodium berghei was maintained in Swiss albino mice by intraperitoneal injection of 0.1 ml of heparinized parasitized blood in each mouse at weekly intervals. The mortality rate of this animal is 100% within 12 days of infection. The parasite has been maintained in the Immunopathology, Laboratory, Department of Pathobiology, Faculty of Science, Mahidol University for several years.

Plasmodium yoelii and Plasmodium chabaudi were obtained from Dr. P. Perlmann of Department of Immunology, University of Stockholm, Denmark and maintained in the Immunopathology Laboratory by intraperitoneal passage (i.p.) in Swiss Albino mice at 10 days intervals. Occasionally, they were frozen in liquid nitrogen using the method of Yoelii et.al. (1963)⁽²⁰⁶⁾. Cryopreserved parasites were

passaged once before used. P.yoelii is normally non-lethal and developing self-limiting infection within 3 weeks. P.chabaudi is partially-lethal in Swiss Albino mice from our experiences.

3. ANTIBODY PREPARATIONS

3.1 Mouse Antiserum to P.berghei

Mouse hyperimmune sera were produced in Swiss Albino mice against P.berghei. Forty mice were inoculated with 1×10^5 parasitized erythrocytes by i.p. Parasitemia was determined by means of Giemsa method. When the parasitemia reached about 5-10% all mice were treated with 5% chloroquine phosphate (1:100) in drinking water and 0.8 mg per mouse intraperitoneally on 5 consecutive days. The parasitemia was decreased and finally absent. Two months later these mice were inoculated again with 1×10^6 parasitized erythrocytes. The antibody titre were determined by indirect immunofluorescent test on blood smear of P.berghei. A titre of 1:4096 or higher was considered to be used as a hyperimmune serum antibody. Pooled antisera to P.berghei were kept at -20°C until used for conjugation with fluorescein isothiocyanate (FITC).

3.2 Mouse Antiserum to P.chabaudi and P.yoelii

The preparation of mouse hyperimmune sera against P.yoelii was the same as P.chabaudi. Forty mice were immunized with 5×10^5 parasitized erythrocytes. Mice

survived from primary infection were intraperitoneally challenged with 25 microlitre of pack parasitized erythrocytes twice a month. The serum titres were determined as above. Antisera were stored at -20°C until used.

3.3 Rabbit Antiserum to Normal Mouse Globulin

Mouse γ -globulin was obtained from normal mouse serum by precipitation with 40% saturated ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$ solution, at 4°C . Two volume of saturated $(\text{NH}_4)_2\text{SO}_4$ solution was added to 3 volume of the serum dropwise with constant stirring. After further stirring for at least 15 min, the globulin was centrifuged at 10,000 rpm for 10 min, then discarded the supernatant. The globulin precipitate was redissolved in a minimum volume of sterile phosphate-buffered saline (PBS, 0.01 M pH 7.1). The globulin solution was dialysed against 3 changes of PBS (2 hr, overnight, and 2 hr) in cold room (4°C). After dialysis, the globulin solution was centrifuged at 10,000 rpm for 30 min to discard any formed precipitate. The supernatant was determined for protein concentration by measuring absorbance at 280 nm (see Appendix). The globulin solution of 5 mg/ml was emulsified with an equal volume of Freund's incomplete adjuvant (Difco laboratories, Detroit, U.S.A.). Rabbit (body weight about 3 kg) was injected with 0.2 ml of the mixture in each footpad by weekly for one

month. Blood was collected from rabbit producing the strong antibody after testing by immunoelectrophoresis (IEP) method (see Appendix). Blood was bled via central ear artery puncture and allowed to clot at room temperature for 3 hr. The serum was recovered by centrifugation at 2,500 rpm for 10 min and stored at -20°C .

3.4 Rabbit Antiserum to Mouse β 1C Globulin

Rabbit antiserum to mouse β 1C globulin was carried out by the method of Mardiney and Müller-Eberhard⁽¹¹⁴⁾ with some modifications. Five hundred milligrams of zymosan (Schwarz/Mann Orangeburg, New York.) were boiled in 50 ml of normal saline solution (NSS) for 30 min in water-bath. The heat-treated zymosan was isolated by centrifugation at 3,000 rpm for 15 min. Eleven millilitres of pooled normal mouse serum was added to the zymosan pellet, mixed and incubated at 37°C for 1 hr with constant stirring. The mouse β 1C was activated and formed insoluble zymosan complement complexes at this temperature. The mixture was centrifuged at 3,000 rpm for 15 min, the supernatant was kept at -20°C for purification of rabbit antimouse β 1C. The zymosan complement complexes were washed sixty times each of 45 ml of cold isotonic Veronal buffer, pH 7.6. Then, 1 ml of zymosan complement complexes (approximately 40 mg/ml) was suspended in 3 ml of Freund's incomplete adjuvant (Difco Laboratories) and 0.1 ml of the mixture was

injected into each footpad of healthy rabbit by weekly for 2 weeks. On the third week, the antiserum was tested for the presence of one strong precipitin line at the B-region by IEP method. Then, the rabbit was bled, the serum was collected and stored at -20°C . If the precipitin line was not strong, continued injection at weekly interval was available. Frequently, continued injection led to the production of heterogenous antisera. This antiserum must be absorbed with appropriated antigen to remove the contaminating antibodies before use.

The purification of rabbit antimouse βLC serum was performed as previously described by Avamease and ternynch⁽³⁾ with some modifications. The supernatant removed from zymosan complement complexes as mentioned above was used as an immunosorbent for absorbed the contaminating proteins. Dropwise of 3 volume of 2.5% glutaraldehyde solution into 10 volume of supernatant with constant stirring and let standing for 3 hr at room temperature without agitation. The protein molecules will cross-linked to one another and form jelly-liked insoluble protein. The insolubilized protein was dispersed in 0.2 M phosphate buffer pH 7.2 (PB) with 18 gage syringe, centrifuged at 3,000 rpm for 15 min and repeated the step for 2-3 times. The immunosorbent was then washed in PB until the supernatant showed no presence of protein. This immunosor-

bent was ready to used for purification of rabbit anti-mouse β 1C serum. An appropriate volume of antiserum was mixed with the immunosorbent. The mixture was shaken gently for 30 min at room temperature and then centrifuged at 3,000 rpm for 15 min at 4°C. The supernatant was tested by IEP against normal mouse serum, only one precipitin line should be observed in β_1 region just beneath the well.

4. PREPARATION OF CONJUGATES : FITC-LABELLED ANTIBODY

Antisera to P.berghei, P.chabaudi, P.Yoelii, mouse β 1C, and mouse γ -globulin, obtained as previously described, were labelled with fluorescein dye by using the method of Nairn⁽¹²⁹⁾. The general procedure was as following. Gammaglobulin was fractionated from each antisera and adjusted the protein concentration to 20 mg/ml with sterile PBS. Dropwise of 2 volumes of 0.5 M carbonate-bicarbonate buffer pH 9 into 1 volume of γ -globulin solution with constant stirring in ice bath. One milligram of fluorescein isothiocyanate (FITC), isomer I (Sigma) was added to 1 ml of globulin solution with continuous stirring for 30 min at 4°C. After conjugation, the unreacted fluorescent material was removed by gel filtration on Sephadex G-25 column in PBS. The column volume was approximately six times of the reaction volume (see Appendix). Gel filtration was carried out in cold room and the first yellow fraction was collected. The conjugated globulin

was absorbed with rat liver powder at a ratio of 100 mg powder per 1 ml original serum. The mixture was shaken for 2 hr at room temperature, then centrifuged at 10,000 rpm for 15 min. The supernatant of conjugate was collected and then concentrated by precipitation with saturated $(\text{HN}_4)_2 \text{SO}_4$ as previously described. The conjugate was then dialysed against PBS with three changes and centrifuged. The supernatant was tested for specificity against appropriated antigen by IEP. Non-specific staining of the conjugate was removed by further absorption twice with rat liver homogenate. Absorption was carried out by shaking 2 volumes of conjugate with one volume of the homogenate each for 2 hr at room temperature. The conjugate was recovered by centrifugation at 10,000 rpm for 20 min. This stock conjugate will be diluted to appropriated dilution when use. Stock conjugate was kept at -20°C .

5. IMMUNOFLUORESCENT STUDY OF TISSUE SECTIONS

Experimental mice were killed under ether anesthesia and blood was taken by heart punctured. Brain, kidney liver spleen and lung were quickly removed and cut into small block about 2 mm thick and $4-5 \text{ mm}^2$. These tissue blocks were rapidly frozen in dry ice. Frozen tissue were sectioned in cryostat (Tissue-Tek[®] Microtome cryostat, Ames Company) at 4 μ thick. The tissue sections were attached to glass slides and then fan-dried for 2 hr before staining.

5.1 Detection of mouse γ -globulin or mouse β LC

Kidney sections were washed in PBS for 15 min and fan-dried for 30 min. The fluorescein-labelled rabbit anti-mouse globulin or mouse β LC was applied by loop technique and incubated in a moist chamber for 30 min. The slides were washed twice in PBS and mounted with buffered glycerin.

5.2 Detection of Malaria Antigen

The sections detected for malarial antigen, after fan drying, were fixed in 2-octanol at -20°C for 30 min. Then these sections were fan dried for an additional hour and stained with conjugated antimalarial antibody as mentioned above.

The slides were then examined under a fluorescent microscope (Reichert, Austria).

6. HISTOPATHOLOGICAL STUDIES

Brains, Livers, kidneys, spleens and lungs were taken from mice killed as the experimental design. The organs were removed within a few minutes of death. The kidney and spleen were fixed in Zenker-formol solution for 2-3 hr and washed in running tap water for 4 hr. These fixed organs were kept in 70% alcohol until processing. Other organs were fixed in 10% neutral buffered formalin. All tissue sections were embeded in paraplast, sectioned at 4-5 μm thick and staining with haematoxylin-Eosin (H & E) for histopathological studies.

7. SERUM TITRE DETERMINATION

The malaria antibody titre was determined by indirect immunofluorescent antibody test (IFA). Blood smears from a mouse in the erythrocytic phase of infection (parasitemia about 10-40%) with each malaria parasite; P.berghei, P.yoelii, and P.chabaudi, were used as a source of malarial antigen. The unfixed blood smears were stored at -20°C up to 6 months without any loss of antigenicity. Antigen slides were removed from the storage, fan dried for 2 hr and fixed in cold acetone for 15 min. After additional 30 min drying serial two folds dilution of the tested sera were applied and incubated in a moist chamber for 30 min at room temperature. The slides were washed twice with PBS each for 5 min of gentle agitation and fan dried. Fluorescein isothiocyanate-labelled anti-mouse γ -globulin was applied and the slides were again incubated for 30 min. The slides were washed with PBS as above, mounted in buffered glycerine and examined under a fluorescent microscope. The highest dilution of antiserum giving unambiguous fluorescent staining of malarial parasites was considered the end-point.

8. DETECTION OF CIRCULATING IMMUNE COMPLEXES BY POLYETHYLENE GLYCOL PRECIPITATION COMPLEMENT CONSUMPTION METHOD (PEG-CC) ⁽¹⁴⁾

Ten millilitres of SRBC in Alsever's solution was centrifuged at 3000 rpm for 5 min, removed the supernatant, buffy coat, and upper layer of erythrocytes. The cells were

washed three times with normal saline solution and resuspended 0.5 ml of packed cells in 9.5 ml of a 1:5 dilution of VB solution. After thoroughly mixing, 200 μ l of cell suspension was mixed with 2.2 ml of 0.1% Na_2CO_3 solution in distilled water and stand for 15 min. The suspension was centrifuged at 3,000 rpm for 5 min and the supernatant was read at O.D. 545. The suitable dilution of cell suspension was calculated from the O.D. of the supernatant to give a final value of 1 as the following equation:

$$V_t = V_i (\text{OD}/1)$$

V_i = the volume before adjustment

V_t = the volume to be adjusted

O.D. = the measured value

The volume was adjusted from 10 ml to V_t with 1:5 dilution of VB and thoroughly mixed and kept for 30-60 min at 4°C.

The sensitization was assayed by mixing 10 ml of the adjusted SRBC suspension with 100 μ l of rabbit antish sheep hemolysin and incubated in water bath at 37°C for 30 min with gentle shaking.

Polyethylene Glycol Precipitation

All samples, glass test tubes, reagents and buffers were precooled in ice bath before starting the test. Fifty microlitres of serum sample and 150 μ l of BB were mixed in glass test tube by a vortex mixer.



One ml of 2.75% PEG was added and mixed, and another 1 ml of 2.75% PEG was added. The tubes were incubated at 4°C for 90 min and centrifuged at 3,000 rpm for 20 min, 4°C. The supernatants were carefully and thoroughly aspirated with a water suction with a bent Pasteur pipette. The precipitates were suspended in 1 ml of 3.5% PEG, mixed, and centrifuged. The supernatants were aspirated as above. The precipitates were redissolved in 260 ul of 1:5 dilution of VB, mixed and shaken for 30 min at 4°C. The suspension were kept overnight at 4°C.

Complement mediated hemolysis

Eighty microlitres of redissolved precipitates were mixed with 80 ul of appropriate diluted complement, mixed immediately after each addition and incubated at 37°C for 30 min with gentle shaking. One hundred microlitres of sensitized SRBC were added in the same as the complement. The tubes were shaken after each addition. After 30 min incubation with gentle shaking in the water bath at 37°C, the tubes were removed and 1 ml of cold PBS was added.

The tubes were centrifuged at 3,000 rpm for 5 min and read the absorbance of the supernatant at wavelength 545 nm.

Normal serum control was also tested.

The results were expressed as OD 545.

OD 545:	0 - 0.250	=	+++
	0.250 - 0.450	=	++
	0.450 - 0.650	=	+
	650 -	=	Negative

9. HEMATOCRIT DETERMINATION

Hematocrit determination was done by the microhematocrit method⁽⁷⁾. Blood of experimental mice were collected in heparinized capillary tubes and centrifuged at 7,000 rpm for 10 min with high speed centrifugation. The hematocrit values were recorded.

10. SERUM BUN DETERMINATION

Serum B.U.N. was assayed following Croker (1967)⁽⁴⁰⁾. Fifty microlitres of serum sample were mixed with 3 ml of oxime solution and 3 ml of acid solution. This solution was boiled in water bath for 15 min and then cooled immediately. After the solution was measured at O.D. 520, the B.U.N. concentration was calculated from the calibrated curve as mg %.

11. URINE PROTEIN DETERMINATION

An appropriate-diluted urine of 0.5 ml was mixed with 2 ml of sulfosalicyclic acid sodium sulfate mixture (SSA-SS reagent) by inverting the tube for 6-8 times. Let the mixture stand at room temperature for exactly 10 min. Then concentration of proteinuria was calculated from the

calibrated curve as mg %.

12. PARASITEMIA DETERMINATION

Blood from tail of experimental mice were smear on glass slides, fixed in 95% methanol stained with Giemsa stain and examined under light microscope at 1000 x magnification. Parasite nuclei were stained red-purple and parasite cytoplasm as blue. A number of parasitized red blood cells (PRBC)/ 10^3 RBC were determined in individual mouse.

RESULTS

1. PARASITEMIA AND COURSE OF INFECTION

The mice had prepatent periods of 3-4 days after intravenously injection with 1×10^5 PRBC. Later, the parasites were detected on peripheral blood smears of all mice (Table 1. and Fig 2). The parasitemia developed rapidly and reach a maximum value of $60^{+12.6}\%$ and $44.7^{+28.3}\%$ on day 14 and day 8 in P.berghei and P.chabaudi infections, respectively. In P.yoelii infection, the parasitemia gradually increased and reached a maximum value of $51.9^{+15.2}\%$ on day 11. After that, the parasitemia of both P.chabaudi and P.yoelii infected groups declined and disappeared from the circulation on day 20.

Mice of all groups became unwell by day 8 of infection. Thereafter, most of them had ruffled fur and move slowly or sat beside the cage and shivering. These signs of illness were prominent at the peak of parasitemia. A sudden death occurred in some mice of all experimental groups of malaria infection as early as day 7 until day 10 of infection. Most mice died on day 9 (Table 7) and some died without any sign of illness before. These early acute death accompanied with generalized signs; including ataxia, paresis, convulsion and coma. No distinct these signs were seen in a P.yoelii infected mouse

died on day 16. The parasitemia of the acute death mice was higher than those killed during infection (Fig. 2). The parasitemia level on day 8 of dead P.berghei infected mice was significant different from mice sacrificed on the same day ($P < 0.05$). But no significant difference was found in P.chabaudi group ($0.05 < P < 0.10$).

Observation of blood smears just before death, all mice showed high proportions of irregular or spurshaped red blood cells with deep Giemsa staining (Fig. 8). High parasitemia with late trophozoites and schizont-infected red cells were also observed.

2. HEMATOCRIT

The mean hematocrit values of each group are summarized in table 1 and fig 3. The hematocrit values of infected mice were in normal range (44-52%) until day 5 and then gradually decreased while the infection advanced. In P.berghei infection, the lowest hematocrit value was $12.7 \pm 1.7\%$ on day 14. The lowest values ($19 \pm 4.5\%$ and $14.6 \pm 3.8\%$) were observed on ay 14 of P.chabaudi and P.yoelii infections, respectively and were higher at later stage of infection.

3. BLOOD UREA NITROGEN (BUN)

The normal BUN value of normal mice was 30.3 ± 7.84 mg% (20-39 mg%). In P.berghei infection mean

BUN value was high as 56.75 ± 31.25 mg% on day 7 and maintained until the end of experiment (day 14). Mean BUN values of P.chabaudi and P.yoelii infected mice were higher by day 11 and day 14, respectively. But the mean BUN levels of both P.yoelii and P.chabaudi infected mice turned to normal range from day 17 onwards (Table 2).

4. PROTEINURIA

The proteinuria value of normal mice ranged from 26 to 55 mg%. Proteinuria of mice infected with P.berghei or P.chabaudi was elevated on day 8 with values of 120 ± 40 mg% and 240 mg%, respectively. In P.yoelii infected mice, proteinuria was increased on day 11. The proteinuria of both P.chabaudi and P.yoelii infected mice maintained at high level until day 14. Thereafter the proteinuria level returned to normal range (Table 2).

5. ANTIMALARIAL ANTIBODY

Anitmalarial antibodies were detected by immuno-fluorescent antibody technique (IFA) as shown in table 1 and fig 4. The antibody titers of infected mice rose up gradually by day 8, 10 and 11 in P.chabaudi, P.berghei and P.yoelii infections, respectively. The maximum antibody titer of P.chabaudi mice was 1:512 and 1:128 in P.yoelii infection on day 20 while in P.berghei infection reach a maximum titer of 1:128 on day 14. No anti-malarial antibody

was detected in P.berghei infected mice died by day 9 (Table 7).

6. CIRCULATING IMMUNE COMPLEXES (CIC)

Serum CIC were detected by polyethylene glycol precipitation (PEG-CC) method. There was no evidence of CIC in normal and infected mice at early stage of infection. Low level (+) of CIC was first detected on day 8 of P.chabaudi and P.yoelii infected mice and on day 10 in P.berghei infection (Table 1). The low level of CIC remained present during patent infection. In P.chabaudi and P.yoelii infections, CIC became undetectable on day 17 onwards. But no CIC presented in the sera of mice died of P.berghei infection (Table 7).

7. IMMUNOFLUORESCENT STUDY

Brains, kidneys, livers, spleens, and lungs of mice infected with P.berghei, P.chabaudi or P.yoelii were examined by immunofluorescence antibody technique for malarial antigen, mouse -globulin or B1C.

A. MALARIAL ANTIGEN

BRAIN : Malarial antigens detected in mice infected with P.berghei, P.chabaudi and P.yoelii were similar in pattern but different in degree of fluorescent intensity as shown in table 3. The malarial antigens were first detected in the choroid plexus of infected mice on day 5 as a corpuscular form. This form of malarial antigen

was found mostly at the early stage of infection (fig 6 and fig 7) and later, granular form was dominant (Fig. 5). The more intense fluorescent malaria antigens were observed in mice with the high parasitemia. The brains of mice that collected a few minutes after death showed high degree of fluorescent intensity, mostly in cerebral cortex, choroid plexus and cerebellar white matter. The malarial antigen was detected in the brain of mice infected with P.berghei until the end of the experiment whereas the fluorescent intensity of antigens in P.chabaudi and P.yoelii infections was decreased on day 17 and absent on day 20. However, P.yoelii infection produced less fluorescent intensity than those of P.berghei and P.chabaudi infections.

KIDNEY, LIVERS, SPLEEN, AND LUNGS : Malarial antigens were first detected in the lung and splenic sinusoidal lining cells on day 4 after infection in all groups. Thereafter, malarial antigens were detected in all kidneys, livers, spleens and lungs sections (Table 4, and 5). Corpuscular forms were found mostly in blood vessels of all organs studied at early stage of infection. Later the granular forms were dominant. In kidney, malarial antigens were found in the glomeruli and in the peritubular blood vessels. Also, the granular form of malarial antigens was found in the Kupffer cell of liver,

splenic sinusoidal lining cells (Fig 8) and in alveolar macrophages of the lung. The degree of fluorescent intensity gradually increased in all organs studied during infection advanced in all groups. Except those of P.chabaudi and P.yoelii infections which the fluorescent intensity of malarial antigens was decreased on day 17 onwards.

B. MOUSE γ -GLOBULIN

The mouse γ -globulin was detected in only kidney organs which were removed from mice killed as schedule. Firstly, it was detected at the glomerular basement membrane with low fluorescent intensity on day 5. Later, the intensity was slightly increased until the end of the experiment in all groups (Table 5). At the later stage of infection the fluorescent intensity of immunoglobulin deposits profound at the mesangial area of the glomeruli.

C. MOUSE β 1C

The mouse β 1C was first detected as low intensity of fluorescent staining on day 8 in P.chabaudi and P.yoelii infection, and on day 10 in P.berghei infection. Diffuse fine granular deposits were slightly increased and persisted until day 20 in P.chabaudi and P.yoelii infection, and on day 14 in P.berghei infection. The fluorescent intensity of mouse β 1C was shown in table 5.

8. HISTOPATHOLOGICAL STUDY

A. BRAIN

Normal brain : Brain is consisted of two main parts; cerebrum and cerebellum. Cerebellum is composed of an inner white matter and an outer gray matter or cerebella cortex. The gray matter has three distinguished layers: an outer molecular layer with few cells, an inner granular layer with numerous intensely stained nuclei cells, and an intermediate layer of Purkinje cells. Cerebral cortex is composed of neurone and neuroglia. Three types of neuroglia are identified: astrocytes are mostly seen near the blood vessels, oligodendroglia are densely stained with smaller size than astrocytes, and microglia with small nuclei scattering throughout the brain. Olfactory lobe of cerebrum is composed of densely stained cells. Choroid plexuses found at the roof of the third and fourth ventricles and the wall of the lateral ventricles lining by specialized cuboidal epithelial cells in a single layer and occupying by many blood vessels. Meninges contain numerous blood vessels.

Pathological Finding of Brain

All experimental mice killed during infections were found less severe cerebral lesions than those died acutely at the early stage of infection.

Brain of P.berghei infected mice : Generally, brain of the sacrificed mice exhibited normal appearance until day 5 of infection. On day 6, few parasitized erythrocytes were seen in the brain capillaries and venules and profound congestion was found on day 8 to day 12 (Table 6). Meningeal blood vessels and choroid plexus of some mice were also congested and infiltrated with few monocytes and PMN. Only few hemorrhages were observed in olfactory lobe, cerebral cortex and cerebellum in some mice. Malarial pigment-laden macrophages were rarely seen.

The brains of mice died acutely mostly developed severe congestion and some petechial hemorrhages. The congestion was found in all blood vessels throughout the brain. Some vessels were plugged by agglutinated red cells (Fig. 11, 12, 13). Hemorrhages were found in 8 out of 12 mice and frequently occurred in the white matter of the cerebellum and cerebral cortex. In the most severe case, hemorrhage also occurred in the choroid plexus blood vessels (Fig. 15). Degeneration of some neuronal cells and glial reaction were also observed (Fig. 14). Mononuclear cells infiltration was rarely seen in meningeal blood vessels and choroid plexus. No thrombosis was seen in all infected mice.

Brain of P.chabaudi infected mice : In sacrificed mice, few parasitized erythrocytes were first detected in

cerebral blood vessels on day 11 and slightly increased on day 14 but finally they disappeared. Mild degree of congestion was observed in cerebral cortex and cerebellum on day 11 and day 14. Petechial hemorrhage with some phagocytic cells was found in cerebral cortex of a mouse on day 14 (Fig. 10). Mostly the brain of P.chabaudi infected mice looked normal excepted one killed on day 17 showed hemorrhage with trace amount of malarial pigment. Brain lesions of acutely dead mice looked similar to those of P.berghei infected mice. Also, karyorrhexis of some astrocytes and degeneration of some neurons were found accompanied with glial reaction.

Brain of P.yoelii infected mice : The pathological findings of brains in the sacrificed mice were similar to those of P.chabaudi infected group. Small number of lymphocytes was found in the choroid plexus at later stage of infection. Two mice died on day 9 and day 16 developed similar lesions as those died of P.berghei infection.

B. KIDNEY

Normal Kidney : The kidney is consisted of two regions; the outer renal cortex containing tubules and glomeruli and the inner medulla composed of peritubular blood vessels and collecting tubules. The functional unit

is nephron, consisting of a glomerulus and urinary tubules. The glomerulus composes of a tuft of capillaries surrounded by Bowman's capsule, is supplied by afferent arteriole and drained out by an efferent arteriole. In mouse a marked sex difference is the type of epithelial cell lining of Bowman's capsule: cuboidal epithelial cells for the male and squamous epithelial cells in the female. The glomerular filtering membrane is consisted of endothelial cells lining along the lumen, glomerular basement membrane and visceral epithelial cells (podocyte). Mesangial area between the glomerular capillary loops is associated with mesangial cell embedding in the mesangial matrix. The proximal convoluted tubule, emerging from the glomerulus, is lined by cuboidal epithelial cell with pronounced brush border. The tubule continues into the medulla as the descending portion of the loop of Henle and returns to the cortex as the ascending portion of distal convoluted tubule. The collecting tubule; continues from the distal convoluted tubules, is mainly located within the medulla.

Kidney of Infected Mice : The main pathological findings of kidney in mice infected with P.berghei, P.chabaudi and P.yoelii were similar but different in the severity of the lesion as shown in table 8. Congestion was apparently seen in the kidneys of mice in all group from day 8 onwards. Interstitial hemorrhages were noted in some

mice of all groups at the peak of parasitemia. Malarial pigment-laden macrophages and parasitized erythrocytes were found increasingly in number in the peritubular blood vessels as the infection progressed. Small number of PMN and monocytes were found in the glomerular capillary lumens. Slight proliferation of mesangial cells and thickening of glomerular capillary wall were observed from day 8 onwards. Excepted for P.chabaudi and P.yoelii infections, the glomerular cells turned to normal in recovered mice. Proximal convoluted tubules showed cloudy swelling and hydropic degeneration in some mice. Dilatation of some distal convoluted tubules accompanied with hyaline casts in some tubules were also observed in some P.berghei infected mice. Histological changes of most collecting tubules were shown as degeneration and necrosis in some areas with inflammatory cells infiltration. Marked increased of mononuclear cell infiltration around the blood vessels was seen from day 8 throughout the course of infection in all groups. These cells comprised lymphocytes, macrophages and plasma cells, the latter increasingly numerous at the late stage of infection (Fig. 16).

The severity of the lesions in recovered mice in P.chabaudi and P.yoelii infections were reduced to normal. However, mononuclear cells infiltration were persisted with marked increase of plasma cells.

C. LIVER

Normal Liver : Liver is divided into lobules each with a central vein at the center. The outer boundaries of the lobules are incompletely demarcated by the portal triads which contain a branch of the hepatic artery, portal vein and bile duct. The bile ducts are lined by regularly arranged cuboidal cells. Liver sinusoids are lined by endothelial cells and Kupffer cells. The hepatic cells are large polygonal cells with a prominent central nucleus or, occasionally two nuclei and one or more nucleoli may be seen. The cellular filtration of mononuclear cells and granulocytes is often seen in the perivascular area.

Pathological Findings of Liver : The pathological appearances of liver were the same in all groups of malarial infections. These included hypertrophy of Kupffer cells filled with malarial pigment, distension of liver sinusoids, mononuclear cell infiltration, and liver necrosis. By day 6 of infection, malaria pigment-laden Kupffer cells were seen in the sinusoids and increased as the infection progressed. Distension of sinusoid with numerous inflammatory cells and foci of coagulative necrosis (Fig. 17) were seen during high parasitemic infection. Panlobular liver necrosis accompanied with PMN and mononuclear cells infiltration were seen in some mice. Thromboarteritis was found in a mouse infected with P.chabaudi (Fig. 18). Extramedullary .

hemopoiesis and megakaryocyte proliferation were found during patent infection and thereafter. Periportal mononuclear cell infiltration was most striking in P.chabaudi and P.yoelii infected mice, especially at later stage of infections.

In recovered mice of P.chabaudi and P.yoelii infections, livers showed mild degree of degeneration, periportal mononuclear cell infiltration and Kupffer cells containing heavy malarial pigment.

D. LUNG

Normal Lung : Lung consists of series of branches bronchioles, alveolar ducts, alveolar sacs and finally alveoli. The alveoli which is the smallest part are lined by epithelial cells and supported by interstitial tissue of the alveolar septa. The alveolar septa consists of few reticular fibers, septal cells, and many capillaries. In normal mice a mild degree of interstitial pneumonia is usually observed.

Pathology of Lung : The general pathological findings of lung in these three malarial parasite infections were similar; including interstitial inflammation, malarial pigment-laden macrophages and aggregation of mononuclear cells. Malarial pigment-laden macrophages were first seen on day 5 in the lung of P.yoelii infected mice

and increasing as the infection progressed in all groups afterwards. Marked interstitial inflammation was observed during the patent infections. Infiltration and aggregation of mononuclear cells in alveolar sac were seen in all groups at later stage of infections. Megakaryocytes were found from day 10 onwards in some of P.berghei infected mice (Fig. 19). A mouse died of cerebral malaria involvement and high parasitemia showed severe pulmonary edema occupied all of alveoli (Fig. 20). But the other infected mice with high parasitemia developed only mild interstitial edema (Fig. 19).

E. SPLEEN

Normal Spleen : Spleen is a vascular organ covered with capsule of dense connective tissue and trabecular project into the spleen pulp. Branches of splenic arteries, veins, nerves, reticular fibers, and trabeculae support as connective tissues in red and white pulp. White pulp consists of lymphocytes, plasma cells and macrophages. Occasionally, germinal centers are seen and surrounded by lymphocytes. A central arteriole occupies the center of nodule. Red pulp consists of primarily of erythropoietic cells, lymphocytes, granulocytes, plasma cells and megakaryocytes. Extramedullary hemopoiesis is commonly found in normal mouse spleen.

Pathology of Infected Mice Spleen : Grossly, splenomegaly developed by day 5 in P.yoelii and P.chabaudi infected mice and by day 8 in P.berghei infection. There after splenomegaly was marked increasing especially in P.chabaudi and P.yoelii infections. The changes in the spleen of infected mice were similar in all groups. Congestion with parasitized erythrocytes involved in both sinusoids and blood vessels of red pulp. Reticuloendothelial cells showed erythrophagocytosis and heavy deposit of pigment. At the later stage of infection extramedullary hemopoiesis and plasma cell differentiation increased. Proliferation of blast cells and germinal center and plasma cell accumulation in the white pulp were found mostly at later stage of P.chabaudi and P.yoelii infection. However, some of P.berghei infected mice exhibited apparent reduction in the white pulp but massive expansion in the red pulp.

Table 1. Mean of parasitemia and hematocrit, and mode of IFA titre and circulating immune complexes (CIC) in mice infected with *P. berghei* (PB) *P. chabaudi* (PC) and *P. yoelii* (PY).

Day of infection	Parasitemia (Mean $\pm$ S.D.)			Hematocrit (Mean $\pm$ S.D.)			IFA Titer			CIC		
	PB	PC	PY	PB	PC	PY	PB	PC	PY	PB	PC	PY
1	N.D.	-	-	47.33 $\pm$ 3.29	46.33 $\pm$ 3.68	N.D.	-	-	-	-	-	-
2	-	-	-	46.33 $\pm$ 1.24	45.3 $\pm$ 2.86	N.D.	-	-	-	-	-	-
3	-	-	-	45 $\pm$ 1.4	45 $\pm$ 2.16	47.66 $\pm$ 1.24	-	-	-	-	-	-
4	1 $\frac{8}{8}$ 1.4 $\pm$ 0.43	-	3.46 $\pm$ 0.25	47.3 $\pm$ 4.10	42.66 $\pm$ 1.24	51.6 $\pm$ 2.35	-	-	-	-	-	-
5	14.5 $\pm$ 2.42	0.69 $\pm$ 0.54	7.9 $\pm$ 3.34	45.33 $\pm$ 2.49	45.66 $\pm$ 2.49	39.6 $\pm$ 0.47	-	-	-	-	-	-
6	22.33 $\pm$ 5.72	N.D.	N.D.	45.66 $\pm$ 2.49	N.D.	N.D.	-	N.D.	N.D.	-	N.D.	N.D.
8	46.6 $\pm$ 5.24	44.66 $\pm$ 28.26	19.6 $\pm$ 7.3	37.3 $\pm$ 0.47	25.33 $\pm$ 6.59	23.66 $\pm$ 2.05	-	1:8	-	-	+	+
10	N.D.	N.D.	N.D.	24.3 $\pm$ 9.8	N.D.	N.D.	1:6	N.D.	N.D.	+	N.D.	N.D.
11	N.D.	39.2 $\pm$ 1.57	51.86 $\pm$ 15.18	N.D.	22.66 $\pm$ 6.59	17.66 $\pm$ 2.62	N.D.	1:16	1:4	N.D.	+	+
12	29 $\pm$ 2.31	N.D.	N.D.	14.33 $\pm$ 4.10	N.D.	N.D.	1:32	N.D.	N.D.	+	N.D.	N.D.
14	60 $\pm$ 12.6	34.8 $\pm$ 6.04	38.13 $\pm$ 7.08	12.66 $\pm$ 1.69	19 $\pm$ 4.54	14.6 $\pm$ 3.77	1:128	1:64	1:8	N.D.	+	-
17	-	15.8 $\pm$ 10.06	12.93 $\pm$ 17.86	-	20.66 $\pm$ 5.90	26.66 $\pm$ 7.93	-	1:128	1:14	-	-	-
20	-	-	-	-	35 $\pm$ 2.5	34.3 $\pm$ 4.92	-	1:512	1:128	-	-	N.D.

N.D. = Not done

Table 2. Blood urea nitrogen (B.U.N.) and proteinuria of mice infected with P. berghei, P. chabaudi and P. yoelii (Mean $\pm$ S.D. of 3 mice)

Day of infection	B. U. N. (mg %)			Proteinuria (mg %)		
	PB	PC	PY	PB	PC	PY
1	N.D.	35.66 $\pm$ 5.72	N.D.	N.D.	N.D.	N.D.
2	N.D.	30 *	N.D.	N.D.	51.25 $\pm$ 11.25 **	N.D.
3	N.D.	34.83 $\pm$ 1.02	17.66 $\pm$ 8.99	N.D.	112.5 *	56.66 $\pm$ 20.54
4	N.D.	39.83 $\pm$ 6.14	17.33 $\pm$ 5.24	N.D.	83.33 $\pm$ 29.33	30 *
5	18.16 $\pm$ 10.12	23 $\pm$ 4.45	19.33 $\pm$ 8.95	70 $\pm$ 10 **	119.16 $\pm$ 25.83	55 $\pm$ 25 **
6	27.16 $\pm$ 4.78	N.D.	N.D.	120 $\pm$ 40 **	N.D.	N.D.
8	N.D.	22.16 $\pm$ 6.45	20.83 $\pm$ 6.45	100 $\pm$ 20 **	200 *	60 *
10	56.75 $\pm$ 8.75	N.D.	N.D.	195 *	N.D.	N.D.
11	N.D.	41.16 $\pm$ 14.37	28.5 $\pm$ 2.54	N.D.	120 $\pm$ 20 **	135 **
12	34 $\pm$ 1 **	N.D.	N.D.	N.D.	N.D.	N.D.
14	53.32 $\pm$ 9.25 **	41.83 $\pm$ 8.87	46.75 $\pm$ 4.25 **	N.D.	150 *	220 *
17		28 $\pm$ 3.56	25.5 $\pm$ 3.18		56.25 $\pm$ 6.25 **	80 *
20		24.25 $\pm$ 1.75 **	35.86 $\pm$ 7.77		N.D.	40 *
Normal range	30.33 $\pm$ 7.84 (20 - 39)	mg %		40 $\pm$ 14.24 (26 - 52)	mg %	

N.D. = Not done
 PB = P. berghei infection PY = P. yoelii infection * = one mouse
 ** = Two mice

Table 3. BRAIN : Immunofluorescent studied on brain tissue sections of mice infected with P.berghei (PB), P.chabaudi (PC), and P.yoelii (PY). The number of positive exp. mice/total number of exp. mice.

Day of infection	Detection of malarial antigen in brain											
	Olfactory lobe			Cerebrum			Choroid Plexus			Cerebellum		
	PB	PC	PY	PB	PC	PY	PB	PC	PY	PB	PC	PY
1	N.D.	0/3	0/3	N.D.	0/3	0/3	N.D.	0/3	0/3	N.D.	0/3	0/3
2	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
4	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
5	0/3	0/3	0/3	0/3	0/3	(1)/3	0/3	0/3	1/3*	0/3	0/3	0/3
6	0/3	N.D.	N.D.	(3)/3	N.D.	N.D.	(2)/3	N.D.	N.D.	(3)/3	N.D.	N.D.
8	(1)/3	3/3*	0/3	3/3*	3/3*	(2)/3	2/3**	3/3*	0/3	3/3*	(3)/3	(1)/3
10	(3)/3	N.D.	N.D.	3/3**	N.D.	N.D.	3/3**	N.D.	N.D.	3/3**	N.D.	N.D.
11	N.D.	3/3*	0/3	N.D.**	3/3	3/3*	N.D.	3/3**	(2)/3	N.D.	3/3**	3/3*
12	(3)/3	N.D.	N.D.	3/3**	N.D.	N.D.*	2/3*	N.D.	N.D.	3/3*	N.D.	N.D.
14	(2)/3	(2)/3	(2)/3	3/3*	(3)/3	3/3*	1/3*	(2)/3	(3)/3	3/3*	2/3*	(3)/3
17		(2)/3	0/3		(3)/3	1/3*		0/3	(1)/3		3/3*	
20		0/3	0/3		0/3	0/3		0/3	0/3		0/3	(1)/3

N.D. = Not done
 () = Trace of fluorescent, staining of malarial antigen
 * = Mild or low fluorescent staining of malarial antigen
 ** = Moderate degree fluorescent staining of malaria antigen

Table 4. Immunofluorescent studied on liver, spleen and lung tissue sections of mice infected with *P. berghei* (PB), *P. chabaudi* (PC) and *P. yoelii* (PY). The number of positive exp. mice/total number of exp. mice.

Day of infection		Detection of malarial antigen on frozen tissue sections by direct FA									
		Liver			Spleen			Lung			
		PB	PC	PY	PB	PC	PY	PB	PB	PY	
1	N.D.	0/3	0/3	N.D.	0/3	0/3	N.D.	0/3	0/3		
2	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3		
3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3		
4	(2)/3	(1)/3	0/3	(2)/3	1/3*	3/3*	3/3*	1/3*	3/3*		
5	(1)/3	(1)/3	3/3*	(3)/3	3/3*	3/3**	(3)/3	3/3*	3/3*		
6	(3)/3	N.D.	N.D.	3/3*	N.D.	N.D.	3/3**	N.D.	N.D.		
8	3/3*	3/3**	3/3*	3/3**	3/3	3/3**	3/3***	3/3	3/3		
10	3/3**	N.D.	N.D.	3/3	N.D.	N.D.	3/3**	N.D.	N.D.		
11	N.D.**	3/3***	3/3**	N.D.***	3/3	3/3	N.D.**	3/3	3/3		
12	3/3**	N.D.	N.D.	3/3***	N.D.	N.D.	3/3**	N.D.	N.D.		
14	3/3**	3/3*	3/3**	3/3***	3/3	3/3***	3/3***	3/3	3/3		
17		(3)/3*	1/3*		3/3**	3/3**		3/3**	3/3*		
20		1/3*	0/3		1/3*	0/3		1/3*	0/3		

N.D. = Not done
 () = Trace of fluorescent staining of malarial antigen
 * = Mild or low positive fluorescent staining of malarial antigen
 ** = Moderate positive fluorescent staining of malarial antigen
 *** = High positive fluorescent staining of malarial antigen

Table 5. KIDNEY : Immunofluorescent studies on kidney tissue sections for malarial antigen, mouse γ -globulin and mouse β 1C in mice infected with P.berghei (PB), P.chabaudi (PC), and P.yoelii (PY). The number of positive exp. mice/total number of exp. mice.

Day of infection	Malarial antigen			Mouse γ -globulin			Mouse β 1C		
	PB	PC	PY	PB	PC	PY	PB	PC	PY
1	N.D.	0/3	0/3	N.D.	0/3	0/3	N.D.	0/3	0/3
2	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
4	0/3	(1)/3	(3)/3	0/3	0/3	0/3	0/3	0/3	0/3
5	(3)/3	(2)/3	3/3	(2)/3	(3)/3	(3)/3	0/3	0/3	0/3
6	3/3*	N.D.	N.D.	3/3*	N.D.	N.D.	0/3	N.D.	N.D.
8	3/3**	3/3***	3/3	3/3*	3/3*	3/3	0/3	(2)/3	(1)/3
10	3/3***	N.D.	N.D.	3/3**	N.D.	N.D.	(3)/3	N.D.	N.D.
11	N.D.	3/3	3/3	N.D.	3/3	3/3	N.D.	3/3	3/3
12	3/3**	N.D.	N.D.	3/3**	N.D.	N.D.	3/3*	N.D.	N.D.
14	3/3**	3/3**	3/3**	3/3**	3/3**	3/3**	3/3**	3/3*	3/3**
17	3/3	3/3*	3/3*	3/3	3/3***	3/3**	3/3	3/3*	3/3*
20		2/3	1/3		3/3	3/3		3/3	(3)/3

N.D. = Not done

() = Trace of fluorescent intensity

* = Low degree of fluorescent intensity

** = Moderate degree of fluorescent intensity

*** = High degree of fluorescent intensity

Table 6. BRAIN : Histopathological findings on tissue sections of mice infected with P.berghei (PB), P.youeli (PY), and P.chabaudi (PC). The number of positive exp. mice/total number of exp. mice.

Day of infection	Hemorrhage			Congestion of blood vessels			Inflammatory cell infiltration			PRBC and/or MPLM		
	PB	PC	PY	PB	PC	PY	PB	PC	PY	PB	PC	PY
1	N.D.	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
2	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
4	0/3	0/3	0/3	0/3	0/3	(1)/3	0/3	0/3	0/3	0/3	0/3	(1)/3
5	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	3/3*
6	0/3	N.D.	N.D.	2/3	N.D.	N.D.	0/3	N.D.	N.D.	3/3	N.D.	N.D.
8	2/3*	0/3	0/3	3/3*	0/3	0/3	0/3	0/3	0/3	3/3*	0/3	(3)/3
10	(1)/3	N.D.	N.D.	3/3*	N.D.	N.D.	(3)/3	N.D.	N.D.	3/3*	N.D.	N.D.
11	N.D.	0/3	(1)/3	N.D.	(1)/3	(1)/3	N.D.	0/3	0/3	N.D.	3/3*	(3)/3
12	2/3*	N.D.	N.D.	3/3*	N.D.*	N.D.	(3)/3	N.D.	N.D.	3/3*	N.D.	N.D.
14	0/3	1/3	(2)/3	(2)/3	2/3*	(2)/3	0/3	0/3	(2)/3*	(3)/3	3/3*	(3)/3
17		0/3	(2)/3		0/3	3/3*	0/3	0/3	3/3*		2/3	(3)/3
20	*	1/3*	0/3		0/3	0/3	0/3	0/3	0/3		2/3	(1)/3

N.D. = Not done
 () = trace or less severity
 * = Mild degree of severity
 ** = Moderate degree of severity
 PRBC = Parasitized red blood cell
 MPLM = Malarial pigment-laden macrophage

Table 8. KIDNEY : Histopathological findings on tissue sections of mice infected with P.berghei (PB), P.chabaudi (PC), and P.yoelii (PY). The number of positive exp. mice/total number of exp. mice.

Day of infection	Glomerular infiltration of and/or MPLM			Mononuclear cells infiltration			Malarial Pigment-laden macrophages			Tubular changes		
	PB	PC	PY	PB	PC	PY	PB	PC	PY	PB	PC	PY
1	N.D.	0/3	0/3	N.D.	(1)/3	0/3	N.D.	0/3	0/3	N.D.	0/3	0/3
2	0/3	0/3	0/3	0/3	0/3	(2)/3	0/3	0/3	0/3	0/3	0/3	0/3
3	0/3	0/3	0/3	(1)/3	0/3	(1)/3	0/3	0/3	0/3	0/3	0/3	0/3
4	0/3	0/3	0/3	0/3	(3)/3	(1)/3	0/3	0/3	0/3	0/3	0/3	0/3
5	(1)/2	0/3	(2)/2	(2)/3	(2)/3	(2)/2	0/2	0/2	(1)/2	0/2	0/3	0/2
6	(3)/3	N.D.	N.D.	2/3	N.D.	N.D.	3/3	N.D.	N.D.	0/3	N.D.	N.D.
8	3/3	3/3	(3)/3	3/3	3/3	3/3	3/3	3/3	3/3	1/3	0/3	(1)/3
10	3/3	N.D.	N.D.	3/3	N.D.	N.D.	3/3	N.D.	N.D.	2/3	N.D.	N.D.
11	N.D.	2/2	3/3	N.D.	2/2	3/3	N.D.	2/2	3/3	N.D.	1/2	3/3
12	3/3	N.D.	N.D.	3/3	N.D.	N.D.	3/3	N.D.	N.D.	+3/3	N.D.	N.D.
14	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	+3/3	2/3	3/3
17		2/2	3/3		2/3	3/3		2/2	3/3		2/2	3/3
20		3/3	(2)/3		3/3	3/3		3/3	(2)/3		2/2	0/3

N.D. = Not done
() = Trace or less severity

* = Mild degree of severity
** = Moderate degree of severity
*** = Severe degree of severity

= lesions associated with tubular degeneration.
= lesions associated with interstitial hemorrhage.

Table 9. LIVER : Histopathological findings on tissue sections liver of mice infected with P.berghei (PB), P.chabaudi (PC) and P.yoelii (PY). The number of positive exp. mice/total number of exp. mice.

Day of infection	Hypertrophy of Kupffer cell with malarial pigment			Extramedullary hemopoiesis			Liver necrosis			Mononuclear cell infiltration		
	PB	PC	PY	PB	PC	PY	PB	PC	PY	PB	PC	PY
1	N.D.	0/3	0/3	N.D.	0/3	(2)/3	N.D.	0/3	0/3	N.D.	(3)/3	0/3
2	0/3	0/3	0/3	(3)/3	(3)/3	(2)/3	0/3	0/3	0/3	(2)/3	(1)/3	(1)/3
3	0/3	0/3	0/3	(2)/3	(3)/3	(2)/3	0/3	0/3	0/3	(3)/3	2/3	(3)/3
4	0/3	0/3	0/3	(3)/3	2/3	(3)/3	0/3	0/3	(2)/3	(1)/3	(3)/3	(2)/3
5	0/3	(3)/3	3/3	(3)/3	(3)/3	3/3	0/3	0/3	0/3	(2)/3	(3)/3	3/3
6	3/3	N.D.	N.D.	3/3	N.D.	N.D.	0/3	N.D.	N.D.	(2)/3	N.D.	N.D.
8	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	2/3	3/3	3/3	3/3
10	3/3	N.D.	N.D.	3/3	N.D.	N.D.	3/3	N.D.	N.D.	3/3	N.D.	N.D.
11	N.D.	3/3	3/3	N.D.	3/3	3/3	N.D.	3/3	3/3	N.D.	3/3	3/3
12	2/2	N.D.	N.D.	2/2	N.D.	N.D.	2/2	N.D.	N.D.	2/2	N.D.	N.D.
14	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
17		2/2	3/3		2/2	3/3		2/2	3/3		2/2	3/3
20		3/3	3/3		3/3	3/3		3/3	2/3		3/3	3/3

N.D. = Not done
 () = Trace or less severity
 * = Mild degree of severity
 ** = Moderate degree of severity
 *** = Severe degree of severity

Table 10. LUNG : Histopathological findings on lung tissue sections of mice infected with P.berghei (PB), P.chabaudi (PC) and P.yoelii (PY). The number of positive exp. mice/total number of exp. mice.

Day of infection	Interstitial inflammation			PMN and/or M Ø in alveoli			MPLM			Nodular or diffuse aggregation of MC			Pulmonary edema		
	PB	PC	PY	PB	PC	PY	PB	PC	PY	PB	PC	PY	PB	PC	PY
1	N.D.*	3/3*	(2)/3	0/3	-	-	-	-	-	(2)/3	-	(1)/3	-	-	-
2	3/3*	3/3*	(1)/3	-	-	-	-	-	-	(3)/3	-	(1)/3	-	-	-
3	(3)/3*	(3)/3*	(1)/3*	(2)/3	-	-	-	-	-	(3)/3*	(1)/3	-	-	-	-
4	3/3*	3/3*	3/3*	(3)/3	-	(1)/3*	-	-	-	3/3*	(2)/3	(2)/3*	-	-	-
5	3/3*	3/3*	3/3**	(2)/3	(3)/3	3/3*	-	-	3/3*	3/3*	(1)/3	3/3*	-	-	-
6	3/3*	N.D.*	N.D.*	3/3*	N.D.*	N.D.*	3/3*	N.D.*	N.D.*	3/3*	N.D.*	N.D.*	N.D.*	N.D.*	N.D.*
8	2/2**	3/3*	3/3***	2/2*	3/3*	3/3	2/3*	2/3*	3/3	(1)/2	(1)/3	(1)/3	3/3*	N.D.*	-
10	3/3**	N.D.*	N.D.*	3/3**	N.D.*	N.D.*	3/3	N.D.*	N.D.*	3/3	N.D.*	N.D.*	3/3*	N.D.*	N.D.*
11	N.D.*	3/3**	3/3**	N.D.*	3/3*	3/3	N.D.*	3/3	3/3	N.D.*	(3)/3	N.D.*	N.D.*	(2)/3	1/3
12	2/2**	N.D.*	N.D.*	2/2**	N.D.*	N.D.*	2/2**	N.D.*	N.D.*	(2)/2	N.D.*	N.D.*	2/2*	N.D.*	N.D.*
14	3/3**	3/3**	3/3**	3/3	3/3**	3/3**	3/3	3/3**	3/3**	3/3*	3/3*	(2)/3**	3/3*	3/3**	2/3*
17		3/3**	3/3**		3/3*	3/3*		3/3*	3/3*		3/3*	2/3**		3/3*	
20		3/3**	3/3**		3/3*	(3)/3		3/3*	3/3*		3/3*	3/3**		-	(2)/3

N.D. = Not done * = Mild degree of severity MPLM = Malarial pigment-laden macrophage
 () = Trace or less severity ** = Moderate degree of severity MC = Mononuclear cell
 *** = High degree of severity M Ø = macrophage

Table 11. SPEEN : Histopathological findings on spleen tissue sections of mice infected with P. berghei (PB), P. chabaudi (PC) and P. yoelii (PY). The number of positive exp. mice/total number of exp. mice.

Day of infection	Hyperplasia of white pulp			MPLM			Extramedullary hemopoiesis			Congestion of Red pulp		
	PB	PC	PY	PB	PC	PY	PB	PC	PY	PB	PB	PY
1	N.D.	0/3	0/3	N.D.	0/3	0/3	N.D.	(3)/3	(3)/3	N.D.	0/3	0/3
2	0/3	0/3	0/3	0/3	0/3	0/3	(3)/3	(3)/3	(3)/3	0/3	0/3	0/3
3	0/3	(1)/3	0/3	0/3	0/3	0/3	(3)/3	(3)/3	(3)/3	0/3	0/3	0/3
4	(2)/3	2/3*	1/3*	0/3	0/3	0/3	(3)/3	(3)/3	(3)/3	(1)/3	(2)/3	0/3
5	(2)/2	3/3*	3/3*	0/3	(3)/3	(3)/3	(2)/2 G	3/3*	3/3*	(2)/2	2/3*	3/3*
6	2/3*	N.D.	N.D.	3/3*	N.D.	N.D.	2/3* G	N.D.	N.D.	3/3*	N.D.	N.D.
8	3/3**	3/3**	3/3**	3/3**	3/3**	3/3**	3/3* G	3/3* G	3/3* G	3/3*	3/3*	3/3**
10	2/2**	N.D.*	N.D.**	2/2***	N.D.	N.D.**	2/2** G	N.D.	N.D.	2/2**	N.D.	N.D.
11	N.D.*	2/2*	3/3	N.D.***	2/2	3/3	N.D.	2/2** G	3/3* G	N.D.	2/2**	3/3
12	3/3*	N.D.*	N.D.*	3/3***	N.D.	N.D.	3/3***	N.D.	N.D.	3/3***	N.D.	N.D.
14	3/3* P	3/3* P	3/3* P	3/3***	3/3***	3/3***	3/3 E	3/3***	3/3***	3/3***	3/3***	3/3***
17		2/2* P	3/3* P		3/3***	3/3***		3/3***	3/3***		3/3***	3/3***
20		3/3*** P	3/3*** P		3/3***	3/3***		3/3 E	3/3 E		3/3**	3/3***

N.D. = Not done
MPLM = Malarial pigment-laden macrophages
() = Trace or less severity
* = Mild degree of severity
** = Moderate degree of severity
*** = Severe degree of severity
G = Granulocytic series are dominant
E = Erythrocytic series are dominant
P = Plasma cells are dominant

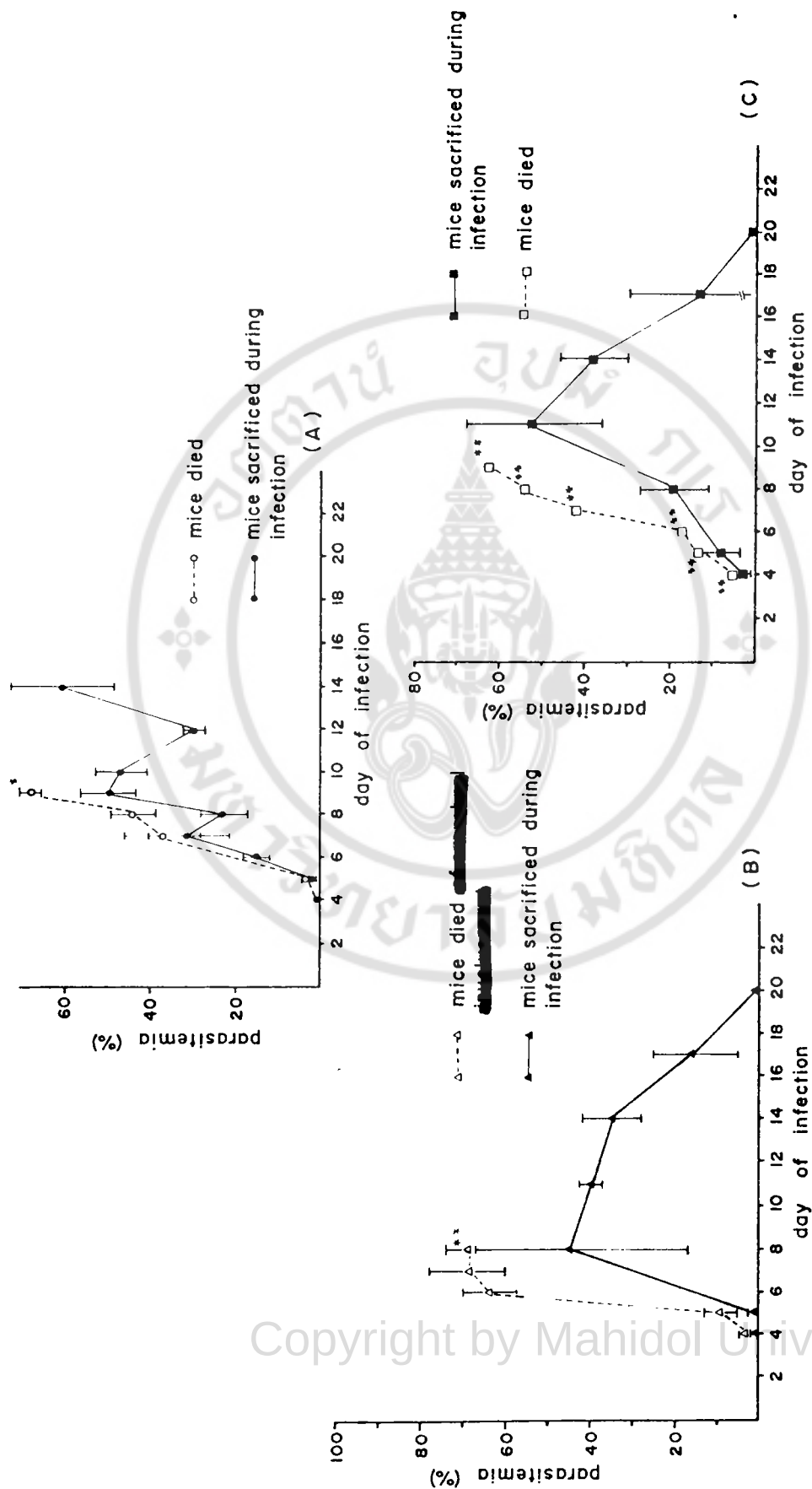


Fig. 2 Percent of parasitemia in mice after inoculation of 10^5 RBC infected with

(A) *P. berghei* (B) *P. chabaudi* and (C) *P. yoelii*. Each point represents the

arithmetic mean $\pm$ SD of 5 mice and 3 mice of died mice group and mice

sacrificed during infection group respectively

* 2 mice are calculated

* 1 mouse is calculated

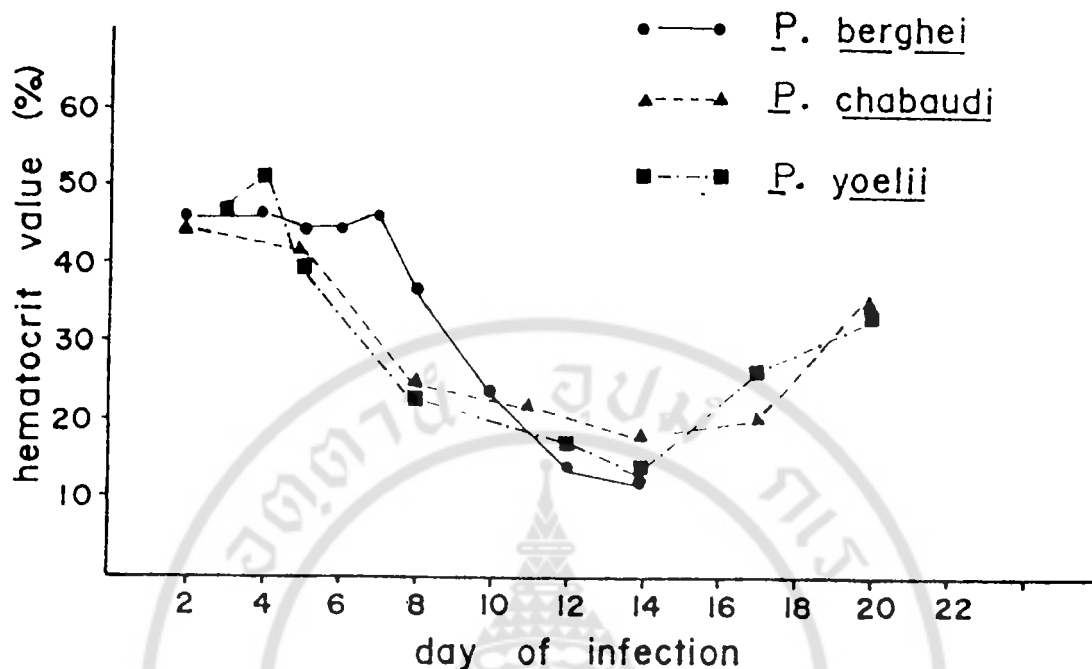


Fig. 3. Mean hematocrit values of mice infected with *P.berghei*, *P.chabaudi* and *P.yoelii*.

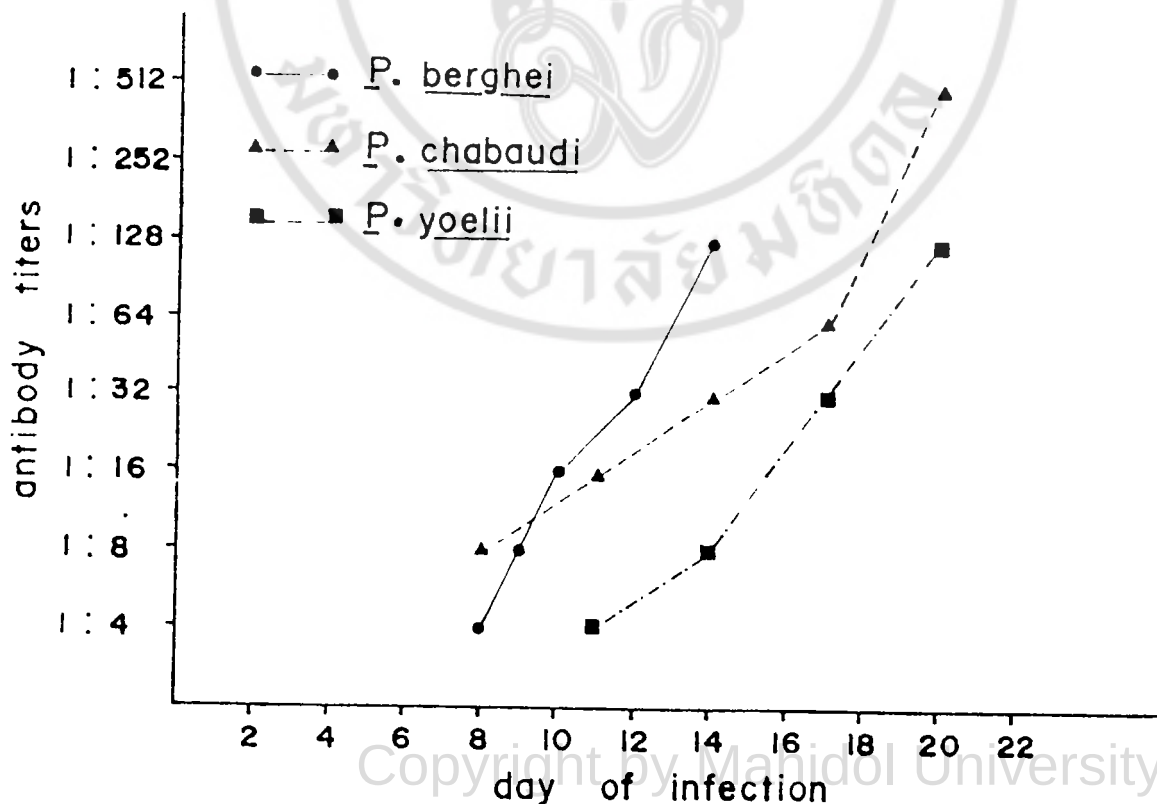


Fig. 4 The mode values of IFA titer of mice infected with *P.berghei*, *P.chabaudi* and *P.yoelii*. Each point represents of three mice.

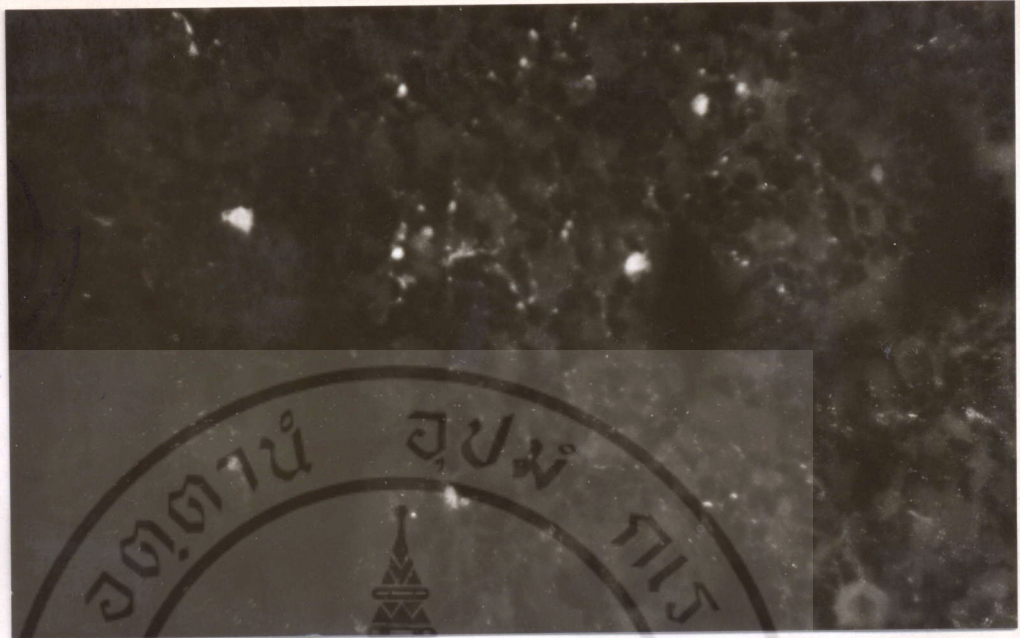


Fig. 5 Brain of P.chabaudi infected mouse sacrificed on day 11 of infection shows granular malarial antigens in the blood vessels of cerebral cortex. (stained with FITC-labelled anti P.chabaudi antibody x 126).



Fig. 6 Brain of P.chabaudi infected mouse sacrificed on day 7 shows mostly corpuscular form of malarial antigens in the choroid plexus. Autofluorescence of brain tissue is seen at the right lower part of the picture. (stained with FITC-labelled anti P.chabaudi antibody x 126).



Fig. 7 Brain of *P.berghei* infected mouse sacrificed on day 9 of infection shows mostly corpuscular form of malarial antigens in the meningeal blood vessels. (stained with FITC-labelled anti *P.berghei* antibody x 126).

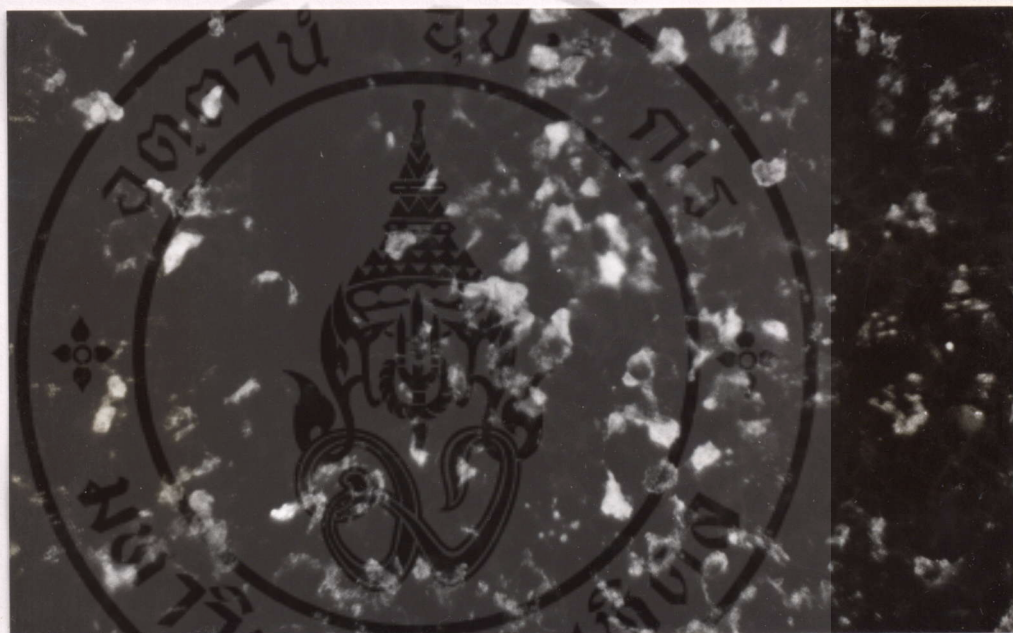


Fig. 8 Spleen of P.yoelii infected mouse killed on day 14 of infection shows granular malarial antigen in the splenic sinusoidal lining cells and macrophages (stained with FITC-labelled anti P.yoelii antibody x 252).

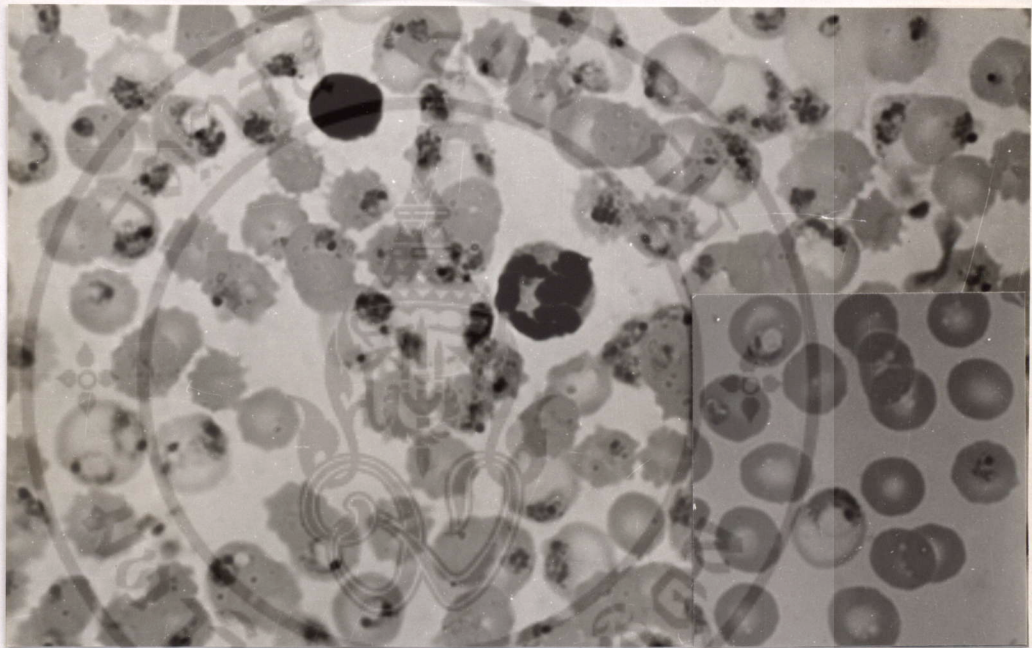


Fig. 9 Blood smear of P.berghei infected mouse with high parasitemia died on day 9 shows irregular or spured shaped red cells in comparison to infected red cells of low parasitemia infected mouse (inset). Heavy parasitemia with late trophozoites and schizonts are seen. (Giemsa stained x 1000).

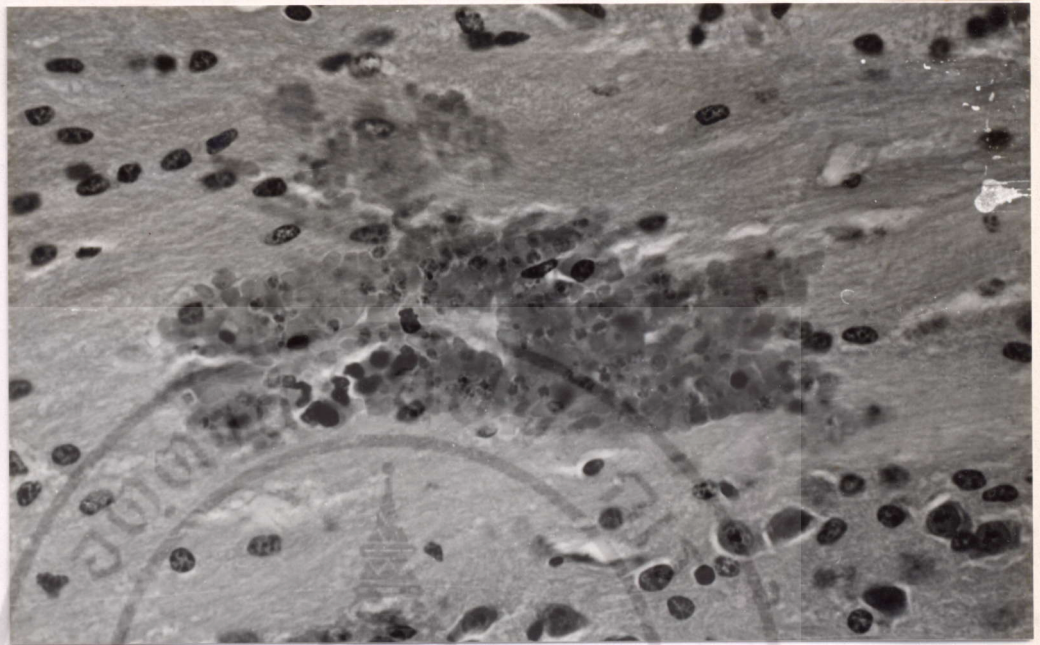


Fig. 10 Brain of P.chabaudi infected mouse sacrificed on day 14 of infection shows petechial hemorrhage of cerebral blood vessel. Some phagocytic cells are seen in the hemorrhagic area. (H & E stained x 400).

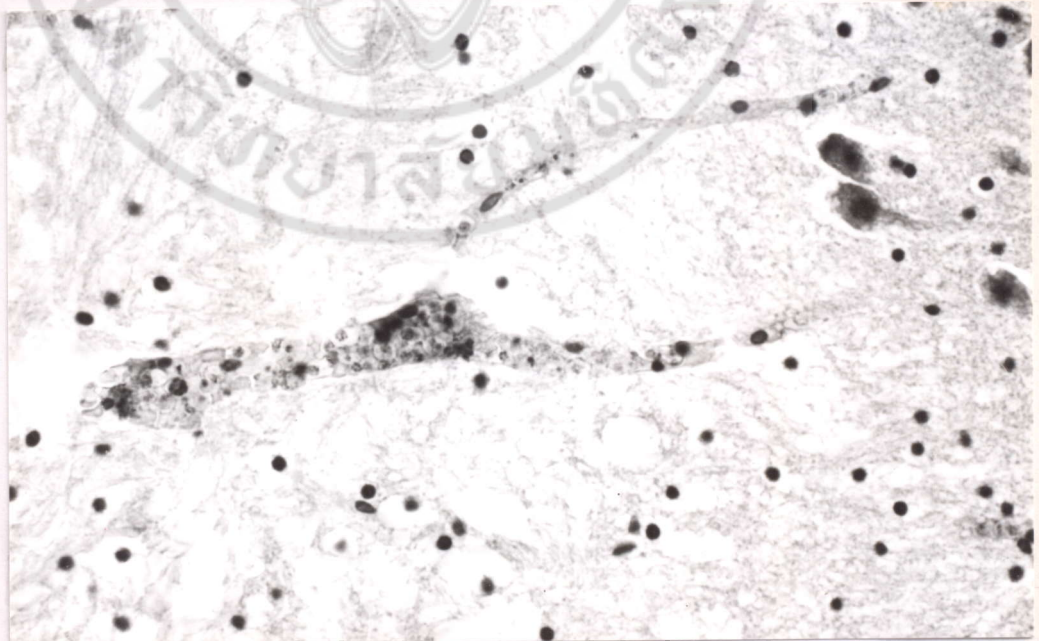


Fig. 11 Brain of P.chabaudi infected mouse died on day 8 of infection shows capillary plugging of agglutinated red cells in the cerebellar white matter. (H & E stained x 400).

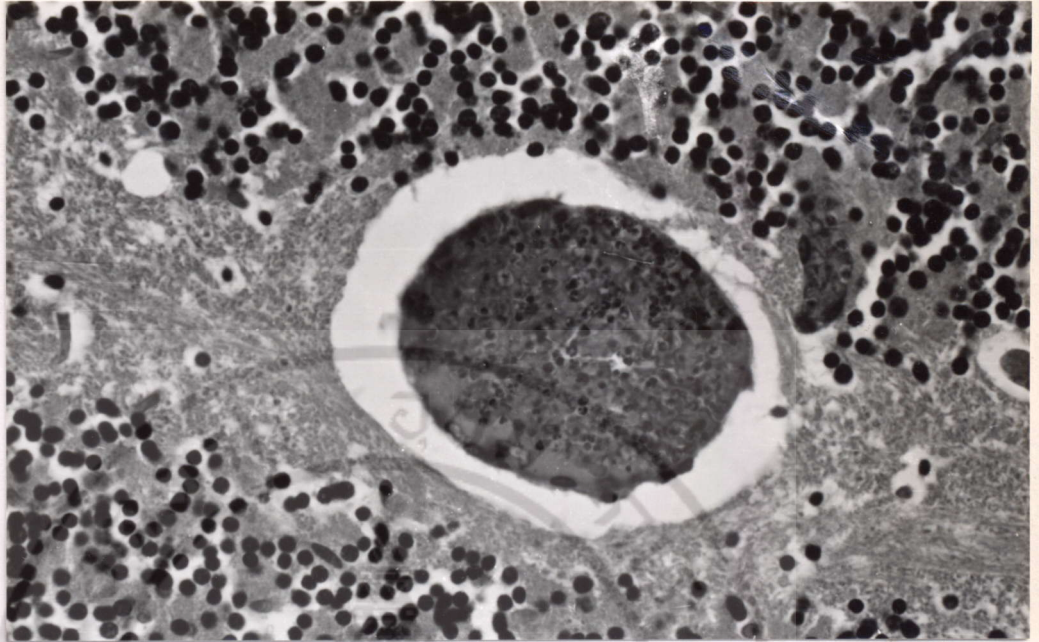


Fig. 12 Brain of P.berghei infected mouse died on day 9 shows severe congestion of blood vessel with agglutinated red cells in the white matter of cerebella folia. (H & E stained x 400).

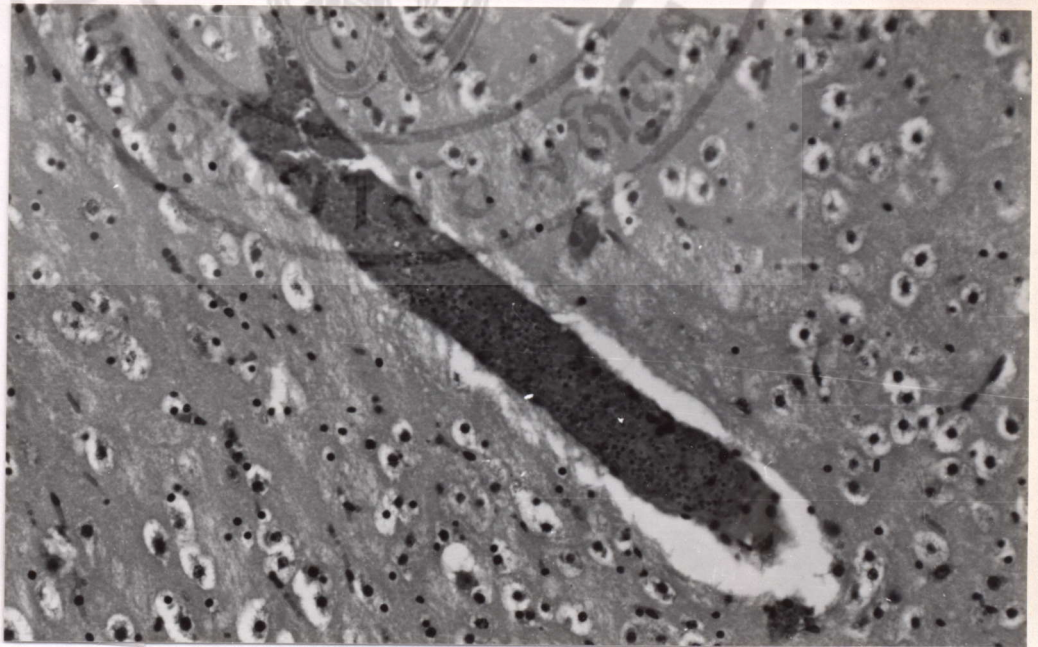


Fig. 13 Brain of P.berghei infected mouse died on day 9 of infection shows capillary blockage with parasitized erythrocyte in cerebral cortex. (H & E stained x 200)

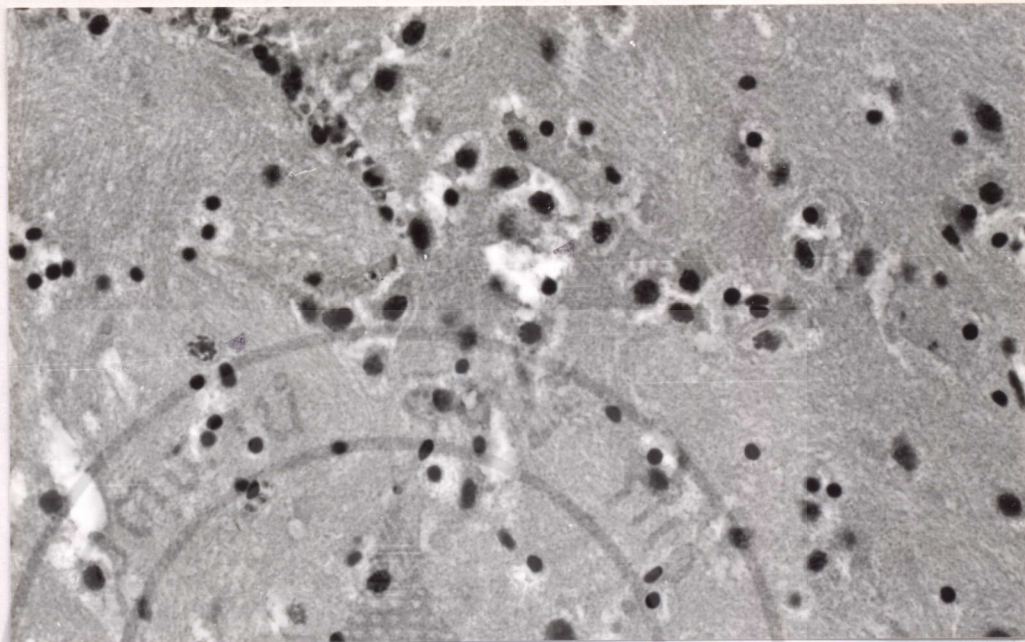


Fig. 14 Neuronal degeneration (arrow) associated with glial reaction was seen in the brain of P.berghei infected mouse died on day 9. (H & E stained x 400).

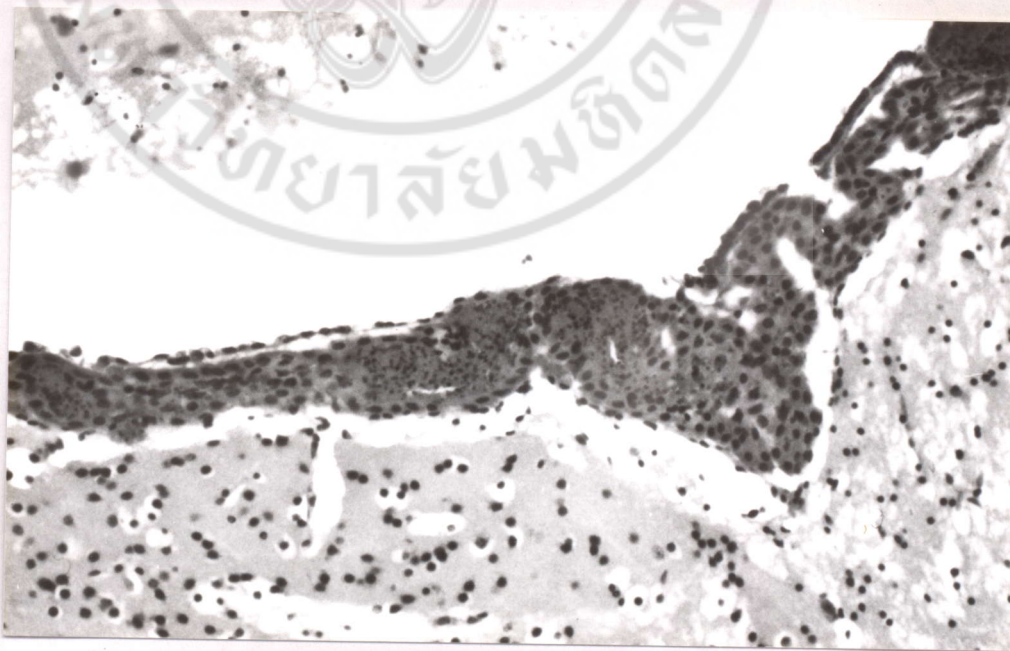


Fig. 15 Choroid plexus of P.berghei infected mouse died on day 9 reveals blood vessel congestion and hemorrhage. (H & E stained x 200).

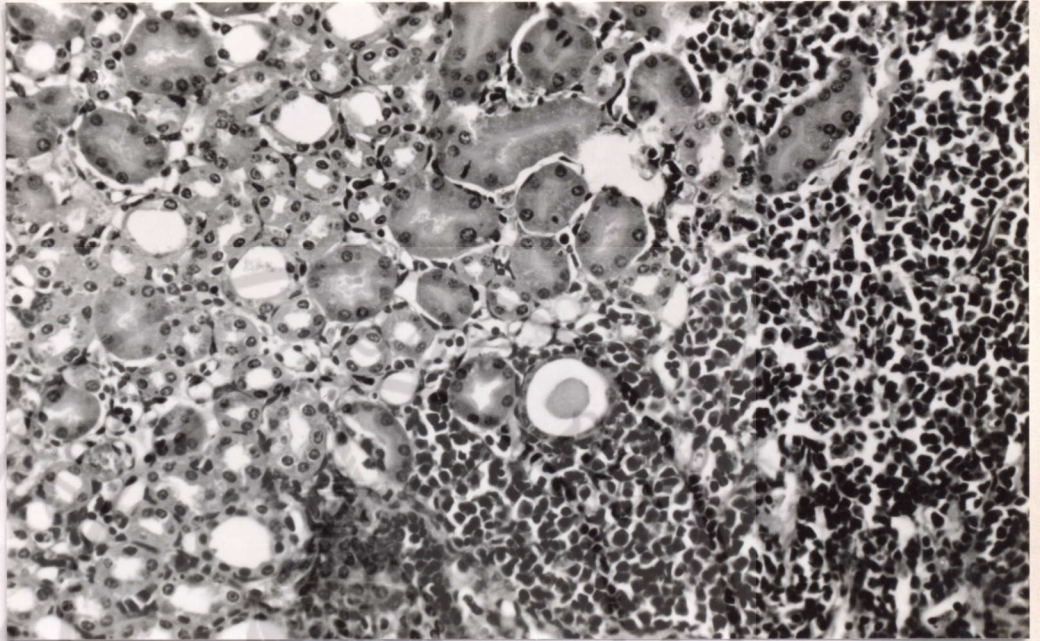


Fig. 16 Kidney from P.berghei infected mouse sacrificed on day 12 shows hyaline cast and dilatation of tubules. Mononuclear cell infiltration is seen at the right corner. (H & E stained x 200).

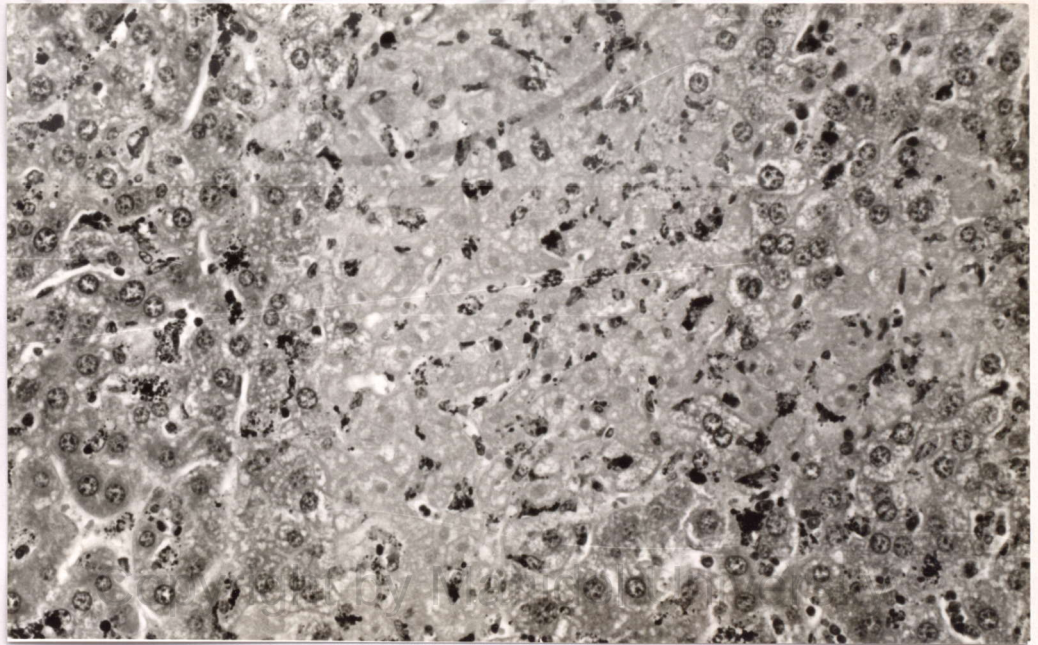


Fig. 17 Liver of P.berghei infected mouse killed on day 14 shows area of coagulative necrosis. Hypertrophy of Kupffer cells with prominent malarial pigment is seen. (H & E stained x 200).

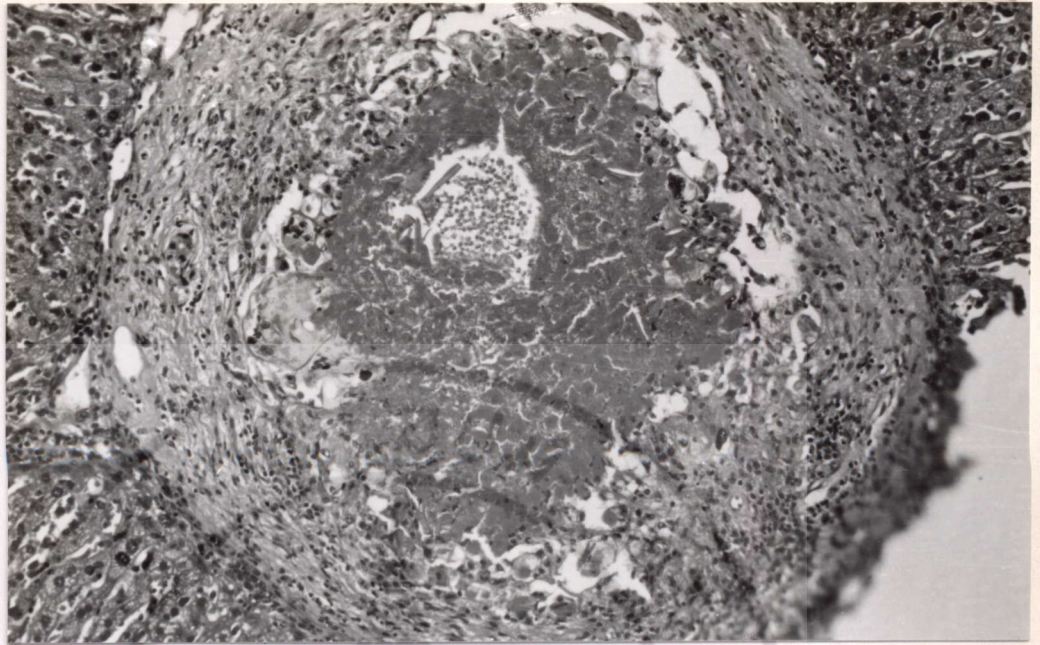


Fig. 18 Liver of P.chabaudi infected mouse died on day 9 shows thromboarteritis accompanied with atrophy of adjacent liver cells. Inflammatory cell infiltration and malarial pigment-laden macrophages are also seen. (H & E stained x 100).

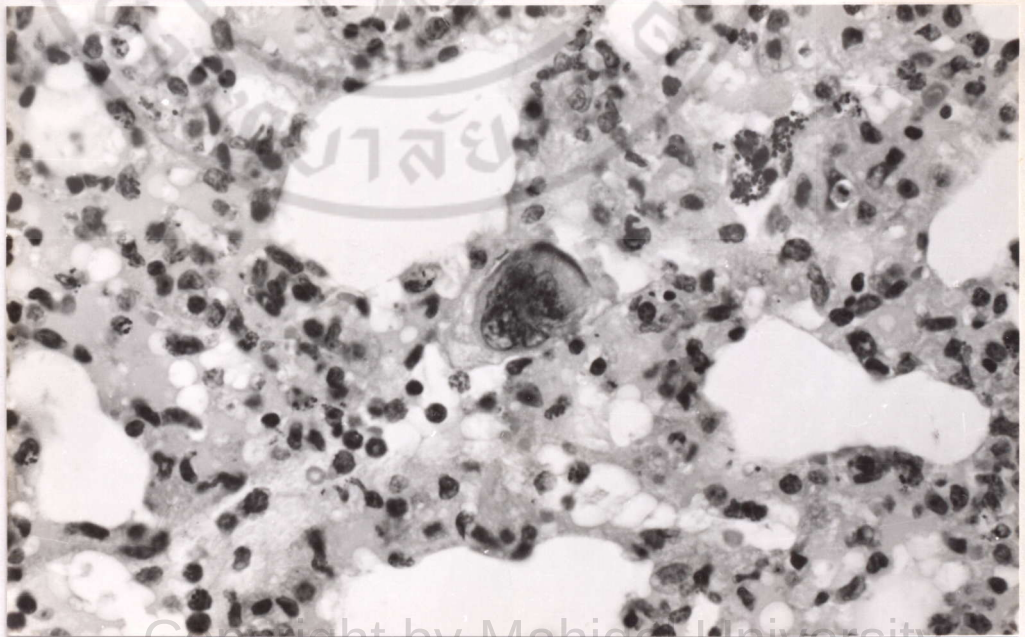


Fig. 19 Lung from P.berghei infected mouse sacrificed on day 12 of infection shows interstitial edema and malarial pigment-laden macrophages. Megakaryocyte is seen in the interalveolar septa. (H & E stained x 400).

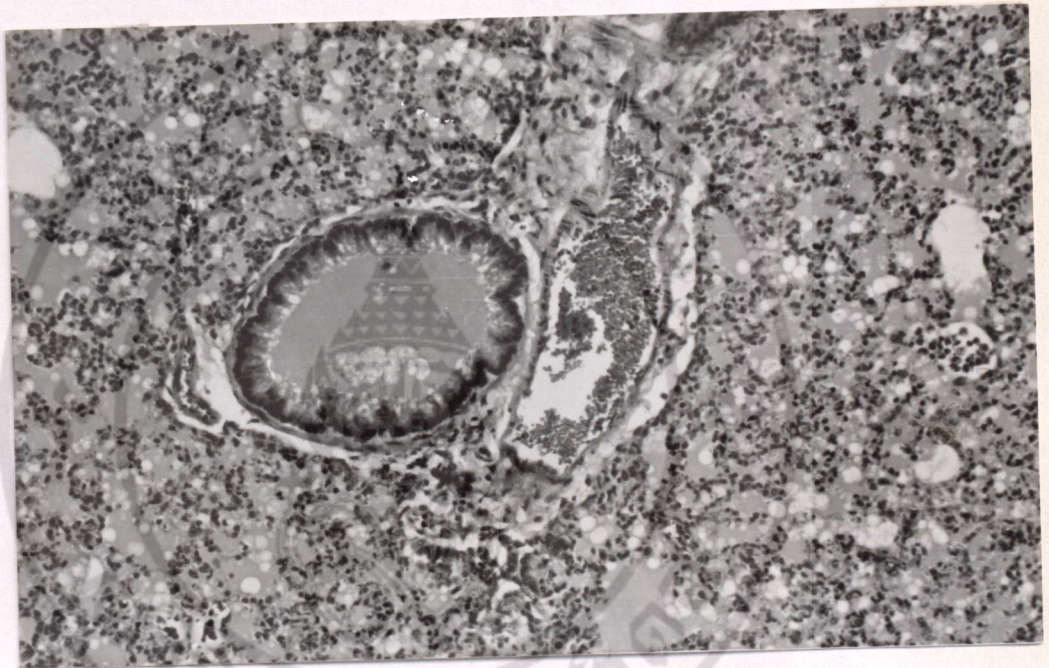


Fig. 20 Lung of P.berghei infected mouse died on day 9 with cerebral complication shows severe pulmonary edema. (H & E stained x 100).

DISCUSSION

Cerebral malaria is a major cause of death in human *Falciparum* malaria^(24,154) and in mice infected with *P.berghei*^(153,112). Variation in susceptibility to malaria infection of different strains of mice have been studied⁽¹¹²⁾. This experiment tried to study the cerebral lesions and factors contributing to the development of cerebral malaria in three different species of murine malaria parasite infections; *P.berghei*, *P.chabaudi* and *P.yoelii*. Generally, *P.berghei* malarial parasite induces fatal infection in all strain of mice while a mild strain of *P.yoelii* parasite posses a self-limiting infection^(57,58,125,191). For *P.chabaudi* parasite, it induce recrudescent infection in some strains of mice after the primary parasitemia has become subpatent⁽¹¹⁰⁾. However this was not found in this study. Mortality rate of *P.berghei*, *P.chabaudi* and *P.yoelii* infections in mice were 30%, 22.5% and 5% respectively. Mostly, infected mice in all experimental groups died by day 9 of infection. Those early dead mice developed sharp rise in parasitemia. Histopathologically, these mice had more severe brain lesions than those killed during the course of infection. Among of the mice sacrificed during infection *P.berghei* cerebral changes than those of *P.chabaudi* and *P.yoelii*

infections. The difference in the virulency of each malaria parasite in induction of cerebral lesions and other lesions may be due to the genetic nature of the parasite^(97,188). A sudden increase of virulence of P.yoelii was observed after injecting mice with along period frozen stablitate of this parasite⁽²⁰³⁾. This variant strain of P.yoelii caused acute fulminating infection in mice accompaied with blockage of cerebral capillaries by sequestered red cells⁽²⁰²⁾. However, its virulency decreased during blood passages⁽¹⁸⁾. In human malaria, only P.falciparum have been known to be a major cause of cerebral malaria. Some aspects of cerebral pathology of infected mice in this study were similar to those described in human cerebral malaria^(45,46,154,183). Congestion of cerebral blood vessels and hemorrhage seen in human cerebral malaria were also found in the mice. Fibrin deposition, gliosis and granuloma formation, which were found in some human cases, were not found in this study. However, the pathological changes seen in human cerebral malaria may be minimal or even absent. The fully changes seen in the fatal human malaria may not be seen developing in mice killed during the course of infections.

The presence of cerebral lesions, in this study, was not directly related to the level of CIC or anemia or antimalaria antibody. The most striking finding was the

rapid increase of parasitemia in mice died at acute stage. This event was also observed by many investigators^(15,89,150,151,197). The rapid increase of parasitemia underwent the development of mature stage of parasites. Blood smears of some mice obtained at the period of coma showed high parasitemia with late trophozoites and schizonts infected red cells. The morphological appearances showed a high proportion of irregular or spurshaped red cells (Fig. 9) more than those of non-cerebral involved mice. These may be due to the alteration of surface membrane of both parasitized and non-parasitized erythrocytes as reported by many investigators^(68,78). The changes of erythrocyte membrane induced a decrease in deformability of erythrocytes in passage through cerebral capillaries. The alteration in the rheologic properties of parasitized erythrocytes may involved in the mechanism of capillary obstruction^(119,120). As the parasitemia developed filterability of parasitized erythrocytes decreased markedly^(119,139). The adherence of parasitized erythrocytes to endothelial cells and to each other and the leakage of blood plasma during malaria infection⁽¹¹³⁾ may enhance the paucity of blood circulation of cerebral capillaries. In P.falciparum and P.coatneyi infections, knob-like protrusions on surface membrane of parasitized erythrocytes have been believed to be the site of adherence

to endothelial cells^(92,104,107) and may involve in the pathogenesis of cerebral malaria^(104,119). In P. knowlesi, it was evident that parasitized erythrocytes were trapped in brain blood vessels⁽²⁾. Frequently, obstruction as suggested by Jerusalem et.al.⁽⁸⁹⁾, occurred in postcapillary venules (PCV). The obstruction of cerebral capillaries and venules led to congestion and/or hemorrhage. The junction between capillaries and PCV has been recognized as the preferable site of rupture of blood vessels⁽⁸⁹⁾. The obstruction and/or hemorrhage of blood vessels gave rise to cerebral tissue anoxia in the beyond area and terminated with sudden death of mice. Glial reaction may be due to the degeneration of neurons⁽⁶³⁾.

The adherence of monocytes to endothelial cells and the releasing of monokines have been proposed to be the cause of endothelial damages^(150,151). But the attachment of monocytes to endothelial cells was not obviously seen in this study. However, by immunofluorescent study, the deposits of granular form of malaria antigen were found in cerebral blood vessels. This suggested that monocytes or other phagocytic cells presented in the blood vessels and may involved, in part, in the pathogenesis of cerebral malaria.

The role of immune response in pathogenesis of cerebral malaria remains obscure⁽⁸⁹⁾. Although there is a

clear evidence that thymectomized or antithymocyte serum (ATS) treated hamsters can prevent cerebral lesions from P.berghei infection^(197,198). The early death with cerebral involvement in this study was not correlated to the level of CIC, as previously reported^(89,112). Even antimalaria antibody was not detected in most mice developing mild cerebral lesions which were sacrificed during the course of infection. This less immune status was also found in human cerebral malaria⁽¹⁷⁸⁾. Many studies showed that the immunoglobulins and malarial antigens existed on the surface of parasitized and non-parasitized erythrocytes of P.berghei infection^(80,105,137). One factor as a marked pathogenesis of cerebral malaria may be intravascular agglutination⁽¹¹²⁾. Also, the agglutination of P.knowlesi parasitized Rhesus monkey erythrocytes by immune serum was reported⁽²²⁾.

Endotoxin, that can be detected during P.falciparum and P.berghei infections⁽¹⁸⁵⁾, has proposed to be one of the cause of endotoxin shock during high parasitemia in acute malarial infection⁽³²⁾. Recently, the endotoxin was found to contribute, in part, in the development of cerebral malaria in complicated P.falciparum infection⁽¹⁷⁸⁾. In this study detection of endotoxin was not accomplished, however, the role of endotoxin in induction of acute death cannot be excluded.

Pathological findings of other organs : Kidney, liver, lung, and spleen, were recorded. Each organ studied in all experimental groups were similar in principle changes, but slightly different in the degree of severity. P.berghei seemed to cause the most severe infection. Whereas the other two malarial parasites, P.chabaudi and P.yoelii, produced less severe lesions and had a tendency to return to normal after the parasite disappeared from the circulation. Typical histopathology of liver was coagulative necrosis, proliferation of malaria pigment-laden Kupffer cells and perivascular mononuclear infiltration. While kidney showed pigment-laden macrophages in glomeruli and peritubular blood vessels and degeneration tubules in some mice. White pulp and red pulp of spleen altered and pigment-laden macrophages presented in alveolar septa of lung. Immunopathological study of kidney showed the deposition of mouse γ -globulin, mouse β 1C in the mesangial area and in the glomerular capillary wall. Corpuscular forms of malarial antigens were found mostly at the early of infection and granular forms were dominant later.

Generally, the histopathological findings of kidney liver, lung and spleen were similar to those described by other investigators^(112,121,167,187). In kidney, acute transient reversible glomerulonephritis occurred during malaria infection with several species^(76,77)

and in association with localization of immune complexes in the glomeruli^(9,11,13,60,90,176). Evidence of immune complexes deposition in the glomeruli was in parallel with the presence of CIC in this study. These immune complexes were believed to be the cause of proteinuria^(12,14,138,145). Interstitial hemorrhages of renal blood vessels may be due to the circulatory stasis and hyperviscosity of blood in microvasculature of renal blood vessels⁽¹⁴⁾. These may lead to renal ischemia and resulted in the degeneration of tubules. Dilation of some distal convoluted tubules in some mice may be due to the obstruction of the tubular lumens. All of these severe renal lesions were observed during heavy parasitemic infections.

Coagulative necrosis of liver cells in this study resulted, in part, from hepatic artery obstruction. Fibrin thrombi were major components of the intravascular obstruction. This suggested that intravascular coagulation did occur. Since alteration of coagulation factors during malaria infection was noted in rodent⁽⁸³⁾ and human malaria⁽¹⁷⁴⁾. One factor that may influence intravascular coagulation is the alteration of red blood cell membrane^(31,68). However, thrombosis may be occurred after intravenously injection of any fluid (Boonpucknavig, personal communication). Hypertrophy of malarial pigment-laden Kupffer cells caused the distension of liver sinusoids and enlargement of the

liver. These results resembled those described in several malaria infection^(112,121,187).

Lung showed mild histological changes with malarial pigment laden macrophages. Interstitial pneumonia was due to the chronic respiratory disease of mice as described by Nelson⁽¹³²⁾. However, interstitial pneumonia in the infected mice was more severe than normal mice. Mild interstitial edema was noted in most high parasitemic mice. Only one acutely dead mouse infected with P.berghei showed severe pulmonary edema (Fig. 20). This may be due to the increase of vascular permeability and leakage of plasma fluid into interstitium and alveoli^(48,193).

In all groups of infections, splenomegaly was well developed, especially in P.yoelii and P.chabaudi infected mice. This was due to the splenic function in blood clearance of parasitized erythrocytes^(147,149,169,199). Also, excessive destruction of parasitized and non-parasitized erythrocytes by spleen lead to anemia^(161,162). This correlated to the increasing of extramedullary hemopoiesis and pigment macrophages. Congestion of red pulp with mostly parasitized erythrocytes was due to the trapping of altered red cells by reticular network of spleen⁽¹⁹⁹⁾. Prominent germinal center of white pulp and proliferation of plasma cells at later stage of infection

paralleled with the rising of antimalarial antibody as described elsewhere (121).

In conclusion, the cerebral lesions occurred in some mice infected with P.berghei, P.chabaudi, and P.yoelii. P.yoelii infection revealed the least severity of the lesions and mortality rate. Some aspects of brain lesions of mice that died acutely resembled those described in human cerebral malaria. In the present study, circulating immune complexes (CIC), antimalaria antibody and anemia were not directly correlated to the presence of cerebral lesions. The sharp rising of parasitemia and the alteration of the membrane of parasitized and non-parasitized erythrocytes were markedly associated with the acute death of infected mice. The pathogenesis of cerebral malaria may be due to the obstruction of capillaries and venules by "agglutinated red cells". This may lead to tissue anoxia and terminated with sudden death of infected mice. However, endotoxin may play an important role in the development of acute death with cerebral complication of infected mice (32,178).

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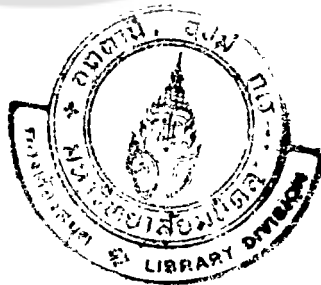
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APPENDIX1. ASSAY FOR COMPLEMENT TITRE (for PEG-CC)

Materials : - Fresh, Guinea Pig serum

- 1:5 dilution of veronal buffer (V.B.)
- Sensitized SRBC (as described in the text)
- microtitre plate

Method

1. Guinea Pig serum was made to X dilution of 1:4, 1:8, 1:10, 1:12, in VB (1:5) in microtitre plate.
2. From these four initial dilutions, each was made serial two folds dilutions.
3. The plate was incubated for 15 min at 37°C
4. Added 1% sensitized SRBC (50 X) into each well and mixed.
5. Incubate the mixture at 37°C for 30 min and at 4°C overnight.
6. Read the hemolysis titres of each of different starting dilutions. The highest dilution of this serum that hemolysis is up to 1:256 will be used in PEG-CC test.
7. Diluted the guinea pig serum in VB (1:5) to the desired dilution and kept in an aliquot of 100 ul at -70°C.

8. Before used of each lot of complement (guinea pig serum), the appropriated dilution is made, usually in the experiment is 1:10 in VB (1:5).

2. IMMUNOELECTROPHORESIS (IEP)

Materials : - 1% Agarose in Veronal buffer (1 g agarose, 99 ml of 0.05 M Veronal buffer pH 8.6)

- Glass slides
- Universal Electrophoretic cell (UE cell) and Voltage + current regulated D.C power supply (Buchler Instruments, Inc.)
- 0.05 M Veronal buffer pH 8.6

Method :

1. Melt 1% agarose in veronal buffer in water-bath and layer the agar on clean glass slides about 3.0-3.5 ml per slide. Allow the molten agar to solidify for 15 min at room temperature, then place in a humidity chamber to prevent drying and keep in a refrigerator.
2. Cut well and trough on the agar and fill the well with antigen sample carefully.
3. Place the agar slides in U.E. cell containing 0.05 M Veronal buffer pH 8.6 and connect each end of the slide to the buffer chambers.

4. Apply the current about 5 m A per slide for 2 hr, then turn off.
5. Remove the slides from the chamber and remove the agar from the trough before fill with antiserum
6. Incubate the slides in the moist chamber at room temperature for at least 6 hr. Observe for the precipitation pattern produced, generally, the patterns should developed sufficiently within 24-36 hr.

3. RAT LIVER HOMOGENATE AND RAT LIVER POWDER

Materials : - Healthy adult rats
- 0.01 M PBS pH 7.1 (cold)
- Acetone
- Homogenizer (Sorvol omni-mixer)

Method :

1. Sacrify the rats by cutting through the neck and remove livers. Blood can be removed from the liver by an intravenous infusion of cold PBS
2. Chopped Livers into pieces and homogenize with 4 volumes of cold PBS by using Sorval omni-mixer.
3. Centrifuge the homogenized liver at 8,000 rpm for 20 min and suspend the pellet in cold PBS.

3.1 RAT LIVER HOMOGENATE

3.1.1 Wash the homogenized liver in PBS until the supernatant is clear.

3.1.2 The pellet is frozen at -20°C overnight, thaw and wash extensively until no hemolysis occur.

3.1.3 A small tube filled with 1 ml of packed liver homogenate is kept at -20°C until use in the ratio of 1:2 of rat liver homogenate to conjugate absorbed.

3.2 RAT LIVER POWDER

3.2.1 The homogenized liver in (3) is washed then resuspended in cold acetone and homogenized again

3.2.2 Remove large aggregates by filtering through a gauze wool

3.2.3 Centrifuge the filter effluent at 8,000 rpm for 20 min and resuspend pellet in acetone

3.2.4 Filter the suspension through a Buchner funnel fitted with vacuum pump and wash with acetone until the preparation is white on drying.

3.2.5 Keep the liver powder in 37°C incubator for 6-12 hr, grind up into powder and store at room temperature.

4. SEPHADEX G-25 COLUMN PACKING

Materials : - Sephadex G-25 (Pharmacia, Upsala, Sweden)
 - Column
 - 0.01 M Phosphate buffered saline, pH 7.1

Method :

The volume of the gel should be six times of the conjugated volume. The height of the packed gel in the column can be calculated by this formula:

$$6 \times V = \pi r^2 h$$

V = volume of conjugate

$$= \frac{22}{7}$$

r = radius of the column used

h = height of gel packed in the column

1. Sephadex G-25 is equilibrated in 0.01 M PBS pH 7.1
2. Carefully layer the gel into the column until reach the desired height.
3. Allow the gel to settle in a cold room overnight before used.

5. PROTEIN CONCENTRATION DETERMINATION

Protein concentration is determined by measuring the absorbance at 280 nm (O.D. 280) and calculated as follow:

$$\text{Protein concentration} = \frac{\text{O.D. 280} \times \text{dilution}}{1.4} \text{ mg/ml}$$

6. ALSEVER'S -GLYCEROL FOR PRESERVATION OF MALARIAL PARASITES (203)

Materials : - Alsever's solution (4.66 g glucose, NaCl 1.05 g, sodium citrate 2 g, and DW 200 ml)
 - AR grade glycerol
 - Millipore filter paper

Method

Prepare Alsever's solution and add glycerol to make 10% solution before use. The solution is filtered through a millipore filter and stored at 4°C

For cryopreservation of malaria parasites, 1 ml of heparinized blood is mixed with 3 ml of Alsever's -glycerol solution and rapidly frozen in acetone-dryice. Store the frozen parasites at -70°C or in liquid nitrogen.

BUFFERS AND WORKING SOLUTIONS

1. 0.5 M Carbamate-bicarbonate buffer pH 9.0

(for conjugation)

<u>Reagents</u>	NaHCO ₃	3.7 g
	Na ₂ CO ₃	0.6 g
	DW	100 ml

Method

1. Dissolve NaHCO₃ and Na₂CO₃ in DW
2. Adjust pH 9.0 with 0.1 N NaOH

Note : The carbonate-bicarbonate buffer should be prepared just before use.

2. Isotonic Veronal buffer pH 7.6 (for β LC preparation)

<u>Reagents</u>	<u>Solution I</u>	NaCl	85.0	g
		Barbitone sodium	3.75	g
		(Na-5, 5-diethylbarbiturate)		
		DW	1400	ml
	<u>Solution II</u>	Barbitone	1.44	g
		(5,5-diethylbarbituric acid)		
		Hot DW	500	ml
	<u>Solution III</u>	Stock solution containing 1 M		
		MgCl ₂ and 0.3 M CaCl ₂	5	ml
		DW up to	2000	ml

Method

1. Mix solution I and II together, let it cool to room temperature
 2. Add solution III and stir
 3. Adjust the pH to 7.6 with 0.1 N NaOH or 1 M HCl.
 4. Add DW to 2000 ml and sterile in autoclave.
 5. Store at 4°C and dilute 1:5 before use.
3. 0.05 M Veronal buffer, pH 8.6 (for IEP)

<u>Reagents</u>	Sodium barbital (NaC ₈ H ₁₁ N ₂ O ₃)	10.30	g
	Diethyl barbituric acid (C ₈ H ₁₂ N ₂ O ₃)	1.84	g
	NaN ₃	0.65	g
	DW		

Method

1. Add approximately 900 ml of DW to a 1 litre volumetric flask and heat.
 2. Add 1.84 g diethylbarbituric acid to hot DW and mix thoroughly to dissolve.
 3. Add 10.3 g sodium barbital and 0.65 NaN_3
 4. Allow the solution to cool at room temperature
 5. Determine the pH, if the pH is below 8.55 adjust with 1 N NaOH, if the pH is above 8.65, adjust with 1 N HCl
 6. Add DW to 1 litre and store in a closed vessel at 4°C
4. 0.01 M Phosphate-buffered saline (PBS) pH 7.1
- | | | | |
|-----------------|---|------|----|
| <u>Reagents</u> | NaCl | 85 | g |
| | Na_2HPO_4 | 13.5 | g |
| | (or $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$) | 27.0 | g |
| | $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ | 3.9 | g |
| | DW | 2000 | ml |

Method

1. Dissolve all reagents in 2000 ml DW
2. Adjust pH to 7.1 with NaOH or HCl and sterile in autoclave
3. Dilute 1:5 for using

5. 10% Neutral buffer formalin

<u>Reagents</u> :	40% formalin	100	ml
	NaH_2PO_4	4	g
	Na_2HPO_4	6.5	g
	DW	900	ml

Method

1. Dissolve NaH_2PO_4 and Na_2HPO_4 in 900 ml of DW
2. Add 100 ml of 40% formalin
3. Adjust to pH 7 with NaOH or HCl

6. Zenker-Formol

<u>Reagents</u> :	DW	100	ml
	Mercuric chloride	50	g
	Potassium dichromate	25	g

Method :

1. Dissolve mercuric chloride in hot DW and cool at room temperature.
2. Add potassium dichromate and store at room temperature just before use add 5 ml of 40% formalin per 100 ml fixative. For mouse organs, fix for 2 hr, then wash in running tap water for 4 hr and keep in 70% alcohol.

7. 0.2 M Phosphate buffer pH 7.2 (PB for purification of rabbit antimouse p1C)

<u>Reagents</u> : $\text{NaHPO}_4 \cdot \text{H}_2\text{O}$	4.42	g
Na_2HPO_4	23.86	g
DW	1000	ml

Method : Dissolve $\text{NaHPO}_4 \cdot \text{H}_2\text{O}$ and Na_2HPO_4 in DW and adjust to the desired pH with 0.1 N NaOH or 1 M HCl.

8. Veronal buffer pH 7.4 (VB) for PEG-CC

<u>Reagents</u> : Sodium barbital	10.2	g
NaCl	88.9	g
CaCl_2	85	mg
MgCl_2	500	mg
NH_4Cl	31.3	ml
DW	2000	ml

Method : Dissolve all reagents, autoclave and store at 4°C after addition of 50 mg NaN_3 per liter. Dilute 1:5 in DW prior to use.

9. Borate buffer pH 8.3 (BB)

Boric acid	6.18	g
Disodium tetraborate	9.53	g
NaCl	4.38	g
NaN_3	1	g
DW	1000	ml

10. Phosphate-buffered saline (PBS) pH 7.2 (for PEG-CC)

NaCl	32.43	g
KH_2PO_4	6.10	g
Na_2HPO_4	24.72	g
DW	1000	ml

Diluted 1:5 in DW prior to use.