Degree of Red blood cell reduction in different strains of semi immune mice infected with *Plasmodium berghei ANKA* after chronic exposure

Gideon Kofi Helegbe

Graduate School of Biomedical Sciences
Institute of Tropical Medicine (NEKKEN)
Nagasaki University, Japan

MARCH 2011

Declaration

The experimental work described in this thesis was done by me, at the Immunogenetics Department, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Japan, under the supervision of Professor Kenji Hirayama. References cited in this work have been fully acknowledged.

.....

Gideon Kofi Helegbe
(Candidate)

.....

Professor Kenji Hirayama
(Supervisor)

Dedication

To

Setor and Setor; and Madam Mansa Margaret Dzade

List of Journal Publications

1. Rate of red blood cell destruction varies in different strains of mice infected with Plasmodium berghei-ANKA after chronic exposure. Helegbe GK, Huy NT, Yanagi T, Shuaibu MN, Yamazaki A, Kikuchi M, Yasunami M, Hirayama K. Malar J. 2009 May 5;8:91.
2. Histopathological studies in two strains of semi-immune mice infected with Plasmodium berghei ANKA after chronic exposure. Helegbe GK, Yanagi T, Senba M, Huy NT, Shuaibu MN, Yamazaki A, Kikuchi M, Yasunami M, Hirayama K. Parasitol Res. 2010 Oct 27

Abbreviations

RBC: Red blood cell

iRBC: Infected RBC

uRBC: Uninfected RBC

SMA: Severe Malaria Anaemia

CM: Cerebral Malaria

PbANKA: *Plasmodium berghei* ANKA

EPO: Erythropoietin

GPI: Glycosyl-phosphotidylinositol

Hz: Haemozoin

HNE: Hydroxy-nonenal

RSP-2: Ring surface protein 2

MO: Macrophage

Hb: Hemoglobin

Ig: Immunoglobulin

IFA: Immunoflourescence assay

HRP: Horse radish peroxidase

IL: Interleukin

TNF: Tumour necrosis factor

HE: Hematoxylin-eosin

Table of Contents

Background

Malaria is a protozoan disease caused by parasites of the genus *Plasmodium*. Four Plasmodium species namely: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and currently *P. knowlesi* have been known to cause infection in man. Of these Plasmodium species *P. falciparum* is observed to be the leading cause of death in the tropics. Annually, about 1.5- 2.7 million deaths are observed mostly due to the *P falciparum* [1]. However, the frequency of the disease is greatly reduced in individuals older than 5 years in spite of persistent infection [2]. This protection is known as clinical immunity, and it is not seen in low-endemic or sporadically exposed areas.

Due to persistent infection of the *Plasmodium* in individuals in the endemic areas, the individual become semi-immune. Despite being semi-immune, individuals still develop the severe forms of the disease such as severe malaria anaemia (SMA) and cerebral malaria (CM). Severe malaria anaemia and CM (with case fatality rate of approximately 19% and >13%, respectively) are observed to be among the leading cause of the associated morbidity during *Plasmodium* infections [3]. The present study focuses on malaria anaemia in mouse model. Unfortunately little is known about the pathogenesis of malaria aneamia despite its high prevalence. Three possible mechanisms have been suggested to explain the pathogenesis of SMA as summarized in Figure 1 [4]. These are:

- Rapture of infected red blood cell (iRBC) due to parasite proliferation [5]
- Immune-mediated dependent destruction of RBC. The immune factors are antibody, complement, T-cells and cytokines. These immune factors have two targets, the iRBC [6] and uninfected RBC (uRBC) [7]
- Suppression of erythropoiesis [8, 9].

The relative contribution of these is still not clear.

It has been suspected for some time that anaemia in malaria may not wholly result from destruction of iRBC but from uRBC as well [10], for example in some other protozoan infections such as kala-azar, anaemia develops although no intra-erythrocytic growth of the parasites occurs [11]. A recent study [12] suggested that part of the haemolytic component of the anaemia of *Plasmodium* results from immune-mediated destruction of uRBC.

Despite the fact that experimental animal models cannot reproduce all the features of human disease, it could be explored to explain some phenomena in human situations. Also animal studies have been useful in understanding the mechanisms of pathogenetic basis of various disease conditions to help in prophylactic or therapeutic interventions. Except for studies by Evans *et al.*, [12], most studies carried out in the mouse model have been on the naïve mice, which is not a true reflection of the situation in the endemic areas. And the anaemia observed in these endemic areas is observed to occur at relatively low parasitaemia. This observation implicates the role of uRBC destruction in the SMA.

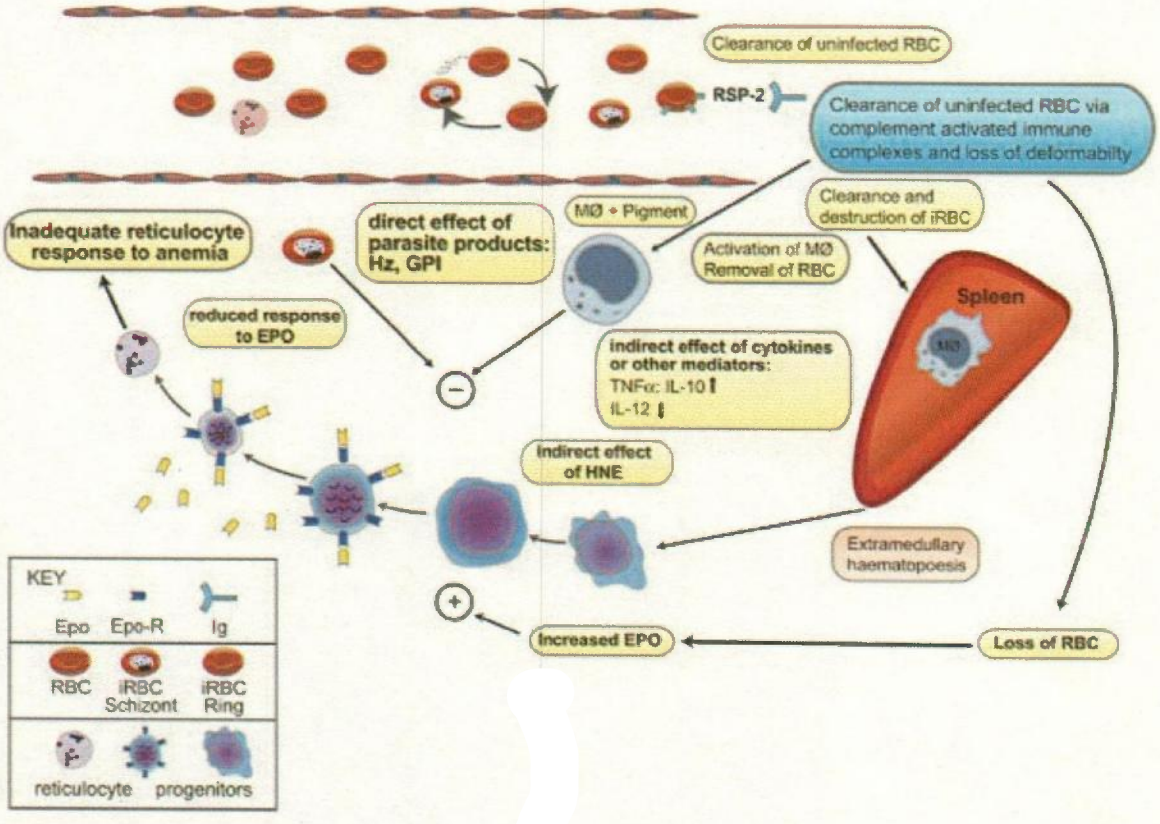

Figure 1: Direct and indirect effects of parasite on the development of malarial anemia. Severe malarial anemia is characterized by destruction of infected red blood cell (iRBC) following schizogony and clearance of both iRBCs and uninfected RBCs. During malarial infection, changes in membrane protein composition occur and the resultant immune complexes of RBCs, Ag, and immunoglobulin (Ig) (e.g., RBC: RSP2: Ig) are cleared by macrophages to the spleen where they become activated. Pigment-containing macrophages may release inflammatory cytokines and other biologically active mediators such as hydroxy-nonenal (HNE). It is possible that malarial pigment or other parasite products may have a direct inhibitory effect on erythropoiesis. Inhibition of erythropoiesis may be at one or more sites in the growth and differentiation of hematopoietic progenitors. Both indirect and direct effects may cause suppression of the bone marrow and spleen resulting in inadequate reticulocyte counts for the degree of anemia. Blue box indicates demonstrated in human infection; pink box, demonstrated in mouse infection; yellow box, demonstrated in both human and mouse infections. Hz indicates hemozoin; GPI, glycophosphatidylinositol anchors of merozoite proteins; Epo, erythropoietin; Epo-R, erythropoietin receptor; MO, macrophage; RSP-2, ring surface protein-2; and Ig, immunoglobulin. [Source: Ref [4]

Furthermore, even though individuals are exposed to the same *Plasmodium* infection they respond differently; some develop the severe forms of the malarial disease and others not, thus implicating host factors. Therefore, to understand the extent to which host factors play a role in the development of SMA, four different strains of mice were used in this study and their response to *Plasmodium berghei ANKA* infection with particular reference to RBC loss was observed, by testing the hypothesis that;

Genetic background can change the levels of destruction of uRBC during malaria infection

The following objective was set to address the above hypothesis;

To observe the levels of uRBC destruction in four different inbred strains of mouse, Balb/c, CBA, NZW and C57BL/6 using semi-immune mouse malaria model

Thus, in this thesis report, the author showed that there is some involvement of host factors in the development of the SMA as there was variation in rate of RBC loss in the four strains of semi immune mice (Chapter 1). And the mechanism by which SMA occurs is observed to be mainly by the destruction of uRBC, as shown by the histology study (Chapter 2).

Chapter One

Rate of red blood cell destruction varies in different strains of mice infected with *Plasmodium berghei-ANKA* after chronic exposure

1.1 Introduction

Malaria continues to claim the life of millions in the tropics and it is reported that 1.5-2.7 million deaths are observed annually mostly due to *Plasmodium falciparum* [1]. Individuals in the endemic regions become semi-immune as a result of the repeated infection [13]. Despite being semi-immune, a significant proportion of these individuals develop the severe forms of malaria disease leading to high mortality and morbidity, with severe malaria anaemia (SMA) as one of the leading causes [3]. However, much remains to be understood of the pathogenesis of SMA.

Central to the proposal to explain the pathogenesis of SMA is the destruction of high numbers of uninfected red blood cells (uRBC) compared with the infected RBC (iRBC) [12], due to the consistent observation of SMA at relatively low parasite burdens of semi-immune individuals in malaria endemic areas [14]. Jakeman *et al* used a mathematical method to evaluate that with one destroyed iRBC, there is 10 destructed uRBCs [15]. The phenomenon of high uRBC destruction at low parasitaemia in the semi-immune is still unclear, but phagocytic cells and/or $CD4^+$ T lymphocytes are thought to play a role [12]. Also, inadequate reticulocyte response has been proposed as being a contributory factor to the SMA, due to an abnormal bone marrow cellularity reflected by low reticulocyte counts in SMA patient [16].

Another process that contributes to the destruction of uRBC is the mechanical mechanism, as indicated by the role of auto-antibodies [17, 18]. Even though elevated anti-erythrocyte ghost antibody levels have been demonstrated to be associated with human malaria infections [19], its association with anaemia and host genetic factors has

not been clarified in the semi-immune. Anti-erythrocyte auto-antibodies reacting with the surface of normal or acetone fixed human erythrocytes have also been reported to occur in *P. falciparum* patients' sera [10, 20] and are thought to be at least in part responsible for the anaemia frequently seen in acutely infected *P. falciparum* patients. Using Direct Coombs antiglobulin test, previous studies proposed a relationship of anti-RBC antibodies in the anaemia seen in *P. falciparum* infections [21, 22].

Although the role of auto-immune mechanism in uRBC destruction resulting in anaemia during malaria has been debated for some time, it is still controversial. While some studies have implicated auto-antibodies such as IgM, IgG and IgA classes [17, 23-26], as having specificity toward uninfected and infected RBCs, thus playing an auto-immune mediated mechanism of uRBC destruction, and others do not [27]. Thus using the rodent model the association between level of auto-antibodies against uRBC ghost and degree of anaemia at low parasite burden in the semi-immune was investigated. Rodent model of SMA as developed by Evans *et al* [12] are uncomplicated by excessive parasite burdens. In contrast, naïve murine malaria infections are hyperparasitaemic, thereby making the associated haemolytic anaemia not be reflective of SMA in the human populations.

Since severe malaria has been found to vary from one individual to another [28], with the implication of host genetic factors, due to variation in number of infected erythrocytes and spleen size in the naïve murine malaria [29, 30], the role of strain specificity in auto immune mediated mechanism of uRBC destruction in the different strains of chronic infected mice was also investigated. Studies have shown that there is a

differential level of auto-antibodies in other diseases such as auto-immune haemolytic anaemia in mice strains [31, 32].

1.2 Materials and Methods

1.2.1 Mice, malaria infections and profiles of SMA

Four strains of mice BALB/c, C57BL/6 (B6), CBA and New Zealand White (NZW) aged 8 weeks supplied by SLC laboratories, Fukuoka, Japan, were injected intraperitoneally (i.p.) with 10^4 *Plasmodium berghei* ANKA–infected RBCs. Parasitaemia and reticulocyte levels were monitored every two days by Giemsa-stained thin blood film and are expressed as a percentage of more than 500 RBCs. Haemoglobin (Hb) was measured in a 96-well plate at 570 nm on Bio-Rad Model 3550 Micro plate Reader as previously described [33]. Four microliter (4 μL) of tail-vein blood was suspended in 1 mL Drabkin reagent (Sigma, St Louis, MO) and absorbance measured, and is expressed as a percentage of baseline levels. Laboratory and animal practices of the Animal Center of Institute of Tropical Medicine (NEKKEN), Nagasaki were adhered to, after the approval from the local ethics committee for animal care and research was obtained.

1.2.2 Generation of semi-immune mice and harvesting of serum

This was a modified method as described elsewhere [12]. Four strains of infected mice were treated at day 6 after infection with chloroquine/ (10 mg/kg intraperitoneally) and pyrimethamine (10 mg/kg intraperitoneally) daily for 6 days. During subsequent rounds of infection, mice were rested for two weeks before being rechallenged with 10^4 *P. berghei* ANKA, then monitored and drug-cured prior to parasitemias reaching 5%. Mice

underwent seven to eight cycles of drug-cured infection before finally being challenged with 10^4 *P. berghei* parasites without treatment. Preliminary studies in the laboratory, in which mortality occurred beyond some minimum days after infection (Figure 2) and data from other studies, in which complications arose as a result of parasitaemia between 25 and 75% [34], influenced the time blood harvested for serum. About 100µL of blood was collected *via* eye vein during the 7th and 8th cycle of infection at low Hb. Two weeks after treatment (7th cycle), blood was collected again for serum. The sera were stored at -30°C until used. The whole set of experiments was performed twice and pooled data are presented.

1.2.3 Preparation of red blood cell (RBC) white ghost membrane

The method used here was based on a previously described one [35] with some modifications. Briefly, heparinized blood (0.5 mL) from uninfected mice was washed with phosphate buffer saline (PBS), pH 7.4 and later haemolysed in hypotonic phosphate buffer (5 mM, pH 8.0). After vigorous shaking, the haemolysate was washed twice for 20 minutes at 15,000rpm. The supernatant was removed by aspiration. The membranes were washed six times with the same haemolysate buffer until the pellet became white, and then washed 2-3 times with Tris-HCl (50 mM, pH 7.2) and finally in PBS. Antigen concentration was determined by BCA protein assay kit (Product number 23227, Pierce Biotechnology, Rockford, USA).

1.2.4 Screening of sera from semi-immune for antibody binding to RBC membrane

Immunofluorescence assay (IFA) was used to check antibody binding to RBC membrane. RBC white ghost membrane prepared above was used as antigen to coat the

IFA slides, fixed in cold acetone and washed in PBS. Goat serum (Chemicon International, CA) diluted 1:100, was used for blocking and incubated at room temperature (RT) for 30 minutes. After washing the goat serum with PBS, the serum samples (primary antibody) were added at different dilutions and incubated at RT for 3 hours. Washing was done thrice in PBS and secondary antibody goat anti-mouse IgG-FITC (Sigma-Aldrich, St Louis, Missouri, USA) diluted 1:50, was added to the slides and incubated for an hour in the dark at RT. The slides were later washed thrice in PBS and observed under fluorescence microscope.

1.2.5 Antibody titer measurement using ELISA

This was a modified method as described previously [36]. The RBC white ghost membrane was used as antigen at a protein concentration of 2µg in 100µl of coating buffer (pH 9.6) per well to coat polystyrene plates (Lot number 091611, Nunc, Copenhagen, Denmark) at 4°C overnight. The plates were washed thrice with 0.05% Tween-20-PBS, then optimum blocking conditions for non-specific binding was achieved using 300µl per well of 0.1% blocking reagent (lot number 13945300, Roche Diagnostics, Mannheim, Germany) -0.1% Tween-20/PBS, pH 7.2, and incubated for 1 hour at 37°C. Plates were washed thrice with PBS containing 0.05% Tween-20. The antigen in coated plates was then reacted with the serum samples obtained from non-infected (as negative control) and infected mice at 1/40 dilutions, in duplicates. After three hours incubation at 37°C, plates were washed five times with 0.05% Tween-20/PBS. Later, 100µL of horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL) was added to each well and incubated for 1 hour at 37°C, then washed five times with 0.05% Tween-20/PBS. For

colour development, 3, 3', 5, 5'-tetramethylbenzidine (TMB, Catalogue number SK-4400, Vector Laboratories, CA, USA) was used and prepared according to the manufacturer's instructions. The reaction was then interrupted at 30 minutes by the addition of 50µl 1N H_2SO_4. Absorbance was read at 450nm in EIA-reader (Bio-Rad, Hercules, CA)

1.2.6 Statistical analysis

Data analysis was done using the GraphPad Prism Version 5.00 for Windows, GraphPad Software, San Diego California, USA, www.graphpad.com. Data are expressed as the mean with standard error of mean (SEM) unless otherwise stated. Data were log transformed to ensure normal distribution before one-way analysis of variance (ANOVA, with Tukey's post-test), were performed. Pearson correlation analysis was performed on the transformed data of variables to compare the relationship between them. Values were considered significant when $p<0.05$

1.3 Results

1.3.1 Parasitaemia-time course, profile of severe malaria anaemia and erythropoietic response in the semi-immune mice strains

Four strains of mice (Balb/c, B6, NZW and CBA) were taken through several cycles of infection with 10^4 *P. berghei* ANKA followed by pyrimethamine and chloroquine treatment to generate semi-immune status. Upon challenge with 10^4 *P. berghei* ANKA after generating semi-immune status (i.e. after the final cycle), similar parasitaemia profiles were observed in different cycles of infection in the same strain, but different responses were observed in the strains (Figure 3). Prepatent period was four days in Balb/c and two days in B6, NZW and CBA. Also there were two peaks of parasitaemia exhibited by the individual Balb/c mice during recovery (Figure 3a). The first peak (0-3.1 % parasitaemia) was observed between days 4 and 12 and the second peak (0-1.2 % parasitaemia) between days 12 and 22. Individual variation in parasitaemia was also observed in the other semi-immune mice, but at relatively higher parasitaemia, Figures 3b-d. Much higher parasitaemia in the individual mice were observed in the NZW (Figure 3d). Even though some mice appeared to resolve their parasitaemia (Figures 3b-d), they could not fully recover and most of them died.

The kinetic profiles of mean blood haemoglobin, reticulocytes, and parasitaemia of four strains were also recorded as shown in Figure 4. One consistent observation made here is that as parasitaemia reached its peak, it was followed by a Hb decrease and an increase of reticulocyte count. Balb/c semi-immune mice resolve their parasitaemia and their Hb improved gradually (Figure 4a). The other strains could not resolve their parasitaemia, hence continued fall in Hb and eventual death (Figure 4b-d). Percentage

of mice in each strain that died was similar to that of Figure 2. The correlation between Hb and parasitaemia was poor in Balb/c and B6 (Figure 5a, b) but significant in NZW and CBA (Figure 5c, d), indicating that the degree of anaemia was independent of the level of parasitaemia in Balb/c, B6 while it is correlated in NZW and CBA.

Haemoglobin levels fell to 47.11%, 28.44%, 30.05% and 25.10% of normal levels in Balb/c, B6, NZW, and CBA, respectively, on days with minimum Hb (Hb_m) at relatively low parasitaemia, Table 1. Interestingly, Hb reduction in Balb/c, B6 and CBA were at relatively lower mean parasitaemia (<4.0%), compared with NZW mice of mean parasitaemia > 7.0%, $p<0.0001$. Moreover, this minimum percentage Hb drop occurred at different period for each semi-immune mice strain, Table 1. During the repeated cycles of infection and treatment, it was observed that relatively higher RBC destruction occurred at later cycles (semi-immune status of mice), but at low parasitaemia in comparison with the first cycle of infection, implying uRBC are destroyed as well and/or inadequate reticulocyte response. With this observation, the extent to which destruction occurred in the semi-immune mice strains at the final cycle and the contribution of parasitaemia was evaluated. It can be seen in Figure 6 that, the mean percentage Hb loss was significantly different statistically in the semi-immune mice strains ($p=0.0005$).

Reticulocyte count was estimated in the semi-immune mice to assess extent of erythropoietic response. During the course of infection and treatment to generate semi-immune status, it was observed that reticulocyte production correlated significantly with %Hb in Balb/c, B6 and CBA, Figure 7. Similar trend was observed in

the final cycle where statistically significant correlation of mean %Hb levels and reticulocyte levels were observed in the mice strains of Balb/c (r^2=0.88, p<0.0001), B6 (r^2=0.89, p=0.0014), CBA (r^2=0.58, p=0.003), and insignificant in NZW (r^2=0.30, p=0.27). The extent of reticulocyte production to Hb loss in Balb/c was two to three times higher than that of B6, CBA and NZW, and statistically significant (Table 1).

1.3.2 Detection of autoantibody to white ghost RBC membrane

The binding of antibody to white ghost RBC in sera of all the semi-immune mice was observed using IFA as shown in Figure 8. To further understand if antibody level is related to extent of Hb loss, anti-RBC auto-antibodies against white ghost RBC was estimated and observed that antibody level in the sera of the mice strains were significantly higher in Balb/c when compared with the other strains, p<0.001 (Figure 9a); thus consistent with the observations made in Table 1 regarding amount of Hb loss. To further evaluate the effect of autoantibody at recovery where parasitaemia was not detected, amount of auto-antibody to white ghost RBC in the sera at recovery of the semi-immune mice was estimated and observed that the titer of autoantibody to RBCs within the strains were statistically different, p<0.0001 (Figure 9b). Furthermore, anti-RBC antibody at recovery was similar to that at low Hb with infection, p> 0.05 for NZW and CBA, whereas that of Balb/c and B6 were significantly different, p<0.001 and <0.05 respectively (Figure 9c). Also auto-antibody at recovery for Balb/c was only statistically significant when compared with that of CBA at recovery.

To further explore the relationship of auto-antibodies with degree of anaemia, anti-erythrocytic antibody was analyzed with extent of Hb loss during the final cycle.

This was to understand further the relationship between the two in all the mice strains. It was observed that the correlation was significant (Figure 10), thus indicating that anti-erythrocytic antibody does play a role in the extent of uRBC destruction.

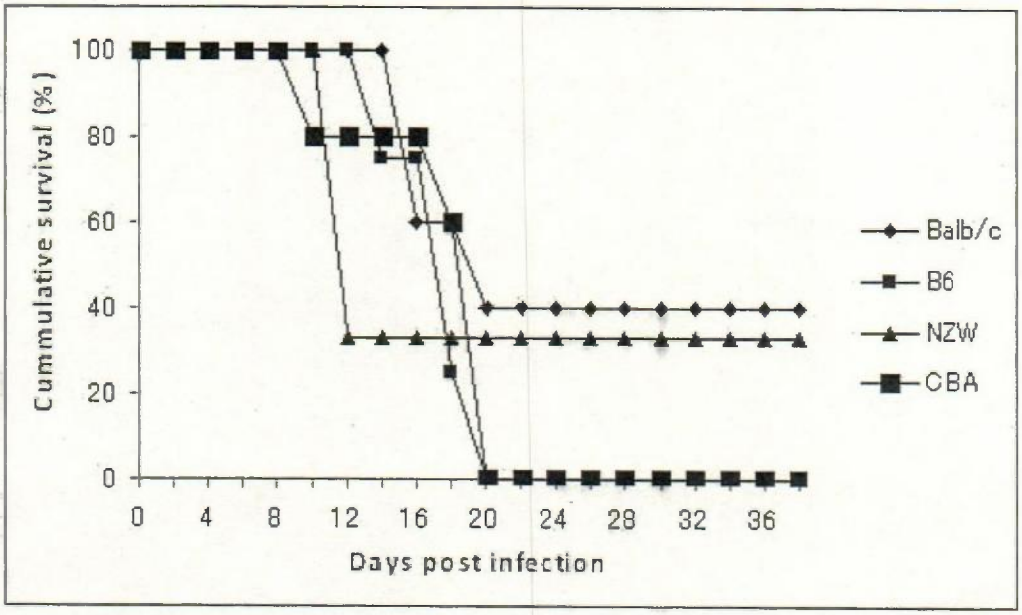

Figure 2: Cumulative survival curve of semi-immune mice.

Death of semi-immune mice were monitored after challenge with 10^4 *P. berghei* ANKA following attainment of semi-immune status in the mice strains without treatment. Balb/c, n = 5; B6, n = 4; NZW, n = 3; CBA, n = 5.

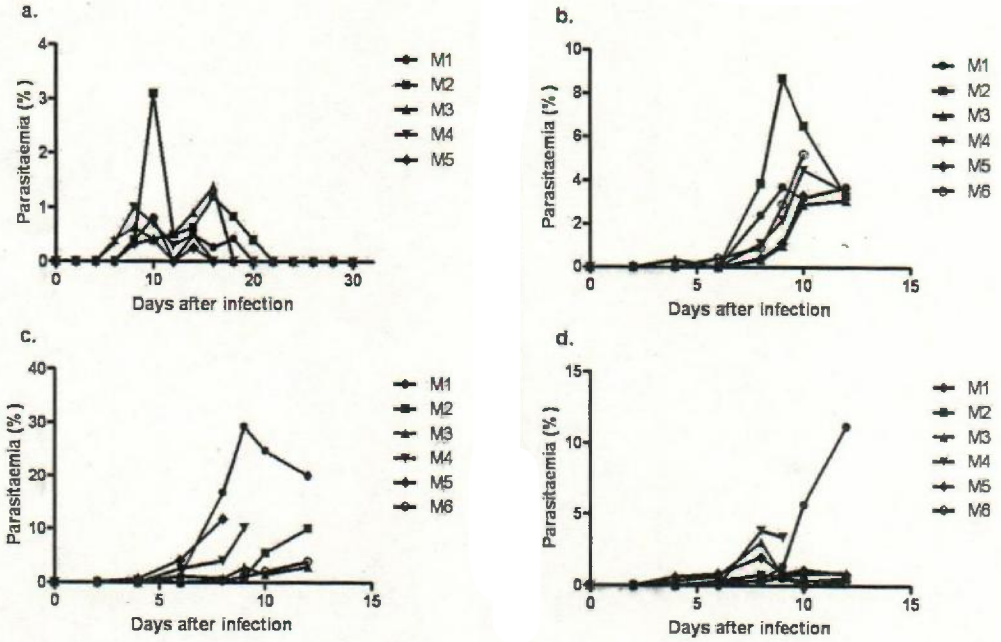

Figure 3: Parasitaemia time course in the semi-immune mice.

Representative data of infected semi-immune mice (a) Balb/c, n =5 (b) B6, n = 6 (c) NZW, n = 6 and (d) CBA mice, n = 6 following infection with 104 *P. berghei* ANKA during the final cycle without treatment.

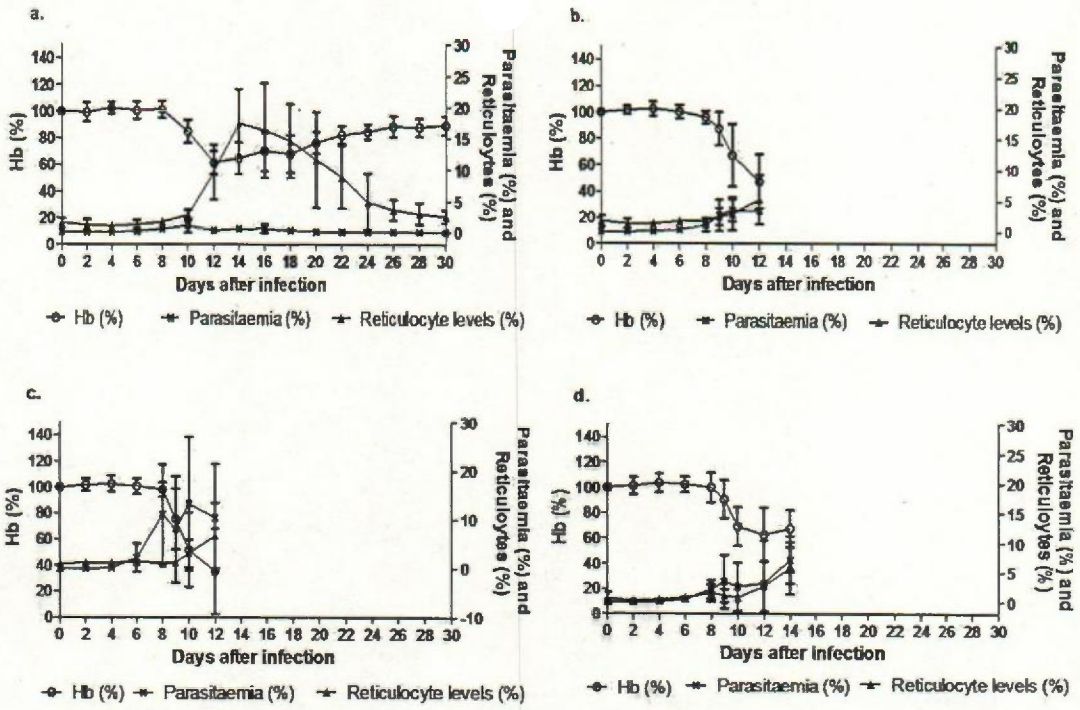

Figure 4: Profile of malaria anaemia in the semi-immune mice.

Mean parasitaemias, reticulocyte levels and Hb in semi-immune mice strains (a) Balb/c, n = 5 (b) B6, n = 11 (c) NZW, n = 8 and (d) CBA, n = 12, after *P. berghei* ANKA infection during the final cycle. These are pooled data from 2 separate experiments and data are represented as mean ± SD.

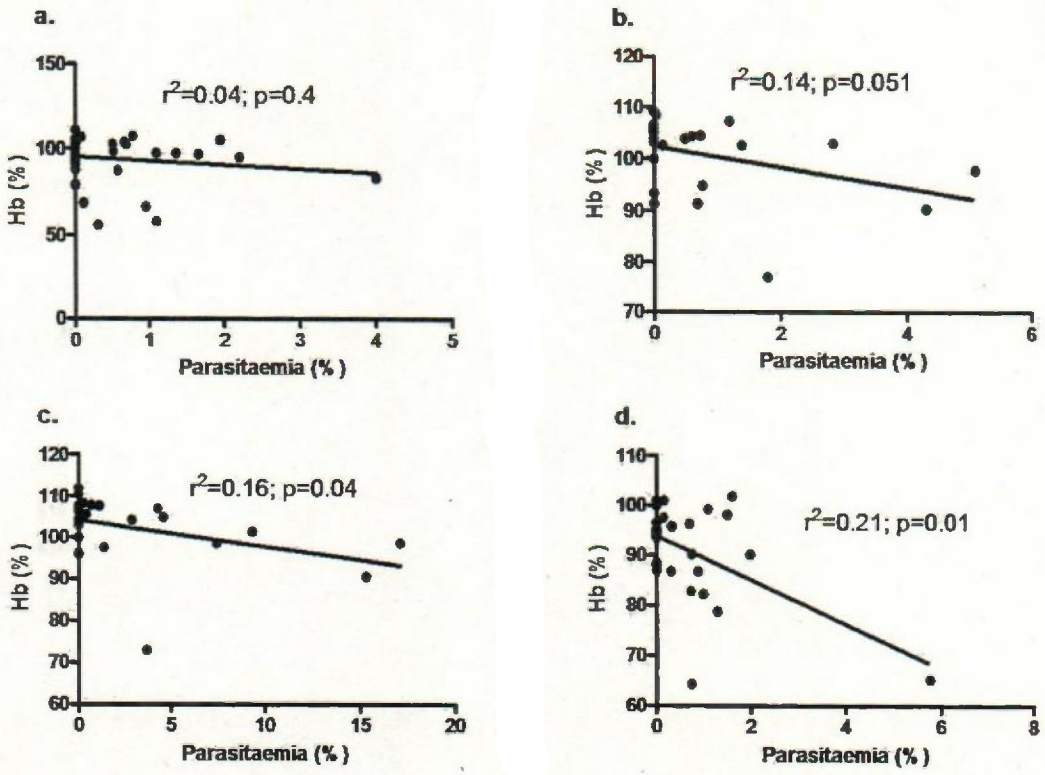

Figure 5: Relationship between Hb and parasitaemia in semi-immune mice strains.

Mice were infected with 10^4 *P. berghei* ANKA to generate semi-immune status in the mice strains. Hb was determined (see text for procedure) and parasitaemia every two days. Hb and parasitaemia were compared in the combined cycles (1–6) to examine the extent of their relationship. (a) Balb/c, n = 5 (b) B6, n = 6, (c) NZW, n = 6 (d) CBA, n = 6. Hb, haemoglobin. These are values of one experiment.

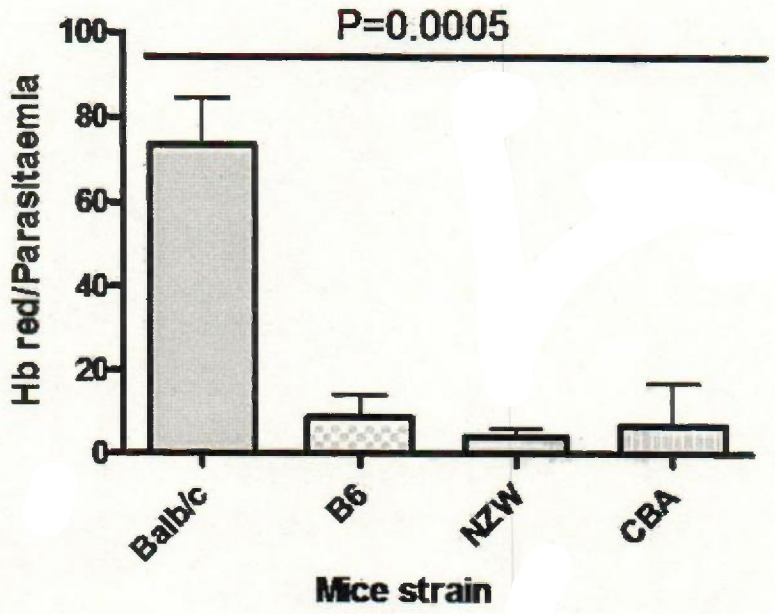

Figure 6: Percent Haemoglobin drop per parasitaemia in the semi-immune mice strains at later cycles.

Hb reduction (Hb red) for each mouse is the difference in the Hb (baseline, 100%) before first cycle infection and that of Hbm during the 7th cycle. To evaluate the extent of Hb reduced per parasitaemia rise, the ratio of the Hb red and parasitaemia at Hbm was calculated. The values are pooled data of two separate experiments. Similar observation was made during 8th cycle. Balb/c, n = 11; B6, n = 11, NZW, n = 8, CBA, n =12. Error bars are standard error of mean (SEM). One way ANOVA was used to analyse data with Tukey post-test, where p values for Balb/c vrs B6, NZW < 0.001, and < 0.01 for CBA. The p values for the others were > 0.05. Hb red, Haemoglobin reduction; Hbm, minimum Haemoglobin value.

Table 1: Magnitude of Hb reduction, peak reticulocyte count and peak parasitaemia in the semi-immune mice strain on day minimum Hb was observed

Parameters	Balb/c	B6	NZW	CBA	P value[a]
n	11	11	8	12	-
Mean Hb reduction (95% CI)	47.11[b] (38.12-56.1)	28.44 (22.82-34.1)	30.05 (16.61-43.5)	25.1 (13.97-36.2)	0.037
Mean Parasitaemia, % (95% CI)	0.64[c] (0.2-1.1)	3.3 (1.6-4.9)	7.5 (5.0-10.1)	3.1 (0.98-6.25)	<0.0001
Mean Reticulocyte level, % (95% CI)	12.45[d] (8.9-15.97)	3.2 (1.9-4.4)	2.0 (0.6-3.4)	3.1 (1.4-4.8)	0.0016
Mean of Reticulocyte level/Hb reduction	0.24[e]	0.11	0.07	0.12	0.003
Mean Period, day (95% CI) at which Hb_m was observed	13.64[d] (12.13-15.15)	10.6 (9.95-11.33)	10.86 (9.2-12.5)	10.9 (10.3-11.5)	0.014

Hb reduction is the difference between %Hb on day prior to first cycle infection and treatment, and day on which Hbm was observed (blood harvested for serum). The data presented here are pooled results of two separate experiments. aOne-way ANOVA with Tukey's post-test. [b]Significantly higher than CBA; Significantly [c] lower, [d]higher than B6, NZW, CBA; [e]Significantly higher than B6, NZW. Hbm, minimum Hb.

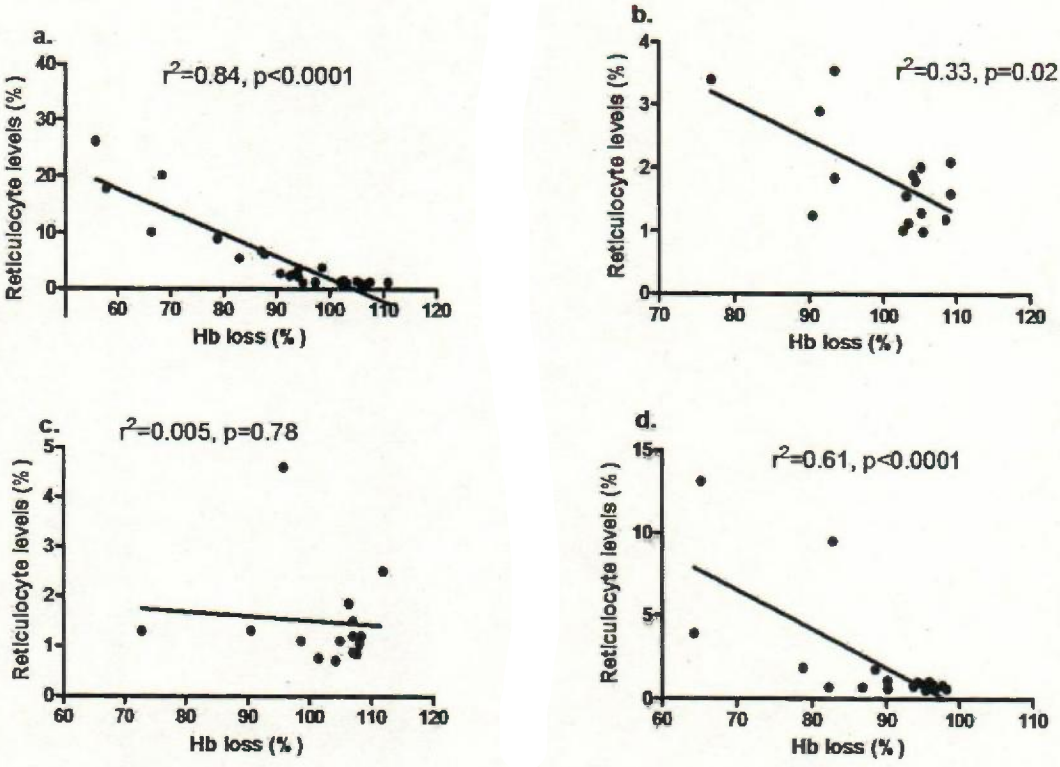

Figure 7: Relationship between Hb and reticulocyte in semi-immune mice strains.

Mice were infected with 104 *P. berghei* ANKA to generate semi-immune status in the mice strains. Hb was determined (see text for procedure) and reticulocyte count every two days. Hb and reticulocyte count were compared in the combined cycles (4–6) to examine the extent of their relationship. (a) Balb/c, n = 5 (b) B6, n = 6, (c) NZW, n = 6 (d) CBA, n = 6. Hb, haemoglobin. These are values of one experiment.

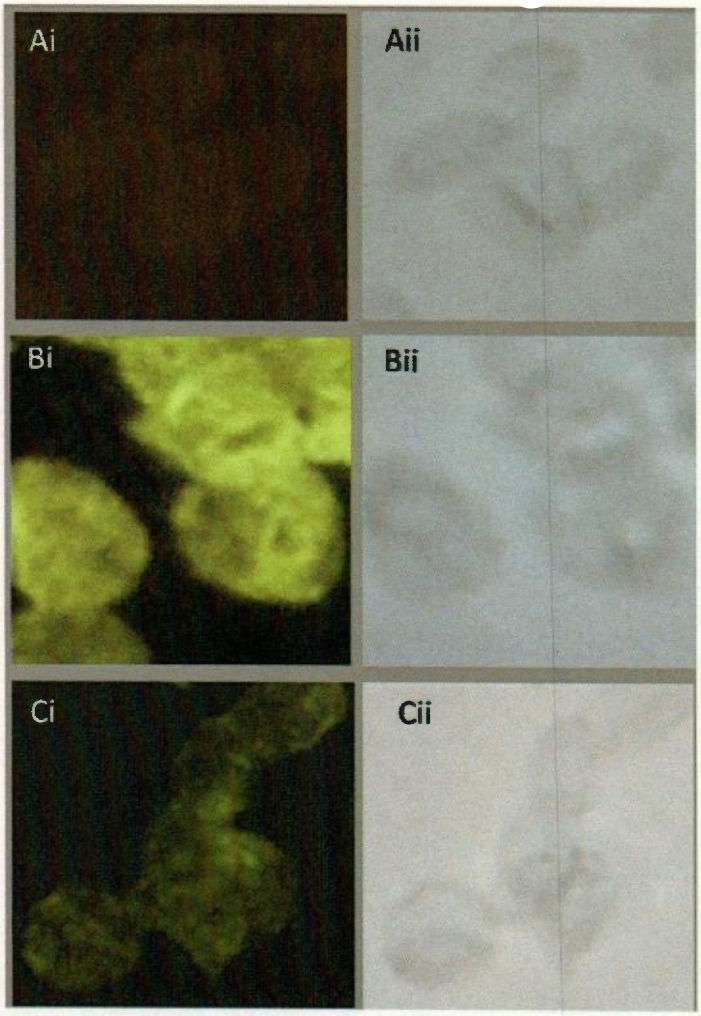

Figure 8: Reactivity of anti-erythrocyte antibody induced by *Plasmodium berghei* ANKA infection by indirect fluorescent antibody test.

(A) Serum of uninfected mouse reacting with white ghost RBCs from a normal uninfected mouse, under fluorescence i, and bright field, ii. (B) Panel representative of serum from a B6 infected semi-immune mice (Balb/c, B6, NZW) and (C) Serum of CBA semi-immune mice reacting with white ghost RBC of a normal uninfected mouse under fluorescence i, and bright field, ii. Serum dilution was at 1:16.

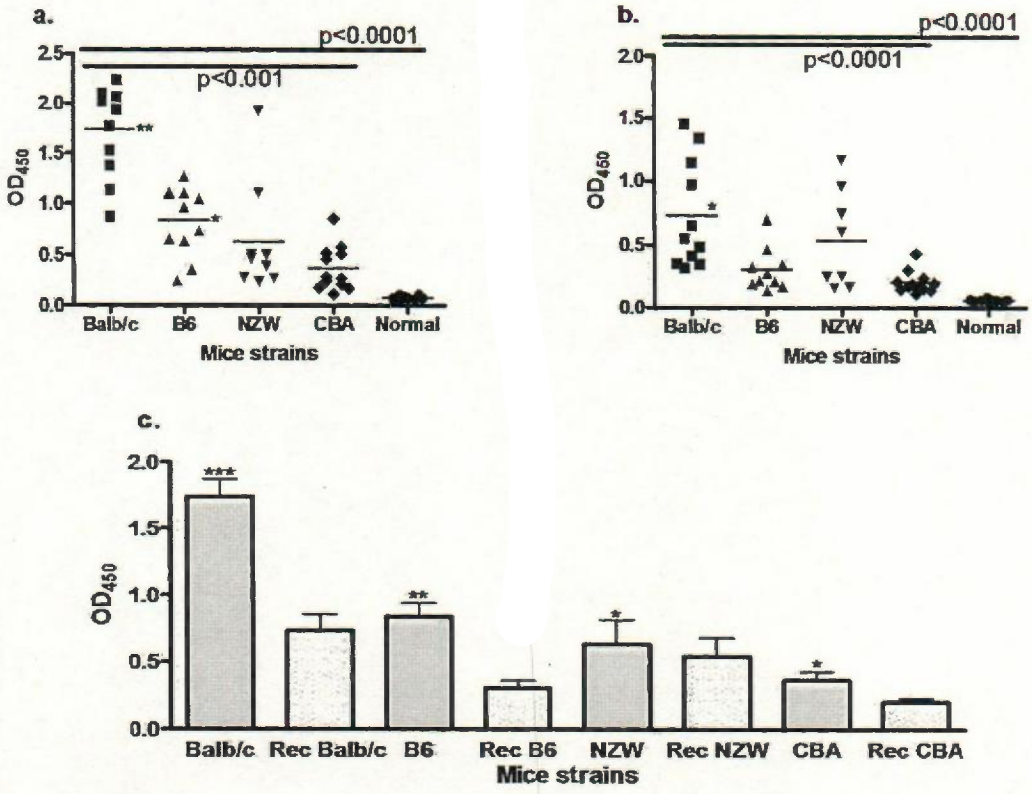

Figure 9: Antibody level of the sera (1:40) from the semi-immune mice strains against white ghost RBC of an uninfected white ghost RBC.

Sera were harvested in each of semi-immune mice strain at 7th cycle of infection (a) and at recovery (b), and (c) when both are compared. The antibody titer was measured via ELISA. Background values were not higher than 0.06 optical density (OD) units and those for control un-infected sera were slightly higher (mean 0.09). Antibody level in (a) and (b) expressed as individual plots, while the mean is represented by the horizontal bar within the individual plots. (a) $^{**}p < 0.001$ when compared with the mean antibody titer of the other mice strains; $^{*}p < 0.05$ when compared with CBA, Tukey's posttest. Balb/c, n = 10; B6, n = 11; NZW, n = 8; CBA, n = 12. (b) $^{*}p < 0.001$ when compared with B6, CBA. Balb/c, n = 8; B6, n =9; NZW, n = 7; CBA, n = 11, Normal, n = 9. (c) Error bars are standard error of the mean (SEM). One-way ANOVA was used to analyze data, $p < 0.0001$. ***/**/*$p < 0.001, < 0.05, > 0.05$ respectively when that at low Hb with infection is compared with that at recovery for each strain. Balb/c, n = 8; B6, n = 9; NZW, n = 7; CBA, n = 11. Results presented here are pooled data of two separate experiments, with duplicates.

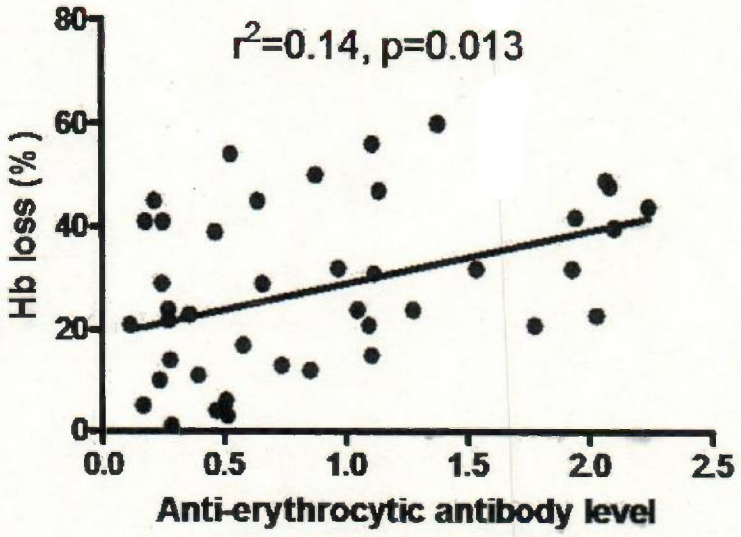

Figure 10: Correlation of anti-erythrocytic antibody with Hb loss in the semi-immune mice strains.

Extent of Hb loss was estimated at the final cycle after challenging with 10^4 *P. berghei* ANKA. Sera were also harvested when minimum Hb was observed at this last cycle, and anti-erythrocytic antibody measured by ELISA. The above are individual values of mice of all the mice strains, n = 42 (Balb/c, n = 11, B6, n = 11; NZW, n = 8; CBA, n = 12), at the final cycle. These are pooled data of two experiments.

1.4 Discussion

In the early stage of malarial infection, destruction of iRBCs is the primary cause of the anaemia [37]. The severity of anaemia with acute *P. falciparum* malaria correlates with density of parasitaemia [38]. However, in the semi-immune studies have observed that malaria anaemia occurs at low parasitaemia [12, 14], and variation in extent of Hb reduction has also been noted in these anaemic individuals. However, the association of this RBC destruction in the semi-immune mice with an immunologic mechanism *via* auto-antibody, and host genetic factors has not been explored. Results from this study shows that auto-antibody may play a role in the destruction of uRBC leading to low Hb in the semi-immune mice at low parasite burden and associated with host genetic factors.

The study here on SMA at low parasitaemia provided a fine opportunity to evaluate extent of uRBC destruction in the semi-immune. The kinetics of blood haemoglobin, reticulocyte levels and parasitaemia showed that Hb improved gradually in Balb/c, even though reticulocyte production in Balb/c was 2-3 times more than the other mice. In as much as inadequate reticulocyte response [16] and destruction of iRBC cannot be excluded, destruction and elimination of uRBC in chronic infected mice may be a major contributory factor resulting in anaemia as observed in another [12] and this study. This is demonstrated during the evaluation of Hb reduction per parasitaemia at the final cycle in the semi-immune mice strains and the observation of Hb loss at a much lower parasitaemia during one of the cycles of infection when compared with the first cycle infection. A recent study has shown that actual parasite numbers may be a major factor in evaluating anaemia than percent parasitaemia [25]. However, in this study only

percent parasitaemia was considered, thus further study to estimate the role of actual parasite number in such a study will be interesting. The kinetics and magnitude of reticulocyte production have been observed to be similar in both phenylhydrazine-induced anaemia and *P. berghei* ANKA infected mice [12], suggesting reticulocyte response was adequate. Inadequate reticulocyte response may be a major factor to low Hb in naïve hyperparasitaemic [39] or acute infections. Another possible mechanism to explain for the observed low Hb during Plasmodium infections is the preference of *P. berghei* ANKA for young erythrocytes/reticulocytes [40, 41]. Thus, at all levels of parasitaemia, more especially when Hb is low, higher proportions of parasitized reticulocytes than parasitized erythrocytes have been shown to occur [39]. Due to this phenomenon, not enough reticulocytes are able to develop into mature RBC, as both infected and uninfected reticulocytes are cleared [39], hence the persistent low Hb in the chronic infected mice despite compensatory erythropoiesis response to haemolytic anaemia.

The destruction of uRBC may be auto-immune mediated [24] due to the high statistical significant anti-RBC ghost antibodies reported in this study and its significant correlation with anaemia. However, it is suspected that the high auto-antibody mediation could be as a result of the RBC destruction. In that sense a lot of antigens are exposed thus enhancing the synthesis of the antibodies especially in Balb/c. The low parasitaemia observed in the Balb/c seems to indicate that its immunity is much more enhanced compared to the other strains. As a result Balb/c is able to control the parasitaemia growth. The high immune status coupled with the high antibody level in Balb/c appeared to be protective but at a cost, resulting in pathology situation of low Hb.

This anti-RBC ghost antibody may lead to sensitization of RBC resulting in immune complex formation during malaria infection at the acute anaemia phase of malaria infection, which has been widely proposed as the cause of RBC destruction [24] and resultant anaemia [42]. Several additional autoantigens have been implicated in the auto-immune disorders occurring during malaria, including modified antigen-antibody complexes [26]. Also, these surface-adherent antigen-antibody complexes initiate complement activation [43, 44] inducing a prehaemolytic or a haemolytic condition, as observed in this study. The entire immune complex may be auto-immune responses leading to elimination of RBCs. The observation of continues fall in Hb after parasite clearance following treatment with antimalarial in this study and others [16], in addition to IFA and ELISA results further support the fact that auto-immune mechanisms may be involved to some extent in the low Hb observed at relatively low parasitaemia. Similar observation was made to give explanation for the low Hb during babesiosis infections in cows [36]. In addition to the IFA result in that study [36], higher anti-erythrocytic auto-antibody to ghost RBC was reported in the naturally infected cows in comparison with the non-infected. A contrasting result was, however, obtained in another study, where lack of association between auto-immune mechanism and RBC in chronic malaria was reported [27]. It is not clear if the different parasite strain used could result in this difference, thus this needs to be investigated further.

Previous work showed that depletion of macrophage delayed the clearance of uRBCs in mice, suggesting a role of macrophage in the destruction of uRBCs [12]. At the onset of malaria infection, macrophage activity is crucial to control level of parasitaemia, via eythrophagocytosis, which is enhanced by opsonization with antibodies and other

immune reactions like complement [43, 44]. However, the over activity can result in pathology (such as low Hb) and sometimes death [45]. Although significantly high anti-RBC autoantibody was observed in the mice strains, which will enhance macrophage activity, it was surprising that comparative Hb drop, was not observed in them as in semi-immune Balb/c. It is possible the macrophage activity may have been impaired or switched off in semi-immune B6, NZW and CBA. The evidence of low Hb drop at relatively higher parasitaemia in these semi-immune strains on one hand and Balb/c on the other could implicate haemozoin; a waste product of haemoglobin may be a contributing factor. In addition to stimulating TNF secretion, it is known to impair macrophage function [46]. The relative higher percent parasitaemia observed in the other semi-immune mice strains other than Balb/c might produce a higher amount of haemozoin, which may impair macrophage function. In addition, haemozoin is also reported to suppress erythropoiesis [47], agreeing well with the data in Figure 4 and Table 1, further supporting that macrophage is suppressed by haemozoin in these strains.

Variation in Hb drop and anti-erythrocytic auto-antibody at low parasitaemia in the semi-immune mice give cause to assume more of host genetic factors are at play. It was realized that in some of the strains more of Hb were lost at relatively much lower parasitaemia, and the possibility of their unique genetic background might play a great role in this various responses. How this affect the variation in Hb loss could be point for further research. This observation goes to establish the fact that despite being exposed to similar plasmodium infections at various times to become semi-immune, the individuals respond differently with some able to withstand the parasite pressure by controlling the

parasite growth and others not, leading to high parasitaemia with anaemia and eventually died. It is postulated that the unique genetic background may be responsible in determining how individuals under the same level of malaria transmission in endemic areas respond differently to uRBC destruction at low parasitaemia. It is of interest to note that, the results shown here, reveals that the immune status of the semi-immune appears to delay peak parasitaemia when compared with the naïve status [12, 48], by 2-5 days depending on the mice strain, suggesting the immune system of the semi-immune has been developed to some extent in that regard, during the repeated infections and treatment. Also, more especially in the other strains, absence of parasites at recovery could imply that the considerable effect it (parasites) exert on its host RBC, which eventually lead to similar alterations as seen in oxidatively damaged normal RBC [49, 50] are no more. Consequently uRBC destruction is minimized.

The rodent model reported in this study is unique as it enables the study and comparison of RBC destruction in different mice strain at the same time. Similar Hb reduction in the semi-immune Balb/c compares with another study [12], and to the knowledge of the authors those of semi-immune B6, NZW and CBA are the first to be reported here. While, some deaths were observed in this study, none was reported in that by Evans *et al*, [12]. It is not clear if the source of parasite could contribute to this. Also one advantage of the rodent model is that Hb loss at relatively low parasitaemia could be studied, which is similar to humans. However, a disadvantage in the model reported in this study is that Hb loss was just about 50% of baseline, where as Hb values < 50% has been observed in infants [51]. It is possible the mice in this study might have become adults after several cycles of infection and treatment to generate the semi-immune

status.

Finally, a study into the possible candidate gene that might be responsible in eliciting the various responses especially of Balb/c on one hand and others such as CBA, by studying into their F1 cross, will be very informative. This will help in understanding further the role of host genetic factors in auto-immune mediated RBC destruction in malaria anaemia at the molecular level.

1.5 Conclusion

Together, results from this study show auto-antibody may play a role in the destruction of uRBC in the semi-immune individuals, as shown in the present mice model. In addition, host genetic factors to some extent influence the outcome of auto-immune mediated mechanism in RBC destruction. This suggests that the host has evolved a mechanism in controlling the degree of RBC destruction, to the benefit of some and detrimental to others. The significance of this study to human malaria of diverse genetic background cannot be overemphasized and warrant further study at the molecular level.

Chapter Two

Histopathological studies in two strains of semi-immune mice infected with *Plasmodium berghei* ANKA after chronic exposure

2.1 Introduction

Immunity plays a critical role in helping individuals fight infection. Thus, immune responses to blood-stage malaria infection are in general deficient, with the need for long term exposure to the parasite to achieve immunity. This results in the development of immunopathological states in some individuals such as cerebral malaria (CM) and severe malaria anemia (SMA) in most cases. However, with increase in age, the frequency of clinical attacks reduces, and after puberty most individuals except for pregnant women present some amount of immunity against clinical stages of malaria [52, 53]. In the holo-endemic area in Ghana, for example, the major cause of malaria death is severe anemia, whereas it is CM in the meso-endemic area. The reason why such difference occurred is not clear; however, it is highly possible that the frequent infection can enhance the destruction of RBC, during infection. This motivated us and others to use a semi-immune malaria model mouse [12, 54] for the analysis of the pathogenesis of severe malaria anemia.

Hb loss in the semi-immune mice occurred at relatively low parasitemia and was speculated to be generated by the destruction of normal uninfected red blood cell (uRBC) [12, 54]. Meanwhile, some studies observed that parasitemia level may confound anemia and thus actual parasite numbers may be related to anemia [55], suggesting that some parasites may be sequestered in some organs and not available in the peripheral blood as determined by percent parasitemia. Histopathological study on these organs will therefore be helpful to elucidate if these organs do sequester iRBC in the SMA model of the semi-immune individual. In most reports, histopathological studies have been carried in naïve animal models [30] and recently by [56]. However,

not much characterization has been carried out in the SMA model of semi-immune mice strains.

The main advantage of the rodent model of SMA as developed by [12] and used successfully in another study [54] is that they are uncomplicated by excessive parasite burdens, which is reflective of the associated hemolytic anemia of SMA in the human populations. Thus, with the variation in rates of RBC destruction observed in the different semi-immune mice strains and for the fact that actual parasite numbers have been implicated in the development of anemia [33], the histopathological studies of major organs responsible for RBC circulation, storage, and clearance has been carried out in two semi-immune mice strains in this study.

2.2 Materials and Methods

2.2.1 Mice, parasites, and infection

With our previous studies of high Hb loss at low parasitemia and high antibody titer in Balb/c, and low antibody titer with low Hb loss at relatively high parasitemia in CBA [54], we chose these two strains for the histopathology study to help understand further the mechanism of uRBC destruction in Balb/c. Thus, the two strains of mice Balb/c and CBA, aged 8 weeks supplied by SLC laboratories, Fukuoka, Japan, were injected intraperitoneally with 10^4 Plasmodium berghei ANKA (PbANKA)- infected RBCs. Parasitemia and reticulocyte levels were monitored every 2 days by Giemsa-stained thin blood film and are expressed as a percentage of more than 500 RBCs. Hemoglobin (Hb) was measured in a 96-well plate at 570 nm on Bio-Rad Model 3550 Micro plate Reader as previously described [33]. Four microliter of tail-vein blood was suspended in 1 mL

Drabkin reagent (Sigma, St Louis, MO) and absorbance was measured and expressed as a percentage of baseline levels. Laboratory and animal practices of the Animal Center of Institute of Tropical Medicine (NEKKEN), Nagasaki were adhered to after the approval from the local ethics committee for animal care and research was obtained.

2.2.2 Generation of semi-immune status in the mice strains

This was a modified method as described elsewhere [12]. Two strains (Balb/c and CBA) of mice infected with 10^4 P. berghei ANKA, were treated at day 6 after infection with chloroquine (10 mg/kg intraperitoneally) and pyrimethamine (10 mg/kg intraperitoneally) daily for 6 days. During subsequent rounds of infection, mice were rested for 2 weeks before being rechallenged with 10^4 *P. berghei ANKA*, then monitored and drug cured prior to parasitemias reaching 5%. To evaluate if mice will be completely immunized, they underwent 14 cycles of drug cured infection before finally being challenged with 10^4 *P. berghei* parasites without treatment. After the final cycle of infection, mice were monitored every other day and on days in which minimum Hb level (Fig. 11) was observed at parasitemia not exceeding 20%; mice were sacrificed and organs taken for histopathological study. Parasitemias exceeding 20% have been shown to complicate malaria anemia [34] and were excluded from analyses.

2.2.3 Histopathological study

The spleen, brain, liver, kidney, lung, heart, and muscle were fixed in 10% formalin until ready to be used. After fixation, the spleen, liver, and brain were cut in transversal sections, while kidney, lung, heart, and muscle were cut in longitudinal sections. These specimens were suspended in absolute alcohol, absolute xylene for 4 days, and

embedded in paraffin. Sections were cut at 3.5 μm, stained with hematoxylin–eosin (HE) and analyzed by light microscopy. Same organs were obtained from uninfected mice and taken through the HE staining to serve as control. Results of histological findings were confirmed by at least an additional person. To further ensure the validity of the results, not less than ten fields per organ with similar microscopic field were observed carefully for histopathological signs of malaria pigment and sequestered iRBCs. Semi-qualitative approach was used to analyze and compare the variation in malaria pigment deposition among the semi-immune mice strains. For each histological section per animal not less than ten microscopic fields at 400× were randomly selected and examined for any histopathological changes, such as malaria pigment deposition and sequestered iRBCs.

2.2.4 Statistical analysis

Data analysis was done using the GraphPad Prism Version 5.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com. Data are expressed as the mean unless otherwise stated. Data were log transformed to ensure normal distribution before t test analysis was performed. Values were considered significant when $p<0.05$.

2.3 Results

2.3.1 Profile of parasitemia and hematological parameters in the semi-immune mice strains Upon challenge with 10^4 *P. berghei ANKA* after semi immune status was attained, it was observed that the two strains (Balb/c and CBA) exhibited different responses (Figure 11). Prepatent period was longer for Balb/c than CBA, with Balb/c recovering fully from the parasitemia, and CBA did not. While mean %Hb reduction kinetics of Balb/c increased to a maximum on day 12 then dropped to a minimum on day 22, the mean %Hb reduction kinetics of CBA decreased continuously and in proportion to parasitemia level (Figure 11). Interestingly, mean parasitemia was lower in Balb/c and higher in CBA (Table 2). The effect of parasitemia on Hb loss, calculated by the ratio mean Hb drop/mean parasitemia (Table 2) was more in CBA (lower ratio 15.9) than in Balb/c (higher ratio 28.7). The kinetic profile of reticulocyte count (Figure 11) revealed erythropoietic response to be slightly better in Balb/c with higher mean peak reticulocyte count of 8.5% and 7.3% in CBA, p=0.76 (Table 2). Splenomegaly was observed in both semi-immune mice, increasing in size between two and three times, than the uninfected mice. Increase in spleen size was significantly higher in Balb/c, p=0.01 (Table 2).

2.3.1 Histopathological study

Spleen: Clear distinction between red and white pulp, resting follicles, and marginal zone was evident in the spleen of normal uninfected control mice (Figure 12). The splenomegaly observed in all the infected semi-immune mice might be as a result of an overall enlargement of the red and white pulp [56] as shown in Figure 12. In addition, the germinal centers in the spleen of semi-immune mice lost their typical structure.

Malaria pigment deposition (indicated arrows) was observed in both strains. However, that of Balb/c was fine while that of CBA were aggregates as shown in Figure 12. By means of the semi-qualitative approach, it was observed that between two and three thick/clumps of malaria pigments were seen at 400× magnification of the CBA spleen (Figure 12), while smaller sizes of the malaria pigments were seen in that of Balb/c. Hypertrophy of the red pulp and intense proliferation of the cells in the resting follicles were also observed in the semi-immune mice.

Liver: The liver sections of the normal uninfected control mice revealed normal, clear architecture, and color, such as sinusoids and Kupffer cells, as shown in Figure 13. Hypertrophy of Kupffer cells are seen in all the semi-immune mice strains in addition to congested sinusoids (Figure 13). Infected RBCs were also not observed, except for some few uRBCs. Meanwhile that of the infected immune mice showed some amount of malaria pigment deposition (indicated arrows) in the sinusoids. Semi-qualitative approach revealed five to eight malaria pigments thicker/clumps in CBA. Those of Balb/c were few in number between 3 and 4, as shown in Figure 13.

Brain: It was also observed that the brain tissues of the normal uninfected control mice did not show any sign of RBC sequestration or leukocytes infiltration. Abnormal features, such as hemorrhages and edema, were not observed as shown in panels of the semi-immune mice strains (Figure 14). Interestingly, the brain tissues of all the semi-immune mice strains did not show any endothelium adherent hyperactivated macrophages.

Kidney: The kidney tissues of the normal uninfected control mice showed normal kidney cells, with clear, clean glomeruli, and uRBC when compared with the semi immune mice, as shown in Figure 15. In addition, no malaria pigment deposition was observed.

Lung: The lung tissues of normal uninfected mice show clear alveolar walls with some few uRBCs. The infected semi-immune mice on the other hand showed some alveolar wall thickening in both Balb/c and CBA, as shown in Figure 16. Similar features such as normal RBCs (uRBCs) were also observed in the alveolar walls and blood vessels of all the mice strains.

Heart: Uninfected control mice showed characteristic heart muscle tissues and with uRBC. And a group of uRBC can be seen in one of the chambers of the heart of Balb/c, as shown in Figure 17. The histopathological finding of the heart muscles of CBA also shows similar features with no sequestration of iRBC (Figure 17).

Muscle: Similar characteristic features were observed in the normal uninfected control mice muscle tissues and the semi-immune mice strains, data not shown. No obvious sequestration of iRBC in the muscle fibers of both semi immune mice strains was observed.

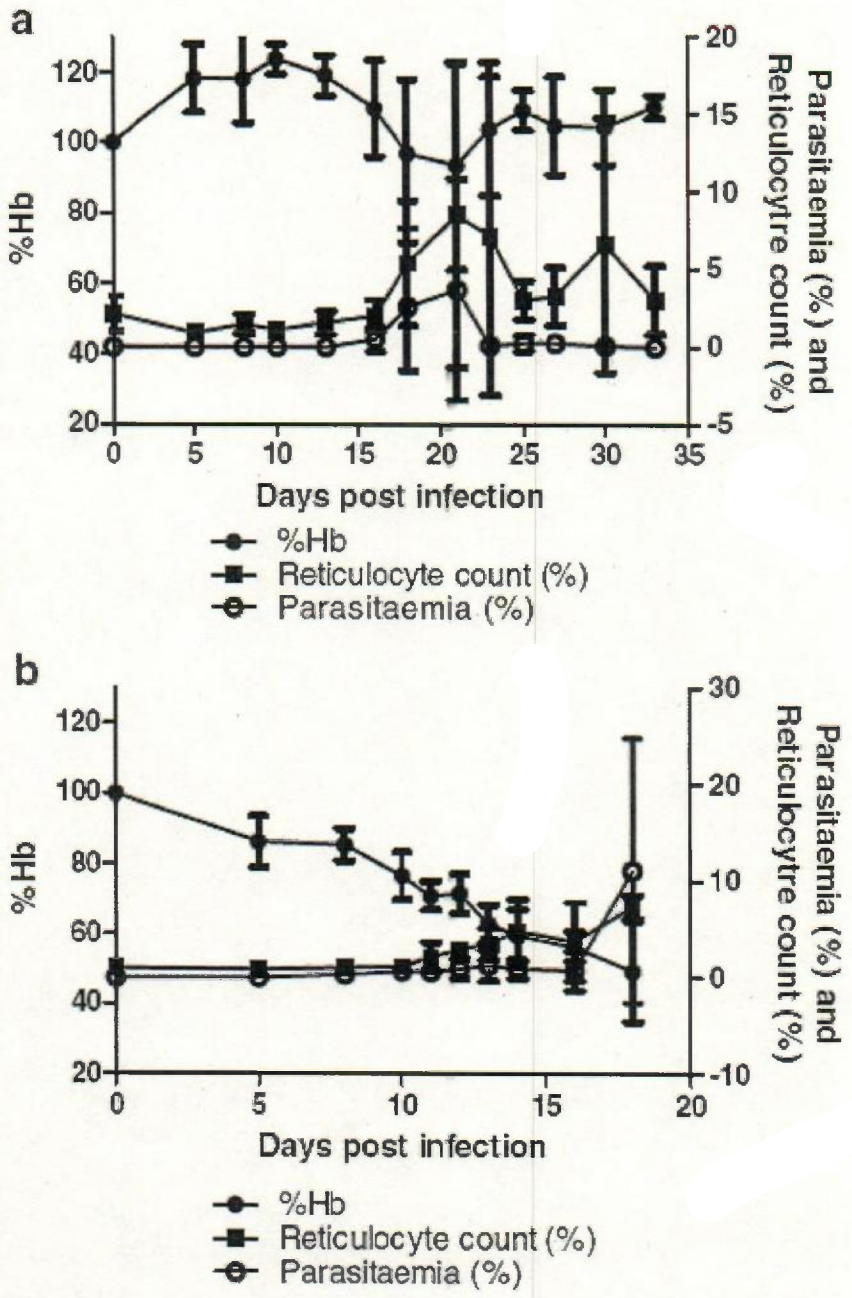

Figure 11 Profile of malaria anemia in the semi-immune mice.

Mean parasitemias, reticulocyte levels, and Hb in semi-immune mice strains a Balb/c, n=6, and b CBA, n=7, after P. berghei ANKA infection during the final cycle. Each mouse showed minimum %Hb level (maximal reduction) on different days. These are data from one experiment, and data are represented as mean±SD

Table 2 Magnitude of Hb reduction, peak reticulocyte count, and peak parasitemia in the semi-immune mice strain on the day minimum Hb was observed

Parameters	Balb/c	CBA	*p* valu
n	6	7	-
Mean Hb reduction,% (SD)	30.5 (29.7)	50.6 (10.5)	0.25
Mean Parasitemia,% (SD)	3.7 (3.2)	11.1 (9.7)	0.22
Mean Hb drop/mean parasitemia ratio (SD)	28.7 (25.6)	15.9 (12.9)	0.57
Mean peak reticulocyte count, % (SD)	8.5 (4.4)	7.3 (0.8)	0.76
Mean spleen size, cm (SD)	3.04 (0.11)	2.75 (0.13)	0.01

Hb reduction is the difference between maximum and minimum %Hb during the last cycle of infection without treatment as shown in Fig. 1. Each mouse showed minimum %Hb level (maximal reduction) on different days. The data presented here are values of one experiment [a] Mann–Whitney test

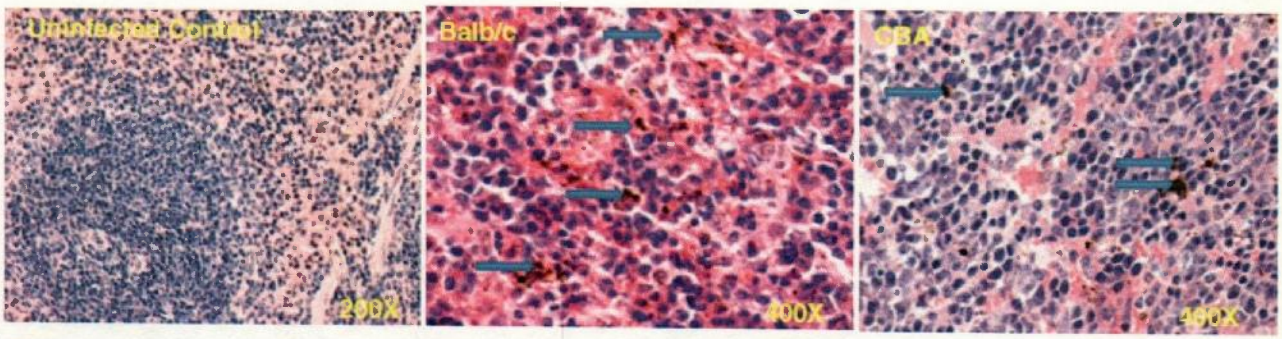

Figure 12 Hematoxylin–eosin (HE) staining of spleen of uninfected control (n=3), semi-immune Balb/c (n=3), and CBA (n=7). The organs were removed when similar minimum %Hb reduction was observed as shown in Fig. 1 (Balb/c, days 18–22, and CBA, days 16–18). Sections of these organs were stained with HE and analyzed with light microscopy. The blue arrows indicate malaria pigment deposition in the spleen

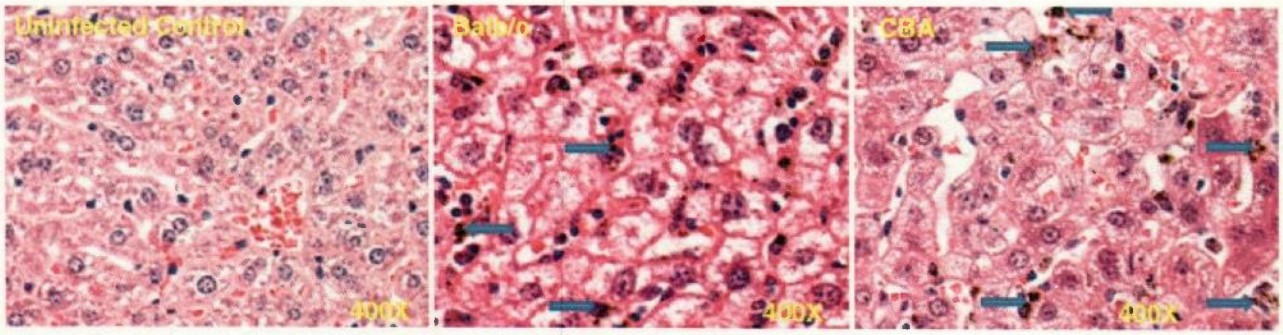

Figure 13 Hematoxylin–eosin (HE) staining of liver of uninfected control (n=3), semi-immune Balb/c (n=3), and CBA (n=7). The organs were removed when similar minimum %Hb reduction was observed as shown in Fig. 1 (Balb/c, days 18–22, and CBA, days 16–18). Sections of these organs were stained with HE and analyzed with light microscopy. The blue arrows indicate malaria pigment deposition in the liver

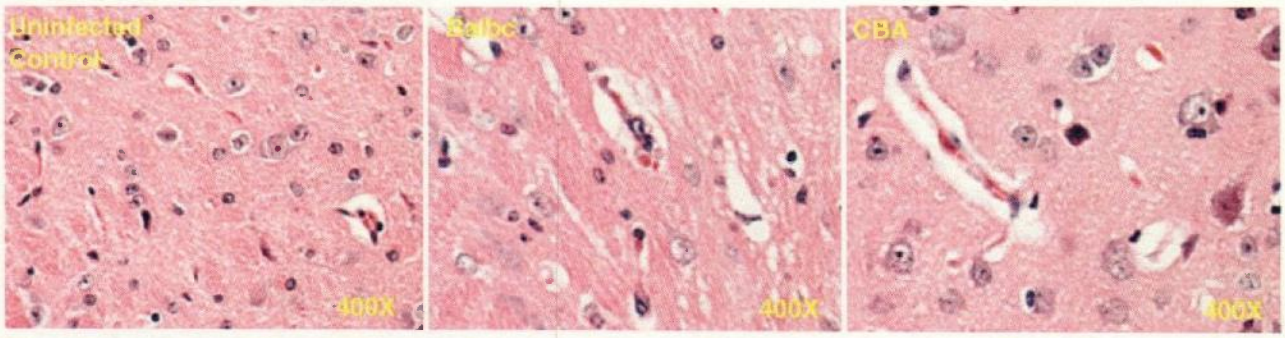

Figure 14 Hematoxylin–eosin (HE) staining of brain of uninfected control (n=3), semi-immune Balb/c (n=3), and CBA (n=7). The organs were removed when similar minimum %Hb reduction was observed as shown in Fig. 1 (Balb/c, days 18–22, and CBA, days 16–18). Sections of these organs were stained with HE and analyzed with light microscopy

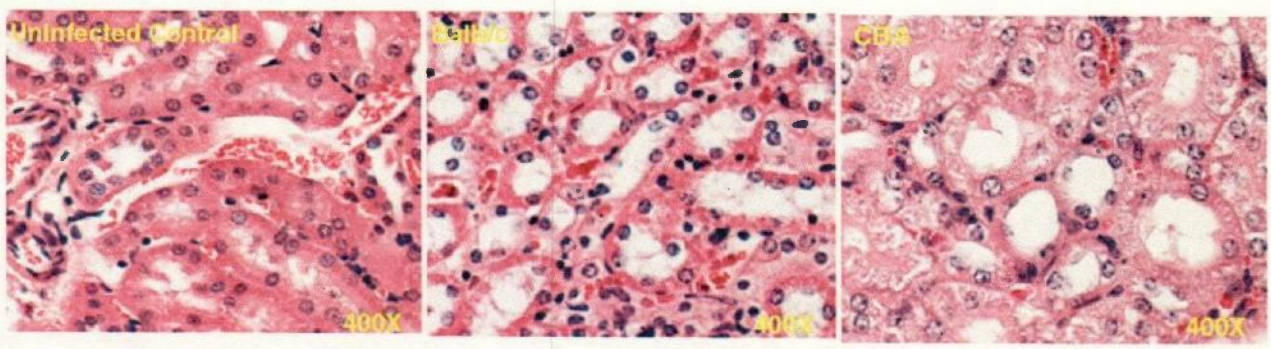

Figure 15 Hematoxylin–eosin (HE) staining of kidney of uninfected control (n=3), semi-immune Balb/c (n=3), and CBA (n=7). The organs were removed when similar minimum %Hb reduction was observed as shown in Fig. 1 (Balb/c, days 18–22, and CBA, days 16–18). Sections of these organs were stained with HE and analyzed with light microscopy

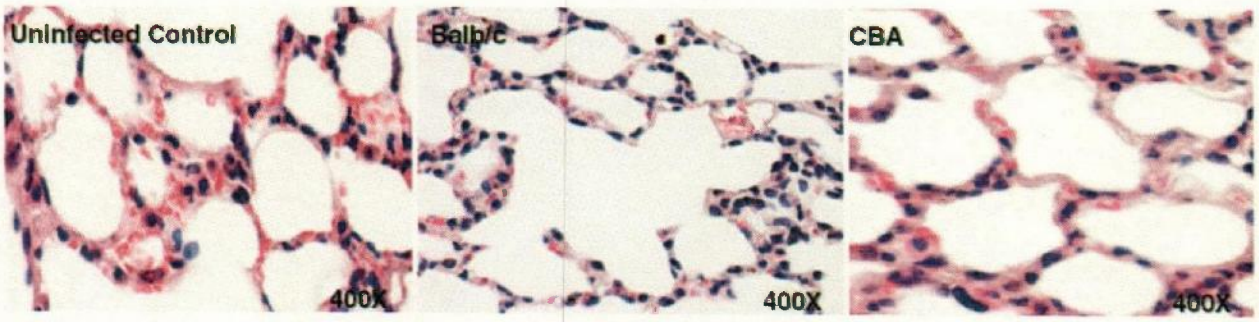

Figure 16 Hematoxylin–eosin (HE) staining of lung of uninfected control (n=3), semi-immune Balb/c (n=3), and CBA (n=7). The organs were removed when similar minimum %Hb reduction was observed as shown in Fig. 1 (Balb/c, days 18–22, and CBA, days 16–18). Sections of these organs were stained with HE and analyzed with light microscopy

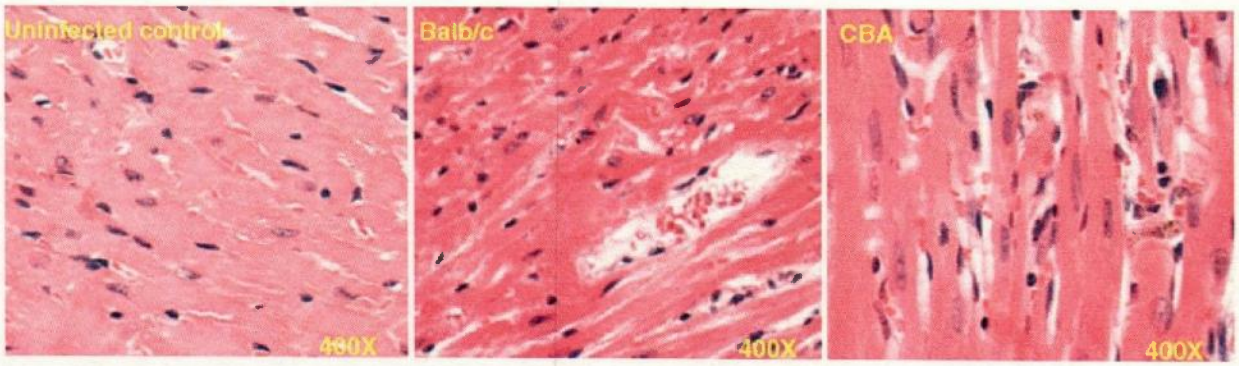

Figure 17 Hematoxylin–eosin (HE) staining of heart of uninfected control (n=3), semi-immune Balb/c (n=3), and CBA (n=7). The organs were removed when similar minimum %Hb reduction was observed as shown in Fig. 1 (Balb/c, days 18–22, and CBA, days 16–18). Sections of these organs were stained with HE and analyzed with light microscopy

2.4 Discussion

This study describes the histopathological changes in some organs of semi-immune SMA model of mice. Variation in malaria pigment deposition has been observed in some of the organs of this semi-immune mice model. Even though PbANKA is regarded as a CM model, its use in the anemia studies of chronic situation [54] shows that this can be adapted for the study of SMA in the semi-immune.

Despite the fact that experimental animal models cannot reproduce all the features of human disease, it could be explored to explain some phenomena in human situations. Also animal studies have been useful in understanding the mechanisms of pathogenetic basis of various disease conditions to help in prophylactic or therapeutic interventions. Repeated infection plays a role in providing protection to the semi-immune mice strains where antibody titer was observed to be high [54]. One of the roles of antibody during malaria infection is that it prevents the cytoadherence of iRBC onto the endothelial walls of the blood vessels [53]. Since antibody titer was observed to increase in semi-immune individuals, it could be an explanation why sequestration was not observed in the brains of the semi-immune but occurred in the naïve. This might contribute to less CM incidence in adults (who have high antibody titer due to long exposure to plasmodium infections) than children in endemic areas. While sequestration in the adipose tissue, lung and spleen [57], and brain hemorrhages [55]have been associated with severe malaria in naïve mice, none is reported in the histopathological sections of the organs of each semi-immune mice strain. Our study, however, did not evaluate any study on the naïve. In addition, the lack of endothelium-adherent hyperactivated macrophages in the brain, a hall mark of acute infection by PbANKA,

could help explain the absence of systemic damage.

Splenomegaly, which was observed in all the infected semi-immune mice, might be a result of overall enlargement of the red and white pulp [56], suggesting hyperactivity of the macrophages. The significant difference in spleen size of the semi-immune mice strains might suggest differential immune response. Closely related to the high parasitemia in CBA is the high malaria pigmentation in the spleen and liver when compared with the other strain. This is consistent with other studies where a higher level of parasitemia results in higher amounts of free hemoglobin leading to free heme released [58-60]. Malaria pigment does not only stimulate TNF secretion, but also impair macrophage function [46]. Thus, this relatively higher malaria pigmentation in CBA could result in higher impairment of macrophage function than Balb/c. Hemozoin do persist in macrophages for some months. Thus, we do not exclude the fact that a considerable amount of the malaria pigment in the histological sections might have been released during previous cycles. Our data presented here, the mean Hb loss/mean parasitemia ratio, being higher in Balb/c than CBA (Table 2), goes further to substantiate the assertion that more uRBC destruction may be involved in the Hb loss in Balb/c. While Hb loss in CBA may be due to direct lyses of iRBC due to its high parasitemia (Table 2), high antibody titer in the Balb/c as reported in an earlier study [54] enhances the sensitization of the RBC (both iRBC and uRBC) resulting in the significant Hb loss in the Balb/c [12, 54]. It is possible, polyclonal B cell activation and proliferation may be higher in Balb/c resulting in the high immunoglobulin production [61].

The inability to observe sequestration in the lungs might be due to the approach used in this study as compared to another [57] where with the use of chemiluminescence sequestration was observed. Furthermore, the extent of malaria pigment deposition in the liver may help explain the hepatic dysfunction during PbANKA infection [62]. However, we are not sure if malaria pigment deposition in the liver during Plasmodium falciparum infection [63, 64] can have any relation with oxidative stress induction as evidenced from reduced glutathione concentration, which correlated with degree of parasitemia [63]. It is also not clear how malaria pigment influence hepatic dysfunction of these semi-immune individuals, but metabolic changes have been implicated in the late stage of naïve Balb/c [65]. Overall, although these semi-immune mice strains seem highly vulnerable to anemia despite low parasitemias, they conversely appear to be quite protected from multi-organ pathology, which is a hallmark of infections in non-immune mice. Thus, further studies exploring the bone marrow activity in relation to these organs' histology need to be carried out to help explain better the anemia and RBC destruction at these low parasitemias of the semi-immune.

2.5 Conclusion

In conclusion, high Hb loss in the SMA model of the semi-immune in Balb/c at low parasitemia might be contributed mainly by uRBC, which was reflected by relatively low percent parasitemia and low malaria pigment deposition. This may therefore not be masked by sequestered iRBC in any of these organs as might be suspected.

References

1. Breman JG, Alilio MS, Mills A: Conquering the intolerable burden of malaria: what's new, what's needed: a summary. *Am J Trop Med Hyg* 2004, 71(2 Suppl):1-15.
2. Le Hesran JY: [The particularities of malaria in the child]. *Med Trop (Mars)* 2000, 60(1):92-98.
3. Murphy SC, Breman JG: Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy. *Am J Trop Med Hyg* 2001, 64(1-2 Suppl):57-67.
4. Lamikanra AA, Brown D, Potocnik A, Casals-Pascual C, Langhorne J, Roberts DJ: Malarial anemia: of mice and men. *Blood* 2007, 110(1):18-28.
5. Glenister FK, Coppel RL, Cowman AF, Mohandas N, Cooke BM: Contribution of parasite proteins to altered mechanical properties of malaria-infected red blood cells. *Blood* 2002, 99(3):1060-1063.
6. McGilvray ID, Serghides L, Kapus A, Rotstein OD, Kain KC: Nonopsonic monocyte/macrophage phagocytosis of Plasmodium falciparum-parasitized erythrocytes: a role for CD36 in malarial clearance. *Blood* 2000, 96(9):3231-3240.
7. Stoute JA, Odindo AO, Owuor BO, Mibei EK, Opollo MO, Waitumbi JN: Loss of red blood cell-complement regulatory proteins and increased levels of circulating immune complexes are associated with severe malarial anemia. *J Infect Dis* 2003, 187(3):522-525.

8. Kurtzhals JA, Adabayeri V, Goka BQ, Akanmori BD, Oliver-Commey JO, Nkrumah FK, Behr C, Hviid L: Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria. *Lancet* 1998, 351(9118):1768-1772.

9. Naik RS, Branch OH, Woods AS, Vijaykumar M, Perkins DJ, Nahlen BL, Lal AA, Cotter RJ, Costello CE, Ockenhouse CF *et al*: Glycosylphosphatidylinositol anchors of Plasmodium falciparum: molecular characterization and naturally elicited antibody response that may provide immunity to malaria pathogenesis. *J Exp Med* 2000, 192(11):1563-1576.

10. Rosenberg EB, Strickland GT, Yang SL, Whalen GE: IgM antibodies to red cells and autoimmune anemia in patients with malaria. *Am J Trop Med Hyg* 1973, 22(2):146-152.

11. Woodruff AW: Mechanisms involved in anaemia associated with infection and splenomegaly in the tropics. *Trans R Soc Trop Med Hyg* 1973, 67(3):313-328.

12. Evans KJ, Hansen DS, van Rooijen N, Buckingham LA, Schofield L: Severe malarial anemia of low parasite burden in rodent models results from accelerated clearance of uninfected erythrocytes. *Blood* 2006, 107(3):1192-1199.

13. Wipasa J, Elliott S, Xu H, Good MF: Immunity to asexual blood stage malaria and vaccine approaches. *Immunol Cell Biol* 2002, 80(5):401-414.

14. Price RN, Simpson JA, Nosten F, Luxemburger C, Hkirjaroen L, ter Kuile F, Chongsuphajaisiddhi T, White NJ: Factors contributing to anemia after uncomplicated falciparum malaria. *Am J Trop Med Hyg* 2001, 65(5):614-622.

15. Jakeman GN, Saul A, Hogarth WL, Collins WE: Anaemia of acute malaria infections in non-immune patients primarily results from destruction of uninfected erythrocytes. *Parasitology* 1999, 119 (Pt 2):127-133.

16. Phillips RE, Looareesuwan S, Warrell DA, Lee SH, Karbwang J, Warrell MJ, White NJ, Swasdichai C, Weatherall DJ: The importance of anaemia in cerebral and uncomplicated falciparum malaria: role of complications, dyserythropoiesis and iron sequestration. *Q J Med* 1986, 58(227):305-323.

17. Voller A: Immunopathology of malaria. *Bull World Health Organ* 1974, 50(3-4):177-186.

18. Waitumbi JN, Opollo MO, Muga RO, Misore AO, Stoute JA: Red cell surface changes and erythrophagocytosis in children with severe plasmodium falciparum anemia. *Blood* 2000, 95(4):1481-1486.

19. Wahlgren M, Berzins K, Perlmann P, Bjorkman A: Characterization of the humoral immune response in Plasmodium falciparum malaria. I. Estimation of antibodies to P. falciparum or human erythrocytes by means of microELISA. *Clin Exp Immunol* 1983, 54(1):127-134.

20. Zouali M, Druilhe P, Gentilini M, Eyquem A: High titres of anti-T antibodies and other haemagglutinins in human malaria. *Clin Exp Immunol* 1982, 50(1):83-91.

21. Facer CA, Bray RS, Brown J: Direct Coombs antiglobulin reactions in Gambian children with Plasmodium falciparum malaria. I. Incidence and class specificity. *Clin Exp Immunol* 1979, 35(1):119-127.

22. Woodruff AW, Ansdell VE, Pettitt LE: Cause of anaemia in malaria. *Lancet* 1979, 1(8125):1055-1057.

23. Adner MM, Altstatt LB, Conrad ME: Coombs'-positive hemolytic disease in malaria. *Ann Intern Med* 1968, 68(1):33-38.

24. Zuckerman A: Autoimmunization and Other Types of Indirect Damage to Host Cells as Factors in Certain Protozoan Diseases. *Exp Parasitol* 1964, 15:138-183.

25. Kreier J, Shapiro H, Dilley D, Szilvassy IP, Ristic M: Autoimmune reactions in rats with Plasmodium berghei infection. *Exp Parasitol* 1966, 19(2):155-162.

26. Topley E, Knight R, Woodruff AW: The direct antiglobulin test and immunoconglutinin titres in patients with malaria. *Trans R Soc Trop Med Hyg* 1973, 67(1):51-54.

27. Wu YL, Yu Q, Li WL, Liu EX: Studies on the mechanism of anemia in rodent malaria. *Proc Chin Acad Med Sci Peking Union Med Coll* 1989, 4(2):102-105.

28. Hananantachai H, Patarapotikul J, Ohashi J, Naka I, Looareesuwan S, Tokunaga K: Polymorphisms of the HLA-B and HLA-DRB1 genes in Thai malaria patients. *Jpn J Infect Dis* 2005, 58(1):25-28.

29. Eling W, van Zon A, Jerusalem C: The course of a Plasmodium berghei infection in six different mouse strains. *Z Parasitenkd* 1977, 54(1):29-45.

30. Adun EH, Williams JS, Meroney FC, Hutt G: Pathophysiology of Plasmodium berghei infection in mice. *Exp Parasitol* 1965, 17(3):277-286.

31. Caulfield MJ, Stanko D, Calkins C: Characterization of the spontaneous autoimmune (anti-erythrocyte) response in NZB mice using a pathogenic monoclonal autoantibody and its anti-idiotype. *Immunology* 1989, 66(2):233-237.

32. Menshikov I, Beduleva L: Evidence in favor of a role of idiotypic network in autoimmune hemolytic anemia induction: theoretical and experimental studies. *Int Immunol* 2008, 20(2):193-198.

33. Lamb TJ, Langhorne J: The severity of malarial anaemia in Plasmodium chabaudi infections of BALB/c mice is determined independently of the number of circulating parasites. *Malar J* 2008, 7:68.

34. Langhorne J, Quin SJ, Sanni LA: Mouse models of blood-stage malaria infections: immune responses and cytokines involved in protection and pathology. *Chem Immunol* 2002, 80:204-228.

35. Huy NT, Serada S, Trang DT, Takano R, Kondo Y, Kanaori K, Tajima K, Hara S, Kamei K: Neutralization of toxic heme by Plasmodium falciparum histidine-rich protein 2. *J Biochem* 2003, 133(5):693-698.

36. Goes TS, Goes VS, Ribeiro MF, Gontijo CM: Bovine babesiosis: anti-erythrocyte antibodies purification from the sera of naturally infected cattle. *Vet Immunol Immunopathol* 2007, 116(3-4):215-218.

37. Menendez C, Fleming AF, Alonso PL: Malaria-related anaemia. *Parasitol Today* 2000, 16(11):469-476.

38. Biemba G, Dolmans D, Thuma PE, Weiss G, Gordeuk VR: Severe anaemia in Zambian children with Plasmodium falciparum malaria. *Trop Med Int Health* 2000, 5(1):9-16.

39. Cromer D, Evans KJ, Schofield L, Davenport MP: Preferential invasion of reticulocytes during late-stage Plasmodium berghei infection accounts for reduced circulating reticulocyte levels. *Int J Parasitol* 2006, 36(13):1389-1397.

40. Singer I: The course of infection with Plasmodium berghei in inbred CF 1 mice. *J Infect Dis* 1954, 94(3):237-240.

41. Collins WE, Jeffery GM, Roberts JM: A retrospective examination of anemia during infection of humans with Plasmodium vivax. *Am J Trop Med Hyg* 2003, 68(4):410-412.

42. Owuor BO, Odhiambo CO, Otieno WO, Adhiambo C, Makawiti DW, Stoute JA: Reduced immune complex binding capacity and increased complement susceptibility of red cells from children with severe malaria-associated anemia. *Mol Med* 2008, 14(3-4):89-97.

43. Helegbe GK, Goka BQ, Kurtzhals JA, Addae MM, Ollaga E, Tetteh JK, Dodoo D, Ofori MF, Obeng-Adjei G, Hirayama K *et al*: Complement activation in Ghanaian children with severe Plasmodium falciparum malaria. *Malar J* 2007, 6:165.

44. Goka BQ, Kwarko H, Kurtzhals JA, Gyan B, Ofori-Adjei E, Ohene SA, Hviid L, Akanmori BD, Neequaye J: Complement binding to erythrocytes is associated with macrophage activation and reduced haemoglobin in Plasmodium falciparum malaria. *Trans R Soc Trop Med Hyg* 2001, 95(5):545-549.

45. Butcher GA: Malaria and macrophage function in Africans: a possible link with autoimmune disease? *Med Hypotheses* 1996, 47(2):97-100.

46. Turrini F, Schwarzer E, Arese P: The involvement of hemozoin toxicity in depression of cellular immunity. *Parasitol Today* 1993, 9(8):297-300.

47. Casals-Pascual C, Kai O, Cheung JO, Williams S, Lowe B, Nyanoti M, Williams TN, Maitland K, Molyneux M, Newton CR *et al*: Suppression of erythropoiesis in malarial anemia is associated with hemozoin in vitro and in vivo. *Blood* 2006, 108(8):2569-2577.

48. Lou J, Lucas R, Grau GE: Pathogenesis of cerebral malaria: recent experimental data and possible applications for humans. *Clin Microbiol Rev* 2001, 14(4):810-820, table of contents.

49. Golenser J, Chevion M: Oxidant stress and malaria: host-parasite interrelationships in normal and abnormal erythrocytes. *Semin Hematol* 1989, 26(4):313-325.

50. Hunt NH, Stocker R: Oxidative stress and the redox status of malaria-infected erythrocytes. *Blood Cells* 1990, 16(2-3):499-526; discussion 527-430.

51. Ong'echa JM, Keller CC, Were T, Ouma C, Otieno RO, Landis-Lewis Z, Ochiel D, Slingluff JL, Mogere S, Ogonji GA *et al*: Parasitemia, anemia, and malarial anemia in infants and young children in a rural holoendemic Plasmodium falciparum transmission area. *Am J Trop Med Hyg* 2006, 74(3):376-385.

52. Baird JK, Masbar S, Basri H, Tirtokusumo S, Subianto B, Hoffman SL: Age-dependent susceptibility to severe disease with primary exposure to Plasmodium falciparum. *J Infect Dis* 1998, 178(2):592-595.

53. Druilhe P, Perignon JL: A hypothesis about the chronicity of malaria infection. *Parasitol Today* 1997, 13(9):353-357.

54. Helegbe GK, Huy NT, Yanagi T, Shuaibu MN, Yamazaki A, Kikuchi M, Yasunami M, Hirayama K: Rate of red blood cell destruction varies in different strains of mice infected with Plasmodium berghei-ANKA after chronic exposure. *Malar J* 2009, 8:91.

55. Amante FH, Stanley AC, Randall LM, Zhou Y, Haque A, McSweeney K, Waters AP, Janse CJ, Good MF, Hill GR *et al*: A role for natural regulatory T cells in the pathogenesis of experimental cerebral malaria. *Am J Pathol* 2007, 171(2):548-559.

56. Martins YC, Smith MJ, Pelajo-Machado M, Werneck GL, Lenzi HL, Daniel-Ribeiro CT, Carvalho LJ: Characterization of cerebral malaria in the outbred Swiss Webster mouse infected by Plasmodium berghei ANKA. *Int J Exp Pathol* 2009, 90(2):119-130.

57. Franke-Fayard B, Janse CJ, Cunha-Rodrigues M, Ramesar J, Buscher P, Que I, Lowik C, Voshol PJ, den Boer MA, van Duinen SG *et al*: Murine malaria parasite sequestration: CD36 is the major receptor, but cerebral pathology is unlinked to sequestration. *Proc Natl Acad Sci U S A* 2005, 102(32):11468-11473.

58. Gramaglia I, Sobolewski P, Meays D, Contreras R, Nolan JP, Frangos JA, Intaglietta M, van der Heyde HC: Low nitric oxide bioavailability contributes to the genesis of experimental cerebral malaria. *Nat Med* 2006, 12(12):1417-1422.

59. Penet MF, Laigle C, Fur YL, Confort-Gouny S, Heurteaux C, Cozzone PJ, Viola A: In vivo characterization of brain morphometric and metabolic endophenotypes in three inbred strains of mice using magnetic resonance techniques. *Behav Genet* 2006, 36(5):732-744.

60. Pamplona A, Ferreira A, Balla J, Jeney V, Balla G, Epiphanio S, Chora A, Rodrigues CD, Gregoire IP, Cunha-Rodrigues M *et al*: Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. *Nat Med* 2007, 13(6):703-710.

61. Donati D, Zhang LP, Chene A, Chen Q, Flick K, Nystrom M, Wahlgren M, Bejarano MT: Identification of a polyclonal B-cell activator in Plasmodium falciparum. *Infect Immun* 2004, 72(9):5412-5418.

62. De-Oliveira AC, Da-Matta AC, Paumgartten FJ: Plasmodium berghei (ANKA): infection induces CYP2A5 and 2E1 while depressing other CYP isoforms in the mouse liver. *Exp Parasitol* 2006, 113(4):256-261.

63. Guha M, Kumar S, Choubey V, Maity P, Bandyopadhyay U: Apoptosis in liver during malaria: role of oxidative stress and implication of mitochondrial pathway. *Faseb J* 2006, 20(8):1224-1226.

64. Kochar DK, Agarwal P, Kochar SK, Jain R, Rawat N, Pokharna RK, Kachhawa S, Srivastava T: Hepatocyte dysfunction and hepatic encephalopathy in Plasmodium falciparum malaria. *Qjm* 2003, 96(7):505-512.

65. Penet MF, Kober F, Confort-Gouny S, Le Fur Y, Dalmasso C, Coltel N, Liprandi A, Gulian JM, Grau GE, Cozzone PJ *et al*: Magnetic resonance spectroscopy reveals an impaired brain metabolic profile in mice resistant to cerebral malaria infected with Plasmodium berghei ANKA. *J Biol Chem* 2007, 282(19):14505-14514.

Acknowledgment

I wish to acknowledge my sincere appreciation to my supervisor Professor Kenji Hirayama for his criticisms, encouragement, and invaluable contribution to the success of this study. I also appreciate the help of Dr. Huy T Nguyen, Dr. Shuaibu N Mohammed, Dr. Mihoko Kikuchi, Professor Michio Yasunami and Assoc. Professor Tetsuo Yanagi for their great help in carrying out this study.

Sincere thanks also go to my laboratory mates for their company and making the study a pleasant one. Special thanks also go to the Ms. Hayashima Junko for her excellent secretarial work at the laboratory, which made my stay in the department less stressful.

I am grateful to the Japanese Government for financial support and an opportunity to experience life in Japan. And finally, I would like to thank the Almighty God for His care and protection throughout the entire programme of my study.