In the name of God, the Beneficent, the Merciful

The immune response and immunization studies in avirulent DK and virulent DS strains of *Plasmodium chabaudi adami* and a synthetic peptide immunization in *P. chabaudi* AS.

A thesis submitted for the degree of Doctor of Philosophy at the University of Glasgow

By

Mohammad Javad Namazi

UNIVERSITY *of* GLASGOW

Mary Calder Laboratories
Division of Infection and Immunity
Faculty of Biomedical and Life sciences
University of Glasgow
Glasgow, G12 8QQ

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Declaration

I declare that this thesis is of my own composition and that the experimental work and research described herein was performed entirely by myself except where expressly stated.

Mohammad Javad Namazi

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Summary

Approximately 40% of the world's population are living at risk of malaria in endemic areas. Malaria kills more people than any other infectious disease except HIV and tuberculosis. Malaria causes more than 300 million clinical cases and between 1.7 to 2.5 million deaths annually. Among effective low-cost strategies for its treatment, prevention and control of the disease there is no routine vaccine against malaria.

In vivo experimental studies of human malaria are ethically limited, mainly because attacks are very unpleasant and *Plasmodium falciparum* infections may be fatal. So, animal models of human malaria such as rodent malaria infections are used for *in vivo* studies. Such studies help to understand better the immune response to malaria in animal models which will facilitate development of protective vaccines against and in the control of the disease in people. However, as the asexual blood stage infection is responsible for almost all morbidity and mortality associated with malaria the majority of studies are focused on this stage.

In the first part of the present study the immune responses in NIH mice against asexual blood stages of either avirulent DK or virulent DS strains of *P. chabaudi adami*, in single or mixed infections, were examined using the ELISA test for the detection and measurment of cytokine and antibody production. The present results showed that the profile of the immune response in all the above infections suggests a sequential Th1/Th2 $CD4^+$ T cell response. Previous studies have shown that there is a sequential Th1/ Th2 response in *Plasmodium chabaudi* AS infection which is reflected in the activation of both cell- and antibody-mediated responses. These findings, therefore, indicate that vaccines which induce both arms of the protecive immune respnse against malaria parasites could be most effective. The sequential Th1/Th2 response was supported by detecting early high levels of IFNγ and IgG2a during the acute phase of the infection and later by elevation of IL-4 and IgG1 levels during the course of infection compared to controls. The levels of IgG2a were at highest levels at or immediately after the peak parasitaemia while the levels of IgG1 increased in later stages in the course of infection. However, a higher level

IFNγ early in the infection indicated a stronger Th1 response in the avirulent DK strain infection compared to the virulent DS strain or mixed infections. On the other hand, in the virulent DS infection a stronger Th2 response with higher IL-4 levels compared to the DK strain and mixed infections was observed in mice treated with chloroquine.

In the mixed infection, an infective dose consisting of 8×10^3 pRBCs of the avirulent DK strain and 2×10^3 pRBCs of the virulent DS strain was used. Despite a relatively low number of pRBCs of the DS strain in the infective dose the peak parasitaemia was significantly higher than that in the single-infection of 1×10^4 pRBCs of the DK strain. The mixed infection also showed a significantly lower peak parasitaemia compared to that in mice given 1×10^4 pRBCs of the DS strain single-infection in untreated mice. So, it may be concluded that a higher peak parasitaemia in the mixed infection compared to the DK single-infection is reflected in a higher replication rate of the DS strain compared to the DK strain during the course of the mixed infecion. However, the virulent DS strain may suppress the immune response or the immune response does not effectively respond to the virulent strain compared to the avirulent strain.

In the second part of the study the effectiveness of passively transferred whole immune serum, purified IgG1 and IgG2a obtained from the DK-infected mice was assessed, in NIH mice challenged with avirulent DK or virulent DS strain. IgG1 and IgG2a were purified with protein A chromatography and their presence was confirmed using SDS-PAGE, ELISA and Western blotting tests. In all passively immunized mice peak parasitaemias were significantly reduced compared to control mice. The kinetics of IgG2a and IgG1 production showed a sequential Th1/Th2 response in the passively immunized mice after challenge. The profile of antibody production was predominantly IgG2a at or immediately after the peak parasitaemia and higher levels of IgG1 later during the course of infection which reflects switching from Th1 to Th2 response.

Cross-reactivity of immune serum and serum IgG1 and IgG2a obtained from mice infected with the avirulent DK strain in mice challenged with the virulent DS strain was also examined. Passive transfer of IgG1 and IgG2a from DK-infected mice into DS-challenged mice resulted in significantly reduced peak parasitaemias in DS-challenged mice compared

to control infected mice. However, some of these passively immunized mice died.

Following passive immunization, surviving mice were challenged with a high infective dose of 1×10^8 pRBCs of the DK strain or the DS strain. The results did not show any parasitaemia in these rechallenged mice indicating protective secondary responses.

The last part of the study examined immunization of NIH mice with synthetic peptides whose amino acid sequences are based on the two multiple gene families of *P. chabaudi* AS. The first family is called *clag* in human *P. falciparum* and *clag*-like genes in animal models such *P. chabaudi* AS. The second family called the *cir* gene family which has homologuos in other rodent malaria parasites such as *P. berghei* and *P. yoelii* and importantly in the human malaria *P. vivax*. These two gene families more particularly *cir* are linked to antigenic variation and are thought to be involved in some aspects of the pathology of malaria through cytoadherence and invasion of erythrocytes by merozoites. The products of *clag* and *cir* genes can be considered as vaccine candidates. In this study three peptides were related to *clag* genes and designated P1, P2 and P3, and the two others were related to *cir* family designated as P4 and P5. The amino acid sequences of P1 is conserved between *clag* 7 and 3 and based on postions 121-134 and 128-141 respectively. The P2 and P3 are specific for *clag* 7 and 3 and their amino acid sequences are based on postions 244-257 and 246-259 respectively. The amino acid sequences of P4 and P5 are based on positions 102-115 for P4 and 174-187 for P5 based on sequenced mRNA of *cir* gene of *P. chabaudi* AS.

Immunization of NIH mice with these synthetic peptides resulted in statistically reduced peak parasitaemias compared to the controls when mice challenged with *P. chabaudi* AS. The results showed that immunization with P1 significantly lowered the peak parasitaemia compared to P2 or P3.

Using the adjuvant Titer max gold did not show more effect in reduction of peak parasitaemia in immunized mice compared to immunization without the adjuvant.

The kinetics of IgG2a and IgG1 production probably indicated a sequential Th1/Th2 activation in the peptide-immunized mice. However, the degree of efficacy afetr peptide immunization did not show complete

protection. In immunized mice the levels of IgG1 and IgG2a were significantly higher compared to control non-immunized mice either post-immunization or post-challenge. In immunized mice the IgG2a and IgG1 levels were higher post-challenge compared to post-immunization. The production of IgG2a and IgG1 was significantly higher in P1-immunized mice compared to P2- or P3-immunized mice. The antibody production in P5-immunized mice was higher than that in P4-immunized mice. In P4- and P5-immunized mice anti-peptide IgG2a levels post-immunization and post-challenge were also significantly higher than that in mice immunized with P1, P2, or P3. The results show that anti-peptide antibodies obtained from sera of immunized mice can specifically recognise similar eptitopes and/or probably cross-reactive epitopes present in the soluble crude antigen preparation of *P. chabaudi* AS parasites. This observation may be considered as an important aspect in immunization with synthetic peptides because these antibodies may be protective in heterologous challenges.
In all fields of this study further experiments are required to determine more accurate knowledge about the immune response in mice which may help to develop important data for comparative immunology between animal models and their human counterparts. In terms of synthetic peptide immunization different formulations or combinations of such peptides which induce protective immunity are essential to design vaccine candidates.

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Abbreviations

Abs	Antibodies
ADCC	Antibody-dependent cell cytotoxity
ADCI	Antibody-dependent cellular inhibition
AMA-1	Apical membrane antigen-1
ANOVA	Analysis variance between groups
APC	Antigen presenting cell
BSA	Bovine Serum Albumin
Ci	Curi
Clag	Cytoadherence linked asexual gene
ConA	Concanavalin A
Cpm	Counts (of radioactive) per minute
CSP	Circumsporozite protein
CTL	Cytolytic T Lymphocyte
DBL-EBP	Duffy binding like erythrocyte binding protein HLA
DCs	Dendritic cell(s)
EBA-175	175-kDalton erythrocyte-binding antigen
EDTA	Ethylenediamence tetraacetic acid
ELI	Expression library immunization
ELISA	Enzyme linked immunosurbent antigen test
ELISPOT	Enzyme linked immunospot test
EGF	Epidermal growth factor
FACS	Fluorescent activated cell sorting
FCS	Foetal calf serum
FP9	Fowlpox strain 9
GPI	Glycosylphosphatidylinositol

G6PD	Gglucose-6-phosphate dehydrogenase
GSS	Gene survey sequences
HbsAg	Hepatitis B surface antigen
HLA	Histocompatibility leukocyte antigen
HMGB1	High-mobility-group box 1 protein
HSPGs	Heparan sulphate proteoglycans
ICAM	Intracellular adhesion molecule-1
IFNγ	Interferon-gamma
Ig	Immunoglobulin
IL-	Interleukin
i.p	Intraperitoneally
i.u	International unit
i.v	Intravenously
kDa	Kilodalton
KLH	keyhole limpet haemocyanin
LPS	Lipopolysaccharide
M	Molar
MAb	Monoclonal antibody
MHC	Major histocompatibility complex molecule
MIM	Multilateral Initiative on Malaria
mg	Milligramme(s)
µg	Microgramme
ml	Millilitre(s)
µl	Microlitre(s)
MSP-1	Merozoite surface protein-1
MVA	Modified Vaccinia Virus Ankara
NANP	Aspargine-alanine-aspargine-proline

NIAID	National Institute of Allergy and Infectious Diseases
NAVDP	Asparagine, Alanine, Valine, Aspartic acid, Proline
NIH	National Institutes of Health of USA
NK cell	Natural killer cell
nm	Nonometer nm
NO	Nitrogen oxide
PABA	Para-aminobenzoic acid
PARP-1	The enzyme poly-(ADP-ribose) polymerase-1
PBS	Phosphate buffered saline
PfEMP1	*P. falciparum* erythrocyte membrane protein1
PfLSA-3	*P. falciparum* liver-stage antigen 3
p.r.	Proliferative responses
pRBCs	Parasitized red blood cell(s)
PMMSA	Principal molecules of the erythrocytic stages of malarial antigen
Pv RBP-1	*P. vivax* reticulocyte-binding protein-1
RFLP	Restriction fragment length polymorphism
RBM	Roll Back Malaria
RESA	Ring-infected erythrocyte surface antigen
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error mean
SSP-2	sporozoite surface protein-2
TBV	Transmission blocking vaccines
T cell	T lymphocyte
Th cell	T helper cell
TGF	Transforming growth factor

TNFα	Tumour necrosis factor-alpha
TRAP	Thrombospondin-related adhesion protein
s.c.	Subcutaneously
SI	Stimulation index
SSP-2	Sporozoite surface protein-2
TRAP	Thrombospondin-related adhesion protein
U/ml	Unit per millilitre
U.N.	United Nations
VCAM-1	Vascular cell adhesion molecule 1
W.H.O.	World Health Organisation

Chapter One

General introduction

1. General introduction

1.1. Malaria

Malaria is derived from an Italian word "mal-aria" which means "bad air". It was thought that malaria comes on the wind from swamps and rivers in marshy lands (Floore, 2004). Probably malaria has been recorded since the beginning of the written word, 6000-5500 B.C. as a deadly fever (Kakkilaya, 2004). The oldest material of malaria in history, to date, is malarial DNA from a Roman site, dating from around AD 450 (Thompson, 2001). Malaria probably originated in Africa (Escalante *et al.*, 1998) and accompanied human migration to the Mediterranean shores, India and South East Asia (McConnell, 2004; Thompson, 2001). Malaria parasites are classified into the genus *Plasmodium*, family *Plasmodiidae*, subclass *Coccidiasina*, and phylum *Apicomplexa* (Escalante *et al.*, 1998). Malaria parasites, at some relatively early stage in their evolution, acquired asexual intracellular replication during adaptation to the tissue of the host, particularly RBCs (reviewed by Carter and Mendis, 2002; West *et al.*, 2000). At the same time certain lines of malaria parasites became adapted to live in insects and achieved a two-host life cycle and the human malaria parasites became adapted to the blood-feeding habits of female *Anopheles* mosquito as an invertebrate host (Epstein, 1999; Nakajima, 1994; Carter, 2002).

Four species of the genus of *Plasmodium* cause human malarias: *Plasmodium vivax*, *P. ovale*, *P. malariae*, and *P. falciparum* of which *P. falciparum* is the cause of almost all mortality (reviewed by Good, 2001).

1.2. Current global picture and problems facing malaria control

Malaria is still one of the most important parasitic diseases with an estimated 40% of the world's population in 101 countries at risk of infection. It kills more people than among other pathogens apart from tuberculosis (W.H.O., 2005). It is reported that there are 300-500 million clinical cases each year with between 1.5 to 2.7 deaths. The main victims are children under 5 years old, pregnant women, immigrants, and refugees (W.H.O., 2005).

Despite many global efforts to eradicate, control, or prevent malaria, the situation is serious and becoming worse. For example, malaria persisted in Sri-Lanka with a massive epidemic in 1968. In Madagascar 25000 deaths were reported in 1988 (Epstein, 1999; Nakajima, 1994). More than 90% of the total malaria incidence and the great majority of deaths occur in tropical African countries. Nine countries are in the second high-risk position, India, Brazil, Afghanistan, Sri-Lanka, Thailand, Indonesia, Vietnam, Cambodia, and China (W.H.O., 2004). Apart from malaria in endemic areas, between 1969 and 1999, 12 countries reported a total of 87 cases of "Airport malaria" in people living near airports. This could be as a result of infected vectors brought in on flights from the endemic areas. For example, Airport malaria has been reported in a publican working close to the London's Heathrow Airport and in four workers at the Amsterdam airport, none of whom ever been out of the two countries (Opperdoes, 1997). In France Airport malaria was found in four people in the vicinity of the Roiss Charles-de-Gaulle airport in Paris during summer 1999 (Jafari *et al.*, 2002).

Due to many problems, such as vector resistance to insecticides, parasite resistance to anti-malarial drugs, uncontrolled immigrations, and easy transportation throughout the world the WHO world global eradication programme was finally abandoned in 1960 (Russell, 2003). Therefore, more investment is required in the fields of control and vaccination research projects particularly when other problems such as inadequate health structure, ethical limitations in human studies and clinical trials, and poor socio-economic conditions are considered (W.H.O., 2004). The total budget from all funds has been raised from $US 85 million in 1995 to more than $US 100 million through the Multilateral Initiative on Malaria (MIM). MIM is beginning to make more effective use of global resources through promoting coordinated activities. The commitment of NIAID, the National Institute of Allergy and Infectious Diseases, notably has increased by more than 150% between 1995 and 1999 and the Wellcome Trust doubled its expenditure (W.H.O., 2004). Malaria victims in endemic areas occupy three of ten hospital beds. In Africa the cost of a single bout of the disease is equal to 10 working days (W.H.O., 2004). In 1998 four United Nations (U.N.) agencies WHO, UNDP, UNICEF, and the World Bank, launched the Roll Back Malaria (RBM) programme in which the main purpose is to halve

the world's malaria burden by 2010 and halving it again by 2015 (W.H.O., 2004). A report has found that all funds from donor countries and World Bank remains at the range of $100 million annually which means that RBM will fail to meet its goal if the budget does not increase (Narasimhan, 2003).

Attempts to develop a successful vaccine, as an effective way for control, have faced many problems such as the complex life cycle of the parasites, many gene families coding for polymorphic antigens, antigenic diversity and variation, and unknown expression and transcription mechanisms of those antigens. Successful progress leading to the RBM goal needs a budget of perhaps $1.5-$2.5 billion annually (W.H.O., 2004).

1.3. Brief history of the discovery of malaria parasite life cycle

Despite the presence of many documents in the ancient history of medicine which describe malaria and its symptoms, particularly its fever, the causative agent was not recognised until the 19th century. Maeckel, a German pathologist, who concluded that the black granules in blood, spleen, and liver of the cadavers were associated with malaria, made the first observation about malaria. It is now known that the black granule was malaria pigment. Thirty years later, Kelsch, a French pathologist, observed parasitized red blood cells in which pigment was visible (Phillips, 1995).

Laveran (1845-1922) on the 6th of November 1880 detected the parasites in the blood of a soldier with malaria symptoms. He saw spheres and crescent forms of the parasites, when examining an infected blood sample. He was, in fact, looking at exflagellation of male gametes of *P. falciparum*, a phenomenon which was later explained by MacCallum in 1897.

Laveran's discovery is one of the milestones in the history of medicine. He called them *Oscilaria*, a term which was replaced by *Haemamoeba* and eventually by *Plasmodium* by Marchiafava and Celli in 1885. In 1884, four years later, Laveran demonstrated the correlation of exflagellation with the existence of malaria parasites to Pasteur and Roux. Leuckart emphasized that malaria transmission might occur through on arthropod in 1877 (Bruce-

Chwatt, 1988). Laveran in 1884 stated that there was a probability of mosquito transmission in malaria infection (Nobelprize.org, 2004).

Marchiafava and Celli saw the asexual multiplicative forms of the parasites in patient's red blood cells in 1885. Golgi, an Italian physician, described the relationship between the paroxysm of fever. Indeed he had observed the release of the merozoites and the infected red blood cells bursting at time of fever (Phillips, 1995; Bruce-Chwatt, 1988). He finally identified *P. vivax* and *P. malariae* in 1885 (Kakkilaya, 2004). The first standard method of staining blood films for recognition of malaria was described in Romanowsky's MD thesis "The parasitology and Therapy of Marsh-fever" in 1891 (Bruce-Chwatt, 1988).

The mystery of the life cycle of the parasites in *Anopheles* and its transmission by mosquito bite was not solved until the 20th of August 1897. At this time Ronald Ross, a British military physician in India, demonstrated the malarial oocyst in the gut tissue of a female *Anopheles* (Bruce-Chwatt, 1988). This observation was published in the Lancet on December 18, 1897 (Phillips, 1995). He concluded that anopheline mosquitoes could be the vectors of malaria, because he had examined the female *Anopheles*, after taking blood meal on the infected patient with crescent shapes in the blood. He observed all stages of bird malaria parasites in the mosquito *Culex fatigens* in 1898.

Human malaria transmission through the mosquito was established in 1898 when the Italian scientist, Giovani Batista Grassi, proved that human malaria was transmitted by only a species of *Anopheles* (The Columbia Encyclopedia, 2001). The complete development cycles of human *P. falciparum* and *P. vivax* was demonstrated by Grassi *et al.* in 1898. Bastianelli and colleagues described malaria stages in *Anopheles claviger* at that time. MacCallum, in Ontario, explained fertilization in 1897 (Bruce-Chwatt, 1988). Koch then described the passage of the ookinete through the gut of the mosquito in 1898. Manson obtained infected anopheline mosquitoes with benign tertian malaria from Bignami and Bastianelli in 1900. The mosquitoes were dispatched to London and the infected mosquitoes bit two people, Manson's son and George Warren, a laboratory assistant. They came down with malaria two weeks later and Manson's son had two relapses of malaria in 1901 (Bruce-Chwatt, 1988). In 1903 Fritz Schaudinn mistakenly described sporozoites directly penetrating red blood

cells when *P. vivax* sporozoites are released from the salivary glands of the infected mosquitoes. Due to the reputation of Schaudinn this misleading observation was not contradicted for three decades (Phillips, 1995). During the 1930s malaria parasites were found in haemopoietic tissues by Huff during his work with bird malaria parasites. James and Tate found *P. gallinaceum* in the brain of chickens and so the term 'exo-erythrocytic' was coined. Raffael described the exo-erythrocytic stage of the bird's malaria, *P. elongatum*, in the reticulo-endothelial cells of the bone marrow and internal organs in 1934 (Phillips, 1995). Mudrow-Reichenow (1950) saw *Plasmodium* in bird's tissues before its invasion of red blood cells. It was termed pre-erythrocytic stages in 1940. Eventually Huff and Coulston described the full life cycle of *P. gallinaceum* in 1944. Shortt successfully identified the pre-erythrocytic stages of *P. cynomolgi*, a monkey *Plasmodium*. The observation of *P. vivax* and *P. falciparum* became thereafter possible with the help of volunteers. Shortt and Garnham showed all stages of human malaria parasites between 1948 and 1950.

At last the complex and mysterious life cycle of malaria parasites was solved after about one century of continuous investigation (Phillips, 1995, and Bruce-Chwatt, 1988).

1.4. Life cycle of *Plasmodium*

The life cycle of *Plasmodium* includes three distinct stages: pre-erythrocytic, erythrocytic stage, and sexual stage as figure 1 shows. These three stages are explained below.

1.4.1. Pre-erythrocytic and liver stage

The motile sporozoites, the infective stages of the parasite, enter the skin when a female *Anopheles* bites the host when taking a blood meal. Sporozoites remain in the peripheral blood for usually up to 45 minutes during which they actively enter the hepatocytes (Ho and White, 1999). However, it is suggested that sporozoites injected directly into the bloodstream invade hepatocytes within a few minutes (Shin *et al.*, 1982). During the biting process the mosquito releases many bioactive substances

like anticoagulants and with them the infective sporozoites enter the bloodstream.

However, it is now clear that most sporozoites do not pass directly into the bloodstream. Mosquitoes may deposit sporozoites in the bite site when they probe the skin of the host to search capillaries in which they would enter through a wound inflicted by the mosquito proboscis (reviewed by Kappe, Kaiser, and Matuschewski, 2003). Another way is that sporozoites can actively migrate through the cells lining capillaries (Matsuoka *et al.*, 2002) and other cells (Mota *et al.*, 2001).

Most mosquito-transmitted sporozoites are deposited as clumps within the skin and there is a delay in the movement away from the bite site either by entry into efferent capillaries or, via lymphatic drainage. In the "taxicab" hypothesis, it is proposed that mosquito-transmitted sporozoites quickly invade macrophages and other leukocytes in skin away from the bite site and are carried through the peripheral lymph nodes, and on to the liver via the draining lymph, a circuitous way, (Krettli, and Dantas, 2000) or enter Kupffer cells, the stationary phagocytic cells of the liver (Vaughan, *et al.*, 1999). This hypothesis has been supported by the fact that *P. berghei* sporozoites can enter and leave macrophages without being destroyed, and the presence of the extremely low percentage of infections in all attempts to cultivate the mammalian sporozoites in hepatocytes (Krettli, and Dantas, 2000). If mosquito-transmitted sporozoites are fortunate enough to enter the trunk they may be sent directly into the liver via the common hepatic artery (Krettli, and Dantas, 2000). Although it is known that intracellular bacteria and parasites typically invade host cells through the formation of an internalization vacuole around the invading pathogen, *Plasmodium* sporozoites have an alternative mechanism to enter cells (Krettli, and Dantas, 2000). It has been observed that breaching of the plasma membrane of the host cell is followed by a rapid repair. In contrast with the formation of a vacuole around the sporozoite, this mode of entry was followed by exit of the parasite from the host cell. Nevertheless, the actual route by which sporozoites reach the liver is still uncertain

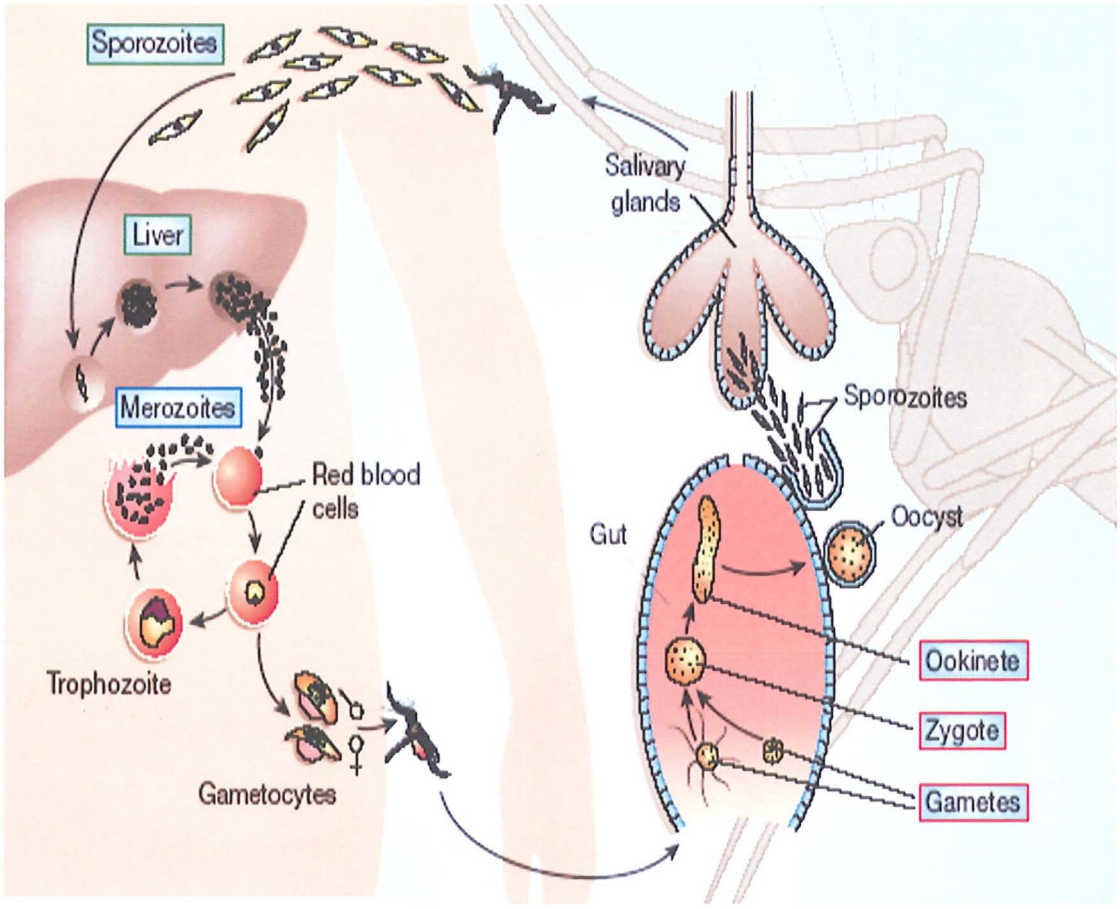


Figure 1. The *Plasmodium* life cycle involves three distinct stages. In the first stage, pre-erythrocytic stage, a female *Anopheles* mosquito inculates sporozoites into a vertebrate host's skin. The sporozoite infects hepatocytes and mature into schizonts which rupture and release merozoites. The second stage, the asexual blood stage starts when emerged merozoites from the liver invade eryhtrocytes and undergo asexual replication. In *P. vivax* the merozoite develops to ring form, trophozoite and the nucleus divides inside the trophozoite forming immature and mature schizonts. The mature schizont bursts and releases new merozoite into the blood stream. During asexual stages some merozoites differentiate to male (microgametocytes) and female (macrogametocytes) gametocytes. The third stage, sexual stage initiates when female mosquitoes take up gametocytes during a blood meal. In the lumen of the mosquito the microgametes fertilize macrogamtes and produce zygote. A zygote changes to a motile ookinete which esides in the wall of the mosquito's gut and develops into an oocyst in which thousands of sporozoites can be produced. These sporozoites are infective stages for a vertebrate host (Menard, 2005).

Sporozoites can stick in the liver in the presence of specific receptors on the cells lining sinusoids (reviewed by Kappe, Kaiser, and Matuschewski, 2003). Sporozoites traverse several hepatocytes in the liver of the host before infection *in vivo* by disruption of the cell membranes and remain in the cytosol without a surrounding parasitophorous vacuole. This migration in the mammalian host is thought to be essential for activation of *Plasmodium* sporozoites to infect host cells and finding suitable hepatocytes for the next stage of the development and completion of the life cycle (Mota *et al.*, 2001; Mota *et al.*, 2002). *Plasmodium* sporozoites home to the liver to infect a single hepatocyte and rapidly grow and divide inside the hepatocyte over the following 5–15 days, depending on species. Eventually, the infected hepatocytes burst and each one releases merozoites into the blood circulation (Hviid, 2004).

Several proteins such as the circumsporozite protein (CSP) and sporozoite surface protein-2 (SSP-2) contribute to the invasion process and are held on the surface of the parasite with glycosylphosphatidylinositol (GPI) as an anchor. For example, CSP binds to heparan sulphate proteoglycans (HSPGs) on the surface of hepatocytes (Frevert, 1994; reviewed by Kappe, Kaiser, and Matuschewski, 2003).

A number of parasites do not divide and remain as hypnozoites inside the hepatocytes in *P. vivax* and *P. cynomolgi*. They are responsible for relapses of malaria, several months or years later (Krotoski *et al.*, 1982).

1.4.2. Erythrocytic stage (asexual blood-stage)

Merozoites, which are released from the mature schizont (in the liver) discharge into the circulation and invade erythrocytes. On the surface of merozoite, each a little more than 2-3μm in diameter, a protein called merozoite surface protein-1 (MSP-1) may be involved in initial recognition of erythrocyte (Chitnis and Blackman, 2000). A protein complex comprising four polypeptides derived from the MSP-1 precursor, in association with two other proteins, encoded by distinct genes uniformly covers the merozoite surface. The bulk of this complex is released at time of invasion (Chitnis and Blackman, 2000).

The proposed invasion mechanism can be summarized as follows:

Initially some adhesion ligands are stable residents on the merozoite surface such as MSP-1. Other components of the associated protein complex may provide the low-affinity interaction with the host cell such as apical membrane antigen-1 (AMA-1). Alternatively some merozoite proteins with significant homology to members of the Duffy binding like erythrocyte binding protein (DBL-EBP) may be involved in this phase.

Secondly reorientation of the bound parasite may be favoured by the presence of higher-avidity ligands clustered around the apical prominence such as *P. vivax* reticulocyte-binding protein-1 (Pv RBP-1).

Thirdly exocytosis of micronemal components such as 175-kDalton erythrocyte-binding antigen (EBA-175) and other DBL-EBPs may provide tight attachment and junction formation.

Finally the parasite may be aided to drive into the nascent parasitophorous vacuole by the effect of anterior-to-posterior trafficking and/or proteolytic shedding of these and other RBC-proteins linked to the action of a sub-pellicular actinomysin motor (Chitnis, and Blackman, 2000). Hence, the apical end of the merozoite contains proteins that have been identified as erythrocytes binding proteins for successful penetration and homing of the parasite (Holder *et al.*, 1994).

The erythrocyte is a suitable host cell for the malaria parasite because there is no major histocompatibility complex molecule (MHC) expression, no internal defence mechanism, contains almost all necessary nutrients for the parasite, and helps the parasite to hide from many immune response mechanisms.

The merozoite begins to grow inside the erythrocyte and the prepatent period begins. The prepatent period refers to the minimum time from infection until the first appearance of the malaria parasite in the infected erythrocyte as seen in (thin) blood smears. It is a character which depends upon the species of parasite and an infective dose (Wiser, 2003).

The parasite replication is initiated by rapid development of a vacuole in the merozoite to form a ring. Growth continues with the disappearance of the vacuole and appearance of pigment as a result of digesting and metabolizing haemoglobin. The parasite then occupies a portion of the host cell and is called the trophozoite. The nucleus of the parasite divides then to form a mature schizont in which numerous daughter cells from within

the body of single parent cell are produced. The number of these new merozoites depends on the species of malaria parasites. Timing for each asexual cycle is also species specific. It is 48 hours in tertian parasites (*P. vivax*, *P. ovalae*, and *P. falciparum*) and 72 hours in quartan (*P. malariae*). This timing is relevant to the cyclical fever, the hallmark of malaria of different species (reviewed by Wipasa *et al.*, 2002).

The parasite goes through a series of asexual erythrocytic cycles. The time of each new generation becomes synchronous in many malaria parasites such as *P. c. adami* and *P. chabaudi* AS which undergo synchronous infections (Kima *et al.*, 1992). Sequestration, a phenomenon in which late trophozoite/schizont stage of some malaria parasites such as *P. falciparum* and *P. chabaudi* stop circulating and adhere to endothelial cells of post capillary venules, usually occurs in the final third of the asexual erythrocytic cycle (Kima *et al.*, 1992). In humans *P. falciparum* infections sequestration could be the first stage of pathologic events contributing to potentially fatal disorders in many organs such as brain (cerebral malaria), heart, kidneys, gut, and liver, but not in *P. chabaudi* infections. So, almost all morbidity and mortality of malaria occurs in the erythrocytic stage (Kima *et al.*, 1992; reviewed by Wipasa *et al.*, 2002). A proportion of new merozoites released from a ruptured schizont is not picked up by the phagocytic cells and invade new erythrocytes (reviewed by Moorthy, Good and Hill, 2004) and some other merozoites then differentiate to become gametocytes (Phillips, 2001).

1.4.3. Sexual stage

Gametocytes are stages infective for the female *Anopheles* mosquito, the vector. Gametocytogenesis usually starts after two cycles of schizogony in blood in humans (Garnham, 1988). Killick-Kendrick suggested that in rodent malaria infection gametocytes might arise directly from merozoites from the liver (Garnham, 1988).

When *Anopheles* takes up the infected blood meal, gametocytes are taken up into the mosquito's gut. The macrogametocyte rapidly forms a macrogamete, but microgametocytogenesis proceeds more slowly through the phenomenon called exflagellation, after ten minutes. The microgamete nucleus usually divides into eight, each of which is motile and has a shape

like a flagellum of 20 μm length. A free exflagellated microgamete fertilizes a macrogamete and a pigmented zygote is formed by fusion of two nuclei. Within 18 hours or less after the blood meal the zygote has elongated to the motile ookinete. The ookinete penetrates the epithelial layer of the midgut with its peritrophic membrane and finally rests between the basal lamina and epithelial cells on the outer surface. The ookinete develops to become an oocyst. The oocyst may be recognized after 24-72 hours after ingestion of the infected blood meal. The nucleus of the oocyst, 6-8 μm diameter, divides repeatedly during the next 7 to 15 days post-infective bite. A mature oocyst usually contains thousands of sporozoites. Sporozoites leave the oocyst into the haemocoelic fluid and migrate to the acinal cells of the salivary glands.
After a day residence in the gland they will have the highest infectivity, which may be decreased with age. An infective bite consists of passing many sporozoites into the vertebrate host such as human (Garnham, 1988).

1.5. Rodent malaria parasites

In vivo studies of *P. falciparum* malaria in humans which lead to an understanding of the immune responses and development of a successful vaccine are very limited. This is because of different obstacles such as ethical problems due to the dangerous outcome of the infection. Although *in vitro* culture of *P. falciparum* is possible (Trager and Jensen, 1976), extrapolating any *in vitro* results to the *in vivo* situation has to be with caution. Although the human parasites are largely host specific, they can infect a few nonhuman primates and monkeys. But using nonhuman primates is also restricted because of availability, and expense (Phillips, 2001). So, animal models of human malaria parasites, which are easy and cheap to maintain such as rodents, are being used. However, malaria parasites in birds or primates were studied prior to rodents. The discovery of rodent malaria parasites in Katanga by Vincke and Lips in 1948 opened a new era in malaria study (Killick-Kendrick, 1978; Kreier, 1977). These parasites have features of human malaria infections and today most of our knowledge of malaria is indebted to theier discovery and use. The mouse also as a rodent host has a well-characterized immune system. So, using

mice provides advantageous information in the study of immune responses against malaria parasite (Taylor-Robinson, 1995).

The first experimental *Plasmodium* infection was studied in an arboreal rodent, *Grammomys surdaster*, which was infected with *P. berghei*. Vincke and Lips (1948) captured two parasitized *G. surdaster* and the blood of these infected rodents was passaged to white rats and mice and both produced infections. The malaria parasite was then isolated and it was named *P. berghei* (Killick-Kendrick; 1978).

Three rodent species may coexist in the same host and locality, *P. yoelii*, *P. vinckei,* and *P. chabaudi* (Landau and Chabaud, 1994). The non-lethal infections by a number of rodent malaria parasites such as *P. chabaudi chabaudi*, *P. c. adami*, *P. yoelii*, and *P. vinckei petteri* can be used as appropriate models to investigate the mechanisms of acquired immunity. All species can be grown in laboratory mice and young rats. Maintenance of above three species and *P. berghei* with a complete life cycle is now possible in the laboratory (NCBI, 2004).

1.5.1. Using *P. chabaudi*

Plasmodium chabaudi was first isolated from a shiny thicket rat, *Thamnomys rutilans*, in the Central African Republic, by Irene Landau and Alain Chabaud in 1965. Two subspecies of this species were discovered by Carter and Walliker in the Congo Republic in 1976 in *T. rutilans* and have been defined as *P. chabaudi chabaudi* and *P. c. adami* (NICB, 2004).

There are several similarities between *P. chabaudi* and *P. falciparum* during their life cycle and the pathology caused in the host (Taylor-Robinson 1995). *P. chabaudi* AS strain has been widely used as an animal model of a *P. falciparum* infection. The similarity of *P. c. chabaudi* AS to *P. falciparum* is in the asexual blood stage where it infects normocytes, undergoes antigenic variation and partial sequestration in resistant inbred strains of mice laboratory including C57Bl/6, and NIH. This parasite also shows recovery from the acute parasitaemia which is followed by one or more recrudescences (Cox, Semoff and Hommel, 1987; Gilks, Walliker and Newbold, 1990; Phillips, Mathers and Taylor-Robinson, 1994). However, it should be borne in mind that in this animal model the host is unnatural and artificial and also the course of infection has some differences in animal

models and humans. For example, in the rodent malaria parasite *P. c. chabaudi* AS, the acute parasitaemia has a higher peak parasitaemia (>30%) than that normally seen in *P. falciparum* (10%). The asexual blood stage of *P. chabaudi* grows synchronously. In *P. chabaudi* the rupture of pRBCs occur between midnight and 3:00 am (Hawking, Gammage and Worms, 1972). So, most merozoites reinvade new red blood cells between midnight and 08:00 (Landau and Chabaud, 1994). Sequestration of schizont-infected red blood cells has been observed in mice infected with *P. c. chabaudi* (Cox, Semoff and Hommel, 1987; Gilks, Walliker and Newbold, 1990). Transmission electron microscopy has showed that in mice infected with *P. c. chabaudi* pRBCs were directly in contact with endothelial cells in the liver (Mota *et al.*, 2000). Using two different modifications of Giemsa's stain showed that *P. c. chabaudi* invades all red blood cells as *P. falciparum* does, regardless of their age, but will prefer to invade metabolically young RBCs (Taylor-Robinson, 1993).

P. chabaudi genome size, with 14 chromosomes, is estimated to be close to that of *P. falciparum* at 25-30 Mb. This genome is also very A/T rich at approximately 80%, and comparable to that of *P. falciparum* (NICB, 2004).

1.5.2. The course of the infection of malaria parasite in rodents

In the natural host, *T. rutilans*, the infection lasts throughout the host's life (Landau and Chabaud, 1994). However, the infection in experimentally infected rodents does not become chronic and cure occurs after a variable length of time (Landau and Chabaud, 1994). Following a natural infection in *T. rutilans* is very difficult because parasites are usually very scanty. The asexual blood stage of the malaria parasite was obtained by sub-inoculation of pRBCs from *T. rutilans* into laboratory mice. This erythrocytic cycle lasts approximately 24 hours in *P. chabaudi*, *P. vinckei*, and *P. yoelii* (Landau *et al.*, 1993).

1.6. Overview of immunity of malaria

Naturally acquired immunity in people who live in endemic areas is very slow to develop and takes between 10-15 years of exposure (Long, 1993; Baird, 1998). Although this immunity controls parasite replication, it can not eliminate the parasite from the blood completely. It is, however, sufficient to prevent death and decrease the impact of malaria after reinfection (Baird, 1998). Immune responses are generally species-, stage-, strain-, and variant- specific (Andrysiak, Collins and Campbell, 1986; Fandeur, and Chalvet, 1998). Individuals in endemic areas frequently have premunition, a semi-immune response, showing periodic parasitaemia and specific antibodies. Premunition is initially reflected in fewer clinical symptoms and eventually in lower parasitaemia and no symptoms (Long, 1993; Long *et al.*, 1994; Baird, 1998). Immune responses involve both antibody-dependent and cell-mediated immunity (reviewed by Wipasa *et al.*, 2002).

Immunity to malaria in the vertebrate host according to Phillips (1995) can be divided into three categories in which "the parasite may either 1) multiply and rapidly kill the host, 2) be quickly controlled and eliminated from the blood of the host, or 3) be reduced to low and usually sub clinical levels after the acute phase and persist for long periods.

The mechanisms and factors that provide resistance or acquired immunity have been explored through population studies, either *in vitro* using human sera and cells, or a variety of animal models in primates and rodent hosts in both *in vitro* and *in vivo* studies (Phillips, Mathers and Taylor-Robinson, 1994; Nardin and Nussenweig, 1993; Long, 1993).

The mechanisms of malaria immunity may be divided as follows: natural resistance to malaria and acquired immunity.

1.6.1. Natural resistance to malaria

Possession of natural resistance in some individuals against malarial infections is largely based on genetic characteristics. The change in host's genome can be developed over the time through mechanisms such as

mutations and selective pressure which may be positive and result in advantages for the host (Liberles and Wayne, 2002).

Malaria infection results from the entry of the parasites into the host cell, first the hepatocyte, and then the erythrocyte. It involves a complex process in which interaction between specific receptors on the host cell membrane and binding sites of the parasite is required. If any cell membrane does not have appropriate receptors, the penetration of the parasite will not occur (Butcher, Mitchell and Cohen, 1973). For instance, absence of *P. vivax* malaria in people who live in West Africa results from a high proportion of Duffy negative individuals. This molecule, as a specific erythrocyte receptor, is necessary for successful entry of *P. vivax* into the host red blood cell (Miller *et al.*, 1976). It is the same for *P. knowlesi* (Miller *et al.*, 1975).

Genetic mutations which are proposed to link to malaria occur almost exclusively in areas where the disease has been a killer. The frequencies of these mutations are balanced to keep them at appropriate levels. So, protective effects against malaria are balanced with the side effects of disease such as sickle cell anaemia and thalassemia (Harder, 2001).

The presence of foetal haemoglobin, a protective factor in new-borns against malaria up to three months, can retard development of *P. falciparum* and give resistance to the parasite (Giardina *et al.*, 1995).
In 1949 Haldane stated the "Malaria Hypothesis". He hypothesized that there is a correlation between malaria endemicity and hereditary as) that haemoglobinopathies such as thalassaemia (Allison, 2004). The hypothesis suggests that malaria applies selective pressure by which a related gene is maintained within a population. For example, in the South West Pacific region there is a geographical correlation between the frequency of alpha $^{+-}$ thalassaemia and the endemicity of malaria (Allen *et al.*, 1997). So, it was concluded that thalassaemia provides some advantages for people who are living in malaria endemic areas.

In addition to thalassaemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and other enzymatic deficiencies can protect against malaria. The resistance will be enhanced in a host who has both thalassaemia and G6PD (Oo *et al.*, 1995).

According to Tishkoff (2001) and her colleagues two variants of G6PD deficiency arose in Africa. They focussed on the two alleles that cause

G6PD deficiency. These deficiencies are known as A and Med, which appeared in African and Mediterranean populations respectively. The A one appeared within up to the past 11760 years ago and the Med one was up to 6640 years ago. Interestingly archaeological evidences confirm that the presence of a dramatic lifestyle change started in Africa 12000 years ago due to severe change in climate and weather which provided favour conditions for malaria spread. So it may be concluded that natural selection spreads this genetic disorder for protecting the population from the fatal malaria in those areas (Tishkoff *et al.*, 2001).

If the merozoite enters the host cell, where the intracellular environment is unfavourable, the parasite's development will fail. For example, *P. falciparum* is unable to cause the severe symptoms in people who have a mutation in their haemoglobin gene by which valine replaces glutamic acid in the β chain of the molecule, which is resistant to breakdown by the parasite proteases and termed HbS (Pasvol, Weatherall, and Wilson, 1980). In a large case-control study in Burkina Faso it was shown that HbS also provides protection against clinical *P. falciparum* in homozygotes with 29% and in heterozygotes with 92% of subjects (Modiano *et al.*, 2001).

Malaria infections are also affected by specific diet or lack of some nutrients. *P. berghei* infection can be suppressed in rats which were fed only milk. Infants that are also fed only milk are resistant because milk has no para-aminobenzoic acid (PABA) which is a necessary growth factor for the parasite (Maegraith, Deegan, and Sherwood, 1952). A diet with cod liver oil allows mice infected with lethal *P. berghei* to live longer. Adding vitamin E reversed the effect (Godfrey, 1957). A vitamin E-deficient diet suppressed the infection in mice infected with lethal *P. yoelii* (Taylor *et al.*, 1997). Low protein foods such as Cassava, yams, sugar cane, and dark lima beans provide some resistance to malaria. The severity of infection with *P. yoelii*, *P. berghei*, and, *P. vinckei* has been shown to be reduced in mice feeding on cassava (Ibekwe and Ugwunna, 1990). Levander (1995) reported that the infected host erythrocyte, the parasite, or both were affected by an antimalarial effect of fish oil through imposing a dietary-induced oxidative stress.

1.6.2. Acquired immunity to malaria

It is now known that early events in the host response during natural immunity to an invasive pathogen influence the development of acquired immunity. So, natural immunity mechanisms play a role in bridging innate resistance and adaptive immunity. Non-specific responses, as essential first defences, are able to activate specific immunological responses via the release of cytokines (Choudhury *et al.*, 2000). Induction of IL-12 secretion, a key cytokine which initiates Th1 responses by triggering IFNγ production from NK and $CD4^+$ T cells (Gazzinelli, 1996), results from stimulation of cells such as macrophages (Sam, and Stevenson 1999; Stevenson *et al.*, 1995) and dendritic cells (DCs) (Ahuja *et al.*, 1998).

Despite repeated exposure during childhood to malaria and evidence of a variety of effector mechanisms to malaria, immunity remains incomplete even in adults (Baird, 1998). Immunity can be lost if these immune people move out of an area of endemicity. It is suggested that repeated exposure is necessary to maintain resistance (Winstanley, 1998). Some important aspects of the immune responses have been identified and are summarised as follows. However, *in vivo* immune response mechanisms in humans are still uncertain:

In endemic areas, where the people are regularly exposed to *P. falciparum*, an acquired immunity develops in most residents. Very young children may also be resistant to malaria due to their maternal antibodies, and/or presence of foetal haemoglobin during the first six months of life (Wellcome Trust and Roll Back Malaria information booklet, 2004). Although an acquired immunity starts in babies, children remain at risk of dying up to 5 years old. There is high mortality and morbidity in children from 6 months to 5 years, particularly in Africa (Schwartlander, 1997). However, with increasing age parasite prevalence, density, and the number of clinical episodes progressively decline (Greenwood *et al.*, 1987).

Mayor *et al.* (2003) reported that there was a significant correlation between multiplicity of infection, multiple populations and clones of a species present in a host, and parasite density in infants, children under 4 years of age and adults. It is also proposed that the relationship between multiple infections and malaria morbidity is age-dependent in highly endemic areas (Smith *et al.*, 1999). Although in adults immunity never is

complete, an effective immunity might develop more quickly after first infections in adulthood than in infants and young children (Baird, 1995). In Indonesia there were no fatalities after two years in adults who moved from the areas often free of malaria to the areas where malaria is endemic (Baird, 1995). This suggests that lifesaving immunity developed in 2 years in adults who were infected due to movement to an endemic area, compared with 5 years in children. So, it is suggested that acquired immune response in adults is an age-dependent phenomenon (Baird, 1995; Jones, 1991).

To achieve an immune response, the host has to experience multiple infections in which a spectrum of different antigens are presented to the immune system. The possible sources of these different variant antigens could be antigen polymorphism and antigenic variation over several years (Langhorne, Mombaerts and Tonegawa, 1995).

As previously indicated rodent malaria parasites as animal models for human malaria, particularly the mouse, have provided valuable and important information in understanding the mechanisms of immunity to *Plasmodium* spp. Despite many features in common that justify using these animal models there are some differences between human and mice in the development of immune responses to malaria infection.

In most mice strains a single infection induced a strong immunity in both self-curing and drug-cured infections (McColm and Dalton 1983; Jarra and Brown 1985) and mice more easily become immune to *Plasmodium* than humans (Favila-Castilo, Monroy-Ostria, and Tapia, 1999) whose immunity develops after several infections over years (Hommel, 1985). However, infected mice, in general, suffer higher parasitaemias than humans (Favila-Castillo, Monroy-Ostria, and Tapia, 1999). The usefulness of mice as an artificial host in malaria studies might be questioned because they have different biological features than humans (Good, 1995) and the parasites are used in mice are also different. Nevertheless, using animal models in the light of limitations in human studies remained the best source of information. The main focus, therefore, in introducing knowledge of the immune responses to malaria in this thesis comes from studies with animal models.

1.6.2.1. Immunity to pre-erythrocytic stage and sporozoites

It is known that most mature T-lymphocytes in mice can be separated into two major subpopulations according to their cell surface glycoproteins, $CD4^+$ or $CD8^+$. $CD4^+$ T cells recognize and can be activated by antigenic molecules presented with class II MHC molecules, expressed on B cells and macrophages (Langhorne *et al.*, 1989). This subpopulation promotes antibody production, cell mediated responses and delayed-type hypersensitivity reactions (Bottomly, 1988). $CD4^+$ T cells in mice are functionally heterogeneous. One subset called T helper-1 (Th1) induces inflammatory reactions via cytokines such as IL-2, IFNγ, and TNF leading to cell-mediated responses. For example, IFNγ plays a central role in protection to liver stage malaria infection by inducing infected hepatocytes to produce NO as a killer of the parasite (Mellouk *et al.*, 1991). The other subset, called T helper-2 (Th2) subset, induces production of IL-4, IL-5, and IL-6 preceding antibody production (Langhorne, 1989).

Vaccination experiments initially with animals and later in humans are the main sources of information about the immune responses to pre-erythrocytic stages (reviewed by Phillips, 2001). The presence of both subsets is necessary for the development of protective immune responses against different life cycle stages of malaria parasites such as *P. chabaudi* AS (Langhorne, 1989). $CD4^+$ T cells act as helper cells for the generation of $CD8^+$ T-cells, which kill the parasites within the hepatocytes (Langhorne, 1989). The liver is thought to be an important multifunctional site in protecting a host against infectious agents such as parasites. Although the liver stage of a malaria infection is silent, immunologically it is significant (Lau, Sacci and Azad, 2001). The liver responds to *Plasmodium* infection by increasing the production of C-reactive protein induced by IL-1 stimulation (Nussler *et al.*, 1991c). Intrahepatic lymphocytes that are primed with *P. yoelii* $CD3^+$ $NK1.1^+$ inhibited parasite growth within the hepatocyte (Pied *et al.*, 2000). During the liver stage the expression of several genes may be altered in the host. Lau and colleagues (2001) showed that expression patterns of several host transcripts were altered and up-regulated in the tissue tested 24 hours after infection during the liver stage of *P. yoelii* infection compared to controls. For example, in

the infected spleen the expression of TNF-α was down- regulated where as the IFNγ gene expression was increased (Lau *et al.*, 2001).

Irradiated sporozoites which are able to invade hepatocytes but are unable to complete their development in the liver (Nussenzweig *et al.*, 1967; Hollingdale and Krzych, 2002) can induce protective immune responses in some malaria parasite infections. The role of cell-mediated including $CD4^+$ T cells response to pre-erythrocytic stage in both humans and mice has been shown (Ferreira *et al.*, 1986; Mellouck *et al.*, 1991; Good and Doolan, 1999). Irradiated sporozoites of *P. berghei* in mice and *P. falciparum*, and *P. vivax* in humans can induce a very strong resistance to a challenge with viable sporozoites (Nussenzweig, *et al.*, 1967; Clyde, 1990). Investigations of the protection mechanisms to pre-erythrocytic stage in immunized individuals have shown a role for protective antibody.

Serum of mice immunized with irradiated sporozoites probably contains invasion-blocking antibodies. This serum passively protects mice against sporozoites challenge (Potocnjak *et al.*, 1980). It has been shown that antibodies against sporozoite surface may also play a role to neutralize the infectivity of sporozoites for hepatocytes, (Good and Doolan, 1999).

Mice lacking B cells but not T cells could be immunized with irradiated *P. berghei* sporozoites, indicating a role for antibody-independent (cellular) immune mechanisms as well as antibody mediated protection (reviewed by Phillips, 2001). Protective immune responses are directed to the parasite-derived peptides expressed on the surface of the infected hepatocyte in which $CD8^+$ T cells were the principal effectors (Hoffman and Franke, 1994). It is hypothesized that activated $CD8^+$ T cells release IFNγ and subsequently up-regulate NO production which can eliminate the parasites in infected hepatocytes (Hoffman *et al.*, 1997; Doolan *et al.*, 1996). In BALB/c mice immunized with irradiated sporozoites and challenged with *P. yoelii* sporozoites parasite-specific $CD8^+$ T cells induce a novel mechanism of adaptive immunity in which NK cell, IFNγ, IL-12, and NO are involved. In these immunized mice blood stage parasitaemia was absent after the challenge (Doolan and Hoffman, 1999). Serum of mice immunized with irradiated sporozoites inhibited both invasion of sporozoites and development of exo-erythrocytes stages *in vitro* (Chatterjee *et al.*, 1996).

Human volunteers protected by immunization with radiation-attenuated *P. falciparum* sporozoites produce $CD8^+$ CTL directed at an epitope on the CSP of *P. falciparum* (Hoffman *et al.*, 1997; Doolan *et al.*, 1996). Specific $CD8^+$ T cells for a peptide derived from the *P. yoelii* CPS (Py CSP) are also can be produced in mice which were primed with *P. yoelii* (Doolan and Hoffman, 1999; Weiss *et al.*, 1990). A distinct mechanism of protection is induced in BALB/c mice by immunization with irradiated sporozoites which is initiated by $CD8^+$ T cells following recognition of parasite-derived peptide-MHC complexes on the surface of infected hepatocytes. This mechanism requires NK cells and is dependent on IFNγ, IL-12, and NO (Doolan and Hoffman, 2000).

A best-characterized antigen and major immunodominant surface antigen of sporozoites is the CSP (Nardin and Nussenzweig, 1993; Nussenzweig and Nussenzweig, 1989). It is known that motility of sporozoite is associated with the secretion of CSP, and it is important for successful penetration into the hepatocyte (Phillips, 1995). CSP induces the production of protective antibodies which are able to recognize the repeat region of CSP and protected recipient mice against a viable homologous sporozoite challenge (Potocnjk *et al.*, 1980; Charoenvit *et al.*, 1991). It is noteworthy that Lopez and colleagues (1996) showed that sera from people living in endemic areas can recognise CSP of *P. falciparum*.

In the CSP there are two highly conserved regions in all different *Plasmodium* species called region I and region II. Both might be parasite ligands for binding to hepatocytes and become possible vaccine targets (Sinnis *et al.*, 1996). Invasion of hepatoma cells by sporozoites was inhibited by anti-region I antibodies (Sinnis and Sim, 1997; Sinnis *et al.*, 1996). It has also been shown that peptides representing region II can inhibit binding of CSP to hepatoma cells and sporozoite invasion (reviewed by Phillips, 2001).

An anti-CSP monoclonal antibody (MAb) gave a very high degree of protection against homologous challenge (Chen, Tigelaar, and Weinbaum, 1977). Although the mechanisms by which antibody inhibits the pre-erythrocytic stages are not clear, it seems that anti-CSP antibodies can inhibit sporozoite penetration into the hepatocytes (Nudelmann *et al.*, 1989, Mazier *et al.*, 1987, Mazier *et al.*, 1986). On the other hand, it has been shown that such antibodies are not involved in blocking penetration by *P.*

falciparum (Mellouk *et al.*, 1986). So, in *P. falciparum* infection it may be suggested that the antibodies may destroy the parasite in the parasitophorous vacuoles in post-penetration events (Mazier *et al.*, 1988).

It has been recently shown that γδ T cells, a minority subset of $CD4^+$ T compared to $CD4^+$ αβT cells in the peripheral blood of individuals (Salerno and Dieli, 1998), function as a component in cell-mediated immunity to experimental malaria (Ho *et al*, 1990) and are increased in number during a malaria parasite infection in humans and rodents (Von der Weid *et al.*, 1996, Nakazawa *et al.*, 1994). It is proposed that γδ T cells are components of early immunity directed against malaria pre-erythrocytic stages. However, they are not required for the induction of an effector αβ T cell immune response generated by irradiated sporozoite immunization (McKenna *et al.*, 2000). γδ T cell-deficient mice show increased liver parasite burden compared with similarly challenged immunocompetent mice at 24 hours post-infection (McKenna, *et al.*, 2000). Although γδ T cells play a role in the protection induced by immunization with irradiated sporozoites (Tsuji *et al.*, 1994), they are not essential for clearing blood stage infection since γδ T cells depleted mice control a *P. chabaudi* blood stage infection (Langhorne, Mombaerts and Tonegawa, 1995). There are a number of possibilities for γδ T cell function. First they can recognize antigens directly, independent of MHC restriction (Morita *et al.*, 1996). Secondly γδ T cells exert their anti-parasitic activity against the infected hepatocytes. It was shown that γδ T cell clone 291-H4 exhibited activity against pre-erythrocytic parasites in hepatocytes in mice, which were challenged with sporozoites (Tsuji *et al.*, 1994, and 1996). So, data support the conclusion that γδ T cells have some role in the control or inhibition of pre-erythrocytic stages of malaria parasites. The role of γδ T cells against asexual blood stages is discussed later.

1.6.2.2. Immune response to asexual blood stages

The asexual erythrocytic stages of the parasite cause all the morbidity, pathology, and mortality from the disease (Phillips, 1994). Efforts have been made towards an understanding of the immune mechanisms which

could facilitate development of a protective vaccine against this stage, which could limit parasite growth, and consequently prevent or minimize clinical disease (reviewed by Wipasa *et al.*, 2002). Immune responses against asexual blood stages of malaria parasites were thought to be partly anti-parasite and partly anti-toxic (Sinton, 1950), but subsequently immunity to asexual blood stages has been redefined as anti-parasite and anti-disease immunity (Playfair *et al.*, 1990).

In mice the immune response to erythrocytic stages of infection is multifactorial and involves both cell-mediated and antibody-dependent mechanisms including the two subsets of $CD4^+$ T cells, Th1 and Th2 (Taylor-Robinson, 1995). During the acute primary parasitaemia inflammatory-type or Th1 cells, produce IFNγ and IL-2, indicating cell-mediated immunity. There is also a release of TNF-α, and reactive oxygen radicals from macrophages (reviewed by Long, 1993). As the infection progresses the frequency of Th2 type lymphocytes increases, providing IL-4 as a growth factor for Th2 cells, and indicating development of antibody-dependent immunity (Langhorne, 1989). A sequential Th1 to Th2 switch has been shown in immunity against some strains of rodent malaria parasites such as *Plasmodium chabaudi* AS (Langhorne 1989). Some investigations however, suggest that T cell-mediated immune responses play a more significant role in resisting the acute blood stage of haemoprotozoan infections than was proposed before. Thus B cell-deficient mice infected with *P. c. adami* resolved their infections without chemotherapy (Grun, and Weidanz, 1981). It was shown that T cells but not B cells adoptively transferred immunity to *P. c. adami* (Cavacini *et al.*, 1989). Nude mice infected with *P. vinkei petteri* and *Babesia microti* were unable to resolve parasitaemia and finally died. Mice rendered B cell deficient by lifelong treatment with anti-μ antibody were able to control acute infections with three haemoprotozoan parasites: *P. c. chabaudi*, *P. vinkei petteri*, and *B. microti* (Cavacini, Parke and Weidanz, 1990).

An imbalance in the Th1 and Th2 responses may modify a host's immune response favouring of an environment in which malaria parasite can persist. Generally in infections caused by intracellular pathogens such as malaria parasites the Th1 response has a key role in controlling diseases, particularly the acute phase. So, in such cases regulatory cytokine such as

IL-12 could play a critical role because its production inhibits IL-4 that promotes a Th2 response (Ahuja *et al.*, 1998). Many studies in *P. chabudi* AS indicated that Th1 responses are initiated early in the course of infection in resistant hosts such as inbred NIH mice (Taylor-Robinson *et al.*, 1993; Taylor-Robinson and Phillips, 1993) and C57BL/6 mice (Langhorne *et al.*, 1989). However, in susceptible A/J mice (Stevenson and Tam 1993) Th2 response were induced earlier.

Th1 cells promote opsonization and phagocytosis via increasing in production of IgG2a (Matsumoto *et al.*, 2000) and IgG3 in mice (Smith *et al.*, 1997) while in humans IgG1 and IgG3 have the main role. These antibodies lower parasitaemia and reduce pathology in people living in endemic areas (Aribot *et al.*, 1996; Bouharoun-Tayoun and Druilhe, 1992). Th2 cells induce IgG1 in infected mice with malaria parasites and in humans promote production of IgG4, which is involved in allergic reactions and control of helmintic infections (Smith *et al.*, 1997).

IFNγ has a central role in protection against asexual blood-stages of *P. chabaudi* AS when either produced endogenously (Su and Stevenson, 2000) or administered exogenously (Curfs *et al.*, 1993). IFNγ gene knockout mice infected with *P. chabaudi* AS showed higher morbidity and severe mortality compared to control mice infected with wild type (Su and Stevenson, 2000). Yoneto *et al.* (1999) showed that splenocytes of mice deficient in inducible nitric oxide synthase (iNOS-/-) produced an amount of IFNγ comparable to that produced by splenocytes of wild type control mouse in *P. berghei* XAT, an irradiation-induced attenuated variant of the lethal strain of *P. berghei* NK65. However, treatment of these mice with neutralizing anti-IFNγ antibody resulted in a lethal outcome. An early NK cell-mediated IFNγ production is implicated in the control of asexual blood stages of *P. chabaudi* (Mohan *et al.*, 1997). Sam and Stevenson (1999) suggested that the consistent presence of higher levels IFNγ and IL-12 might contribute to the polarization of Th cell response in resistant B6 mice. This study showed that there is a significantly higher production of IL-12 p70 by splenic macrophages in resistant B6 mice compared to macrophages in susceptible A/J mice *in vitro*.

TNFα is a pyrogenic factor responsible for fever and is incriminated in the pathology of cerebral *P. falciparum* malaria. The parasite, *P. falciparum*, induces TNFα release from monocytes during acute phase of the infection (Curfs *et al.*, 1993). TNFα has both protective and pathologic roles in malaria infections as high levels of TNFα mRNA expression in the spleens of C57Bl/6 mice correlate with resistance to *P. chabaudi* AS infection, and neutralizing anti-TNFα antibodies in resistant mice abrogate immunity (Jacobs, Radzioch, and Stevenson, 1996). In contrast, reducing serum TNFα in mice infected with *P. berghei* by neutralizing the IFNγ results in protection from experimental cerebral malaria (Grau *et al.*, 1989b). In humans high levels of TNFα in children with *P. falciparum* malaria have been also shown to correlate with hypoglycaemia and a high mortality rate (Grau *et al.*, 1989a).

Transforming growth factor-β (TGF-β) is thought to play a role as a major immunomodulatory cytokine in the successful control of malaria (Omer, and Riley, 1998). TGF-β induces protective immune responses, leading to slower parasite multiplication early in the infection and down-regulates pathology of infection thereafter, in BALB/c mice. In lethal infections with *P. berghei* circulating TGF-β was low. However, it was at significant levels in resolving infections with *P. yoelii* and *P. c. chabaudi* (Omer, and Riley, 1998).

In contrast to $CD4^+$ T cells it is thought that $CD8^+$T cells have a minor role in asexual blood stage immunity (Langhorne, 1989). For example, it has been shown that β2-microglobulin-deficient (β2-m0/0) mice, which are genetically blocked from expressing MHC class I, resolved *P. c. adami*, *P. yoelii* 17X, and *P. c. chabaudi* AS infections in the virtual absence of $CD8^+$ T cells. So, the results showed that $CD8^+$ T cells are not essential in suppression of murine malaria and that the suppression mechanism is not MHC class I restricted (van der Heyde *et al.*, 1993). Podoba and Stevenson (1991) showed that in *P. chabaudi* AS infection in C57BL/6 mice, depletion of $CD8^+$ T cells with monoclonal anti-$CD8^+$ T cells antibodies had no effect on the early course of parasitemia or on the level of peak parasitemia. They showed that mice experienced two recurrent bouts of parasitemia during the later stage of the infection and elimination of the parasite required time more than 5 weeks. Further, $CD8^+$ T cells have been

implicated in the pathology of *P. berghei* acute phase infection due to localized overproduction of TNFα, principally in the liver (Waki *et al.*, 1992). Some investigations have shown that $CD8^+$ T cell responses in acute malaria may suppress the production of IL-2 and its receptor expression (Ho *et al.*, 1988; Troye-Blomberg *et al.*, 1985).

On the other hand, there are suggestions that $CD8^+$ T cells have a protective function in control of asexual blood stage of malaria parasites (Weidanz, Melancon-Kaplan and Cavacini, 1990). In this regard cytotoxic $CD8^+$ T cells have been found, as well as $CD4^+$ T cells, to mediate in elimination of infected hepatocytes *in vitro* and *in vivo* (Doolan and Hoffman, 1997).

Interaction and regulation between Th1 and Th2 responses are involved in effective control in malaria parasites such as *P. chabaudi* infection. Th0 are designated as T cells which are able to produce both cytokines of Th1 and Th2 T cells (Romagnani, 1996). They will differentiate to be either Th1 or Th2 cells, depending on the immediate microenvironment. Stimulated naïve $CD4^+$ T cells first produce IL-2 and differentiate into either Th1 or Th2 phenotypes (Mosmann and Sad, 1996). DCs, macrophages, and B cells are known as inducers in the differentiation of Th1 or Th2 when appropriate cytokines are present (Mosmann and Sad; 1996; Banchereau *et al.*, 2003). There are three necessary identified signals for T cell activation and differentiation. Contact between T cell and peptide/MHC Class II complexes on the surface of APCs provides the first signal (Muller, Jenkins and Schwartz, 1989). The presence of the co-stimulatory molecules or cytokines such as IL-2 is an essential signal 2 (Lafferty *et al.*, 1988) and finally signal 3 derived as a co-stimulatory factor by APCs or via adjuvant which may usually control differentiation of T cells (Curtsinger *et al.*, 1999).

It is shown that in humans, DC1 derived from peripheral monocytes (pDC1), induces Th1 differentiation and DC2, derived from $CD4^+CD3^-CD11^-$ plasmacytoid cells (pDC2), induces Th2 differentiation (Rissoan *et al.*, 1999). In an *in vitro* study Seixas *et al.* (2001) showed that *P. chabaudi* infection in the mouse directly activates DCs to elicit cytokines which induce Th1 response. Moreover, Bruna-Romero and Rodriguez (2001) showed that DCs initiate a protective immune response against the liver stage through activation of $CD8^+$ and $CD4^+$ T cells in *P. yoelii* infection.

They incubated the mature DCs with peptides containing the identified H-$2K^d$-specific $CD4^+$ and $CD8^+$ T cells epitopes from the CSP of *P. yoelii*. These DCs were transferred to naïve BALB/c mice and specific activation of anti-CS $CD4^+$ and $CD8^+$ T cells was measured using an ELISPOT assay for the production of IFNγ. When mice immunized by adoptive transfer of DCs loaded with the CS-derived $CD8^+$ epitope and challenged with *P. yoelii* they showed a much lower level of parasite RNA in the liver compared to mice transferred with DCs alone or DCs loaded with the $CD4^+$ epitope. The activation of Th1 response has been demonstrated in *P. yoelii* infection in which $CD11b^+$ cells, as APCs, could present the antigens of the parasite and production of Th1 cytokines by $CD4^+$ T cells (Luyendyk *et al.*, 2002). The migration of substantial DCs, $CD11c^+$, cells within 5 days p.i. in C57BL/6 mice infected with *P. chabaudi* AS blood stages was shown in an *in vivo* study (Leisewitz *et al.*, 2004). This migration was from marginal zone or red zone of the spleen to the white pulp which was rich in $CD4^+$ T cells. Leisewitz *et al.*, (2004) suggested that an early activation and engagement of DCs in infection could be critical to regulate the subsequent immune response.

Reports have shown that γδ T cells also have protective or immunoregulatory effects during the acute blood phase of malaria (Langhorne, Mombaerts, and Tonegava, 1995). During *P. vivax* (Perera *et al.*, 1994) and *P. falciparum* infections in humans proliferative responses showed that a subset of γδ T cells is increased (Goerlich *et al.*, 1991). It has been proposed that γδ T cells may play a role in the control of asexual blood stage of *P. falciparum* infection *in vivo* (Elloso *et al.*, 1994). Flow cytometry showed that γδ T cells are able to inhibit replication of the blood stages of *P. falciparum* parasites in a dose-dependent manner *in vitro* (Goerlich *et al.*, 1991). Free merozoites are targets rather than parasitized red blood cells (pRBCs) and this inhibition requires contact between γδ T cells and merozoites (Elloso *et al.*, 1994). The expansion of γδ T cells in spleen of mice infected with *P. chabaudi* has also been reported (Minoprio *et al.*, 1989; van der Heyde *et al.*, 1995). Resolution of the *P. chabaudi* acute phase may be related to the increase in γδ T cells in normal mice or with chronic infections in B cell-deficient mice (Seixas and Langhorne, 1999). Both NK and γδ T cells contribute to the early IFNγ and TNF-α

response 24 hours after non-lethal *P. yoelii* infection (Choudhury *et al.*, 2000). Seixas and Langhorne reported (1999) that in double knockout mice, deficient in both B cells (μ-MT) and γδ T cells, there is a markedly elevated parasitaemias compared to the single knockout mice deficient in B cells in a *P. chabaudi* AS infection. C57Bl/6 mice depleted of γδ T cell with MAbs were unable to suppress their infections (Van der Heyde *et al.*, 1995). So, it appeared that $CD4^+$ αβT cells alone could not mediate early resolution of the infection. On the other hand, it has been suggested that there is no effective contribution for γδ T cells to protect infected mice because depletion of γδ T cells did not alter parasiatemia, anaemia or survival rates of mice infected with avirulent *P. c. adami* or virulent *P. c. chabaudi* CB (Sayles and Rakhmilevich, 1996). So, it seems that further investigations are required to determine the role of γδ T cells in protection against malaria (reviewed by Wipasa *et al.*, 2002).

Antibody has been found as a major component of the protective immune response to the erythrocytic stages of *Plasmodium* in passive transfer experiments (Cohen, McGregor, and Carrington 1961). The efficacy of treatment of non-immune infected patients with immunoglobulin G from protected individuals has been shown in human malaria infection (Cohen, McGregor and Carrington, 1961). Sabchareon *et al.* (1991) showed that there is a protection of passively transferred IgG from African donors to Thai patients with *P. falciparum* infection. The asexual blood stage parasitaemia declined and clearance of parasites and symptoms was as fast or faster than that in drug cured patients (Sabchareon *et al.*, 1991). Control of a patent parasitaemia of *P. falciparum* has been reported using passive transfer of IgG from immune adults (Druilhe and Perignon, 1994). In endemic areas people who survived childhood infection develop immune responses in which parasites are maintained at low levels, and symptoms absent. This immunity results from production of specific antibodies to different antigens such as *Plasmodium falciparum* erythrocyte membrane proteine 1 (PfEMP-1) (Bull *et al.*, 1998; Newbold, 1999; Piper *et al.*, 1999) or against antigens expressed on the surface of merozoites (Hirunpetcharat *et al.*, 1997).

The critical role of B cells has been demonstrated in B cell deficient mice (μ-MT mice). These mice are able to reduce a primary acute infection of *P.*

c. chabaudi AS to low levels, but they are unable to eliminate parasites. In these mice the infection was remained chronic and unresolved and characterized by relapsing peaks of parasitaemias up to 30 to 50% of pRBCs (von der Weid, Honarvar, and Langhorne, 1996). The chronically infected mice were able to clear their infection when they were injected with B cells from immune donor mice (von der Weid, Honarvar, and Langhorne, 1996). This observation confirms the previous findings in which anti-IgM-treated BALB/c mice were unable to eliminate a primary infection of *P. c. chabaudi* AS completely and the infection remained chronic at a low level and switching from a Th1 to a Th2 response failed to occur (Cavacini, Parke, and Weidanz, 1990; Taylor-Robinson and Phillips, 1994; Taylor-Robinson and Phillips, 1996). In addition, μ-MT mice, which were cured of a first infection showed a secondary infection when they were rechallenged with *P. c. chabaudi* AS. However, the study showed that in the wild type control mice a secondary infection was seen only in a transient low patent parasitaemia. These results suggested that B cell-dependent-mechanisms play a crucial role in protection in secondary infections (von der Weid, Honarvar, and Langhorne, 1996).

Antibodies may have different roles in protection such as blocking invasion of merozoites into erythrocytes, promoting the uptake of opsonized merozoites (reviewed by Wipasa *et al.*, 2002), neutralizing malaria toxins, and preventing sequestration of *P. falciparum* (Phillips, 2001; Long, 1993). It was also shown that the adherence of the mature infected erythrocytes to the small blood vessels can be prevented by anti PfEMP-1 antibodies, and agglutination of those erythrocytes can be promoted (reviewed by Good, 2001). However, it seems that not all antibodies are protective as it is reported that polyclonal antibodies (Abs) specific to merozoite surface protein-2 (MSP-2), but not MAbs for the same antigen, enhance invasion of multiple merozoites into erythrocytes (Ramasamy, Ramasamy and Yasawardena, 2001; Ramasamy *et al.*, 1999).

Specific antibodies importantly play a role in activation of antibody-dependent cellular inhibition (ADCI) to control parasitaemia. Antibodies are capable of promoting a monocyte-dependent inhibition of parasite growth *in vitro* (Druilhe and Pregnon, 1994). At the time of schizont rupture phagocytes bound to cytophilic specific antibodies via FC receptor contact with some component of merozoites and inhibit parasite growth

through releasing soluble mediators which are able to block the division of surrounding parasites (Bouharoun-Tayoun *et al.*, 1990; Aucan *et al.*, 2000; Tebo, Kremsner, and Luty, 2001).

There is a correlation between antibody levels, mainly IgGs, and the degree of protection to asexual blood stage antigens in human (Astagneau *et al.*, 1995; Piper *et al.*, 1999) and mice (Hirunpetcharat *et al.*, 1997). This protection is also antibody isotype-dependent. Protective immunity to *P. falciparum* has been reported in *Saimiri sciureus* monkeys which were treated with immune *Saimiri* antibodies (Gysin, Fandeur and Pereira,da Silva, 1982). Gysin *et al.* (1996) demonstrated that passive transfer of an IgG preparation obtained from immune African donors had a strong protective effect in *Saimiri* monkeys, which were infected with *P. falciparum*. Cytophilic IgG subclasses IgG1 and IgG3 but not non-cytophilic IgG2 are involved in this protection (Groux *et al.*, 1990; Bouhaoun-Tayoun, and Druilhe, 1992). However, in a study in Burkina Faso, Aucan and colleagues (2000) suggested that high levels of IgG2 were associated with low risk of infection, contributing to parasite clearance. In contrast, high IgG4 was associated with high risk to infection suggesting that IgG4 may block the protective effect of cytophilic antibody (Aucan *et al.*, 2000).

Passive transfer of MAb 302 that reacts with the C-terminal cysteine-rich region of *P. yoelii* MSP-1 (Burns *et al.*, 1989) protected mice against challenge infection with the lethal strain (17XL) of *P. yoelii* (Majarian *et al.*, 1984). In a passive transfer study Spencer Valero and colleagues (1998) evaluated the ability of the MAbs against *P. yoelii* MSP-1 in protection of mice against a blood stage challenge of *P. yoelii* YM. They showed that some MAbs mediated substantial reduction in parasitaemia in BALB/c mice which were passively transferred with such MAbs and infection was cleared.

Passive transfer of both IgG1 and IgG2a from hyperimmune serum obtained from drug cured BALB/c mice conferred protection to *P. c. chabaudi* AJ, inhibiting the emergence of new ring forms in BALB/c mice (Cavinato *et al.*, 2001). They suggested that the merozoites released from ruptured schizonts are the main targets for antibodies prior to red blood cell invasion (Cavinato *et al.*, 2001). It was because in primary infected mice the generation of new ring forms was inhibited with treatment of

hyperimmune serum, but the number of schizont-infected erythrocytes were not altered and also treatment with purified IgG1 and IgG2a decreased reinvasion in drug cured mice challenged with a high dose of the parasite.

Different antigens, when administered under different conditions, routes or doses, may selectively induce humoral or cellular immune responses (Parish, 1972). Infective dose is thought to have an influence on the balance between Th1/Th2 responses in *P. chabaudi* infection (Taylor-Robinson, and Phillips, 1998). Although in *P. chabaudi* AS, the importance of the balance between Th1 and Th2 subsets and their characteristic cytokines is well known (Taylor-Robinson, 1995), the initial activation and expansion of either Th1 or Th2 and the elements involved *in vivo* are not fully understood. In resistant NIH mice Th1 was upregulated with an increasing infective dose while Th2 responses were downregulated. The reverse effect was seen in susceptible A/J mice, with a high-level of IL-4 production and elevated Th2 activation correlating with a rising infective dose (Taylor-Robinson and Phillips, 1998). The study supported the view that IFNγ is a key cytokine for the induction of protective immune responses in mice in the acute phase because of its significant production in resistant mice regardless of infective dose (Taylor-Robinson, and Phillips, 1998).

Two different groups of CB9F1 mice infected with *P. chabaudi* AS were cured with chloroquine when parasitaemia reached either to 0.2% or 5.9 % on day 5 p.i. and day 7 p.i. respectively. Mice which had suffered low parasitaemia showed good immunity to homologous reinfection. However, in mice which were not treated and suffered full parasitaemia challenge with a heterologous *P. yoelii* 17XL resulted in an acute parasitaemias with no development of immunity (Favila-Castilo, Monroy-Ostria, and Tapia, 1999). In mice that suffered low parasitaemias the level of IgG2a was higher than those that suffered a full parasitaemias in *P. chabaudi* AS infection. Splenomegaly was not seen in mice that suffered low parasitaemias, but splenectomy diminished their immunity to homologous reinfection (Favila-Castilo, Monroy-Ostria, and Tapia, 1999).

Although inflammatory cytokines play a role in the control of the disease, it is now widely accepted that overproduction or uncontrolled activity of such inflammatory cytokines are involved in cause of the acute phase of such disease (Clark and Cowden, 2003). The interaction between

these cytokines as an important part of the innate immune system and the pathogen may determine the pathology of the disease (reviewed by Clark *et al.*, 2004). Proinflamatory cytokines such as TNF, IL-1, lymphotoxin, NO, carbon monoxide, and overactivity of the enzyme poly-(ADP-ribose) polymerase 1 (PARP-1) and presence of high-mobility-group box 1 (HMGB1) protein in the circulation are also involved to promote pathologic events as studied in *P. falciparum* malaria (reviewed by Clark *et al.*, 2004).

1.6.2.3. Antigenic variation and cytoadherence

Antigenic variation is a feature of some malaria parasites such as *P. falciparum* (Newbold, 1999) which may be identified as the ability of a pathogen to vary its antigens either during or between infections, enabling the occurrence of persistent or recurrent infections.

Brown and Brown (1965) demonstrated that in *P. knowlesi* infection in rhesus monkeys recrudescent parasites from a single original inoculum, but not a clone, differed in the antigens they expressed on the infected erythrocytes surface compared with the infecting population (Brown and Brown, 1965). Expressing different variant antigens on the surface of the infected red blood cells occurs within a parasite clone through switching the expression of different variant genes (reviewed by Good, 2001).

PfEMP1, a variant antigen, is a cytoadherence molecule encoded by the *var* gene family. This gene family provides expression of variant PfEMP1 which binds to receptors on the endothelial cells such as CD36 and intracellular adhesion molecule one (ICAM-1) and sequester to capillary vessels. So, as a result of sequestration pRBCs stop circulating and the parasite can escape from the splenic clearance (Borst *et al.*, 1995; Berendt, 1993; reviewed by Newbold, 1999). So, these variant antigens play a role as a survival strategy for the parasite.

Saul (1999) proposed that the primary role for variant antigens is to generate an immune response, which regulates parasite growth and thereby establishes a chronic infection. Chronicity is an important consequence of antigenic variation by which parasitaemia and severity are modified (Saul, 1999). Chronicity offers an evolutionary advantage to the parasite: an increased probability of transmission to a new host (Snounou, Jarra and

Preiser, 2000). So, the chance of uptake of gametocytes by a mosquito vector is improved if the infection is extended. For instance, *P. falciparum* parasites have been detected 1-3 years after primary infection (Eyles, and Young, 1951). Antigenic variation evolved in the first instance to ensure gametocyte survival (reviewed by Kyes, Horrocks and Newbold, 2001) and these variant antigens with cytoadherence feature, therefore, may be also accounted as virulence factor. This hypothesis seems to be attractive in *P. falciparum* because gametocyte development is prolonged and the developing gametocyte spends about 7 days in the bone marrow as a specialized location (reviewed by Kyes, Horrocks and Newbold, 2001). It is also proposed that the role of the cytoadherence is ensuring that the spleen destroys those parasites failing to express variant antigens (Saul, 1999).

Immune responses can be hampered in *P. falciparum* and *P. chabaudi* infection because they undergo antigenic variation (Phillips *et al.*, 1997) providing that the parasites evade from effector clearance mechanisms. In humans, studies have shown that even in local communities of restricted size there are a large number of circulating variants of *P. falciparum* (Bull *et al.*, 1998; Giha *et al.*, 1999a; Giha *et al.*, 1999b). It was therefore, concluded that the chronic infection could be maintained by the serial expression of different antigenic types and the immune response was associated with the development of variant-specific opsonizing antibodies (Brown and Brown, 1965; Brown and Hill, 1974). In a *P. falciparum* infection Bull and colleagues (1999) showed that a specific antibody to a particular antigenic variant dramatically reduced the subsequent chance that the individuals would become clinically ill if they were reinfected with the same variant. However, the presence of cross-reactive antibodies to specific variant antigens has been reported (Marsh and Howard, 1986). If these variant antigens are considered as vaccine targets, the immune system of adults has to recognize a high proportion of those antigens. Induction of cross-reactive antibodies may increase the efficacy of such vaccines (reviewed by Kyes, Horrocks and Newbold, 2001).

Cytoaherence also is an important virulence and pathogenic factor contributing to the manifestations of malaria, the disease (Baruch *et al.*, 1997). Adhesion of erythrocytes infected with *P. falciparum* to vascular endothelium and to uninfected red blood cells (rosetting) may be involved in the pathogenesis of severe malaria (Chen *et al.*, 1998). In humans

accumulation of the parasite in large numbers in different organ beds or causing occlusions in the small blood vessels of those organs (Holt *et al.*, 1999; Bourke *et al.*, 1996) damages those organs leading to severe disease or death (reviewed by Kyes, Horrocks and Newbold, 2001).

In rodent malaria, erythrocytes infected with *P. c. chabaudi* AS adhere to CD36 *in vitro* (Mota *et al.*, 2000). Pre-treatment of rat endothelial cells with IFNγ, up-regulates expression of ICAM-1 and VCAM-1, as possible receptors for pRBCs (Faveeuw *et al.*, 2000). Mota and colleagues (2000) also showed that erythrocytes infected with *P. c. chabaudi* AS bound to CD36 particularly pRBCs containing mature trophozoites and schizonts in a IFNγ-dependent manner. Many similar features of sequestration of *P. c. chabaudi* model and *P. falciparum* have been revealed by *in vivo* characterization of the interaction between *P. c. chabaudi* AS and the tissues of different organs (Mota *et al.*, 2000).

It has been shown that a region at the right end of chromosome 9 in *P. falciparum* is implicated in the binding of the infected red blood cells to the endothelial cell receptor CD36 (Holt *et al.*, 1999). Bourke and colleagues (1996) observed that the deletion of the right end of chromosome 9 during prolonged *P. falciparum in vitro* cultivation is associated with loss of ability to cytoadhere to melanoma cells and greatly lowered gametocyte production. The relevant gene in this region is called cytoadherence linked asexual gene (*clag*), and is expressed in erythrocytic stage of some malaria parasites such as *P. falciparum*. The first gene characterizing *clag* is identified on chromosome 9 and its protein product (CLAG9) was implicated in cytoadhesion of *P. falciparum* pRBCs to host endothelial cells (Holt *et al.*, 1999). Targeted gene disruption of *clag* resulted in great reduction of the binding of *P. falciparum*-infected erythrocytes to CD36 (Trenholme *et al.*, 1999; Holt *et al.*, 1999). Other *clag* genes on chromosomes 2 and 3 have also been described in *P. falciparum*, and designated *clag*2, *clag*3.1 and *clag*3.2 and two *clag*-like genomic DNA sequences were identified in *P.vivax* (Gardiner *et al.*, 2004; Holt *et al.*, 2001).

Some recent studies have created some doubts about the role and products of *clag* genes. Holt *et al.*, (1999) indicated other possible roles for *clag* other than cytoadherence to endothelial receptors. They suggested that *clag* may be involved in other processes of cellular adhesion such as binding the

sporozoite to hepatocytes or in the binding of merozoites to erythrocytes. Recent studies have proposed other possible locations for products of the *clag* gene. Kaneko *et al.* (2001) suggested that some translated proteins of members of *clag* gene family such as proteins encoded by *clag*3.1 and *clag*3.2 genes were associated with a protein assembly in rhoptries. Antisera were used in Western blotting and immunofluorescence experiments from mice immunized with peptides specific for *clag*9 products, showed that these *clag*9 products are localized to the rhoptry of *P. falciparum* (Gardiner *et al.*, 2004). It is, therefore, proposed that *clag*9 products are involved in trafficking of adhesion molecules or in the remodelling of the erythrocytes so that these proteins can be trafficked to the right location where they can participate in invasion into the new red blood cell (Gardiner *et al.*, 2004). Ling *et al.* (2004) indicated that *clag*9 product is part of the RhopH complex on the surface of merozoite. After erythrocyte invasion by the merozoite *clag*9 product transfers to the ring stage and still associated with RhopH complex, a high-molecular-mass protein complex of merozoites (rhoptry). So, it may be suggested that the primary role for the complex is remodelling the pRBCs after invasion by the merozoite, as the results also did not show direct role in cytoadherence and sequestration.

A wider examination of the *clag* genes using current sequence databases has shown several *clag*-like genes in other *Plasmodium* species. In *P. chabaudi* a single *clag*-like gene was identified which corresponds to part of exons 8 and 9 and intron 9 of *P. falciparum clag* sequences (Holt *et al.*, 2001). Two *clag*-like genes were shown in *P. yoelii* and several fragments of *clag*-like sequences were identified for *P. berghei* (Holt *et al.*, 2001).

Janssen and colleagues have discovered a major gene family, *cir*, in the rodent malaria parasite, *P. chabaudi*, using genome survey sequencing (2001). They have also identified homologues of this family in two other rodent malaria parasites, *P. yoelii* (*yir*) and *P. berghei* (*bir*). More importantly these gene families are homologous to the *vir* gene family in the human malaria parasite *P. vivax* (Janssen *et al.*, 2002). *Vir* gene family encodes an immunovariant protein (del Portillo *et al.*, 2001).

1.6.2.4. Immunity to sexual stages

It is thought that both antibody-dependent and antibody-independent mechanisms have protective roles against sexual stages in different host-parasites combinations (Hoffman, 1996). Protection to sexual stages may prevent fertilization when the protective factors come in with the blood meal in the midgut of the mosquito (reviewed by Phillips, 2001).

Acquired immunity to sexual stages has been discussed as transmission blocking immunity, and cross-immunity between asexual and sexual stages. It has been reported that some specific monoclonal antibodies to PfEMP-1 have cross-reactivity and can agglutinate multiple strains (Gamain, Miller, and Baruch, 2001). In *P. falciparum* and *P. vivax* in man, and *P. cynomolgi* infection in monkey transmission blocking activity may reduce infectivity of gametocytes to mosquitoes, both by antibody, and cytokines such as IFNγ, TNFα and NO (Phillips, 1995).

Measuring the production of IgG antibody to PfEMP-1 in residents from an area where malaria is endemic, Papua New Guinea showed that there is an increase in anti-PfEMP-1 prevalence with age. This mirrors the decline in both the prevalence and the density of asexual and transmission stages in erythrocytes (Piper, Roberts and Day, 1999). In addition to IgG role in reducing the density of asexual stages it may have an immunoregulatory role in the production of gametocytes, either by reduction of their proliferation or by an effect on gametocyte maturation (Piper, Roberts and Day, 1999).

1.7. Immunization studies

Immunization studies help to recognise and evaluate vaccine candidates that may provide protection against malaria infections.
Immunization could be a major way for controlling and preventing infectious diseases such as malaria. The main goal is to identify, and/or synthesize protective vaccines against malaria for all people who live in high-risk situations, particularly in endemic areas. Vaccination is one of the most important components of new strategy presented in 1992 to W.H.O as Roll Back Malaria (RBM) for malaria control.

(http://www.mja.com.au/public/issues, 2004). In endemic areas children up to five years are at high risk of death. So, in endemic areas a vaccine with 50% efficacy for one to five years could substantially reduce all-cause child mortality (http://www.mja.com.au/public/issues, 2004). Vaccines against malaria would be a cost-effective public health tool to reduce the burden of disease, and will be an essential component of successful global control. Effective vaccines, as a group, represent the single most cost-effective public health intervention (W.H.O., 2004).

Almost all efforts in the field of malaria vaccine development are directed to *P. falcipaum* as the most dangerous human malaria parasite (Reviewed by Phillips, 2001). Although many advanced vaccines against a variety of pathogens are available, there is no routine vaccine for malaria. However, today the hope is that an effective vaccine will be available within the next 7-15 years. Sites for development and evaluation include USA, Colombia, Switzerland, Australia, Papua New Guinea, Gambia, and Tanzania (Perry, 2001; Wellcome Trust and Roll Back Malaria information booklet, 2004).

An effective vaccine theoretically should mimic but accelerate natural immunity processes. In areas endemic for malaria natural immunity (reviewed by Good, 2001) is induced and developed by multiple exposure to parasites over the years (Baird, 1998). However, a non-natural immunity theoretically refers to immune mechanisms induced by a vaccine which do not need to any great extent by natural exposure and could be highly effective (reviewed by Good, 2001). Moreover, this immunity may be induced following recognition of antigens which are not normally exposed to the immune system naturally. During natural immunity acquiring specific antibodies against different variants of PfEMP-1 takes several years in an individual who lives in an endemic area. Whereas a vaccine designed based on immunogenic epitopes of PfEMP-1 may induce non-natural immunity. These immunogen epitopes could be cryptic epitopes which are not recognized after infection but can be presented as immunogenic components to immune system in non-native forms and can be used as a part of multivalent vaccine (reviewed by Good, 2001).

In current researches malaria vaccines are divided into three types: pre-erythrocytic vaccines, asexual blood stage vaccines, and sexual or transmission blocking vaccines (TBV) (Anders and Saul, 2000; reviewed by Phillips, 2001). Pre-erythrocytic, anti-sporozoite, vaccines are designed to

prevent infection. Anti-asexual blood stage vaccines are designed to reduce severity and complicated manifestations of the disease i.e, reducing morbidity and mortality among children up to five years in Africa. Several vaccine candidates are currently undergoing clinical and field-testing (reviewed by Webster and Hill, 2003). Finally, TBV vaccines are designed to stop the development of the parasite in the mosquito, and thereby reducing or eliminating transmission of the disease in humans.

1.7.1. Immunization and vaccines against pre-erythrocytic stages

Immunization with irradiated sporozoites in humans and animal model systems can induce sterile immunity to sporozoite challenge (Doolan and Hoffman, 1999). The first vaccination of human volunteers using irradiation-attenuated sporozoites of *P. falcipaum* and *P. vivax* induced complete protection (Clyde, 1975; Clyde, 1990). This level of vaccine efficacy created a strong hope that an effective malaria vaccine would be developed (Anders, and Saul, 2000). But it is not practical because for vaccination of each individual a huge number of infected mosquitoes are required (reviewed by Phillips, 2001).

Although the irradiated-sporozoite based-vaccines can elicit both protective antibody-dependent and cell-mediated immunity to malaria (Rodrigues, Nussenzweig and Zavala, 1993; Nussenzweig and Nussenzweig, 1989), providing irradiated-sporozoites on a large scale is not yet possible. Therefore, only using subunit vaccines or synthetic peptides would provide possibility to obtain amounts of selected immunogenic malaria antigens that may be used in immunization studies (Bruna-Romero *et al.*, 2001).

The important epitopes identified on particular malarial protein molecules could be isolated and sequenced. These epitopes can be synthesised and may be used as potent vaccines. The synthetic peptide vaccines, which would be small and soluble, have to be attached to a carrier protein or hapten to make them more immunogenic. The appropriate orientation of the immunogen and the attached carrier protein, are the important keys for favourable presentation to the immune system for effective elicitation of the immune response.

Polyclonal T cells are able to kill the liver stage of *P. yoelii* by prior immunization of donor mice with Py1 *in vitro*. Py1 is a synthetic peptide that corresponds to a T-helper epitope within the CSP of *P. yoelii*. BALB/c and C57BL/6 mice immunized with Py1 also showed specific T-cell proliferation and antibody production (Renia *et al.*, 1991).

The importance of a single epitope for inducing an immune response has been demonstrated in immunized B10 (I-Ab) mice with the recombinant baculovirus-expressed *P. falciparum* CSP. The lymph node cells of the immunized mice were challenged *in vitro* with a series of overlapping synthetic peptides. Only a single epitope aspargine-alanine-aspargine-proline (NANP)n, from CSP was immunodominanat. This peptide could also reproducibly elicit a significant proliferative response from immunized lymph node cells *in vitro* (Good *et al.*, 1990). However, there was only limited success in phase I and phase II clinical trials with synthetic and recombinant vaccines containing the B-cell epitope NANP (Engers, Bergquist and Modabber, 1996).

According to Roggero and colleagues (1995) long polypeptides of 104 and 102 amino acids corresponding to N- or C-terminal of *P. falciparum* CSP induced neutralizing antibodies in mice which prevented *P. falciparum* sporozoites penetrating into hepatoma cells, HepG2-A16, *in vitro*. CS-specific T helper cells and CTLs could be obtained after a single immunization *in vitro* in the lymph node cells culture (Blum- Tirouvanziam *et al.*, 1994). The results confirmed the possibility of generating CSP-specific MHC class I-restricted T cell responses due to their adequate processing and presentations in the context of MHC class I (Eberl *et al.*, 1999). In a following study it was confirmed that immunization of BALB/c mice with *P. berghei* CSP 242-310 induced high titres of anti-peptide antibodies which also recognize the native *P. berghei* CSP (Roggero *et al.*, 2000).

A chimaeric protein mixture, consisting of a fusion between the CSP and the hepatitis B surface antigen (HBsAg) that is expressed in yeast has been designed by GlaxoSmithKline and the US Army (Kester *et al.*, 2001). This mixture can form a HBsAg particle in the presence of unmodified HBsAg which is called the RTS,S vaccine (Richie and Saul, 2002; Kester *et al.*, 2001).

The vaccine furthest along in testing is known as "RTS,S/AS02A". In this vaccine ASO2A is an experimental oil in water adjuvant containing monophosphryl lipid A (3D-MPL) and the saponin QS21. RTS,S vaccine with ASOA adjuvant elicits a better protective response (Richie and Saul, 2002). This vaccine has proved safe in children during trials in the Gambia in which the efficacy during the first 9 weeks of follow-up was estimated to be 71 % but decreased to 0 % over the next 6 weeks (Bojang *et al.*, 2001). A more developed RTS,S vaccine, a hybrid product containing repeat and C-terminus of CSP regions, fused to the hepatitis B surface antigen vaccine in a complex adjuvant mixture (reviewed by Phillips, 2001) was able to reduce prevalence of parasitaemia in adults by 65% for two months in a field trial in Gambia. However, Alonso *et al.* (2004) also evaluated the efficacy of RTS,S/ASO2A vaccine in Mozambique reporting that this subunit vaccine confers protection in children aged 1-4 years against both infection and range of clinical illness caused by *P. falciparum*. There were 57.7% efficacy for severe malaria and 45% for extending time to first infection in two cohorts of children aged 1-4 years in a phase IIb randomized controlled trail (Alonso *et al.*, 2004).

Among antigens in the liver stage *P. falciparum* liver-stage antigen 3 (PfLSA-3) has been shown to display promising antigenic, immunogenic, and protective properties in Aotus monkeys (Perlaza *et al.*, 1998) and chimpanzees (Ben Mohamed *et al.*, 1997; Daubersies *et al.*, 2000). PfLSA-1 DNA immunization also induces potent Th1 responses with protection against heterologous *P. yoelii* challenge in mice (Sauzet *et al.*, 2001).

More recently clinical trials are being run based on another strategy called prime-boosting in which the host can be primed by a DNA-vaccine followed with boosting with a recombinant modified antigen. This immunization strategy has been evaluated in murine, non-human primate, and human studies (reviewed by Dunachie and Hill, 2003). For example, in murine malaria priming with plasmid-DNA encoding the entire *P. berghei* CS antigen followed by a boosting immunization with recombinant modified vaccinia virus Ankara (MVA) carrying the same antigen induced strong level of $CD8^+$ T cells which was associated with an increase observed efficacy against *P. berghei* sporozoite challenge from 0-20 % to 80-100 % (Schneider *et al.*, 1998).

In humans priming the immune response with a DNA vaccine and heterologous boosting of the response with recombinant MVA induced high levels of specific CTL response (reviewed by Webster and Hill, 2003). Moorthy *et al.* (2004) reported that in a prime-boost vaccination no clinically relevant laboratory abnormalities and no severe or serious adverse events related to vaccination were seen in 29 Gambian men aged 18-45 years. A single-boost after the final vaccination expanded the effector T cell pool to a similar or higher numbers than that after the primary vaccination. This vaccine includes thrombospondin-related adhesion protein (TRAP) construct, includes $CD8^+$ and $CD4^+$ T cell epitopes from pre-erythrocytic *P. falciparum* antigens and three carriers of construct-plasmid DNA and 2 recombinant MVA and fowlpox strain 9 (FP9). A series of heterologous prime-boosting immunization trials are underway in Oxford, England. For example in a DNA-vaccine followed by boosting with a MVA vaccine an excellent safety profile for the vaccines was seen in over 150 subjects in total (Moorthy and Hill, 2002). Studies in sub-Saharan Africa showed that the most potent inducers of circulating effector T cells seen to date were DNA/MVA and FP9/MVA regimens (Moorthy *et al.*, (2004).

1.7.2. Immunization and vaccines against asexual blood-stages

The immune response to the asexual erythrocytic stage of malaria is least well understood. So, immunization and vaccine development studies face more challenges than to other stages (reviewed by Good, 2001). The lack of an established human challenge, the limitation of available animal models, and unclear protection mechanisms are examples of such challenges (reviewed by Moorthy, Good and Hill, 2004). However, as almost all symptoms and deaths of malaria come from this stage, it is therefore an important target for a vaccine (reviewed by Good, 2001). Immunization against asexual blood stages needs special necessary requirements such as high titres of antibody because parasites reside within erythrocytes and immune clearance mechanisms have access to pRBCs only for a short time (Mahanty, Saul and Miller, 2003).

An effective vaccine against this stage must limit growth of the parasite, because the level of parasitaemia is in general proportional to the severity of malaria (Miller, Good, and Milon, 1994).

Asexual blood-stage vaccines may be classified into two groups, anti-invasion and anti-complication vaccines (reviewed by Moorthy, Good and Hill, 2004).

The most interesting anti-invasion vaccine candidates for asexual stages are MSP-1 and AMA-1 antigens. Both have homology in all *Plasmodium* spp including rodent models and this has allowed their vaccine potential to be assessed in several animal models (Anders, and Saul, 2000).

MSP-1 is known as a vaccine candidate due to its role in the initial recognition and invasion of RBCs (reviewed by Wipasa *et al.*, 2002) and as a target for the immune response. A fragment of MSP-1, MSP-1_{19}, including two C-terminal epidermal growth factor (EGF) like-domain, has been shown to induce inhibitory antibodies targeting the first of the EGF domain and subsequently reduces growth of *P. falciparum in vitro* (Chappel, and Holder, 1993). As this fragment is not a strong immunogen the larger MSP-1_{42} polypeptide that is cleaved to generate MSP-1_{19} and a 33 kDa part, has been examined. These polypeptides were found to be safe, immunogenic and induce antibodies that inhibits invasion *in vitro* (Anders, and Saul, 2000).

A recombinant protein vaccine comprising *P. falciparum* ring erythrocyte surface antigen (PfRESA), MSP-1, and MSP-2 formulated in an oil-based adjuvant, has been tested in Papua New Guinea between 1998 and 1999. This vaccine reduced *P. falciparum* density in children by 62% without any harmful side effect (Genton *et al.*, 2003). The vaccine potential of MSP-4/5 is being studied and has shown protection in immunized mice to challenge with *P. yoelii* YM. This antigen has some common structural feature with PfMSP-4 and PfMSP-5 (Anders, and Saul, 2000).

Ling and colleagues (1994) expressed the C-terminal parts of the *P. yoelii* EGF-like domains in MSP-1 in bacteria. Immunization of mice with the above recombinant protein induced a protection against a challenge of *P. yoelii*. It is known that structural determinants of MSP-1 which are formed by two EGF-like modules together are critical for the immunogenicity of the protein (Ling, Ogun and Hoder, 1995). Mice were only protected when they were immunized with both modules of the EGF-like domains but not

with a single module (Ling, Ogun and Holder, 1995). In this regard, the specificity of the immune response was shown in BALB/c mice immunized with the two EGF-like modules from MSP-1 of *P. yoelii* YM strain. These mice were protected against a homologous but not a heterologous *P. yoelii* sporozoite challenge (Renia *et al.*, 1997).

Burns *et al.* (2003) used a combined formulation of C-terminal EGF-like domain of PcMSP-1, a fragment functional in merozoite invasion, and the ectodomain of PcAMA-1, a fragment contributing to erythrocyte binding activity as a vaccine candidate in C57BL/6J mice. They showed that immunization with both PcAMA-1 and PcMSP-1 induced a high level of protection to *P. c. adami* challenge, although efficacy was dependent on antigen dose, adjuvants, and immunization protocols.

Among anti-complication vaccines PfEMP-1, the main ligand for adherence, sequestration and subsequence severity of malaria, and glycosil phosphatidyl inositol (GPI) have been assessed and showed protection from severe disease (reviewed by Moorthy, Good and Hill, 2004). However, these did not show clear pathway in use of such vaccines because this findings were not yet reproducible by other investigators.

Although a large numbers of blood stage antigens have been identified, the number of human clinical trials is too few. This situation causes delays to rapid progress. So, the capacity for vaccines testing in endemic regions needs to be expanded (Mahanty, Saul and Miller, 2003).

1.7.3. Immunization against sexual stages and TBV vaccines

TBVs may only interrupt, and reduce the transmission of malaria. Such vaccines are unable to induce protective immunity. TBVs can prevent the transmission of malaria by inducing antibodies against antigens of sexual stages in humans which act in the blood meal in the gut of mosquitoes and subsequently reduce deaths in most malaria endemic areas (Carter *et al.*, 2000; Carter, 2001). Constructs of TBV candidates for both *P. falciparum* and *P. vivax* have been successfully tested in animal systems (Carter 2001).

Antigen candidates for TBV vaccines are: *P. falciparum*-11 (Pfs11), Pfs230, Pfs25, and Pfs28 molecules (Carter *et al.*, 2000). Two highly characterized antigens, including *P. vivax*-25 (Pvs25) and Pfs25, have been

tested. It has been shown that Pvs25 produced in *Sacccharomyces cerevisiae* elicits antibodies in mice, rabbits, and non-human primates. These antibodies can efficiently block transmission in membrane feeding assays. There is also preclinical data for Pfs25 expressed in *P. pastoris* (Ballou *et al.*, 2004). Most target antigens that are being currently studied are: (a) pre-fertilization antigens in the gametocyte, (b) post-fertilization antigens expressed on the zygote, and (c) late-midgut-stage antigens such as chitinase which is required for ookinete penetration through peritrophic membrane (reviewed by Phillips, 2001).

Despite the fact that post-fertilization antigens would not be exposed to the immune response in the patient and so antibody responses would not be boosted after natural infection, some post fertilization antigens such as Pfs25 have been examined (reviewed by Phillips, 2001). Antibodies against Pfs25, Pvs25, and Pvs28 have been shown to block transmission of *P. falciparum* (Kaslow, 1997) and *P. vivax* to mosquitoes completely (Hisaeda *et al.*, 2000). Immunogenicity in non-human primates has been tested for Pvs25 (Arevalo-Herrera and Herrera, 2001). Phase I clinical trials have been conducted at the Malaria Vaccine Development Unit, National Institutes of Health (NIH, USA) (reviewed by Hisaeda and Yasutomo, 2002).

Regarding late-midgut-stage antigens such as chitinase it has been found that inhibition of chitinase can block both parasite infectivity in mosquitoes and transmission (Shahabuddin *et al.*, 1993). The ookinete has to recognise two types of receptors on the midgut epithelium and surface. So, these receptors may be potential TBVs (reviewed by Phillips, 2001).

1.7.4. Multivalent vaccines

Multivalent vaccines, which are comprised from optimized different antigen components and their sequences, are necessary to mimic the naturally acquired resistance to malaria in people (Rainczuk *et al.*, 2003a and b). Optimal components of a multivalent vaccine sequences can be identified using a method called expression library immunization (ELI) (Smooker *et al.*, 2000). This technique enables screening of a pathogen's genome and determining vaccine candidates (Smooker *et al.*, 2000; Melby

et al., 2000). ELI with a *P. c. adami* genomic library significantly protects mice against asexual blood-stage of a lethal *P. c. adami* DS challenge (Smooker *et al.*, 2000). In a DNA vaccination using three different libraries of *P. c. adami* DS T cells responses specific to native malarial antigens or epitopes of the parasite were determined. Sera obtained from mice vaccinated with genomic libraries promoted the opsonization of *P. c. adami*-infected erythrocytes by murine macrophages *in vitro*. Over three-vaccine trials protection after lethal challenge with *P. c. adami* DS ranged from 33 to 50%. These results showed that protective epitopes or antigens were expressed within the libraries and that ELI induces responses specific to *P. c. adami* malaria.

Mueller and collegues (2005) has developed a genetically modified *P. berghei* which may use as a protective experimental malaria vaccine. They disrupted a targeted gene, UIS3, which is essential for early liver-stage development in *P. berghei* and obtained a uis3-deficient sporozoite. These genitically-modified sporozoites are able to infect hepatocytes but are unable to establish asexual blood stage infection *in vivo* in C57BL/6 mice. Immunization with uis3-deficient sporozoite induced complete protection in mice challenged with wild sporozoite of *P. berghei*. So, as UIS3 of rodent malaria parasite and human *P. falciparum* show 34% amino acid sequence identity, this principle study indicates possibility of development attenuated malaria parasites which may open another feasible hope to develop an effective malaria vaccine.

1.8. Projects aims

The aims of the present study were first to carry out a detailed examination of the immune responses of mice infected with either virulent or avirulent *P. chabaudi adami*, single and mixed-infections, and secondly an investigation of the protective role of IgG subclasses, IgG1 and IgG2a, in those infections through passive immunization and finally evaluation of the efficacy of some synthetic peptides as possible vaccine candidates in NIH mice immunized against *P. chabaudi* AS challenge.

Comparison of the immune responses in NIH mice in avirulent and virulent infections.

A better understanding of immune responses against malaria parasites, particularly asexual blood stage of the parasite is one of the main factors to facilitate efforts leading to control of the disease through vaccine development. The present study has aimed to determine the nature of and compare the immune responses in mice infected with avirulent DK, or virulent DS strains of *P. c. adami* and mixed infections of both strains. The association between IgG2a production and CD^+ Th1 and IgG1 and Th2 responses were also examined as previous studies have suggested (Taylor-Robinson *et al*, 1993; Smith and Taylor-Robinson, 2002; Stevenson, 1988; Langhorne *et al.*, 1989). This included monitoring the course of infection, parasitaemia levels, cytokines and specific IgG subclasses productions in the acute and chronic phases of infections. In the virulent infections studies were done in either drug-treated or untreated mice.

It is reported that the size of the infective dose influences in balance between Th1/Th2. Taylor-Robinson and Phillips (1998) showed that in resistant NIH mice infected with *P. chabudi* increasing the infective dose enhanced the Th1 response and IFNγ and reduced Th2 and IL-4 production. Favila-Castilo, Monroy-Ostia and Garci-Tapia (1999) reported that mice that suffered low parasitaemias developed good immune response to homologous reinfection when ascending parasitaemias were stopped by treatment. In the present study the effect of a low infective dose in induction of the immune response and the dynamic of the course of infection compared to that in mice given a higher infective dose in the virulent DS infection were also examined.

Determination of the immune responses when NIH mice were infected with an mixed infective dose containing both *P. c. adami* DK and *P. c. adami* DS

Mixed infection studies may develop more useful knowledge about the immune response dynamics of the infection or alteration in immune responses (Snounou *et al.*, 1992). In the present study the immune response of NIH mice to a mixed infection consisting avirulent DK, and virulent DS

strains was examined. This helps to identify whether there is any cross-reactions or alteration in immunity in context of sequential Th1 and Th2 responses.

The effect of in host-competition between two different strains in a mixed infection on proliferation rate of each parasite may be evaluated by comparison of peak parasitaemia between mixed and single infection of those parasites (De Roode *et al.*, 2003). So, the results presented here examined any effect of a low proportion of virulent DS strain in a mixed infective dose including the DK strain in the outcome of the course of their mixed infection.

The amelioration of clinical course of *P. falciparum* has been shown in humans when previously infected with *P. vivax* (Maitland *et al.*, 1997). The present study also examined any amelioration in the outcome of infection with the virulent DS when the avirulent DK strain is present in the infective dose.

The effects of the passive transfer of sera and purified IgG subclasses from avirulent strain in both virulent and avirulent infections

Many experiments in animal models have confirmed that passive transfer of serum (reviewed by Phillips, 2001) and specific-parasite IgGs (Smith and Taylor-Robinson, 2002; Narum *et al.*, 2000) can confer protection to asexual blood stage of malaria in humans or rodent malaria. Using passive transfer experiments the present study examined the degree of the protectivity of immune serum and purified IgG1 and IgG2a in either avirulent or virulent infections. However, as the immune serum or purified IgG subclasses were obtained from mice infected with DK strain. So, using these sera or IgGs provide determination of any cross-reactivity in recipient mice which were challenged with the virulent DS.

Purified IgG1 and IgG2 were also obtained from sera collected on two different days p.i. day 15., after the resolution of peak parasitaemia or on day 55 pi. determination of differences effects of above purified IgGs on the course of infection in avirulent or virulent infections in terms of collection time was examined.

Peptide immunization

A member of *clag* gene family of *P. falciparum* showed high similarity to one of the gene survey sequences (GSS) in *P. chabaudi* (Janssen *et al.*, 2001). Janssen *et al.*(2002) also identified another gene family in rodent malaria prasites such as *cir* in *P. chabaudi* which is homologous to *vir* in *P. vivax*. The features of *clag* gene products make them important contributors to pathology of malaria through cytoadherence, sequestration and also probably invasion to red blood cells(Ling *et al.*, 2004; Gardiner *et al.*, 2004; and Holt *et al.*, 2001). Trenholme *et al.* (2000) suggested that *clag*9 product might be a candidate molecule for an anti-disease vaccine. So, it may be concluded that in *P. chabaudi*, antigens encoded by *clag* genes may have the same roles.

The present studies has aimed to evaluate immunization of mice with the synthetic peptides which were designed and produced based on two families *clag* and *cir* (see 1.6.2.3) gene families in rodent malaria parasite, *P. chabaudi* AS in NIH mice. The present study provides information about potency or efficacy of such peptides as vaccine candidates. These peptides may be used in multivalent vaccines or use in other vaccination strategies.

Chapter Two

Materials and Methods

2.1. Mice

Female outbred NIH mice were used and purchased from Harlan (Bicester, UK). They were kept in the Joint Animal Facility, (JAF), at 20-24 C° and 50-60% humidity. They were brought to the JAF between 4-6 weeks of age, and acclimatized for a minimum one week when their weight was approximately 25g. Depending on the experiment the mice were kept in artificial light from 1900 to 0700 and fed with standard diet (Beekay Universal Ltd, UK) before infection and on diet CRM (S. & S. Scotland Ltd) after infection.

2.2. Parasites

A new focus of rodent malaria parasites was found in captured thicket rats, *Thamnomys rutilans*, in forest galleries in Brazaville in 1966 (Adam *et al.*, 1966). Three species of *P. yoelii*, *P. vinckei*, and *P. chabaudi* may coexist in the same host, *T. rutilans*.
P. chabaudi AS was also isolated from adult *T. rutilans* from La Maboke, Central African Republic, in 1969 (Walliker, Carter and Morgan, 1971). In this study *Plasmodium chabaudi adami* DK (avirulent), *P. c. adami* DS (virulent), and *P.chabaudi* AS were used. They were all obtained from Professor David Walliker (University of Edinburgh, UK).

2.3. Parasite maintenance

For long term storage the parasites have been maintained by cryopreservation. The infected blood with usually 15-25% parasitaemia containing ring stages, the most useful stage for freezing and thawing, was collected into sodium heparin (1000 i.u./ml, Evans Medical Ltd.) in phosphate buffered saline (PBS, pH 7.2) at 10 i.u. heparin per ml of blood from mice. The infected blood was diluted 1:1 with a solution of sorbitol-glycerol (38% glycerol, 2.9% sorbitol, and 0.63% NaCl). The mixture was then aliquoted, 0.3 ml into each cryopreservation ampoules (Griener, UK), labelled with the WEP code and a number, and was snap frozen by immersing in liquid N_2 (-196 C°), and stored in canisters.

For experimental purposes the parasites were maintained by sub-passage through susceptible mice (see below).

2.4. Recovering parasites from cryopreservation (liquid N_2)

The capsule containing the cryopreserved infected blood was taken from liquid N_2 and the blood thawed in a 37 C° water bath (Gallencamp) or by hand. To the thawed blood 0.3 ml 17% sorbitol was added dropwise. The mixture was then inoculated intraperitoneally (i.p.) into a mouse.

2.5. Challenge infection (from the subpassage-infected mouse)

Infected mice were sacrificed in a CO_2 chamber and bled by cardiac puncture. Infected blood was added into a syringe which contains sodium heparin (1000 i.u./ml) in PBS (pH 7.2) at 10 i.u. heparin per ml of blood from mice. The parasitaemia of the donor mouse was determined by examination of a Giemsa's (Gurr BDH Ltd, England) stained thin blood smear. The infected blood was subsequently diluted to give the required concentration of the parasitized red blood cells (pRBCs). Here, each infective dose was 1×10^4 pRBCs for *P. c. adami* DS and 1×10^5 pRBCs for *P. c. adami* DK in 0.25 ml PBS i.v. For passive immunisation experiments 1×10^4 pRBCs were used for both strains. In the mixed infection experiments the infective dose was $1x10^4$ pRBCs containing 8×10^3 pRBCs of *P. c. adami* DK and 2×10^3 from *P. c. adami* DS. In this latter experiment some mice were infected with *P. c. adami* DS at 2×10^3 pRBCs as a control group for determination of the immune response in mice when they were infected with a low infective dose.
For reinfection, mice were injected with 1×10^7 to 1×10^8 pRBCs, i.p depending on the experiment. For intravenous (i.v.) inoculation mice were previously warmed in a warm box. Non-infected red blood cells were added to the diluent of PBS to avoid spontaneous lysis of infected RBCs at a very low concentration.

2.6. Chloroquine treatment

In some cases sub-curative dose of chloroquine (chloroquine disulphate salt, Sigma) was given i.p. to the mice infected with virulent DS parasites. Mice weighing 25 grams were received 0.1ml chloroquine at 6mg/ml (24mg/Kg).

2.7. Determination of parasitaemia

Parasitaemia was determined by daily examination of Giemsa's stained thin blood smear viewed under oil immersion (Gurr, BDH, England). The stain was diluted to 10 times in Giemsa's buffer pH 7.2. Blood smears were normally taken between 08.00-11.00 hours each day by piercing the tip of the tail with a needle (needle was replaced for each group). A drop of the infected blood was placed at one end of a glass microscope slide (BDH, England), smeared, dried at room temperature (RT), fixed in 100% methanol (Analar, BDH, England), and then stained for 10-15 minutes.

2.8. Presentation of parasitaemia

For each day, the parasitaemia was determined for each mouse as the percentage of infected erythrocytes. The mean percentage parasitaemia of each group was then calculated. The mean parasitaemia was also expressed as the geometric mean (mean $\log_{10}$ pRBCs per 10^5 RBCs) for each group.

2.9. Collection of serum

Mice were sacrificed in a CO_2 chamber and bled by cardiac puncture. The blood was allowed to clot at RT and was then put into the cold room for at least one-hour to give better contraction of the clot. The serum was separated from contaminating RBCs by centrifugation for 5 minutes at 6000g. Normally the sample was then aliquoted into 20µl volumes in small Eppendorf tubes (Griener, UK), labelled, and stored at -20C° until required. In some cases serum was aliquoted in larger volumes.

For collection of small serum samples the mice were warmed in warm box, and blood was collected (up to 100µl) by pricking the end of the tail. The number of bleeds for each mouse was equal within each group, and of a frequency to avoid inducing anaemia from repeated bleeding of an individual mouse.

2.10. Spleen cell culture

Whole spleen cell suspensions from infected or naïve mice were separately cultured with pRBCs, RBCs, medium, or concanavalin A (Con A, Sigma,UK) at equal numbers and volume. Con A is a polymeric plant component, commonly used as a non-specific polyclonal T cell activator and a mitogen (Zhang *et al.*, 1996). All spleen cell samples were prepared in medium of RPMI 1640 (GIBCO/ Technology, UK) supplemented with 10% foetal calf serum (FCS) as complete medium, (Lab. Tech. International, UK). ConA (Sigma, stock at 1mg/ml) was added at 5µg/ml (final concentration 0.5µg per well).

Spleen cell culture contains several steps as follows:
Mice were sacrificed and spleen aseptically removed into complete RPMI 1640 in a Class II hood. The spleen was then teased gently through a tea strainer in a petri dish (Griener, UK) in complete RPMI 1640. The resultant suspensions including splenocytes were harvested with a sterile Pasteur pipette allowing tissue debris to sediment. All separated spleen cell suspensions were centrifuged for 10 minutes at 1200 g at 4 C°, and resuspended in 0.3 ml of Boyle's solution which was prepared at ratio of 1:9 from one volume of 0.17 M tris-HCl pH 7.65 and nine volumes from 0.16 M ammonium chloride to lyse erythrocytes. After 3 min. incubation at RT the spleen cells were centrifuged at 1200 g for 5 min at 4 C°, washed three times with RPMI or PBS, and the pellets rsuspended in 10 ml of complete RPMI 1640.

Adjusting the number of spleen cells: The number of viable splenocytes was calculated by diluting 10µl of the resuspended washed spleen cells in 190µl of 0.2% Trypan blue (Sigma, USA) in PBS. Dead cells could not exclude the dye. The suspensions were incubated for 2-3 min at RT and examined using a Neubauer Haematocytometer under phase contrast

microscope (Diavert Leitz, Germany). The cell concentrations were adjusted to 5×10^6 viable cells /ml. The cell suspension was transferred into triplicate wells of sterile 96 well flat-bottom tissue culture plates (IWAKI, Japan). The final volume of each well was 200µl. All experimental wells were set up as described.

Adjusting the number of pRBCs and RBCs

Parasitized red blood cells were used as an antigen and uninfected red blood cells as a control. They were washed two times with PBS to remove all the plasma. The parasitaemia was determined to calculate the percentage pRBCs. The total number of RBCs was adjusted at the same number when they were added to each well in a 96 well culture plate. ConA was added at 5µg/ml (final concentration was 0.5µg/well) as a positive control and complete RPMI 1640 medium was used as negative control. The plates were then incubated for 72 hours in 5% CO_2 in air in a 37 C° incubator (Flow laboratory, UK). The supernatants from each well were then collected and stored at -20C° until required for enzyme linked immunosurbent antigen test (ELISA) quantification of cytokine production.

2.11. Splenocytes proliferation assay

To evaluate proliferative responses of splenocytes a separate plate was set up. Whole spleen cells suspensions from infected or naïve mice were separately cultured with either pRBCs, RBCs, medium, or ConA at 200µl per well. The samples were prepared in complete medium RPMI 1640. The number of spleen cells was 5×10^6/ml. After 60 hours incubation, 0.5µCi (37KBq) [^{3}H] thymidine (Amersham) was added to each well, and the plate was incubated for a further 12-18 hours. The cells were harvested on a filter Mats (ICN Biomedical. Inc., U.S.A) and later in the study on a printed filtermat (WALLC, Finland) by a harvester machine (Flow Laboratories, Norway and later Tomtec, Hamden, CT). Uptake of [^{3}H] thymidine was measured by liquid scintillation (Opstiscint Hisafe, Wallac, England) in a beta-scintillation counter detecting the beta activity present in each sample and presented as count per minute (c.p.m). All samples were

set up in triplicate to calculate a proliferative response as an arithmetic mean.

Stimulation index

Stimulation index presents the ratio of the proliferative responses (p.r.) between the test samples, spleen cells from infected mice, and control groups. This test shows the effect of secondary response of T cells present in the cultures when they expose to identify pRBCs. The degree of this proliferation can be compared with controls including spleen cells from naïve mice exposed to pRBCs or naïve RBCs. In this study stimulation index was calculated by using the formula below:
SI = p.r. pRBCs+f/ p.r m+f, or p.r ConA+f/ p.r m+f.
In this formula pRBCs+f is the p.r. of splenocytes from infected mice exposed to the specific antigens, pRBCs, divided by the response of the same splenocytes exposed to medium only. ConA+f is p.r. for splenocytes from infected mice exposed to ConA divided by the response of the same splenocytes exposed to medium only.

2.12. Preparation of pRBCs and RBCS lysates

In normal light condition schizogony in *P. chabaudi* usually occurs around 01:00. However, when infected mice are kept under a reversed light-dark cycle condition (12 hours of light between 019:00 to 07:00) schizogony occurs between 11:00 and 13:00. Mice were kept in reverse light because the pRBCs can be collected in the morning between 08:30 and 10:30 hours when the parasites are mostly at the late ring/trophozoite stage. *P. c. adami* DS and DK strains and also *P. chabuadi* AS antigens were prepared as crude antigens from whole blood cells enriched for mature trophozoite/schizont pRBCs, using the method described by McDonald & Sherman (1980). Infected mice were bled when the parasitaemia was between 25 to 40% and most parasites were at the late ring/trophozoite stage and then cultured *in vitro* to trophozoite/schizont stage. The heparinized (10 i.u./ml) blood was washed twice in 5% foetal calf serum (FCS) in RPMI 1640 medium, and resuspended to a 10% haematocrit in the same medium and cultivated using the candle jar method

of Trager and Jensen (1976). Volumes of 1.5 ml of the 10% w/v suspension in medium were dispensed in 35-mm petri dishes (cell-cult, Sterilin). The petri dishes were placed in a humidified glass dessicator, the candle was lit and the lid put on with the stopcock open until the flame extinguished when the stopcock was immediately closed providing a gas phase of approximately 3% CO_2 and 15-17% O_2. The parasites were cultured until they had reached the late trophozoite/scizont stage which normally takes two hours. The maturation process was followed by examining Giemsa's stained thin blood smear every 45 minutes. The parasites were then harvested, washed in 5% FCS RPMI 1640 medium (200g for 10 min), and resuspended to their original volume in sterile PBS. The suspensions were run through Whatman CF11 powdered cellulose columns (Beutler *et al.*, 1976) to remove leukocytes. The filtrate was subsequently collected and washed with PBS. The pellet, containing the parasites, was restored in PBS to its original volume and then freeze-thawed five times. Each freeze-thawing cycle entailed snap freezing the blood cells in liquid N_2 and then immediately defrosting using a waterbath at 37 C°. The rapid transition of the temperature causes fracturing of the RBCs and release of the parasites' components. The freeze-thawed preparation was centrifuged at 1500g for 10 min., and the supernatant was collected. This is termed the pRBCs lysate and was stored in 50µl aliquots at-20C° until required. Non-infected red blood cells lysate was similarly prepared and used as a control antigen.

2.13. Determination of Protein Concentration

The protein concentration of lysates prepared from both pRBCs and RBCs was determined. This technique was also used for determining protein concentration of fractions, prepared in peptide conjugation and also for purified immunoglobulins, which were used in passive immunization experiments. The method was adapted from Sedmak and Grossberg (1977) using Coomassie Protein Assay reagent (Pierce).

Standards of known protein concentration were prepared from 2mg/ml stock of Bovine Serum Albumin (BSA, Pierce chemical Co.). Serial dilutions were made with a range of 2.5 to 40µg/ml in PBS. The unknown protein concentration of lysates, either pRBCs or RBCs, was diluted at 1:100, 1:1000, and 1:10000 in PBS. From each standard and sample

150µl/well were pipetted into separate wells of a 96 well plate. Coomasie proteins assay reagent (Pierce) was then added (150µl/well) to each well. The plate was shaken for 30 seconds using a plate shaker. The plate was then read in an ELISA reader at 600 nm (Biolinx Dynatech). The protein concentration was determined by plotting the results against the standard curve.

2.14. Determination of optimal antigen concentration

Determination of optimal antigen concentration was done for all ELISA tests for antibody levels during the study. Lysates of pRBCS and RBCs were diluted at 1:2, 1:20, and 1:200µg/ml in coating buffer NaHCO3, 0.1M, pH 8.2 (Analar, BDH, England). These different lysate dilutions were coated at 50µl/well in a 96 well plate, and incubated (Dynex, Immulon, 4HX, USA) overnight at 4 C°. The plates were subsequently washed 3 times with 0.05% Tween (BDH, England) in PBS pH7.2, and then non-specific binding sites were blocked with 10% FCS PBS at 200 µl per each well, covered, and incubated for 1 hour at 37 C°. The plate was then washed two times with 0.05% Tween/PBS, and 50 µl/well of dilutions of sera at 1:100, 1:1000, and 1:10000 in 10% FCS PBS from immune and control mice were added, covered, and incubated at 37 C° for 3 hours. After washing 4 times with 0.05% Tween/PBS, 50 µl/well Biotinelated anti-mouse IgG at 1:100000 (whole molecule, Sigma) in 10% FCS PBS were added, covered, and incubated at 37 C° for 1 hour. Washing 6 times with 0.05% Tween/PBS followed, and then diluted Streptavadin-peroxidase (1:1000), at 100µl/well in PBS 10% FCS were added, covered, and incubated for 1 hour at 37 C°. The plate was then washed 8 times with 0.05% Tween/PBS, after which 3,3`,5,5`-tetramethylbenzidine (TMB, microwell KPL, USA) substrate at 100µl/well were added and the colour (blue) allowed to develop. The plate was read at 630 nm with reference filter of 405 nm using Biolinx Dynatech software. The plate was covered to prevent evaporation, and blocking was for reduction of non-specific binding. Each sample was tested in triplicate and the blank wells contained

10% FCS PBS. For determination of optimal concentration of conjugated peptides the same process was carried out.

2.15. Cytokine analysis by ELISA (sample from supernatants)

Purified anti-mouse cytokine, either interferon-gamma, IFNγ, or interleukine-4, IL-4, (PharMingen, U.S.A) monoclonal antibody, was diluted in 0.1M NaHCO3 pH8.2 as a coating buffer at 2μg/ml. The antibody solution was added at 50μl to each well in a 96-well plate, covered, and incubated overnight at 4C° in the cold room. The plates were subsequently washed 3 times with 0.05%Tween in PBS pH7.2, and then blocked with 10% FCS PBS at 200 μl per each well, covered, and incubated for 1 hour at 37 C°. After blocking, the plate was washed two times with 0.05% Tween/PBS. Serial dilutions of recombinant cytokine standards (for IFNγ stock was at 1mg/ml, and for IL-4 stock was at 0.5mg/ml, PharMingen) and the culture supernatants were added to the wells at 50μl/well, covered, and incubated at 37C° for 3 hours. After incubation the plate was washed 4 times with 0.05% Tween/PBS, and 50μl/well of Biotinelated rat anti mouse cytokine (IFN, and IL-4, at 2μg/ml PharMingen) in 10% FCS PBS were added, covered, and incubated at 37C° for 1 hour. After washing 6 times, diluted Streptavadin-peroxidase (1:1000), 100 μl/well in 10% FCS PBS 1:1000 were added, the plate covered, and incubated for 1 hour at 37C°. The plate was then washed 8 times with 0.05% Tween/PBS, and then 100 μl/well of TMB substrate were added and the colour allowed to develop. The plate was read at 630 nm with reference filter at 405 nm using Biolinx Dynatech software.

2.16. Cytokine analysis by ELISA (samples from sera)

The technique was followed as previously described except for sera dilutions. Sera were diluted at 1:5 for IFNγ and at 1:2 or neat for IL-4 if there was sufficient serum.

2.17. Determination of specific antimalarial IgG

Lysates of pRBCs and RBCs (control) were diluted to 20μg/ml as antigen in coating buffer NaHCO3, 0.1M, pH 8.2., coated, covered, and incubated in a 96 well plate (Dynex, Immulon, 4HX, USA) at 50μl/well overnight at 4C°. The plate was washed 3 times with 0.05%Tween in PBS pH 7.2, blocked with 10% FCS PBS at 200 μl per each well, covered, and incubated for 1 hour at 37C°. Two washes followed with 0.05% Tween/PBS, and then 50μl/well of either 1:100 or 1:1000 dilution of the sera in 10% FCS PBS from the infected and control mice were added, covered, and incubated at 37 C° for 3 hours. After washing 4 times with 0.05% Tween/PBS, 50μl/well anti-mouse IgG biotinelated (whole molecule), at dilution of 1:100000 in 10% FCS PBS were added, the plate covered, and incubated at 37 C° for 1 hour. The plate was washed 6 times with 0.05% Tween/PBS, and then diluted Streptavidin-peroxidase (1:1000), 100 μl/well in PBS 10% FCS were added, covered, and incubated for 1 hour at 37 C°. Washing 8 times with 0.05% Tween/PBS followed, and then 100 μl/well TMB substrate were added and the colour allowed to develop. The plate was read at 630 nm with reference filter at 405 nm using Biolinx Dynatech software. For detecting the IgG subclasses, IgG1 and IgG2a, the same technique was used. However, the concentrations of secondary biotinelated anti IgG1 or IgG2a were at 2μg/ml and added at 50μl/well in 10% FCS PBS.

For determination of anti-peptide antibodies 1mg of each peptide was conjugated to 1mg BSA in 0.5 ml conjugated buffer for coating on the ELISA plates. This conjugation was carried out because peptide does not stick on the ELISA plate. Each conjugated peptide with BSA was diluted at 20 μg/ml in coating buffer as described above.

2.18. Passive immunization with serum

2.18.1. Preparation of immune serum

Mice were infected with *P. c. adami* DK and their sera were collected for use in passive immunization experiments. Mice were infected with *P. c. adami* DK at $1x10^5$ pRBCs and divided into two groups.
On day 15 post-infection mice of the first group were sacrificed and sera were collected. Mice in the second group were reinfected at $1x10^7$ pRBCs on day 40 p.i., and their sera were collected two weeks later on day 55 p.i.

2.18.2. Passive immunization and challenge of NIH mice

Two different groups of mice were infected with *P. c. adami* DK at $1x10^4$ pRBCs and immediately each mouse given 500µl of immune serum from either day 15 or 55 p.i.,
To determine any cross-reaction, other mice were infected with *P. c. adami* DS at $1x10^4$ pRBCs and passive immunization was carried out as above. Mice in the control group were given 500 µl of PBS. Previous work has been shown that the course of infection is the same in mice given PBS as those given normal mouse serum.

2.19. Synthetic peptide immunization

Synthetic peptides:
Peptides for immunization were commercially prepared by Genosphere biotechnologists according to the order of Dr M. Barrett University of Glasgow. These peptides contain sulfhydryl as free binding site by which they can be conjugated with a carrier protein and so their amino acid sequences are artificially initiated with cystein. The sequences of amino acids of these linear peptides are based on *clag* 7 and 3 of *P. chabaudi* AS and are shown below (see Chapter Six):
P1: CYAKKPITQLRYGKT
P2: CQSHFTINYRIRQVI

P3: CSGRVLPRPLYDELQ

P4: CKINQHPNKKFGTND

P5: CSQKASEFVKSFKEL

A set of experiments was set up as follows to determine immune responses in mice immunised with the synthetic peptides.

2.19.1. Preparation of conjugated peptides

Although small molecules, haptens, such as small peptides are able to interact with products of an immune response, they cannot stimulate an immune response. So, coupling peptides with a carrier protein make them immunogenic which can induce specific immune response. In this study mariculture keyhole limpet haemocyanin, mcKLH (imject maleimide activated immunogen conjugation Kit, Pierce) and BSA (Pierce) were used as carriers.

A standard protocol for hapten-carrier conjugation with the imject activated immunogen kits was done as follows:

Up to 2mg of sulfhydryl-containing peptide were dissolved in 200 to 500µl of conjugation buffer (see Appendix). For quantitation of the conjugation small amount (10-20 µl) of this peptide solution has to be saved.

2mg of pre-activated carrier protein (one vial in the kit) were dissolved with 200 µl deionised distilled water. The peptide may be added as a solid to the activated carrier solution if it is freely soluble. To obtain complete and efficient coupling a molar excess of hapten over the carrier protein is required. So, because the synthetic peptides have a molecular weight more than 2000 Dalton, then 2mg (~1µmole) of hapten was added to 2mg of carrier protein, which has ~ 0.7µmoles of maleimide group.

Both solutions of the peptide and mcKLH were mixed immediately and allowed to react for 2 hours at RT.

Purification of conjugation by gel filtering:

Removing ethylenediamine tetraacetic acid, EDTA, (Sigma) carried over from the activated carrier proteins is a necessary step because it is an anti-coagulant substance and should not be injected into laboratory animals. This process contains the following steps:

The contents of one bottle of dry blend purification buffer salt (0.083 M sodium phosphate buffer, 0.9 M NaCl, pH 7.2 with proprietary stabilizer) were dissolved in 60ml degassed, deionised water. Any unused buffer can be stored at 4C°.

The top and bottom caps from one D-salt™ desalting column were removed allowing storage solution to drain.

A D-salt™ cross-linked dextran gel filtration column was used. Gel is suspended in 0.02% sodium azide as a preservative. Molecular weight cut-off for the column is 5000. Sodium azide will also be removed as well as EDTA in the final conjugates prior to their use as immunogens.

The column was washed with 3 to 5 column volumes of purification buffer. The hapten-carrier mixture, 0.5ml, was applied directly to the top of the disc. Sometimes mcKLH formed a precipitate during conjugation. When this happened the precipitated material was centrifuged, the supernatant collected and applied to the desalting column and the precipitated was saved. After collecting fractions the precipitate was combined with the collected fractions. When the reaction volume was greater than 0.5 ml, a second desalting column for another 0.5ml sample size was used.

Each fraction was separately collected in a tube in which 0.5ml of purification buffer was added. Absorbance of each fraction was measured at 280nm using a spectrometer (Thermospectronic, Heyiosγ, U.K.) to find the fractions containing the conjugate. The hapten-carrier conjugate was in the first absorbance peak detected. The dead volume of a 5ml-gel filtration column is approximately 2.0ml. So, the conjugate-containing fractions will begin to elute after this volume. All fractions containing acceptable levels of the conjugate were pooled. They can be stored at -20C° until required.

2.19.2. Quantitation of conjugation

The degree of successful conjugation between hapten and carrier protein was examined using a microwell plate protocol as following:

Ellman's reagent (5,5'-dithobis-[2-nitrobenzoic acid], Pierce) can react with sulfhydryl groups to produce a chromophore with maximum absorbance at 412nm.

Using the protocol a standard curve was made with known quantities of cysteine (Sigma). For this 10µl of varying concentrations of cysteine were added in place of the peptide to wells containing 200µl of conjugation buffer. The total number of peptide sulfhydryl groups present before and after conjugation was estimated by this assay. The cysteine produced a similar response to a peptide containing one free sulfhydryl group. A comparison of the absorbance of all samples after modification with Ellman's reagent gaves an estimate of the number of free sulfhydryls present on the peptides before and after conjugation. Because disulfide bonds can form during even short-term storage of the peptide, the initial assay of the non-conjugated peptide was done immediately after preparation. The same assay was carried out for the prepared conjugated peptides, which had been reacted for 2 hours at RT. So, the assay was done separately for non-conjugated and conjugated peptide as follows:
Conjugation buffer was added at 200µl to each appropriate well of a 96-well plate (Dynex, Immulon, 4HX, USA). For wells which were used as blanks 210µl of conjugation buffer were added to the wells.
Hapten solution was added at 10µl to each of the wells containing buffer.
A solution of Ellman's reagent was made (1mg/ml reagent in buffer), 20µl of this solution were added to each well with peptide and blank. The plate was incubated for 15 minutes at RT. Absorbance of all wells was determined at 412 nm using Biolinx Dynatech Software.
The same protocol was carried out to measure the absorbance of conjugated peptide after they had been prepared.

2.19.3. Peptide immunisation

Adjuvants promote the immune response in immunization protocols. In this study Titermax gold adjuvant was used (Sigma) instead of Freund's complete because the latter is not recommended for use in mice.
Titermax gold adjuvant has a water-in-oil formulation in which oil forms the continuos phase in the emulsion. It is considerably easier to emulsify than Freund's adjuvant, resulting in less viscosity which makes it easier to inject through small needles. To prepare 1.0ml of the emulsion containing

immunogen and adjuvant, 0.5ml of immunogen is required. So, a ratio of 1:1 water-in-oil emulsion is usually optimum.
For mixing of the immunogen and titermax an emulsifier (Ultra-Turrax T25, Janke& Kunkel GMBH & Co. KG) was used as follows:
After Titermax gold was vortexed, 0.5ml was loaded into small plastic Bijoux. The immunogen suspension was pushed into the Titermax gold tube. The amount of immunogen was divided into two equal volume and each volume (0.25ml) was pushed into the tube contains the adjuvant. The mixture was emulsified using a dispersing tool attached to the emulsifier which rotates from 8000 to 24000 per minute. Each course of emulsifying was run for one minute. The procedure, emulsifying, was continued until the thick emulsion developed. The mixing was complete if a drop of the emulsion did not disperse on the surface of water.

2.19.4. Injection and bleeding mice

Day 0: An initial injection of 75µg immunogen per mouse (150µl of the emulsion) was made. The route of injection was i.p.
Day 14: The first boost was done with the same sample size.
Day 21: Mice were bled from the tail vein and antibody response was tested using ELISA. T second boost was also injected at the same dose, and ELISA test was done one week later.

2.19.5 Challenge with the parasite

Two weeks after the final boost the mice were challenged with 1×10^5 pRBCs i.p. of *P. chabaudi* AS. Parasitaemia was monitored by daily examination of Giemsa's stained thin blood smear. Sera from the challenged mice were collected regularly to follow antibody levels.

2.20. Passive immunisation with IgG subclasses purified from serum

To determine the role of IgG1 and IgG2a subclasses in control of infections in mice infected with two strains of *P. c. adami* DK and DS, both IgG subclasses were purified from whole serum using protein A chromatography. Each mouse in test group was injected 50µg of IgG1 or IgG2a in 200µl of PBS.

2.20.1. Preparation of immune serum

Immune serum was obtained by infecting NIH mice with *P. c. adami* DK described earlier (see passive immunization with serum, 2.18.).

2.20.2. Isolation and purification IgG1 and IgG2a from serum

For purification of the specific IgG1 and IgG2a from serum an affinity chromatography technique was used as described below.
Recombinant protein A Sepharose (Amersham Biosciences, U.K.) is derived from a *Staphylococcus aureus* and contains five regions that bind to the Fc region of IgG. In a column, protein A is immobilized to Sepharose as an affinity ligand and free regions can bind to the Fc of antibody. Two molecules of IgG can be bound to one molecule of immobilised protein A. Native protein A and recombinant have the same specificity for the Fc region, but the recombinant protein A has been engineered to include a C-terminal cysteine enabling a single-point coupling when the protein is immobilised to Sepharose. Therefore, single-point coupling often provides an enhanced binding capacity. In this study HiTrap™ rProtein A flow fast was used (Amersham Bioscinces, U.K.). HiTrap rProtein A FF column, made of polypropylene, is prepacked ready to use in an affinity chromatography. It provides separations to purify antibodies from different sources such as serum.

Purification: Buffers were prepared using deionised distilled water and were filtered by passing them through a 0.45μm filter.
In pH dependent chromatography which was used in the present study different IgG subclasses were bound to the column depending on different pH of the buffers. IgGs bind to the protein A over a wide pH range in the column. Recommended buffers and their pH were set as follows to separate and purify IgG1 and IgG2a subclasses:
Binding buffer: 20 mM sodium phosphate monobasic (Sigma), pH7 was for IgG2a.
For mouse IgG1 sodium chloride (AnalaR, BDH) up to 4 M was added to the binding buffer, 20 mM sodium phosphate, to achieve efficient binding, pH 8.
Elution buffer: a solution of 20mM tri-sodium citrate (AnalaR, DDH) was used. For IgG2a, pH 4, and for IgG1 pH 5.8 to 6 were used.
As a safety measure, to preserve the activity of acid liable IgG when using very acidic elution buffers, 60-200μl of 1 M Tris-HCl, pH 9.0 were added to the eluted fractions as they were collected. This results in an approximately neutral final pH.

Sample preparation: All samples were adjusted to the composition of the binding buffer. The samples were diluted with binding buffer and centrifuged immediately before being applied to the column. This prevents clogging of column when large volumes of serum are loaded.

Applying samples: A peristaltic pump (Pharmaci fine chemicals, p-1, U.K.) was used for operating the column. A flow rate of 1 ml/min was used according to the manufacturer's instruction. The collection tubes were prepared by adding 60-200µl of 1 M Tris-HCl, pH 9.0 to each tube.

The pump tubes were filled with binding buffer. The stopper was removed and the column was connected to the pump. Adding buffer into the column through pump was drop to drop to avoid introducing air into the column. The twist-off end was removed. The ethanol preservative was washed out with at least 5 column volumes of distilled water or binding buffer.
The column was regenerated with at least 5 column volumes of relevant elution buffer according to each subclass of IgG. For each subclass a separate column was used avoiding any contamination and non-specific binding. The columns were connected in series because higher capacity was required.
The column was equilibrated with 5-10 column volumes of binding buffer. The sample, 1ml, was applied by pumping it onto the column and washed with 5-10 column volumes of binding buffer until no material appears in the effluent. Interaction between the IgG1 and the ligand is weaker than IgG2a so, for IgG1 washing was not more than 5 times avoiding any decrease in the yield.
The protein of interest, IgG1 or IgG2a was eluted with 5 column volumes of relevant elution buffer.

Storage: The columns were washed with five column volumes of 20% ethanol to prevent microbial growth using the supplier stopper, sealed, and stored them at 4 C°.

Protein concentration: Protein concentration tests were done for all purified samples using Coomassie plus reagent (see2.13.).

2.20.3. Concentration and buffer exchange of the IgG fractions

The samples (purified IgG fractions) were collected in sodium citrate as an elution buffer. To make samples safer the buffer was exchanged with PBS. This was carried out using ultrafree-4 centrifugal filter and tube units (Millipore Corporation, U.K.). This device has 4ml capacity with the cut off molecular weight of 30000 for its membrane. All molecules with a higher molecular weight cannot pass through the membrane of the tube.
A 4ml sample was poured into the Ultra centrifugal filter unit and placed into the tube included. The assembly was centrifuged at 2800 g.
The device was removed and the sample was recovered from the bottom of the concentrate pocket with a pipette.
The concentrated sample was then diluted to 4 ml by PBS, pH 7.4, so that the buffer will be exchanged. This process was usually carried out three times. Finally the sample contained IgG fraction in PBS and would be ready for immunization. All recovered fractions for each sample were pooled and protein concentration test was performed.

2.20.4. Confirmation of the presence of IgG1 and IgG2a

The eluted fractions were pooled and the presence of both IgG1 and IgG2a was evaluated separately using three different techniques a) sodium dodecyl sulphate, (ICN, biomedicals, Inc. U.S.A) polyacrylamide (Amresco, U.S.A) gel Electrophoresis, SAD-PAGE, b) ELISA, and 3) Western blotting.

2.20.4.1. SDS-PAGE (mini gel)

The samples: The samples were a) standard IgG1 and IgG2a (PharMingen, U.K.), b) rainbow™ coloured protein molecular weight marker (Amersham Pharmaciabiotech, U.K.), and c) isolated fractions of IgG1 and IgG2a.

Fractionation of the samples: The samples were fractionated and isolated by vertical slab electrophoresis using SDS-PAGE. The composition of separating and stacking gels are detailed in the Appendix.
First the separating gel was loaded. The stacking gel was then poured and immediately a clean Teflon comb was inserted in the gel. The prepared SDS-PAGE gel was 8% (w/v) acrylamide.

Loading the samples: The samples were mixed with sample buffer (see Appendix) at a volume ratio of 2:1, 20µl of each sample and 10µl of the sample buffer, and heated at 100 C° for at least 5 minutes using a hot plate (Grant, UBD, England). The samples were then centrifuged at 8500 g for 1 minute in a microcentrifuge. After removing the comb the wells were washed with deionised water to remove any unpolymerised acrylamide and the samples were loaded at 15-20µl into the bottom of the wells using a Hamilton microlitre syringe. The samples were separated on the gel at 120 V using an electric power supply (Bio-Rack, U.K). Running the gel at 120 V was continued until the colour of the sample buffer reached the bottom of the resolving gel.

Staining the gel: The glass plate was removed from the apparatus and the gel was stained usually overnight in Coomassie brilliant blue, which contains methanol/acetic acid solution for fixing. The gel was destained using a destainng buffer (see Appendix). The gel can be autoradiographed or used to establish a Western blot.

2.20.4.2. Western blotting

Western blotting is an extremely useful technique for the identification and quantitation of specific proteins in mixtures when they are not radiolabeled. This technique was used to confirm the presence of the purified IgG subclasses. In this technique electrophoretically separated components are transferred from a gel to a solid support. The purified IgG was attached to the solid phase and the secondary antibody was used to determine the specific reaction between the target antibody and the secondary antibody. The reaction was detected using ECL system.
Western blotting contains different steps as follows:

Preparation of the samples by SDS-PAGE: The samples were run on the gel using SDS-PAGE technique as described above. The gel was rinsed and any beads of liquid were removed by wiping.

Transfer of proteins from SDS-polyacrylamide gel to solid phase: The gel can be transferred to the solid support, which is here a nitro cellulose membrane (Millipore, U.K.). The membrane orientated by placing a cut on the left-hand bottom corner. The proteins transferred from the gel become covalently bound to the membrane. After transfer the glass plates holding the gel are removed and briefly the gel is soaked in water or transfer buffer. The gel and its attached membrane were sandwiched between pieces of Whatman 3MM paper, usually three pieces were used for each side of the membrane. The gel was orientated so that the mark on the membrane corresponds to the bottom left-hand corner of the gel. All the papers and the membrane were soaked in a transfer buffer (seeAppendix) containing Tris, glycine, and methanol. The sandwich was then placed between graphite plate electrodes, with the membrane on the anodic side. All air bubbles must be squeezed out because transfer of the protein would be affected. The apparatus, a tank containing the gel and the membrane, was connected to the power supply as described before. To transfer the proteins of the gel on the membrane the power supply a voltage of 20 V may be applied overnight. For more rapid transfer a current of 400m Amperes for 1 hour can be applied.

Temporary staining: After turning off the electric current the membrane was washed with PBS and stained with Ponceau S (Sigma) which takes up to 10 minutes. This staining checks whether electrophoretic transfer is complete. The membrane was then washed with PBS and the rainbow™ marker (see 2.20.4.1), purified standard IgG1, and IgG2a transferred onto membrane.

Blocking the membrane: Non-specific binding was blocked on the membrane. For this the membrane was packed in a plastic bag with PBS supplemented with 10 % FCS and put on a shaker (Grant. Boeker, England) for at least 1 hour at 37C° or overnight at 4C°.

Adding the secondary antibody: The membrane was washed 3 times with 0.05% Tween 20 in PBS and biotinelated anti-mouse antibody conjugated 2μg/ml as the secondary antibody.

Adding extraavidin proxudase: After three times washing with 0.05% Tween 20 in PBS extravidin peroxidase was added at 1:1000 and the memrane was put on RT for 1 hour.

Detection: Detection: Specific anti-mouse IgG1 or IgG2a reactive bands were visualized with the ECL detection system (Amersham, UK) which is 10 times more sensitive than other colorimetric methods. Briefly, The ECL chemiluminescence reagent was added onto the membrane as instructed by the manufacturer after 3 times washing with 0.05% Tween 20 in PBS. This elicits a peroxidase-catalyzed oxidation of luminol and subsequently ECL, where the proxidase labeled protein is bound to the antigen on the membrane. The resulting light is detected on the film (X-ray) in minutes, or often seconds. The molecular weight of the detected bands can be measured by comparing them with rainbow™ coloured protein molecular weight marker (Amersham Pharmaciabiotech, U.K.).

2.21. Statistical analysis

The results presented here are expressed as standard error mean (±SEM) for each set of values in each experiment. SEM can be obtained when standard deviation divides by square root of the sample size for each time point at which a number of sample were tested. SEM is reversibly correlated with the sample size and it shows that the sample size was enough at each time point for statistical analysis. However, when SEM is too smal for any time pont it is not shown.

When comparison between two groups at the same time point is considered, student t-tests were performed i.e., comparison of mean % prasitaemia between two groups at the time of peak parasitaemia.

For identifying significant differences when data were compared between more than two groups, data were compared and analysed using Two-way, or One-way ANOVA tests. When two different variables are considered i.e., OD and time, Two-way ANOVA was performed. One-way ANOVA was performed when only one variable i.e., OD was considered. Two-way or One-way ANOVA show whether or not there are significant differences between all tested groups. If the above tests show any significant difference between all groups then significant difference between two particular groups, whether test sample or control, can be evaluated with

appropriate post-tests. In the present study for Two-way ANOVA, Bonferroni and for One-way ANOVA, Tukey's tests were performed as relevant post-tests. A significant result was considered to be when the P value was < 0.05 in all experiments. For performing all statistical tests software Prisem 3 was used.

Chapter Three

Comparison of the immune response in NIH mice infected with the avirulent DK and the virulent DS strains of *Plasmodium chabaudi adami.*

3.1. Introduction

Asexual blood stages of malaria parasites are responsible for almost all morbidity and mortality of the disease (reviewed by Wipasa, 2002). So, a better understanding of the immune responses against these stages is an important step towards development of effective vaccines to reduce clinical disease and prevent death.

Acquired immunity to the asexual blood stages of malaria in mice involves both cell-mediated and antibody-dependent mechanisms through activation of two subsets of $CD4^+$ T cells, Th1 and Th2 (De Souza, 1997). In avirulent self-resolving infection of *P. chabaudi* AS and *P. c. adami*, a sequential Th1/Th2 response was observed of which Th1 subset is responsible for control of the acute phase of the patent parasitaemia and Th2 is activated when infection becomes chronic (Langhorne, 1989; Smith and Taylor-Rabinson, 2003; Stevenson and Tam, 1993). In a virulent infection of *P. yoelii*, a failure to induce adequate activation of both Th1 and Th2 subsets resulted in a fatal outcome. (De Souza *et al.*, 1995 and 1996).

In mice infected with *P. chabaudi* AS, IgG2a is a predominant antibody during the primary ascending parasitaemia followed by up-regulation of IgG1 in the chronic phase as a result of $CD4^+$ Th1 to Th2 switching (Taylor-Robinson and Phillips, 1994; Taylor-Robinson and Smith, 1999). So, it seems that antibody-dependent immune response is responsible for the elimination of the *P. chabaudi* AS after the acute phase (Smith and Taylor-Robinson, 2003; McDonald and Phillips, 1980).

In *P. chabaudi* AS infection, high levels of IFNγ production were observed before the peak parasitaemia in resistant C57Bl/6 mice (Meding *et al.*, 1990). In susceptible BALB/c mice, infection with non-lethal *P. yoelii* resulted in an immediate IFNγ response while infection with lethal *P. yoelii* did not. However, in resistant CBA/J mice an initial burst of IFNγ was observed in both lethal and non-lethal infections (Shear *et al.*, 1989).

Smith and Taylor-Robinson (2003) compared the immune responses in mice infected with two close genetic matches of paired parasites from virulent and avirulent strains: lethal *P. chabaudi* 7/F1 with non-lethal *P. chabaudi* AS and lethal *P. yoelii* 17XL with non-lethal *P. yoelii* 17XNL strains. They assessed the induction of humoral immunity by the different

parasites in a genetically homogeneous host population, NIH mice. Non-lethal infections were characterized by significant early and late up-regulation of IgG2a and IgG1, respectively. In contrast, for lethal infections, a slower, reduced IgG2a response correlated with a rapidly fatal outcome prior to any significant synthesis of IgG1. It is, therefore, proposed that the sequential up-regulated synthesis of parasite-specific IgG2a (cytophilic) and IgG1 (non-cytophilic) is associated with immunity to blood stage malaria infections and is influenced by the prevailing Th1/Th2 cytokine balance in mice (Abbas, Murphy and Sher, 1996).

In an immunization study in which BALB/c mice were drug-cured and challenged with a high inoculum of ring-infected erythrocytes or mature trophozoites of *P. c. chabaudi* AJ, parasitaemia did not decline until the time of erythrocyte rupture (Cavinato *et al.*, 2001). This suggests that effector mechanisms operate immediately before reinvasion. The same result was seen when mice were challenged with schizont-pRBCs. So, the results support the idea that merozoites and/or mature schizonts from *P. c. chabaudi* AJ are the principal targets of effector mechanisms in drug-cured mice. Cavinato *et al.* (2001) also showed that purified IgG1 or IgG2a from hyperimmune serum, detected by flow cytometry, limited re-invasion when mice were treated with these antibodies. However, IgG2a had a stronger protectivity. This study also demonstrated a sequential up-regulation of specific anti-parasite IgG2a and IgG1.

Cytokines are the principal mediators of the induction and regulation of immune responses. For example, it is known that T cell-originating IFNγ is central to the induction of IgG2a production while IL-4 has the same role for production of IgG1 (Stavnezer, 1996). Cytokines have important roles as co-stimulatory signals in the affinity maturation process during B-cell differentiation to develop an antibody response (Garraud *et al.*, 1997). So, the influences of IFNγ and IL-4 in terms of IgG2a and IgG1 production in malaria infection refers to their functions as co-stimulators. An early significant production of IgG2a by mice infected with non-lethal *P. yoelii* 17XNL is probably related to a very early IFNγ synthesis in infection with the avirulent parasite (De Souza *et al.*, 1997). IFNγ up-regulates the expression of FcγRI on macrophages resulting in an increase of IgG2a-mediated opsonization (Boehm *et al.*, 1997).

Biologically active IFNγ is a homodimer glycoprotein of two 21 to 24-kDa subunits (Greenlund *et al.*, 1993). This molecule can bind to a specific receptor expressed on all nucleated cells (Valente *et al.*, 1992). The main sources for IFNγ are activated Th1 $CD4^+$ T cells (Mosmann and Coffman, 1989), NK cells and $CD8^+$ cytotoxic T cells (Sad, Marcotte and Mosmann, 1995).

IFNγ is a cell-mediated immunity factor (Paul and Seder, 1994) with a major immunoregulatory role such as in the differentiation of $CD4^+$ T cells to the Th1 phenotype (Paul and Seder, 1994). Briefly, stimulated cells, such as macrophages, produce IL-12 that induces production of IFNγ by naïve $CD4^+$ T cells in a positive feedback loop in which Th1 differentiation is maintained. Thus, the IFNγ produced from $CD4^+$ Th1 or IL-12-activated NK cells induces the production of IL-12 by macrophages creating a constant stimulus for Th1 differentiation. So, IFNγ appears to be a secondary cytokine to IL-12 which is a director of cell-mediated immunity (Trinchieri, 1995).

IFNγ also plays an important role in resistance to asexual blood stages of malaria by killing parasites through phagocytosis by activated macrophages (Ockenhouse and Shear, 1984). IFNγ also activates macrophages to release reactive oxygen molecules. Activated macrophages destroy intra-erythrocytic *P. yoelii* by oxygen-dependent mechanisms when incubated with supernatants containing IFNγ *in vitro* (Ockenhouse and Shear, 1984). Administration of recombinant IFNγ (rIFNγ) to mice infected with *P. c. adami* showed a more rapid degeneration of intra-erythrocytic parasites and a decline in the parasitaemia (Clark, 1987). These observations are consistent with the concept that T cell-dependent mediators are central to the immune responses in which parasites are killed inside circulating red blood cells. Stevenson and colleagues (1990) showed that neutralizing endogenous IFNγ impairs resistance to *P. chabaudi* AS infection. However, this neutralization does not completely abrogate resistance to the infection. A protective role for IFNγ has been demonstrated by infecting IFNγ gene knockout (GKO) C57BL/6 mice with *P. chabaudi* AS. These mice had higher morbidity and mortality compared to controls (Su and Stevenson, 2000; Yoneto *et al.*, 2001).

Choudhury *et al.* (2000) suggested that the immune response in mice infected with non-lethal *P. yoelii* is probably driven by an early production of IFNγ and TNFα depending on the presence of both NK and γδT cells and are essential for the control of acute phase of parasitaemia. Early IFNγ production also contribute to the control of the primary parasitaemia in self-resolving infections of non-lethal *P. yoelii* and *P. chabaudi* in BALB/c mice (Clark *et al.*, 1987; Hoffman, 1997; De Souza *et al.*, 1997). Early NK cell-mediated IFNγ production implicates in the control of asexual blood stages of *P. chabaudi* infection (Mohan, Moulin and Stevenson, 1997).

The principal pleiotropic cytokine of a $CD4^+$ Th2 response is IL-4, an approximately 20-kDa glycoprotein which acts on various components of the immune system such as T and B cells (Paul and Seder, 1994). The principal cellular source of IL-4 is the $CD4^+$ Th2 subset (Taylor-Robinson, 1995). IL-4 induces B cell differentiation and IgG1 production (Snapper and Paul, 1987). Stimulation of B cells with IL-4 results in an increase in expression of MHC class II (Noelle *et al.*, 1984) and IL-4 receptor molecules (Ohara and Paul, 1988). IL-4 enhances antigen presentation by macrophages (Stuart, Zlotnik and Woodward, 1988; Zlotnik *et al.*, 1987), promotes proliferation of precursors of cytotoxic T cells, and thier differentiation into CTL (Trenn *et al.*, 1988; Widmer and Grabstein, 1987).

It is suggested that susceptibility may direct to induce different Th type response in resistant and susceptible mice during acute phase of infection (Jacobs, Radzioch and Stevenson, 1995). During the acute phase, the spleen cells from susceptible mice produce high levels of IL-4 and IL-10, and low levels of IFNγ in *P. chabaudi* infection (Jacobs, Radzioch and Stevenson, 1995; Langhorne, 1989). It has been shown that early IL-4 production in susceptible A/J mice (Jacobs, Radzioch and Stevenson, 1995) was coincident with severe and fatal acute primary parasitaemia (Stevenson and Tam, 1993). Jacobs and colleagues (1995) also reported that addition of recombinant murine IL-4 or IL-10 diminished the ability of splenic macrophages recovered from mice infected with *P. chabaudi* AS to produce NO in response to lipopolysaccaride (LPS), showing that a Th2 response early in infection may suppress NO production by splenic macrophages and leave mice more susceptible to the infection. In contrast, it seems that there is a correlation between resistance and early IL-12 production through

direct activation of DC by the parasite, *P. chabaudi*AS (Seixas *et al.*, 2001) which colud be followed by an early Th1 response development.

Inducing both Th1 and Th2 responses through immunization is an important aim for malaria control (i.e. vaccination against the parasite) because both humoral and cellular responses are required for resolution of infection with *P. chabaudi* (Langhorne, Quin and Sanni, 2002; Weidanz *et al.*, 1999). Moreover, Rainczuk *et al.* (2003) showed that an expression library immunization (ELI) of BALB/c mice resulted in both IFNγ and IL-4 production when mice challenged with lethal *P. c. adami* DS as a stringent test for evaluation of vaccine efficacy (Anders *et al.*, 1998; Crewther *et al.*, 1996; Smooker *et al.*, 2000).

The experiments presented in this chapter were carried out to determine and compare the immune response induced in NIH mice against the avirulent DK and the virulent DS strains of *P. c. adami* which are genetically close, but differ in virulence in rodent malaria infections.

The influence of virulence on the course of single-infections of lethal or non-lethal malaria parasites was also studied. The effect of a low infective dose on the course of infection in the virulent *P. c. adami* DS infection was also examined.

3.2. Results

3.2.1. Parasitaemia

3.2.1.1. *P. c. adami* DK

The parasitaemia was monitored daily. The blood smears were usually collected between 9:30 to 11:30 am from the tail. In all experiments, parasitaemia is expressed both as mean percentage and the log geometric mean parasitaemia (Log_{10} pRBCs for 10^5 RBCs) which better indicated presence of the parasites at very low density.

Figure 3.1. shows the course of infection in NIH mice infected with 1×10^5 pRBCs of *P. c. adami* DK i.v. The infection was followed for 64 days. The peak parasitaemia, with a maximum of 23.85%, which represents the acute phase of the infection, was on day 10-post infection (p.i.). The

parasitaemia then quickly decreased to sub-patent levels on day 15 p.i. A recrudescent parasitaemia was seen between days 38 and 43 p.i. No parasites were observed after this time up to the last day of the experiment.

3.2.1.2. *P. c. adami* DS (in sub-curative treated mice)

Figure 3.2 shows the parasitaemia in mice were infected i.v. with 1×10^4 pRBCs of *P. c. adami* DS. Mice were treated with a sub-curative dose of chloroquine at 24 mg/Kg on day 8 p.i. The infection was followed until the day 50 p.i. The peak parasitaemia was observed on day 8 p.i., the day in which mice were treated, with a maximum of 46.08%. Parasitaemia declined to sub-patent levels on day 15 p.i. A recrudescence was detected between days 18 and 24 p.i (Figure 3.2a and 3.3a).

3.2.1.3. *P. c. adami* DS (in untreated mice)

To compare the course of infection and immune responses between treated and untreated mice with the virulent infection, mice were infected with 1×10^4 pRBCs of *P. c. adami* DS and were not treated with chloroquine. The parasitaemia peaked to 68.73% on day 10 p.i. and a recrudescent parasitaemia was observed between days 21 and 25 p.i. There was a significantly higher peak parasitaemia (t-test $P < 0.005$) in mice left untreated compared to the treated group (Figure 3.2b and 3.3b).

To determine the effect of a low infective dose on the course of the virulent infection, six NIH mice were infected i.v. with 2×10^3 pRBCs of *P. c. adami* DS (Figure 3.4). The peak parasitaemia was 54.45% on day 10 p.i. The first day in which parasite was seen was day 5 p.i. This shows that there was a one day delay compared to mice infected with the higher infective dose (Figures 3.2. 3.3 and 3.4). The pre-patent period was extended in mice infected with a reduced infective dose. The number of mice died will be discussed below (see % Survival rate).

3.2.1.4. % Survival rate

Survival curves are used as a measure of the outcome of infection. For example, the effect of virulence on the outcome of infection can be evaluated when similar hosts are infected with two parasites which differ in virulence. In this regard, survival curves show delay in death and host's survival versus death. Survival curves may also be used as a relative measure in evaluation of efficacy of a vaccine candidate (Jacobs, Radzioch and Stevenson, 1996; Omer and Riley, 1998; Rainzuk *et al.*, 2003). Survival curves are usually expressed as the percentage of survivors (% survival). Here, the % survivals were calculated to present the outcome of virulent *P. c. adami* DS infections when the mice were either treated, left untreated or infected with a low infective dose.

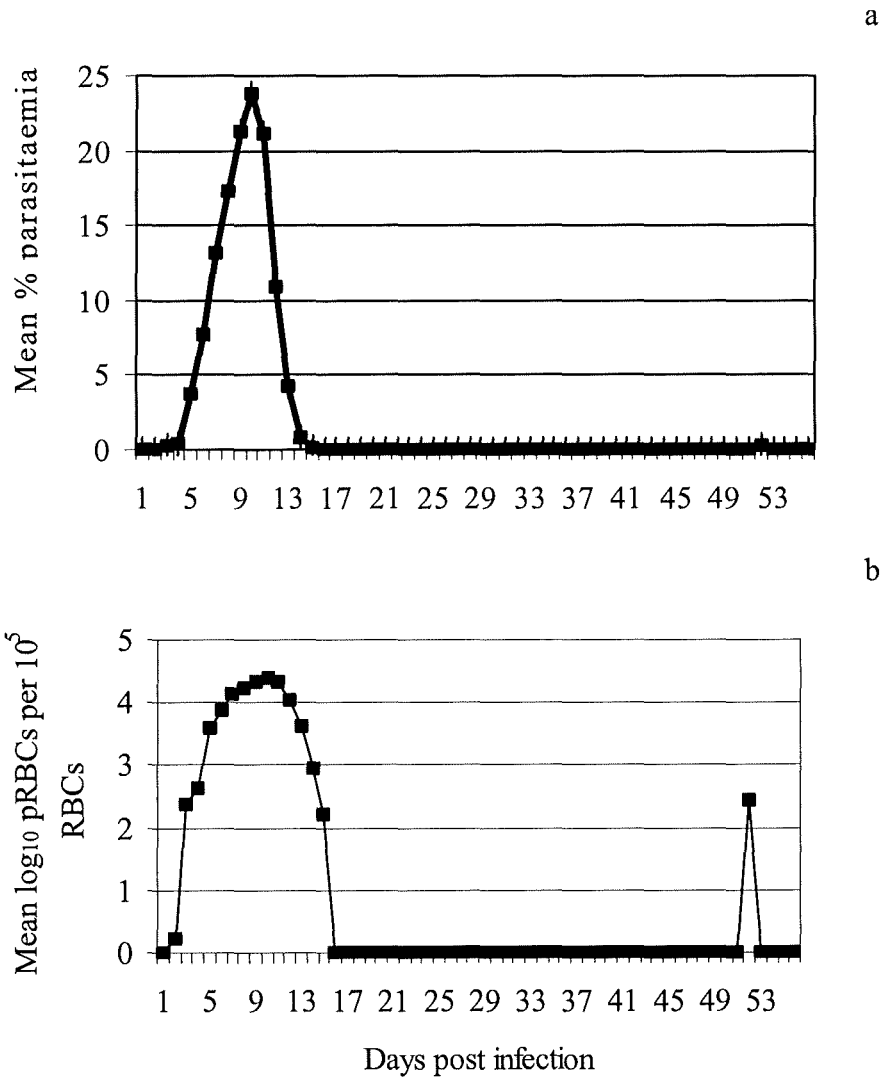


Figure 3.1. The course of infection in NIH mice infected with 1×10^5 pRBCs of *P. c. adami* DK. Sample size was 21 mice.

Each data point is a mean % of parasitaemia. For each time point ± SEM has been calculated (For details see 2.21). However, SEM is not shown when it is too small.

a) Mean percentage of parasitaemia. The peak parasitaemia was 23.85 % on day 10 p.i.

b) The mean $\log_{10}$ of parasitaemia per 1×10^5 pRBCs.

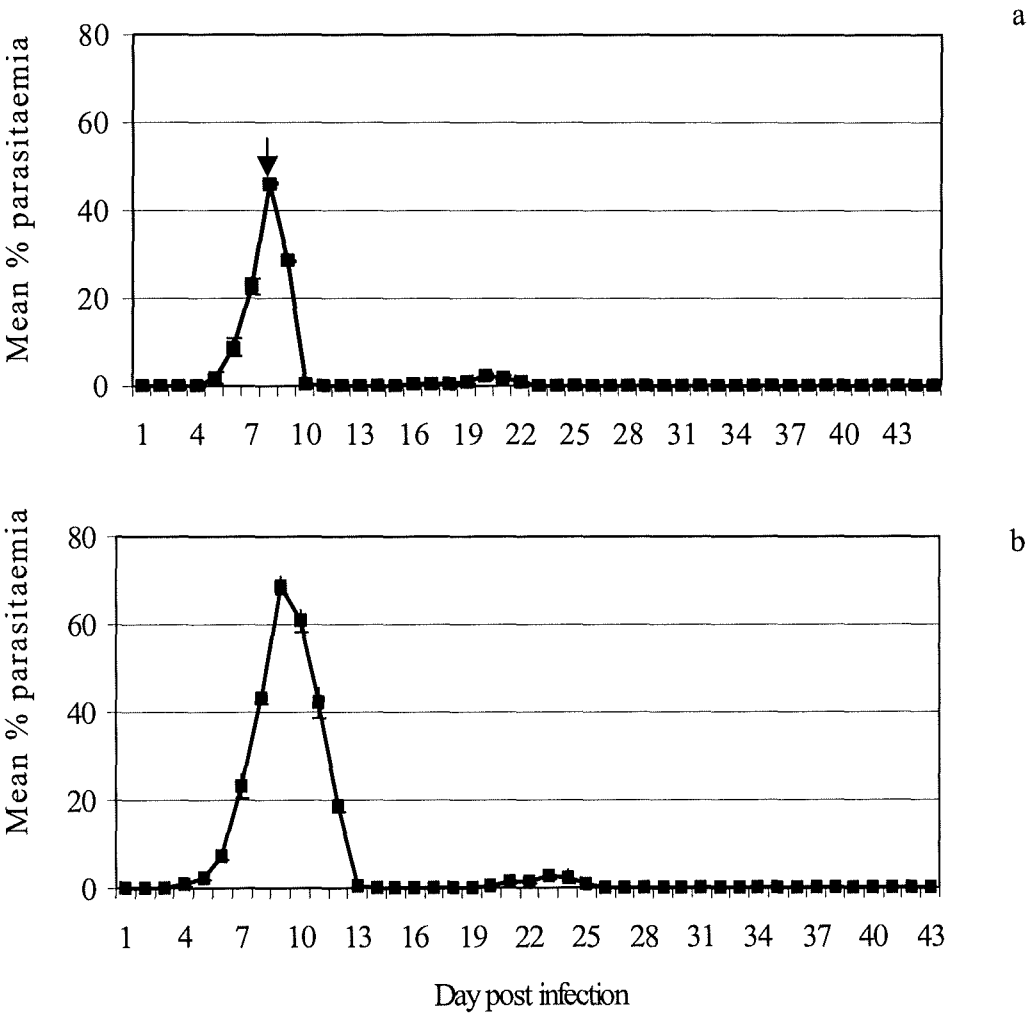


Figure 3.2. The course of infection in NIH mice infected with *P. c. adami* DS.

Each data point is a mean % of parasitaemia. For each time point ± SEM has been calculated (For details see 2.21). However, SEM is not shown when it is too small.

a) Mice infected with 1×10^4 pRBCs and treated with a sub-curative dose of chloroquine at 24mg/Kg on day 8 p.i as the arrow shows. The sample size was 21 mice. The peak parasitaemia was 46.08 % on day 8 p.i. A recrudescent parasitaemia was seen between day 18 and 24 p.i.

b) Mice infected with 1×10^4 pRBCs of *P. c. adami* DS and left without treatment. The sample size was 21 mice. Six mice were killed before the peak parasitaemia and nine mice died thereafter. The peak parasitaemia was 68.73 % on day 10 p.i. A recrudescent parasitaemia was seen between days 21 and 25 p.i.

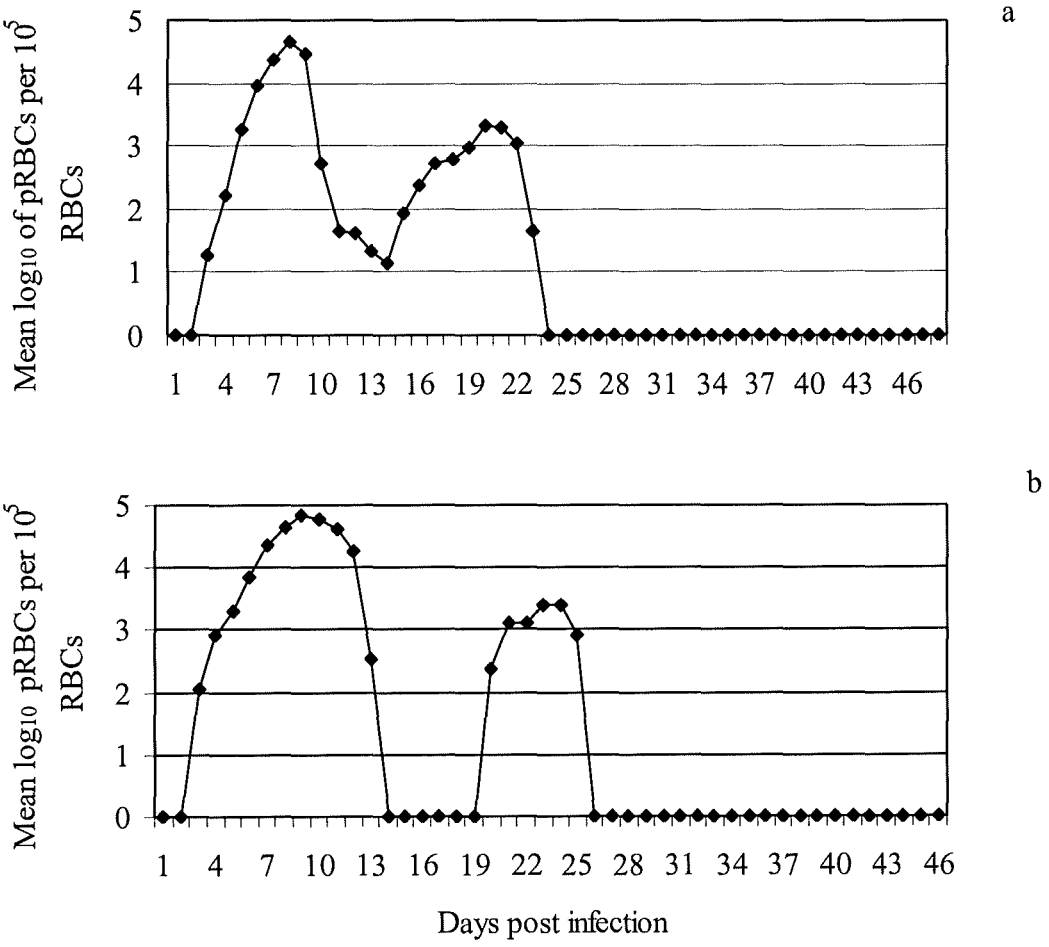


Figure 3.3. The course of infection in NIH mice infected with *P. c. adami* DS.

The mean $\log_{10}$ of parasitaemia per 1×10^5 pRBCs is shown.

a) Mice infected with 1×10^4 pRBCs and treated with sub-curative dose of chloroquine at 24mg/Kg on day 8 p.i. as the arrow shows.

b) Mice infected with 1×10^4 pRBCs of *P. c. adami* DS and left without treatment.

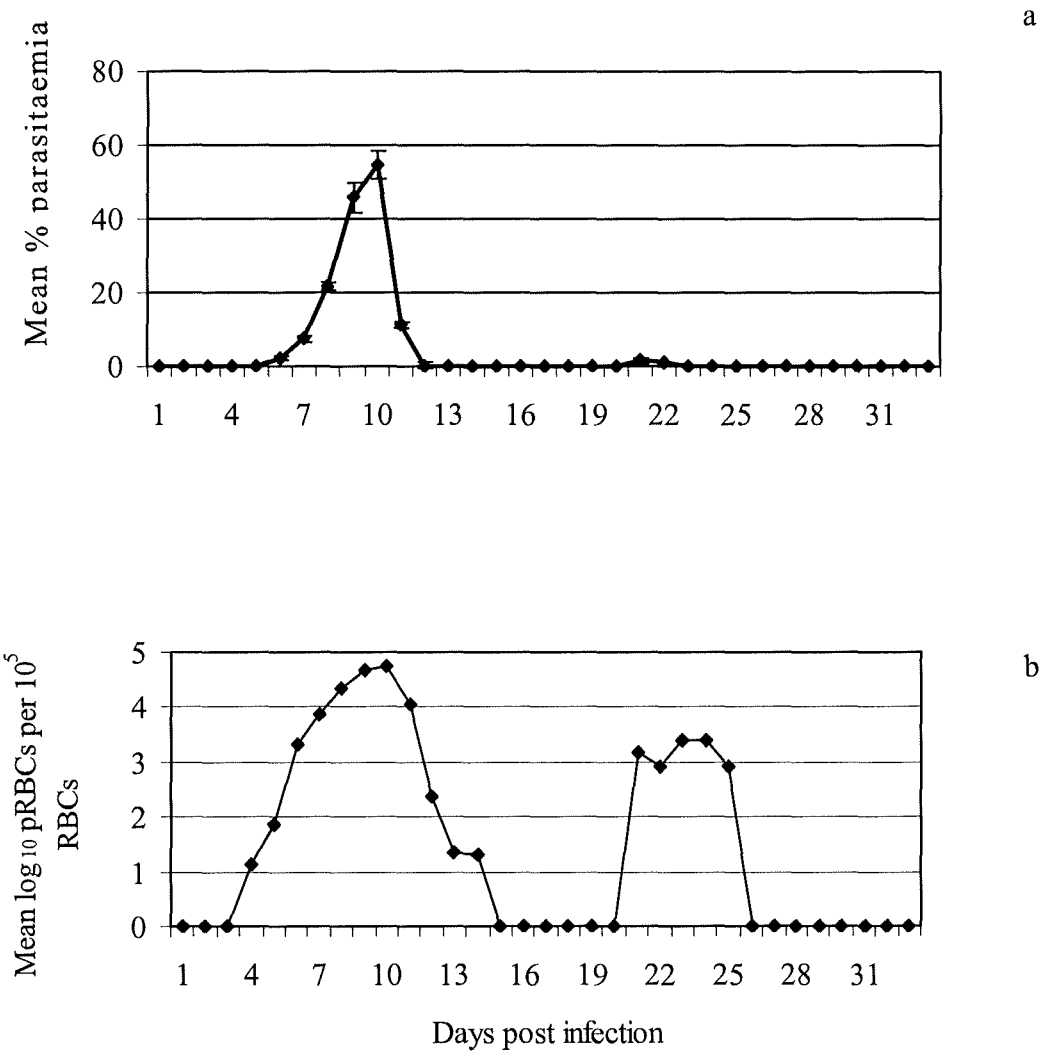


Figure 3.4. The course of infection in NIH mice infected with a low infective dose, 2×10^3 pRBCs *P. c. adami* DS. The sample size was 6 mice. Each data point is shown as the mean % of parasitaemia. For each time point ± SEM has been calculated (For details see 2.21). However, SEM is not shown when it is too small.

a) Mean % of parasitaemia. The peak parasitaemia was 54.45 % on day 10 p.i. Two mice of six died.

b) Mean $\log_{10}$ pRBCs per 1×10^5 RBCs.

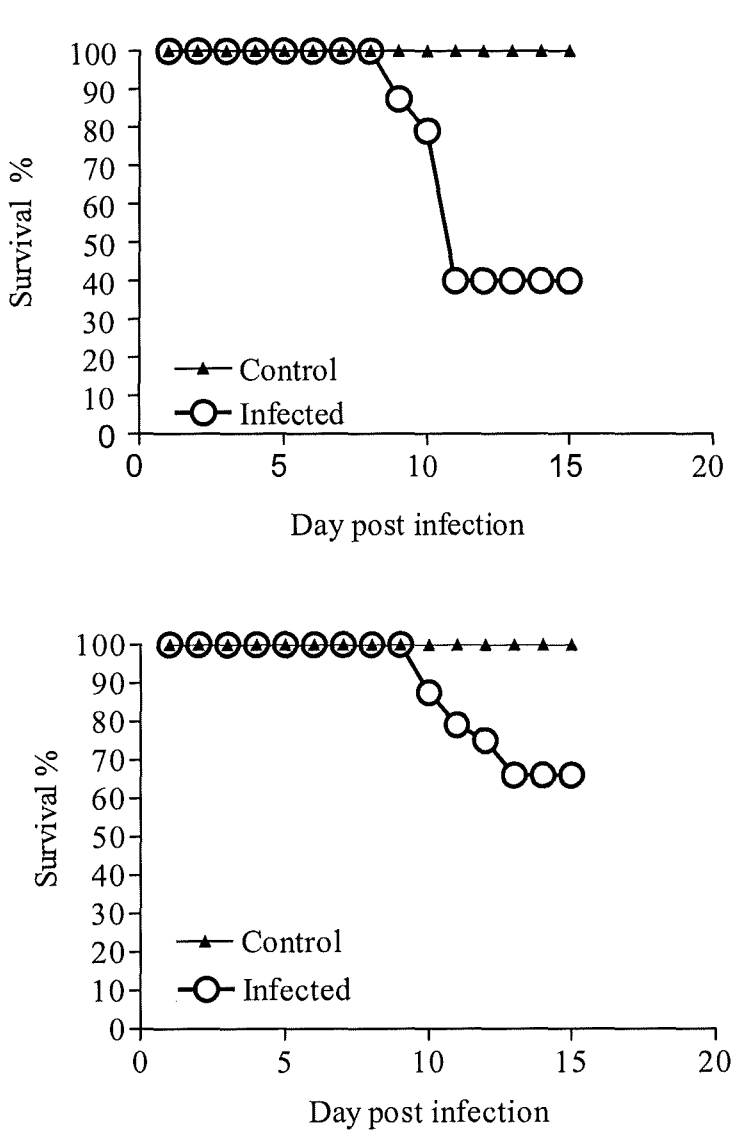


Fig. 3.5. Percentage survival in mice infected with *P. c. adami* DS.

a) Mice infected with 1×10^4 pRBCs. The course of infection was monitored for 50 days. Twenty one mice were infected initially, of which six mice were killed for spleen cell culture and were withdrawn from the calculation for % survival. From the 15 remaining mice nine died. The % survival was 40 %.

b) Six mice were infected with 2×10^3 pRBCs. The course of infection was monitored for 58 days p.i. Two mice died and % survival was 66%.

There were no deaths in the avirulent *P. c. adami* DK infections. In the virulent infections no death was seen in the treated group. In the untreated group 21 mice were infected with *P. c. adami* DS. Six mice were killed before the peak parasitaemia for cytokine analysis using spleen cultures. Therefore, they were withdrawn from the calculation for % survival. From the 15 remaining 9 mice died and 40% of mice survived (Figure 3.5, a). However, in another control group in which 6 mice infected with the same pRBCs only one mouse survived (16.5% survival rate, data not shown). In the low-infective dose infection two mice died on day 13 p.i. which shows a three-day delay in death compared to the untreated mice which were infected with a higher infective dose. The % survival for six mice infected with a low infective dose was 66.% (two of six mice, Figure 3.5, b).

3.2.2. Splenocyte proliferation

The proliferative response of the splenocytes was evaluated for splenocytes cultured from infected and naïve mice as previously described in Chapter Two. Splenocytes from both infected and naïve mice were cultured at different time points as indicated in Figures 3.6 to 3.8. The suspensions of spleen cells at 5×10^5 cells/well (100µ) from each mouse were incubated *in vitro* with pRBCs, naïve RBCs, Con A, or medium only. They were pulsed with 0.5 µCi (37 KBq) of tritiated [^{3}H] methyl thymidine and the proliferative response of the splenocytes in all groups was determined as described in Chapter Two.

3.2.2.1. Splenocyte proliferation in *P. c. adami* DK infection

The proliferative responses were evaluated after 72 hours of culturing splenocytes. This experiment was carried out at each time point at which splenocytes were cultured for cytokine analysis. The experiment showed that (Figure 3.6.) the spleen cells from naïve mice which were exposed to ConA had the highest (Tukey's test, $P < 0.0001$) proliferative response compared to all other groups for all time points during the experiment. The

second highest responses were observed in splenocytes from infected mice exposed to ConA and the third highest levels of responses were seen for splenocytes from infected mice which were restimulated with pRBCs. The control groups, except splenocytes exposed to ConA, included splenocytes from infected mice exposed to medium only, splenocytes from infected mice exposed to naïve RBCs, splenocytes from naïve mice exposed to either pRBCs or RBCs.

3.2.2.2. Splenocyte proliferation in *P.c. adami* DS infections

The proliferative responses in mice infected with *P. c. adami* DS and treated or left untreated were examined (Figures 3.7 and 3.8). The highest (Tukey's test, $P < 0.0002$) levels of proliferation compared to other groups were seen in splenocytes from naive mice exposed to ConA, the second highest responses were observed in splenocytes from infected mice exposed to ConA, and the third highest levels were also seen in splenocytes from infected mice restimulated with pRBCs as described above (3.2.2.1). However, the results showed that in untreated mice, the proliferative responses were lower (Tukey's test P, < 0.001) than that in mice were treated at the peak parasitaemia.

3.2.2.3. Stimulation index (SI)

The stimulation index is the ratio of the p.r. between the test and control groups as described in Chapter Two. Generally, the results showed that lower responses were observed after resolution of the peak parasitaemia and they rose again after the recrudescent parasitaemia (Figures 3.9 and 3.10). However, as shown above at the peak parasitaemia, the proliferative responses were low compared to other time points. In the virulent infections the stimulation indices in the treated and untreated mice were significantly different (Two-way ANOVA, $P < 0.005$). Splenocytes from mice infected with the virulent DS and treated showed the lowest levels compared to the other groups in these experiments (Figures 3.9 and 3.10).

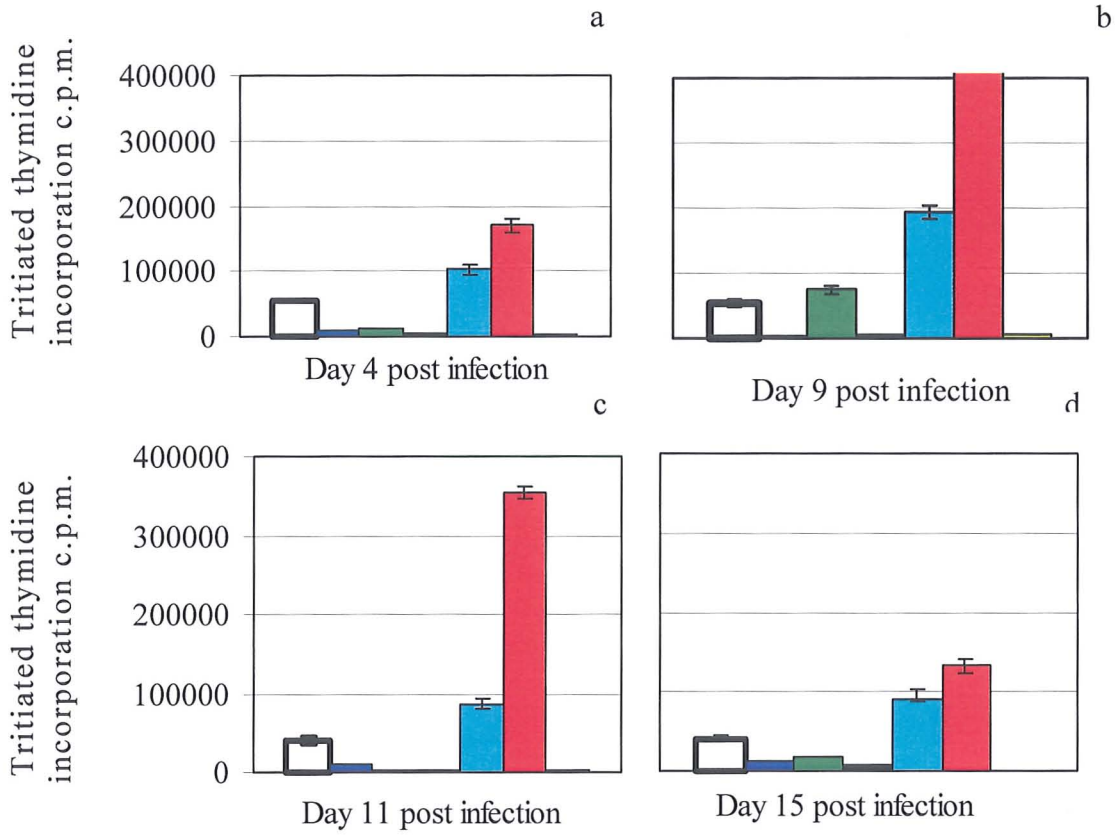


Figure 3.6. (a to d). The proliferation responses of splenocytes from mice infected with 1×10^5 pRBCs of *P. c. adami* DK and naïve control mice. Three mice were sacrificed at each time point and splenocytes of each mouse (5×10^6 cells/ml) were separately cultured and stimulated with 5×10^5 of pRBCs/well, 5×10^5 of RBC/well, ConA at 0.5 µg/ml, or medium only. Before harvesting all suspensions were pulsed with tritiated thymidine for 12-18 hours. Each data point is the mean ± SEM for an experiment performed in triplicate. However, SEM is not shown when it is too small.

- p+f : Splenocytes from infected mice exposed to pRBCs.
- m+f: Splenocytes from infected mice cultured in medium only.
- r+f: Splenocytes from infected mice exposed to RBCs.
- p+c: Splenocytes from naive mice exposed to pRBCs.
- r+c: Splenocytes from naïve mice exposed to naive RBCs.
- ConA+f: Splenocytes from infected mice stimulated with ConA.
- ConA+c: Splenocytes from naïve mice stimulated to ConA.

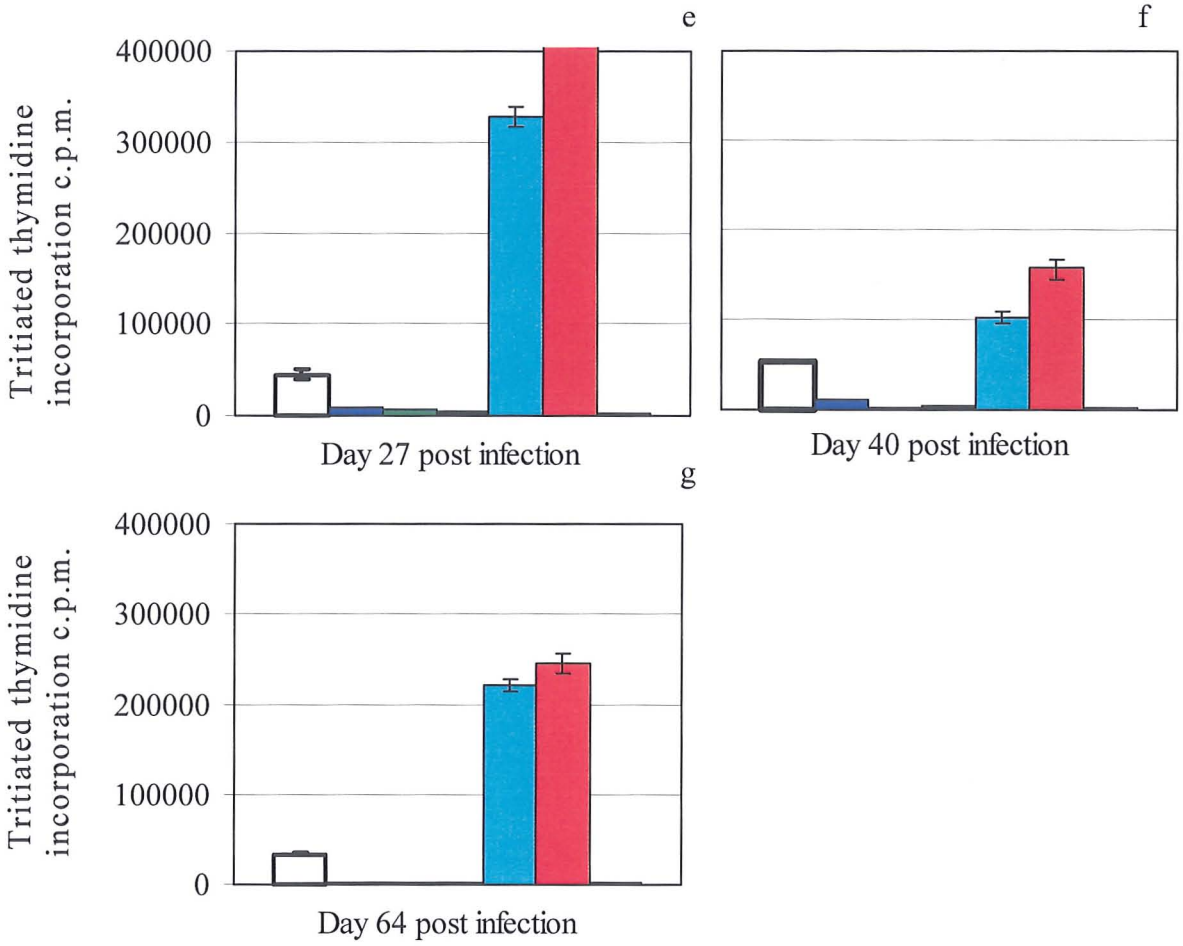


Figure 3.6. (e to g). The proliferation responses of splenocytes from mice infected with 1×10^5 pRBCs of *P. c. adami* DK and naïve control mice. Three mice were sacrificed at each time point and splenocytes of each mouse (5×10^6 cells/ml) were separately cultured and stimulated with 5×10^5 of pRBCs/well, 5×10^5 of RBC/well, ConA at 0.5 µg/ml, or medium only. Before harvesting all suspensions were pulsed with tritiated thymidine for 12-18 hours. Each data point is the mean ± SEM for an experiment performed in triplicate. However, SEM is not shown when it is too small.

- p+f : Splenocytes from infected mice exposed to pRBCs.
- m+f: Splenocytes from infected mice cultured in medium only.
- r+f: Splenocytes from infected mice exposed to RBCs.
- p+c: Splenocytes from naive mice exposed to pRBCs.
- r+c: Splenocytes from naïve mice exposed to naive RBCs.
- ConA+f: Splenocytes from infected mice stimulated with ConA.
- ConA+c: Splenocytes from naïve mice stimulated to ConA.

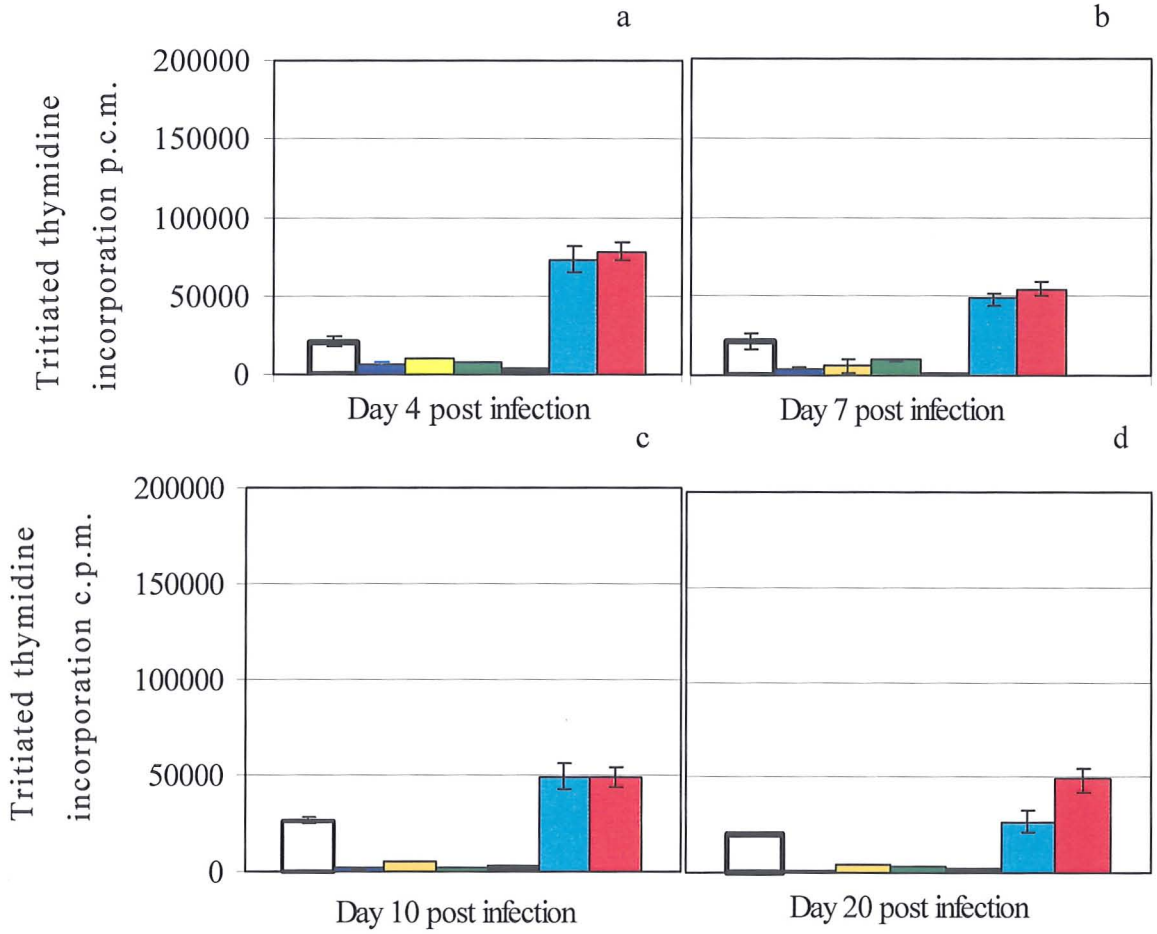


Figure 3.7. (a to d). The proliferative responses of splenocytes from mice infected with 1×10^4 pRBCs of *P. c. adami* DS or naïve mice.
Mice were treated with sub-curative dose of chloroquine. Three mice were sacrificed at each time point and splenocytes of each mouse (5×10^5cells/well) were separately cultured and stimulated with 5×10^5 pRBCs/well, 5×10^5 RBC/well, ConA at 0.5 µg/ml, or medium only. Before harvesting all suspensions were pulsed with tritiated thymidine for 12-18 hours. Each data point is the mean ± SEM for an experiment performed in triplicate. However, SEM is not shown when it is too small.

- p+f: Splenocytes from infected mice exposed to pRBCs of the parasite.
- m+f: Splenocytes from infected mice in medium only.
- r+f : Splenocytes from infected mice exposed to RBCs.
- p+c: Splenocytes from naïve control mice exposed to pRBCs.
- r+c: Splenocytes from naïve mice exposed to RBCs.
- ConA+f: Splenocytes from infected mice exposed to ConA.
- ConA+c: Splenocytes from naïve mice stimulated with ConA.

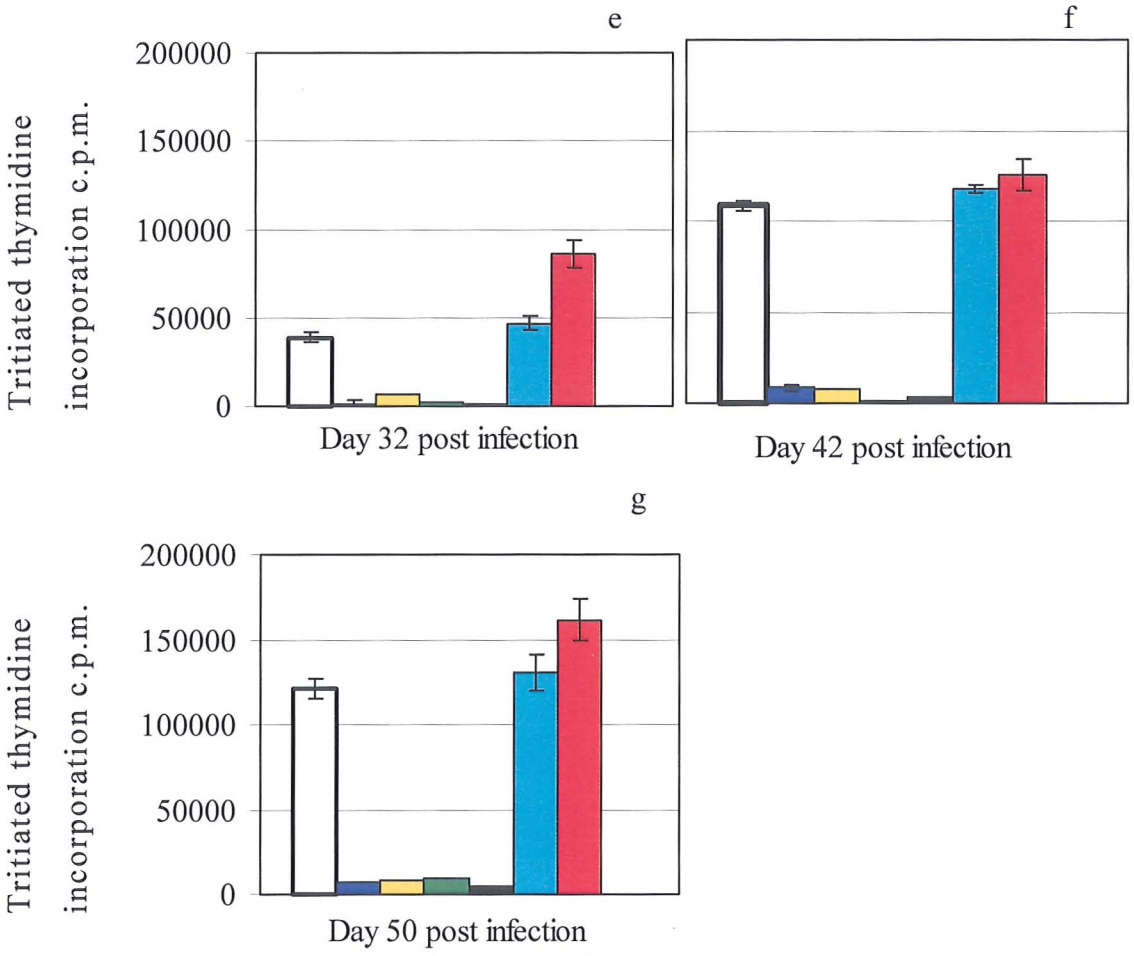


Figure 3.7. (e to g). The proliferative responses of splenocytes from mice infected with 1×10^4 pRBCs of *P. c. adami* DS and naïve mice.
Mice were treated with sub-curative dose of chloroquine. Three mice were sacrificed at each time point and splenocytes of each mouse (5×10^6cells/ml) were separately cultured and stimulated with 5×10^5 pRBCs/well, 5×10^5 RBC/well, ConA at 0.5 µg/ml, or medium only. Before harvesting all suspensions were pulsed with tritiated thymidine for 12-18 hours. Each data point is the mean ± SEM for an experiment performed in triplicate. However, SEM is not shown when it is too small.

- p+f: Splenocytes from infected mice exposed to pRBCs of the parasite.
- m+f: Splenocytes from infected mice in medium only.
- r+f : Splenocytes from infected mice exposed to RBCs.
- p+c: Splenocytes from naïve control mice exposed to pRBCs.
- r+c: Splenocytes from naïve mice exposed to RBCs.
- ConA+f: Splenocytes from infected mice exposed to ConA.
- ConA+c: Splenocytes from naïve mice stimulated with ConA.

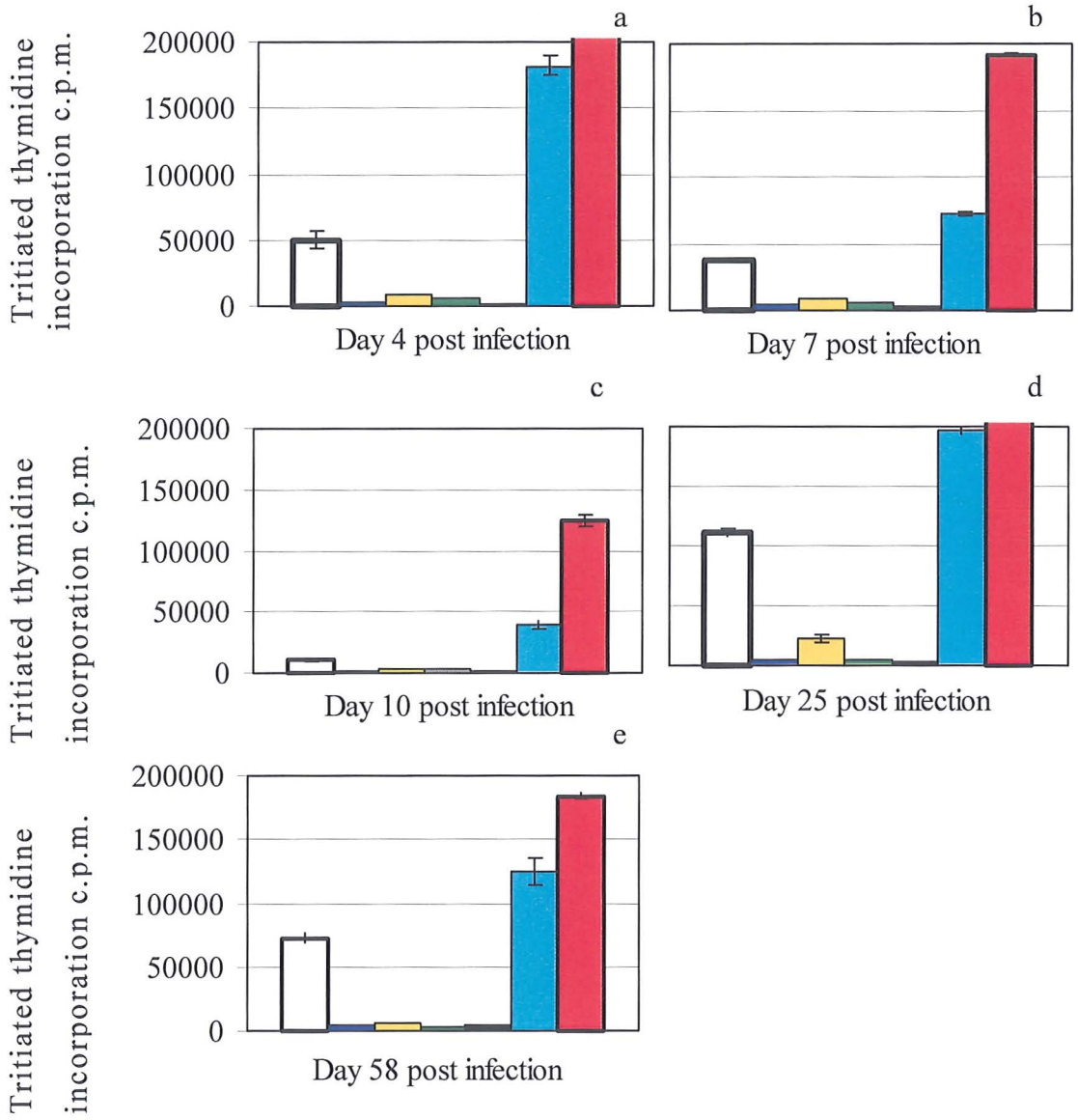


Figure 3.8. (a to e). The proliferation responses of splenocytes from untreated mice infected with 1×10^4 pRBCs of *P. c. adami* DS and naïve mice. Three mice were sacrificed at each time point and splenocytes of each mouse were separately cultured and stimulated with 5×10^5 of pRBCs/well, 5×10^5 of RBC/well, ConA at 0.5 µg/ml, or medium only. Before harvesting all suspensions were pulsed with tritiated thymidine for 12-18 hours. Each data point is the mean ± SEM for an experiment performed in triplicate. However, SEM is not shown when it is too small.

- p+f : Splenocytes from infected mice exposed to pRBCs.
- m+f : Splenocytes from infected mice in medium only.
- r+f: Splenocytes from infected mice exposed to RBCs.
- p+c: Splenocytes from naïve control mice exposed to pRBCs.
- r+c: Splenocytes from naïve mice exposed to RBCs.
- ConA+f: Splenocytes from infected mice stimulated to ConA.
- ConA+c: Splenocytes from naïve mice stimulated to ConA.

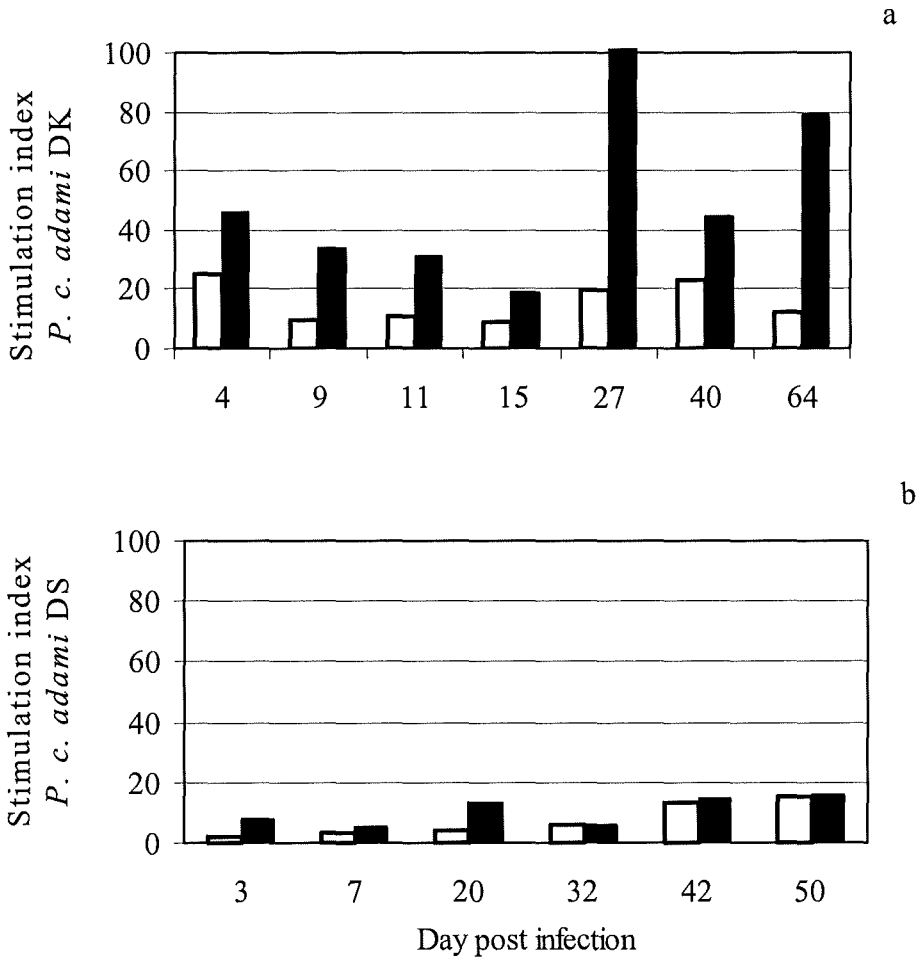


Figure 3.9. Stimulation index (SI) for proliferative responses.
Three mice were sacrificed at each time point and their splenocytes were cultured and pulsed with tritiated thymidine for 12-18 hours before harvesting.

□ p+f/m+f: The proliferative response of the spleen cells from infected mice exposed to pRBCs divided by the proliferative response of spleen cells from infected mice cultured in medium only.

■ ConA+f/m+f: The proliferative response of the spleen cells from infected mice exposed to ConA divided by the proliferative response of the spleen cells from infected mice cultured in medium only.

a) SI for mice infected with 1×10^5 pRBCs of *P. c. adami* DK

b) SI for mice infected with 1×10^4 pRBCs of *P. c. adami* DS. Mice treated with a sub-curative dose of chloroquine on day 8 p.i.

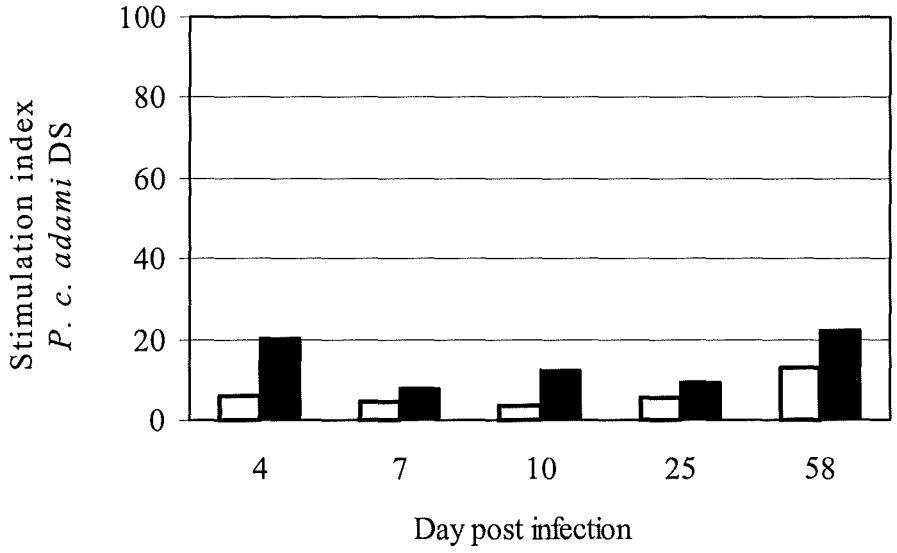


Figure 3.10. Stimulation index for mice infected with 1×10^4 pRBCs of *P. c. adami* DS.

Mice were not treated. Three mice were sacrificed at each time point and their splenocytes were cultured and pulsed with tritiated thymidine for 12-18 hours before harvesting.

□ p+f/m+f: The proliferative response of spleen cells from infected mice exposed to pRBCs divided by the proliferative response of spleen cells from infected mice cultured in medium only.

■ ConA+f/m+f: The proliferative response of spleen cells from the infected mice exposed to ConA divided by the proliferative response of spleen cells from infected mice cultured in medium only.

3.2.3. Cytokine analysis

The IFNγ and IL-4 concentrations were measured in supernatants from *in vitro* cultures of splenocytes derived from mice during the course of infections of the virulent and the avirulent *P. c. adami* parasites. Three mice from each group were sacrificed and splenectomized at each time point. Naïve mice were also killed and splenectomized at the same time. Spleen cells were separately cultured as previously described in Chapter Two and supernatants of each culture were obtained after three days (72 hours) and examined for the cytokines as described in Chapter Two.

3.2.3.1. IFNγ in *P. c. adami* DK and *P. c. adami* DS infections

The present results showed that in mice infected with the avirulent DK strain there was an early production of IFNγ with the highest level on the first sampling day, day 4 p.i, and it remained at high levels until the peak parasitaemia (Figure 3.11). On day 11 p.i., a day after the peak parasitaemia, IFNγ sharply declined (Figure 3.11). IFNγ levels gradually increased thereafter, particularly after the recrudescence (Figure 3.11). The results showed that the levels of IFNγ significantly were different (Two-way ANOVA, $P < 0.0001$) between all tested groups. The levels of IFNγ in infected mice were significantly higher (Tukey's test, $P < 0.0001$) compared to its levels in naïve control mice on the first sampling day (Figure 3.11). In mice infected with *P. c. adami* DS, and treated with chloroquine, the highest level of IFNγ was seen in the cultures of spleen cells recovered on the first sampling day, day 3 p.i. The lowest level was measured on day 10 p.i. which was coincident with the peak parasitaemia. Increases in IFNγ levels were observed after the recrudescent parasitaemia (Figure 3.12). The production of IFNγ in the untreated mice infected with the DS strain was also examined. In this group, the highest level of IFNγ was recorded on the first sampling day, on day 4 p.i. The level of IFNγ was at the lowest level at peak parasitaemia. It increased later after the recrudescence (Figure.3.13).

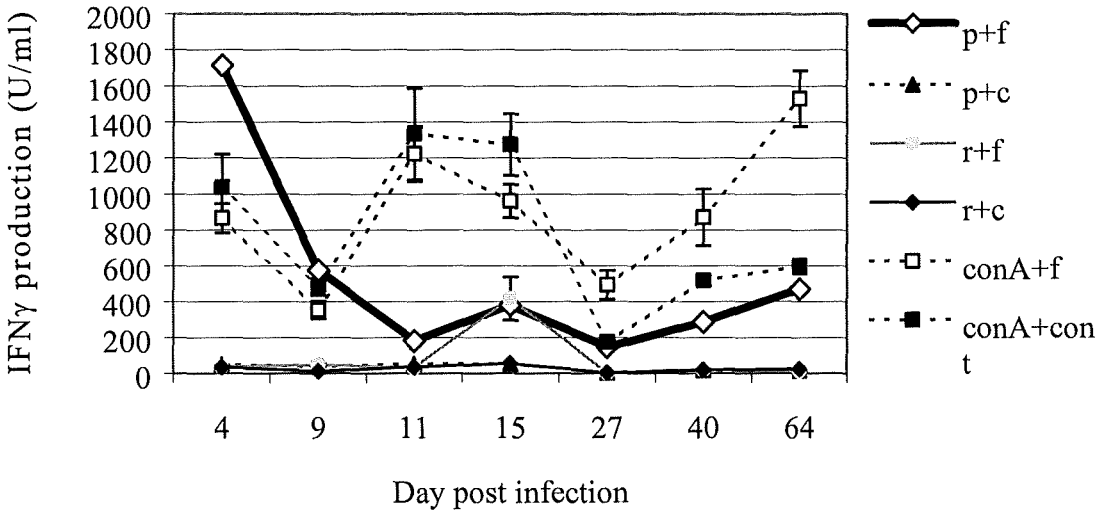


Figure 3.11. IFNγ production in NIH mice infected with 1×10^5 *P. c. adami* DK.

The course of infection was monitored for 64 days. At each time point three mice were sacrificed and their spleens were individually cultured. Supernatants from splenocytes cultures for each mouse were assayed separately by ELISA. Each data point is the mean ± SEM for an experiment performed in triplicate. However, SEM is not shown when it is too small.

p+f: Supernatant from infected mouse splenocytes culture exposed to pRBCs.

p+c: Supernatant from naïve mouse splenocytes culture exposed to pRBCs.

r+f: Supernatant from infected mouse splenocytes culture exposed to naïve RBCs.

r+c: Supernatant from naïve mouse splenocytes culture exposed to naïve RBCs.

ConA+f: Supernatant from infected mouse splenocytes culture exposed to ConA.

ConA+c: Supernatant from naïve mouse splenocytes culture exposed to ConA.

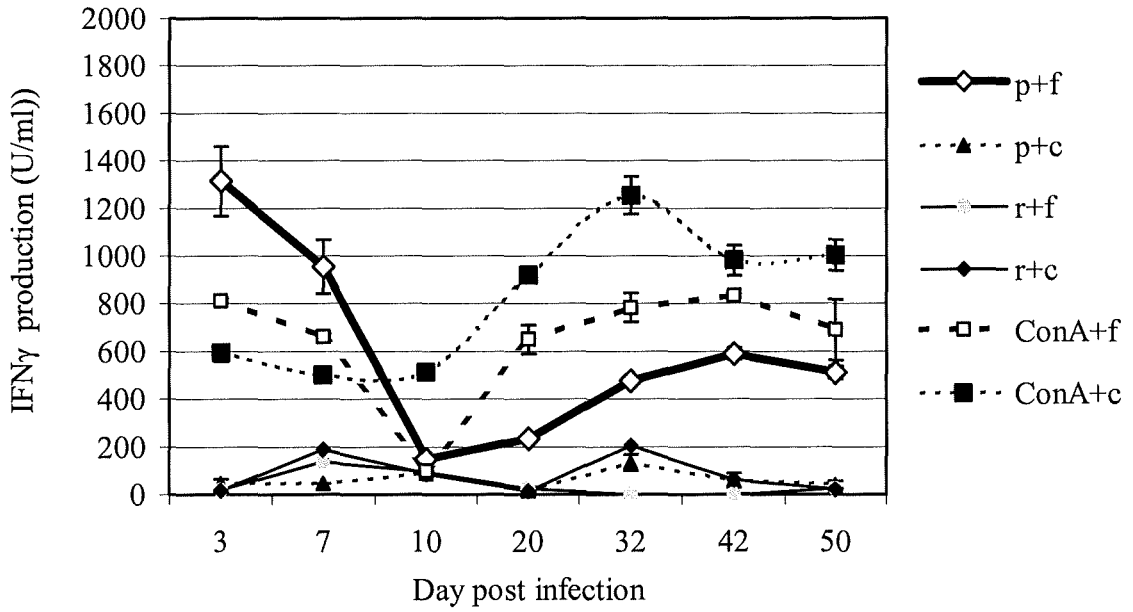


Figure 3.12. IFNγ production in NIH mice infected with 1×10^4 pRBCs *P. c. adami* DS.

Mice were treated with a sub-curative dose of chloroquine (24mg/Kg). The course of infection was monitored for 50 days. At each time point three mice were sacrificed and their spleens were individually cultured. Supernatant of splenocytes cultures for each mouse was separately assayed by ELISA.

Each data point is the mean ± SEM for an experiment performed in triplicate. However, SEM is not shown when it is too small.

p+f: Supernatant from infected mouse splenocytes culture exposed to pRBCs.

p+c: Supernatant from naïve mouse splenocytes culture exposed to pRBCs.

r+f: Supernatant from infected mouse splenocytes culture exposed to RBCs.

r+c: Supernatant from naïve mouse splenocytes culture exposed to RBCs.

ConA+f: Supernatant from infected mouse splenocytes culture exposed to ConA.

ConA+c: Supernatant from naïve mouse splenocytes culture exposed to ConA.

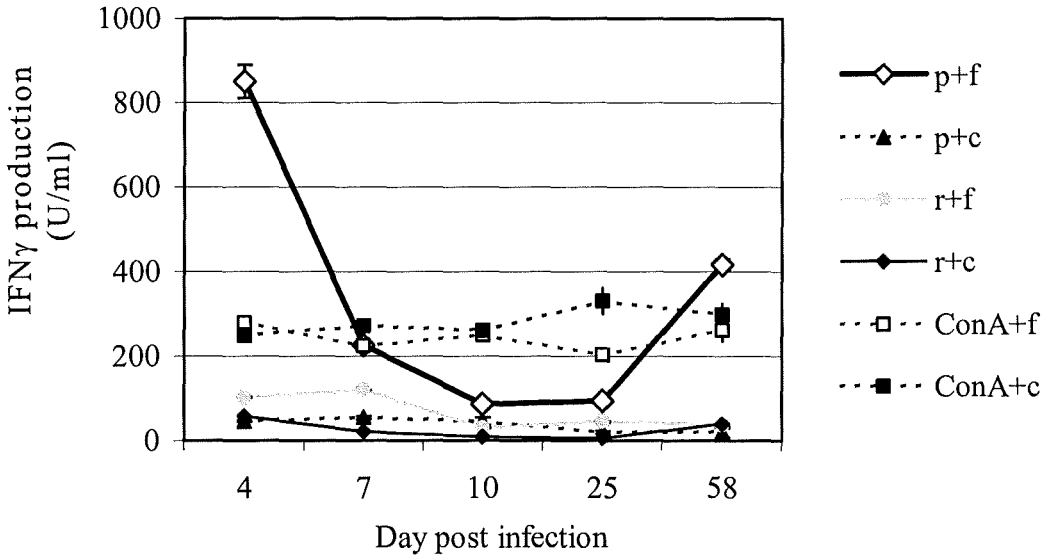


Figure 3.13. IFNγ production in NIH mice infected with 1×10^4 pRBCs. *P. c. adami* DS.

Mice left untreated. The course of infection was monitored for 58 days. At each time point three mice were sacrificed and their spleens were individually cultured. Supernatant of splenocytes cultures for each mouse was assayed separately by ELISA. Each data point is the mean ± SEM for an experiment performed in triplicate. However, SEM is not shown when it is too small.

p+f: Supernatant from infected mouse splenocytes culture exposed to pRBCs.

p+c: Supernatant from naïve mouse splenocytes culture exposed to pRBCs.

r+f: Supernatant from infected mouse splenocytes culture exposed to naïve RBCs.

r+c: Supernatant from naïve mouse splenocytes culture exposed to naïve RBCs.

ConA+f: Supernatant from infected mouse splenocytes culture exposed to ConA.

ConA+c: Supernatant from naïve mouse splenocytes culture exposed to ConA.

3.2.3.2 IL-4 production in *P. c. adami* DK and *P. c. adami* DS infections

The present results demonstrated that in both avirulent and virulent malaria infections, IL-4 levels increased later during the observation period. In the early period of observation, in both virulent and avirulent infections, IL-4 did not increase above background control levels (Figures 3.14, 3.15 and 3.16).

The present data showed that in the DK-infected mice, there was initially a gradual increase in IL-4 production and then a sharp increase when the infection became chronic. The highest level was detected on day 64 p.i which was significantly higher (Tukey's test, $P < 0.001$) compared to the IL-4 levels for all time points except day 40 p.i. (Figure 3.14).

In mice infected with *P. c. adami* DS, and treated with chloroquine, IL-4 production gradually increased and the highest level was measured on the last sampling day, day 50 p.i. Increases were detected after day 32 p.i. which were after the recrudescent parasitaemia and when the infection had become sub-patent. There was significantly higher IL-4 production (Tukey's test, $P < 0.0001$) for splenocytes from infected mice restimulated with pRBCs for days 42 and 50 p.i. compared to IL-4 level from splenocytes derived from naïve mice and exposed to pRBCs at the same time (Figure 3.15).

In the untreated mice surviving from infection with *P. c. adami* DS, IL-4 production had the same profile as shown above. However, in comparison with the treated group, the IL-4 production had higher levels in the first sampling days and it was significantly lower (Tukey's test, $P < 0.0001$) than that in the last two sampling days (Fig 3.15 and 3.16).

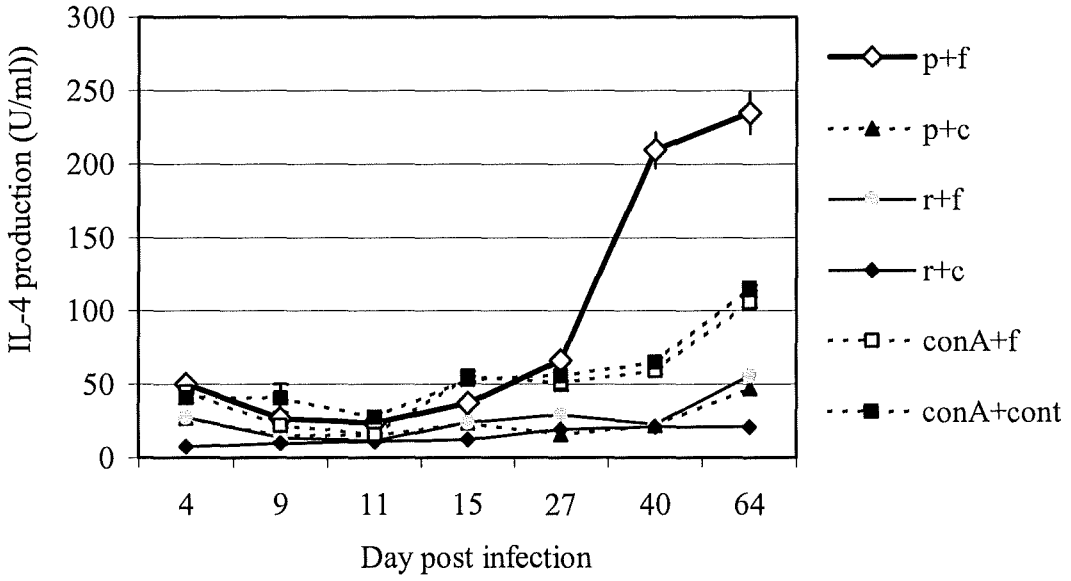


Figure 3.14. IL-4 production in NIH mice infected with 1×10^5 pRBCs *P. c. adami* DK.

The course of infection was monitored for 64 days. At each time point three mice were sacrificed and their spleens were individually cultured. Supernatant of splenocytes cultures for each mouse was assayed separately by ELISA. Each data point is the mean ± SEM for an experiment performed in triplicate. However, SEM is not shown when it is too small.

p+f: Supernatant from infected mouse splenocytes culture exposed to pRBCs.

p+c: Supernatant from naïve mouse splenocytes culture exposed to pRBCs.

r+f: Supernatant from infected mouse splenocytes culture exposed to naïve RBCs.

r+c: Supernatant from naïve mouse splenocytes culture exposed to naïve RBCs.

ConA+f: Supernatant from infected mouse splenocytes culture exposed to ConA.

ConA+c: Supernatant from naïve mouse splenocytes culture exposed to ConA.

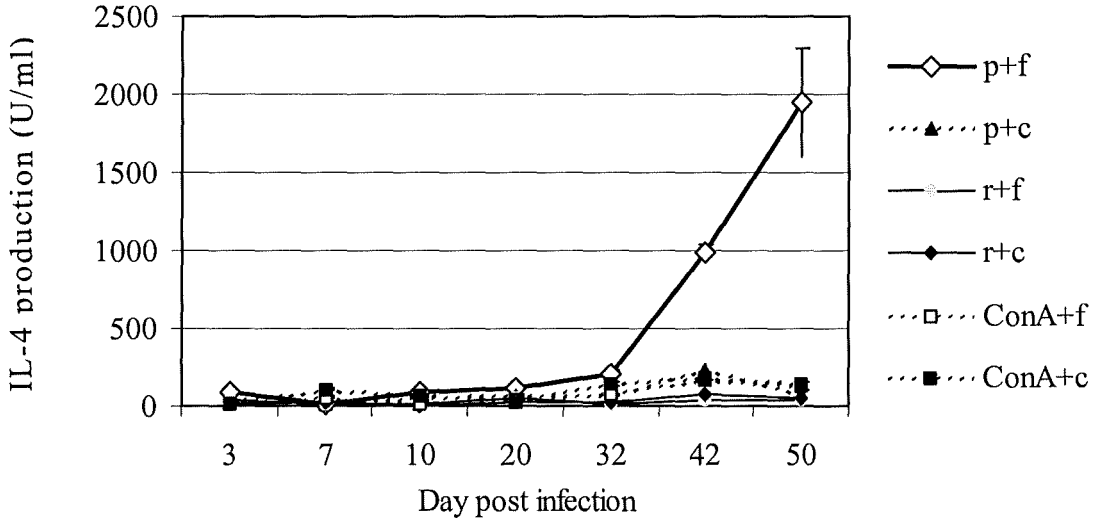


Figure 3.15. IL-4 production in NIH mice infected with 1×10^4 pRBCs of *P. c. adami* DS.

Mice treated with a sub-curative dose of chloroquine. The course of infection was monitored for 58 days. At each time point three mice were sacrificed and their spleens were individually cultured. Supernatant of splenocytes cultures for each mouse was assayed separately. Each data point is the mean ± SEM for an experiment performed in triplicate by ELISA. However, SEM is not shown when it is too small.

p+f: Supernatant from infected mouse splenocytes culture exposed to pRBCs.

p+c: Supernatant from naïve mouse splenocytes culture exposed to pRBCs.

r+f: Supernatant from infected mouse splenocytes culture exposed to naïve RBCs.

r+c: Supernatant from naïve mouse splenocytes culture exposed to naïve RBCs.

ConA+f: Supernatant from infected mouse splenocytes culture exposed to ConA.

ConA+c: Supernatant from naïve mouse splenocytes culture exposed to ConA.

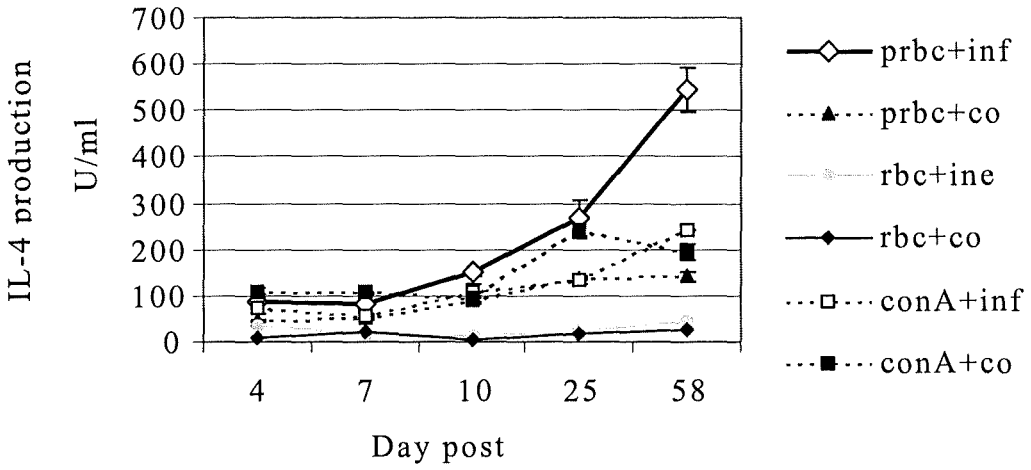


Figure 3.16. IL-4 production in NIH mice infected with 1×10^4 pRBCs of *P. c. adami* DS.

Mice left untreated. The course of infection was monitored for 58 days. At each time point three mice were sacrificed and their spleens were individually cultured. Supernatant of splenocytes cultures for each mouse was assayed separately. Each data point is the mean ± SEM for an experiment performed in triplicate by ELISA. However, SEM is not shown when it is too small.

p+f: Supernatant from infected mouse splenocytes culture exposed to pRBCs.

p+c: Supernatant from naïve mouse splenocytes culture exposed to pRBCs.

r+f: Supernatant from infected mouse splenocytes culture exposed to naïve RBCs.

r+c: Supernatant from naïve mouse splenocytes culture exposed to naïve RBCs.

ConA+f: Supernatant from infected mouse splenocytes culture exposed to ConA.

ConA+c: Supernatant from naïve mouse splenocytes culture exposed to ConA.

3.2.3.4. Specific antimalarial IgG (whole molecule) production in *P. c. adami* DK and DS infections

Parasite-specific IgG (whole molecule) production in mice infected with the parasites and control naïve mice was determined by the ELISA test. Sera were collected from at least 3 mice at each time point, usually every three days. Sera from the test or control groups were assayed for parasite specific IgG (whole molecule), and parasite-specific IgG1 and IgG2a subclasses. The control groups were as follows: the level of IgG in sera from infected mice reacted to lysate of naïve RBCs, and sera from naïve mice reacted to lysate of pRBCs, or naïve RBCs. Lysates of pRBCs, for both the DK and the Ds strains, and naïve RBCs were prepared.

In the avirulent infection, parasite-specific IgG (whole molecule) increased immediately after the peak parasitaemia, declining thereafter and increasing after the recrudescence (Figure 3.17, a). Parasite-specific total IgG (whole molecule) levels in the infected mice were significantly higher (Tukey's test, $P < 0.001$) compared to all control groups on the last sampling days (days 54, 59 and 64 p.i.).

In mice infected with the virulent DS strain, and treated with chloroquine, parasite-specific IgG (whole molecule) increased with the highest levels at the peak parasitaemia and it levels remained high for the rest of observation period (Figure 3.17, b).

The same profile of total IgG production was seen in mice infected with the DS strain and left untreated (Figure 3.18, a). However, the levels of IgG were lower compared to treated mice except for the last sampling day which was comparable to the treated mice.

In general, the total parasite-specific IgG (whole molecule) levels in sera taken from the treated DS-infected mice were higher from day 10 p.i. onwards compared to IgG levels in the DK-infected mice (Figure 3.17).

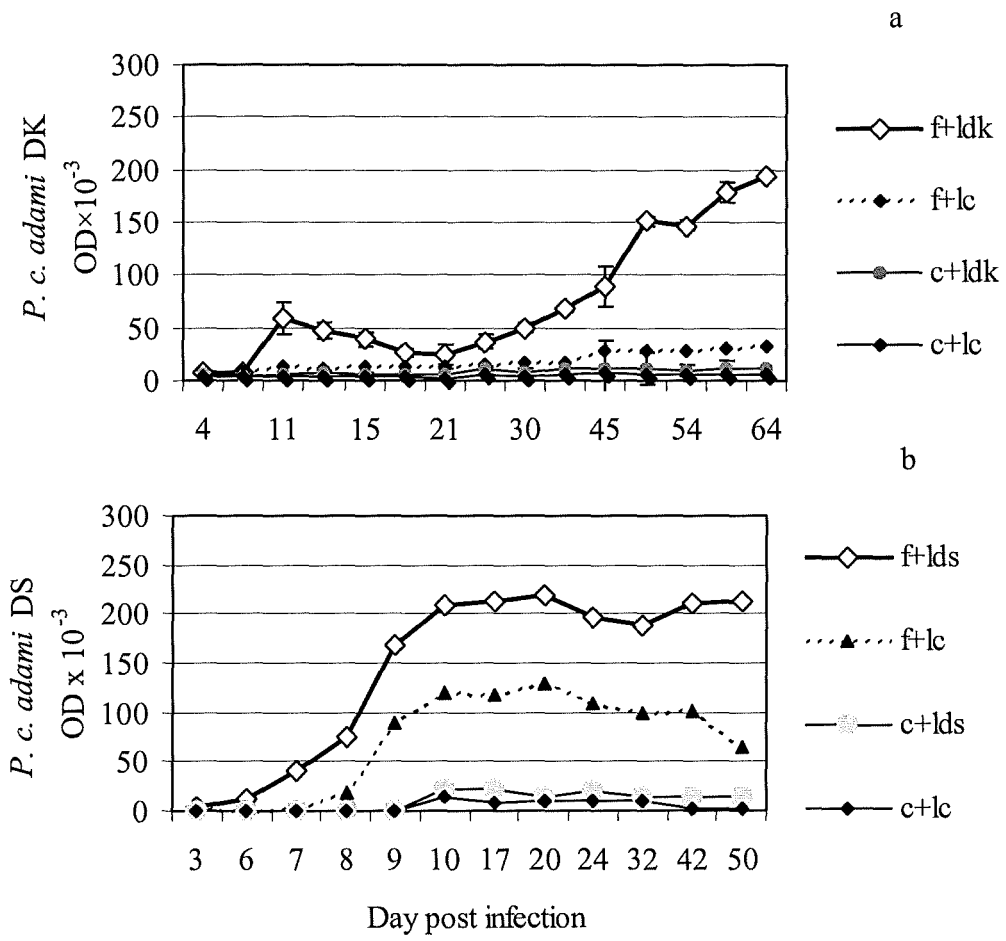


Figure 3.17. Specific total IgG (whole molecule) antibody production in NIH mice infected with *P. c. adami* DK or *P. c. adami* DS.

a) IgG (whole molecule) production in non-lethal *P. c. adami* DK infection.

b) IgG (whole molecule) production in lethal *P. c. adami* DS where mice treated with sub-curative dose of chloroquine. Each data point is the mean ± SEM for an experiment performed in triplicate by ELISA. However, SEM is not shown when it is too small.

f+ldk: Sera from infected mice with the DK strain and reacted with the lysate obtained from pRBCs of *P. c. adami* DK.

f+lds: Sera from infected mice with the DS strain and reacted with the lysate obtained from pRBCs of *P. c. adami* DS.

f+lc: Sera from DK-infected mice or from DS-infected mice reacted with the lysate obtained from naïve control mice as separately shown above.

c+ldk or c+lds: Sera from naïve control mice reacted with the lysate obtained from pRBCs of the DK or the DS strains.

c+lc: Sera from naïve control mice reacted to the lysate obtained from naïve control mice.

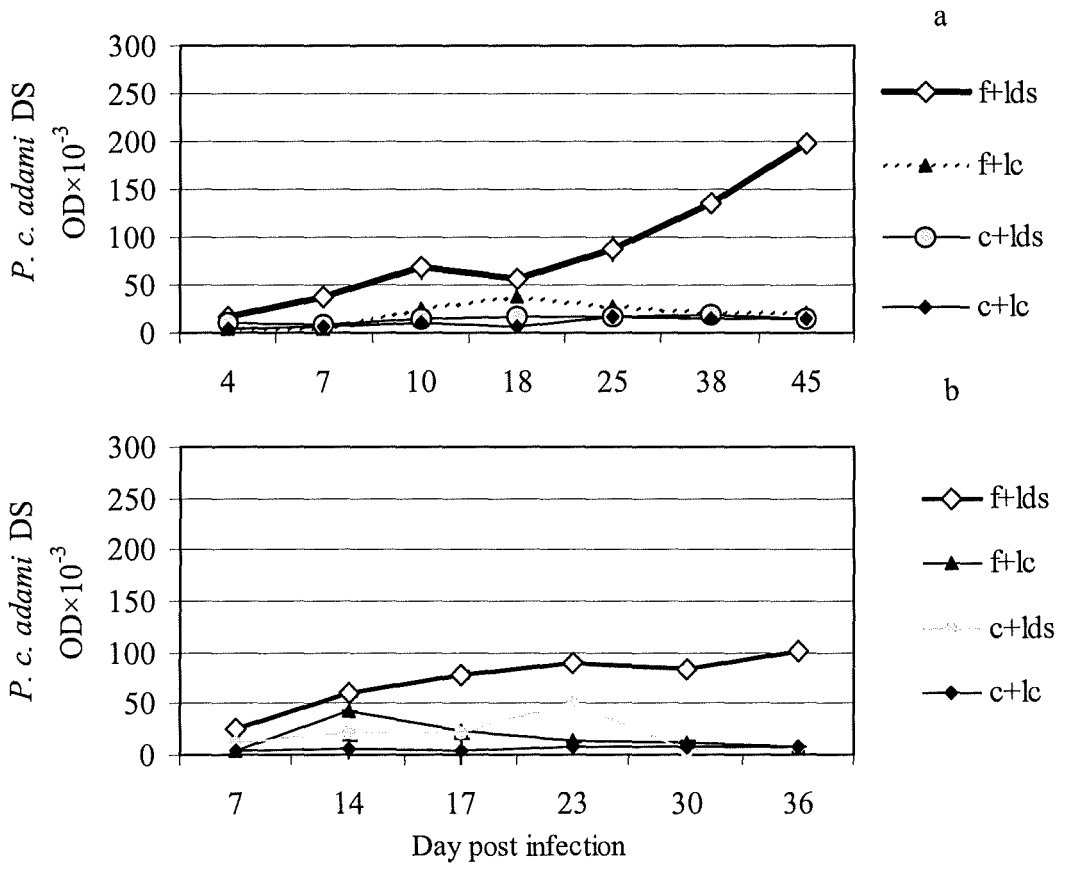


Figure 3.18. Specific total IgG (whole molecule) antibody production in NIH mice infected with *P. c. adami* DS.

Each data point is the mean ± SEM for an experiment performed in triplicate by ELISA. However, SEM is not shown when it is too small.

a) IgG (whole molecule) production in mice infected with 1×10^4 *P. c. adami* DS and left untreated.

b) Specific IgG (whole molecule) antibody production in NIH mice infected with a low infective dose of 2×10^3 pRBCs of *P. c. adami* DS.

f+lds: Sera from infected mice with the DS strain and reacted with the lysate obtained from pRBCs of *P. c. adami* DS.

f+lc: Sera from DS-infected mice either with 1×10^4 or 2×10^3 pRBCs reacted with the lysate obtained from naïve control mice as separately shown above.

c+lds: Sera from naïve control mice reacted with the lysate obtained from pRBCs of the DS strain.

c+lc: Sera from naïve control mice reacted to the lysate obtained from naïve control mice.

3.2.3.5. Specific antimalarial IgG1 production in *P. c. adami* DK and DS infections

In mice infected with avirulent *P. c. adami* DK, although the levels of IgG1 increased, particularly after the peak parasitaemia, antibody production only increased sharply on the last sampling days when usually infection became chronic (Figure 3.19.). The increase of specific IgG1 later in the course of infection (chronic phase) indicates activation of a Th2 response. There were significantly different (Two-way ANOVA, $P < 0.001$) levels of IgG1 between the groups. The IgG1 levels were significantly higher (Tukey's test, $P < 0.01$) in serum from mice infected with the parasite when reacted with pRBCs lysate compared to the serum from naïve mice reacted with pRBCs for all time points except for the first three sampling days.

In the chloroquine-treated mice infected with the DS strain, specific IgG1 did not increase significantly during the acute phase, but increased after the recrudescence and subsequently remained at high levels during the observation period. A significant difference (Tukey's test, $P < 0.005$) was seen for specific-IgG1 in serum from infected mice reacted with pRBCs lysate compared to the control group in which serum from infected mice reacted with naïve RBCs except for the first three sampling days (Figure 3.20, a). The difference was also significant (Tukey's test, $P < 0.002$) between IgG1 in serum from infected mice reacted to pRBCs compared to IgG1 in serum from naïve mice reacted to pRBCs.

In surviving mice infected with the DS strain and left untreated increases in parasite-specific IgG1 were detected on the last sampling days (days 38 and 45 p.i., Figure 20, b). There were significantly different (Two-way ANOVA, $P < 0.05$) levels of specific IgG1 between all tested groups. In general, the same profile was seen for specific IgG1 production in mice infected with either non-lethal or lethal strains (Fig.3.19 and 3.20).

The results showed that in mice infected with a low infective dose the production of IgG1 was not as high as seen in other experimental groups of DS-infected mice (Figure 3.21). However, the profile of IgG1 production was the same, as higher levels of the parasite-specific IgG1 were observed on the last sampling days.

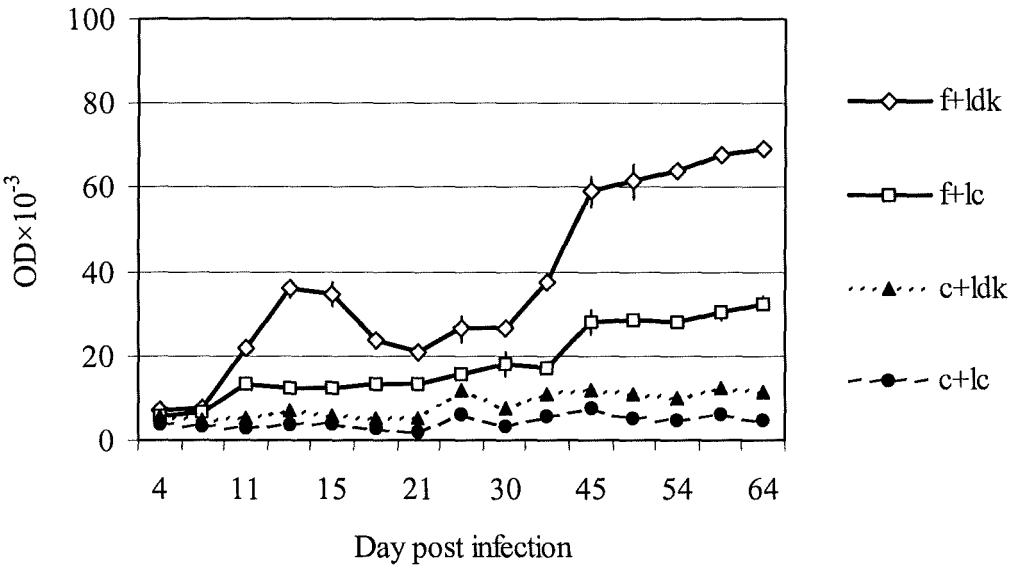


Figure 3.19. Specific anti-parasite IgG1 production. Mice infected with 1×10^5 pRBCs of *P. c. adami* DK.

Each data point is the mean ± SEM for an experiment performed in triplicate by ELISA. However, SEM is not shown when it is too small.

f+ldk: Sera from infected mice reacted with the lysate obtained from pRBCs of *P. c. adami* DK.

f+lc: Sera from infected mice reacted with the lysate obtained from naïve control mice.

c+ldk: Sera from naïve control mice reacted with the lysate obtained from pRBCs of the *P. c. adami* DK.

c+lc: Sera from naïve control mice reacted with the lysate obtained from naïve control mice.

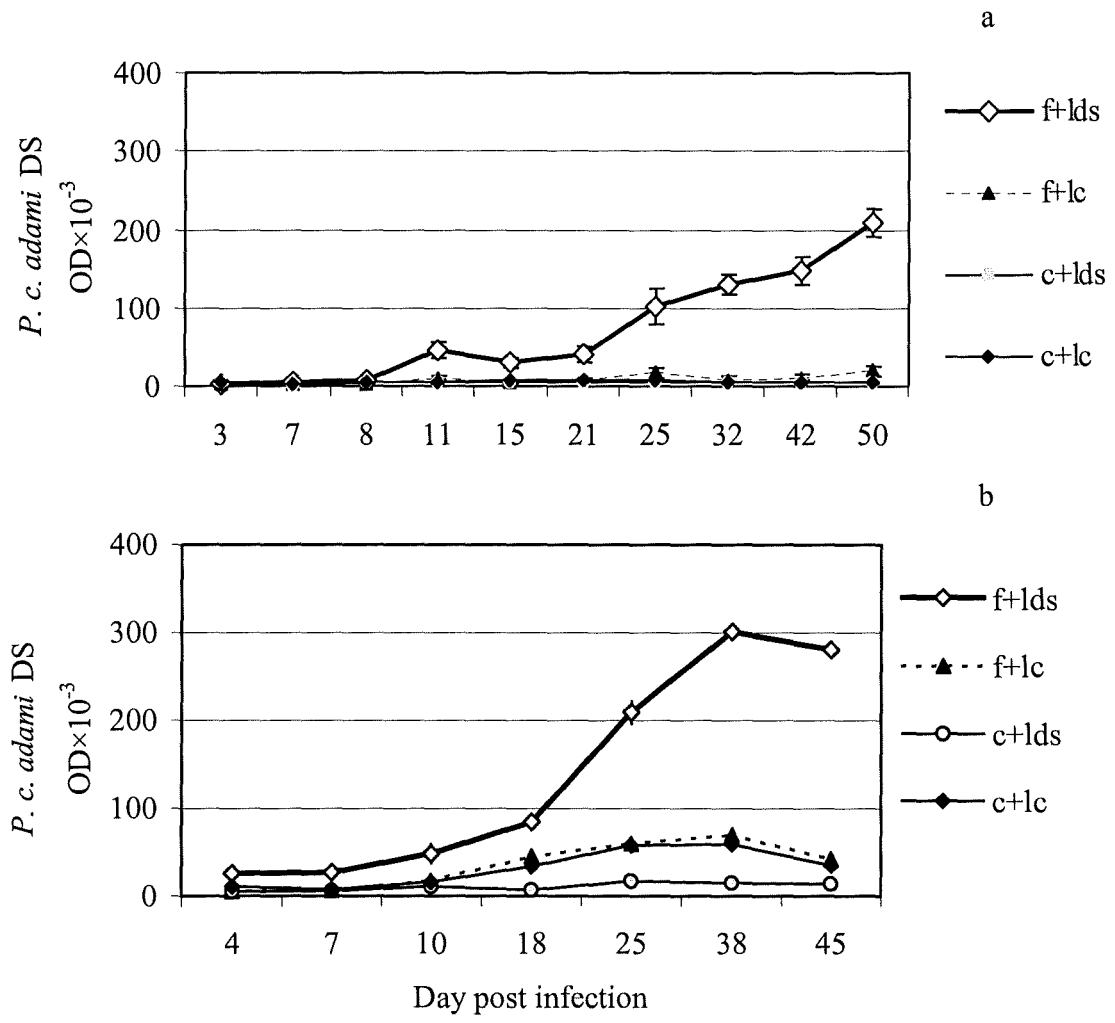


Figure 3.20. Specific anti-parasite IgG1 production in mice infected with 1×10^4 pRBCs of *P. c. adami* DS.

Each data point is the mean ± SEM for an experiment performed in triplicate by ELISA. However, SEM is not shown when it is too small.

a) Specific IgG1 production when mice treated with sub-curative dose of chloroquine.

b) Specific IgG1 when mice left untreated.

f+lds: Sera from DS-infected mice either treated or untreated reacted with the lysate obtained from pRBCs of *P. c. adami* DS as separately shown above.

f+lc: Sera from DS-infected mice either treated or untreated reacted with the lysate obtained from naïve control mice as separately shown above.

c+lds: Sera from naïve control mice reacted with the lysate obtained from pRBCs of the DS strain.

c+lc: Sera from naïve control mice reacted with the lysate obtained from naïve control mice.

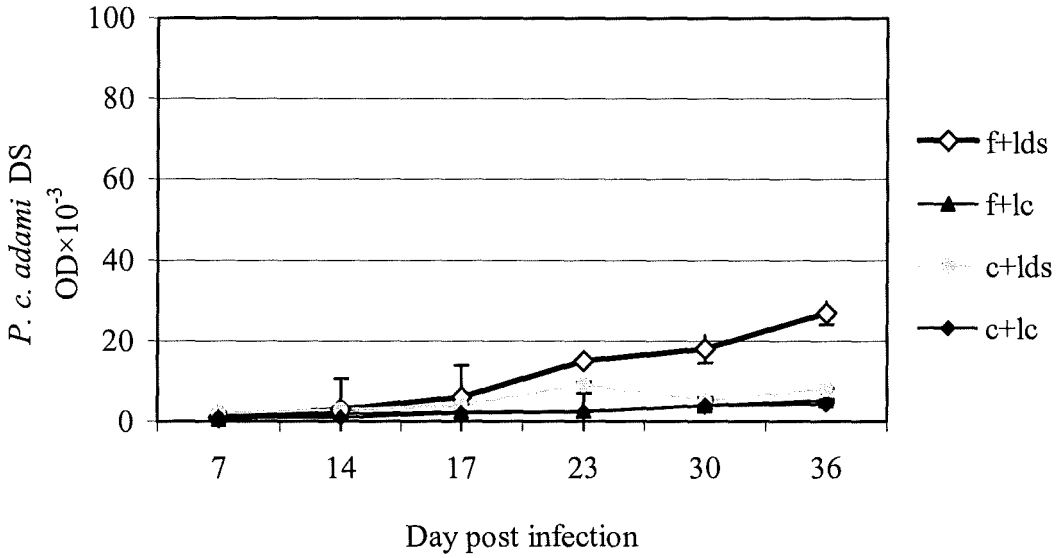


Figure. 3.21. Specific anti-parasite IgG1 production in mice infected with a low infective dose of 2×10^3 pRBCs of *P. c. adami* DS.

Each data point is the mean ± SEM for an experiment performed in triplicate by ELISA. However, SEM is not shown when it is too small.

f+lds: Sera from infected mice reacted with the lysate obtained from pRBCs of P. c. adami DS.

f+lc: Sera from infected mice reacted with the lysate obtained from naïve control mice.

c+lds: Sera from naïve control mice reacted with the lysate obtained from pRBCs of the DS strain.

c+lc: Sera from naïve control mice reacted with the lysate obtained from naïve control mice.

3.2.3.6. Specific antimalarial IgG2a production in *P. c. adami* DK and *P. c. adami* DS infections

Sera of infected and control mice were also assayed for specific IgG2a production. In *P. c. adami* DK infection, specific-IgG2a sharply increased at or immediately after the peak parasitaemia, declining thereafter and rose again during the later stages of the observation period. The results showed that the increase of parasite-specific IgG2a, an indicator of Th1 response, was almost coincident with the peak parasitaemia. There were significantly different (Two-way ANOVA, $P < 0.001$) levels of IgG2a between all groups. When IgG2a was examined in serum from infected mice reacted with pRBCs and RBCS, the levels of parasite-specific IgG2a in the infected group were significantly higher (Tukey's test, $P < 0.01$) than that in the control group for two time points, daya 10 and 11 p.i (Fig. 3.22).

In *P. c. adami* DS infection, when mice were treated, the anti-malarial specific IgG2a increased during the acute phase, slightly decreased thereafter and rose again particularly on days 42 and 50 p.i, the last two sampling days.

In the virulent DS infection, when mice were left untreated, the levels of specific IgG2a were significantly different compared to the treated mice (Two-way ANOVA, $P < 0.05$). But the profile of IgG2a production did not differ. There were higher levels of IgG2a production in mice surviving in the untreated group compared to treated mice for all time points from day 7p.i onward. The results show that the induction of IgG2a production in the untreated group was significantly (Two-way ANOVA, $P < 0.01$ for all time points after day 10 p.i) greater than that in the treated group from day 10 p.i. onward (Figure 3.23).

The production of parasite-specific IgG2a in mice infected with a low infective dose of the DS strain was detected at a relatively high level after the peak parasitaemia and showed the same profile as seen in mice infected with a higher dose (Figure 3.24). There were significantly higher (Tukey's test, $P < 0.01$ for all time points except the first sampling day, day 7 p.i.) levels of IgG2a in serum from mice infected with a low infective dose compared to other control groups. The results indicated that there is a

similar profile for the specific IgG2a production in both non-lethal and lethal infections (Figure. 3.22 and 3.23).

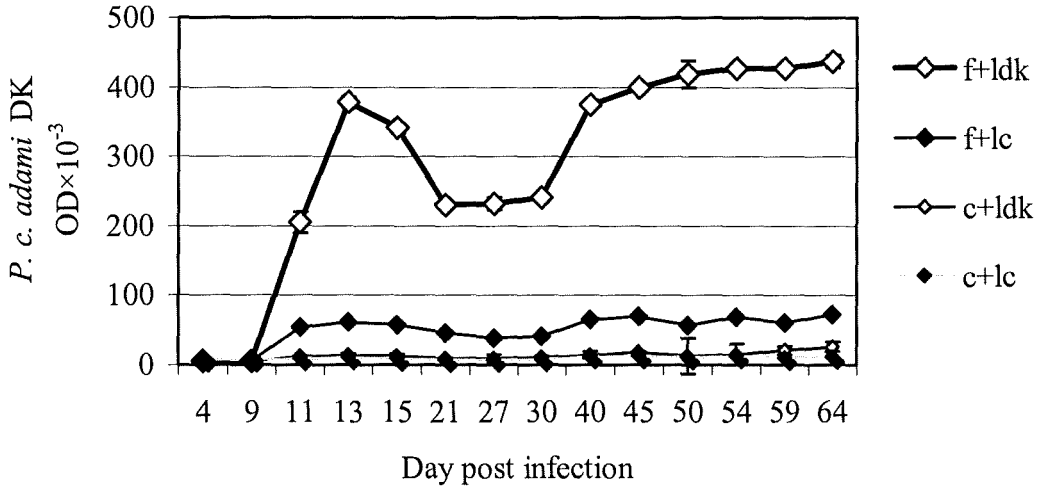


Figure 3.22. Specific anti-parasite IgG2a production in mice infected with 1×10^5 pRBCs of *P. c. adami* DK.

Each data point is the mean ± SEM for an experiment performed in triplicate by ELISA. However, SEM is not shown when it is too small.

f+ldk: Sera from infected mice reacted with the lysate obtained from pRBCs of *P. c. adami* DK.

f+lc: Sera from infected mice reacted with the lysate obtained from naïve control mice.

c+ldk: Sera from naïve control mice reacted with the lysate obtained from pRBCs of the DK strain.

c+lc: Sera from naïve control mice reacted with the lysate obtained from naïve control mice.

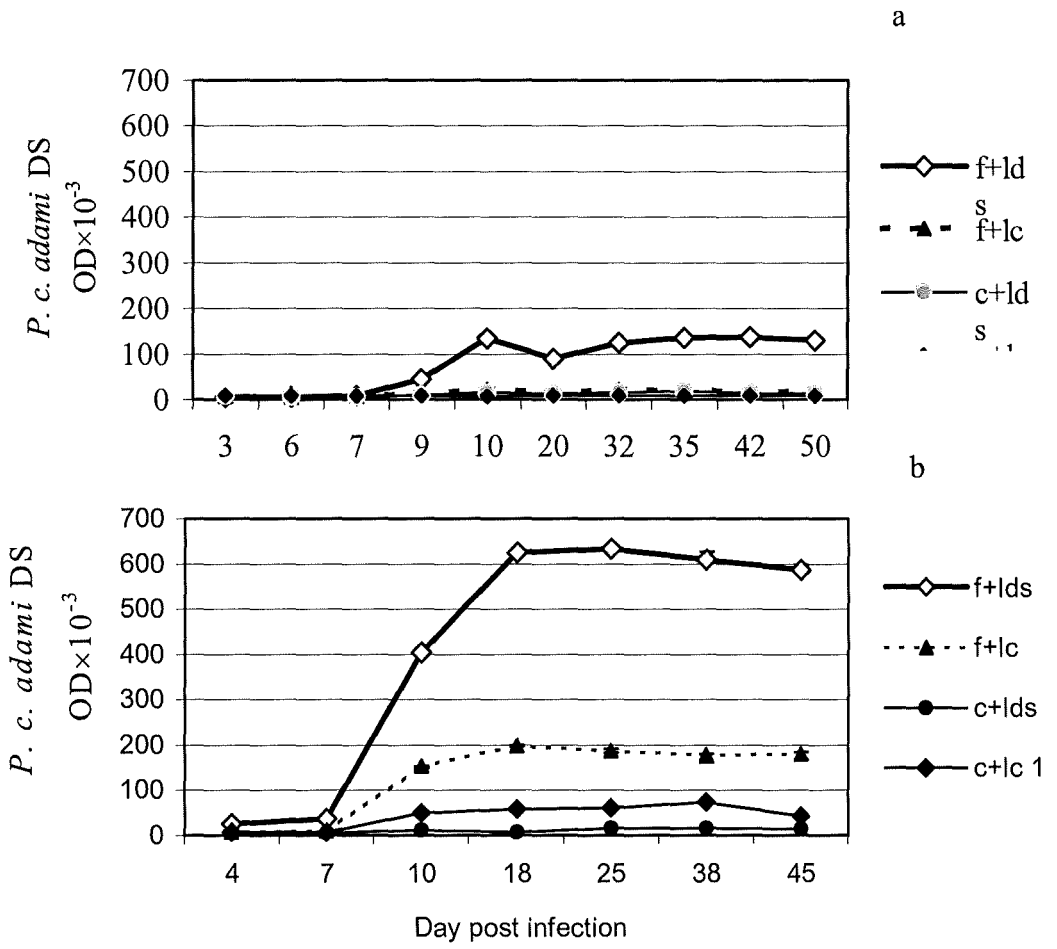


Figure 3.23. Specific anti-parasite IgG2a production in mice infected with 1×10^4 pRBCs of *P. c. adami* DS.

Each data point is the mean ± SEM for an experiment performed in triplicate by ELISA. However, SEM is not shown when it is too small.

a): Mice treated with a subcurative dose of chloroquine.

b): Mice were left untreated.

f+lds: Sera from infected mice reacted with the lysate obtained from pRBCs of *P. c. adami* DS.

f+lc: Sera from infected mice either treated or untreated reacted with the lysate obtained from naïve control mice as separately shown above.

c+lds: Sera from naïve control mice reacted with the lysate obtained from pRBCs of the DS strain.

c+lc: Sera from naïve control mice reacted with the lysate obtained from naïve control mice.

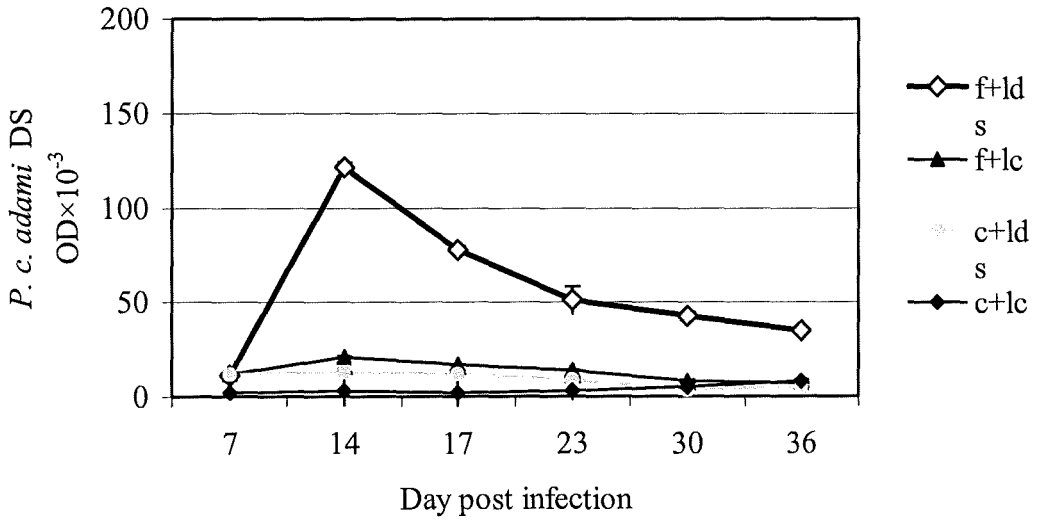


Figure 3.24. Specific anti-parasite IgG2a production in mice infected with a low infective dose at 2×10^3 pRBCs of *P. c. adami* DS.

Each data point is the mean ± SEM for an experiment performed in triplicate by ELISA. However, SEM is not shown when it is too small.

f+lds: Sera from infected mice reacted with the lysate obtained from pRBCs of *P. c. adami* DS.

f+lc: Sera from infected mice reacted with the lysate obtained from naïve control mice.

c+lds: Sera from naïve control mice reacted with the lysate obtained from pRBCs of the DS strain.

c+lc: Sera from naïve control mice reacted with the lysate obtained from naïve control mice.

3.3. Discussion

This study examined the profile of the immune response in NIH mice infected with avirulent *P. c. adami* DK or virulent *P. c. adami* DS. To approach these aims, the course of infection, production and kinetics of selected cytokines, and parasite-specific antibodies were examined.

Monitoring the course of infections showed that in the non-lethal DK infection the peak parasitaemia was on day 10 p.i. In the treated mice infected with the DS strain, the peak parasitaemia was observed on day 8 p.i., the day on which mice were treated with chloroquine. In the lethal DS infection, when the mice were left untreated, the peak parasitaemias was also on day 10 p.i despite the fact that in the virulent DS-infected mice, the infective dose was 10-fold lower than that in the avirulent DK-infected mice. This suggests that in the virulent DS infection the parasite density increases more rapidly due to the higher replication rate compared to the DK avirulent infection. In mice infected with a low infective dose (2×10^3 pRBCs) peak parasitaemia was as the same time as seen in untreated mice infected with a higher infective dose.

The effect of infective dose on the developing parasitaemia, the course of infection and its outcome has been investigated. In humans, the influence of inoculum size on disease severity has not been fully understood (Glynn and Bradley, 1995). In rodents, Timms and colleagues (2001) suggested that the inoculating dose affects malaria parasite dynamics, as larger infective doses induced earlier and higher mortality than did lower infective doses. They reported that C57Bl6J mice infected with the virulent *P. c. chabaudi* BC died about a day earlier for every 10-fold increase in the infective dose. The present results showed that in the virulent DS infection when mice infected with 1×10^4 pRBCs and left untreated the % survival was only 40 % (Fig3.2). In contrast, 100 % survival rate has been reported in NIH mice infected with 10^2-10^6 pRBCs of *P. c. chabaudi* AS (Taylor-Robinson and Phillips, 1998). Timms and collegues (2001) also reported that a proportion of mice that died from 1×10^4 pRBCs of *P. c. chabaudi* BC infection was > 0.4. In the present study (see Chapter Six) mice infected with *P. chabaudi* AS survived when infective dose was 1×10^4 pRBCs despite the presence of a high peak parasitaemia. These observations and

differences in surviving mice show that strain-specific host-parasite interactions impact on the outcome of infection. For example, in the present study this difference shows that *P. chabaudi* AS infection is a non-lethal infection in NIH mice. Large infective dose results in earlier symptoms in mice (Cox, 1966) and in humans (Glynn, 1994). On the other hand, the results presented here showed that a low infective dose , 2×10^3 pRBCs, resulted in one-day delay in the appearance of the parasitaemia and a three-day delay in the time taken to death were seen compared to that in mice infected with the higher dose (Figures 3.2 and 3.4, and 3.5). However, this delay did not prevent mice from the fatal outcome of the infection as two mice out of six died. In an immunization study in which BALB/c mice challenged with 1×10^5 pRBCs of lethal *P. c. adami* DS over the three trials, only 2 of 63 control animals have survived infection (3.2%) which show a very high mortality of the DS strain (Smooker *et al.*, 2000). In the present study, the mortality was not as high as seen in BALB/c mice in Smooker and collegues' study (2000). One reason probably is a lower infective dose, 10^4 pRBCs, compared to the infective dose used in the Smooker's study.

It is also thought that infective dose affects Th1 and Th2 sequential activation. Taylor-Robinson and Phillips (1998) showed that increasing the infective dose of *P. c. chabaudi* AS in susceptible A/J mice results in elevated Th2 responses that lead to a fulminant parasitaemia whereas increasing the infective dose in resistant NIH mice enhances IFNγ, and reduces IL-4 production, promoting a Th1 response. Although in the present study neither avirulent nor virulent infection was examined in any susceptible mice, the profile of the immune response, a sequential Th1/Th2 response, in resistant NIH mice was not altered even in the low-infective dose infection. Taken together, these observations support the idea that dose affects disease severity by altering the time that host needs to control parasite density before the threshold for clinical disease is reached (Timms *et al.*, 2001; Marsh, 1992). This indicates that any intervention that reduces infective dose will have an effect on the severity of the disease.

The present results showed that recovery from the primary peak parasitaemia during *P. c. adami* DK and DS infections in NIH mice is associated with a Th1 response, with early high levels of IFNγ and high levels of IgG2a at or immediately after the peak parasitaemia. The

predominant Th1 cell-mediated response was followed by a Th2 response with high levels of IL-4 and IgG1, particularly in the last sampling days which is usually coincident with the chronic phase of the disease. In general, the present findings are in agreement with previous studies in which both Th1 and Th2 subsets of the $CD4^+$ T cells (Podoba and Stevenson, 1991; Taylor-Robinson *et al.*, 1993; De Souza *et al.*, 1997) have been shown to be crucial for the resolution of the acute primary parasitaemia in the *P. c. adami* model (Kima *et al.*, 1992) and for elimination of self-resolving infection of *P. chabaudi* AS (Langhorne, 1989; Stevenson & Tam, 1993). Early production of IFNγ is also proposed to be a characteristic feature in non-lethal *P. yoelii* infection (de Souza *et al.*, 1997) and for the resolution of the primary parasitaemia in *P. yoelii* infection (Choudhury *et al.*, 2000). Moreover, in *P. c. chabaudi* AS infection, splenocytes produced high levels of IFNγ *in vitro* during ascending parasitaemia and low levels during descending primary parasitaemia (reviewed by Taylor-Robinson, 1995). No early increase in production of IL-4 was seen in either lethal infection of *P. yoelii* YM or in non-lethal infection of *P. chabaudi* AS (de Souza *et al.*, 1997). The observation of increased IL-4 and IgG1 later in the course of the infection, in the present study, was also in agreement with the suggestion that switching from a Th1 to a Th2 response may be due to activation of a feedback mechanism by which immune response quickly returns to a homeostatic situation in which anti-IFNγ inflammatory cytokines such as IL-4 are important (Saul, 1998).

The present results did not show any difference in the profile and timing of IFNγ and IL-4 productions in non-lethal and lethal infections in NIH mice (Figures. 3.11 to 3.16).

Susceptibility of the host is proposed to be an important factor in the induction of Th1 or Th2 after the first exposure to the pathogen. High levels of expression of mRNA of IFNγ and low levels of mRNA of IL-4 were also seen in resistant C57BL/6 mice infected with *P. chabaudi* AS in the early phase of infection which was correlated with the protection against asexual blood stages (Jacobs, Radzioch & Stevenson, 1996). Indeed, Jacobs and colleagues (1996) found significantly higher levels of

mRNA of IFNγ in the splenocytes of resistant B6 mice compared to susceptible A/J mice. In contrast, they showed that in susceptible A/J mice infected with the same parasites, there was an increase of TNFα mRNA levels in the liver and excessive levels of TNFα in serum later during infection, with a higher level of IL-4 mRNA which showed a correlation between the presence of these cytokines and susceptibility to parasite infection.

The results presented here showed the levels of IFNγ on the first sampling days were higher in the avirulent infection compared to the virulent infection. This shows that, in the same host, the avirulent infection may induce stronger Th1 response compared to the virulent infection. This also indicates that the virulent DS strain in the NIH mouse model could modify IFNγ production to a lower level. In respect of IL-4 production, in the avirulent DK infection the IL-4 levels were significantly lower than in the virulent DS infections, probably due to the induction of stronger Th2 response in DS-infected mice compared to DK-infected mice. Further investigations are required to identify the features and factors involved in virulence and resulted deaths in the DS strain infections such as weight loss, irregulation in host's temperature, and rapid multiplication of the parasite.

The present study showed that, regarding to the time of sampling, early high levels of IFNγ declined to the low levels during the time of peak parasitaemia in both non-lethal DK and lethal DS infections. Previous studies also showed that sharplty rising of IFNγ in plasma (Slade and Langhorne, 1989) or in stimulated splenocytes with the parasite or ConA 1 or 2 days beforet the peak parasitaemia follwed with a very decline and not to rise again (stevenson *et al*, 1990, Taylor-Rabinson and Phillips, 1994). However, the present results shows a biphasic production of IFNγ as sharply decline of IFNγ at the pak parasitaemia increased again later during the course of infection. Based on the present results the second wave of IFNγ increase particularly after recrudescence could be as a result of boosting of the immune response by a new variant of the parasite. Related studies about the kinetics of the immune response which are briefly

discussed below may explain biphasic production of IFNγ. In the case of malaria, splenic macrophages following stimulation by malaria parasite release IL-12 (Sam and Stevenson, 1999) which trigers IFNγ production from NK cells (Gazzinelli, 1996). So, the innate immune response as the first line of defence induces IFNγ production leading to protective consequences against the parasite through release of inflammatory cytokines and antibodies which promotes opsonization and phagocytosis (Seder and Paul, 1994; reviewed by Wipasa *et al.*, 2002; reviewed by Taylor-Robinson, 1995). In addition, direct activation of bone marrow-derived DCs, which were co-incubated with purified schizont-stage of *P. chabaudi* AS, has been shown to produce cytokines such as TNFα within 30 minutes, followed by IL-6, IL-12p40 and p70 which subsequently promote a Th1 response. However, at a very early time, this direct activation of DCs was independent of the presence of T cells (Seixas *et al.*, 2001). Therefore, in the present study, non-specific immunity may be a source of the early high levels of IFNγ measured on the first sampling days followed by activation of a Th1 response later. But why IFNγ levels decreased at the time of peak parasitaemia may be justified by different mechanisms. In this respect, an optimum antigen load in the initial days of infection leads to higher production of IFNγ whereas increase of antigen load over the optimum reduces IFNγ production indicating an effect of a negative feedback mechanism. The present study showed this event, over-loading of antigens, might be coincident with the time of peak parasitaemia at which IFNγ was sampled (Figures 3.11, 3.12, and 3.13).

Another possible explanation could be referred to the situation in which the present microenvironment provides switching from Th1 to Th2 by production of Th2 cytokines such as IL-4 and IL-10 which downregulate Th1 cytokines such as IFNγ in not very later stages of infection (reviewed by Sher *et al.*, 1992; Swain *et al.*, 1990; Le Gros *et al.*, 1990). So, activation of Th2 subset in a cross-regulation activity could be coincident with the time of peak parasitaemia at which IFNγ was sampled and showed the lowest level.

The importance of presence of spleen in cell-mediated immunity against crisi forms of *P. chabaudi* AS in C57BL/6 mice was demonstrated by

Stevenson, Tam, and Rae (1990) using splenectomized mice prior to infection. In this regard, Leisewitz *et al.* (2004) showed that DCs, with CD11c marker, in C57BL/6 mice infected with *P. chabaudi* AS, are invloved in in the T cell activation by upregulation of required co-stimulatory molecules such as CD40, CD54, and CD86. These DCs migrated from the marginal zone of the spleen into the $CD4^+$ T cell area within 5 days after the parasites entered the bloodstream. These DCs expressed intracellular IFNγ with a peak on day 5 p.i., 2 days earlier than the peak expression in other cells such as macrophages as shown by FACS technique. It seems, therefore, that DCs not only actively engaged in the earliest phases of malarial infection *in vivo* but also produce a Th1 cytokine, IFNγ. Yadava *et al.* (1996) previously showed that in *P. c. adami* infection, pRBCs were trapped in red pulp in which macrophages and T cells have anti-parasitic function.

The present results showed that high levels of IFNγ and IgG2a did not occur simultaneously, as very high levels of IgG2a coincided with a low level of IFNγ at the peak parasitaemia. This indicates that IFNγ previously activated IgG2a production process and declined itself as a result of a self-limiting property. Previous studies also reported high levels of IgG2a, induced by Th1 and IFNγ, are associated with the immune response with a peak on day 12 p.i. in NIH mice infected with *P. c. chabaudi* (Taylor-Robinson and Philips, 1994) and in immunized mice challenged with *P. yoelii* (Matsumoto *et al.*, 2000). Regarding the present results about timing and kinetics of production of IFNγ and IgG2a as Th1 markers, it may be assumed that the immune response in *P. c. adami* DS and DK starts to balance inflammatory and anti-inflammatory responses around the time of peak parasitaemia. This could be as a result of producing Th2 cytokines such as IL-4, which began to increase after the peak parasitaemia. Thus, Th2 cytokines also promote their inhibitory effects on Th1 response. Nevertheless, according to the present results, understanding which mechanisms and effector cells are involved in the decrease of IFNγ in splenocytes taken at the time of the peak parasitaemia merits more investigation. For example, measuring other cytokines involved in the early events of the immune response before, at and after peak parasitaemia may

help to give more realistic interpretations about kinetics of IFNγ production at these times. This measurement may include levels of cytokines which are produced in spleen cell cultures and/or measuring levels of expression of mRNA of such cytokines in relevant tissues such as the spleen.

The regulation of IgG2a and IgG1 isotypes is influenced by the prevailing Th1-Th2 cytokine balance (Abbas, Murphy and Sher, 1996). In agreement with this idea the present results showed that parasite-specific IgG2a sharply increased at or immediately after the peak parasitaemia, declined thereafter, and then rose thereafter (Figure 3.22 and 3.23). Previous studies also confirmed that when parasitaemia peaks specific IgM and IgG2a can be detected and rise to the highest levels 1 or 2 days after the peak parasitaemia (Taylor-Robinson and Phillips, 1994). The same profile for parasite specific IgG2a was shown in the self-resolving infections of *P. chabaudi* AS and *P. yoelii* (Smith and Taylor-Robinson, 2003). Su and Stevenson (2000) also showed that more IgM and IgG2a, and less IgG1, were produced in wild type of C57BL/6 mice compared to IFNγ GKO mice. However, in the present study IgM was not measured. In contrast, Smith and Taylor-Robinson (2003) showed that in lethal infections there is a correlation between fatal outcome and slower, reduced IgG2a. However, in the present results the levels of IgG2a increased again later during the course of infection in surviving mice in the lethal DS strain infection.

The present study indicated that elevated levels of IgG1 and IL-4 in the chronic phase of the infection were also accompanied with high levels of IgG2a (Figure. 3.19 to 3.21). These results are in agreement with other studies in which IgG1, as the effective specific IgG subclass in the secondary phase of infection, is present when high levels of expression of IgG2a and IgG2b are also observed (Akanmori, Kawai and Suzuki, 1996).

As indicated earlier the present results showed that IL-4 increased in the later stage of the course of infection in all experiments in both avirulent and virulent infections as a marker for switching from Th1 to Th2 response (Langhorne, 1989; Langhorne *et al.*, 1989; Stevenson and Tam, 1993). Taylor-Robinson and Phillips (1994) also suggested that there is a switch from Th1 to Th2 in NIH mice infected with *P. c. chabaudi* AS, typified by sustained production of IL-4 and IgG1 in *P. c. chabaudi* infection

(McDonald and Phllips, 1978). In addition, the present results suggests that the presence of IL-4 is not essential for primary control of parasitaemia as von der Weid *et al.* (1994) and van der Heyde *et al.* (1997) also suggested. This was also noted by Balmer, Alexander and Phillips (2000) when they observed that lack of IL-4 does not significantly alter the outcome of infection, whereas presence of IFNγ is a crucial requirement for development protective immunity to a primary *P. chabaudi* infection. Moreover, IL-4 deficient mice recovered from *P. chabaudi* and *P. yoelii* infections (Balmer, Alexander and Phillips, 2000). On the other hand, in IFNγ receptor deficient mice, the primary parasitaemia to sub-patent levels failed and a high mortality rate was observed. In these mice, IgG2a levels decreased and the absence of IFNγ receptor provides an appropriate microenvironment for elevation of Th2 responses (Balmer, Alexander, and Phillips, 2000). The present study supports this idea that sequentially raised IgG2a and IgG1 is consistent with sequentially raised IFNγ and IL-4 against asexual blood stages in mice infected with either the DK the DS strains. So, the present data indicate that there is a sequential predominance of Th1 and Th2 cytokines and production of associated IgG2a and IgG1 isotypes in immunity against avirulent, DK, and virulent, DS of *P. c. adami* strains. This study also suggests that there is a normal functioning immune system in which both arms of Th1 and Th2 provide flexibility and balance to control the parasites during the course of infection. However, in the virulent infection the predominant Th1 response is not adequate to prevent all mice from death. This study shows much more rapid growth of the DS strain and greater virulence despite the same profile of the immune response compared with the avirulent DK strain infection. This encourages for further investigation to determine what are the virulence factors in the DS strain which differ from the DK strain. Further investigations are also required to identify susceptible stage of DK or DS parasites to specific antibodies or other effector mechanism following infection.

These investigation also help to determine which stage of the parasite are the main targets for the stage-specific antibodies and also provide evidence that how and when these specific antibodies are produced and circulated. This can be achieved by using molecular approaches such as FACS

technique to determine how stage-specific epitopes of antigens are presented to the immune system components for inducing Ab-dependent or cellular immunity.

Chapter Four

Determination of the immune response in NIH mice with mixed infections of avirulent, *Plasmodium chabaudi adami* DK, and virulent *P. c. adami* DS.

4.1. Introduction

Mixed malaria infection studies in both humans and rodents provide information towards a better understanding of the kinetics of the course of infection, its impact on clinical outcome (Black *et al.*, 1994), and the immune response induced compared with that in single-species infections. Mixed infection is common under natural conditions of malaria transmission and it has been reported in all vertebrate hosts of *Plasmodium* (reviewed by Richie, 1988). In humans, harbouring mixtures of different species and different genotypes of the same species of malaria parasites (Babiker *et al.*, 1991) is common in malaria-endemic regions. Despite a lack of direct evidence for cross-species immunity in humans, it has been thought that there is an interplay between density-dependent regulation and clearance rates of individual parasite populations in the species interactions (Bruce *et al.*, 2000). Epidemiological studies in Vanuatu, a South Pacific Melanesian island, where the four human malaria species are endemic, showed that there is a biological interaction between the dominant species, *P. falciparum* and *P. vivax* (reviewed by Maitland, Williams and Newbold, 1997). They suggested that a *P. vivax* infection may modulate i.e, ameliorate subsequent infection with *P. falciparum*. In mixed infections it is proposed that when the majority population is cleared by species or genotype-specific response (Brown, 1990), the minority populations could expand and the sequential episodes of infection can be generated (Bruce *et al.*, 2000). In a study in the Ivory Coast, it was shown that there is a relationship between mixed infections and malaria fever in the children of a village where *P. falciparum* and *P. malariae* are endemic (Black *et al.*, 1994). The study showed that symptomatic children presented with fewer mixed infections and had also less past exposure to *P. malariae* than symptom-free children. Children, who were symptom-free, had more mixed infections of *P. falciparum* and *P. malariae*.

Cross-species immunity has been shown in murine malaria parasites. For example, there is a cross-species immunity in two pairs of; (1) *P. berghei* and *P. yoelii*; and (2) *P. vinckei* and *P. chabaudi* (Cox and Voller, 1966; McColm and Dalton, 1983). In murine infections the course and pathological effect of a single infection may be altered by the presence of another species of murine malaria parasite (reviewed by Taylor, Walliker

and Read, 1997a; Snounou *et al.*, 1992). The specificity of clearance mechanisms was examined by reinfection of *P. c. chabaudi* AS-and *P. y. yoelii* A-infected CBA/Ca mice with clones of *P. c. chabaudi* (CB and IP-PCI strains), *P. c. adami* DS, *P. vinckei lentum* DS, and *P. berghei* ANKA (Jarra and Brown, 1989). The study showed that mice pre-immunized with *P. c. chabaudi* AS or *P. y. yoelii* A resolved re-infection with the homologous parasites. However, *P. c. chabaudi* AS pre-immune mice showed higher parasitaemia when reinfected with heterologous CB and IP-PCI strains. The former mice showed 100% mortality when re-infected with virulent *P. y. yoelii* YM (Jarra and Brown, 1989). Similarly an enhanced, uncontrolled parasitaemia with several deaths was evident in mice pre-immune to *P. y. yoelii* A or *P. c. chabaudi* AS when reinfected with *P. berghei* ANKA (Jarra and Brown, 1989). However, they showed that the mortality in mice pre-immunized with *P. y. yoelii* A, after challenge with *P. y. yoelii* YM, was less than that in control mice infected with the lethal *P. y. yoelii* YM infection. Parasite clearance was also delayed in mice pre-immune with *P. y. yoelii* A or *P. c. chabaudi* AS when reinfected with *P. c. adami* DS, or *P. c. chabaudi* CB or IP-PCI compared with mice challenged with homologous parasites. These results demonstrated that clearance mechanisms, the sudden fall in parasitaemia after peak parasitaemia, of the acute parasitaemia are predominantly mediated by species-and strain-specific responses (Jarra and Brown, 1985, 1989). However, there is also much evidence for cross-immunity between different rodent malaria parasite species. It has been demonstrated that mice that had recovered from *P. chabaudi* infection not only were resistant to challenge with the homologous parasite but also they showed resistance to a fatal *P. vinckei* infection (Cox and Voller, 1966; Yoeli *et al.*, 1966). Cox and Voller (1966) also observed that mice recovered from *P. vinckei* infection were immune to challenge with *P. chabaudi* in addition to the homologous strain.

The infection dynamics of each of two parasite lines in a mixed infection has been determined throughout the parasitaemia, using a DNA hybridization assay, in mice infected with cloned lines from two different parasite species or strains (Snounou *et al.*, 1989 and 1992). This technique involves preparing Southern blotted DNA, isolated from daily blood smears and probing it with a DNA probe, PCsv4.1, a probe derived from *P. c.*

chabaudi genomic library. This can detect a restriction fragment length polymorphism (RFLP) specific for each of the parasite lines from two different parasites. They studied cloned lines of the following parasites: *P. c. chabaudi* AS and CB, *P. c. adami* DS, *P. berghei* ANKA, *P. vinckei* lentum DS, and *P. y. yoelii* A strains. When CBA/Ca mice were simultaneously inoculated with a mixture of cloned lines of *P. c. chabaudi* and *P. yoelii* no deaths were observed compared to the control groups infected with a single line of each parasite in which low but consistent mortality figures were observed (Snounou *et al.*, 1992). There were slight differences in the parasitaemias and mortality when mice were infected with mixed infections of four combinations of two strains from *P. c. chabaudi* (AS and CB) and one strain of *P. c. adami* DS compared with single infections of these malaria parasites. For example, in the presence of *P. c. chabaudi* CB strain, the parasitaemias of *P. c. chabaudi* AS and *P. c. adami* DS were reduced. Mixed infection did not alter the course of *P. yoelii* infection, whereas *P. chabaudi* parasitaemia was depressed when inoculated with another species. However, in the presence of either *P. chabaudi* or *P. berghei* the parasitaemia of *P. vinckei* was higher than as a single infection. In *P. chabaudi* recrudescences were depressed when *P. yoelii* was present. These results show that the outcome of mixed infections is influenced by the composition of the infecting parasite populations.

It may be proposed that interactions between clones within mixed infections are complex and have a significant effect on both infectiousness and the transmission success of individual clones (Taylor and Read, 1998). It was shown that mixed-clone infections of *P. chabaudi* are more infectious to mosquitoes than single-clone infections. They show a rise in oocysts greater than the sum of the single-clone infections in mosquitoes and subsequently higher chance of transmission (Taylor, Walliker, and Read, 1997a and 1997b).

Relative frequency of the clones of *P. chabaudi* ER and *P. chabaudi* CR, changed dramatically during the course of a mixed infection in mice, depending on their ratio in the initial infective dose (Taylor and Read, 1998). Taylor and colleagues (1998) reported that mixed-clone infections caused greater virulence than single-clone infections as assessed by weight loss and parasite replication. They have suggested that the immune responses to mixed infections are more costly compared with a single

infection. This immune response is consistent with higher virulence by destroying more erythrocytes and more production of TNFα which are associated with severity of the disease (Wattavidanage *et al.*, 1999). However, more investigations are required to understand the mechanisms involved in host/parasite and parasite/parasite interaction and acquisition of immunity (Snounou *et al.*, 1992).

In this part of the present study investigation of the course of infection and the immune response in a mixed infection of avirulent and virulent strains of *P. c. adami* were the main aims. The parasitaemia, the cytokine production, and the production of parasite-specific antibodies were examined. Mice were simultaneously infected with a mixed infective dose of one virulent and one avirulent malaria parasite strains at a fixed ratio. The results show the kinetics of the immune response, cross-immunity and reaction between two strains in a resistant rodent host. As the immune responses are compared between single and mixed infections so any altered immune response and effects of inter-host reaction of avirulent and virulent strains of *P. c. adami* may be determined.

4.2. Results

4.2.1. Parasitaemia

The parasitaemia was monitored daily as previously described (Chapters Two and Three). The blood samples were usually collected between 9:30 and 11:30 am from the tail. In all experiments parasitaemia is expressed as both arithmetic and the geometric (Log_{10} pRBCs for 10^5 RBCs) means. At each time point percentage of parasitaemia is presented for at least 12-15 mice except for those occasions in which there were fewer mice surviving in each experiment.

NIH mice were infected with a mixed infective dose of 1×10^4 pRBCs of *P. c. adami* DK and *P. c. adami* DS at ratio of 5:1. The infective dose, therefore, consisted of 8×10^3 pRBCs of *P. c. adami* DK and 2×10^3 of *P. c. adami* DS. Inoculation was i.v. in 0.25ml of PBS. The course of infection was monitored for 58 days. The survival rate was 100%. The parasitaemia in mixed infection peaked at 40.28% on day 10 p.i. (Figure 4.1.). A recrudescence in the mixed infection was observed between days 20 and day 26 p.i. There was also a significantly lower (t-test $P < 0.0001$) parasitaemia in the mixed infection compared to the single-infection of *P. c. adami* DS when mice were left untreated. Despite a relatively small infective dose (i. e. 2×10^3 pRBCs) of the virulent strain the parasitaemia peaked significantly higher than that in the single-infection with avirulent *P. c. adami* DK in which the peak was 23.85% (t-test $P < 0.0001$). The peak parasitaemia was not significantly different between mice infected with the mixed infection and mice infected with the single-infection of *P. c. adami* DS when the latter mice were treated with sub-curative dose of chloroquine (Figure 3.1 to 3.3 and 4.1). Although the number of pRBCs of *P. c. adami* DS inoculated in the mixed infection and in the single-infection with a low infective dose was the same (2×10^3), the peak parasitaemia in mice infected with a single low infective dose was significantly (t-test $P < 0.0045$) higher than that in the mixed infection (Figure 3.4 and 4.1).

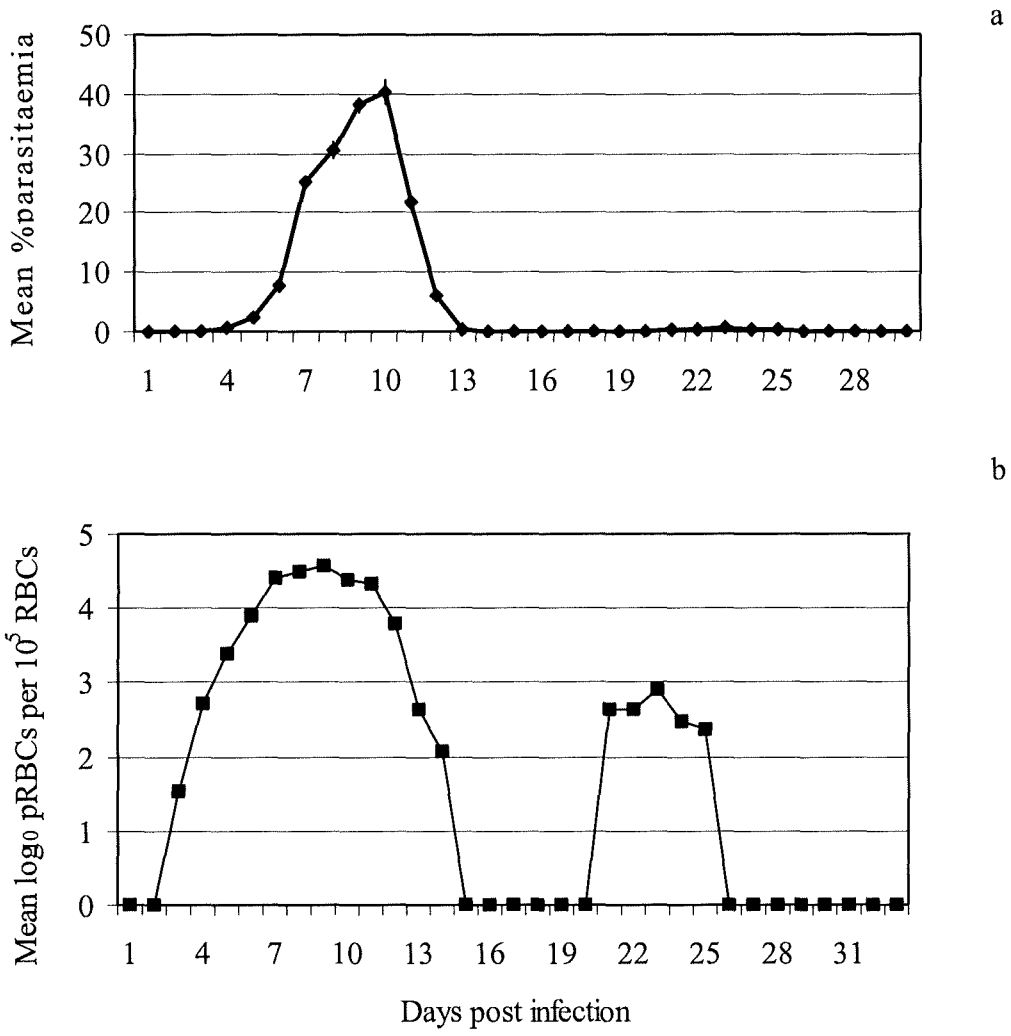


Figure 4.1. The course of infection in NIH mice infected with a mixed infective dose of 1×10^4 pRBCs of *P. c. adami* DK and DS (Ratio 5:1). The sample size was 18 mice.

Each data pont is shown as mean % of parasitaemia for all mice. Howevr, at each time points three mice were killed and exluded from the mean calculation. For each data point ± SEM has been calculated. However, SEM is not shown when it is too small.

a) Mean percentage of parasitaemia.

b) The mean $\log_{10}$ of pRBCs for 1×10^5 RBCs.

4.2.2. Splenocyte proliferative responses

The proliferative responses of the splenocytes in mice infected with a mixed infection of virulent and avirulent parasites were evaluated as previously described in Chapter Two. The levels of proliferative responses are separately presented for each time point at which mice were killed and their spleens collected for spleen cells cultures (Figures 4.2 and 4.3). Splenocytes from infected mice were restimulated with pRBCs. The control groups included splenocytes from infected or naïve mice which were exposed to naïve RBCs, ConA, or medium only.

The highest level of proliferation response was observed for splenocytes from the naive mice exposed to ConA. This result was similar to those observed in the single infections as previously described (see Chapter Three, Figures 3.6, 3.7 and 3.8). The proliferative responses of splenocytes from infected mice that were re-stimulated with pRBCs were also significantly higher (Tukey's test, $P < 0001$) compared to all controls except for splenocytes from naïve and infected mice exposed to ConA.

Stimulation index (SI)

Stimulation index presents the ratio of the p.r. between the test sample, restimulated with the mixed pRBCs, and control groups as previously described in Chapter Two. The highest indices were seen on day 4 p.i. in which restimulated splenocytes from infected mice proliferated >20 times more than the same splenocytes exposed only to the medium. The lowest of the proliferative responses were observed on days 7 and 10 p.i. at and after the peak parasitaemia. Levels of proliferative responses rose thereafter and higher proliferative responses were observed after recrudescent parasitaemias on day 25 p.i. (Figure 4.4).

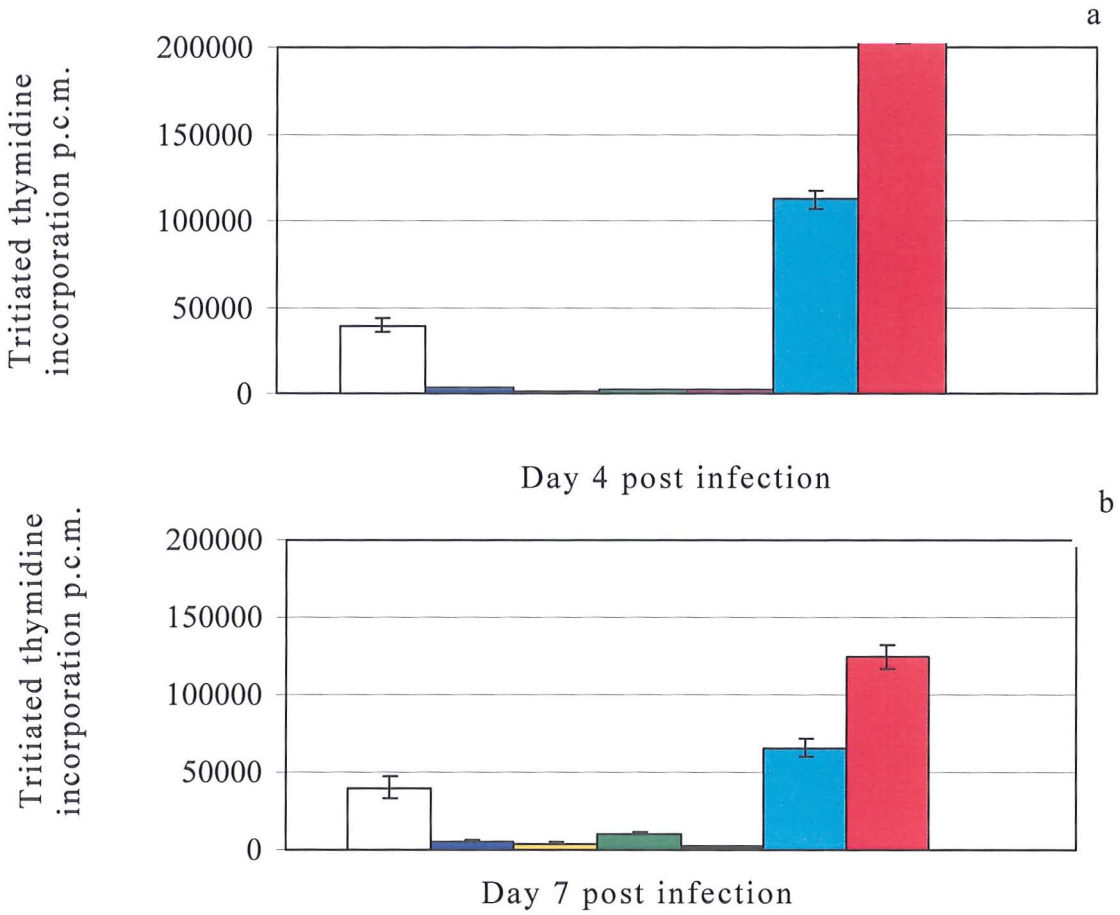


Figure 4.2. (a and b). The proliferative response of splenocytes from mice infected with a mixed infection of 1×10^4 pRBCs of *P. c. adami* DS and DK (ratio: 1/5).

Three mice were sacrificed at each time point and splenocytes of each mouse (5×10^6 cells/ml) were separately cultured and stimulated with 5×10^5 pRBCs/well, 5×10^5 naive RBCs/well, ConA at 0.5 µg/ml, or medium only. Before harvesting all suspensions were pulsed with tritiated thymidine for 12-18 hours.

Each data point is the mean ± SEM for an experiment performed in triplicate. However, SEM is not shown when it is too small.

- p+f: Splenocytes from infected mice exposed to pRBCs.
- m+f: Splenocytes from infected mice cultured in medium only.
- r+f: Splenocytes from infected mice exposed to naïve RBCs.
- p+c: Splenocytes from naïve mice exposed to pRBCs.
- r+c: Splenocytes from naïve mice exposed to naive RBCs.
- ConA+f: Splenocytes from infected mice stimulated with ConA.
- ConA+c: Splenocytes from naïve mice stimulated with naïve RBCs.

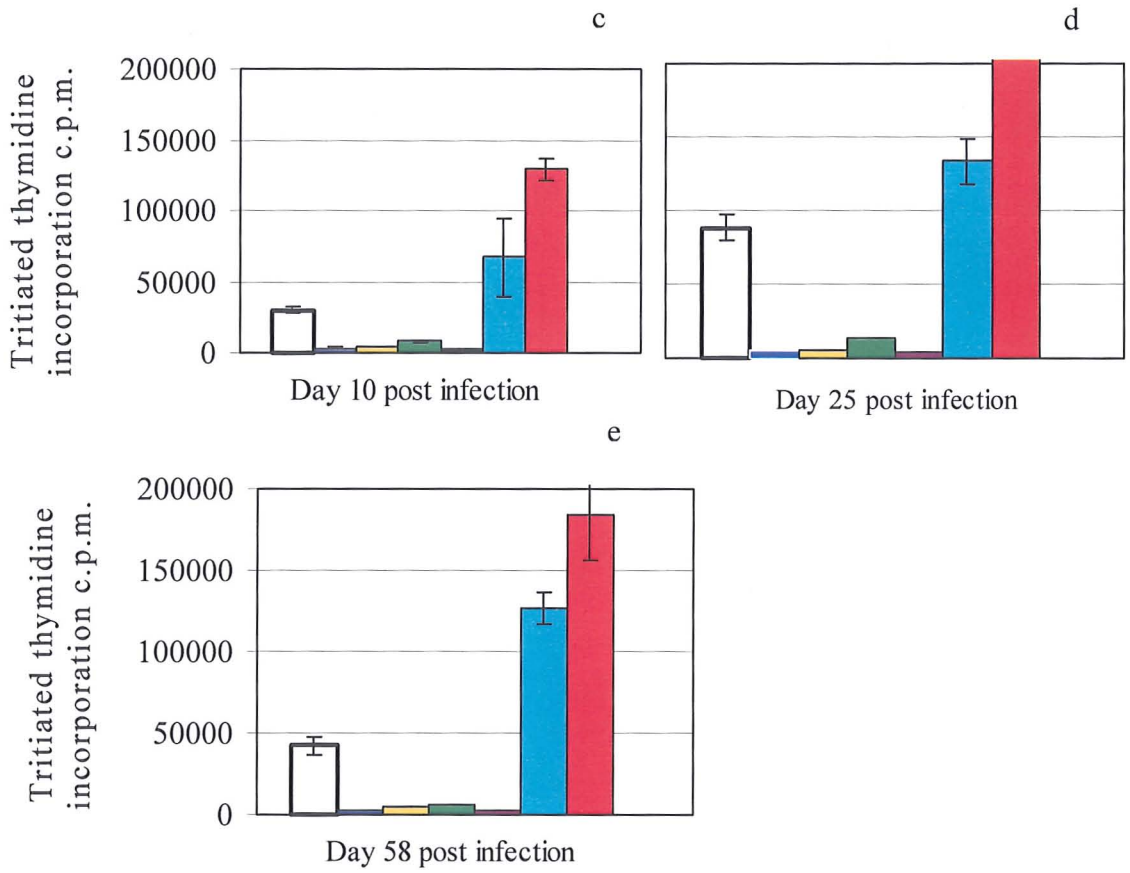


Figure 4.3. (c to e). The proliferative response of splenocytes from mice infected a mixed infection of 1×10^4 pRBCs of *P. c. adami* DS and DK (ratio: 1/5).

Three mice were sacrificed at different time points and splenocytes of each mouse (5×10^6 cells/ml) were separately cultured and stimulated with 5×10^5 of pRBCs/well, 5×10^5 of RBCs/well, ConA at 0.5 µg/ml, or medium only. Before harvesting all suspensions were pulsed with tritiated thymidine for 12-18 hours.

Each data point is the mean ± SEM for an experiment performed in triplicate. However, SEM is not shown when it is too small.

- p+f: Splenocytes from infected mice exposed to pRBCs.
- m+f: Splenocytes from infected mice cultured in medium only.
- r+f: Splenocytes from infected mice exposed to naïve RBCs.
- p+c: Splenocytes from naïve mice expose to pRBCs.
- r+c: Splenocytes from naïve mice exposed to naive RBCs.
- ConA+f: Splenocytes from infected mice stimulated with ConA.
- ConA+c: Splenocytes from naïve mice stimulated with naïve RBCs.

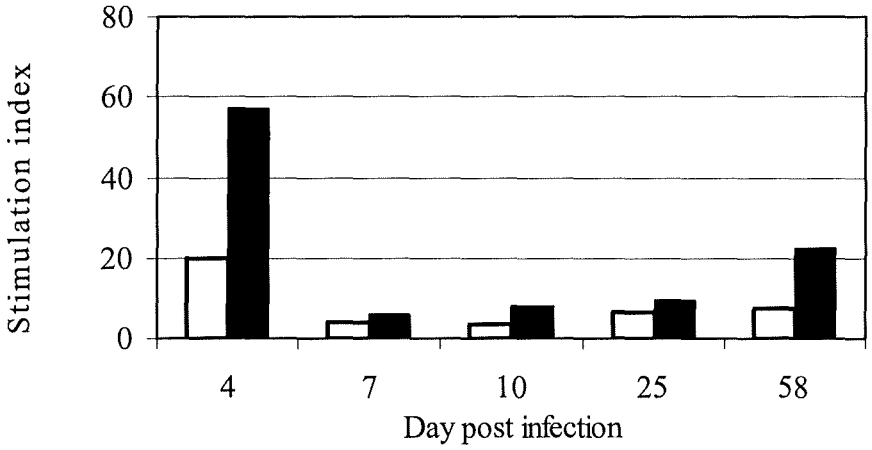


Figure 4.4. Stimulation index for mice infected with a mixed infection of 2×10^3 pRBCs of *P. c. adami* DS and 8×10^3 pRBCs of *P. c. adami* DK (ratio: 1/5).

Three mice were sacrificed at different time point and their splenocytes were cultures and pulsed with tritiated thymidine for 12-18 hours before harvesting.

□ p+f/m+f: The proliferative response of spleen cells from infected mice exposed to pRBCs divided by the proliferative response of spleen cells from infected mice exposed to medium only.

■ ConA+f/m+f: The proliferative response of spleen cells from the infected mice exposed to ConA divided by the proliferative response of spleen cells from infected mice exposed to medium only.

4.2.4. Cytokine analysis

The IFNγ and IL-4 concentrations were measured in the supernatants of cultures of splenocytes *in vitro* from mice infected with a mixed infective dose of virulent and avirulent strains. Spleens of infected and naïve mice groups were removed at each time point. Each spleen was separately cultured as previously described in Chapters Two and Three. Supernatants of the cultures were collected after three days (72 hours) for test for the cytokines, using an ELISA test and the results presented as U/ml.

4.2.4.1. IFNγ in mixed infection of *P. c. adami* DK and *P. c. adami* DS

The early production of IFNγ as indicator of a Th1 response has been previously observed in rodent malaria (see Chapters One and Three). Cytokine production was investigated in NIH mice infected with the mixed infection as described above. The course of infection was monitored for 58 days.

There were significant differences (Two way ANOVA, $P < 0.0001$) in IFNγ production for splenocytes between all tested groups. The early production of IFNγ in splenocytes from mice restimulated with pRBCs, collected on day 4 p.i. was significantly (Tukey's test. $P < 0.0001$) higher compared to all control groups (Figure 4.5). The Level of IFNγ declined to the lowest level at the peak parasitaemia (Figure 4.5) which was similar to its level in the single-infections (Chapter Three). The production of IFNγ rose after the recrudescent parasitaemia and remained at high levels up to the last sampling day.

There were significant differences (Two-way ANOVA, $P < 0.0001$) in IFNγ levels between the the mixed infection and single-infections of both avirulent and virulent strains when IFNγ was analysed among all the groups. The IFNγ level was significantly higher (Tukey's test, $P < 0.001$) in mice infected with non-lethal infection of *P. c. adami* DK compared with the IFNγ level in the mixed infection on the first sampling day (Figure 3.11 and 4.5).

The levels of IFNγ production in the mixed infection was also significantly (Tukey's test, $P < 0.0001$) lower compared to IFNγ levels in the lethal infection of *P. c. adami* DS in which mice were treated. The levels of IFNγ between the mixed infection and the single-infection of lethal *P. c. adami* DS when mice left untreated did not show a significant difference (Figure 4.5 and 3.13). However, the present results showed that the profile of IFNγ production in the mixed infection compared to the single-infections of both avirulent and virulent strains is the same.

4.2.4.2. IL-4 production in mixed infection of *P. c. adami* DK and *P. c. adami* DS

The kinetcs of the IL-4 production in the mixed infection was similar to that seen in the single-infections (see Chapter Three). In the mixed infection the levels of IL-4 were significantly higher (Tukey's test, $P< 0.0001$) than that in controls in the last sampling days compared to early stages in the course of infection. The present data showed that the IL-4 production in the mixed infection was initiated earlier compared to the single infections as higher levels of IL-4 were seen in the earlier times p.i. compared to the single infection. This was approximately coincident with the peak parasitaemia (Figure 4.6). Statistical tests showed that IL-4 level in the mixed infection was significantly lower than that in the single infections of *P. c. adami* DS, either in mice left untreated (Tukey's test, $P < 0.001$) or mice which were sub-curatively treated, (Tukey's test, $P < 0.001$) at all time points. However, IL-4 production in the mixed infection was not significantly (Two-way ANOVA, $P < 0.001$) different to that in the non-lethal infection of *P. c. adami* DK.

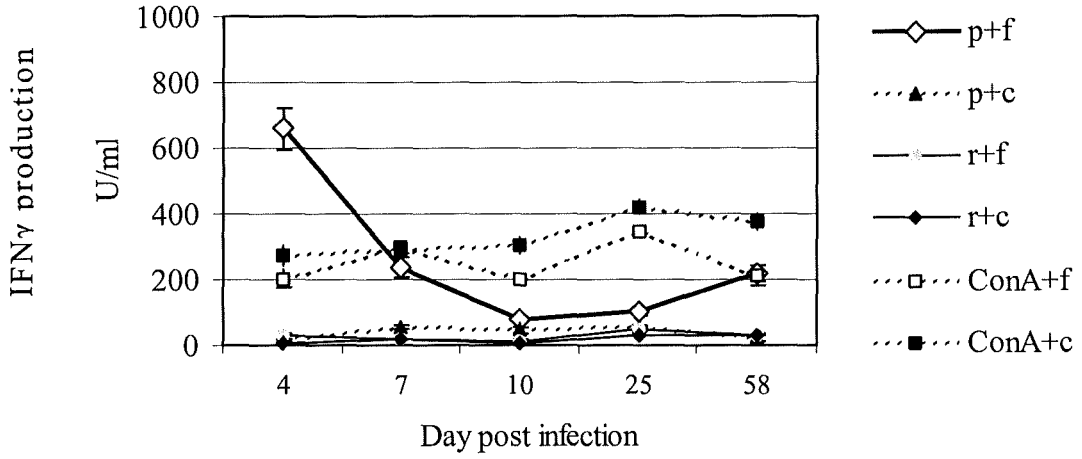


Figure 4.5. IFNγ production in NIH mice infected with a mixed infection of 2×10^3 pRBCs of *P. c. adami* DS and 8×10^3 pRBCs of *P. c. adami* DK (ratio: 1/5).

At each time point three mice were sacrificed and their spleens were individually cultured. Supernatant of splenocytes cultures for each mouse was assayed separately.

Each data point is the mean ± SEM for an experiment performed in triplicate by ELISA. However, SEM is not shown when it is too small.

p+f: Supernatant from infected mice splenocytes culture exposed to pRBCs.

p+c: Supernatant from naïve mice splenocytes culture exposed to pRBCs.

r+f: Supernatant from infected mice splenocytes culture exposed to naïve RBCS.

r+c: Supernatant from naïve mice splenocytes culture exposed to naïve RBCS.

ConA+f: Supernatant from infected mice splenocytes culture exposed to ConA.

ConA+c: Supernatant from naïve mice splenocytes culture exposed to ConA.

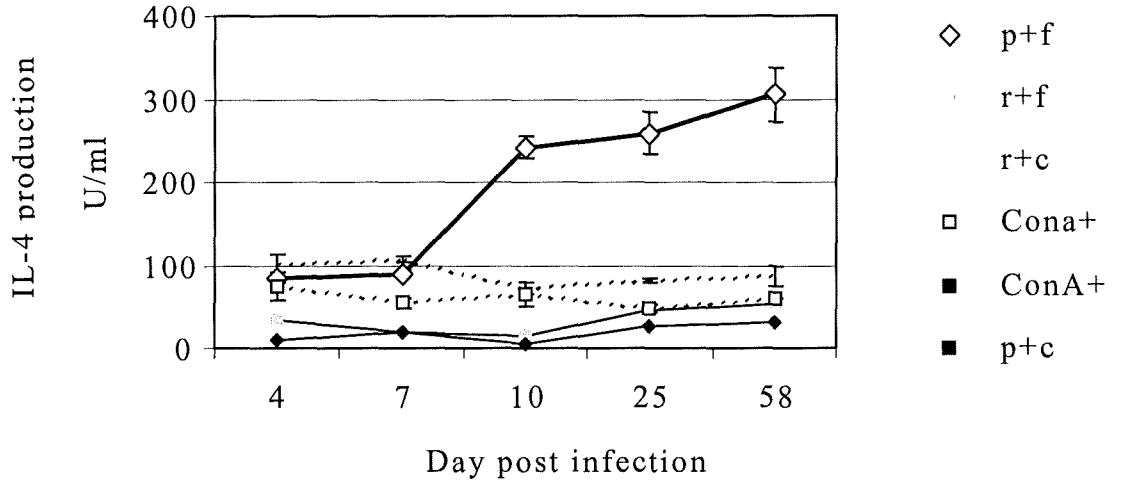


Figure 4.6. IL-4 production in NIH mice infected with a mixed infection of 2×10^3 pRBCs of *P. c. adami* DS and 8×10^3 pRBCs of *P. c. adami* DK (ratio: 1/5).

At each time point three mice were sacrificed and their spleens were individually cultured. Supernatant of splenocytes culture for each mouse was assayed separately. Each data point is the mean ± SEM for an experiment performed in triplicate in ELISA test. However, SEM is not shown when it is too small.

p+f: Supernatant from infected mice splenocytes culture exposed to pRBCs.

p+c: Supernatant from naïve mouse splenocytes culture exposed to pRBCs.

r+f: Supernatant from infected mouse splenocytes culture exposed to naïve RBCS.

r+c: Supernatant form naïve mouse splenocytes culture exposed to naïve RBCS.

ConA+f: Supernatant from infected mouse splenocytes culture exposed to ConA.

ConA+c: Supernatant from naïve mouse splenocytes culture exposed to ConA.

4.2.5. Specific antimalarial IgG antibody production during mixed infection

The parasite-specific production of IgG antibodies in the sera of infected mice and control naïve mice was determined by ELISA test as described in Chapters Two and Three. Sera were assayed for IgG whole molecule, and IgG1 and IgG2a subclasses. Lysates of pRBCs were prepared from mice infected with the same ratio of infective dose as in the mixed infections and from naïve RBCs.

4.2.5.1. Parasite-specific IgG whole molecule in the mixed infection

An increase in the production of parasite-specific IgG (whole molecule) was observed before peak parasitaemia and remained at high levels thereafter during the observation period. The levels of total IgG were significantly higher (Tukey's test, $P < 0.0001$) than that in control groups. The highest level of IgG (whole molecule) was seen on the last sampling day, on day 45 p.i (Figure 4.7) compared to all other time points.

4.2.5.2. Parasite-specific IgG1 in the mixed infection

IgG1 production begins to rise after the peak parasitaemia (Figure 4.8). However, its production showed a sharp increase on day 18 p.i. and then gradually increased from day 25 p.i until the end of the experimental peroid. (Figure 4.8).

IgG1 production in infected mice was significantly different (Two way ANOVA, $P < 0.0089$) between all groups (Figure 4.7). On the last sampling day, day 45 p.i., the levels of IgG1 was assessed between different groups. There was significant (Tukey's test $P < 0.0001$) difference between mice infected with the mixed infection and the avirulent DK strain for IgG1. However, the IgG1 level was significantly higher (Tukey's test, $P < 0.001$) in mice infected with the mixed infection compared to that levels in mice infected with the virulent DS strain when mice treated. The IgG1 levels in the mixed infection were also significantly higher (Tukey's test, $P < 0.001$)

compared to IgG1 levels in mice infected with a low infective dose of the DS strain.

4.2.5.3 Parasite-specific IgG2a in the mixed infection

Sera of mice infected with a mixed infection and control naïve mice were also assayed for IgG2a production. In the mixed infection the specific-IgG2a began to increase after day 7 p.i. and sharply increased immediately after the peak parasitaemia, and remained at high levels thereafter through the observation period (Figure 4.9). There were significant differences (Two way ANOVA, $P < 0.0001$) in serum IgG2a levels between all tested groups (Figure4.9). Comparison of IgG2a levels showed that there were significantly higher levels (Tukey's test, $P < 0.0001$) of IgG2a in the mixed infection compared to the avirulent *P. c. adami* DK infection (Figures 4.9 and 3.22).

The IgG2a levels were also significantly higher (Tukey's test, $P < 0.0004$) in the mixed infection than that in the virulent *P. c. adami* DS infection when mice treated or when mice infected with a low infective dose (Tukey's test, $P < 0.0003$) (Figures 4.8, 3.23, and 3.24). There was no significant difference for IgG2a in the mixed infection and mice infected with the DS strain and left untreated (Figures 4.8 and 3.23).

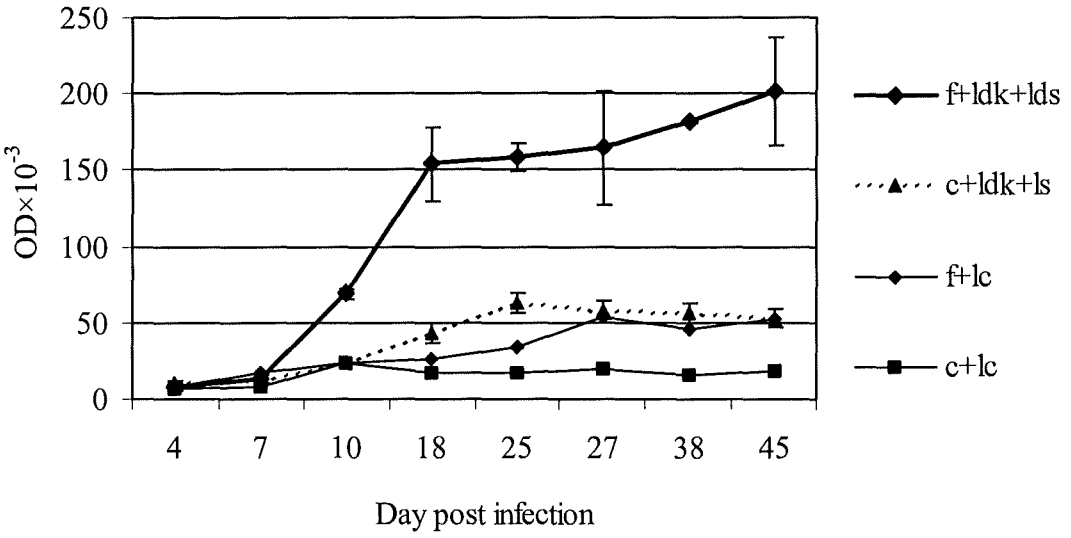


Figure 4.7. Parasite-specific total IgG (whole molecule) production.
Mice infected with a mixed infection of 2×10^3 pRBCs of *P. c. adami* DS and 8×10^3 pRBCs of *P. c. adami* DK (ratio: 1/5).
Sera from both the infected mice and naïve mice were assayed by ELISA test. Each data point is the mean ± SEM for an experiment performed in triplicate. However, SEM is not shown when it is too small.
f+lds+ldk: Sera from infected mice reacted with the lysate from mixed pRBCs of *P. c. adami* DK and *P. c. adami* DS.
c+lds+ldk: Sera from naïve control mice reacted with the lysate from the mixed pRBCs.
f+lc: Sera from infected mice reacted with lysate from naïve mice.
c+lc: Sera from naïve control mice reacted with the lysate from naïve control mice.

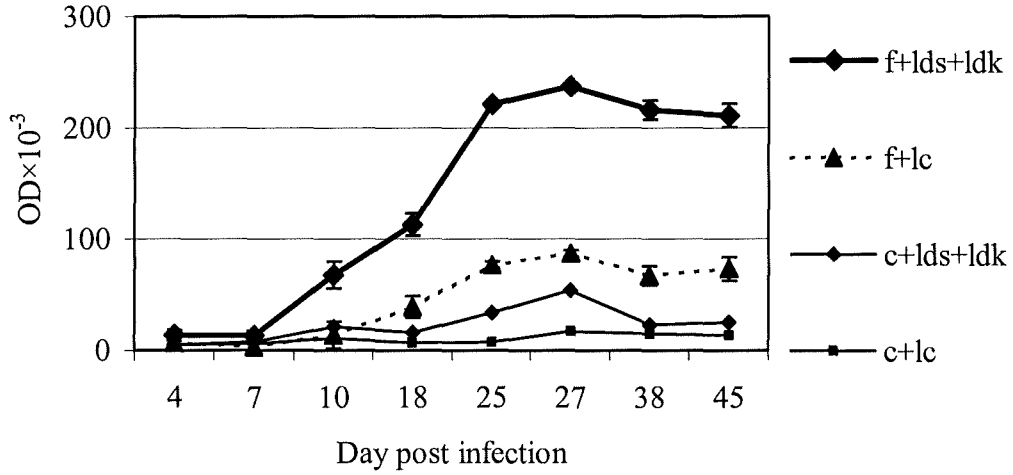


Figure 4.8. Parasite-specific IgG1 production in the mixed infection.
Mice infected with a mixed infection of 2×10^3 pRBCs of *P. c. adami* DS and 8×10^3 pRBCs of *P. c. adami* DK (ratio: 1/5).
Sera from both the infected mice and naïve mice were assayed by ELISA test.
Each data point is the mean ± SEM for an experiment performed in triplicate. However, SEM is not shown when it is too small.
f+lds+ldk: Sera from infected mice reacted with the lysate from mixed pRBCs of *P. c. adami* DK and *P. c. adami* DS.
c+lds+ldk: Sera from naïve control mice reacted with the lysate from the mixed pRBCs.
f+lc: Sera from infected mice reacted with lysate from naïve mice.
c+lc: Sera from naïve control mice reacted with the lysate from naïve control mice.

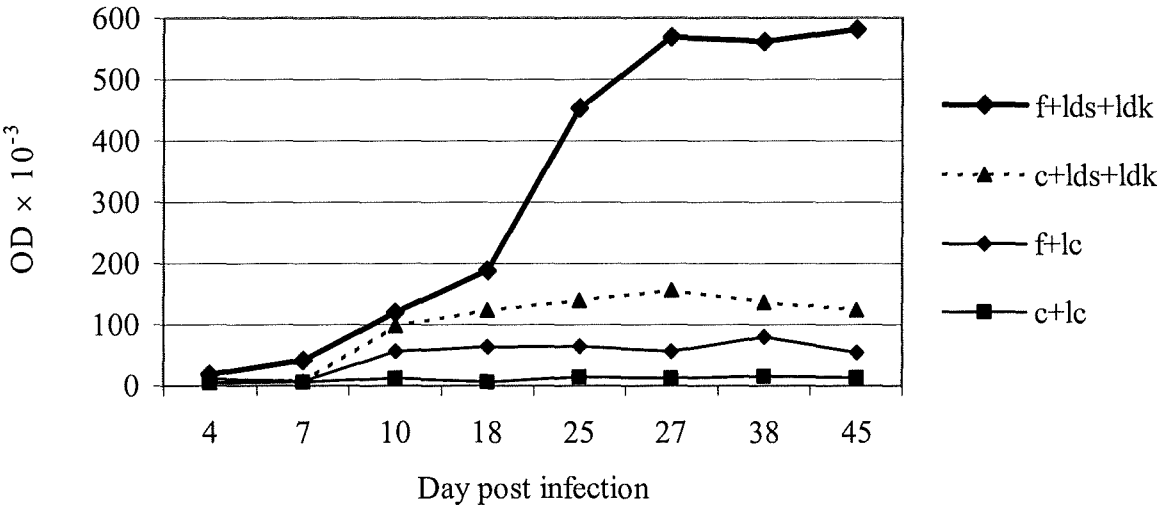


Figure 4.9. Parasite-specific IgG2a production in the mixed infection. Mice infected a mixed infection of 2×10^3 pRBCs of *P. c. adami* DS and 8×10^3 pRBCs of *P. c. adami* DK (ratio: 1/5). Sera from both the infected mice and naïve mice were assayed by ELISA test.

Each data point is the mean ± SEM for an experiment performed in triplicate. However, SEM is not shown when it is too small.

f+lds+ldk: Sera from infected mice reacted with the lysate from the mixed pRBCs of *P. c. adami* DK and *P. c. adami* DS.

c+lds+ldk: Sera from naïve control mice reacted with the lysate from the mixed pRBCs.

f+lc: Sera from infected mice reacted with lysate from naïve mice. c+lc: Sera from naïve control mice reacted with the lysate from naïve control mice.

4.3. Discussion

Evaluation of the immune responses in the mixed infection of avirulent *P. c. adami* DK and virulent *P. c. adami* DS was the main aim of this part of the study. Comparison between the immune responses in mixed infection and their related single-infections of each strain was also included.

As previously indicated there was no mortality as a result of *P. c. adami* DK single-infection.On the other hand, the outcome of infection with the virulent, *P. c.adami* DS was fatal. Studies showed a range of mortality between 3.2% (Smooker *et al.*, 2000) and 20% to 50% (Jarra and Brown, 1985) in infections with the virulent *P. c.adami* DS strain. These results also shows that mortality is influenced by the infective dose. For example, smooker *et al.* (2000) inoculated 1×10^5 pRBCs whereas Jarra and Brown (1985) used between 1×10^3 and 1×10^5 pRBCs. Most deaths in the virulent infection occurred during or immediately after the initial peak parasitaemia. The results presented here (Chapter Three) also showed that infective dose influences outcome of infection as there was between 60% to 34% mortality in mice infected with 1×10^4 or 2×10^3 pRBCs of the virulent DS strain respectively. However, it should be bearing in mind that the group size in the low-infective dose infection was only six mice. Nevertheless, in the present study the outcome of the mixed infection was not fatal despite the presence of a high parasitaemia. This peak parasitaemia was significantly higher compared to the peak parasitaemia in the avirulent DK strain infection. On the other hand, in the mixed infection the parasitaemia peaked at 40 % that was significantly lower than that in the virulent DS strain single-infections either in untreated mice infected with 1×10^4 or in mice infected with 2×10^3 pRBC of the parasite. However, peak parasitaemia in the mixed infection was not significantly different to peak parasitaemia in mice infected with 1×10^4 pRBCs of *P. c. adami* DS and treated with chloroquine. According to the present results, in the mixed infection it may be proposed that the presence of the avirulent DK strain may partially contribute in control of the virulent DS strain. It should be particularly considred when a low-infective dose of the virulent DS strain, 2×10^3 pRBC, causes a higher parasitaemia comapred to that in the mixed infection. This control is reflected by the reduced peak parasitaemia and no

death in the mixed infection comapred to the outcome of the single-DS infections.

Cross-immunity has been also suggested to contribute in control of mixed infections (Cox and Voller, 1966; Jara and Brown, 1989). Although extrapolation from their results to the present results should be done with caution, the cross-immunity between the avirulent DK and the virulent DS strains may partially contribute to the survival of the mice in the mixed infection. Regarding to the cross-immunity, it has been reported that in the presence of *P. c. chabuadi* CB the parasitaemia of *P. c. chabuadi* AS and *P. c. adami* DS was reduced and a lower parasitaemia was observed with *P. c. adami* DS when it was present with *P. c. chabuadi* AS (Snounou *et al.*, 1992). Taken together, the present results suggest that the presence of the avirulent DK parasite may alter the virulence of the DS strain resulting to a non-fatal mixed infection with a lower peak parasitaemia and no mortality (Figures 3.1 3.4 and 4.1). However, it also appeared that the virulent DS strain had probably a higher rate of proliferation, as a virulence factor, compared to the avirulent DK strain because the peak parasitaemia in the avirulent single-infection reached only to 24% even when the inoculum was 1×10^5 pRBCs whereas in the mixed infection parasitaemia peaked up to 40.28% when the number of pRBCs of the DS was only 2×10^3 in the mixed infective dose. In the present study the actual proportion of each strain during asexual blood stage was not examined and needs to be investigated.

A higher replication rate and increase in virulence in mixed infection of two cloned lines of *P. c. chabaudi*, ER and CR, compared to their single-infections was shown by Taylor, MacKinnon and Read (1999). They suggested that a higher replication rate in more virulent parasite shows that in mixed-genotype infections natural selection should favour higher levels of virulence than that in single-infections. It has been also proposed that in *P. chabaudi* virulence is positively and genetically related to replication rate (Mackinnon and Read, 1999). So, in the present results although increase in virulence was observed due to higher peak parasitaemia compared to the non-lethal infection, no death shows that this increase did not result in a lethal outcome. Nevertheless, the present results show that even if the proportion of the virulent DS strain in the infective dose is low, four folds less than the avirulent DK strain, the peak parasitaemia as a virulence factor may increase.

In agreement with previous studies (reviewed by Wipasa *et al.*, 2002; Langhorne *et al.*, 1989; Taylor-Robinson *et al*, 1995) the present results showed that there were consistent early increases of parasite-specific IgG2a with IFNγ and later increases of IgG1 with IL-4 in the mixed infection which reflects a sequential Th1/Th2 response. The results presented here showed that the IFNγ production was at the highest levels early during the course of infection compared to its levels therafter p.i. However, the IFNγ production in the mixed infection was lower than that in all other sinle-infections either in non-lethal or lethal ones. This profile of IFNγ production was followed by increase of IL-4 levels later particularly in the last sampling days. However, there were significantly higher levels of IL-4 in the first sampling days compared to the IL-4 levels in both single infections of the DK strain and DS strains at the same time points when mice were left untreated. This shows that in the mixed infection IL-4 initated to rise earlier than that in the single-infections. Taken together, a sequential Th1/Th2 responses in the mixd infection shows slightly differences compared to the DK and the DS single-infections. In this respect, a stronger induction of a Th1 in the single-infections comapred to Th1 response in the mixed infection was seen. However, a Th2 response was seen stronger in the mixed infection comapred to that in the single-infectios. The lower production of IFNγ compared to the single-infections on the first sampling day (Figures 3.11, 3.12, 3.13, and 4.5) may be justified because of the presence of higher level of IL-4 (Figures 3.14, 3.15, 3.16, and 4.4.6), as an inhibitor for release of IFNγ (reviewed by Saul, 1998).

As the ratio of infective dose was not varied during the study, further studies in which different ratios are used may provide to understand better role of interactions between two strain in terms of the immune response, infection dynamics, and virulence alterations in mixed infections. For example, using different ratios of avirulent and virulent strains in the same host may show the effect of each strain upon the other. Molecular biology techniques i.e. PCR may help to distinguish the proportion of each strain in a mixed infection during monitoring of the course of mixed infection. In addition, using different susceptible or resistant host models may lead to more clarification of mechanisms which are involved in mixed infections.

Chapter Five

The effect of passive transfer of purified IgG subclasses on avirulent *Plasmodium chabaudi adami* DK and virulent *P. c. adami* DS infections in NIH mice and their cross reactivity

5.1. Introduction

Passive immunization studies play an important role in understanding the immune response and also provide useful source of information for all vaccine development studies (Gysin *et al.*, 1996, Garraud, Mahanty, and Perraut, 2003). In a passive immunization encountering the host with a parasite induces antibody which can be collected and transfer to another susceptible host. The roles of these antibodies i.e. in a malaria parasite infection are to help the clearance of parasites, to limit disease, or prevent parasite invasion into the host cells (Garraud *et al.*, 2003). Some studies in humans have shown that treatment of non-immune infected patients with antibodies from protected individuals is effective (Cohen, McGregor, and Carrington, 1961; Sabchareon *et al.*, 1991; Cavinato *et al.*, 2001). Antibodies could be also involved in ADCC or antibody-mediated phagocytosis. In *P. falciparum* infection immunity to the blood stage of malaria is associated with protective-type antibodies of certain classes and subclasses. So, sera of donors that are previously exposed to the parasite contain antibodies which are not only markers of infections but also are effectors in protection (Garraud, Mahanty, and Perraut, 2003).

In animals serum of the squirrel monkey *Saimiri sciureus* after a drug-controlled infection of *P. falciparum* contains antibodies specific to the parasite. Passively transfer of this immune serum has been shown to substantially mediates in protection of *Saimiri sciureus* against asexual erythrocytic stages of *P. falciparum* (Groux *et al.*, 1990).

In rodent models it has been demonstrated that antibodies taken from mice recovered from *P. yoelii*, *P.berghei*, or *P. chabaudi* infections can transfer protection to naïve recipient mice against homologous or heterologous challenges of parasites (Freeman and Parish, 1981; Jarra *et al.*, 1986). In a preliminary passive transfer experiment it was demonstrated that sera from immunized and untreated C57BL/6J mice infected with *P. chabaudi* were more protective for naïve mice than serum from non-immune mice (McDonald and Sherman, 1980).

As a result of passive immunization experiments it is known that among antibody classes IgG antibodies have a major role in the control of asexual blood stage of malaria parasites in humans (Cohen, McGregor and Carrington, 1961; Sabchareon *et al.*, 1991; Bouharoun-Tay-oun *et al.*,

1990), in primate (Gysin, Fandeur and Pereira da Silva, 1982), and in rodent models (Majarian *et al.*, 1984; Jarra *et al.*, 1986; Jarra and Brown, 1989). Druihle *et al.* (1997) showed that passive transfer of IgG from immune adults contributes to control of patent parasitaemia of *P. falciparum*. It was evident that the IgG1 and IgG3 antibody-mediated responses protect humans against the asexual blood stages of *P. falciparum* (Druihle *et al.*, 1997; Aucan *et al.*, 2001). In mouse model IgG2a and IgG1 subclasses have been shown to have a similar role against *Plasmodium* spp (Smith *et al.*, 1997; Rotman *et al.*, 1998; Matsumoto *et al.*, 2000; Cavinato *et al.*, 2001). Bouharoun-Tayou *et al.* (1990) showed the capability of pool African IgG to confer protection in Thai patients. Gysin *et al.* (1996) also showed that the same pool IgG obtained from African donors were able to inhibit parasitaemia in *Saimiri* monkeys acutely infected with two different strains of *P. falciparum*. They showed that the inhibition of development of the parasite was a dose-dependent. When squirrel monkeys received 60 mg/Kg of IgG every day for 5 days the parasitaemia was dropped at 1000 folds compared to the control. Whereas with the same regiment of the treatment, the drop in parasitaemia was moderiate in monkeys received 30 mg/Kg, and in monkeys received only 15 mg/Kg the drop was weak.

Evaluation of the efficacy of MAbs against specific antigen targets has been examined in passive immunization studies. For example, the ability of MAbs produced against MSP-1 to suppress blood-stage parasitaemia of a lethal *P. yoelii* YM challenge was assessed. Of the three MAbs that were most effective for suppressing parasitaemia after passive immunization, two were IgG3 and the other one was IG2a (Spencer Valero *et al.*, 1998).

One of the principal molecules of the erythrocytic stages of malarial parasites (PMMSA), a 230-kDa of PMMSA of *P. yoelii*, can be recognized by a MAb designated MAb 302 (Burns *et al.*, 1989). Passive transfer of MAb 302 provided protection against *P. yoelii* challenge in mice. The variant specific protective capacity of MAb 302 has been also shown as the MAb recognizes PMMSA of three of five *P. yoelii* lines. There is also some similarities between this rodent PMMSA and the PMMSA of human *P. falciparum*. So, this similarity could be important in the construction of malaria vaccines because it shows that using similar antigens may induce response to other species of parasite (Burns *et al.*, 1989). A dramatic delay, six to eight days, in onset of parasitaemia, the most defining feature of the

efficacy of passively transferred specific antibodies, was shown in BALB/c mice administered with anti-MSP-1_{19} MAb (Vukovik *et al.*, 2000). MAb 302, which was produced from hybrid cells of spleen cells immune to17X strain of *P. yoelii* and fused with P3X63Ag8 myeloma cells reacted with the merozoites of nonlethal *P. yoelii* 17X and lethal 17XL strains and passively protected mice against challenge with the lethal variant 17XL. While, all control mice were not passively given the antibody died (Majarian *et al.*, 1984).

Identification of the asexual erythrocytic stages susceptible to parasite-specific IgG is an important contribution in vaccine development studies. This could clarify mechanism by which antibodies interfere with *Plasmodium* growth and propabably control the parasitaemia. Cavinato *et al.* (2001) showed that in a passive transfer study using antibodies that were purified from hyperimmune serum taken from mice infected with virulent and synchronous *P. c. chabaudi* AJ, the effector mechanisms including specific IgG2a and IgG1, operated immediately prior to reinvasion of red blood cells. This observation was confirmed because treatment of infected mice with the hyperimmune serum inhibited the generation of new ring forms but did not alter the number of schizont-infected erythrocytes (Cavinato *et al.*, 2001).

Passive immunization studies also provide evidence for presence of immune cross-reactivity between species or within strains of a species. Sharing the same antigens is an important reason for the occurrence of cross-reactivity and immunity between species as Bray and El-Nahal (1966) found shared antigens for *P. berghei* and *P. yoelii*. Cross-immunity was also examined by Cox and Voller (1966) between *P. berghei*, *P. yoelii*, *P. chabaudi*, and *P. vinckei*. They demonstrated that there is a cross-protection between the rodent malaria parasite species as *P. berghei* infection induces protection against *P. yoelii* but not vice versa. This may be due to the greater virulence of *P. berghei*. The study showed that pre-immune mice recovered from *P. chabaudi* infection resisted challenge with *P. vinckei* and mice recovered from infection with *P. vinckei* were immune to challenge with *P. chabaudi*. It was also demonstrated that passive transfer of pooled human IgG obtained from immune donors resulted *in vivo* anti-parasite activity in *Saimri* monkeys infected with isolates of *P. falciparum* (Gysin *et al.*, 1996).

In a passive immunization experiment sera from CBA/Ca mice infected and then reinfected with *P. c. chabaudi* AS or CB or superinfected with *P. berghei* KSP-11, were injected into naïve syngenic recipients and subsequently challenged with homologous or heterologous parasites. Transfer of these sera mediated some or all of the following changes in the course of infection in the recipient mice: (a) an extension of the pre-patent period (b) more delay in the time taken for parasitaemia to reach 2% compared with the controls (c) a reduced peak parasitaemia (d) and a protraction of the initial peak parasitaemia. The study showed that although sera taken from superinfected mice and to a lesser extent from mice reinfected once after recovery from a primary infection had species-specificity feature, a degree of cross-reactivity was also observed (Jarra *et al.*, 1986).

Although infection with *P. c. adami* DS is normally lethal between 7 to 10 days p.i., mice immune to *P. c. chabaudi* AS after challenge with *P. c. adami* DS showed clearance between days 12 and 25 p.i. However, this clearance was slower than that in the same immune mice challenged with *P. c. chabaudi* AS. Mota and colleagues (2001) showed above evidence for cross-reactivity. They showed species specificity by demonstrating that serum from *P. c. chabaudi* AS hyperimmune CBA/Ca mice reacted with antigens released from disrupted pRBCs from the homologous parasite. They prepared then hyperimmune serum of *P. chabudi* CB or *P. berghei* (KSP-11) by repeated infection with homologous parasites in CBA/Ca mice and observed that *P. c. chabaudi* CB and *P. berghei* KSP-11 hyperimmune serum contained cross-reactive antibodies to antigens of *P. c. chabaudi* AS.

The present study aimed to monitor the course of infection, determie the kinetics of antibody production, and examine the presence of cross-reactivity in NIH mice challenged with eithr avirulent *P. c. adami* DK or virulent *P. c. adami* DS in passive immunization experiments. In the present study both serum or IgG subclasses, IgG1 and IghG2a, obtanined from the DK-infected mice. The present study focused to determine and compare the consequences of passive tansferr of serum or IgG subclasses in avirluent or virulent infections. The profile of antibody production after reinfection of immune mice was also examined.

It is also not investigated if there are any differences in the effect of IgG subclasses that are collected at different time points during the course of

infection. Therefore, the present study investigated differences in the efficacy of purified IgG subclasses from sera which were collected at two different time points in mice challenged with either the avirulent DK or the virulent DS strains of *P. c. adami* strains. IgG2a and IgG1 were obtained on day 15 p.i which was coincident with the resolution of the primary-acute parasitaemia or on day 55 p.i after recrudescent parasitaemia and when the infections usually became chronic. The study also evaluated possible cross-reactivity of these purified antibodies. As IgG2a and IgG1production are consistent with Th1 or Th2 immune responses respectively so, the profile of these IgG subclasses production with consideration of Th type response was also examined.

5.2. Results

In order to determine the efficacy of passively transferred whole sera for protection against either avirulent *P. c. adami* DK or virulent *P. c. adami* DS immune sera were taken from mice infected with 1×10^5 pRBCs of *P. c. adami* DK. These immune sera were collected at two time points, day 15 and day 55 p.i. so that to investigate if there are any differences in the effect of whole sera taken from two different time points which were coincident with the time of declining of the primary peak parasitaemia and the chronic phase of the infection respectively. Mice whose sera collected on day 55 were reinfected on day 40 p.i to boost antibody level. NIH mice were infected with 1×10^4 pRBCs of DK or DS strains and injected with immune serum thereafter. The infected mice in each test were divided into two groups. Group one received 500µl serum from day 15 p.i. and the second group received serum from day 55 p.i. The control groups were infected with the same dose of either avirulent or virulent parasite.

In the present study the efficacy of purified specific IgG1 and IgG2a were also examined. So, the course of infection in mice infected with 1×10^4 pRBCs of either avirulent *P. c. adami* DK or virulent *P. c. adami* DS and passively immunized with purified IgG1 or IgG2a was determined. The antibody subclasses were obtained from pooled sera collected from mice infected with the avirulent DK strain as described in Chapter Two. Immune sera were collected on days 15 and 55 p.i. and IgG1 and IgG2a were

fractionated and purified using chromatography as described in Chapter Two.

5.2.1. The course of infection in mice infected with *P. c. adami* DK and passively immunized with whole serum

The effect of passively transferred immune serum on the course of infection in mice infected with *P. c. adami* DK was examined. Different groups were used in the experiment as follows.

In group 1 mice were infected with 1×10^4 pRBCs of *P. c. adami* DK and immediately injected with 0.5 ml serum collected on day 15 p.i.

In group 2 mice were infected with 1×10^4 pRBCs of *P. c. adami* DK and immediately injected with serum collected on day 55 p.i.

In the control groups mice were infected with 1×10^4 pRBCs of the DK strain and were immediately injected with 0.5 ml PBS or left witout any treatment.

Figure 5.1. shows that in the control group parasitaemia peaked at 21.83% on day 10 p.i. and resolved to subpatency by day 15 p.i. In mice given pooled sera collected on day 15 p.i., the parasitaemia peaked at 13.13% which was significantly lower (Tukey's test, $P< 0.007$) than that in the control group. In this treated group parasitaemia sharply decreased after the peak but increased again two days later and thereafter from day 15 and resolved by day 18 p.i. In mice immunized with pooled sera collected on day 55 p.i. the peak parasitaemia was 6.23 % which was also significantly lower (Tukey's test, $P< 0.0006$) than that in the control group. In this group peak parasitaemia was seen on day 13 p.i. which was three-days later than the control group. In mice passively immunized with sera from day 55 p.i., the primary parasitaemia was resolved by day17 p.i. The results, therefore, showed that sera from day 55 p.i. had more protectivity compared to sera from day 15 p.i. because there was a significant lower (Tukey's test $P < 0.011$) peak parasitaemia in mice given sera from day 55 p.i. compared to peak parasitaemia in mice given sera from day 15 p.i. The recrudescent parasitaemia was first seen in mice immunized with sera from day 55, secondly in mice immunized with sera from day 15 p.i and finally in the control group.

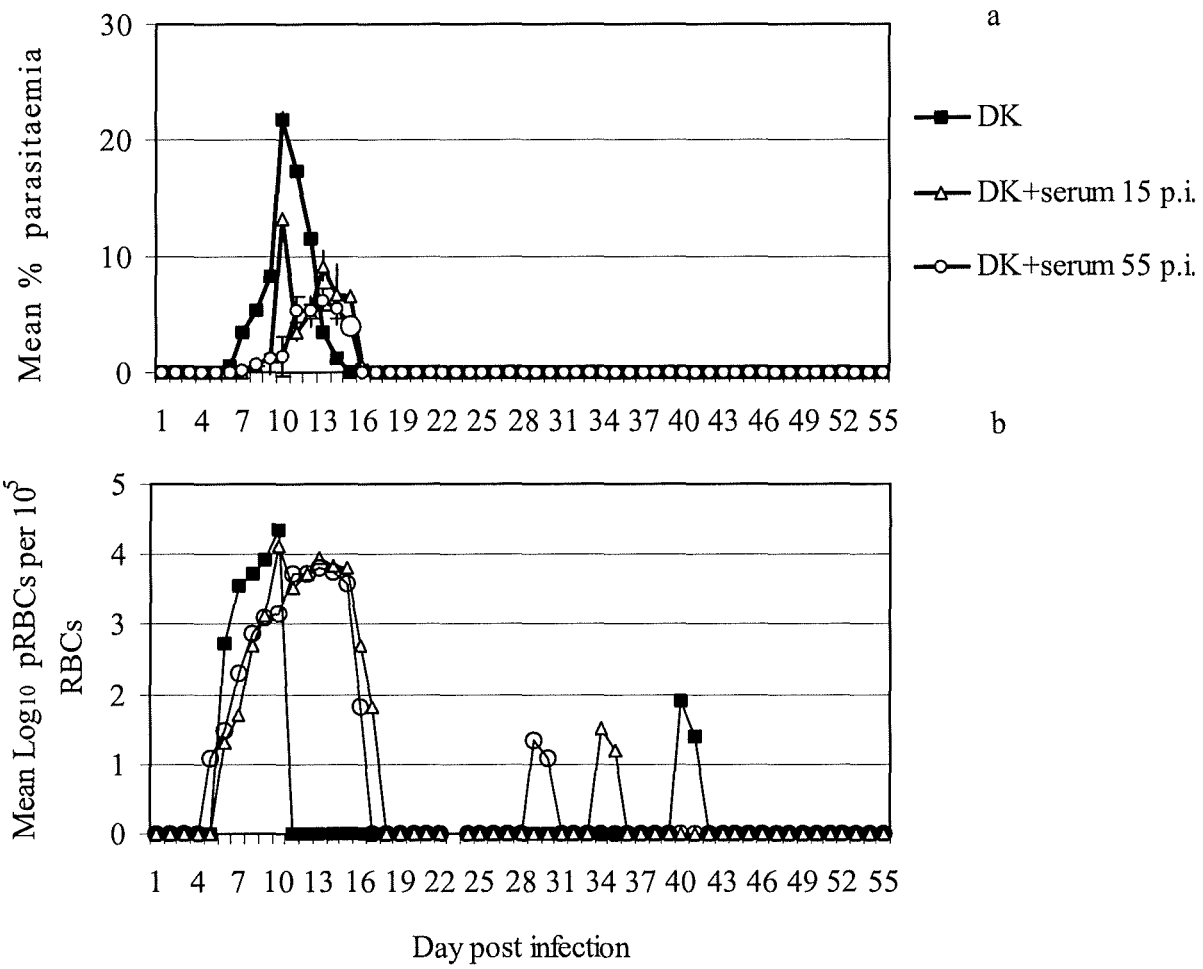


Figure 5.1. The course of infection in NIH mice infected with *P. c. adami* DK.

Mice infected with 1×10^4 pRBCs of *P.c. adami* DK and immediately injected with 0.5 ml of serum. Control group was given only PBS.

Each group included six mice. Serum collected from mice infected with *P. c. adami* DK.

Data points are shown as the mean % ± SEM. However, SEM is not shown when it is too small.

a) Mean % of parasitaemia.

b) Mean $\log_{10}$ pRBCs per 10^5 RBCs.

DK: Mice infected with *P.c. adami* DK and injected 0.5 ml PBS as control group.

DK+serum 15p.i: Mice infected with *P.c. adami* DK and injected with 0.5 ml serum collected on day 15 p.i.

DK+serum 55p.i: Mice infected with *P.c. adami* DK and injected with 0.5 ml serum collected on day 55 p.i.

5.2.2. The course of infection in mice infected with *P. c. adami* DS and passively immunized with whole serum

The presence of cross-reactivity in sera collected from mice infected with avirulent strain DK and passively transferred to mice infected with the DS strain was examined. The experiment was performed as described above (5.2.1.).

Figure 5.2. shows that in the control group which were infected with the DS strain the parasitaemia peaked at 54.5%. In mice passively immunized with pooled sera collected on day 15 p.i. and infected with the DS strain the parasitaemia peaked to 39.1%, which was significantly lower (Tukey's test $P < 0.007$) than that in the control group. The peak parasitaemia was 31.12% in mice injected with sera from day 55. This peak was also significantly lower (Tukey's test $P < 0.006$) compared to the control group. Although the peak parasitaemia in mice given sera collected on day 55 p.i. was lower compared to the peak in mice given sera from day 15 p.i., this difference was not significant. No death was seen in mice passively immunized either with immune serum from day 15 or day 55 p.i. and challenged with the virulent DS strain. So, the results indicated the presence of an effective cross-reactivity in serum from the DK strain in control of primary peak parasitaemia in mice infected with the DS strain. Although in the control group in which mice given PBS no death was seen, in the untreated group four of six mice died (33.3% survival rate).

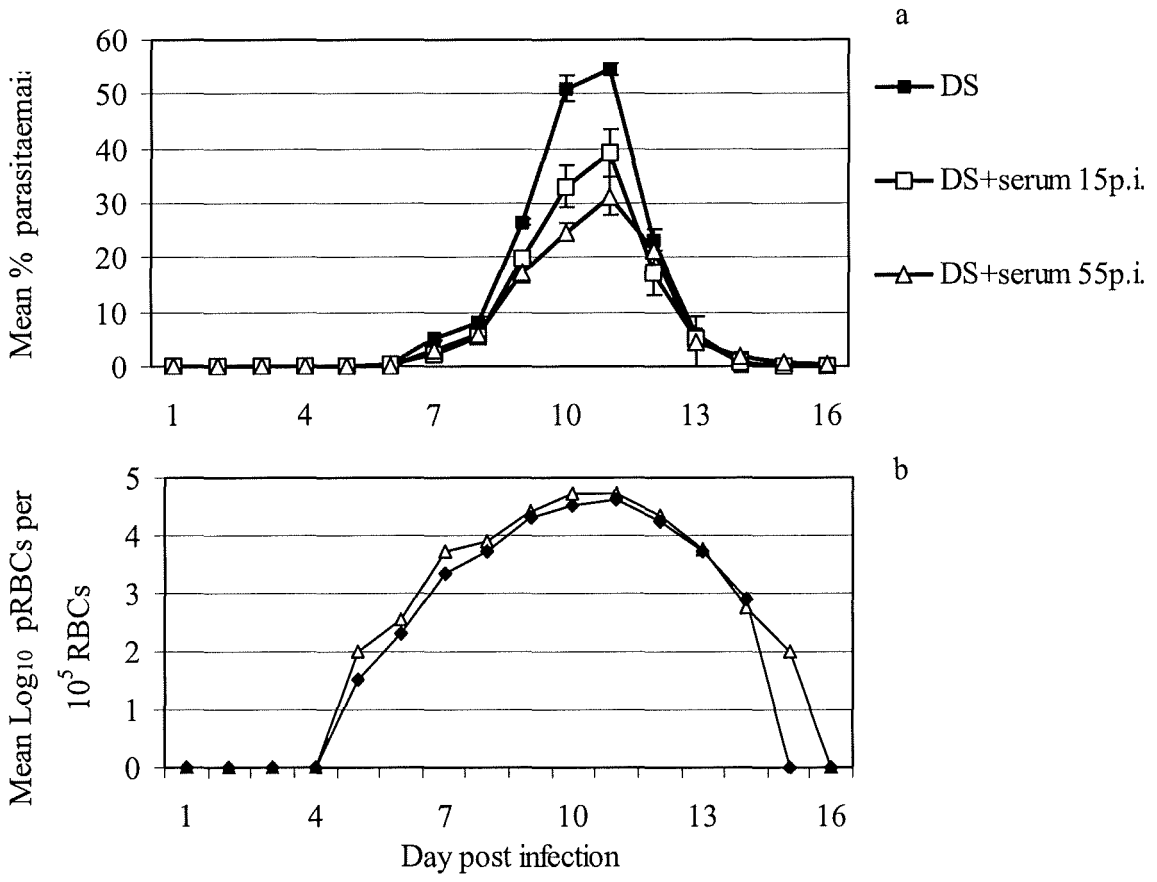


Figure 5.2. The course of infection in NIH mice infected with *P. c. adami* DS.

Mice infected with 1×10^4 pRBCs of *P.c. adami* DS and immediately injected with 0.5 ml serum. Control group given only PBS. Each group included six mice. Serum collected from mice infected with *P. c. adami* DK for immunization.

Data points are shown as the mean % ±SEM. However, ±SEM is not shown when it is too small.

a) Mean % of parasitaemia.

b) Mean $\log_{10}$ pRBCs per 10^5 RBCs.

DS: Mice infected with *P.c. adami* DS and injected with 0.5 ml PBS.

DS+serum 15p.i: Mice infected with *P.c. adami* DS and injected with 0.5 ml serum collected on day 15 p.i.

DS+serum 55p.i: Mice infected with *P.c. adami* DS and injected with 0.5 ml serum collected on day 55 p.i.

5.2.3. The effects of passive transfer of purified parasite-specific IgG1 and IgG2a on the course of infection in avirulent and virulent infections

The efficacy of passive transfers of purified IgG1 and IgG2a on the course of infection in mice infected with either avirulent or virulent strains was examined. The presence of IgG1 and IgG2a in pooled sera was determined before the purification using ELISA. The purification process has been detailed in Chapter Two. The presence, specificity and concentration of purified parasite-specific IgG1 and IgG2a were examined using standards of IgG1 and IgG2a by ELISA, SDS-PAGE, and Western-blotting (Chapter Two, Figure 5.3.).
The protocol for passive immunization was as follows:
Different groups of NIH mice were infected with 1×10^4 pRBCs and each mouse was immediately injected with either purified IgG1 or IgG2 at 50µg per mouse with PBS in 200µl volume per injection. The injection was repeated for the next day with the same concentration. However, a group of mice infected with the DS strain were given passive immunization three times. The third time injection of IgG for this group was on day 9 p.i. In each group the course of infection and antibody levels before immunization and after challenge were examined.

5.2.3.1. The course of infection in mice infected with *P. c. adami* DK and passively immunized with purified IgG1

Figure 5.4. shows that peak parasitaemia in the control group injected only with PBS peaked at 28.25% whereas in mice immunized with IgG1 from day 15 or 55 p.i the peak was 16.18% and 17.34% respectively. Peak parasitaemias were significantly lower (Tukey's test $P < 0.001$) between the immunized and control mice. Despite the lower peak parasitaemia in mice given IgG1 from day 55 p.i, there was no significant difference for peak parasitaemia so the effects of IgG1 collected on two different time points did not show different effect. In the immunized groups two days delay in time taken to reach to the peak parasitaemia was seen.

5.2.3.2. The course of infection in mice infected with *P. c. adami* DK and passively immunized with purified IgG2a

The peak parasitaemias in mice immunized with IgG2a from day 15 or day 55 p.i. were 16.4% and 16.89% respectively. The peak parasitaemia was 28.25% in the control group. There were significant differences (Tukey's test, $P < 0.001$) for the peak parasitaemia between mice immunized with parasite-specific IgG2a from either day 15 or 55 p.i. and the control group. No significant difference was seen in respect of the effect of IgG2a from day 15 or 55 p.i. (Figure 5.5.).

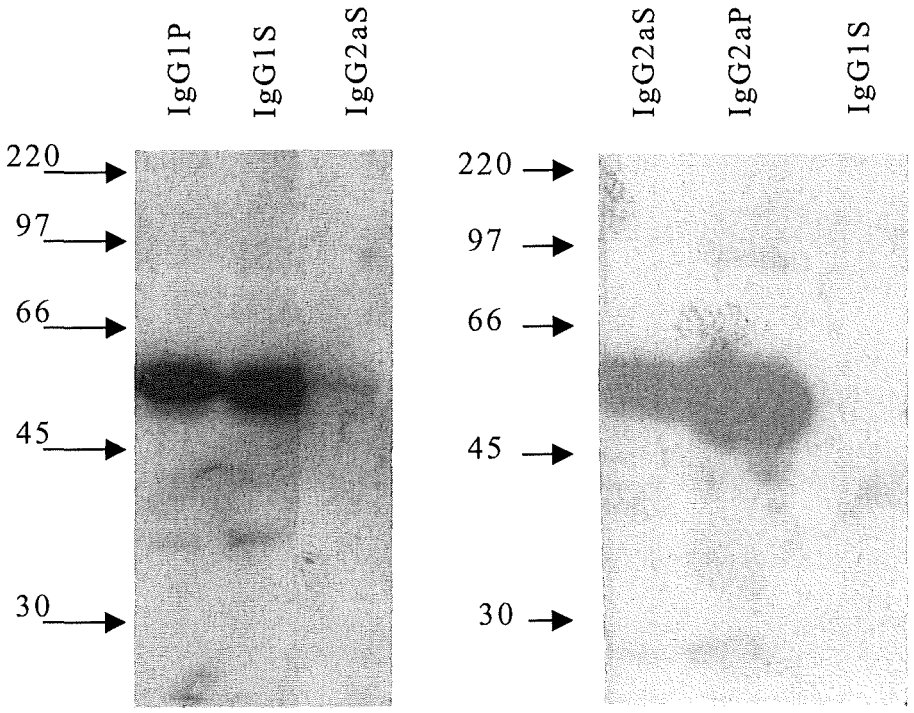


Figure 5.3. Determination and confirmation of the presence of purified IgG1 and IgG2a from serum of mice infected with *P.c. adami* DK.
IgG1 and IgG2a were isolated and purified using protein A chromatography. Purified IgG1 and IgG2a were resolved on SDS-PAGE and specificity of each subclass was determined using Western blotting. The detection system was Enhanced chemiluminescence (ECL). The rows which are in the left of the figure show the molecular weight of proteins of the Rainbow™ marker.
a): IgG1S: Th purified standard IgG1 transferred on to the membrane and detected using ECL in Western blotting.
IgG1P: The purified IgG1 from mice infected with *P.c. adami* DK transferred on to the membrane and detected using ECL in Western blotting.
IgG2aS: The reaction between standard IgG2a and non-specific secondary anti-IgG1.
b): IgG2aS: The purified standard IgG2a transferred on to the membrane and detected using ECL in Western blotting.
IgG2aP: The purified IgG1 from mice infected with *P.c. adami* DK transferred on to the membrane and detected using ECL in Western blotting.

IgG1S: The reaction between standard IgG1 and non-specific secondary anti-IgG2a.

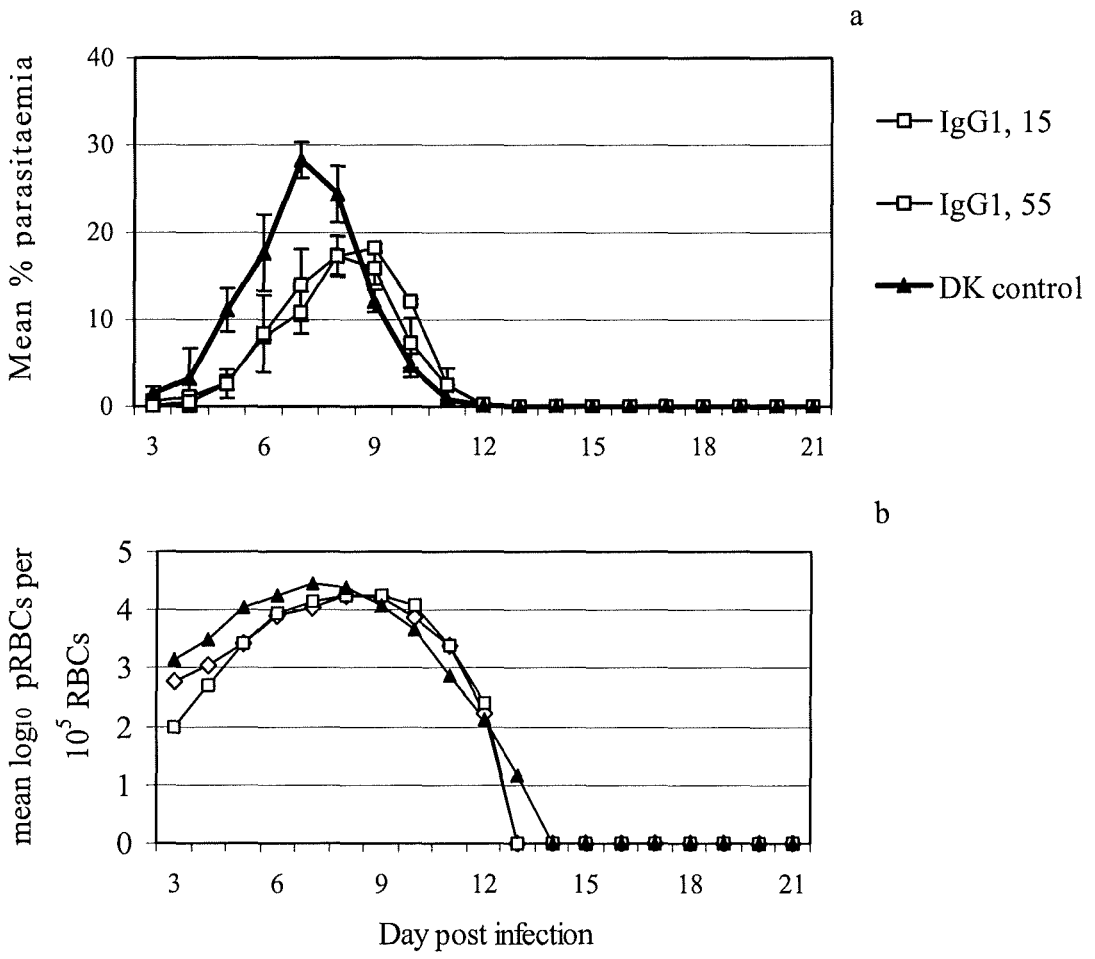


Figure 5.4. The course of infection in NIH mice infected with *P. c. adami* DK and immunized with purified IgG1.

Mice infected with 1×10^4 pRBCs of *P.c. adami* DK and passively immunized with IgG1. Each group included six mice.

IgG1 was injected two times, immediately after the infection and 24 hours later in 0.2ml PBS. Data points are shown as the mean % ± SEM. However, ±SEM is not shown when it is too small.

a) Mean % of parasitaemia.

b) Mean $\log_{10}$ pRBCs for 10^5 RBCs.

DK control: Mice infected with *P. c. adami* DK and injected with 0.2 ml PBS as control group.

IgG1, 15 p.i: Mice infected with *P. c. adami* DK and injected with IgG1 collected on day 15 p.i.

IgG1, 55p.i: Mice infected with *P. c. adami* DK and injected with IgG1collected on day 55 p.i.

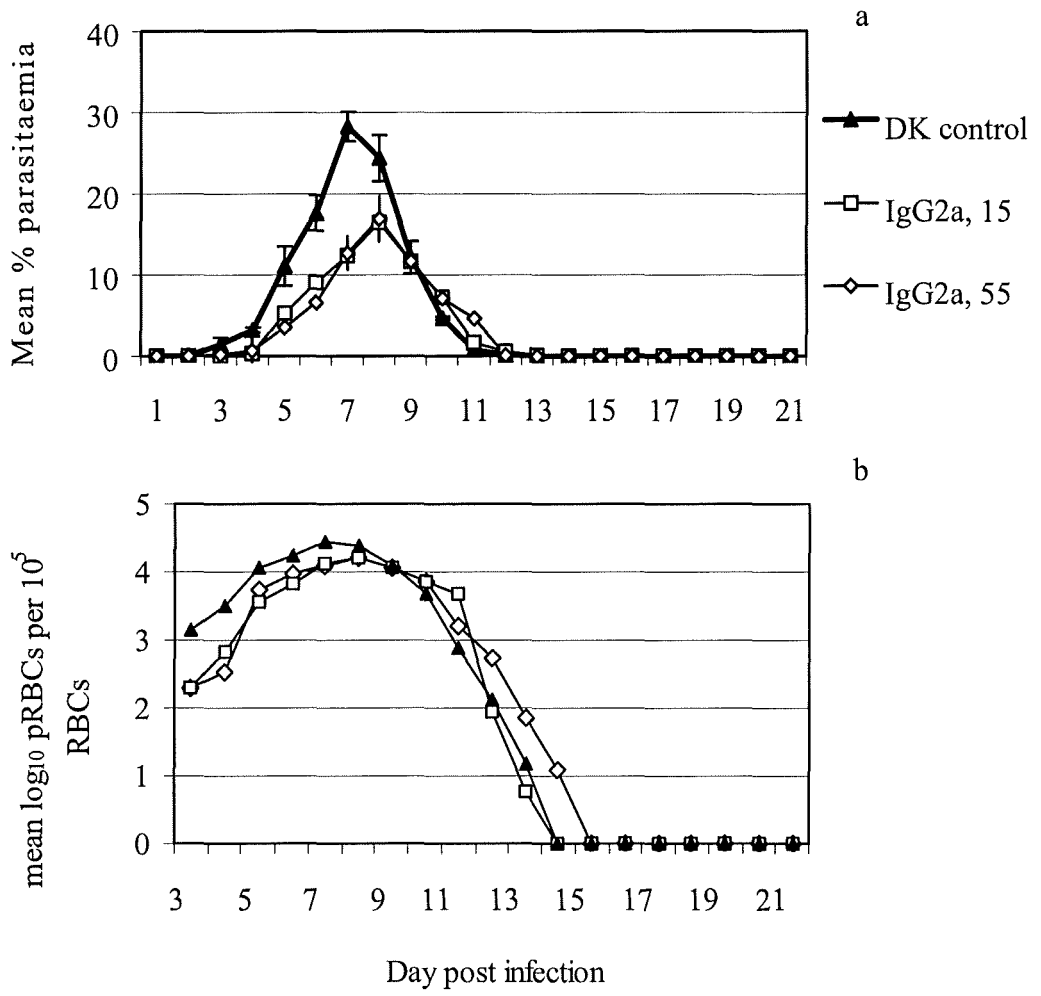


Figure 5.5. The course of infection in NIH mice infected with *P. c. adami* DK and immunized with purified IgG2a.

Mice infected with 1×10^4 pRBCs of *P.c. adami* DK and passively immunized with IgG2a. Each group included six mice.

IgG2a was injected two times, immediately after infection and 24 hours later in 0.2ml PBS. Data points are shown as the mean % ± SEM. However, ±SEM is not shown when it is too small.

a) Mean % of parasitaemia.

b) Mean $\log_{10}$ pRBCs for 10^5 RBCs.

DK control: Mice infected with *P. c. adami* DK and injected with 0.2 ml PBS as control group.

IgG2a, 15p.i: Mice infected with *P. c. adami* DK and injected with IgG2a collected on day 15 p.i.

IgG2a, 55p.i: Mice infected with *P. c. adami* DK and injected with IgG2a collected on day 55 p.i.

5.2.3.3. Cross-reactivity of IgG subclasses in virulent infection of *P. c. adami* DS

Cross-reactivity of specific IgG subclasses purified from mice infected with avirulent *P. c. adami* DK was evaluated in a virulent infection. Therefore, mice were infected with *P. c. adami* DS and immunized with IgG1 or IgG2a to determine the presence of cross-reactivity by the purified IgGs.

5.2.3.4. The course of infection in mice infected with *P. c. adami* DS and passively immunized with purified IgG1

Figure 5.6. shows that there were significant differences (One way ANOVA, $P < 0.0001$) for the peak parasitaemia between all tested groups. The peak parasitaemia in the control group was 56.93 % and it was 47 % in mice immunized with parasite-specific IgG1 from day 15 p.i. The peak was 46.9 % in mice given IgG1 from day 55 p.i. In the both immunized mice the peak parasitaemias were significantly lower (Tukey's test, $P < 0.001$) than that in the control group. This result confirmed the presence of cross-reactivity of these antibodies against the DS strain. Despite a lower peak parasitaemia in mice immunized with IgG1 from day 55 p.i, no significant difference was seen for the effect of IgG1 from day 15 or 55 p.i on the peak parasitaemia.

5.2.3.5. The course of infection in mice infected with *P. c. adami* DS and passively immunized with purified IgG2a

Cross-reactivity of IgG2a was also examined in mice immunized with IgG2a from day 15 or 55 p.i (Figure 5.7.). The peak parasitaemia in control group was 56.93 %. Parasitaemia in mice immunized with IgG2a from day 55 p.i and in mice immunized with IgG2a from day 15 p.i. peaked to 47.95 % and 48.32 % respectively. The peak parasitaemias were significantly lower (Tukey's test, $P < 0.0001$) in the immunizecd mice compared to the control group. There was no significant difference between IgG2a from either day 15 or day 55 p.i.

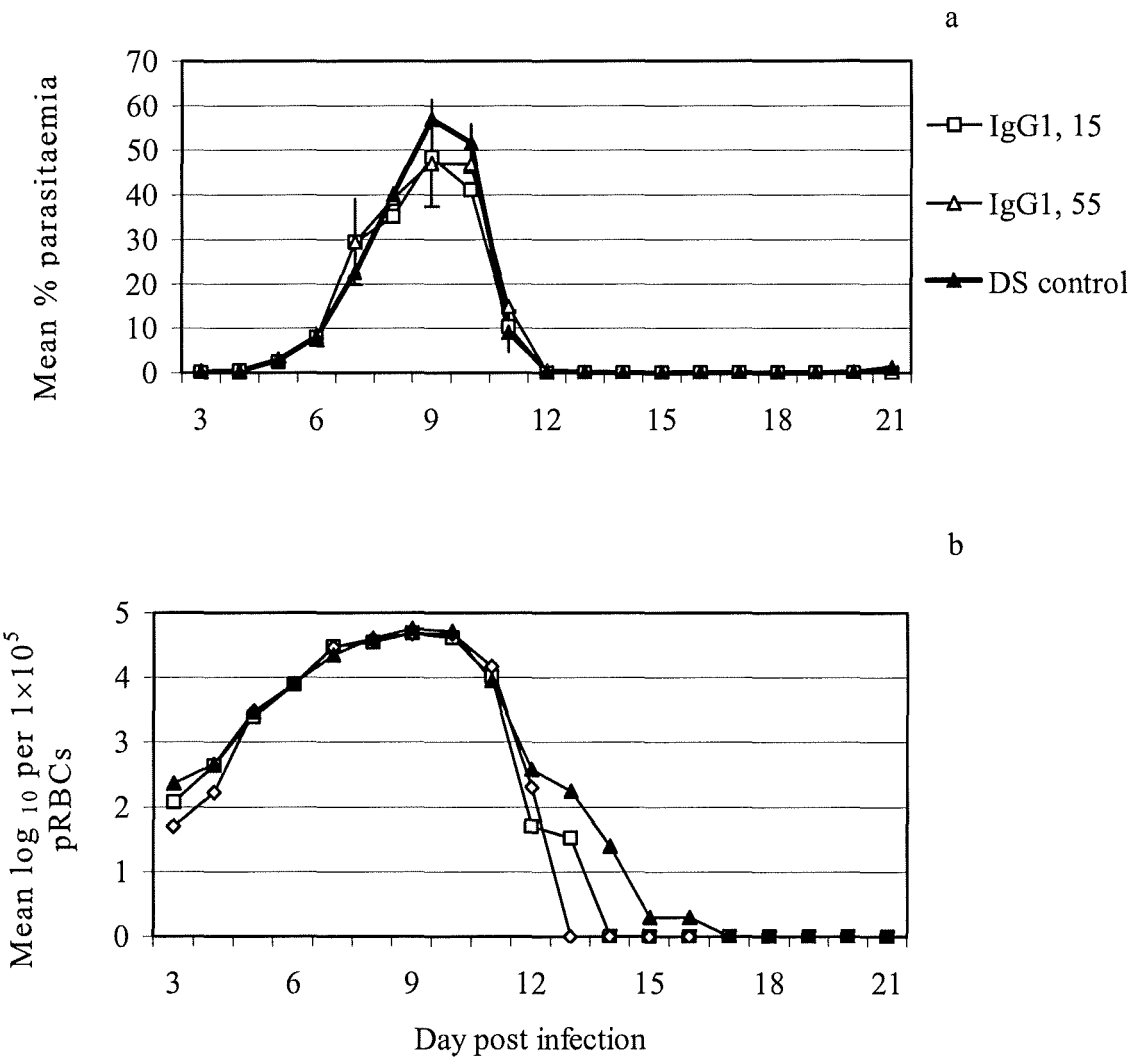


Figure 5.6. The course of infection in NIH mice infected with *P. c. adami* DS and passively immunized with purified IgG1 from the DK strain.
Mice infected with 1×10^4 pRBCs of *P.c. adami* DS and passively immunized with IgG1. Each group included six mice.
IgG1 was injected two times, immediately after infection and 24 hours later in 0.2 ml PBS. Data points are shown as the mean % ±SEM. However, ±SEM is not shown when it is too small.
a) Mean % of parasitaemia.
b) Mean $\log_{10}$ pRBCs for 10^5 RBCs.
DK control: Mice infected with *P.c. adami* DS and injected with 0.2 ml PBS as control group.
IgG1, 15p.i: Mice infected with *P.c. adami* DS and injected with IgG1 collected on day 15 p.i.
IgG1, 55p.i: Mice infected with *P.c. adami* DS and injected with IgG1 collected on day 55 p.i.

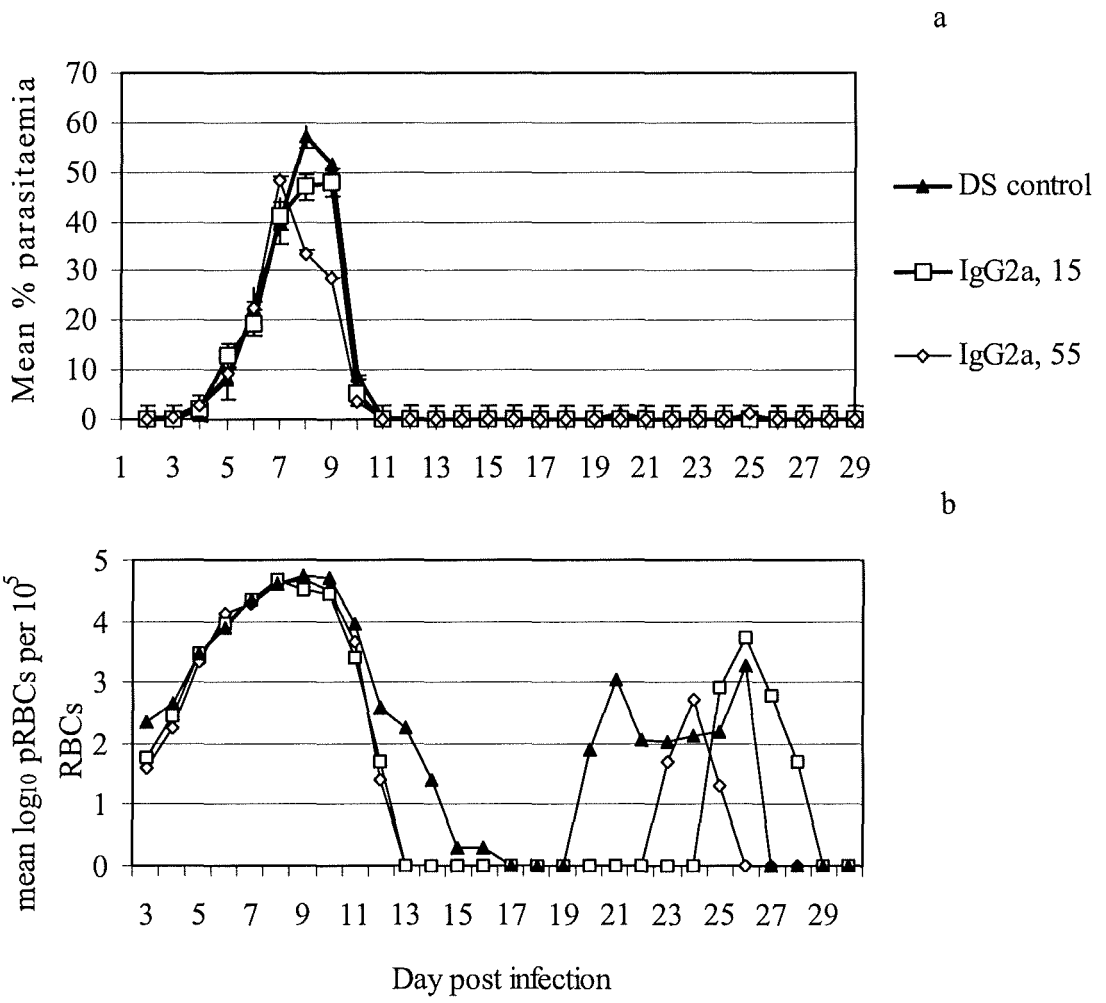


Figure 5.7. The course of infection in NIH mice infected with *P. c. adami* DS and passively immunized with purified IgG2a from the DK strain. Mice infected with 1×10^4 pRBCs of *P.c. adami* DS and passively immunized with IgG2a. IgG2a was injected two times, immediately after infection and 24 hours later, 0.2 ml on each occasion. Data points are shown as the mean % ± SEM. However, ±SEM is not shown when it is too small. Each group included six mice.

a) Mean % of parasitaemia.

b) Mean $\log_{10}$ pRBCs for 10^5 RBCs.

DS control: Mice infected with *P.c. adami* DS and injected with 0.2 ml PBS as control group.

IgG2a, 15 p.i: Mice infected with *P.c. adami* DS and injected with IgG2a collected on day 15 p.i.

IgG2a, 55 p.i: Mice infected with *P.c. adami* DS and injected with IgG2a collected on day 55 p.i.

5.2.3.6. Comparison of peak parasitaemia and death in different groups of mice infected with *P. c. adami* DS or DK and passively immunized with purified IgG1 or IgG2a.

Figure 5.8. and 5.9. have summarized comparison between peak parasitaemias in different groups of mice immunized with sera, IgG1, or IgG2a in both avirulent and virulent infections.

No death was seen in immunized mice with serum challenged with the DK strain. In mice immunized with sera from DK-infected mice cross reactivivity protected mice from death.

Immunization with parasite-specific IgG1 or IgG2a reduced parasitaemia and provided protection against the challenge with the parasite in both avirulent or partially in virulent challenges. However, this protection was not as high as seen for the transfer of whole serum. However, in the virulent challeneg despite significantly reducing parasitaemia, cross-reactivity did not show noteworthy protective effect on the outcome of the infection and the survival rate as some immunized mice died. Figure 5.9. also shows that three times passively immunization results in more reduction in peak parasitaemai compared to two-time transfer. However, third time immunization does not prevent mice from death (Table 1 and 2).

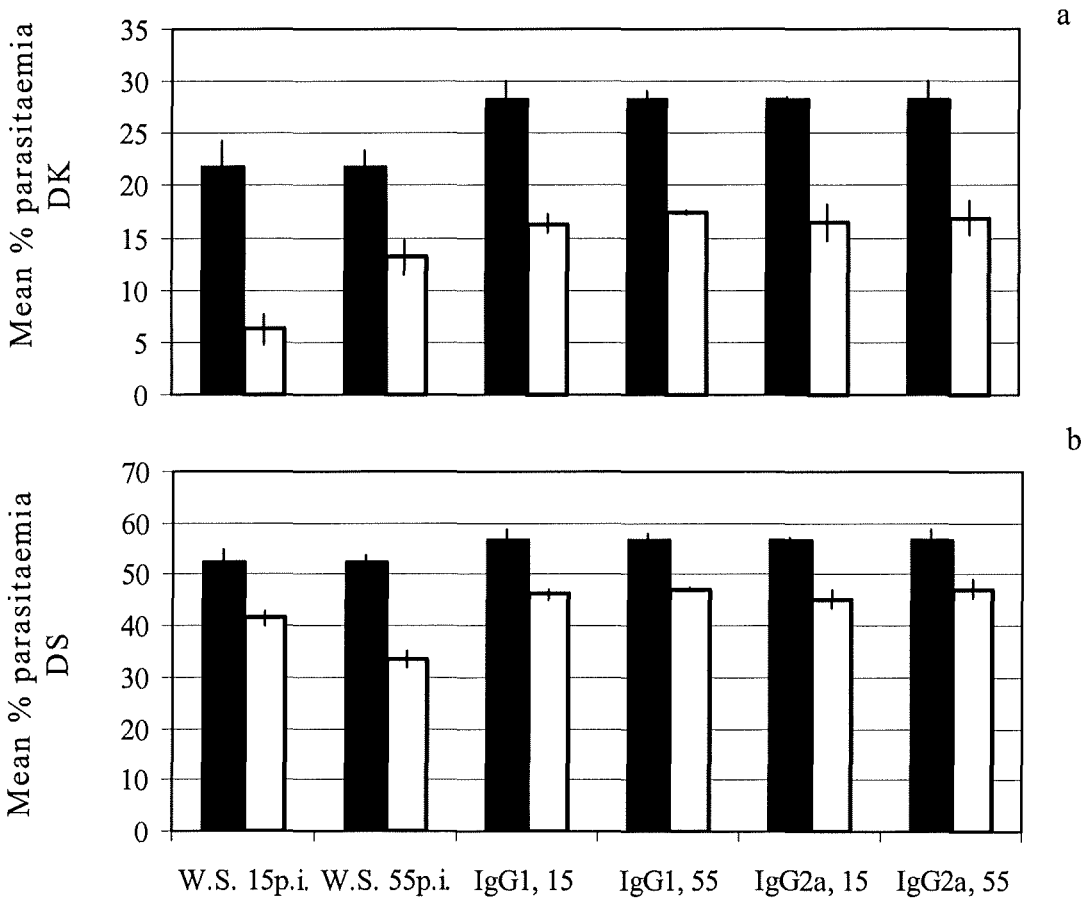


Figure 5.8. Comparison of the peaks of parasitaemias in passively immunized mice with IgG subclasses.

a) Different groups of mice infected with *P.c. adami* DK and passively immunized with whole serum (W.S.) and purified IgGs as described in figures 5.3 and 5.4. The parasitaemia is represented as the mean ± SEM.

b) Different groups of mice infected with *P. c. adami* DS and passively immunized with whole serum and purified IgGs as described in figures 5.5 and 5.6.

■ Control group: mice infected with the parasite and injected with PBS.

□ Immunized groups are as described in previous figures.

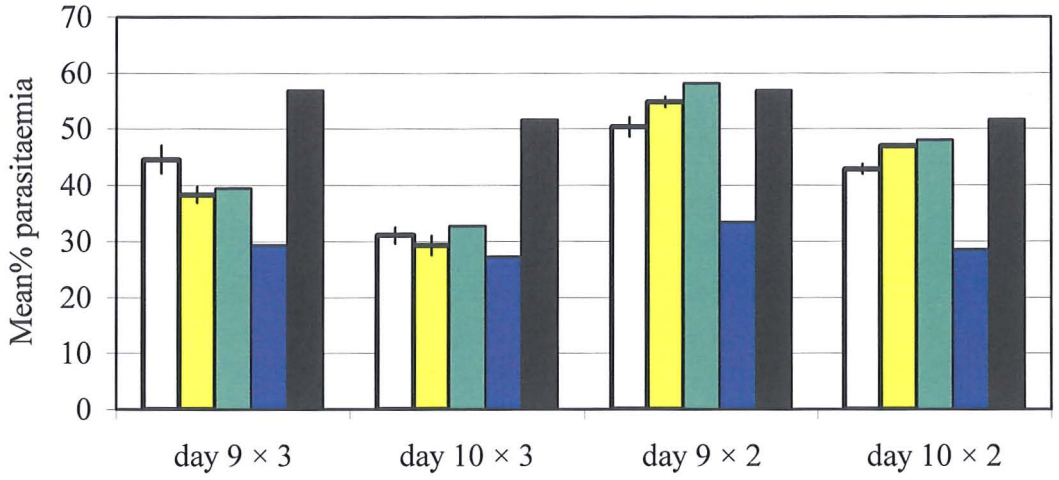


Figure 5.9. Comparison of the parasitaemias in mice infected with *P.c. adami* DS and passively immunized with IgG1 and IgG2a, two or three times.

One group of mice was injected three times. The injection immediately was administered on day 9 p.i. after taking blood for determination of parasitaemia.

The figure compares the mean parasitaemias for two respective days, day 9, and day 10 p.i. between two groups.

- Immunized mice with IgG1 from day 15 p.i.
- Immunized mice with IgG1 from day 55 p.i.
- Immunized mice with IgG2a from day 15 p.i.
- Immunized mice with IgG2a from day 55 p.i.
- Control mice infected with the parasite and injected only PBS.

Table 1. shows the deaths and surviving rates of mice after passively transfer of either IgG2a or IgG1 in the virulent DS strain challenge for all groups of mice on day 9 and 10 p.i. For all mice given antibodies three times, the peak parasitaemia significantly (t-test, $P < 0.0001$) was lower compared to the groups given IgG injections two times (Figure 5.9.). However, for mice given IgG2a from day 55 p.i the peak parasitaemia was not significantly different compared to mice immunized twice. Although mice immunized three times showed reduced parasitaemia compared to mice immunized twice, this reduction did not affect survival rates. Table 2 shows that mice immunized twice with IgG2a from day 55 p.i. had the best survival rate because only two mice of six died. The results did not suggest that transfer of additional antibodies around the peak parasitaemia reduces death after challenge with the virulent DS strain.

Dayp.i.	IgG subclass	Peak	Peak	Significant Difference
Immunization		× 2	×3	
Day 9	IgG1, 15 p.i.	50.41 %	44.63%	$P < 0.0028$
	IgG1, 55 p.i.	54.86 %	38.36%	$P < 0.0001$
Day 10	IgG1, 15 p.i.	42.91 %	31.1%	$P < 0.0012$
	IgG1, 55 p.i.	46.96 %	29.3%	$P < 0.0001$
Day 9	IgG2a, 15 p.i.	58.16 %	39.46%	$P < 0.0001$
	IgG2a, 55 p.i.	33.38 %	29.28%	$P < 0.0012$
Day 10	IgG2a, 15 p.i.	47.95 %	32.7%	$P < 0.0001$
	IgG2a, 55 p.i.	28.5%	27.27%	$P < 0.07$

Table.1. Comparison of peak parasitaemias in mice passively immunized with IgG1 or IgG2a and challenged with the virulent *P. c. adami* DS strainfrom either day 15 or 55 p.i.

IgG subclasses	Death in mice given IgG two times	Death in mice given IgG three times
IgG1, day 15 p.i.	3	2
IgG1, day 55 p.i.	All dead	4
IgG2a, day 15 p.i.	3	3
IgG2a, day 55 p.i.	2	3
Control group	1	1

Table 2. Deaths in mice immunized with purified IgG1 or IgG2a compared to the control group. Each group had six mice which were challenged with *P. c. adami* DS. Mice passively were given antibodies for two or three times when the third time injection was coincident with the peak parasitaemia.

5.2.4. Parasite-specific antibody production post-immunization with IgG1 and IgG2a in avirulent infection.

Production of specific IgG subclasses in mice passively immunized with IgG1 or IgG2a was examined. Blood samples were collected from tail at different time points, usually every three days, and levels of IgGs were measured by ELISA. The control groups included mice infected with the same pRBCs and left untreated and also naïve mice given only PBS.

5.2.4.1. Parasite-specific IgG1 production in mice infected with *P. c. adami* DK and passively immunized with IgG1.

The IgG1 production in mice infected with the DK strain and passively immunized with IgG1 from either day 15 or 55 p.i. increased as the course of infection proceeds (Figure 5.10.). The overoall pattern of the IgG1 production was the same as seen in non-immunized mice (Chapter Three) in which IgG1 increased later in the course of infection. The levels of IgG1 were not significantly different compared to IgG1 in the control group in which infected mice were not treated with IgG1. However, the levels of IgG1 in immunized mice were significantly (Tukey's test, $P < 0.0004$) higher compared to PBS-treated mice.

5.2.4.2. Parasite-specific IgG1 production in mice infected with *P. c. adami* DK and passively immunized with IgG2a

Figure 5.11. shows that, in general, the levels of IgG1 in mice immunized with IgG2a were very low. However, its level was significantly higher (Tukey's test, $P < 0.0005$) than that in the PBS-treated control mice.

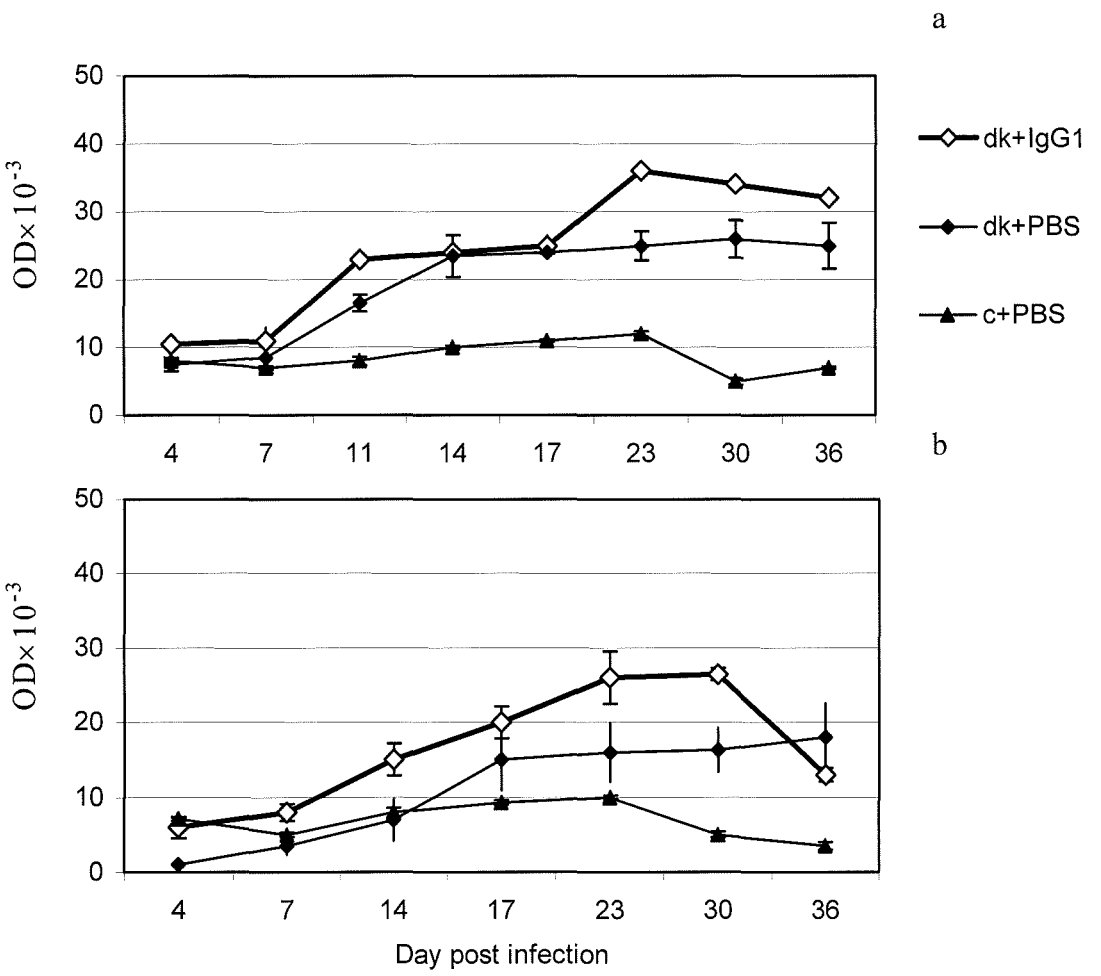


Figure 5.10. Parasite-specific IgG1 production in NIH mice infected with 1×10^4 pRBCs of *P. c. adami* DK and passively immunized with IgG1. IgG1 was injected two times at 50 μg, immediately after infection and 24 hours later, in 0.2 ml on each occasion. The levels of IgG1 are shown as the mean ± SEM. However, ±SEM is not shown when it is too small.

a) IgG1 production in mice passively immunized with IgG1 from day 15 p.i.

b) IgG1 production in mice passively immunized with IgG1 from day 55 p.i.

dk+IgG1: Infected mice passively immunized with IgG1.

dk+PBS: Infected mice left without passive immunization and were injected with PBS

c+PBS: Naïve mice were injected with PBS only.

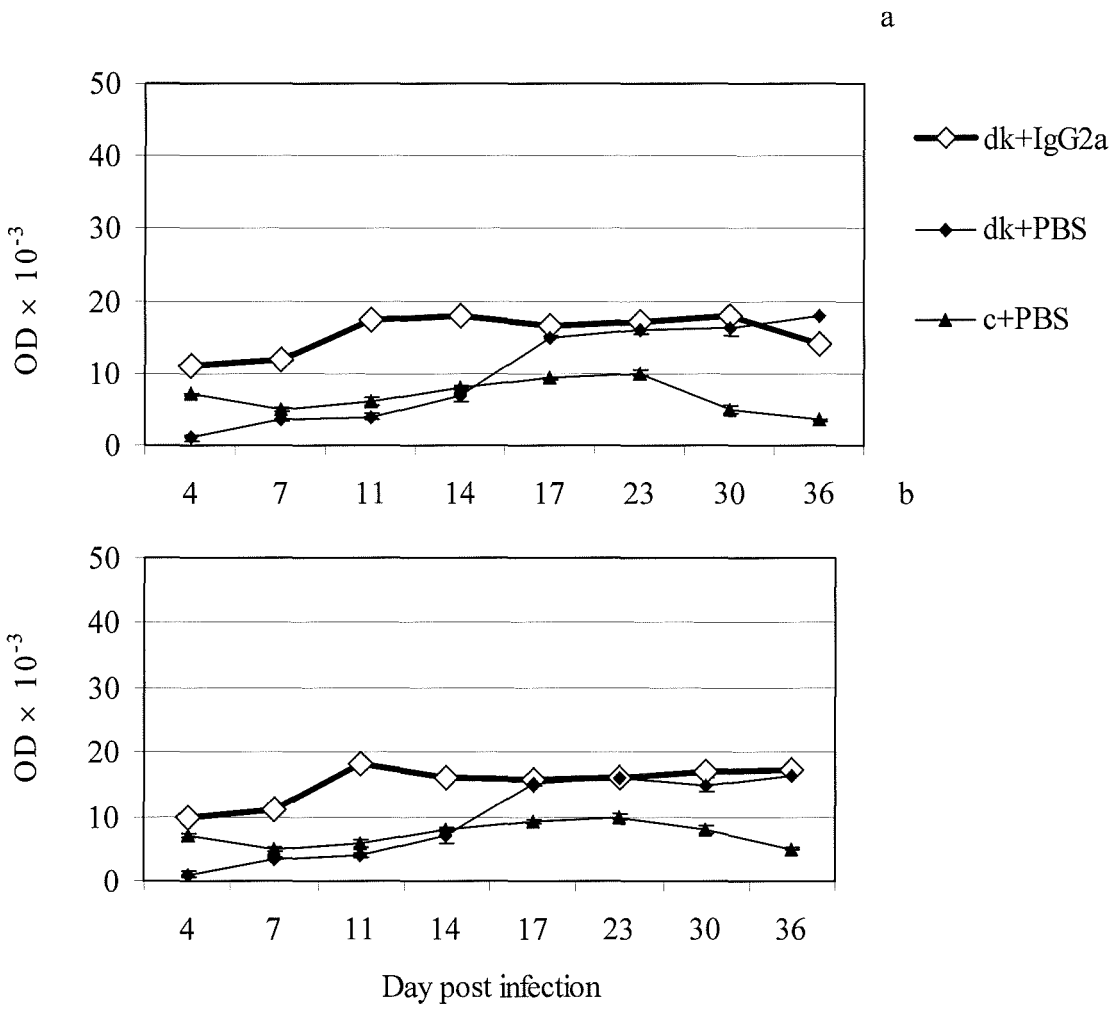


Figure 5.11. Parasite-specific IgG1 production in NIH mice infected with 1×10^4 pRBCs of *P. c. adami* DK and passively immunized with IgG2a. IgG2a was injected two times at 50 µg, immediately after infection and 24 hours later, in 0.2 ml PBS on each occasion.

The levels of IgG1 are shown as the mean ± SEM. However, ±SEM is not shown when it is too small.

a) IgG1 production in mice immunized with IgG2a from day 15 p.i.

b) IgG1 production in mice immunized with IgG2a from day 55 p.i.

dk+IgG2a: Infected mice immunized with IgG2a.

dk+PBS: Infected mice left without immunization and were injected with PBS

c+PBS: Naïve mice were injected with PBS.

5.2.4.3. Parasite-specific IgG2a production in mice infected with *P. c. adami* DK and passively immunized with IgG1.

Figure 5.12. shows that IgG2a particularly increased after the peak parasitaemia. The levels of IgG2a was significantly different between the tested groups (Two-way ANOVA, $P < 0.0007$ and $P < 0.0009$ for days 15 and 55 p.i. respectively). However, statistically there were no significant differences for IgG2a levels between mice immunized with IgG2a and infected mice left without immunization. Whereas, the levels of IgG2a in mice passively immunized with IgG1 (Figure 5.12 a) from days 15 or 55 p.i were significantly higher (Bonferroni, $P < 0.0036$ for IgG1 from day 15 p.i. and $P < 0.00019$ for IgG1 from day 55 p.i.) compared to IgG2a in naïve mice given only PBS.

5.2.4.4. Parasite-specific IgG2a production in mice infected with *P. c. adami* DK and passively immunized with IgG2a.

The same observations for IgG2a productions were seen in mice immunized with IgG2a with mice infected with theDK strain (Chapter three), as its level increased after the peak parasitaemia (Figure 5.13.). There was no significant difference for IgG2a between mice immunized with IgG2a from day 15 p.i and mice infected with the DK strain and given PBS (Figure 5.13 b). However, this IgG2a level was significantly lower (Bonferroni, $P < 0.016$) in mice given IgG2a from day 55p.i compared to mice infected with DK and given PBS (Figures 5.13 a and b). The levels of IgG2a in mice given IgG2a from either day 15 or 55 p.i. were significantly higher (Bonferroni $P < 0.0001$) than that in naïve mice given only PBS.

5.2.5. Parasite-specific IgG1 and IgG2a production post-immunization with IgG1 and IgG2a in the DS virulent infection

For determination of any cross-reactivity of purified IgG subclasses (obtained from mice were infected with the avirulent DK strain) in the

virulent DS infection the same experiments were performed as described previously (3.2.4.).

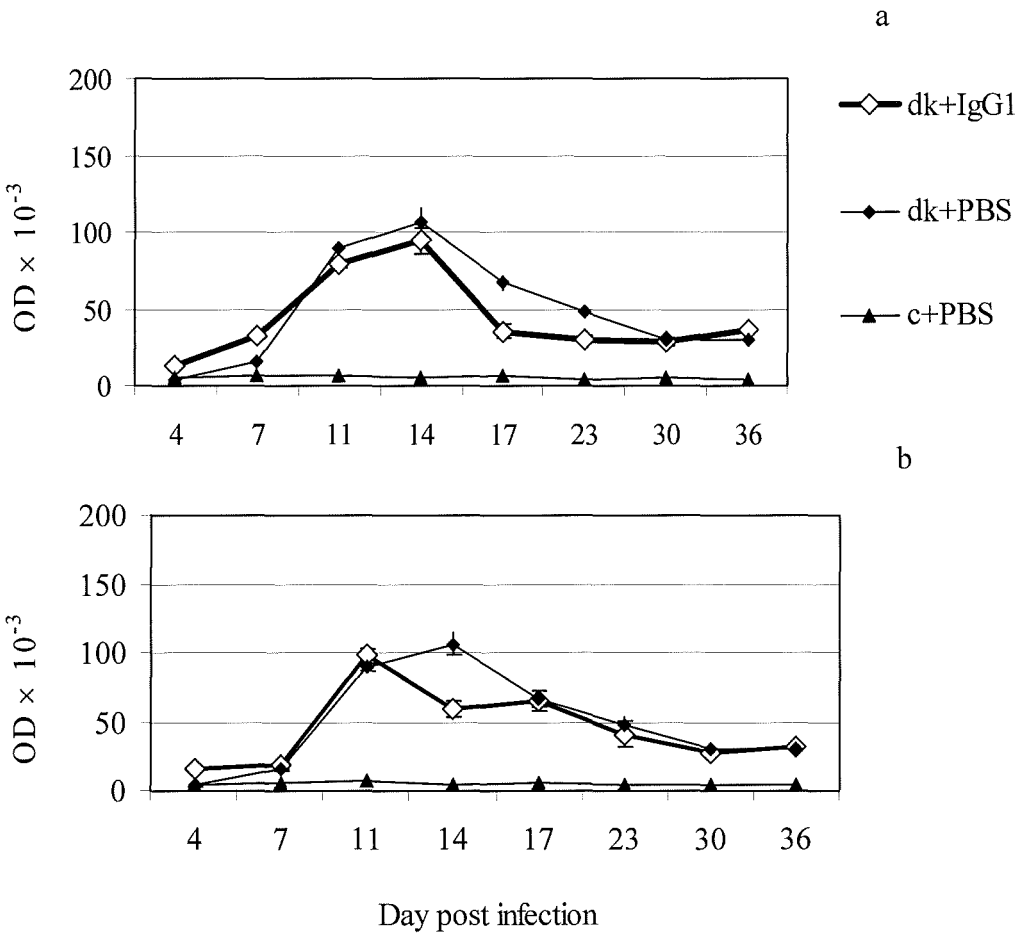


Figure 5.12. Parasite-specific IgG2a production in NIH mice infected with 1×10^4 pRBCs of *P. c. adami* DK and passively immunized with IgG1.

IgG1 was injected two times at 50 µg , immediately after infection and 24 hours later in 0.2 ml PBS at each occasion.

The levels of IgG2a are shown as the mean ± SEM. However, ±SEM is not shown when it is too small.

a) IgG2a production in mice passively immunized with IgG1 from day 15.

b) IgG2a production in mice passively immunized with IgG2a from day 55 p.i. and challenged with the parasite.

dk+IgG1: Mice were challenged with the parasite and passively immunized with IgG1 from days 15 or 55 pi.

dk+PBS: Infected mice left without passive immunization and were injected with PBS.

c+PBS: Naïve mice were injected with PBS.

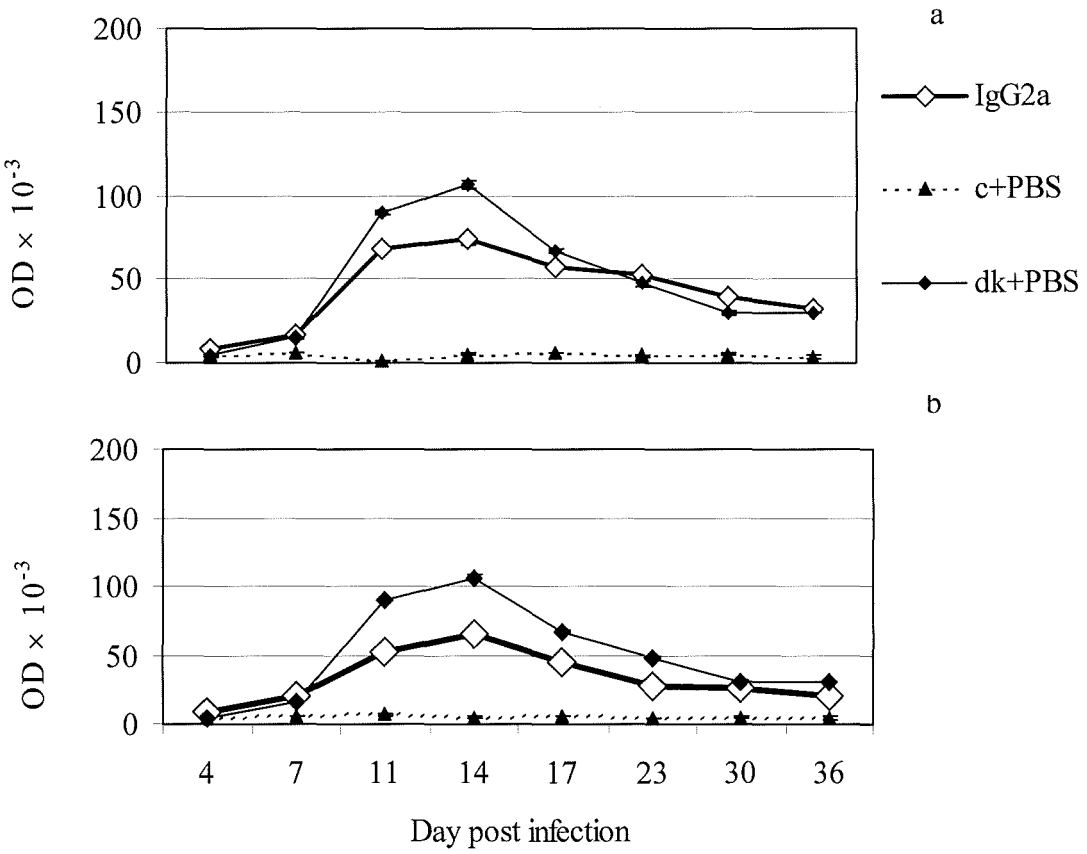


Figure 5.13. Parasite-specific IgG2a production in NIH mice infected with 1×10^4 pRBCs of *P. c. adami* DK and passively immunized with IgG2a. IgG2a was injected two times at 50 µg, immediately after infection and 24 hours later in 0.2 ml PBS at each occasion.

The levels of IgG2a are shown as the mean ± SEM. However, ±SEM is not shown when it is too small.

a) IgG2a production in mice passively immunized with IgG2a from day 15 and challenged with the parasite.

b) IgG2a production in mice passively immunized with IgG2a from day 55 p.i. and challenged with the parasite.

IgG2a: Mice were challenged with the parasite and passively immunized with IgG2a from days 15 or 55 pi.

dk+PBS: Infected mice left without passive immunization and were injected with PBS.

c+PBS: Naïve mice were injected with PBS.

5.2.5.1. Parasite-specific IgG1 production in mice infected with *P. c. adami* DS and passively immunized with IgG1.

The production of IgG1 in mice immunized with IgG1 from day 15 or 55 p.i. showed the same pattern of increase during the course of infection. There were significant differences (Two-way ANOVA, $P < 0.01$) for IgG1 production between all tested groups (Figure 5.14). Significantly higher IgG1 (Bonferroni test, $P < 0.032$) was observed in mice immunized with IgG1 from day 15 p.i compared to mice infected with the DS strain and given only PBS. There was also a significant difference (Bonferroni test, $P< 0.0016$) for the specific IgG1 between immunized mice and PBS-treated mice. The level of IgG1 in mice immunized with IgG1 from day 55 p.i were significantly higher (Bonferroni test, $P < 0.0038$) than that in mice infected with the DS strain and given PBS. Due to death of four mice in immunized group the production of IgG1 could not be measured after 12 days.

5.2.5.2. Parasite-specific IgG1 production in mice infected with *P. c. adami* DS and passively immunized with IgG2a.

Mice immunized with IgG2a from either day 15 or 55 p.i. did not show significant (Boferroni test, $P >0.05$) difference in IgG1 levels compared to control groups (Figure 5.15.).

5.2.5.3. Parasite-specific IgG2a production in mice infected with *P. c. adami* DS and passively immunized with IgG1.

Significantly lower (Bonferroni test, $P < 0.014$) levels of IgG2a were seen in mice immunized with IgG1 from day 15 p.i compared to infected mice given only PBS over time except on day 4, 7, and 36 p.i. This result shows that (Figure 5.16. a) mice immunized with IgG1 did not produce more specific IgG2a production compared with non-immunzed mice. Figure 5.16.b shows that there was a lower level of IgG2a in mice immunized with IgG1 from day 55 p.i compared to non-immunized mice infected with the parasite. In this latter group no mice survived after day 12 p.i. so, following the IgG2a production was not possible (Figure 5.16 b).

5.2.5.4. Parasite-specific IgG2a production in mice infected with *P. c. adami* DS and passively immunized with IgG2a.

Figure 5.17. shows that there were significant differences for IgG2a production between all tested groups either in mice that were passively given IgG2a from day 15 p.i.(Two-way ANOVA, $P < 0.0087$) or from day 55 p.i (Two-way ANOVA, $P < 0.0024$). However, there was no significant difference for IgG2a between immunized mice and control mice which were infected with the DS strain and left without immunization. For both groups of mice immunized with either IgG2a from day 15 or 55 p.i. there were significantly higher (Bonferroni $P < 0.015$ for day 15 and $P < 0.013$ for day 55 p.i.) levels of IgG2a compared to IgG2a in mice injected with PBS only.

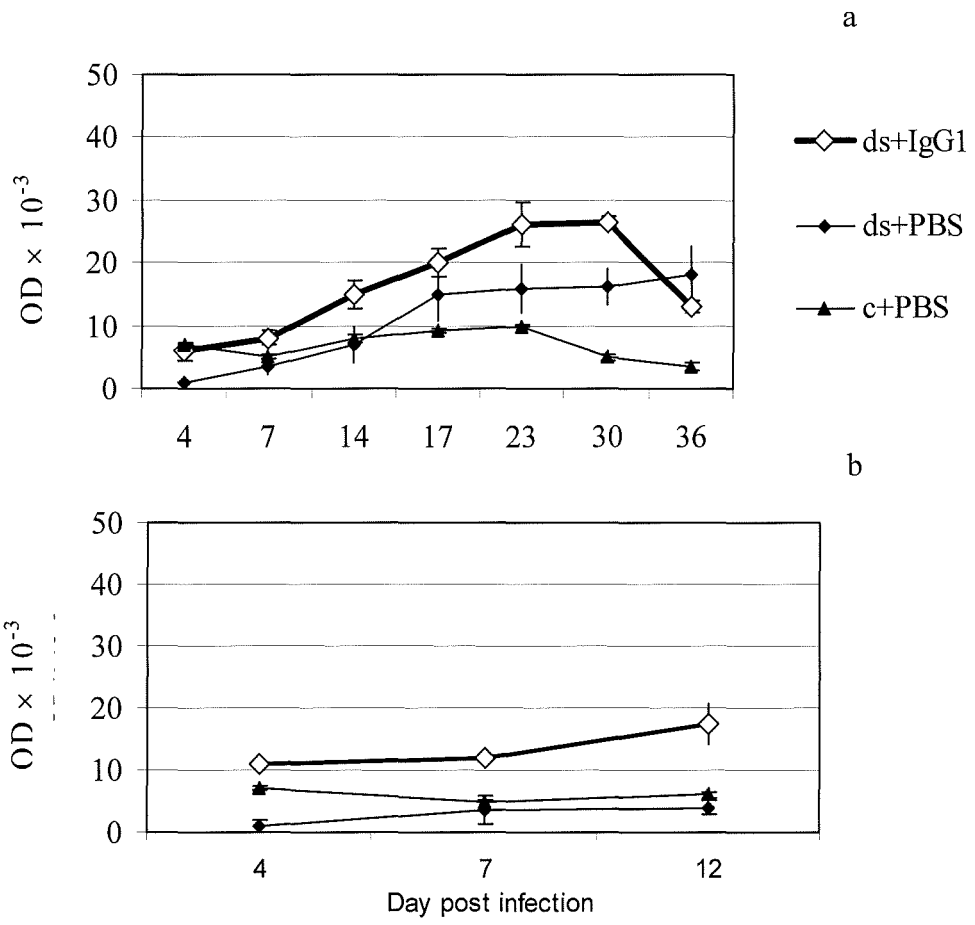


Figure 5.14. Parasite-specific IgG1 production in NIH mice infected with 1×10^4 pRBCs of *P. c. adami* DS and passively immunized with IgG1. IgG1 was injected two times at 50 μg, immediately after infection and 24 hours later in 0.2 ml PBS on each occasion.

The levels of IgG1 are shown as the mean ± SEM. However, ±SEM is not shown when it is too small.

a) IgG1 production in mice infected with the parasite and passively immunized with IgG1 from day 15 p.i.

b) IgG1 production in mice infected with the parasite and passively immunized with IgG2a from day 55 p.i.

ds+IgG1: Mice were infected with the DS strain and passively immunized with IgG1 from days 15 or 55 p.i.

ds+PBS: Infected mice left without passive immunization and were injected with PBS.

c+PBS: Naïve mice were injected with PBS.

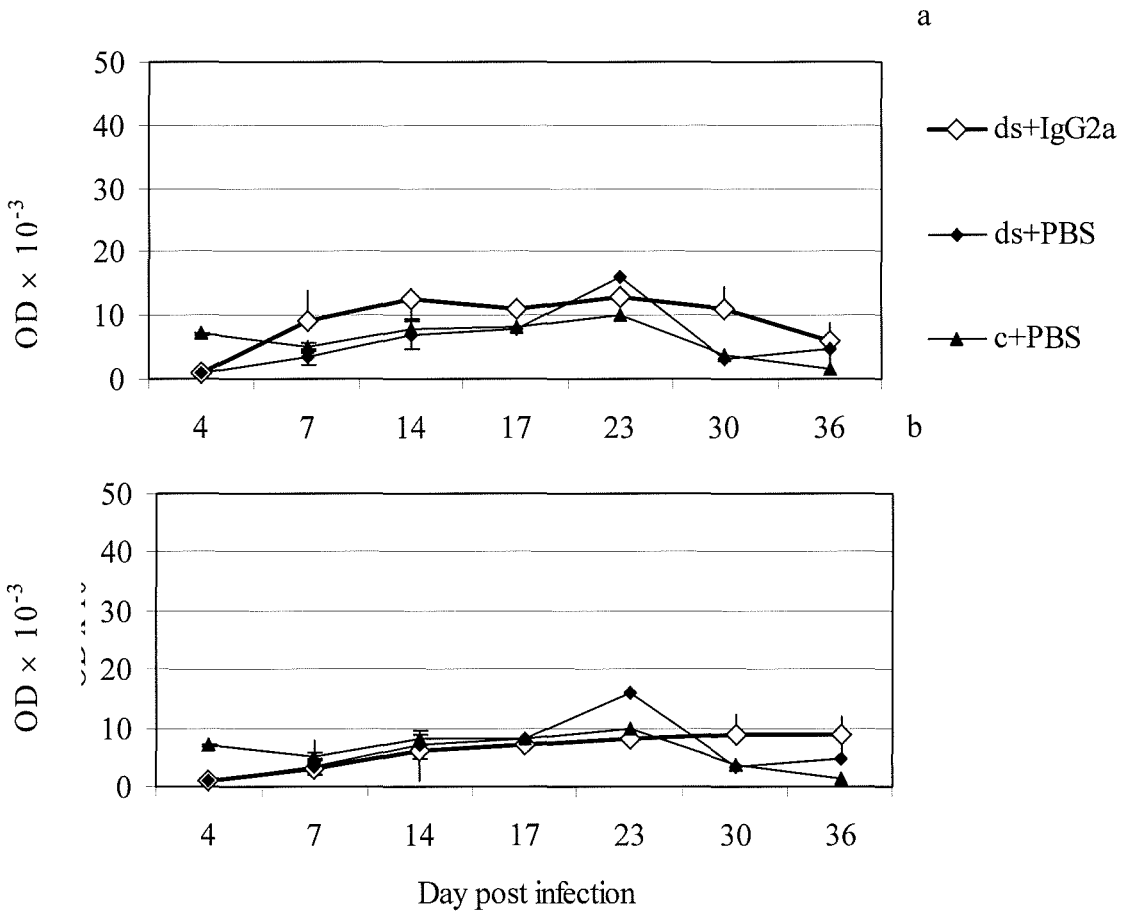


Figure 5.15. Parasite-specific IgG1 production in NIH mice infected with 1×10^4 pRBCs of *P. c. adami* DS and passively immunized with IgG2a. IgG2a was injected two times at 50 µg, immediately after infection and 24 hours later in 0.2 ml PBS on each occasion.

The levels of IgG1 are shown as the mean ± SEM. However, ±SEM is not shown when it is too small.

a) IgG1 production in mice infected with the parasite and passively immunized with IgG2a from day 15 p.i.

b) IgG1 production in mice infected with the parasite and passively immunized with IgG2a from day 55 p.i.

ds+IgG2a: Mice were infected with the DS strain and passively immunized with IgG2a from day 15 or 55 p.i.

ds+PBS: Infected mice left without passive immunization and were injected with PBS.

c+PBS: Naïve mice were injected with PBS.

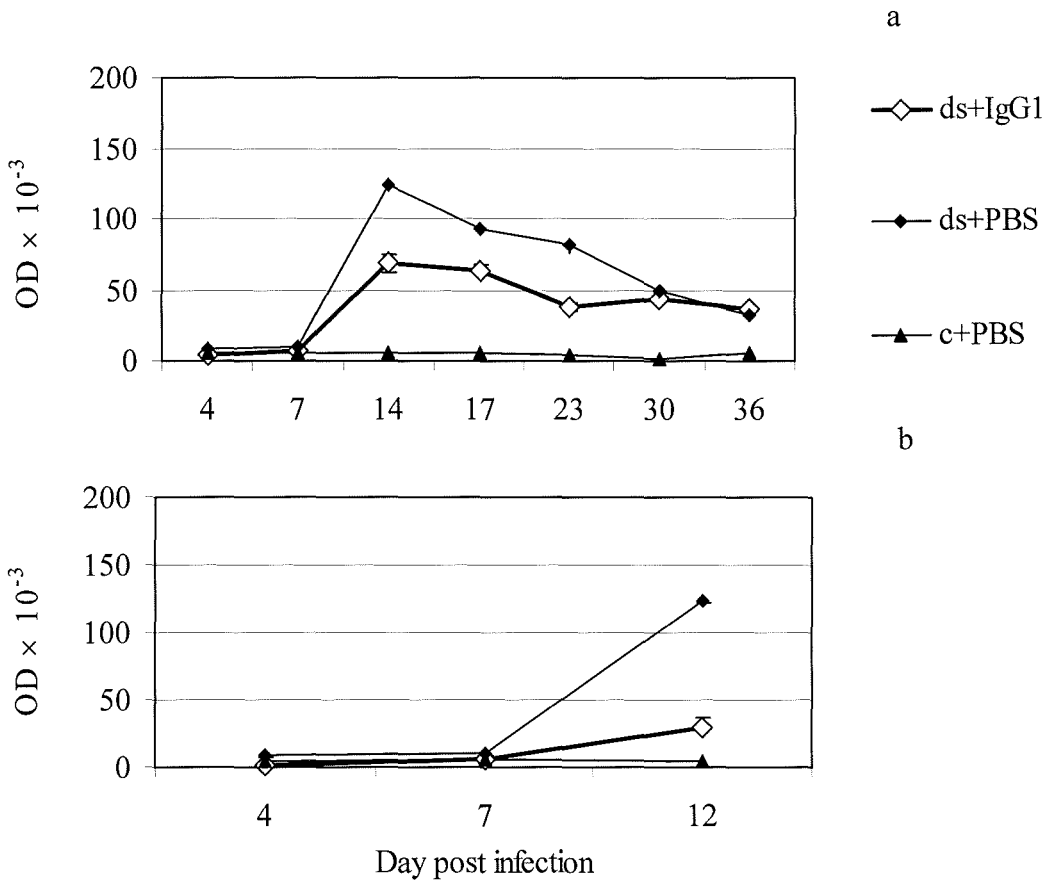


Figure 5.16. Parasite-specific IgG2a production in NIH mice infected with 1×10^4 pRBCs of *P. c. adami* DS and passively immunized with IgG1. IgG1 was injected two times at 50 µg, immediately after infection and 24 hours later in 0.2ml PBS on each occasion.

The levels of IgG2a are shown as the mean ± SEM. However, ±SEM is not shown when it is too small.

a) IgG2a production in mice infected with the parasite and passively immunized with IgG1 from day 15 p.i.

b) IgG2a production in mice infected with the parasite and passively immunized with IgG1 from day 55 p.i.

ds+IgG1: Infected mice with the DS strain and were passively immunized with IgG1.

ds: Infected mice left without passive immunization and were injected with PBS.

c+PBS: Naïve mice were injected with PBS.

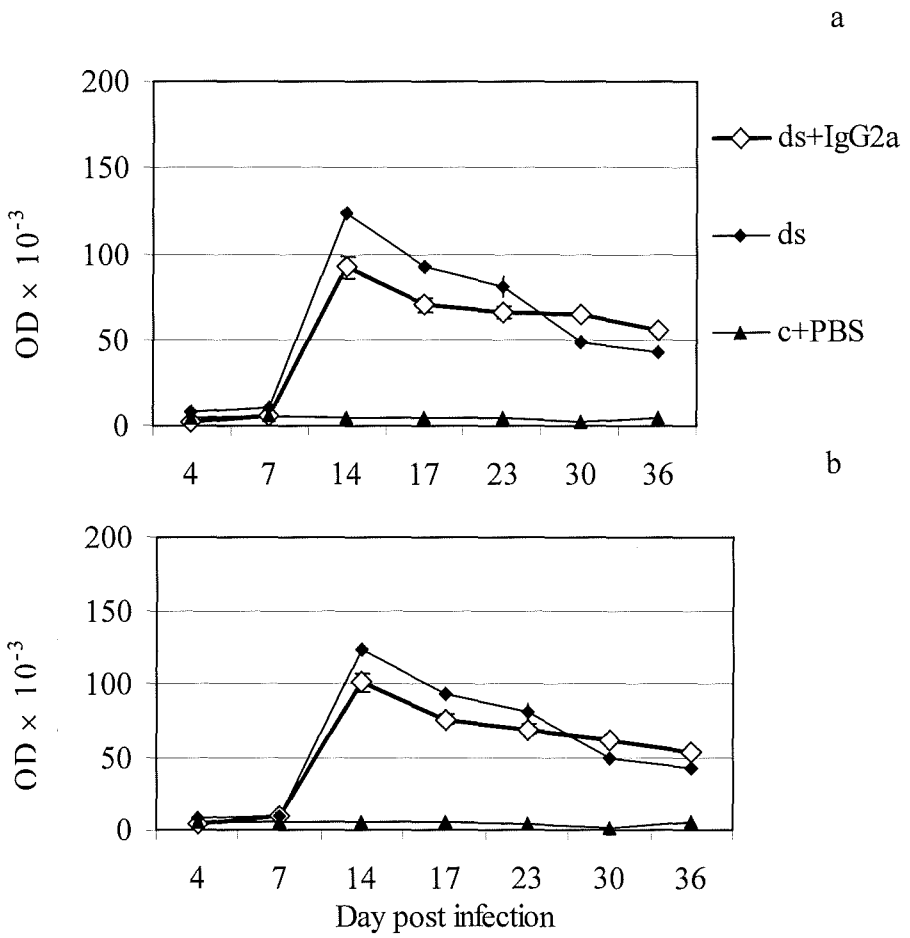


Figure 5.17. Parasite-specific IgG2a production in NIH mice infected with 1×10^4 pRBCs of *P. c. adami* DS and passively immunized with IgG2a. IgG2a was injected two times at 50 µg, immediately after infection and 24 hours later in 0.2 ml PBS.

The levels of IgG2a are shown as the mean ± SEM. However, ±SEM is not shown when it is too small.

a) IgG2a production in mice infected with the parasite and passively immunized with IG2a from day 15 p.i.

b) IgG2a production in mice infected with the parasite and passively immunized with IgG2a from day 55 p.i. .

ds+IgG2a: Infected mice with the DS strain passively immunized with IgG2a from day 15 or 55 p.i.

ds: Infected mice left without passive immunization and were injected with PBS.

c+PBS: Naïve mice were injected with PBS.

5.2.6. Parasite-specific antibody production after the re-challenge in infected mice passively immunized with IgG subclasses.

All mice challenged with either avirulent *P. c. adami* DK or virulent DS strains and immunized with IgG subclasses as described above were rechallenged with a high dose of 1×10^8 pRBCs of either *P. c. adami* DK or *P. c. adami* DS i.v. These rechallenges were performed on day 42 p.i. The course of infections and parasite-specific antibody were examined, as described above for 25 days p.i.

5.2.6.1. The course of infection in immunized mice rechallenged with *P. c. adami* DK.

Mice immunized with either IgG1 or IgG2a in the above experimental groups were rechallenged with 1×10^8 pRBCs of *P. c. adami* DK on day 42 p.i. No parasitaemia was observed except in mice immunized with IgG2a from day 15 p.i. This parasitaemia was 3.16 % on day 3 p.i and it was 0.125 % on day 4 p.i. No parasites were detected thereafter in all groups. No pasrasitaemia was seen in the non-immunized control group and rechallenged with the parasite.

5.2.6.2. The course of infection in immunized mice rechallenged with *P. c. adami* DS

The same procedure was performed for virulent *P. c. adami* DS strain. All immunized mice surviving from challenge with the DS strain were rechallenged with 1×10^8 pRBCs of *P. c. adami* DS. No parasitaemia was observed except in the control group that was infected with DS and injected only with PBS. Mice in this group (five mice survived) showed parasitaemia in the days immediately after the challenge. It was 0.015% on day1 p.i., 1.026% on day 2 p.i., and 0.55% on day 3 p.i and no parasites observed therafter.

5.2.6.3. Parasite-specific IgG1 and IgG2a production after the re challenge in mice infected with avirulent *P. c. adami* DK.

Parasite-specific IgG1 and IgG2a production were examined after rechallenge in all groups of mice.

5.2.6.3.1. Parasite-specific IgG1 production in mice rechallenged with a high infective dose of 1×10^8 pRBCs of avirulent *P. c. adami* DK.

Figure 5.18a shows that there were no significant differences for IgG1 levels in mice immunized with IgG1 from day 15 or day 55 p.i. which were rechallenged with a high infective dose compared to non-immunized control mice rechallenged with the same number of pRBCs. However, there were significantly higher levels of IgG1 (Bonferroni test, $P < 0.0087$) in the immunized group compared to naïve PBS-treated mice.
Figure 5.18.b shows that mice immunized with IgG2a from day 15 did not show significantly higher production of IgG1 compared to mice immunized with IgG2a from day 55. Mice immunized with either IgG2a from day 15 or 55 p.i. showed significantly higher (Bonferroni test, $P < 0.01$ and $P < 0.0051$ for day 15 and 55 p.i. respectively) levels of IgG1 compared to naïve PBS-treated mice.

5.2.6.3.2. Parasite-specific IgG2a production in mice rechallenged with a high infective dose of 1×10^8 pRBCs of avirulent *P. c. adami* DK.

The overall production of IgG2a after rechallenge was significantly (Tukey's test, $P < 0.0001$) higher than that when immunized mice infected for the first time. No significant differences were seen for IgG2a between rechallenged mice immunized with IgG1 from day 15 and 55 p.i. However, in those mentioned groups the IgG2a levels were significantly higher compared to PBS-treated mice (Bonferroni tst, $P < 0.004$ and $P < 0.0001$ for days 15 and 55 p.i respectively) (Figure 5.19a).

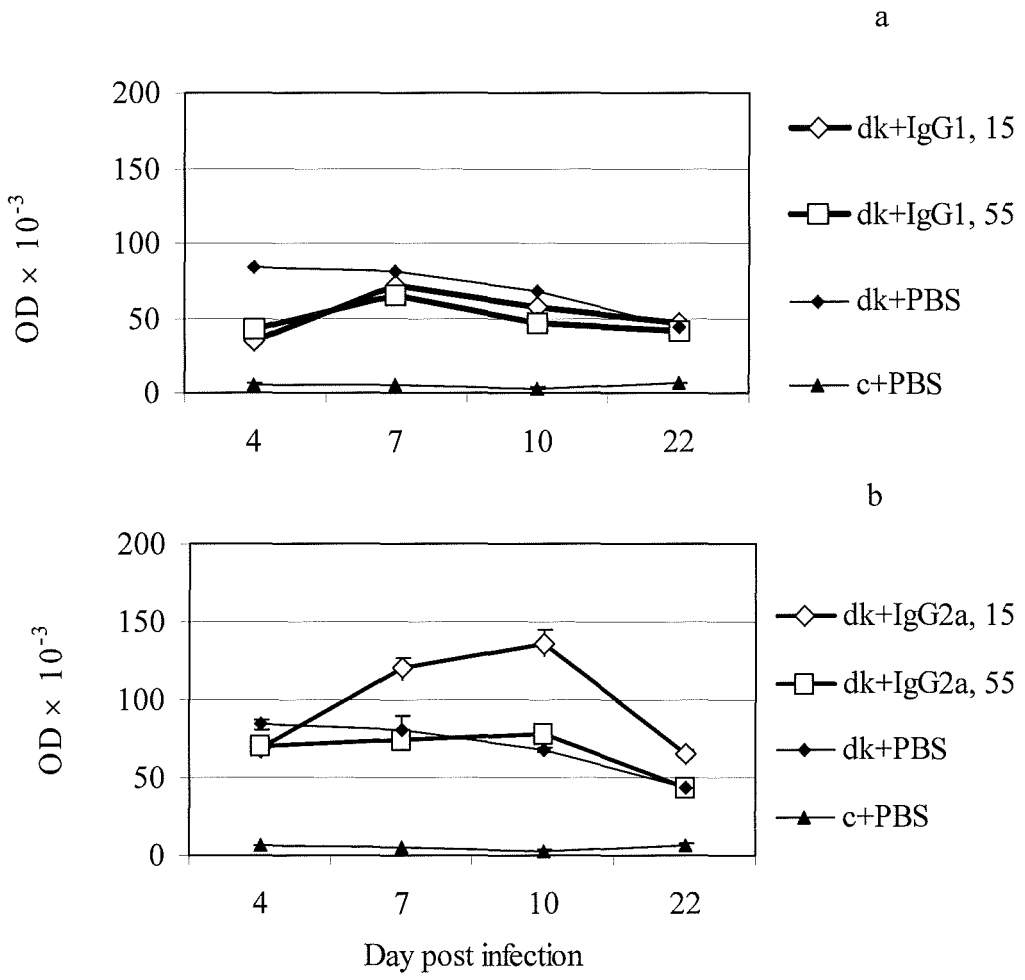


Figure 5.18. Parasite-specific IgG1 production after the rechallenge with 1×10^8 pRBCs of *P. c. adami* DK.

The levels of IgGs are shown as the mean ± SEM. However, ±SEM is not shown when it is too small.

a) IgG1 production in mice immunized with IgG1 from day 15 or 55 p.i. and rechallenged with the parasite.

dk+IgG1, 15 and dk+IgG1, 55: Mice passively immunized with IgG1 from days 15 or 55 p.i. and rechallenged with the parasite.

b) IgG1 production in mice passively immunized with IgG2a from day 15 or 55 p.i. and rechallenged with the parasite.

dk+IgG2a, 15 and dk+IgG2a, 55: Mice passively immunized with IgG2a from days 15 or 55 p.i. and rechallenged with the parasite.

dk+PBS: Mice infected and left without passive immunization and were injected with PBS.

c+PBS: Naïve mice were injected with PBS.

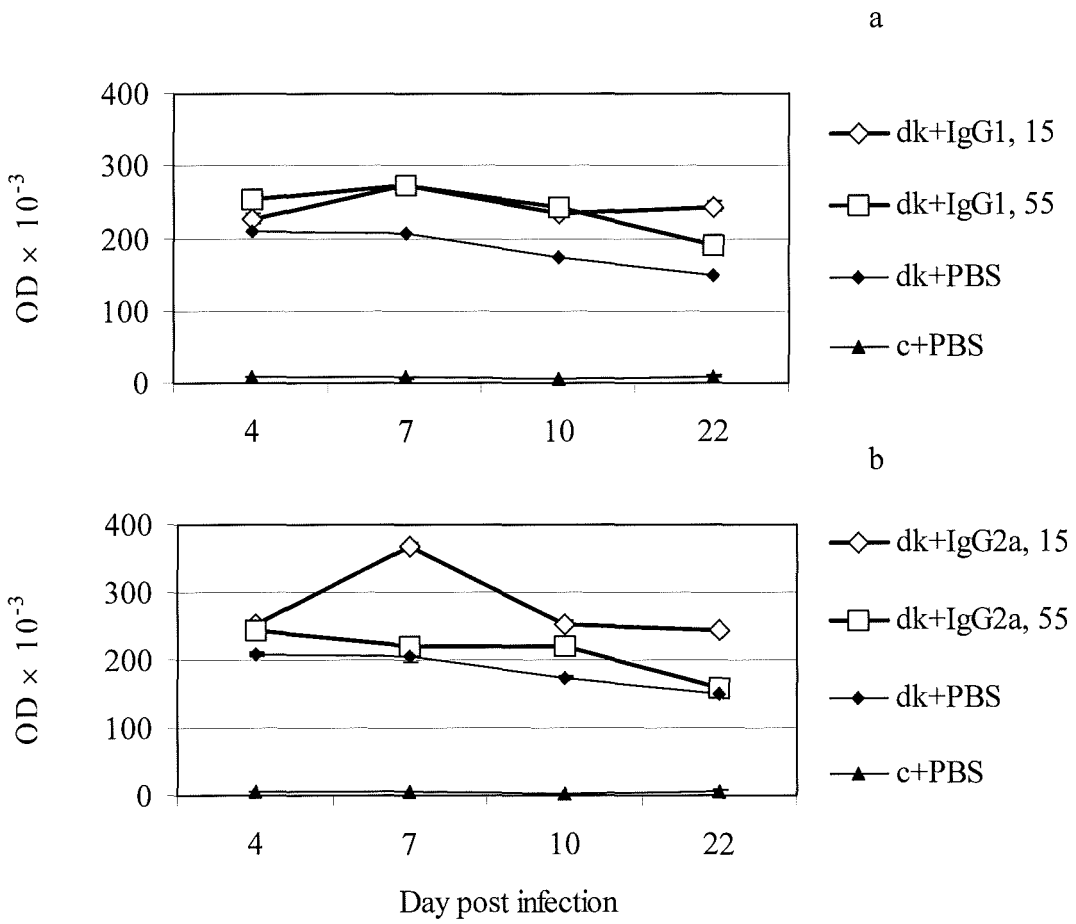


Figure 5.19. Parasite-specific IgG2a production after rechallenge with 1×10^8 pRBCs of *P. c. adami* DK. The levels of IgGs are shown as the mean ± SEM. However, ±SEM is not shown when it is too small.

a) IgG2a production in passively immunized mice with IgG1 from day 15 or 55 p.i. and rechallenged with the parasite.

dk+IgG1, 15 and dk+IgG1, 55: Mice passively immunized with IgG1 from days 15 or 55 p.i. and rechallenged with the parasite.

b) IgG2a production in passively immunized mice with IgG2a from day 15 or 55 p.i. and rechallenged with the parasite.

dk+IgG2a, 15 and dk+IgG2a, 55: Mice passively immunized with IgG2a from days 15 or 55 pi. and rechallenged with the parasite.

dk+PBS: Mice infected and left without passive immunization and were injected with PBS.

c+PBS: Naïve mice were injected with PBS.

5.2.6.4. Parasite-specific IgG1 and IgG2a production in mice rechallenged with a high infective dose of 1×10^8 pRBCs of virulent *P. c. adami* DS.

Cross-reactivity of IgG subclasses and IgG2a and IgG1 production were examined in all surviving mice which were rechallenged with a high infective dose of 1×10^8 pRBCs of *P. c. adami* DS. The experiments were performed as described earlier (above) for the avirulent DK strain.

5.2.6.4.1 Parasite-specific IgG1 production in mice rechallenged with a high infective dose of 1×10^8 pRBCs of virulent *P. c. adami* DS

Figure 5.20.a shows that the levels of IgG1 in mice immunized with either IgG1 or IgG2a from either day 15 or day 55 p.i were not significantly different after the rechallenge.

5.2.6.4.2. Parasite-specific IgG2a production in mice rechallenged with a high infective dose of 1×10^8 pRBCs of avirulent *P. c. adami* DS.

Figure 5.21a. shows that there were no significant differences in IgG2a levels between mice immunized with IgG1 from either day 15 or day 55 p.i after rechallenge. The difference between all these sample groups and non-infected group was significant (Two-way ANOVA, $P < 0.0001$). But, there were significantly higher (Bonferroni test, $P < 0.016$ and $P < 0.014$ for days 15 and 55 p.i. respectively) levels of IgG2a in these two immunized groups compared to the control group that was only rechallenged with a high infective dose of the parasite.

Figure 5.21.b also shows that when mice immunized with IgG2a there were no significant differences between the sample groups and the control group after rechallenge.

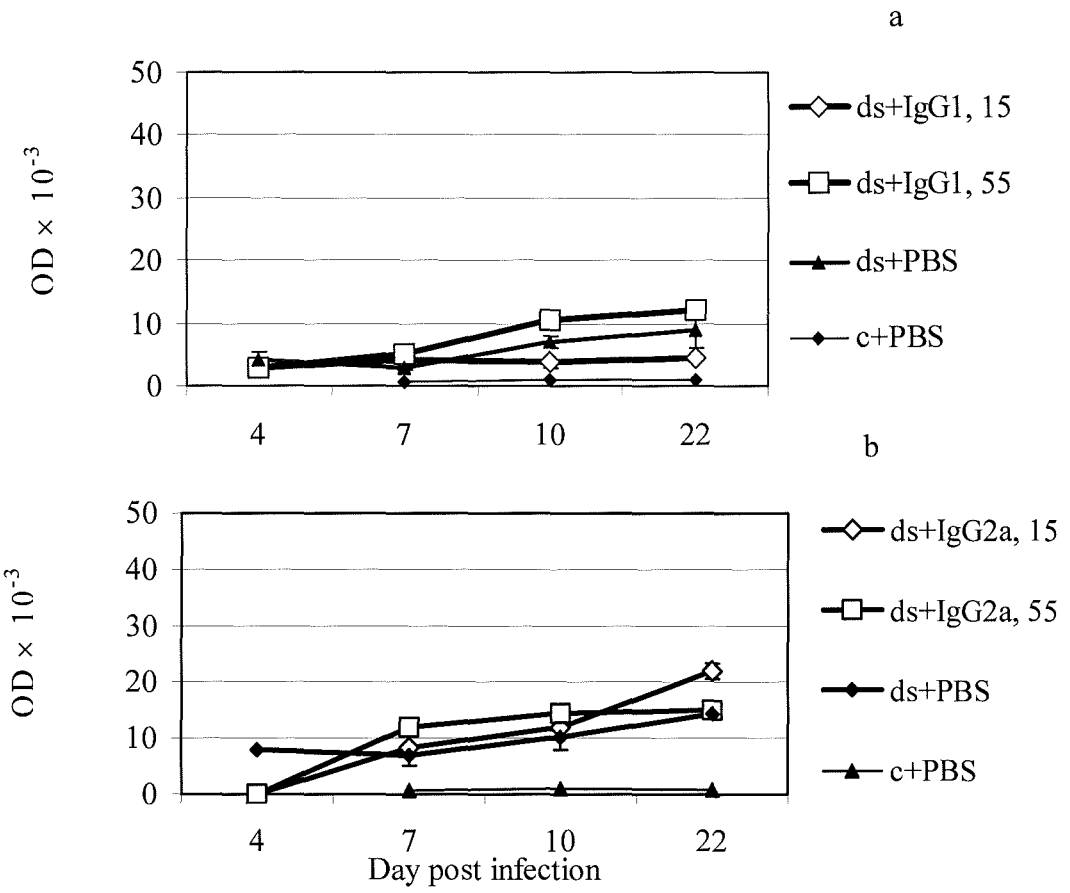


Figure 5.20. IgG1 production after the rechallenge with 1×10^8 pRBCs of virulent *P. c. adami* DS.

The levels of IgGs are shown as the mean ± SEM. However, ±SEM is not shown when it is too small.

a) IgG1 production in mice passively immunized with IgG1 from day 15 or 55 p.i. and rechallenged with the parasite.

ds+IgG1, 15 and ds+IgG1, 55: Mice passively immunized with IgG1 from days 15 or 55 p.i. and rechallenged with the parasite.

b) IgG1 production in mice passively immunized with IgG2a from day 15 or 55 p.i. and rechallenged with the parasite.

ds+IgG2a, 15 and ds+IgG2a, 55: Mice passively immunized with IgG2a from days 15 or 55 pi. and rechallenged with the parasite

ds+PBS: Mice infected and left without passive immunization and were injected with PBS.

c+PBS: Naïve mice were injected with PBS.

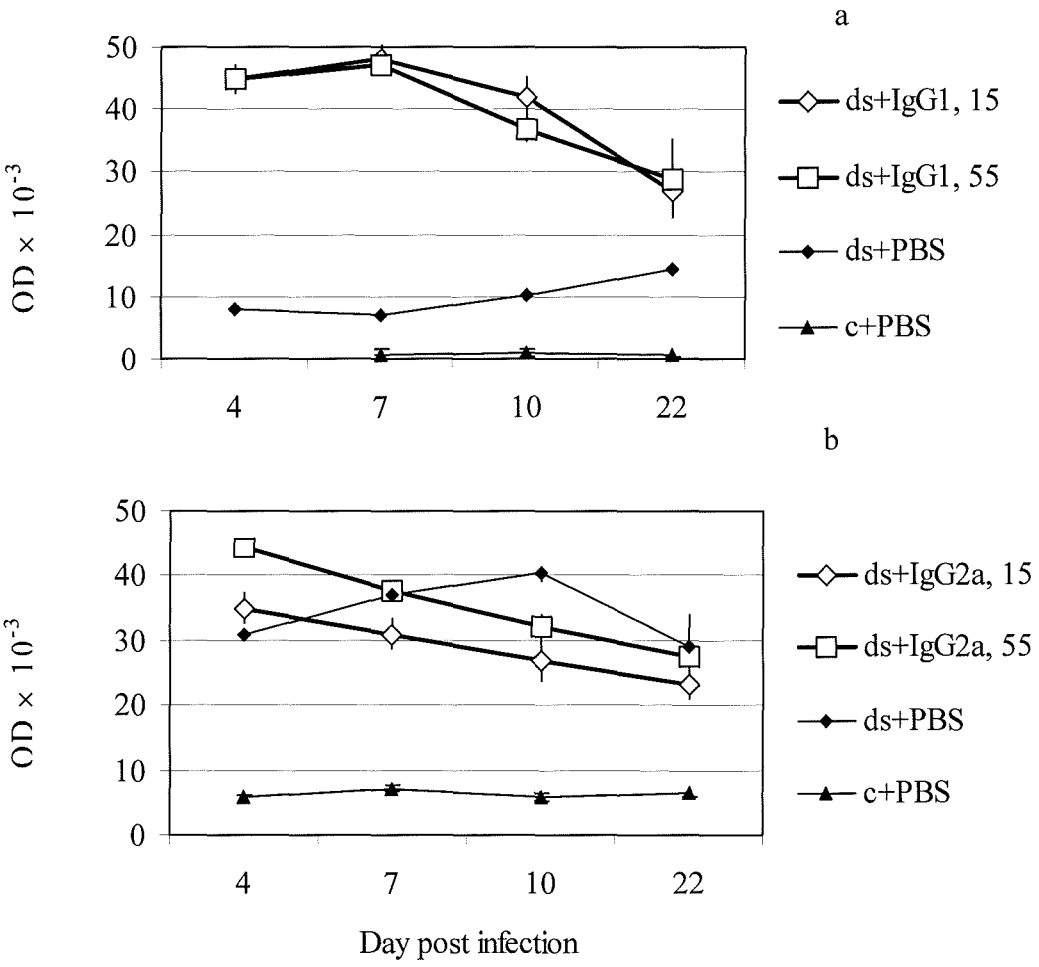


Figure 5.21. IgG2a production after the rechallenge with 1×10^8 pRBCs of virulent *P. c. adami* DS. The levels of IgGs are shown as the mean ± SEM. However, ±SEM is not shown when it is too small.

a) IgG2a production in mice passively immunized with IgG1 from day 15 or 55 p.i. and rechallenged with the parasite.

ds+IgG1, 15 and ds+IgG1, 55: Mice passively immunized with IgG1 from days 15 and 55 p.i. and rechallenged with the parasite.

b) IgG2a production in mice passively immunized with IgG2a from day 15 or 55 p.i. and rechallenged with the parasite.

ds+IgG2a, 15 and ds+IgG2a, 55: Mice passively immunized with IgG2a from days 15 and 55 pi. and rechallenged with the parasite.

ds+PBS: Mice infected and left without passive immunization and were injected with PBS.

c+PBS: Naïve mice were injected with PBS.

5.3. Discussion

The present study examined the effect of passive immunization by transferring whole serum or purified parasite-specific IgG1 and IgG2a subclasses on the course of infection and the kinetics of antibody production in avirulent and virulent infections in NIH mice before and after immunization. The examination of the cross-reactivity of IgG subclasses, IgG1 and IgG2a, obtained from mice infected with the avirulent DK strain in the virulent DS infection was also carried out. Possible differences in effectiveness of IgG subclasses which were purified from sera collected at two different time points, day 15 or 55 p.i., were also evaluated.

The results demonstrated that passive transfer of whole serum from DK-infected mice not only contributed in protection against homologous infection but also has cross-reactivity against the virulent *P. c. adami* DS infection. Although within malaria species protective immune responses have elements which are specific for the cloned line of parasites, cross-immunity between cloned lines of parasites derived from different isolates of the same species has been shown (Jarra and Brown 1985). Moreover, Mota *et al.* (2001) showed that hyperimmune sera from *P. c. chabudi* CB and *P. berghei* KSP-11 infected mice contained cross-reactive antibodies to the antigens of *P. chabaudi* AS strain. Cross-reactivity was also evident in mice challenged with *P. c. chabudi* AS which received immune sera from mice infected or reinfected with *P. c. chabudi* CB (Jarra *et al.*, 1986). In agreement with above results the present study also provided evidence of cross-reactive antibodies in serum from mice infected with the DK strain against the DS strain which indicated the presence of similar antigens or epitopes between the two strains.

In the present study passive transfer of sera taken on day 55 p.i showed significantly more effectiveness than serum taken on day 15 p.i probably due to the stronger secondary response compared to the primary antibody response following recrudescent parasitaemia and reinfection. Moreover, the cross-reactivity of these sera, from DK-infected mice, effectively prevented mice from death in the virulent DS infection. This is in agreement with the observation by Jarra and Brown (1989) in which they showed that in *P. c. chabaudi* AS pre-immune mice the initial growth of heterologous *P. c. chabaudi* CB, IP-PCI, or *P. c. adami* DS parasites

reduced when mice reinfected with those parasites. However, they showed that reinfection of AS pre-immune mice with the homologous parasite did not change the kinetics of the infection compared to control mice infected with *P. chabaudi* AS only. The present study showed that mice passively immunized with sera obtained from the DK-infected mice, survived from challenge with 1×10^4 pRBCs of the heterologous virulent DS strain. The present study showed that immunized mice surviving from a rechallenge with a higher dose, 1×10^8 pRBCs of either the DK or the DS strains, had developed significant immunity during their initial controlled challenges. In this regard, in mice rechallenegd with the DK-strain only one group showed parasitaemia up to 0.125 % on day 4 p.i. In mice rechallenged with the DS strain surviving mice in control group showed a parasitaemia which only peaked to 0.55% until day 3 p.i. In both rechallenged groups no parasites were seen thereafter. This substantial reduction in the peak parasitaemia in immunized mice rechallenged with the DS strain could be due to a strong and effective secondary response (Figure 5.17, 18, and 20). The observation which supports a stronger secondary response is that passively immunized mice with either IgG1 or IgG2a produced significantly higher levels of IgG2a and IgG1 after rechallenge with 1×10^8 pRBCs particularly on the first two sampling days, day 4 and 7 p.i (Figure 5.18 and 5.19) compared with IgG levels after the first challenge with lower infective dose, 1×10^4 pRBCs.

McLean, Pearson and Phillips (1982) suggested that only immune sera taken just after resolution of the primary and recrudescent peak parasitaemia of a *P. c. chabaudi* infection were protective. In agreement with their study the present result confirmed that sera taken after resolution of the primary peak parasitaemia, on day 15, and sera taken after recrudescent parasitaemia, on day 55 p.i., showed protectivivty.

The results presented here showed that there was a delay to reach to 2% parasitaemia and a prolongation of parasitaemia in passively immunized mice challenged with the avirulent DK strain. The same effect was reported by Jarra and colleagues (1986) as they showed that mice challenged with *P. chabaudi* CB and given serum from mice reinfected or superinfected with *P. chabaudi* AS showed an extension to reach to 2% parasitaemia and a significant reduction in parasitaemia between day 3 and 9 p.i. and lower

virulence compared to the controls. However, this delay or prolongation was not seen in the present study in mice passively immunized with sera from the DK-immune mice and challenged with the virulent-DS strain.

The present study showed that peak parasitaemias were significantly reduced in mice passively immunized with IgG1 from days 15 or 55 p.i. post challenge with the DK strain. An extension of the time taken to reach to 2% parasitaemia, protraction of the primary parasitaemia, and a delay in resolution were also observed. The present results also showed that peak of parasitaemias in mice immunized with IgG1 or IgG2a obtained from DK-immune mice were significantly lower than that in control mice after challenge with the virulent DS strain indicating cross-reactivity of those transferred IgGs. However, the efficacy between IgGs collected either on day 15 or on day 55p.i was not significantly different (Figures5.6 and 5.7).

Although this study confirmed that the presence of specific IgG antibodies in sera play a role in control of infection in passively immunized mice, identifying the role of other antibody isotypes such as IgM needs more investigation.

It was also observed that in the virulent DS-challenged mice three times passively immunization significantly lowered peak parasitaemias compared to mice passively immunized two times (see Table 2, Figure5.7 and 5.8). However, degree of mortality was not affected by more passive immunization around the time of peak parasitaemia. This could be as a result of other factors involved in death such as a rapid replication rate of the DS strain, massive distruction of RBCs, irregulation in temperature, and loss weight. So, the presence of more IgG could not compensated the effect of such factors to save the host.

In the present results the kinetics of parasite-specific IgG2a and IgG1 production indicates a sequential Th1/Th2 immune response in passively immunized mice in both avirulent and virulent challenge as previous studies suggested (Smith and Taylor-Robinson, 2003; Snapper and Paul, 1987). These results are not altered due to passive immunization as they are similar to the immune reponse in single-infections of the DK and DS strains when mice were not immunized (Chapter Three) in which the immune response is characterized by early and later significant up-regulation of cytophilic IgG2a and non-cytophilic IgG1, respectively. White *et al.* (1991) suggested that IgG2a is responsible for modulating

parasitaemia as mice passively received IgG2a did not develop patent infection until day 10 to 12 and parasitaemia peaked therafter. In the present study, this suggested modulation of peak parasitaemia was not observed in passively immuniozed mice challenged with the virulent DS strain. However, passively immunized mice challenged with the avirulent DK strain showed modulation in parasitaemia by showing a delay in time taken to reach to the peak parasditaemia compared to the controls (Figure5.4 to 5.7).

Identification of the asexual blood stages susceptibile to induced antibodies also is another important issue in passive immunization studies. Cavinato *et al.* (2001) suggested that merozoites are the main targets for specific antibodies. These antibodies may control reinvasion of new merozoites. It is proposed that a substantial proportion of antibodies in human serum from immune individuals inhibit merozoite invasion (O'Donnell *et al.*, 2001). It is suggested that protection through inhibiting invasion should be associated with the presence of specific antibodies to particular epitopes (Okech *et al.*, 2004). So, investigating the role of fine specificity of antibodies seems to be more important than their simple prevalence or titre to determine their protective efficacy. For example, Okech and colleagues (2004) demonstrated that there is a significant correlation between antibody specificity and protection against malaria infection and high-density parasitaemia in human *P. falciparum* infection. Regarding asexual blood stages of malaria parasite, it is therefore, possible to design some passive immunization studies in which targets of transferred specific antibodies can be determined. Findings of such epitopes and targets can help to identify functional mechanisms by which antibodies implicate in immunity such as opsonization of infected red blood cells by macrophages. In addition, those investigations may determine how transferred antibodies such as IgG subclasses affect the immune response against specific antigen(s). Based on the present results it cannot be stated that what are the mechanisms of clearance of the DK strain parasites following passive immunization with IgGs and or which cross-epitopes may be recognized by transferred IgGs in the DS-challenged mice following passive immunization. However, reduced peak parasitaemia and no detectable parasites post-challenge could suggest that replication rate of the parasites, even the DS strain was affected by the transferred antibodies.

This also could be a sign of effective control of reinvasion by neutralizing new merozoite. Nevertheless, more studies are required to determine what are the susceptible stages of the DK or DS strains, as relevant targets for the these IgGs. For identifying and distinguishing which antigens are actually recognized by the purified transferred antibodies techniques such as FACS may be used. The protective effects of passive immunization have been investigated with purified MAbs against specific antigens of rodent malaria parasites such as MSP-1 or stage-specific merozoite antigens (Majarian *et al.*, 1984; Spencer Valero *et al.*, 1998; Freeman, Trejdosiewicz, and Cross, 1980). In the present study, although, the effect of MAbs was not examined, purified parasite-specific IgG1 or IgG2a showed degree of protection against avirulent and virulent challenges. So, further investigations may determine any protection role for passively transfer of MAb prepared from these subclasses against the DK and DS strains of *P. c. adami* as a suitable model of rodent malaria parasite. These investigations also help how to determine specific targets of those MAbs among asexual blood stages. This could also include identifying more effective MAb which has cross-reactivity and variant-specificity in diffent species. Identifying of target antigens or epitopes for these cross-reactive MAbs is also important in vaccine design studies.

The present study provided useful information to understand better the kinetics of specific antibody production after immunization and rechalleneg. It also provide some information regarding determination of possible cross-reactivity of antibodies between avirulent and virulent infections which are basically important in vaccine development studies.

Chapter Six

Synthetic peptide immunization: the effect of immunization of NIH mice on the course of infection after challenge with *Plasmodium chabaudi* AS

6.1. Introduction

Synthetic peptide immunization seem to be an attractive strategy for antigen delivery. Synthetic peptides are relatively easy to obtain in large quantities, with high purity, and can be made immunogenic by coupling with suitable carrier proteins and/or adjuvants (Tsuji and Zavala, 2001). Using these vaccines is safe because they are not infectious. Synthetic peptides also do not integrate into the chromosomes of immunized host preventing possible undesirable mutations that could result from using DNA-based vaccines (Tsuji and Zaval, 2001).

In rodent malaria, the efficacy of T cells elicited by peptide immunization showed that peptide immunization may lead to the preferential activation of $CD4^+$ and $CD8^+$ T cells in C57BL/6 mice and $CD8^+$ T cells in BALB/c mice (Renia *et al.*, 1991). They demonstrated that parasite elimination was directly mediated by these cells when *in vitro* T-cell activity was evaluated on cultured hepatic stages of *P. yoelii*. So, they suggested that $CD4^+$ and $CD8^+$ T cells primed with the peptides could be cytolytic for the hepatic stage of malaria parasites. Moreover, it is possible to induce protective $CD4^+$ T cell responses in mice immunized with *P. c. adami* proteins with molecular mass of 25 to 40 kDa and challenged with the parasite (Kima, Srivastava and Long, 1992). A single subcutaneous injection of 50 μg of a large soluble synthetic peptid which corresponds to the N- and the C-terminal domains 22-125 and 289-390 of *P. falciparum* 7G8 isolate can elicit CS-specific CTL *in vivo* with one boosting injection in BALB/c mice (Blum-Tirouvanziam *et al.*, 1994). So, attempting to identify peptides with high immunogenicity and smaller molecular weight is thought to be a desired aim for further vaccine researches.

One important aim in immunization against malaria parasites is that what type of immune response should be generated which elicits both B- and T-cell responses as necessities in protection at different stages. The effectiveness of malaria synthetic peptide vaccines against antigens expressed in sporozoites and/or liver stages is correlated with the presence of parasite-derived epitopes that elicit $CD4^+$ T cells (Tsuji and Zavala, 2001). These highly immunogenic vaccines could function both as a source of cytokines to induce both antibody-dependent and cell-mediated immune response to control infections (Tsuji and Zavala, 2001). It has been shown

that synthetic peptide vaccines in mice are capable of inducing high titres of anti-repeat of CSP antibodies protective against sporozoite challenge of *P. berghei* (Zavala *et al.*, 1987).

Immunization with a single synthetic peptide-vaccine, the tetramer repeat region of the *P. falciparum* CSP $(NANP)_3$, elicited relatively low titres of anti-repeat antibodies in volunteers (Herrington *et al.*, 1987). So, it seems that a multi-stage vaccine may be more effective if liver-stage and blood-stage antigens were combined into a single formulation, because if parasites escape control at the liver stage they could be eliminated by immunity targeted against blood-stages. Mice immunized with the repeat region of *P. berghei* CSP conjugated to tetanus toxoid developed high titres of antibodies to native CSP on the *P. berghei* sporozoite and the magnitude of the antibody response was correlated with level of protection (Zavala *et al.*, 1987). To avoid risk of carrier toxicity in such vaccines multiple antigen peptides (MAPs) were developed (Tam *et al.*, 1990). An example of these vaccines contains repeated B cell epitopes from PyCSP and two T helper epitopes that induce high levels of anti-PyCSP antibodies and protects inbred or outbred mice challenged with sporozoites of *P. yoelii* (Wang *et al.*, 1995). Chai *et al.* (1992) showed that immunization with MAP containing a *P. berghei* CSP repeat synthesized in tandem with a T helper epitope, induces a long-lasting immune response, and elicits secondary antibody and T cell responses in pre-infected mice.

In humans, it has been demonstrated that following immunization with a MAP possessing minimal T and B cell epitopes, high levels of parasite-specific antibodies were elicited in 10 out of 12 volunteers with known class II genotypes (Nardin *et al.*, 2000). The synthetic peptide was composed of only five amino acids (NAVDP) and was able to elicit parasite-specific antibody titres comparable to multiple exposures to irradiated *P. falciparum*-infected mosquitoes (Nardin *et al.*, 2000). Immunization of BALB/c mice with three doses of another MAP vaccine which was constructed as a combination of four branches of amino acids 57 to 70 of CSP linked to a lysine-glycine core and lipofectin as an adjuvant, induced T cell proliferation and a peptide-specific CTL response (Franke *et al.*, 2000). Ak *et al.* (1993) showed that a synthetic peptide based on the *P. yoelii* CSP major repeat and conjugated to KLH induced protective antibodies against *P. yoelii* challenge. Howeve, they showed that a specific

antibody subclass is not required for protection. Bharadwaj *et al.* (1998) designed a synthetic peptide vaccine containing a motif which is highly conserved in the CSP and the thrombospondin-related anonymous protein (TRAP) of different *Plasmodium* species. This motif is shown to be crucially involved in the sporozoite invasion of hepatocytes. It has been also shown that antibodies raised against this motif which was originally from CSP of the 7G8 clone of *P. falciparum* inhibit merozoite invasion of erythrocytes. They showed that immunization with a linear multi-epitopic construct, a 60-residue (P60) peptide, containing the conserved motif sequence, elicited anti-P60 antibodies that can block *P. falciparum* merozoite invasion in a dose-dependent manner. They also showed that more than 60% of immunized mice survived a heterologous challenge with a lethal strain of *P. yoelii*. It also induced significant levels of cytokines such as IL-2, IFNγ, and IL-4 in BALB/c mice. So, their study suggested that an appropriate medium-sized synthetic peptide can generate a specific immune response to critical epitopes in an antigen and could be part of a multicomponent malaria vaccine.

Cryptic epitopes are defined as epitopes that induce T cells which recognize the immunizing peptides only and not antigens naturally processed by APC or infected cells (Franke *et al.*, 2000). These epitopes can be used as peptide-based vaccines which elicit protein-specific helper T cells in *in vivo* assays (Good *et al.*, 1990). However, a cysteine-containing peptide motif designed by Bharadwaj *et al.* (1998) also represented a cryptic epitope during natural infection. It has been shown that antibodies against this motif were capable of inhibiting merozoite invasion of erythrocytes (Bharadwaj *et al.*, 1998).

Long polypeptides such as the N-terminal amino acids 22-125 and the C-terminal 289-390 regions of the CSP of *P. falciparum* can induce neutralizing antibody responses in two population in South America and Africa. These antibodies can prevent *P. falciparum* sporozoite penetration *in vitro* (Roggero *et al.*, 1995). In rodents, immunization of BALB/c mice with a long synthetic polypeptide of 69 amino acids, encompassing the C-terminal region of CSP, induced immune responses including high titres of anti-peptide antibodies which recognized the native *P. berghei* CSP, specific-CTL response for this polypeptide, and partial $CD8^+$ T cell protection against sporozoite challenge (Roggero *et al.*, 2000).

With regard to immunization against blood-stage, Spf66, a synthetic multi-component asexual blood stage peptide vaccine containing three synthetic peptides corresponding to fragments of the PfMSP1 and the repeat region of CSP (Patarroyo *et al.*, 1988), is the only vaccine to reach phase III clinical trials (Kaur *et al.*, 2002). This vaccine induced protection against *P. falciparum* challenge in *Aotus* monkey (Patarroyo *et al.*, 1987). However, in human clinical trials, its efficacy was between 35% in South America (Valero *et al.*, 1996), 31% in children aged between 1-5 years in Tanzania (Alonso *et al.*, 1994). In Gambian trials it was shown that anti-SPf66 antibodies decayed and fell to 5-10% of maximum levels within four months (D'Alessandro *et al.*, 1996). In other clinical trials of Spf66 and their following up no more than 8% efficacy was observed in Gambian infants (D'Alessandro, 1996; Bojang *et al.*, 1997 and 1998).

In rodent malaria, Holder and Freeman (1981) first showed that immunization with an affinity-purified protein, MSP-1, induced a high level of protection against *P. yoelii* infection in mice. It has been demonstrated that immunization with recombinant polypeptides containing the 19-kDa region from *P. yoelii* MSP-1 primarily induced antibody-dependent responses that protect mice against asexual parasite challenge (Calvo, Daly, and Long, 1996; Daly and Long, 1995). Burns and colleagues (2003) demonstrated that immunization of C57BL/6 mice with recombinant antigens, including the 54-kDa ectodomain of AMA-1 and the 42-kDA C-terminal of MSP-1, both from *P. c. adami* 556KA, induced a high level of protection against *P. c. adami* infection. They showed that immunization with each antigen alone elicited the same level of IgG. They did not find more efficacy compared to each antigen alone when both peptides were used in a formulated combination. Moreover, immunization of rabbits with peptides derived from AMA-1, designated J1 to J7, induced high titres of anti-peptide antibodies which were reactive with native AMA-1. The peptides J1, J3, and J7 were the most reactive ones. Anti-peptide antibodies in human were isolated from plasma samples of people who exposed to chronic malaria reacted with J1 and J7 peptides using immobilized peptide immunoadsorbents. Anti-peptide antibodies were also obtained from white rabbits which were immunized with peptide-KLH conjugates emulsified in 0.5 ml of Freund's complete adjuvant. The study also showed that both humans and rabbit specific-antibodies against J1 and J7 peptides are

capable of inhibiting the invasion of erythrocytes by *P. falciparum* merozoites (Casey *et al.*, 2004).

Moreover, it is known that different fragments of known antigens, such as MSP-1, induce different levels of protection. Tian *et al.* (1997) examined efficacy of different fragments of MSP-1 in immunization of mice against *P. yoelii* and in *Aotus* monkeys against *P. falciparum*. They found that recombinant 19-kDa C termini from both *P. falciparum* in *Aotus*, and *P. yoelii* in mice, induced protective responses after immunization with Freund's adjuvant. However, other fragments, such as a 42-kDa C-terminal region, induced partial protection (Tian *et al.*, 1997; Chang *et al.*, 1996). Other parts of MSP-1 did not have efficacy as good as those previously described. Mice immunized with a recombinant protein containing two EGF-like molecules of MSP-1 of *P. yoelii* may be protected by a MSP-1-restricted T cell response, and to a lesser extent, IgG2a antibodies (Ling *et al.*, 1997).

In some other vaccine studies single antigen immunization with purified native or recombinant antigens have been the main aim. Immunization with AMA-1 or MSP-1 in rodent and monkey malaria models was investigated (Anders *et al.*, 1998; Crewther *et al.*, 1996; Daly and Long, 1995; Hodder, Crewther, and Anders, 2001). Burns *et al.* (2003) showed that in a single antigen immunization experiment with recombinant PcAMA-1 or PcMSP-1, specific IgG1 was predominant and showed significant protection against *P. c. adami* 556KA challenge by significant reductions in peak parasitaemia in immunized C57BL/6 mice compared to controls. The profile of the results was the same when mice immunized with three different adjuvants. However, immunization with combined formulations of recombinant PcAMA-1 and PcMSP-1_{42} induced protection against *P. c. adami* infection at a high level (Burns *et al.*, 2003) which was comparable to single antigen immunizations. Immunization with bacterial plasmids (Ling, Ogun, and Holder, 1995) used to express a recombinant protein containing two EGF-like modules domains, individually and in combination, induced protective and specific antibody responses to the targeted proteins of *P. yoelii* MSP-1.

The mechanisms required for sterile immunity in malaria infection are not completely known. A role for antibody is shown by the passive transfer of polyclonal anti-repeat CSP antibodies derived from MAP immunized rodents which can protect naïve recipients against sporozoite challenge of

P. yoelii and *P. berghei* (Wang *et al.*, 1995; Reed *et al.*, 1997; Nardin *et al.*, 2000). On the other hand, $CD4^+$ T cell responses mediate protection through release of IFNγ which inhibit intracellular liver stages of the parasite (Ferreira *et al.*, 1986). This response can be induced in murine *P. yoelii* malaria by MAP immunization (Migliorini, Betschart, and Corradin, 1993; Wang *et al.*, 1996).

Many new potential vaccine candidates such as products of the *rifin*, *stevor*, and *clag* genes have been identified (Hoffman *et al*, 2002). Regarding to *clag* gene family in *P. faciparum* (see Chapter One), some *clag*-like genes are also identified in rodent malaria parasites such as *P. chabaudi*, *P. yoelii* and *P. berghei* (Holt *et al.*, 2001). Three synthetic peptides designated P1, P2, and P3 are used in the present study. The amino acid sequences of these peptides are chosen based on genes *clag* 7 and 3 of *P. chabaudi* AS. The amino acid sequences of P1 is conserved between *clag* 7 and 3 and are absed on postions 121-134 and 128-141 respectively. The P2 and P3 are specific for *clag* 7 and 3 respectively. The amino acid sequences of P2 and P3 are based on postions 244-257 and 246-259 respectively (Personal communication and NCBI web site). Janssen and colleagues (2002) identified a highly conserved multigene family in three different rodent malaria parasites, *P. yoelii*, *P. berghei*, and *P. chabaudi* which has homologues in the human malaria *P. vivax* (Janssen *et al.*, 2002). This gene family has been named *cir* as it was discovered in *P. chabaudi*. The homology of *cir* with *P. vivax* (*vir* gene family) shows the importance of *P. chabaudi* as a model for the human malaria infections. Two *cir*-related synthtetic peptides used in the present study are designated P4 and P5. The location of amino acid sequences are between positions 102-115 for P4, and between 174-187 for P5 based on sequenced mRNA of *cir* gene in *P. chabaudi* AS.

A strategy for blood-stage malaria vaccines is focused on disrupting or preventing cytoadherence (reviewed by Brown, 1999). As *P. chabaudi* shows antigenic variation (McLean, Pearson and Philips, 1982) which involves in cytoadherence and pathology of malaria (Smith *et al.*, 1995), the synthetic peptides as the products of *clag* and *cir* genes were included in the interests of the present study. Of *clag* genes the *clag* gene 9 of *P. falciparum* has been cloned in yeast artificial chromosomes and mapped with sequence tagged sites (Holt *et al.*, 1999) and its protein product is

identified as having a role in cytoadherence and designated CLAG9. However, Ling *et al.* (2004) showed that CLAG9 is a part of RhopH complex casting doubts on direct role for CLAG9 in cytoadherence. Thus they suggested that RhopH may have multiple functions including, with respect to CLAG9, a contribution to the mechanism of cytoadhesion such as invasion of sporozoite and merozoite into hepatocytes or erythrocytes resectively.

In the present study five small synthetic peptides, each has 15 amino acids, were used in conjugation with KLH as carrier protein for immunization against the rodent malaria parasite *P. c. chabaudi* AS. These peptides are based on *clag* and *cir* gene families and were synthesized based on *P. c. chabaudi* AS genome sequencing. Figure 6.1a and b shows the amino acid sequences of these synthetic peptides based genomic data of *P. chabaudi* AS from NIBC in which positions of each peptide can be seen.

In the present study comparison of the effects of immunization with the above synthetic peptides in protection against *P. chabaudi* AS challenge was examined. This aim helps to determine the most immunogenic peptide which may be involved for future designing a putative single or multiple immunogenic peptides for inducing more protective immune response.

The present study, therefore, was designed to develop knowledge about some of the functional properties of the synthetic peptides based on above genes. The course of infection, the profile of the antibody response were examined in NIH mice immunized with the indicated synthetic peptides and challenged with *P. chabaui* AS.

6.2. Results

Different groups of NIH mice, as described below, were used in this set of the experiments. The size of each group was six NIH mice.The inoculum was equal proportion (50/50 %) of each synthetic peptide conjugated with KLH, and adjuvant, Titermax (150µl of the emulsion containing 75µg of the conjugated peptide per mouse). In the control group mice were injected with 150µl adjuvant and PBS (50/50 %). Inocula were injected i.p. according to the manufacturer's instruction (Pierce, USA).

There were two test groups for immunization:

a) Mice immunized with each synthetic peptide conjugated to KLH plus the adjuvant.

b) Mice immunized with each peptide conjugated to KLH only.

In the control group mice were infected with the parasites and injected adjuvant plus PBS. The immunization protocol is detailed in Chapter Two. For P1, P2, and P3 an initial injection and two boosting injections were performed with the same amount of peptide at one-week intervals. Three boosting injections for P4 and P5 were given. Mice were challenged with 1×10^5 pRBCs of *P. chabaudi* AS, two weeks after the last boosting injection. The courses of infection were monitored in each group as well as in the control group. Specific anti-peptide and anti-parasite anibodies (reaction between immunized and control group to lysate of pRBCs of the parasite) were evaluated with ELISA after each boosting injections, and also after the parasite challenge. As control, reactions of serum from naïve mice to each peptide coated in ELISA plate or the lysate of *P. chabaudi* AS were also measured. Reactions between sera from immunized and control mice to lysate of naïve mice for detection of specific antibodies were examined. However, due to very low levels their related results have not shown.

6.2.1. Immunization of NIH mice with P1, P2, or P3.

The courses of infection, anti-parasite, and anti-peptide IgG (whole molecule), IgG1, and IgG2a in mice immunized with P1, P2, or P3 were examined as described above.

6.2.1.1. The course of infection in mice immunized with P1, P2, or P3

Figures 6.2 and 6.3 show the course of infection in NIH mice immunized with three different peptides based on *clag* genes products. In all immunized mice, statistically significant lower (Tukey's test, $P< 0.001$ for all groups) peak parasitaemias were observed compared to controls. In mice immunized with conjugated peptides without adjuvant peak parasitaemia was significantly lower (Tukeys's test, $P < 0.001$) compared to mice immunized with the conjugated peptide plus adjuvant (analysis was performed for peak parasitaemias between different groups of immunized mice). So, the results showed that using adjuvant had no significant effect on reduction of peak parasitaemia. The peak parasitaemia in non-immunized control mice was 56.4% while mice immunized with P1 showed the lowest peak parasitaemia with 41.99% between the immunized mice. This latter peak was significantly lower (Tukey's test, $P < 0.01$ for P1 vs P2, and $P < 0.001$ for P1 vs P2+adj) than that in mice immunized with P2, P2 plus adjuvant, and P3 plus adjuvant. No significant difference was seen for peak parasitaemia in mice immunized with P1 or P3 without adjuvant.

```
                 *       900          *       920
gi|5652481  : FKKIQQSFFSHRRNDAVSMNNIFFFNVRPNYSRLPKK :  925
clag7       : CKKLEGNSGSHRRNDSTSITNIYMFNVSKNYSLLSKE :   41
clag3       : CRKLEGKAGSHRRNDSTSLTNIYMLNVSKNYSRLSKE :   48

                    *       940          *       960
gi|5652481  : ERYQEIHESLASRFFEKTLFSIFHIMFVIKISKNVDK :  962
clag7       : ARIKEIDDSMKSKFLAKTLYSVLHTIFSIKMNKQLVE :   78
clag3       : ARIKELDDSMKSKFLGKTLYTVLHAMFSTQINKNLNE :   85

                       *       980          *      100
gi|5652481  : LDAIYGKANMLRMVVHEEPHLRFEYLYNGSMLDSLLN :  999
clag7       : LDKYYSKAKFIGLSANQKAYYTYAYAHYGSIIDTITN :  115
clag3       : LDKDYAKAKLIGLTMDEDAYFKVAFVHYGSIIDSITN :  122

                     0          *      1020          *
gi|5652481  : VFFPLYIKKPSVQLKYGKTFILANMFrlsSELFGIYD : 1036
clag7       : SLMPLYAKKPITQLRYGKTFIFANHFGFVAQVYGILK :  152
clag3       : SLMPLYAKKPITQLRYGKTFIFANYFGFIAQIYGILK :  159

                    1040          *      1060          *
gi|5652481  : LNNLSMLCEYQAITGANFYSFKKLSEFIDRKFVPfvl : 1073
clag7       : LNNMKMLCEHQAIASANNYSIEKKRQFGAKKIFPIVT :  189
clag3       : LNNMKMLCEHQAIVSANSYSMEKKIQYFLKKIFAIVT :  196

                       1080          *      1100          *
gi|5652481  : ggCIMKIRNIVNNPgqsaweafkaravndglitftvl : 1110
clag7       : YFAVLRAYrvylyPLEYNYWTSEVVFAKKNFFHLTIY :  226
clag3       : YFAVLRVYSVIDEPMWWTFWAGQAAFGRQSFFHIMMY :  233

                          1120          *      1140
gi|5652481  : gkTLYMSGNLLYRHIYFFPNNLIEELQKQTEWIEKYE : 1147
clag7       : Y-SMHMGTLLWLEvadcfqshftinyrirqviclcrv :  262
clag3       : Y-TMHMGVTIWSESGRVLPRPLYDELQDQASDMFIPE :  269

                    *      1160          *      1180
gi|5652481  : KDNKPstfyidipaliatfhsvsiktfLYALSLHYSF : 1184
clag7       : hqrndpLHSFTQTHVFDMINLFTYLFSVYNVLRWYAF :  299
clag3       : PPEKPeLHAFHQNYVFGLVIAFTYLFSVYATFRWYVF :  306
```


Figure 6.1a: An alignment of *clag* clones with the peptides sequenced highlighted.

The amino acid sequences of *clag* clones are derived from genomic sequences. The amino acid sequences of P1 which are conserved between *clag* 7 and 3 and are based on positions 121-134 and 128-141 respectively. The P2 and P3 are specific for *clag* 7 and 3 respectively. The amino acid sequences of P2 and P3 are based on positions 244-257 and 246-259 respectively (Personal communication and NCBI web site).

```
                    *         20          *
cir2_mRNA_  : RSFIIMNKDLCDVIKGIDDLIEVEVKAEGIETIRDELF :  38
cir_1_mRNA  : RSFIIMNKDLCDVIKGIDDSIEVEVKAEGIETIRDELF :  38

              40          *         60          *
cir2_mRNA_  : NTYCPTNKGGKMRQQGQDGYCIGYSETVISAFIHLQET :  76
cir_1_mRNA  : NTYCPTNKGGKMRQQGQDGHCIGYSETVISAFIYLQET :  76

                80          *        100          *
cir2_mRNA_  : LKNNDSQKKLDRDKLAQYAILWLSYKINQHPNQTFGTN : 114
cir_1_mRNA  : LKSNYSPEELESDKPAQYAILWLSYKINQHPNKKFGTN : 114

                 120          *        140          *
cir2_mRNA_  : DIYNNFKQYGYWNRKHNNYIEQIKKYVDIKDMTKLHEA : 152
cir_1_mRNA  : DIYNNFKKYNYWNLNHNNYIEQIKKYVDIKDMTKLHEA : 152

                      160          *        180          *
cir2_mRNA_  : FILLCNMYTEIDENKSNCTKCSQKASEFVKKFEILNDD : 190
cir_1_mRNA  : FILLCEMYTEFNDEIKNCTKCSQKASEFVKSFKELNDN : 190

                     200          *        220
cir2_mRNA_  : PNHIKDSPYSQILLTLSKDYDNFKNCCNKKKGESCDFP : 228
cir_1_mRNA  : SNHIDGSSYSQILLTLSKDYDNLKNKCDN--GQSSNFP : 226

                *        240          *        260
cir2_mRNA_  : SLPQISPKKSFAQNSLESPGHTSGHNSEDISSKSPMAN : 266
cir_1_mRNA  : SLPPIKPTKSSTQNNIEA----SVQLSEYKPSSSSVEN : 260

                   *        280          *        300
cir2_mRNA_  : KLIPGLLIFAAIPVFLGIAYKYSLFGFDKQRHRQYLRE : 304
cir_1_mRNA  : ALIPVLSIFVAIPVFLGIAYKYSLFGFDKQRHRQYLRE : 298

                         *        320
cir2_mRNA_  : KIKKIKNKMASYV------ : 317
cir_1_mRNA  : KIKKIKNKMASYV------ : 311
```

1

Figure 6.1b: An alignment of *cir* clones with the peptides sequenced highlighted.

The amino acid sequences of *cir* derived from sequenced mRNA. The amino acid sequences of P4 and P5 are based on positions 102-115 and 174-187 respectively (Personal communication and NCBI web site).

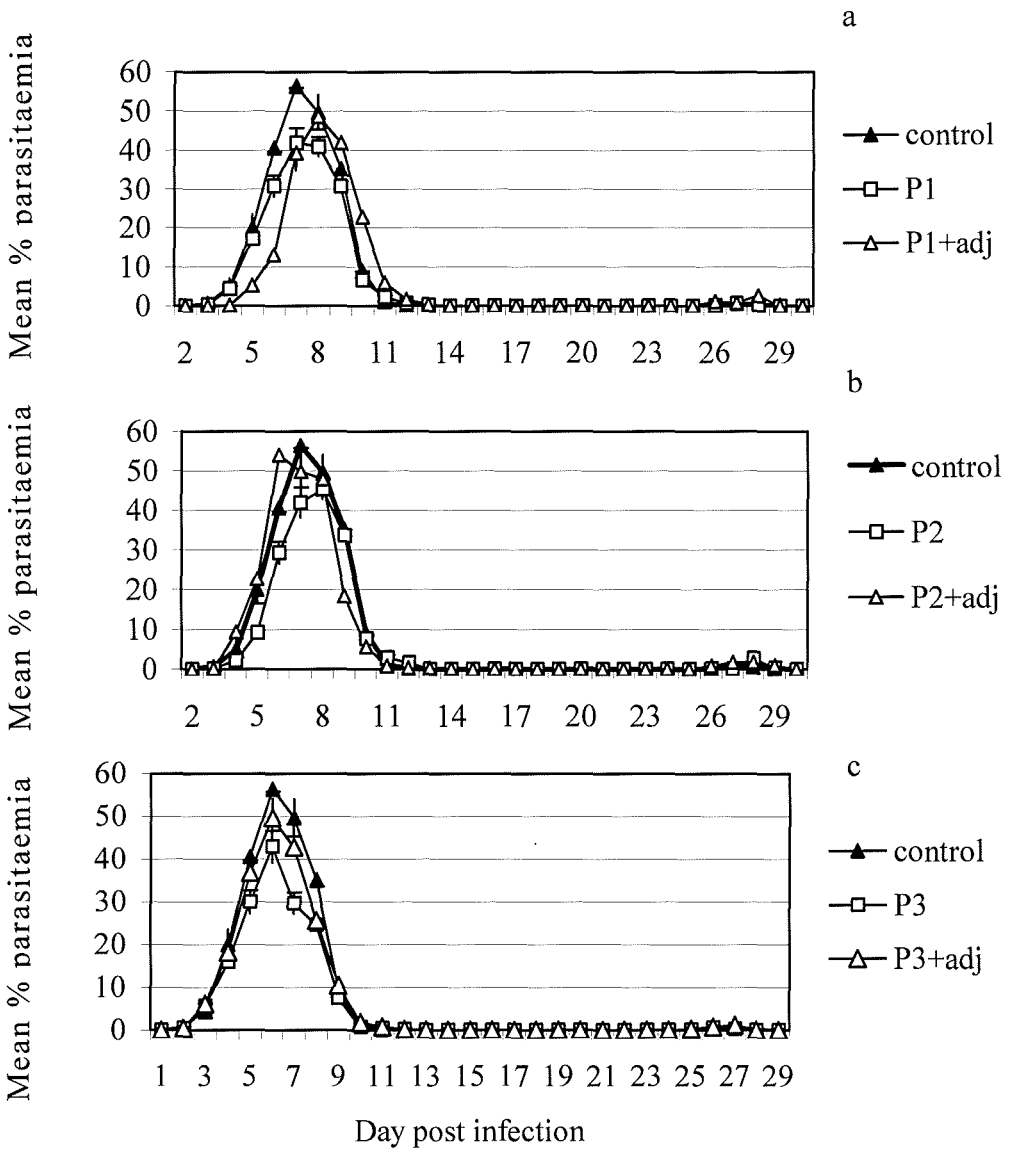


Figure 6.2.The course of infections in mice immunized with three different synthetic peptides based on *clag* gene family. Each group included six mice.

The levels of parasitaemia are shown as the mean % ± SEM for each time point. However, ±SEM is not shown when it is too small.

Immunization included initial injection followed by two boosting injections of the same size at 14 day intervals.

Mice were challenged with $1x10^5$ pRBCs of *P. c. chabaudi* AS i.p. and parasitaemia was monitored for 30 days p.i.

P1+adj, P2+adj, and P3+adj: Mice were immunized with the conjugated peptides 1, 2, or 3 to KLH and the adjuvant respectively.

P1, P2, and P3: Mice were immunized with conjugated peptides 1, 2, or P3 to KLH.

Control: mice were injected with adjuvant and PBS.

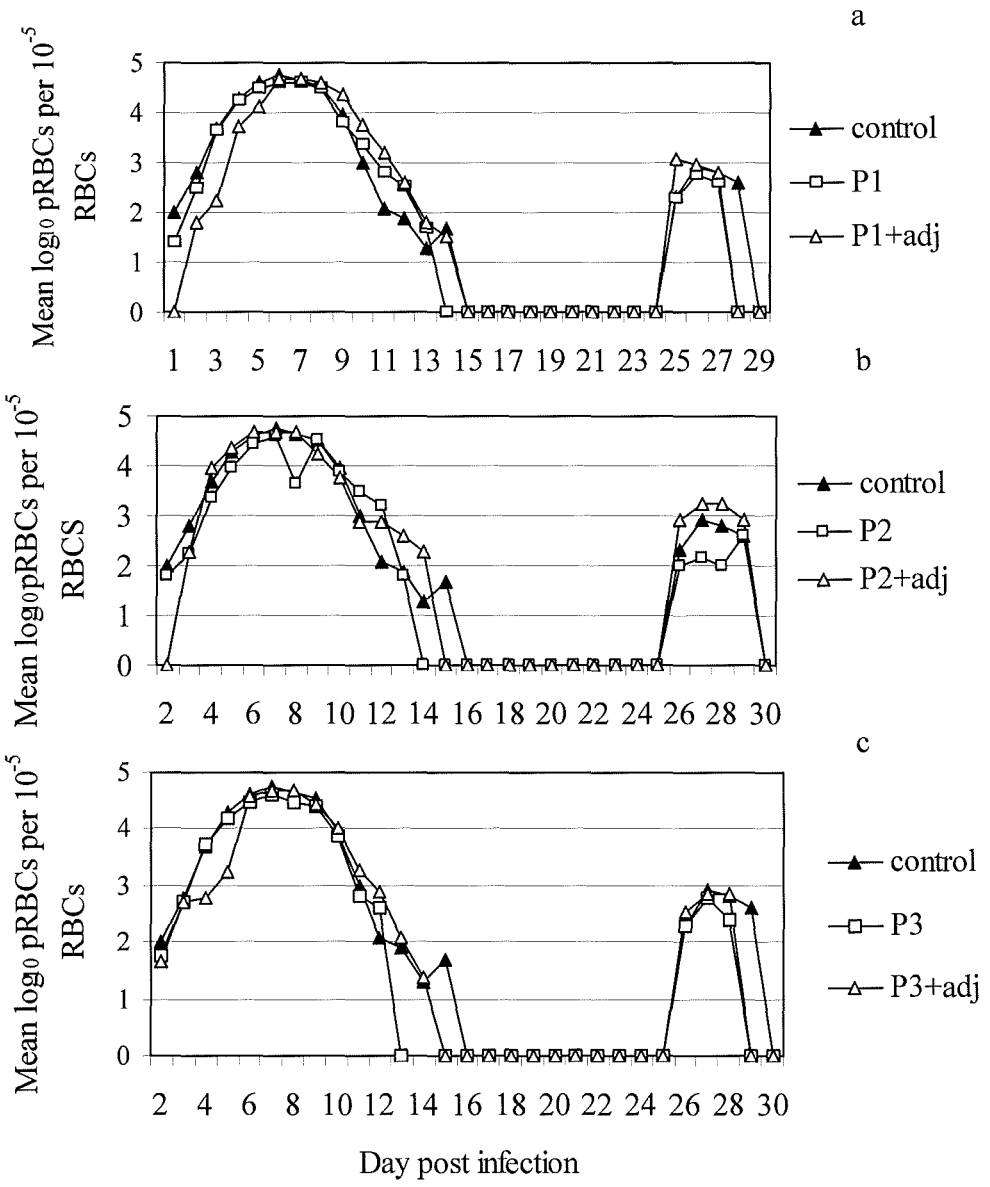


Figure 6.3.The course of infections in mice immunized with three different synthetic peptides based on *clag* gene family. Each group included six mice.

The levels of parasitaemia are shown as mean $\log_{10}$ pRBCs per 10^5 RBCs.

Immunization included initial injection followed by two boostings of the same size at 14 day intervals.

Mice were challenged with $1x10^5$ pRBCs of *P. c. chabaudi* AS i.p. and parasitaemia was monitored for 30 days p.i.

P1+adj, P2+adj, and P3+adj: Mice were immunized with the conjugated peptides to KLH and the adjuvant separately.

P1, P2, and P3: Mice were immunized with conjugated peptides to KLH.

Control: Mice were injected with adjuvant only.

6.2.1.2. Anti-peptide IgG (whole molecule) post-immunization and post-challenge in mice immunized with P1, P2, or P3

Anti-peptide IgG (whole molecule) levels were examined as described in Chapter Two. To determine specific anti-peptide antibody, each peptide was conjugated with BSA and coated on ELISA plates. The IgG levels were significantly higher (Bonferroni test, $P < 0.0001$) in immunized mice compared with that in control mice injected with adjuvant only (Figure 6.4). Mice immunized with P1 and P2 produced higher levels of IgG compared to mice immunized with P3. For example, in mice immunized with P1 or P2 anti-peptide IgG (whole molecule) was significantly higher (Bonferroni test, $P < 0.01$, and $P< 0.05$ respectively) than that in mice immunized with P3. When the results analysed for each time point, it is shown that mice immunized with P1 induced significantly higher levels (Tukey's test, $P < 0.001$ for both day 19 and 21 post-immunization) of anti-peptide IgG (whole molecule) than mice immunized with P2 or P3.

Figure 6.5 shows that anti-peptide IgG (whole molecule) post-challenge increased over the time and it was differed significantly between all tested groups (Two way ANOVA, $P < 0.0001$). In mice immunized with P1 post-challeneg specific anti-peptide IgG (whole molecule) levels were significantly higher (Tukey's test, $P < 0.001$ for all days post-challenge) than that in mice immunized with P2 or P3.

6.2.1.3. Anti-parasite IgG antibody (whole molecule) post-immunization and post-challenge in mice immunized with P1, P2, or P3

To determine that whether anti-peptide antibodies present in sera of immunized mice can react with natural crude parasite antigens, soluble lysate of *P. chabaudi* AS was coated on ELISA plates and reactions between serum from immunized mice and the lysate were examined. This test shows that anti-peptide antibodies, which have been induced following immunization, may recognize similar antigens or epitopes to the synthetic peptide present in the lysate of the parasite. This indicates the presence of cross-reacting epitope reactions. This test was performed for both post-immunization and post-challenge time points.

Figure 6.6 shows that anti-parasite IgG were significantly different (Two way ANOVA, $P < 0.0001$) between all tested groups post-immunization. Anti-parasite IgG in mice immunized with P1 was significantly higher (Tukey's test, $P < 0.001$) compared to anti-parasite IgG levels in mice immunized with P2 or P3. Figure 6.6 shows that after the second boosting no increase was seen in levels of IgG in immunized mice.

Regarding post-challenge, anti-parasite IgG level in mice immunized with P1 was significantly higher (Tukey's test, $P < 0.05$ for days, 7, 14 and 21 in both samples) than that in mice immunized with P2 or P3. However, there were no significant differences for IgG levels between mice immunized with P2 or P3 (Figue 6.7.)

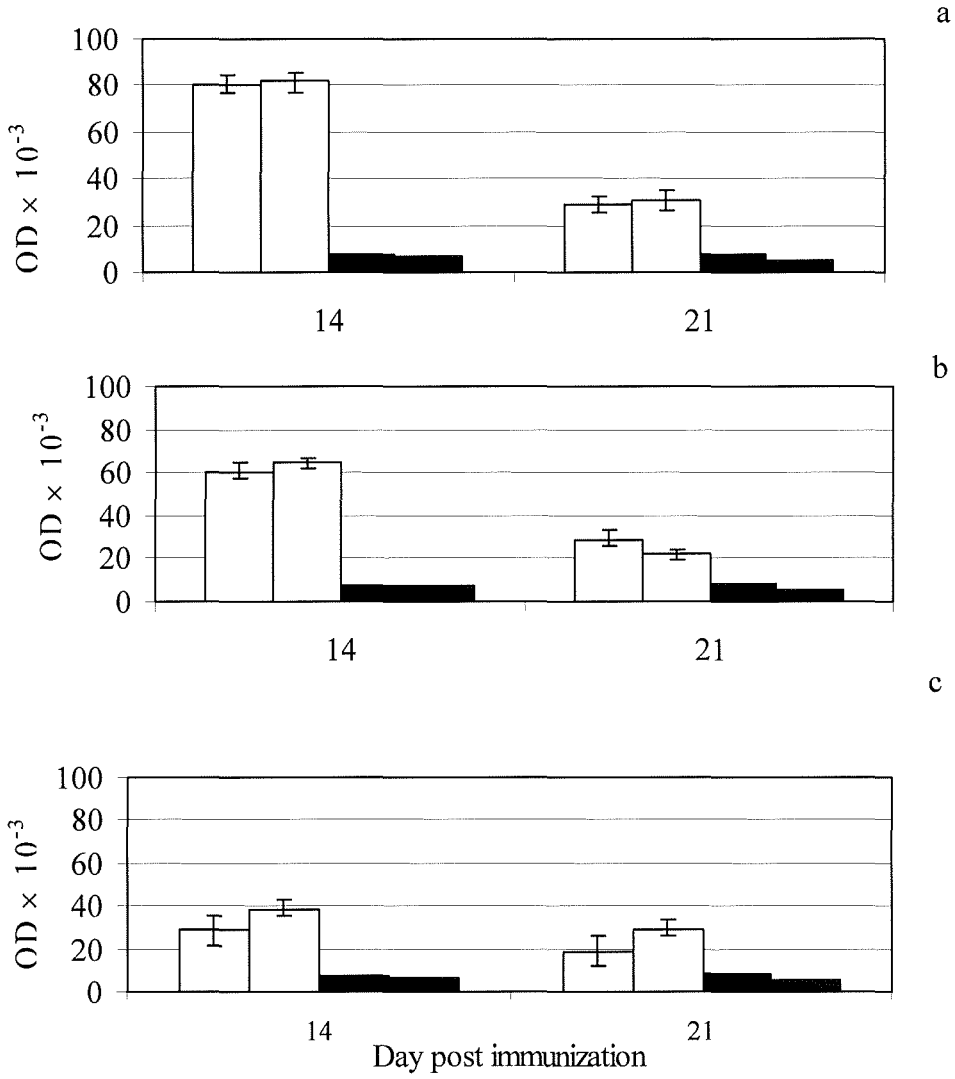


Figure 6.4. Anti-peptide IgG (whole molecule) production post-immunization in mice immunized with synthetic peptides 1, 2, or 3.
An initial injection of emulsion was followed with two boosting injections and anti-peptide IgG was examined. Each peptide was conjugated to BSA for coating on ELISA plate. Sample sera and control serum were reacted to each conjugated peptide coated on ELISA plates. Levels of specific IgG are shown as mean ± SEM. However, ±SEM is not shown when it is too small.

a) Anti-peptide IgG level post-immunization for P1

b) Anti-peptide IgG level post-immunization for P2

c) Anti-peptide IgG level post-immunization for P3

▨ Sera of mice immunized with peptide conjugated to KLH.

□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.

■ Sera of mice injected with adjuvant only.

▩ Sera of naïve mice reacted with the coated peptides.

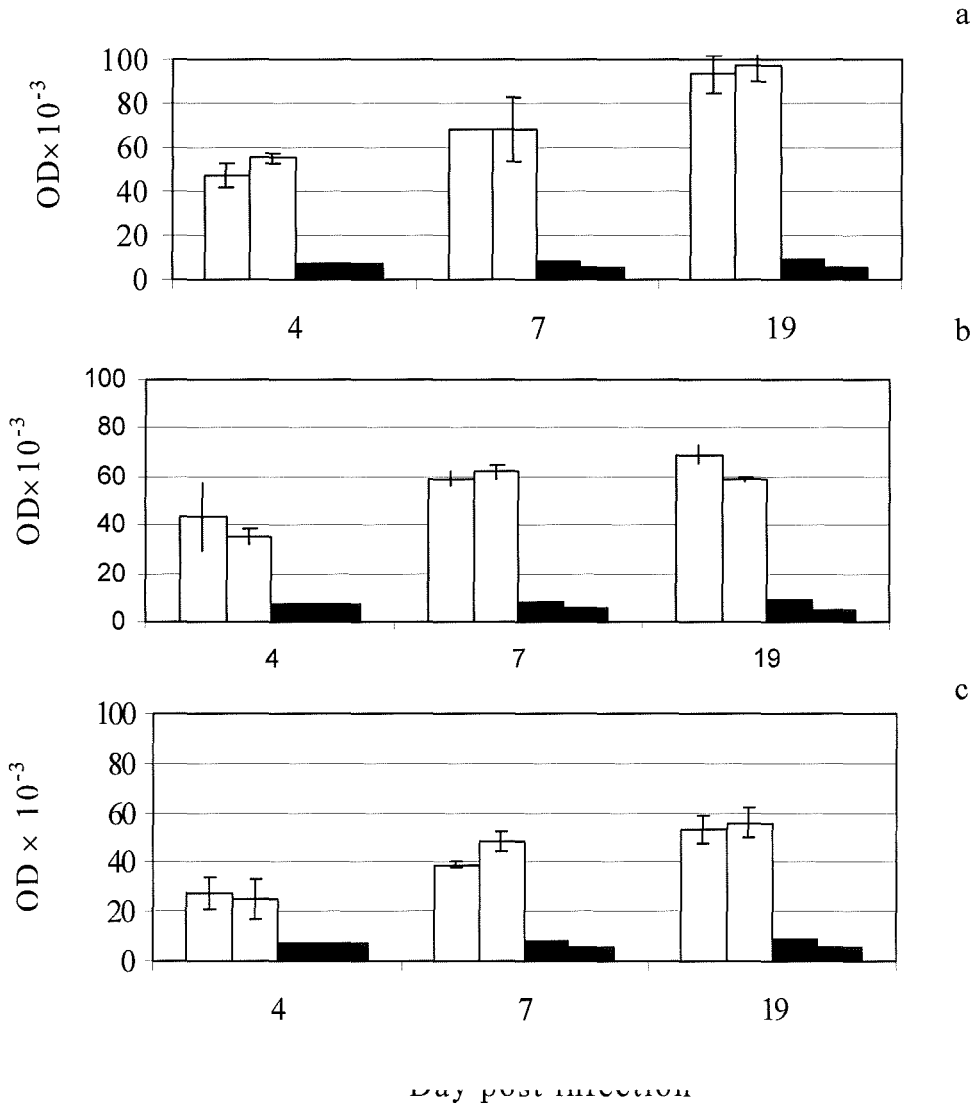


Figure 6.5. Anti-peptide IgG (whole molecule) production post-challenge in mice immunized with synthetic peptides 1, 2, or 3.

An initial injection of emulsion was followed with two boosting injections and anti-peptide IgG was examined. Each peptide was conjugated to BSA for coating on ELISA plate.

Sample sera and control serum were reacted to each conjugated peptide coated in ELISA plate. Levels of specific IgG are shown as mean ± SEM. However, ±SEM is not shown when it is too small.

a) Anti-peptide IgG level post-challenge for P1

b) Anti-peptide IgG level post- challenge for P2

c) Anti-peptide IgG level post- challenge for P3

▨ Sera of mice immunized with peptide conjugated to KLH.

□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.

■ Sera of mice injected with adjuvant only.

▩ Sera of naïve mice reacted with the coated peptide.

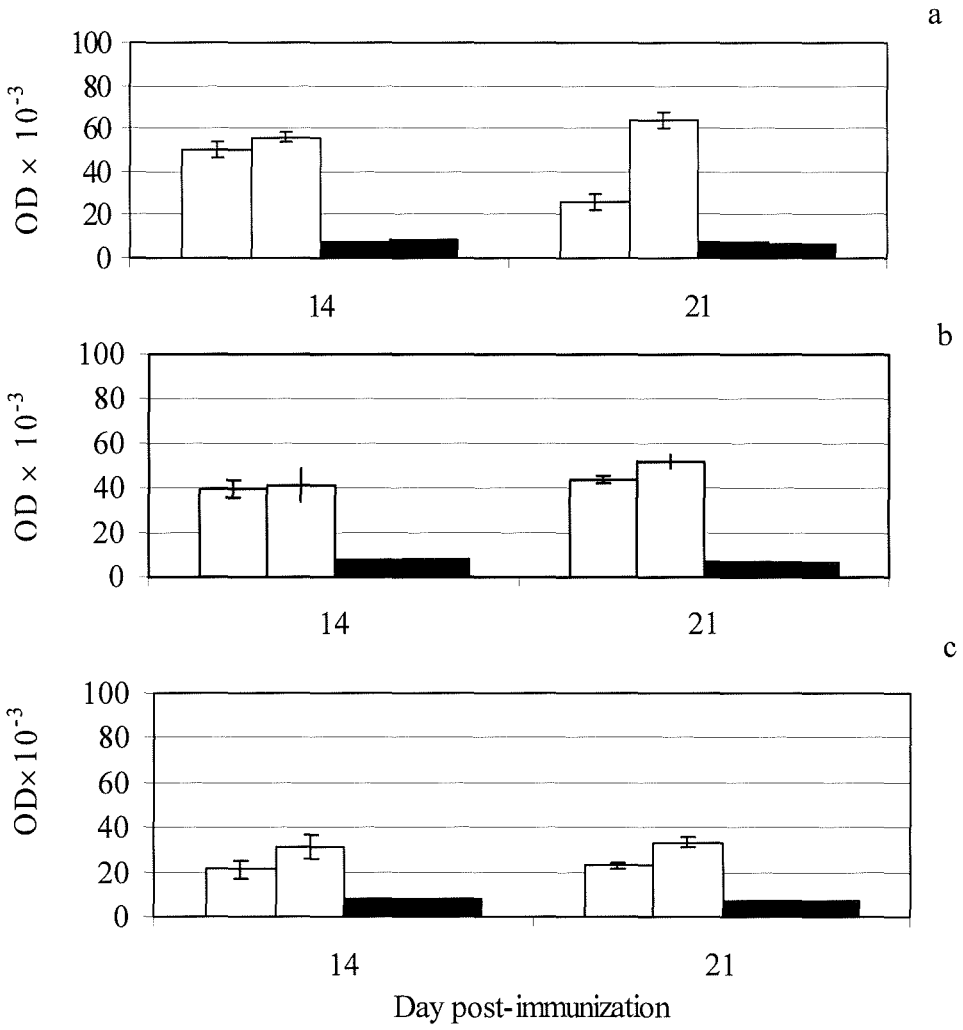


Figure 6.6. Anti-parasite IgG (whole molecule) production post-immunization in mice immunized with the synthetic peptides 1, 2, or 3. An initial injection of emulsion was followed with two boosting injections and anti-parasite IgG was examined. Levels of specific IgG are shown as mean ± SEM. However, ±SEM is not shown when it is too small.
Lysate of pRBCs (obtained from mice infected with *P. c. chabaudi* AS) was coated on the ELISA plates.

a) Anti-parasite IgG level post-immunization for P1

b) Anti-parasite IgG level post-immunization for P2

c) Anti-parasite IgG level post-immunization for P3

▨ Sera of mice immunized with peptide conjugated to KLH.

□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.

■ Sera of mice injected with adjuvant only.

▩ Serum of naïve mice reacted to lysate of infected mice.

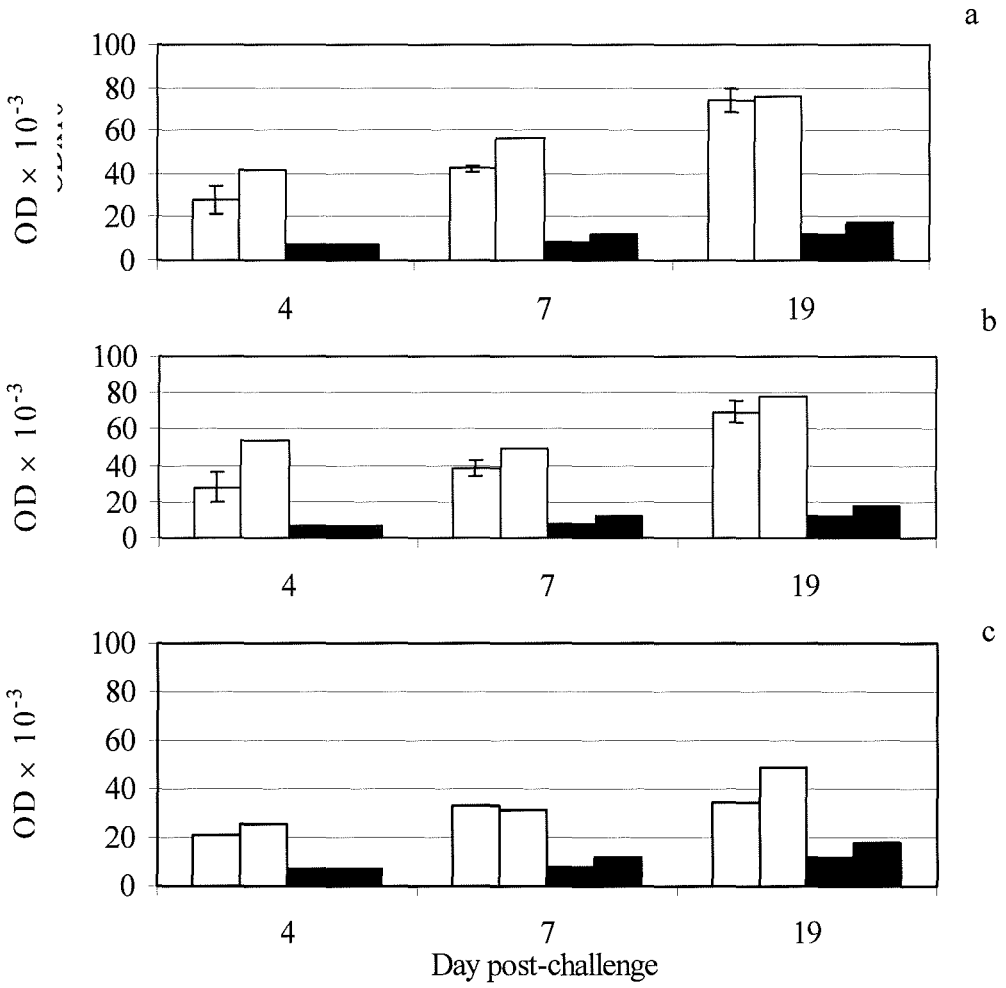


Figure 6.7. Anti-parasite IgG (whole molecule) production post-challenge in mice immunized with the synthetic peptides 1,2,or 3.

An initial injection of emulsion was followed with two boosting injections and anti-parasite IgG was examined. Levels of specific IgG are shown as mean ± SEM. However, ±SEM is not shown when it is too small.

Sample sera and control serum reacted to Lysate of pRBCs which was coated on ELISA plate.

a) Anti-parasite IgG level post-challenge for P1

b) Anti-parasite IgG level challenge for P2

c) Anti-parasite IgG level challenge for P3

▨ Sera of mice immunized with peptide conjugated to KLH.

□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.

■ Sera of mice injected with adjuvant only.

▩ Serum of naïve mice reacted to lysate of infected mice.

6.2.1.4. Anti-parasite IgG1 production post-immunization and post-challenge with P1, P2, or P3

Anti-parasite IgG1 was examined in all immunized mice as described earlier to determine the presence of any cross-epitope reactions. These reactions show that sera from immunized mice contain anti-peptide IgG1 which can recognized epitope(s) which are similar to the synthetic peptide in the soluble lysate of the parasite. Due to limited amounts of the synthetic peptide anti-peptide IgG1 was not examined.

Figure 6.8 shows that there were significant (One-way ANOVA, $P < 0.0001$) differences in anti-parasite IgG1 levels between all groups. Mice immunized with the peptides showed significantly higher (Tukey's test, $P < 0.001$) level of IgG1 than that in control groups including mice given only adjuvant. Mice immunized with P1 induced significantly higher (Tukey's test, $P < 0.001$ for P1 v P2, P1 v P1+adj, P1 v P3+adj and $P < 0.01$ for P1 v P3) levels of IgG1 than mice immunized with P2 or P3. Immunization with P3 or P3+adj induced significantly higher (Tukey's test, $P < 0.001$) level of IgG1 compared to IgG1 levels in mice immunized with P2. However, no increase was observed after the second boost.

Figure 6.9 shows that post-challenge anti-parasite IgG1 increased over time in mice immunized with P1, P2, and P3. However, mice immunized with P1 showed a significantly (Tukey's test, $P < 0.001$) higher level of IgG1 compared to mice immunized with P2 and P3. The increases of IgG1 in mice immunized with P2 or P3 did not significantly differ over time post-challenge.

6.2.1.5. Anti-peptide IgG2a production post-immunization and post-challenge in mice immunized with P1, P2 or P3

Anti-peptide IgG2a antibodies were examined in all immunized mice post-immunization and post-challenge as described in Chapter Two and above.

Figure 6.10 shows that the IgG2a level were significantly different (Two-way ANOVA, $P < 0.0001$) between all tested groups. The highest level of IgG2a was detected for mice immunized with P1 and the lowest level was for mice immunized with P2. The P2 immunization also induced lower

IgG2a levels compared to P3. In other words the levels of IgG2a in mice immunized with P1 or P3 were significantly higher (Tukey's test, $P < 0.001$) than those in mice immunized with P2.

The anti-peptide IgG2a post-challenge also significantly differed (Two-way ANOVA, $P < 0.0014$ for OD and $P < 0.0038$ for time) between all the tested groups (Figure 6.11). Figure 6.11 also shows that immunized mice with the peptides produced significantly higher (Bonferroni test, $P < 0.0001$) levels of IgG2a post-challenge compared to its levels post-immunization. Mice immunized with P1 showed the highest level of IgG2a, particularly on the last sampling day, day 19 post-challenge, which was significantly higher (Tukey's test, $P < 0.001$ and $P, < 0.01$ for P2 and P3 respectively) compared to IgG2a in mice immunized with P2 or P3. No significant differences were observed between mice immunized with P2 and P3.

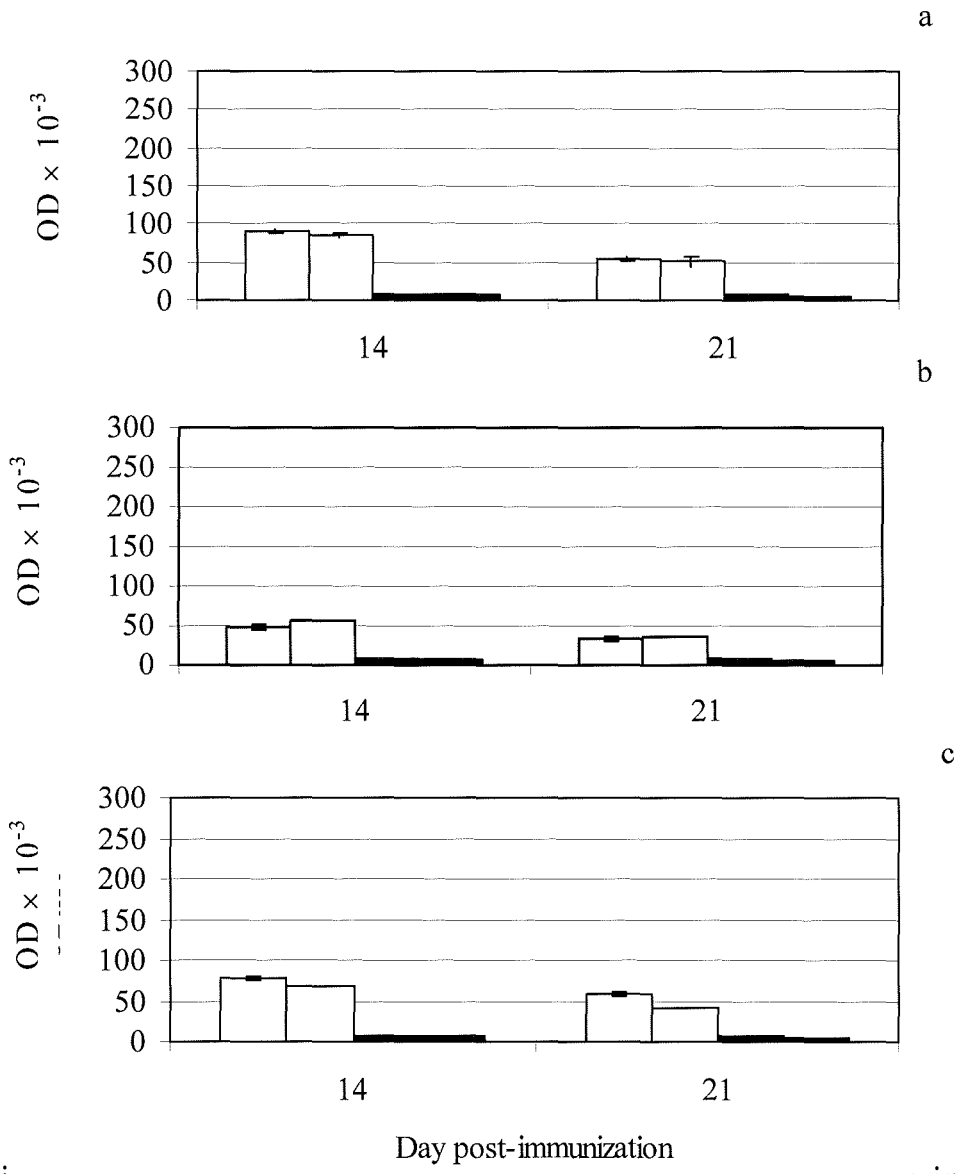
The profile of anti-peptide IgG2a production showed that IgG2a rose after the peak parasitaemia with the highest level of antibody being detected on day 19 post-challenge. The presence of high levels of IgG2a on day 4 and 7 post-challenge indicates presence of a secondary immune response.

6.2.1.6 Anti-parasite IgG2a production post-immunization and post-challenge in mice immunized with P1, P2 or P3

Figures 6.12 shows that sera from mice immunized with P1, P2, or P3 induced anti-parasite IgG2a that indicated these sera recognized cross-epitopes present in the soluble crude antigen of the parasite. The levels of anti-parasite IgG2a post-immunization were significantly (One-way ANOVA $P < 0.0001$) different between all tested groups. These levels of IgG2a in mice immunized with the peptides were significantly higher (Bonferroni test, $P < 0.0001$) than that in the control groups. Anti-parasite IgG2a levels increased over time post-immunization in all the groups. However, the increase in mice immunized with P1 was significantly higher (Tukey's test $P, < 0.00$) compared to mice immunized with P2 or P3.

In respect of post-challenge IgG2a production, figure 6.13 shows that mice immunized with P1, P2, or P3 induced significantly higher (Tukey's test, $P < 0.002$) levels anti-parasite IgG2a compared to IgG2a in controls.

Comparison of IgG2a levels between mice immunized with P1, P2, or P3 showed that there were significantly higher (Tukey's test $P < 0.002$) levels of IgG2a on day 7 post-challenge in mice immunized with P3. These levels of IgG2a in mice immunized with P1 was higher than that in mice immunized with P2, but this was not significantly different. The results also showed that the levels of IgG2a post-challenge were significantly higher (Tukey's test $P < 0.002$) than that in post-immunization particularly on the last sampling day.



Fi n in mice immunized with the synthetic peptides 1,2, or 3.

An initial injection of emulsion was followed with boosting injections and anti-parasite IgG1 was examined. Levels of specific IgG1 are shown as mean ± SEM. However, ±SEM is not shown when it is too small.

Lysate of pRBCs (obtained from mice infected with *P. c. chabaudi* AS) was coated on the ELISA plates.

a) Anti-parasite IgG1 level post-immunization for P1

b) Anti-parasite IgG1 level post-immunization for P2

c) Anti-parasite IgG1 level post-immunization for P3

▨ Sera of mice immunized with peptide conjugated to KLH.

□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.

■ Sera of mice injected with adjuvant oly.

▩ Serum of naïve mice reacted to lysate of infected mice.

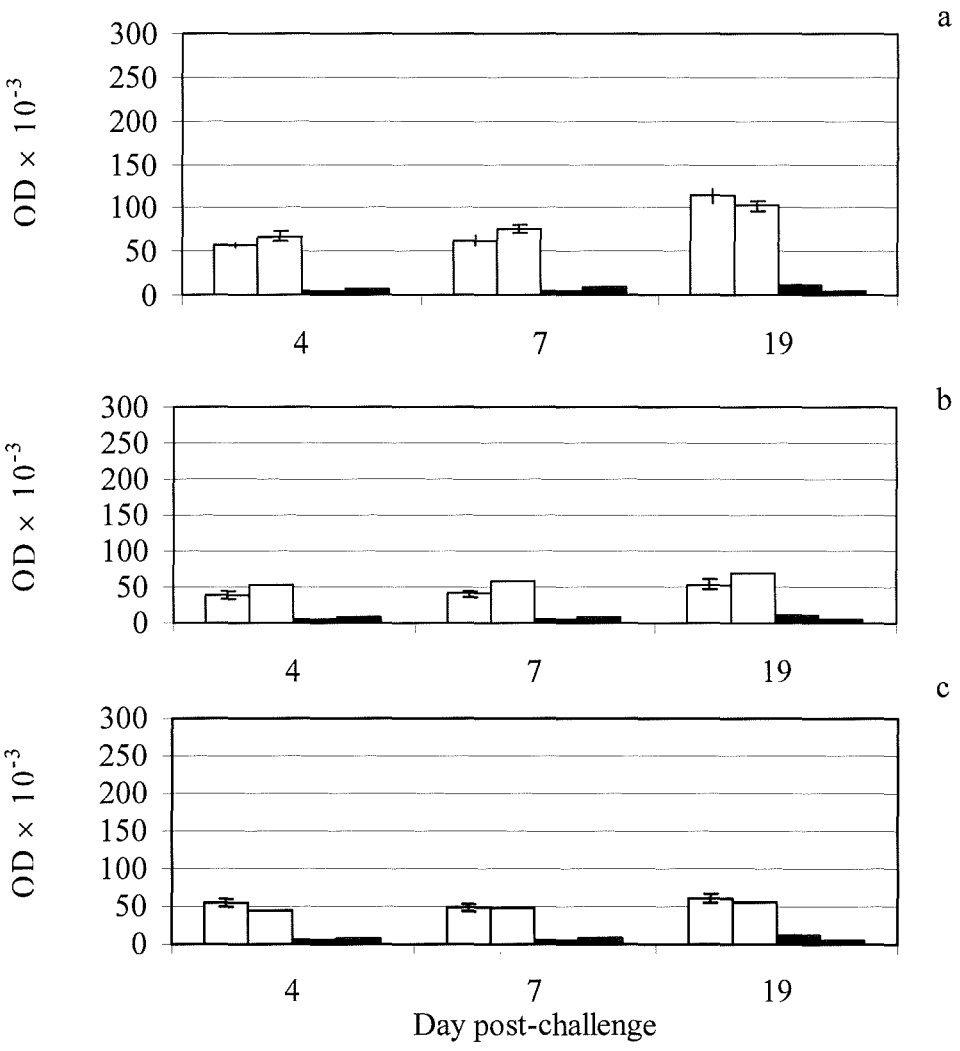


Figure 6.9. Anti-parasite IgG1 production post-challenge in mice immunized with the synthetic peptides 1,2, or 3.

An initial injection of emulsion was followed with boosting injections and anti-parasite IgG1 was examined. Levels of specific IgG1 are shown as mean ± SEM. However, ±SEM is not shown when it is too small.

Lysate of pRBCs (obtained from mice infected with *P. c. chabaudi* AS) was coated on the ELISA plates.

a) Anti-parasite IgG1 level post-immunization for P1

b) Anti-parasite IgG1 level post-immunization for P2

c) Anti-parasite IgG1 level post-immunization for P3

⊡ Sera of mice immunized with peptide conjugated to KLH.

□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.

■ Sera of mice injected with adjuvant only.

▩ Serum of naïve mice reacted to lysate of infected mice.

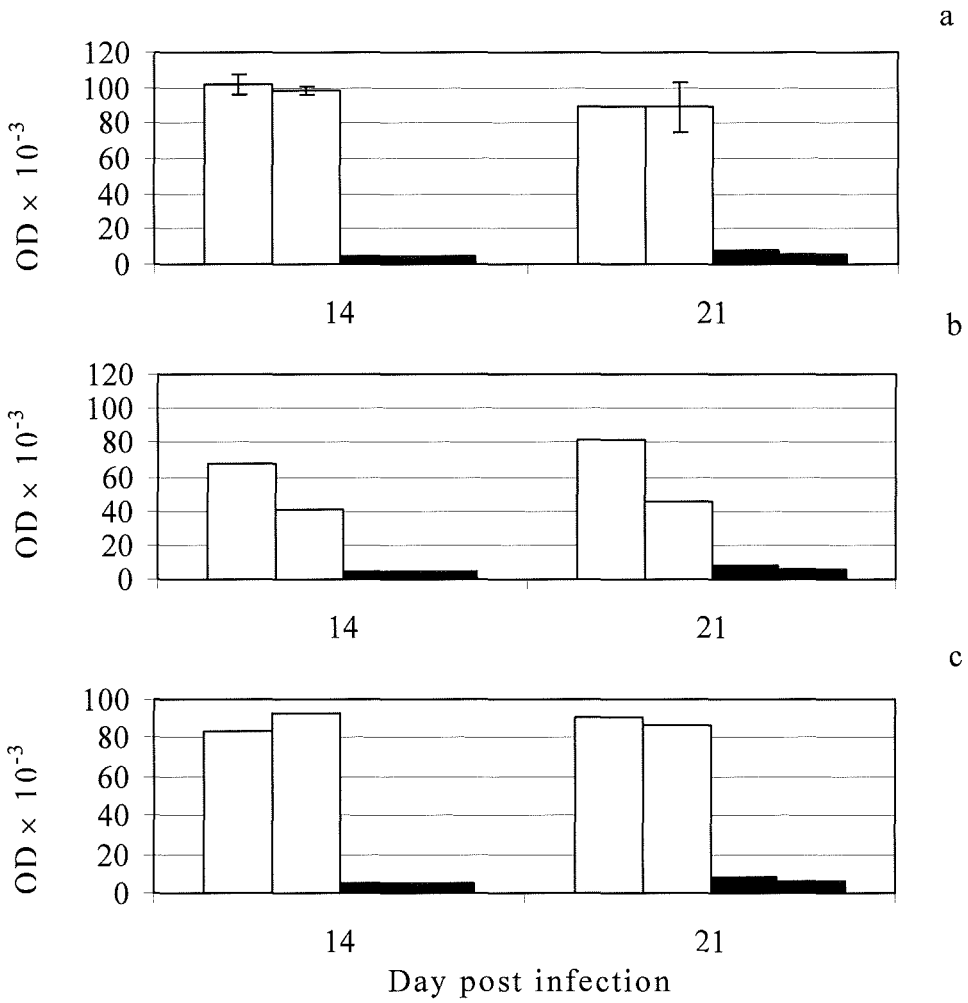


Figure 6.10. Anti-peptide IgG2a production post-immunization in mice immunized with synthetic peptides 1, 2, or 3.

An initial injection of emulsion was followed with two boosting injections and anti-peptide IgG2a was examined. Levels of specific IgG2a are shown as mean ± SEM. However, ±SEM is not shown when it is too small.

Each peptide was conjugated to BSA for coating on ELISA plate. Sample sera and control serum were reacted to each conjugated peptide coated on ELISA plate.

a) Anti-peptide IgG2a level post-immunization for P1.

b) Anti-peptide IgG2a level post-immunization for P2.

c) Anti-peptide IgG2a post-immunization for P3.

▨ Sera of mice immunized with peptide conjugated to KLH.

□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.

■ Sera of mice injected with adjuvant only.

▩ Serum of naïve mice reacted to the coated peptides.

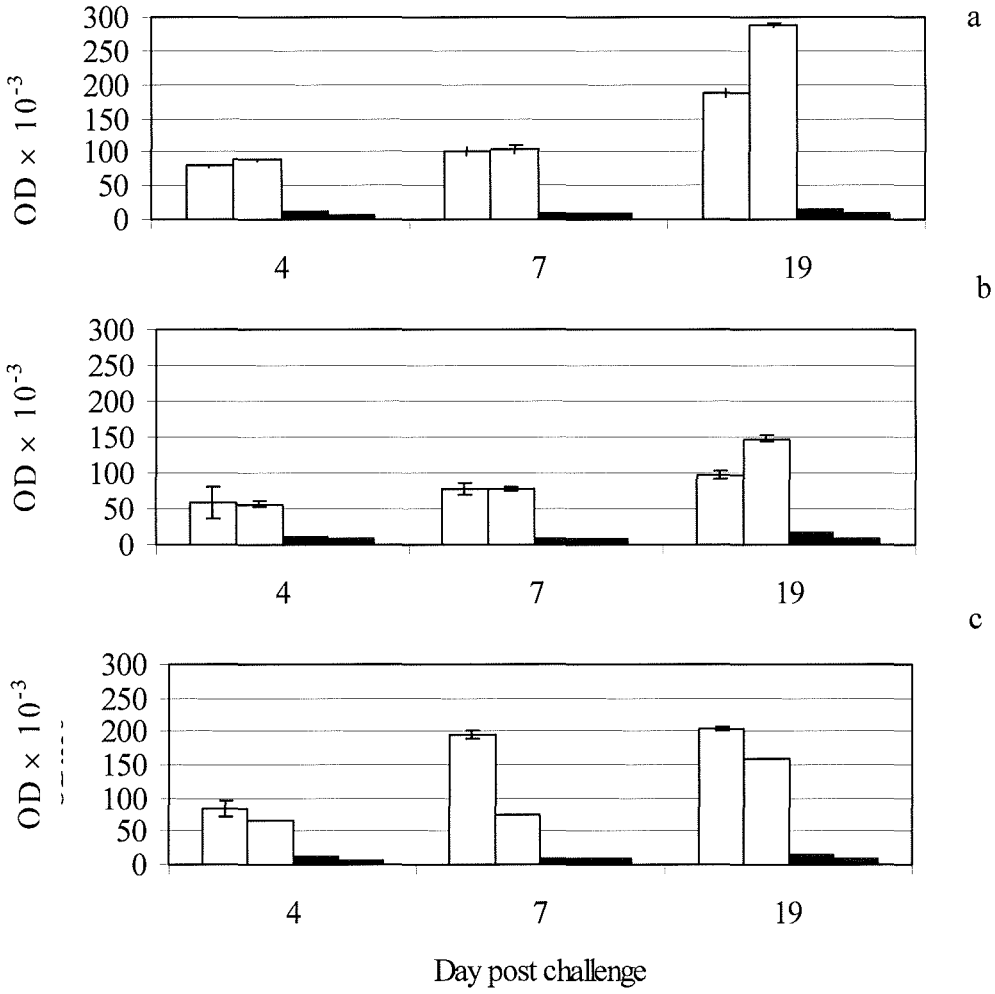


Figure 6.11. Anti-peptide IgG2a production post-challenge in mice immunized with synthetic peptides 1, 2, or 3.

An initial injection of emulsion was followed with two boosting injections and anti-peptide IgG2a was examined. Levels of specific IgG2a are shown as mean ± SEM. However, ±SEM is not shown when it is too small.

Each peptide was conjugated to BSA for coating on ELISA plate. Sample sera and control serum were reacted to each conjugated peptide coated on ELISA plate.

a) Anti-peptide IgG2a level post-challenge for P1

b) Anti-peptide IgG2a level challenge for P2

c) Anti-peptide IgG2a level challenge for P3

▨ Sera of mice immunized with peptide conjugated to KLH.

□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.

■ Sera of mice injected with adjuvant only.

▩ Serum of naïve mice reacted to the coated peptides.

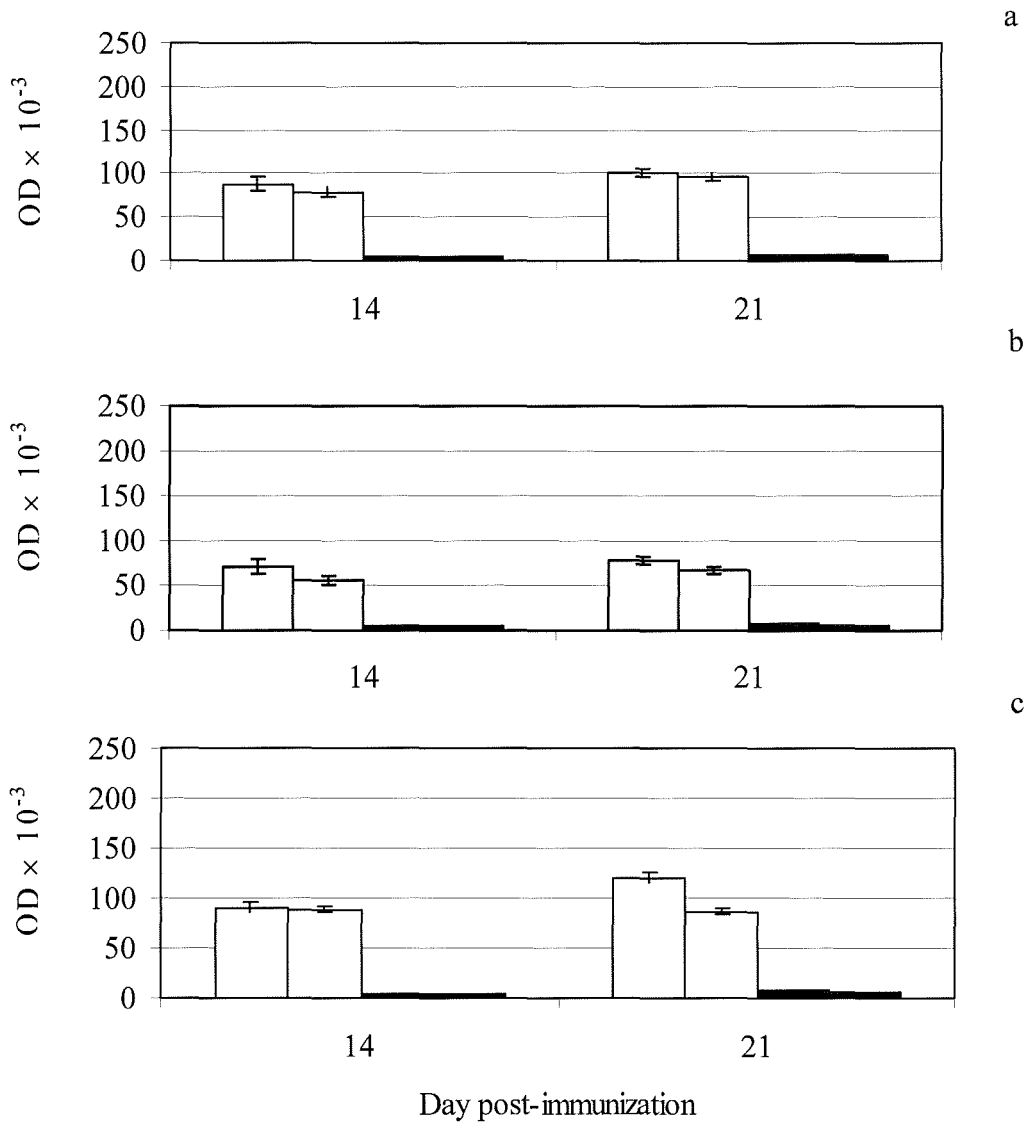


Figure 6.12. Anti-parasite IgG2a production post-immunization in mice immunized with synthetic peptides 1, 2, or 3.

An initial injection of emulsion was followed with boosting injections and anti-parasite IgG2a was examined. Lysate of *P. chabaudi* AS was coated on ELISA plate. Levels of specific IgG2a are shown as mean ± SEM. However, ±SEM is not shown when it is too small.

a) Anti-parasite IgG2a level post-challenge for P1

b) Anti-parasite IgG2a level challenge for P2

c) Anti-parasite IgG2a level challenge for P3

▨ Sera of mice immunized with peptide conjugated to KLH.

□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.

■ Sera of mice injected with adjuvant only.

▩ Serum of naïve mice reacted to lysate of infected mice.

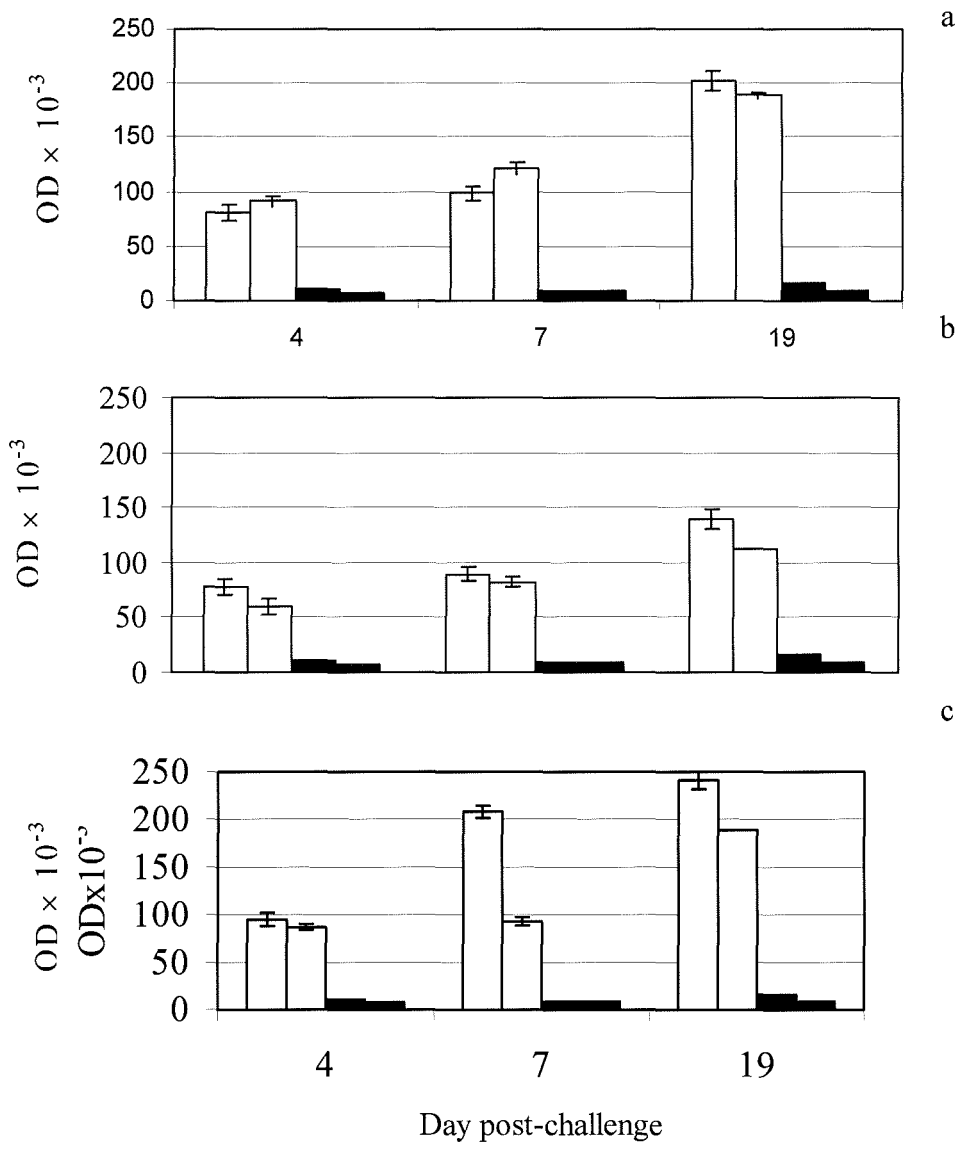


Figure 6.13. Anti-parasite IgG2a production post-challenge in mice immunized with synthetic peptides 1, 2, or 3 related to the *clag* genes. An initial injection of emulsion was followed with boosting injections and anti-parasite IgG2a was examined. Lysate of *P. chabaudi* AS was coated on ELISA plate.

Levels of specific IgG2a are shown as mean ± SEM. However, ±SEM is not shown when it is too small.

a) Anti-parasite IgG2a level post-challenge for P1

b) Anti-parasite IgG2a level challenge for P2

c) Anti-parasite IgG2a level challenge for P3

▨ Sera of mice immunized with peptide conjugated to KLH.

□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.

■ Sera of mice injected with adjuvant only.

▩ Serum of naïve mice reacted to lysate of infected mice.

6.2.2. Immunization of NIH mice with P4 or P5.

As described above the same set of experiments were carried out to determine the course of infection and antibody production in mice immunized with P4 and P5 which are synthesized basde on the *cir* gene family in *P. chabaudi* AS.

6.2.2.1. The course of infection in mice immunized with P4 or P5 (*cir* gene products)

Mice were separately immunized with two synthetic peptides, P4 or P5. The peak parasitaemia in the control group was 68.36%. The peak parasitaemias were 65.7% and 59.08% in mice immunized with P4 plus adjuvant and P4 without adjuvant respectively. For mice immunized with P5 plus adjuvant the peak was 57.23% and for mice immunized with P5 only it was 61.8% (Figures 6.14 and 6.15). Immunization of mice with the peptides resulted in statistically significant lower peak parasitaemias (Tukey's test, $P < 0.001$ for P4 vs control, P5 and P5+ad vs control and $P < 0.05$ for P4+adj vs control) compared to the control group. The results showed that mice immunized with P5 plus adjuvant had a significantly lower (Tukey's test, $P < 0.001$ for all groups) peak parasitaemia compared to the other immunized groups.

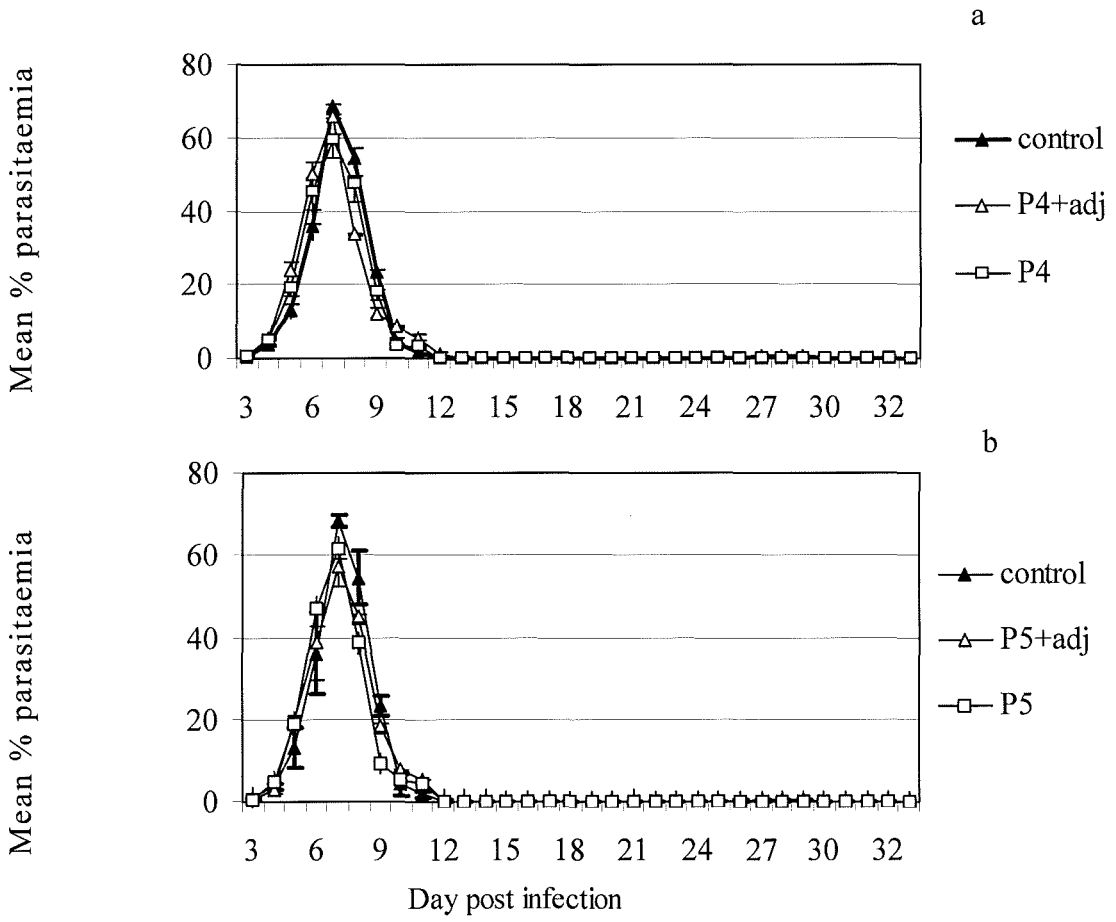


Figure 6.14. The course of infections in mice immunized with two different synthetic peptides based on *cir* gene family. Each group included 6 mice. The levels of parasitaemia are shown as the mean % ± SEM for each time point. However, ±SEM is not shown when it is too small.
Immunization included initial injection followed by three boosting injections of the same size at 14 day intervals.
Mice were challenged with $1x10^5$ pRBCs of *P. c. chabaudi* AS i.p. and parasitaemia was monitored for 32 days p.i.
P4+adj, and P5+adj: Mice were immunized with conjugated peptides 4, or 5 to KLH and the adjuvant respectively.
P4, and P5: Mice were immunized with conjugated peptides 4, or 5 to KLH.
Control: Mice were injected with adjuvant only.

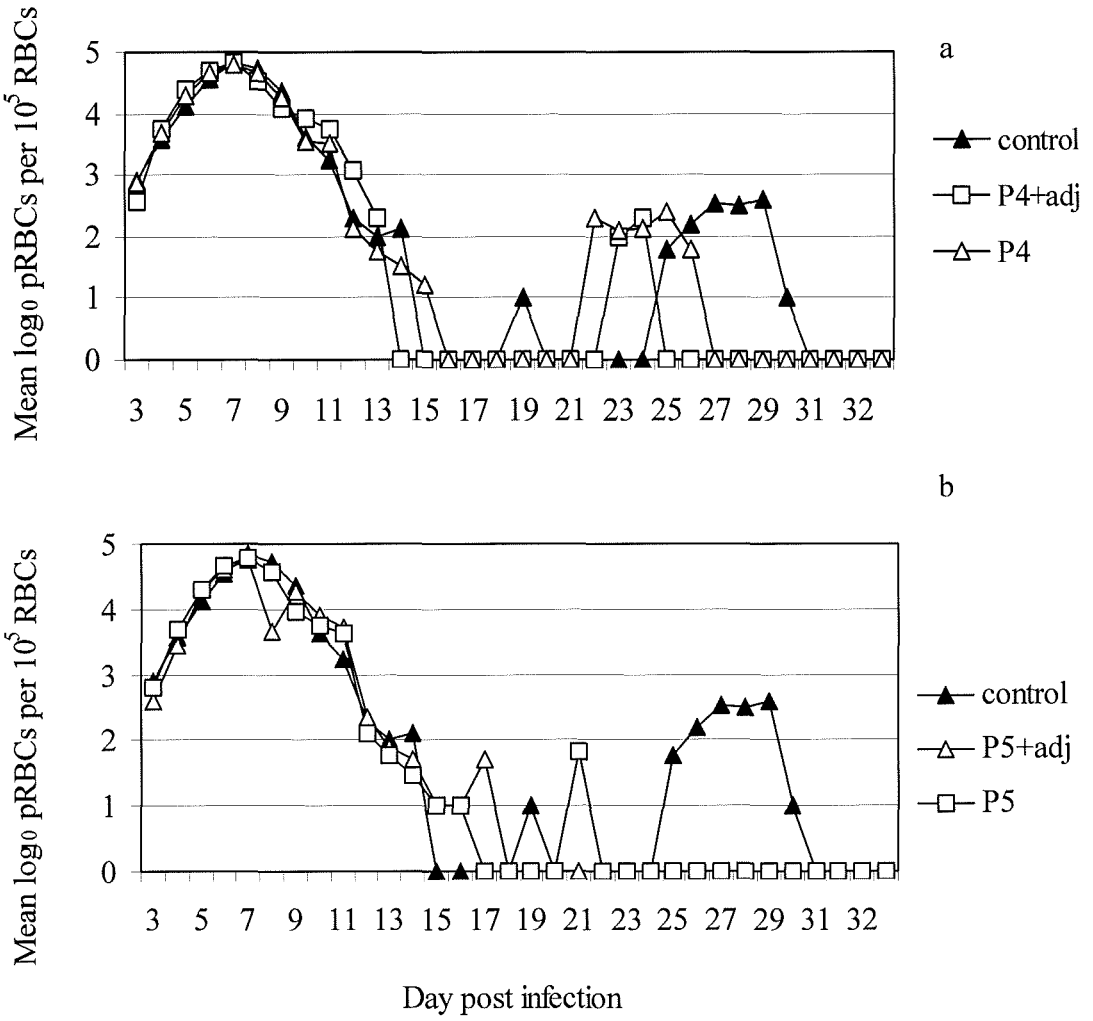


Figure 6.15. The course of infections in mice immunized with two different synthetic peptides based on *cir* gene family. Each group includes 6 mice. The levels of parasitaemia are shown as mean $\log_{10}$ pRBCs per 10^5 RBCs. Immunization was included initial injection followed by three boosting injections at the same size by 14 days intervals.

Mice were challenged with $1x10^5$ pRBCs of *P. c. chabaudi* AS i.p. and parasitaemia was monitored for 32 days p.i.

P4+adj, and P5+adj: Mice were immunized with conjugated peptides 4, or 5 to KLH plus the adjuvant.

P4, and P5: Mice were immunized with conjugated peptides 4, or 5 to KLH.

Control: Mice were injected with adjuvant only.

6.2.2.2. Anti-peptide IgG (whole molecule) production post-immunization and post-challenge in mice immunized with P4, or P5

Specific anti-peptide IgG (whole molecule) was evaluated in mice immunized with P4 and P5 when conjugated peptides were coated on ELISA plates.

The results demonstrated (Figure 6.16) that in mice immunized with P4 or P5 the anti-peptide IgG (whole molecule) level post-immunization increased over time. Two-way ANOVA test showed that there were significant differences ($P < 0.0001$) for IgG (whole molecule) between all tested groups. The levels of anti-peptide IgG were not high at the first sampling days, day 14 and 21 post-immunization, but these levels increased later during the experimental period. Mice immunized with P5 showed significantly higher (Tukey's test, $P < 0.05$) levels of anti-peptide IgG (whole molecule) compared to mice immunized with P4 and the highest level was measured on the last sampling days, day 28 and 36 post-immunization. The profile of total anti-peptide IgG production post-immunization with P4 and P5 was in agreement with the profile of the total anti-peptide IgG production in mice immunized with P1, P2, or P3 which generally shows that the IgG1 level rose later after immunization.

Determination of the levels of anti-peptide IgG (whole molecule) post-challenge showed (Figure 6.17) significantly higher (Tukey's test, $P < 0.01$) levels of IgG in the first sampling day compared to its level at the same time point post-immunization. This indicates the presence of a stronger secondary antibody response compared to its level before the challenge. There were also significantly different (One-way ANOVA, P, 0.005) levels of anti-peptide IgG between all groups post-challenge. Although these levels of IgG (whole molecule) did not increase enormously thereafter, there were significantly higher (Tukey's test, $P < 0.005$) levels of anti-peptide IgG in the last sampling day compared to the first sampling day. Mice immunized with P5 also showed significantly higher (Tukey's test, $P < 0.001$ for both time points) levels of anti-peptide IgG compared to anti-peptide IgG levels in mice immunized with P4 at the first and the last sampling days, day 4 and 19 post-challenge.

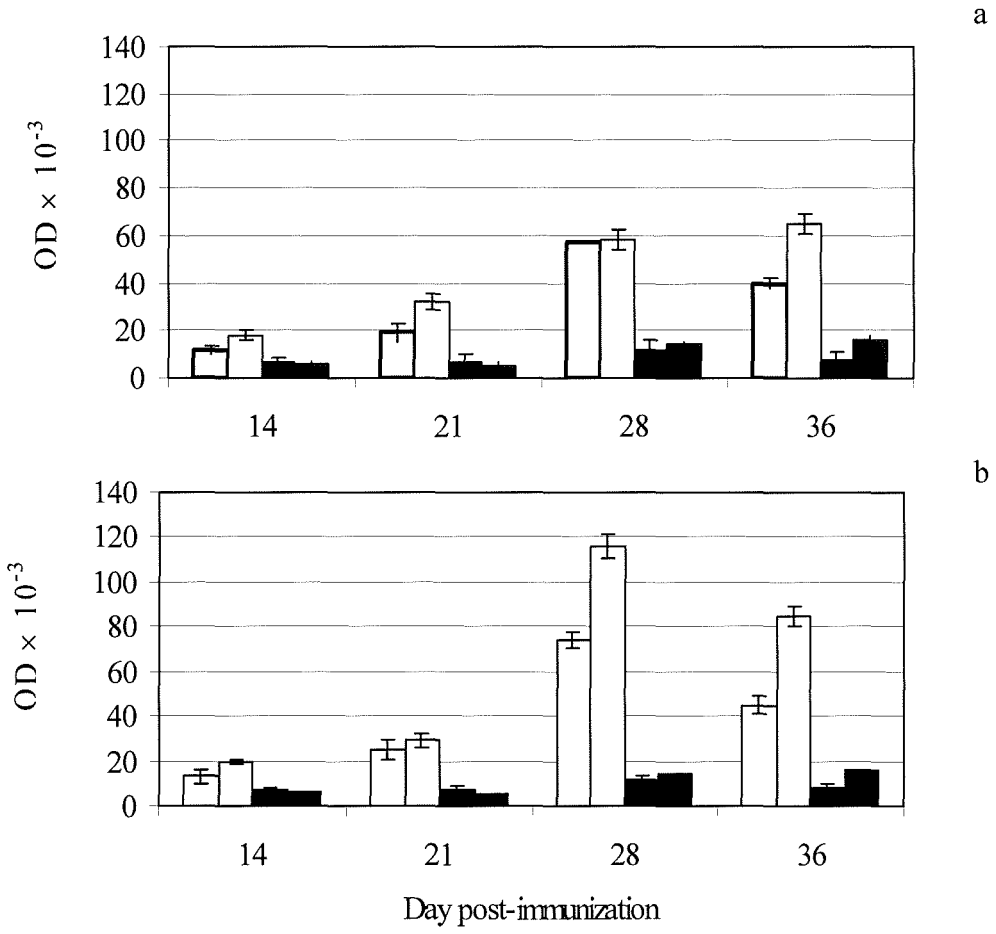


Figure 6.16. Anti-peptide IgG (whole molecule) production post-immunization in mice immunized with synthetic peptides 4 or 5.
An initial injection of emulsion was followed with three boosting injections and specific anti-peptide IgG was examined. Levels of specific IgG are shown as mean ± SEM. However, ±SEM is not shown when it is too small. Each peptide was conjugated to BSA and coated on ELISA plate.
a) Anti-peptide IgG level post-immunization for P4.
b) Anti-peptide IgG level post-immunization for P5.
▨ Sera of mice immunized with peptide conjugated to KLH.
□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.
■ Sera of mice injected with adjuvant only.
▩ Serum of naïve mice reacted to the coated peptides.

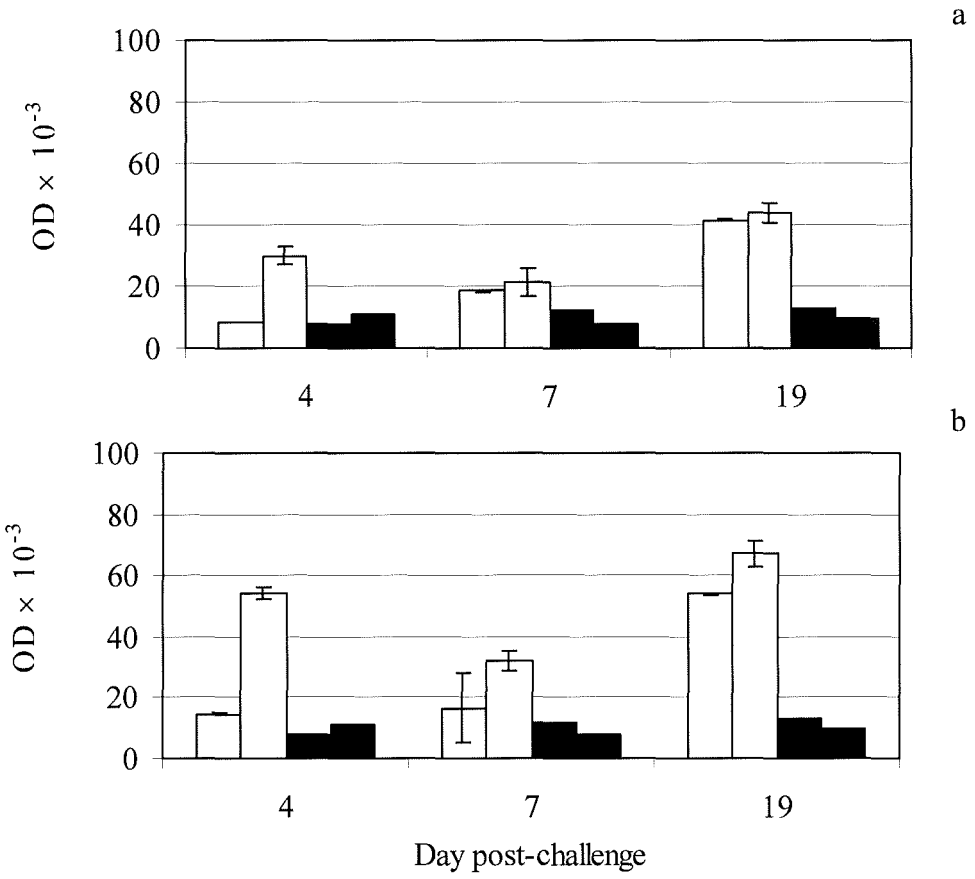


Figure 6.17. Anti-peptide IgG (whole molecule) production post-challenge in mice immunized with synthetic peptides 4 or 5.

An initial injection of emulsion was followed with three boosting injections and specific anti-peptide IgG was examined. Levels of specific IgG are shown as mean ± SEM. Each peptide was conjugated to BSA and coated on ELISA plate. However, ±SEM is not shown when it is too small.

a) Anti-peptide IgG level post-challenge for P4.

b) Anti-peptide IgG level post-challenge for P5

▨ Sera of mice immunized with peptide conjugated to KLH.

□ Sera of mice immunized with peptide conjugated with KLH and adjuvant.

■ Sera of mice injected with adjuvant only.

▩ Serum of naïve mice reacted to the coated peptides.

6.2.2.3. Anti-parasite IgG antibody (whole molecule) post-immunization and post-challenge in mice immunized with P4 or P5

To determine any reaction between sera from immunized mice and soluble lysate of the parasite total anti-parasite IgG levels were measured in mice immunized with P4 and P5 post-immunization and post-challenge. The results showed the similar profiles of increasing anti-parasite IgG levels over the time post-immunization as seen in mice immunized with P1, P2, or P3 (Figures 6.6 and 6.18). Figure 6.18 also shows that the levels of IgG were significantly different (Two-way ANOVA, $P < 0.0001$ for OD and $P < 0.002$ for time) between all the groups. The level of anti-parasite IgG on the last sampling day, day 36, was significantly higher (Tukey's test, $P < 0.001$) compared to its levels on the first two sampling days. In general, the results did not show significant differences on the last sampling day between mice immunized with P4 or P5. However, these results confirmed that the antibodies produced in immunized mice could recognize native antigens from lysate of the parasite.

The levels of anti-parasite IgG (whole molecule) in mice immunized with P4 and P5 were measured post-challenge when lysate of the *P.chabaudi* AS was coated on ELISA plates (Figure 6.19). The levels of anti-parasite IgG were significantly different (Two-way ANOVA, $P < 0.0001$for OD and $P < 0.0058$ for time) between all tested groups. The results showed that mice immunized with P4 produced significantly higher (Tukey's test, $P < 0.001$ for days 4 and 7 and $P < 0.01$ for day 19 post-challenge) IgG (whole molecule) levels compared to mice immunized with P5 at all time points.

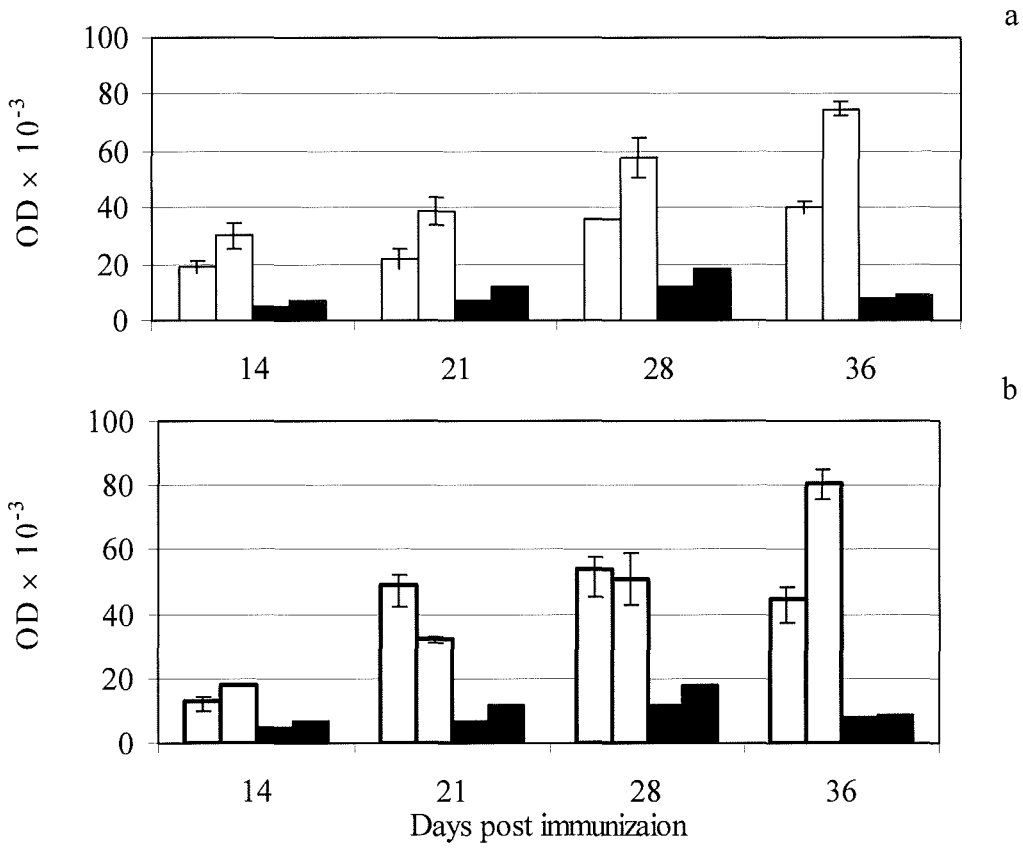


Figure 6.18. Anti-parasite IgG (whole molecule) production post-immunization in mice immunized with the synthetic peptides 4 or 5.
An initial injection of emulsion was followed with three boosting injections and anti-peptide IgG was examined. Levels of specific IgG are shown as mean ± SEM. However, ±SEM is not shown when it is too small.
Sample sera and control serum were reacted to Lysate of pRBCs which was coated on ELISA plate.
a) Anti-parasite IgG level post-immunization for P4.
b) Anti-parasite IgG level post-immunization for P5.
▨ Sera of mice immunized with peptide conjugated to KLH.
□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.
■ Sera of mice injected with adjuvant only.
▩ Serum of naïve mice reacted to lysate of infected mice.

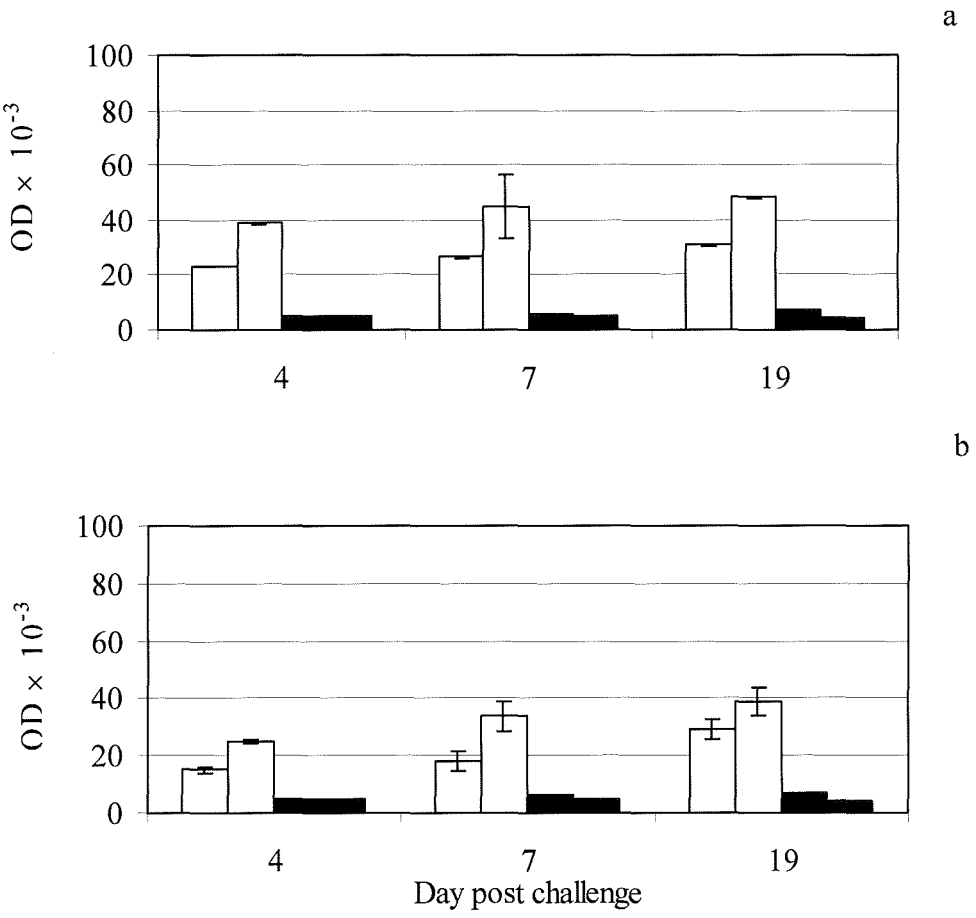


Figure 6.19. Anti-parasite IgG (whole molecule) production post-challenge in mice immunized with the synthetic peptides 4 or 5.

An initial injection of emulsion was followed with three boosting injections and anti-peptide IgG was examined. Levels of specific IgG are shown as mean ± SEM. However, ±SEM is not shown when it is too small.

Sample sera and control serum were reacted to Lysate of pRBCs which was coated on ELISA plate.

a) Anti-parasite IgG level post-challenge for P4.

b) Anti-parasite IgG level post challenge for P5.

▨ Sera of mice immunized with peptide conjugated to KLH.

□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.

■ Sera of mice injected with adjuvant only.

▩ Serum of naïve mice reacted to lysate of infected serum.

6.2.2.4. Anti-parasite IgG1 production post-immunization and post-challenge in mice immunized with P4 or P5

The levels of anti- parasite IgG1 were significantly different (Two-way ANOVA, $P < 0.0002$) between all the groups and increased over time post-immunization (Figure 6.20). Mice immunized with P4 or P5 produced significantly higher (Tukey's test, $P < 0.005$ for P4 v adj, P5 v adj, and P4 and P5 v mice non-immunized) levels of IgG1 compared to the control group.

Regarding to post-challenge figure 6.21 shows that there were significant (Two-way ANOVA $P < 0.014$ for OD and $P < 0.0024$ for time) differences for anti-parasite IgG1 between all the groups. Mice immunized with either P4 or P5 induced significantly higher (Tukey's test, $P < 0.001$) levels of IgG1 than that in non-immunized mice or mice given only adjuvant. Figure 6.22 also showed there were significantly higher (Tukey's test $P < 0.01$ for P4 v P5, $P < 0.0.001$ for P4 v P5+adj, and P4+adj v P5+adj) levels of IgG1 in mice immunized with P5 compared to mice immunized with P4 post-challenge.

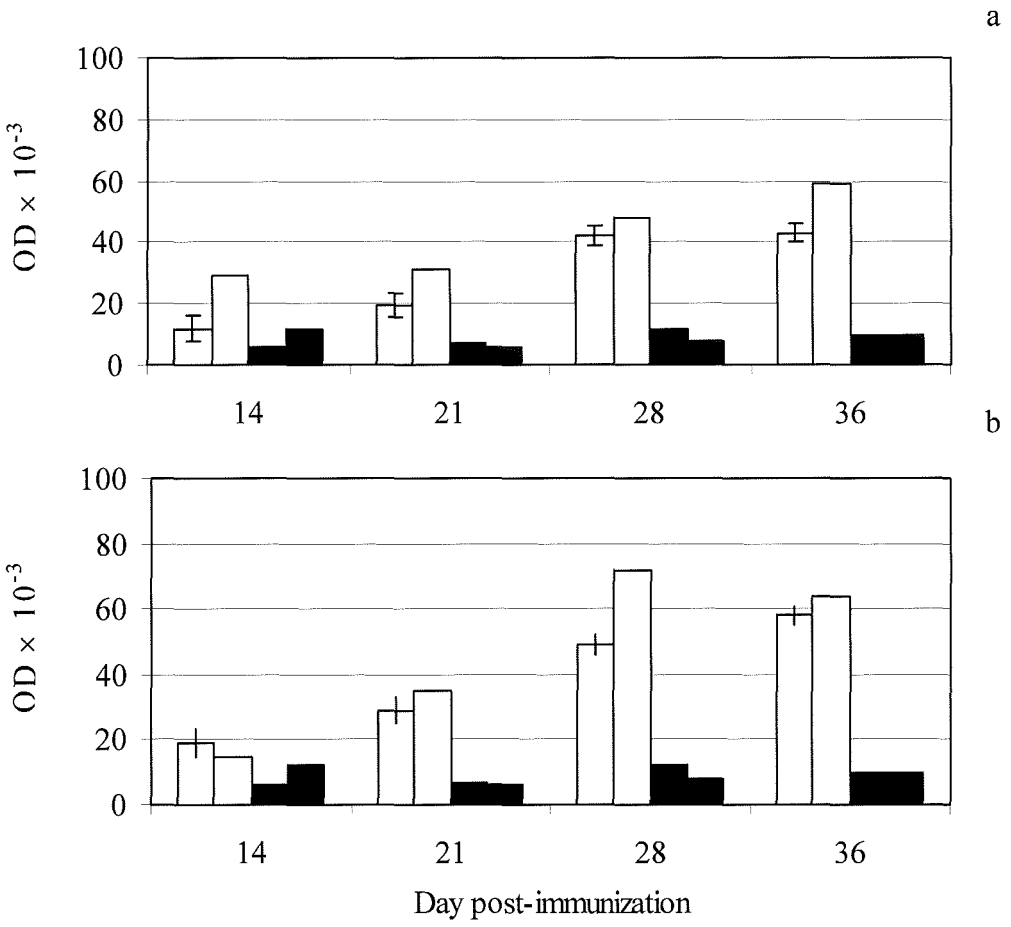


Figure 6.20. Anti-parasite IgG1 production post-immunization in mice immunized with the synthetic peptides 4 or 5 related to the *cir* genes.
An initial injection of emulsion was followed with three boosting injections and anti-peptide IgG1 was examined.
Levels of specific IgG1 are shown as mean ± SEM. However, ±SEM is not shown when it is too small.
Sample sera and control serum were reacted to Lysate of pRBCs which was coated on ELISA plate.
a) Anti-parasite IgG1 level post-challenge for P4.
b) Anti-parasite IgG1 level post challenge for P5.
▨ Sera of mice immunized with peptide conjugated to KLH.
□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.
■ Sera of mice injected adjuvant only.
▩ Serum of naïve mice reacted to lysate of infected mice.

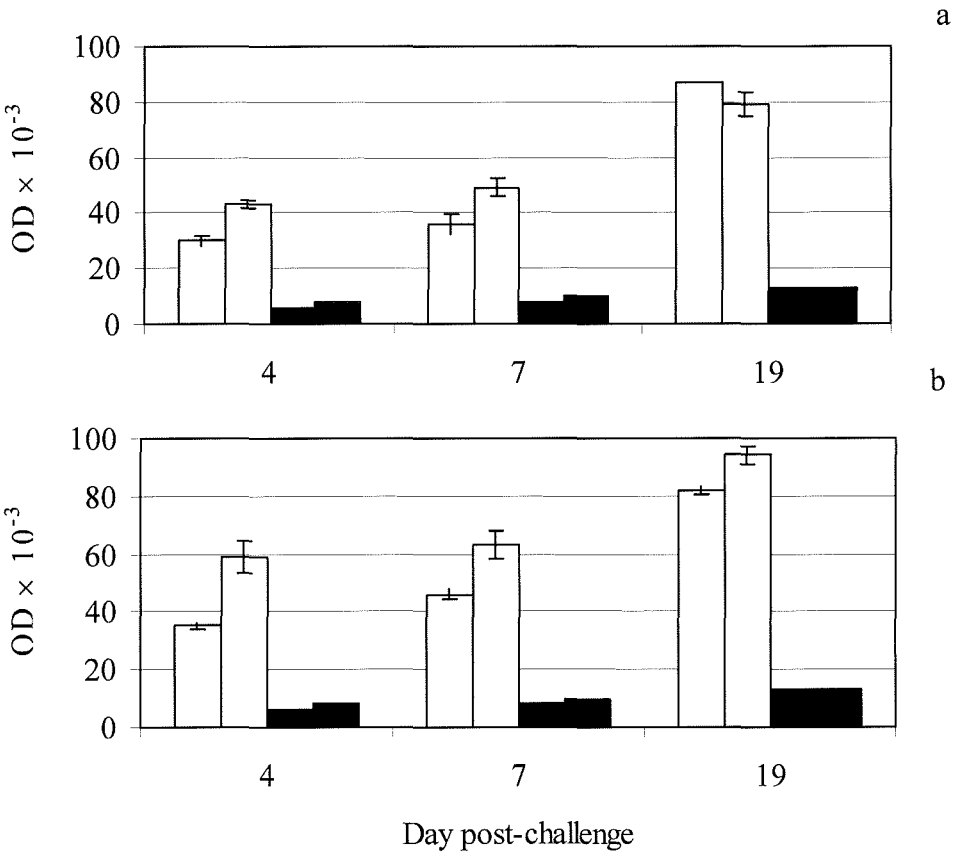


Figure 6.21. Anti-parasite IgG1 production post-challenge in mice immunized with the synthetic peptides 4 or 5 related to the *cir* genes.
An initial injection of emulsion was followed with three boosting injections and anti-peptide IgG1 was examined. Levels of specific IgG1 are shown as mean ± SEM. However, ±SEM is not shown when it is too small.
Sample sera and control serum were reacted to Lysate of pRBCs which was coated on ELISA plate.

a) Anti-parasite IgG1 level post-challenge for P4.

b) Anti-parasite IgG1 level post challenge for P5.

▨ Sera of mice immunized with peptide conjugated to KLH.

□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.

■ Sera of mice injected with adjuvant only.

▩ Serum of naïve mice reacted to lysate of infected mice.

6.2.2.5. Anti-peptide IgG2a production post-immunization and post-challenge in mice immunized with P4 or P5

The levels of anti-peptide IgG2a were determined in mice immunized with P4 or P5 as described above. Figure 6.22 shows that anti-peptide IgG2a increased over time during the observation period. The results showed that there is an enormous production of anti-peptide IgG2a in mice immunized with P4 or P5 over the observation period post-immunization. This shows that immunization with P4 or P5 induced higher production of anti-peptide IgG2a compared to all other immunized groups. The levels of IgG2a were significantly different (Two-way ANOVA, $P < 0.0001$ for OD and $P < 0.0368$ for time) between all tested groups. The results also demonstrated that anti-peptide IgG2a levels in immunized mice were significantly higher (Tukey's test, $P < 0.001$ for all days post-immunization) than that in control groups at all time points.

The Figure 6.23 shows the levels of anti-peptide IgG2a post-challenge in mice immunized with P4 or P5. The results indicated significantly different (Two-way ANOVA, $P < 0.015$ for OD only) levels of anti-peptide IgG2a between all tested groups. The anti-peptide IgG2a levels in immunized mice were significantly higher (Tukey's test, $P < 0.0001$ for all time points) than that in control groups. Mice immunized with P5 produced significantly higher (Tukey's test, $P < 0.001$ for all time points) levels of anti-peptide IgG2a levels compared to mice immunized with P4.

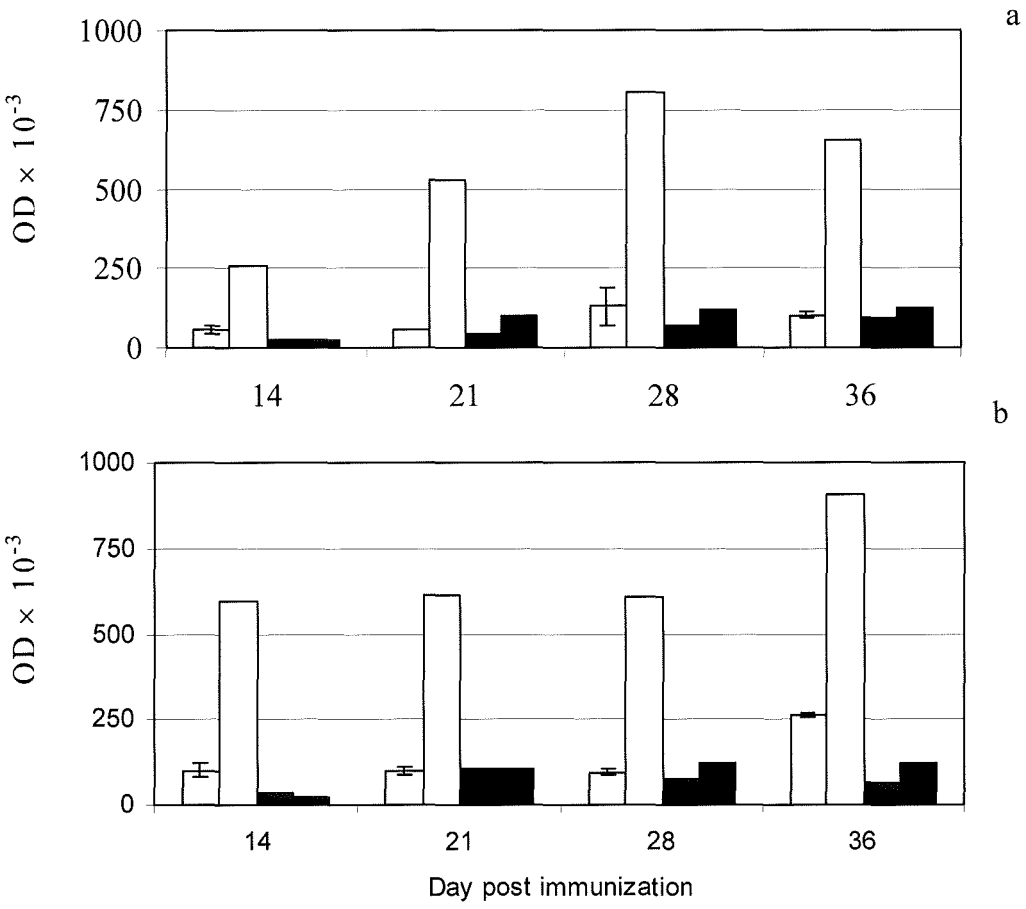


Figure 6.22. Anti-peptide IgG2a production post-immunization in mice immunized with synthetic peptides 4 or 5 related to the *cir* genes.
An initial injection of emulsion was followed with three boosting injections and specific anti-peptide IgG2a was examined. Levels of specific IgG2a are shown as mean ± SEM. However, ±SEM is not shown when it is too small. Each peptide was conjugated to BSA and coated on ELISA plate.
a) Anti-peptide IgG2a level post-immunization for P4.
b) Anti-peptide IgG2a level post-immunization for P5.
⊡ Sera of mice immunized with peptide conjugated to KLH.
□ Sera of mice immunized with peptide conjugated with KLH and adjuvant.
■ Sera of mice injected with adjuvant only.
▩ Serum of naïve mice reacted to the coated peptides.

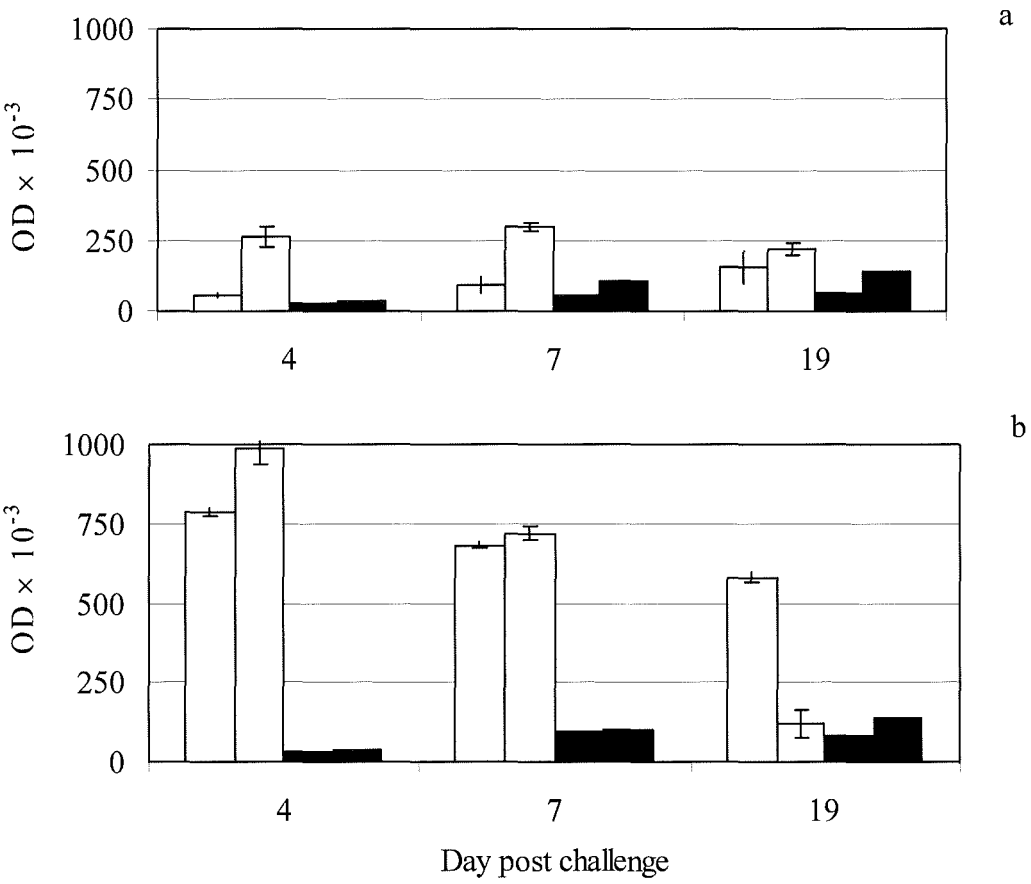


Figure 6.23. Anti-peptide IgG2a production post-challenge in mice immunized with synthetic peptides 4 and 5 related to the *cir* genes.
An initial injection of emulsion was followed with three boosting injections and specific anti-peptide IgG2a was examined. Levels of specific IgG2a are shown as mean ± SEM. However, ±SEM is not shown when it is too small. Each peptide was conjugated to BSA and coated on ELISA plate.
a) Anti-peptide IgG2a level post-immunization for P4
b) Anti-peptide IgG2a level post-immunization for P5.
⊡ Sera of mice immunized with peptide conjugated to KLH.
□ Sera of mice immunized with peptide conjugated with KLH and adjuvant.
■ Sera of mice injected with adjuvant only.
▩ Serum of naïve mice reacted to the coated peptides

6.2.2.6. Anti-parasite IgG2a production post-immunization and post-challenge in mice immunized with P4 or P5

To determine if there are any cross-reacting epitope reactions in mice immunized with P4 or P5, the levels of parasite-specific IgG2a were determined post-immunization and post-challenge. Sera from immunized mice reacted to lysate of the parasite.

Figure 6.24 shows that the levels of anti-parasite IgG2a in immunized mice were significantly higher (Tukey's test, $P < 0.001$ for all time points) than that in control mice.

Anti-parasite IgG2a in immunized mice were also determined post-challenge (Figure 6.25). The same profile of the results as above was seen. Significantly different (Two-way ANOVA, $P < 0.0091$ for time only) levels of anti- parasite IgG2a were detected between all tested groups over the experimental period. Figure 6.25 also shows that anti- parasite IgG2a levels on the last sampling day were higher than that in two first sampling days. In general, the results confirmed that anti-peptide IgG2a antibodies in sera from immunized mice may recognize epitopes present in crude antigen of *P. chabaudi* AS.

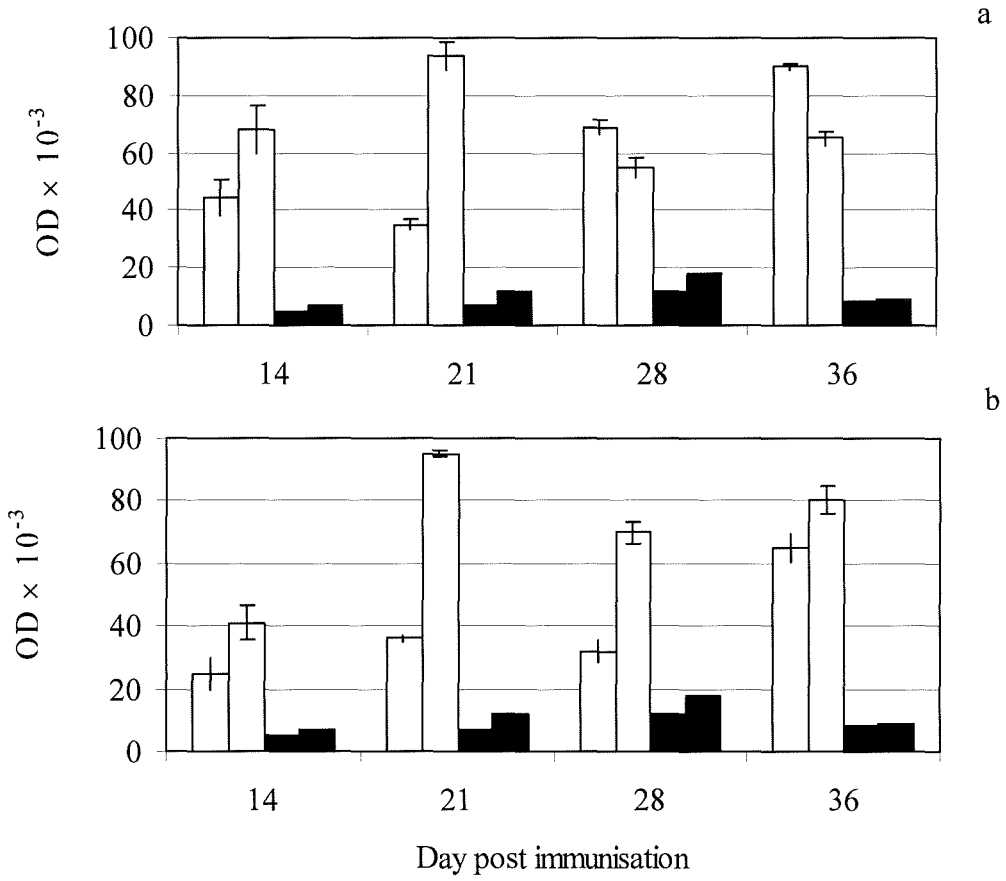


Figure 6.24. Anti-parasite IgG2a production post-immunization in mice immunized with synthetic peptides 4 or 5 related to the *cir* genes.

An initial injection of emulsion was followed with three boosting injections and specific anti-peptise IgG2a was examined.

Levels of specific IgG2a are shown as mean ± SEM. However, ±SEM is not shown when it is too small.

Sample sera and control serum were reacted to Lysate of pRBCs which was coated on ELISA plate.

a) Anti-parasite IgG2a level post-immunization for P4.

b) Anti-parasite IgG2a level post-immunization for P5.

▨ Sera of mice immunized with peptide conjugated to KLH.

□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.

■ Sera of mice injected with adjuvant only.

▩ Serum of naïve mice reacted to lysate of infected mice.

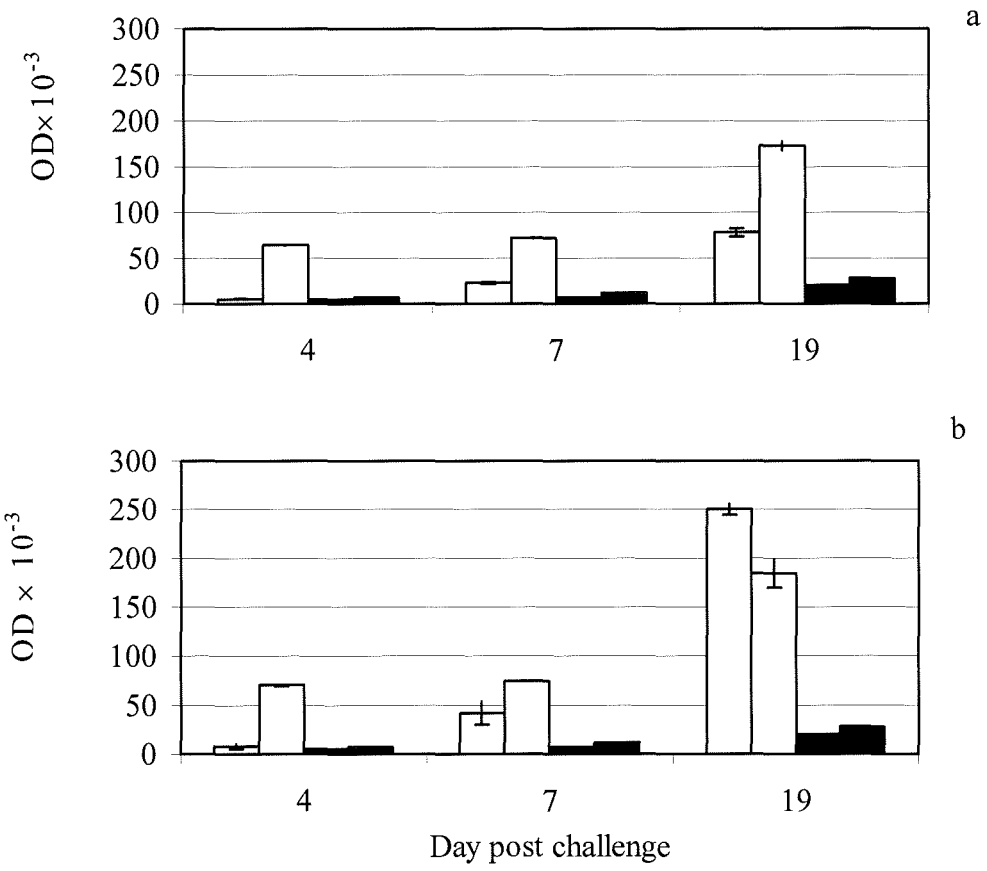


Figure 6.25. Anti-parasite IgG2a production post-challenge in mice immunized with synthetic peptides 4 or 5 related to the *cir* genes.
An initial injection of emulsion was followed with three boosting injections and specific anti-peptise IgG2a was examined. Levels of specific IgG2a are shown as mean ± SEM. However, ±SEM is not shown when it is too small. Sample sera and control serum were reacted to Lysate of pRBCs which was coated on ELISA plate.

a) Anti-parasite IgG2a level post-immunization for P4.

b) Anti-parasite IgG2a level post-immunization for P5.

⊡ Sera of mice immunized with peptide conjugated to KLH.

□ Sera of mice immunized with peptide conjugated to KLH and adjuvant.

■ Sera of mice injected with adjuvant only.

▩ Serum of naïve mice reacted to lysate of infected mice.

6.3. Discussion

In the present study examination of the efficacy of immunization of NIH mice with different peptides which are synthesized based on *clag*-like or *cir* genes of *P. chabaudi* AS was the main aim. To approach to this aim monitoring of the course of infection in immunized mice after the challenge with *P. chabaudi* AS, detection and quantifying of anti-peptide antibody and reactivity of sera from immunized mice with the lysate of *P. chabaudi* AS were examined. The two gene families, *clag* and *cir* are thought to be involved in antigenic variation and cytoadherence of the parasite, *P. chabaudi* (reviewed by Kyes, Horrocks and Newbold, 2001; Janssen *et al.*, 2001, and 2002). So, the products of these two gene families may be interested as potential vaccine candidates. Although relationship between *cir* gene and its homologous *vir* gene in *P. vivax* has been identified (Janssen *et al.*, 2002), the functional properties of *cir* and its relationship with human *P. falciparum rif* and *vir* gene families in terms of immune response merits further studies. Determination of these properties would be invaluable tools which help to predict parasite/host interactions (Janssen *et al.*, 2004). Regarding to the products of *clag* genes, multiple functions in terms of cell adhesion properties (Ling *et al.*, 2004) such as invasion of erythrocytes by merozoite have been proposed (Holt *et al.*, 2001; Gardiner *et al.*, 2004). It has been also shown that *clag* gene products are involved in remodelling of red blood cells after invasion by merozoite as part of complex proteins in rhoptery (Ling *et al.*, 2004).

The present study showed that immunization of mice with the synthetic peptides resulted in statistically significant reductions in peak parasitaemias..

In general, the results showed a partial efficacy and a degree of protection induced by above synthetic peptides in the immunized NIH mice. The lowest peak parasitaemia was seen in mice immunized with P1. The peak parasitaemia in mice immunized with P2 was also significantly lower than that in mice immunized with P3. Comparison between P4 and P5 showed that in mice immunized with P5 peak parasitaemia was significantly lower compared to mice immunized with P4 (Figure 6.15 and 6.14).

One important index of efficacy of vaccine candidates such as peptides is the delay in the onset of parasitaemia which may reduce of initial

parasitaemia. This could be a possible mechanism to ameliorate the severity of the disease in an endemic area (Ballou *et al.*, 2004). In human *P. falciparum* infection, it is proposed that a two-day delay in the time to patent parasitaemia would represent approximately an eight fold reduction in the number of sporozoites that develop through hepatic schizogony and subcequently reduction in the number of merozoites released from an infected hepatocytes (Ballou *et al.*, 2004). In the present study, immunized mice were not challenged with sporozoites. So, reduction of the parasite in the liver was not examined. Nevertheless, reduced peak parasitaemias and kinetics of the antibody production in immunized mice indicate that the synthetic peptide immunization can partially control multiplication of the parasite during asexual blood stages following challenge with pRBCs of *P. chabaudi* AS. In the present study only mice immunized with P1 plus adjuvant showed one-day delay in pre-patent period as pRBCs were observed from day 5 p.i. However, this delay did not result in the lowest peak parasitaemia among the immunized mice as the lowest peak parasitaemia was seen in the P1-immunized mice without adjuvant.

In the present study, the effect of immunization with synthetic peptides with and without adjuvant was examined. Adjuvants are generally known as inducers for higher immune responses (Hioe *et al.*, 1996). The adjuvant formulation (Playfair and Souza 1986) and route of immunization (Kenney *et al.*, 1989) have profound effects on the stimulation of immune responses and efficacy of protection (Ling *et al.*, 1997). However, overall according to the present results using Titermax adjuvant did not show more effectiveness in terms of inducing stronger immune response or significantly lowering parasitaemia compared to other groups which were not immunized with the adjuvant (Figures 6.15). Immunization without adjuvant may be advantageous because it can prevent the host from side effects caused by adjuvant. For example, due to undesirable side effects of complete Freund's adjuvant, it is not recommended for use in animals such as mice (http://research.uiowa.edu/animal/?get=adjuvant, 2005). Nevertheless, some studies have shown that using adjuvant in immunization process may elicit more effective immune responses. For example, using incomplete Freund's adjuvant in immunization of mice with synthetic peptides induced CTL responses (Aichele *et al.*, 1990; Zhou *et al.*, 1992). Attempts to develop new adjuvants have shown that using Montanide

ISA720 (Scalzo *et al.*, 1995), QS-21 (Newman *et al.*, 1992), and peptides admixed with lipid molecules (Borges *et al.*, 1994) induced CTL responses. Immunization with synthetic lipopeptides such as CS 252-260, a class I H-$2k^d$-restricted epitope from *P. berghei* CSP, admixed with ISA 720, or POE lipid molecules stimulated a strong CTL response in BALB/c mice (Hioe *et al.*, 1996). Immunization of BALB/c mice with the recombinant C-terminal fragment of *P. yoelii* MSP-1 mixed with three adjuvants being developed for human use (liposome, SBAS2.1, and SBA2) can protect against a lethal infection of *P. yoelii* (Ling *et al.*, 1997). On the other hand, it has been shown that immunization with Lipopeptides without adjuvant induced strong systemic B and T cell and CTL responses in mice (BenMohammed *et al.*, 1997 and 2002a). BenMohammed *et al.*, (2002a), however, also reported that immunization with peptides in Montanide induced $CD4^+$ T cell proliferative responses, IFNγ, and antibody responses in chimpanzees. So, the controversial issue of using adjuvant still requires further investigation. Although the present study did not show any advantage for using Titermax as an adjuvant, using other adjuvants, or combination between different adjuvants needs further investigation.

The effect of different routes of administration has also been widely examined. In BALB/c mice, immunized with CS peptide emulsified in Montanide ISA720 CTL, responses were evident only when the immunogen was delivered subcutaneously (s.c.) and not i.p. (Hioe *et al.*, 1996). Using parenteral injections, intranasal or sub-lingual delivery of lipopetides, can induce B and T cell and CTL immune responses significantly higher than that in parallel experiments in which the same antigens were injected s.c. (Ben Mohamed *et al.*, 2002b). Ben Mohamed *et al.* (2002b) also showed that intranasal or sub-lingual delivery of synthetic lipopeptides without adjuvant induce strong systemic immune responses in mice including antibody and T cell responses, confirming successful efficient delivery to the central lymphoid system. This mode of antigen delivery also showed a preferential Th1 response. In the present study, the route of immunization was i.p. and this route was not compared to other possible routes. So, one further aspect for expansion of the present immunization study could be comparison of the effect of different administration routes.

The present results showed that immunization of mice with the synthetic peptides resulted in significant higher anti-peptide antibodies production

compared to the controls after challenge with *P. chabaudi* AS. Positive reactions between sera obtained from immunized mice and natural soluble crude antigens, lysate of the parasite have shown that there are similar epitopes in the whole molecules of the soluble lysate of the parasite which can be recognized by serum anti-peptide antibodies. These antibodies including specific anti-parasite IgG2a and IgG1 in immunized mice shows that peptide immunization may elicit specific antibodies which have reactivity with native whole antigens of the parasite. This could be an important aspect in immunization with synthetic peptides because these antibodies may be protective in heterologous challenges. In addition, the conserved regions of the appropriate formulation of such peptides may induce protective immune responses in heterologous species of malaria parasites. The presence of cross-reactive epitopes of malaria parasite was previously evident as sera of people living in endemic areas contained antibodies which can agglutinate pRBCs from various strains and isolates (Marsh and Howard, 1986; Bull *et al.*, 1998; Giha *et al.*, 1999 a and b). Giha *et al.* (1999b) showed high levels of broad-specificity antibodies and capacity of individuals to increase antibody responses to variant surface antigens such as PfEMP-1. Gamain *et al.* (2001) showed that two MAbs to the cysteine-rich interdomain region of PfEMP-1 can recognize 9 of 10 of multiple *P falciparum* strains expressing variant PfEMP-1. The present study suggests that anti-peptide antibodies, post-immunization, may recognize epitopes of products of *clag* or *cir* genes on the surface of merozoites or parasitized erythrocytes. So, finding peptides or combination of peptides which induce protective antibodies against variant antigens of malaria parasites such as those antigens involved in merozoite invasion merits more investigations.

Previous studies showed that high titres of anti-repeat and anti-sporozoite antibodies can be produced along with inducing effector T cells in BALB/c mice immunized with two synthetic CS-peptides (residues 20-39 and 57-70) (Migliorini, Betschart and Corradin1993). The present results also show that the kinetics of specific IgG2a and IgG1 which are significantly higher than that in control mice indicates a sequential Th1/Th2 activation. In general according to the present results a sequential Th1/Th2 response is supported by increases in IgG2a levels at or immediately after peak

parasitaemia and producing higher levels of IgG1 later during the experimental period in immunzed mice compared to controls.
In addition, this profile was seen post-immunization and post-challenge. However, significant higher levels of IgG2a and IgG1 post-challenge compared to post-immunization indicated a stronger secondary response.

In general, it is known that the immune responses are remarkably restricted and focused on one or a few immuno-dominant epitopes (Franke *et al.*, 2000). However, T cell responses can be induced by immunization with whole antigens in which dominant epitopes are recognized by T cells (Sercarz *et al.*, 1993). So, although challenge with the whole live parasite introduces many different antigens to the components of the immune response, anti-peptide antibodies present post-immunization in the present study may neutralize the known epitopes which were present in the whole antigens.

The amino acid sequence of the synthetic peptide is also another important aspect for use in immunization. Sequence of amino acids of a synthetic peptide can mimic the immunological function of the native epitopes within native antigens. This encourages identifying appropriate amino acid sequences as inducers for protective immune responses. Recognition of epitopes of the processed products of MSP-1, such as MSP-1_{42} and MSP-1_{19}, by antibodies has been identified and thought to be involved in the protective response against malaria (Blackman *et al.*, 1990; Chappel and Holder 1993; Burns *et al.*, 1989). So, taken together, in the present study increases in antibody levels after the challenge could be as a result of inducing specific antibodies against epitopes including a few amino acids or whole peptide which were designed for immunization. Techniques such as FACS may help to identify how DCs or other APCs present these epitopes to the immune system and which epitopes are presented. Synthetic peptides may be expressed and presented in DCs using plasmids which express interested peptides. Co-culturing of DCs with peptides can also provide peptide presentation to the immune system particularly to $CD4^+$ T cells. When synthetic peptide(s) appear on the surface of the DCc then using anti-fluorescent-labelled anti-peptides antibodies can recognize and distinguish DCs bearing each peptide and then antigen-specific response of $CD4^+$ T cells can be investigated to clarify ability of the induced immune response. In this regard, Casey *et al.* (2004)

also found some peptides that can mimic conformational B-cell epitopes on AMA-1. These peptides were used in immunization of mice and subsequently mice responded by producing anti-peptide antibodies which are involved in inhibiting merozoite invasion of erythrocytes.

The ability of peptides to mimic some known parasite antigens has been recently shown (Casey *et al.*, 2004; Adda *et al.*, 1999; Demangel *et al.*, 1998; Stoute *et al.*, 1995). This would be a good reason for using peptides in designated vaccines that stimulate immune responses in both cellular and antibody branches. On the other hand, the identified differences in the amino acid sequences of vaccine candidates may explain the lack of protection in heterologous challenge, as Renia *et al.* (1997) reported that a recombinant C-terminal fragment of *P. yoelii* MSP-1 (strain YM) protects mice against homologous but not heterologous strain 265BY challenge.

Timing between boosting injections has been thought to be another factor for achieving long lasting and complete protection. Bruna-Romero *et al.* (2001) observed that when boosting was given to mice after 2 weeks the number of activated $CD8^+$ and $CD4^+$ T cells were considerably lower than that seen at 8 weeks suggesting either lower activation or higher apoptosis may be the reason. In the present study a standard protocol was followed in which each boosting injection was administered at one-week intervals. The Bruna-Romer (2001) observation encourages investigating different schedules in terms of the timing of boosting injections.

The importance of the coupled peptides with carrier proteins and orientation of the peptide was evident when the magnitude of the anti-sporozoite antibody response was directly correlated with protection (Zavala *et al.*, 1987). The peptide concentration contained in the conjugate vaccine is also critical for induction of protective immunity. Reed *et al.* (1996) showed that immunization with *P. berghei* CS repeats coupled to BSA, at peptide to protein ratios of 6:1, 55:1, or 170:1, protected 20, 50, and 100% of the immunized mice, respectively. In the present study KLH was used as a carrier protein and another carrier proteins was not examined. For further investigation the route of injection, using different adjuvants, dose of immunogen and the number of boosting injections may be considered as Burns *et al.* (2003) reported that only one dose of Pc AMA-1 and Pc MSP-1 combination resulted in 40-fold reduction in parasitaemia in immunzed mice compared to controls.

Determination of which stage may be targeted by the immune response could be also another aim for further investigation, as Bharadwaj *et al.* (1996) proposed that linear multipeptides elicited immune responses which block merozoite invasion.

The induction of protective $CD8^+$ and $CD4^+$ T cell responses by peptide immunization has always been an important aim in vaccine studies (Tsuji and Zavala, 2001). These studies provide critical information about the recognition and identification of the most immunogenic epitopes and subsequent generation of protective Th1 and Th2 responses. The present results demonstrate that single peptide immunization can reduce the peak parasitaemia and induce anti-peptide and anti-parasite antibodies which might be consistent with a sequential activation of Th1/Th2 response. The profile of IgG (whole molecule), IgG1 and IgG2a production was in general similar to antibody response in mice infected with *P.c. adami* DK and DS strains. So, the present results provide further information on the effectiveness of synthetic peptide immunization to induce both antibody-dependent and cell-mediated responses. Further studies can determine the mechanisms involved in control of the asexual blood stages of the parasites induced by peptide immunization. Theses mechanisms can be studied in rodent malaria models such as *P. c. chabaudi* AS which are close in some features to *P. falciparum* infection (Hoffman *et al.*, 2002).

Chapter Seven

General Discussion

7.1. Introduction

The present studies included the determination and comparison of the immune responses in NIH mice infected with avirulent *P. c. adami* DK and virulent *P. c. adami* DS and mixed infections of the two. Evaluation of the efficacy of passive immunization with purified IgG subclasses, IgG1 and IgG2a, in NIH mice challenged with the DS or DK strains, and the efficacy of immunization with synthetic peptides in NIH mice challenged with *P. chabaudi* AS were also investigated.

7.2. The immune response in single-infections

In the present study the profile of the immune responses in both virulent DS and avirulent DK infections of *P. c. adami* was shown to be a sequential Th1/Th2 $CD4^+$ T cell response. This finding was supported by early production of IFNγ and IgG2a and a later elevation of IL-4 and IgG1 during the course of infection. Some other studies have also proposed that there is a sequential Th1 and Th2 response against asexual blood stages of rodent malaria parasites such as *P. chabaudi* AS (Langhorne *et al.*, 1989 and 1998; Taylor-Robinson 1995), *P. c. adami* (Kima *et al.*, 1992), and *P. yoelii* (Matsumoto *et al.*, 2000). They showed that the profile of a sequential Th1/Th2 response is indicated by the presence of a predominantly IgG2a response during the primary ascending parasitaemia followed by an increase of IgG1 during the chronic phase of the infection. This could be as a result of switching from Th1 to Th2 when the primary parasitaemia enters resolution as Taylor-Robinson and Phillips (1994) observed in *P. chabaudi* AS infection.

It is also proposed that a Th1 response may develops following activation of DCs stimulated with the malaria parasite to produce IL-12 which promotes IFNγ production by NK cells and $CD4^+$ T cells (Seixas *et al.*, 2001). A sharp rise in IFNγ production was seen in spleen cells stimulated *in vitro* with pRBCs two or three days before peak primary parasitaemia (Stevenson *et al.*, 1990; Taylor-Robinson and Phillips, 1994). The early burst of IFNγ in the spleen is shown to be associated with response to lethal or non-lethal infections of *P. yoelii* in resistant CBA/J mice (Shear *et*

al.,1989). However, they showed that in susceptible BALB/cBy mice only the non-lethal infection of *P. yoelii* showed an initial burst of IFNγ. Early IFNγ production contributes to protection through T cells and NK cells as nude and NK cell-depleted mice showed 50% reduction in IFNγ levels compared to normal mice (de Souza *et al.*, 1997). They also showed that in non-lethal *P. chabaudi* and lethal *P. berghei* and *P. yoelii* infections, IFNγ amount increased from day 7 p.i. and day 5 p.i respectively. In this regard, Langhorne *et al.* (1989) and Stevenson and Tam (1993) showed that spleen cells of resistant B6 mice produce high level of IFNγ within the first week followed by IL-4, IL-5, and IL-10 from 2 to 4 weeks p.i. in *P. chabaudi* AS infection. In the present study, a similar finding was observed. So, it may be concluded that resistance in NIH mice is mediated by an early IFNγ-dependent response followed by a Th2 response in avirulent DK or in virulent DS strains of *P. c. adami* infections. However, in untreated DS-infected mice IFNγ levels were significantly lower compared to avirulent DK strain.

The role of IFNγ-mediated IgG2a response is shown in the control of the primary acute phase of parasitaemia in *P. chabaudi* infection which indicates IgG2a as the main IgG subclass in protection (reviewed by Wipasa, 2002; Stevenson *et al.*, 1990; Smith and Taylor-Robinson, 2003). A correlation between outcome of an infection in death and a slower developing and reduced IgG2a level is also suggested (Smith and Taylor-Robinson, 2003). Although the present results confirmed the protective role of IgG2a in avirulent self-resolving DK strain infection, no reduced level of IgG2a in the virulent DS strain infection was seen which could be correlated to fatal outcome of the DS strain infection. Nevertheless, it is clear that in the virulent DS infection the presence of high levels of IFNγ and IgG2 were not able to protect all mice from death.

In the present study biphasic production of IFNγ was seen. The maximum levels of IFNγ were seen on the first sampling day, day 4 i.p., followed by a sharp decline during the peak parasitaemia and a gradual rise thereafter, particularly after the recrudescence. However, this second increase was not as high as the first elevation and it was significantly lower compared to the levels on the first sampling day. In agreement with the present observation biphasic production of IFNγ has been found in resistant CBA/J mice in

non-lethal and lethal *P. yoelii* 17X infection (Shear *et al.*, 1989). They showed that an early peak of IFNγ was followed by a second peak which usually occurs after the primary resolution of the infection. However, the present results showed that the profile of cytokine production, as markers for Th1 (IFNγ) or Th2 (IL-4), were not changed in resistant NIH mice infected with either avirulent DK or virulent DS strains.

It is known that $CD4^+$ Th2 cells specifically contribute to protection of mice infected with *P. c. chabaudi* through a strong IgG1 response (Taylor-Robinson *et al.*, 1993). Although there is a need for further investigation to identify how and when switching from a Th1 to a Th2 dependent response occurs, it has been shown that there is a significant specific IgG1 response to *P. c. chabaudi* around the time of recrudescence (reviewed by Taylor-Robinson, 1995). In agreement with this observation, the present results showed that specific IgG1 against both avirulent DK and virulent DS strains increased later during the course of infection particularly around the time of recrudescence (Figures 3.19-21) alongside the highest levels of IL-4 (Figures 3.14-16). Although levels of IgG1 and IL-4 were high on the last sampling days, the levels of IgG2a were also high at these time points, probably due to a parasite recrudescence and new variants of the parasite. So, it may be proposed that a recrudescent parasitaemia may play a role as a stimulator for inducing an appropriate secondary response in the presence of memory cells as a crucial factor for the host to clear the parasite (Garraud *et al.*, 2003).

The present study also showed that virulence of *P. c. adami* DS strain did not change the profile of the immune response when mice infected with either with 1×10^4 or a lower infective dose of 2×10^3 pRBCs. However, mice infected with the lower infective dose showed one-day delay in appearance of the parasitaemia and three days in time taken to death.

7.3. The immune response in the mixed infection

This study also examined possible cross-reactivity and parasite/parasite interactions in mixed infections of two DK and DK strains of *P. c. adami*. Higher virulence was observed in mixed-clone infection compared to single-clone infection when weight loss and reduction in mean red blood cells count were measured (Taylor, Mackinnon, and Read, 1998). On the

other hand, the study of Taylor and collegues suggested that replication rate and parsite densities were not always higher in mixed infection. Regarding the results presented here mixed infection showed a higher virleunce compared to the DK single-infection when peak parasitaemias were considered. The present results indicated that a low proportion of the virulent DS strain in an infective inoculum caused a significantly higher peak parasitaemia compared to avirulent DK single-infection. However, in the mixed infection peak parasitaemia was significantly lower than that in the DS single-infection when mice were left untreated. A similar effect was suggested by Snounou *et al.* (1992) as they showed that parasitaemia of a *P. c. adami* DS infection was reduced in the presence of *P. c. chabaudai* AS. So, from the present results, it may be concluded that replication rate of *P. c. adami* DS was higher than that of *P. c. adami* DK in the mixed infection. Moreover, as no death was seen in mice infected with the mixed inoculum, it may be, therefore, assumed that the presence of avirluent DK parasite may contribute to partial control of the virulent parasite in the mixed infection.

In the present study the profile of sequential Th1/Th2 was also observed in the mixed infection. The consistent production of IgG2a and IgG1 with their associated signal cytokines supports this conclusion. However, in mixed infections the Th2 response seemed to be stronger as significantly higher production of IL-4 was observed compared to the DS single-infections (Chapter Three).

Studying host/parasite and parasite/parasite interactions and competitions in mixed infection comapred to single-infections may provide better understanding of importance of virulence and its alterations particualry when different ratios of parasites differed in virulence are examined. Molecular approaches may show the actual ratio of each virulent and avirulent parasites in the resulted parsitaemia during course of a mixed infection. This can show how virulent or avirulent strains interact or compete to each other in the same host and so, what would be the outcome of the mixed infection. For example, Soutern blotted DNA isolated from daily blood samples can be probed with a DNA probe which detects a restriction fragment length polymorphism for each parasite (Snounou *et al.*, 1992). Nested PCR has been also used to detect malaria parasites particularly in the case of low parasitaemias and mixed infections, and has

been shown to be more sensitive than microscopy (Snounou *et al.*, 1993; Humar *et al.*, 1997, Zakeri *et al.*, 2002). In addition, an evaluation of the first commercial available standardized real-time PCR in which DNA binds to a dye such as Sybre as molecular probe or hybrids labelled with fluorescent probes gave results which were more rapid, sensitive and specific compared to nested PCR (Farcas *et al.*, 2004). Cheesman *et al.* (2003) demonstrated that real-time quantitative PCR can distinguish and quantify genetically different malaria parasite clones in a mixed infection. Real-time PCR can show that the amount of product generated is proportional to the amount of template in the original sample. So, in real-time PCR all reactions can be caught at the time of their logarithmic phase (Roberts, Clark and Friedman, 2005). Such techniques may help to answer the question what is the actual replication rate of each parasite in the mixture.

It is also possible to identify the influence of mixed infection on infectivity and transmission which are critical criteria in the control of malaria (Taylor, Walliker, and Read, 1997a). Referring to this important issue it was reported that mixed-genotype infections including two clones of *P. chabaudi* CR and ER were more infectious to mosquitoes than single-infection (Taylor, Walliker, and Read, 1997a). Moreover, Taylor and Read (1998) showed that virulence was enhanced in the mixed infection of two clones of *P. chabaudi* as transmission stage densities were significantly higher than that in single-infections and mice infected with two clones had more weight loss and lowered blood cell count. On the other hand, de Roode *et al.* (2004) showed that in a mixed infection of *P. chabaudi*, when CBA/Ca mice were left untreated, the sensitive AS clone which is resistant to the antifolate drug pyrimethamine, competitively suppressed the resistant AJ clone. This suppression resulted in lower asexual blood stage parasite densities and also reduced transmission to the mosquito vector. In the present study, although the DS strain, sensitive to chloroquine, maintained its replication rate, the peak parasitaemia in the mixed infections of DS and DK strains was significantly lower than that in the DS strain single-infection possibly due to the presence of the avirulent DK strain. Further experiments may help to identify virulence determinants such as parasite replication rate, weight loss and blood count along with transmission rates in mixed infections.

7.4. The efficacy of passive immunization with sera and purified IgG1 and IgG2a

The present study also examined the efficacy of passive transfer of serum and purified IgG1 and IgG2a in both virulent and avirulent infections. Sera and antibodies were obtained from DK-infected mice at two different time points, day 15 and day 55p.i. When these sera or IgG subclassess separately transferred to mice challenged with the avirulent DK strain, mice showed delay and significant reduction in the parasitaemia (Figures 15a and b) compared to the controls. These sera, IgG1, and IgG2a also exhibited cross-reactivity in mice challenged with the virulent DS strain as significant reductions in peak parasitaemia in immunized mice compared to control non-immunized mice were seen. Serum collected on day 55 p.i. showed greater protective activity, indicating the presence of more effective specific antibodies at this time point. However, in mice immunized with IgG1 or IgG2a collected on day 55 p.i. although peak parasitaemias were lower than that in mice passively immunized with antibodies from day 15 p.i., the differences were not significant in all groups.

Although passive immunization with IgG1 or IgG2a prevents a fulminating parasitaemia in the avirulent DK, and partially in the virulent DS challenges, this passive immunization cannot induce complete protection. Despite the observation of the effect of passive immunization, the lethal outcome of DS-infected mice did not alter in passively immunized mice (seeTable 2. Chapter Five). However, the present results show that mice rechallenged with a high dose, 1×10^8 pRBCs, of either the DK or the DS strains induced a significantly stronger secondary response compared to the primary response. In this regard, immunized mice previously challenged with the virulent DS strain also survived from rechallenge.

In the present study, as reported in previous studies (Langhorne *et al.*, 1989; Taylor-Robinson, 1995), the same patterns for kinetics of production of IgG1 and IgG2a were observed after passive immunization which reflects there is a sequential Th1/Th2 response in paasively immunized mice challenged with the parasite.

7.5. The efficacy of synthetic peptide immunization in NIH mice challenged with *P. chabaudi* AS

Active immunization of mice with single synthetic peptides was the final part of this study. The five synthetic peptides used were synthesized based on *P. chabaudi* AS genome data. Three peptides were related to *clag* gene family which is present in *P. falciparum* and for which there is a homologous in *P. chabaudi* genome. The other two peptides were based on *cir* gene family which is now known in several *Plasmodium* species such as *P. vivax* and *P. berghei* (for more details see Chapter Six). The results presented here showed that synthetic peptide immunization resulted in statistically significant reduced peak parasitaemias in immunized mice challenged with *P. chabaudi* AS and they induced anti-peptide and anti-parasite antibodies, which indicates the presence of cross-reactive epitopes in a whole soluble lysate of the parasite which can be recognized by anti-peptide antibodies.

Some studies, as discussed in Chapter Six, showed that synthetic peptides that were designed based on immunogenic molecules of asexual blood stage such as MSP-1 and AMA-1, can induce degrees of protection against rodent malaria parasites (Calvo, Daly and Long, 1996; Tian *et al.*, 1997; Burns *et al.*, 2003). In terms of sporozoite challenge, Ak *et al.* (1993) showed that passive immunization with three different IgG subclasses i.e, IgG1 and IgG2b, which were induced by synthetic peptides based on the *P. yoelii* CS major repeat, (QGPGAP)4 conjugated with KLH as protein carrier in BALB/c mice, were protective. It was also observed that transfer of NYS1 (a MAb against the *P. yoelii* CSP) is completely protective against sporozoite challenge (Charoenvit *et al.*, 1991). So, such results encourage expanded studies of synthetic peptide immunization in animal models particularly when peptides with a small sequence and good immunogenicity are used.

Synthetic peptide immunization is known to induce good antibody responses and protection (AK *et al.*, 1993; Nardin *et al.*, 2000). However, their capacity to induce T-cell mediated responses, particularly $CD8^+$ T cell, is limited. In agreement with those studies the present study also showed significantly higher levels of IgGs produced in peptide-immunized mice after the challenge. As IgG2a and IgG1 subclasses are known as Th1

and Th2 response markers respectively, the present results showed significantly higher production of both IgG2a and IgG1 in immunized mice post-immunization and post-challenge compared to non-immunized mice with the same kinetics which indicates a sequential Th1/Th2 response. However, the levels of antibodies, particularly IgG2a post-challenge, were higher than that in post-immunization indicating a stronger seconday response. For help to better understanding in terms of any changes in induction of Th1 and Th2 response further studies using different peptides or combinations and hosts with different susceptibility should be examined.

The present study mainly focused on determination of the course of infections and profile of antibody responses before and after peptide immunization. However, differences between the effects of each single-synthetic peptide in immunized mice were examined to distinguish which peptide is more potent for inducing a stronger control of parasitaemia and anti-parasite antibody production. The present results showed that P1 and P5 were more potent in reducing peak parasitaemia or inducing higher antibody production compared to other peptides in immunized mice.

For improvement in immunization with synthetic peptides Jones *et al.*, (1999) suggested that using synthetic oligodeoxynucleotides containing CpG motifs enhanced the immune response in *Aotus* monkeys to the synthetic peptide PADRE 45, a synthetic peptide containing amino acid sequences derived from the CSP of *P. falciparum*. They showed that the immune responses, including antibodies against sporozoites, were significantly greater than those seen in animals receiving the oligodeoxynucleotide without CpG motifs. The CpG motifs are sequences based on immunostimulatory bacterial DNA sequences. The use of peptide mimitopes, peptides showing amino acids sequences which diverge from the native antigen but mimic their conformation in inducing antibodies against the native antigen (Stout *et al.*, 1995) are also being studied. These mimitopes can be obtained by chemical synthesis (Lam *et al.*, 1991; Houghten *et al.*, 1991). So, this is another possibility for designing peptide based vaccine or subunit vaccine through exploiting screens of mimitopes which induce immune response against sporozoite challenge.

Taken together, the synthetic peptides were used in the present study because of their relevance to cytoadherence as one of the important aspects of malaria immunopathology. In this regard route of injection, adjuvant

formulation, number of boosting, choosing the best adjuvant, and carrier protein in different immunization protocols should be considered.

In respect of using adjuvants it is thought that adjuvants may enhance long lasting humoral and cellular immune responses. It was shown that adjuvants such as saponin and pertussis are able to induce protective IgG2a (ten Hagen *et al.*, 1993; Daly and Long, 1996) against blood stage malaria. So, it seems that using different delivery system containing different antigen/adjuvant formulation is a crucial step in malaria vaccine design. On the other hand, some studies have shown that strong systemic Th1 response can be induced following immunization with a synthetic lipopeptide derived from *P. falciparum* LSA-1 or LSA-3 using mucosal route of injection such as intranasal or sub-lingual without adjuvant (Ben Mohammad *et al.*, 2002b). Using adjuvant still remains a controversial issue. However, peptide immunization without adjuvant seems to be an approach which is safer, non-invasive, and free of side effect. In the present study, Titermax (Chapter Two and Six) was used as an adjuvant. The adjuvant was emulsified with synthetic peptide which was linked to KLH as a carrier protein. However, the present study did not show any significant advantage for using Titermax for inducing stronger immunity when peak parasitaemia and the course of infection were compared in mice immunized with or without adjuvant. Further investigation is required to identify if there is any antigen/Titermax formulation which could be more advantageous for these particular peptides.

It is known that peptide-based vaccines are more efficient than naked DNA or recombinant vectors and would be safer because there is less risk of pathogenic effect or mutations and no need for a cold chain (Tsuji and Zavala, 2001). So, more investigation for developing peptide-based vaccines for enhancement of their efficacy in animal models and subsequently facilitate improvement in human malaria control strategies are required.

7.6. Comments

The studies reported in this thesis provided information in four different areas in which further investigation could be planned. The first area was determination and comparison of the profile of the immune responses in

mice infected with avirulent and virulent rodent malaria parasites. Although a sequential Th1/Th2 response was observed, further investigations, particularly molecular approaches, may help to identify which mechanisms regulate the immune response, what is the role of APCs and DCs in presenting antigens when difference between two strains in terms of virulence is considered. Measuring the other mediators such as IL-12, IL-10, TNF, and NO would help to show a more accurate profile of the immune response in *P. chabaudi* as an appropriate model for human *P. falciparum* infection using new techniques such as cytokine flow cytometrey with FACS.

In the second area, mixed infection study showed the same profile of immunity. Investigation to determine what is the nature of the interaction between virulent and avirulent parasite populations in the host and how these interactions, such as biological and ecological competitions, balance replication rates and affect induction of the immune response could be expanded. Investigating the effects of different ratios of each strain and determination of the proportion of each strain during the course of infection may lead to an understanding of the mechanisms by which the hosts respond to a mixture of parasites.

The third area was the passive transfer of IgG1 and IgG2a in mice challenged with both avirulent and virulent strains. This part of the study merits further investigation at the molecular level to identify the kinetics of cytokine and antibody response particularly in mice recovered from first infection and exposed to the rechallenge.

In the fourth area of study peptide immunization showed some degree of protection and reduction in parasitaemia in immunized mice. However, in this field further investigation can develop the knowledge of possible effects of synthetic peptides in inducing more protective immunity. For example, molecular approaches may help to identify mechanisms of peptide presentation by DCs and where this presentation takes place. Different formulations or combination of these peptides using different adjuvant systems may also be considered. Using these peptides in other vaccine strategies, such as multivalent vaccines or MAP vaccines, could be investigated to reach to the best ones in terms of inducing the most protective immune response not only in *P. chabaudi* AS but also against other strains if these peptides potentially have had cross reactivity.

Appendix

Recipe for separating gel (for 10ml) in SDS-PAGE

Reagents	Amount
Bis / Acrylamide (30%)	2.7 ml
Resolving buffer (see Table 2, Tris pH 8.8)	2.5 ml
Sodium dodecyl sulphate (SDS) (8% w/v)	0.1 ml
Double distilled water (dd Water)	4.6 ml
Ammonium persulphate (APS) (10% w/v)	0.1 ml
TEMED	0.006 ml

Resolving buffer

Reagent	Final Concentration	Amount
Tris base	3 M	36.3 g
dd H_2O		60 ml
HCl (1 M)		pH was adjusted to 8.8
dd H_2O		exactly to100 ml final

Store at 4°C

Recipe for stacking gel (for 5ml)

Reagents	Amount
Acrylamide mix(30%)	0.83 ml
1.0M (Tris pH 6.8)	0.63 ml
SDS (10% w/v)	0.05 ml
dd Water	3.4 ml
APS (10% w/v)	0.05 ml
TEMED	0.005 ml

Electrophoresis buffer

Reagent	Final Concentration	Amount
Tris base	0.5 M	6 g
dd H_2O		60 ml
HCl (1 M)		pH was adjusted to 6.8
dd H_2O		exactly 100 ml

Store at 4°C

Sample buffer 2X

Reagents	Amount
Stacking buffer (see Table 4)	2.0 ml
dd H_2O	4.0 ml
Glycerol	1.6 ml
SDS (10%)	3.2 ml
Mercaptoethanol	0.8 ml
Bromophenol Blue	a few grains
Total volume	11.6 ml

Storage solution

Reagent	Final Concentration	Amount
4X Resolving buffer*	0.37 M	50 ml
10% SDS	0.1% (v/v)	2 ml
dd H_2O	to 200 ml	to 200 ml

SDS electrophoresis buffer

Reagent	Final Concentration	Amount
Tris base	25 mM	60.5 g
Glycine	192 mM	288.2 g
SDS	0.1%	20 g
dd H_2O	20 liter	20 liter

Buffers for purification of IgG1 and IgG2a

a: Binding Buffer (Sodium phosphate, 20mM)

2.4g $Na_2HPO_4.12H_2O$ (solution A)
2.83g $Na_2HPO_4.2H_2O$ (solution B)
For 100ml (0.1 M) of both solution of A and B, 57.7ml of solution A was mixed to 42.3ml of solution B. Distilled water was mixed to the above mixture up to 500ml for 20mM final concentration for pH 7.
For pH 8 93.2ml of solution A was mixed to 6.8ml of solution B and water then added up to 500ml.
For mouse IgG1 sodium chloride (AnalaR, BDH) up to 4 M was added to the binding buffer, 20 mM sodium phosphate.

b: Elution Buffer (20mM tri-sodium citrate buffer)

19.21g Citrate acid (0.1M) (solution A)
29.41g Sodium citrate (0.1M) (solution B)
For IgG2a, pH 4, and for IgG1 pH 5.8 to 6 were used.
For different pH (up to 500ml of the buffer) different volume of each solution was mixed to another one to reach desired pH. For example;
330ml of solution A was mixed with 170ml of solution B for pH 4.
124ml of solution A was mixed with 376ml of solution B for pH 5.8.

c: Purification buffer salt

49.9g NaCl (0.9M), pH 7.2 with proprietary stabilizer 0.083 M PBS were dissolved in 60ml degassed, deionised water.
Any unused buffer can be stored at 4C°.

d: Transfer Buffer

14.42 g	Glycine	(192mM)
3.03 g	Tris	(25mM)
1.0 g	SDS	
200 ml	Methanol	(20% v/v methanol)

Made up to 1 litre with deionised and distilled water. PH adjusted between pH 8.1-8.4

Phosphate buffered saline (pH 7.2)

60.0 g	$Na_2HPO_4.12H_2O$
13.6 g	$Na_2HPO_4.2H_2O$
8.5 g	NaCl

Made up to 1 litre with de-ionised and distilled water.

Giemsa's Buffer

3.0 g	Na_2HPO_4
0.6 g	KH_2PO_4

The pH was adjusted to pH 7.4 and made up to 1 litre with de-ionised distilled water.

Giemsa' stain

Giemsa' stain (Gurr BDH Ltd) was diluted 1:5 in Giemsa's buffer.

Coomassie Blue Stain

250.0 ml	Methanol
100.0 ml	Acetic acid
10.0 ml	Glycerol
1.0 g	Coomasie Blue

Made up to 1 litre with de-ionised and distilled water.

Coomassie Blue Destain

250.0 ml Methanol
100.0 ml Acetic acid
10.0 ml Glycerol
Made up to 1 litre with de-ionised and distilled water.

Trypan Blue (for viability test)

0.1 g Trypan Blue powder
5 ml PBS
Then filter.

Coating Buffer (Bicarbonate and Carbonate Sodium)

4.39 g $NaHCO_3$
5.3 g Na_2CO_3
0.2 NaN_3
The pH was adjusted to pH 9.4 with NaOH and made up to 1 litre with deionised distilled water.

PBS/Tween

0.5ml Tween 20 (polyoxyethylene sorbitan monolaurate)
Made up to 1 liter with PBS>

Tris Buffered Saline (TBS)

9 g NaCl
1.6 g Tris HCl
The pH was adjusted to pH 7.6 with HCL and made up to 1 litre with de-ionised distilled water.

RPMI 1640 stock medium

10.39g RPMI 1640 powdered medium (with L-glutamine) (Gibco)
5.94g N2-hydroxyethylpiperazine-N-2 ethane sulphonic acid (HEPES) (25mM)

Incomplete RPMI 1640 medium

To up to 1 litre with deionized and distilled water, filter sterilized (Millipore/Gelman filter 0.22μm size) and pH adjusted to pH 7.2

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