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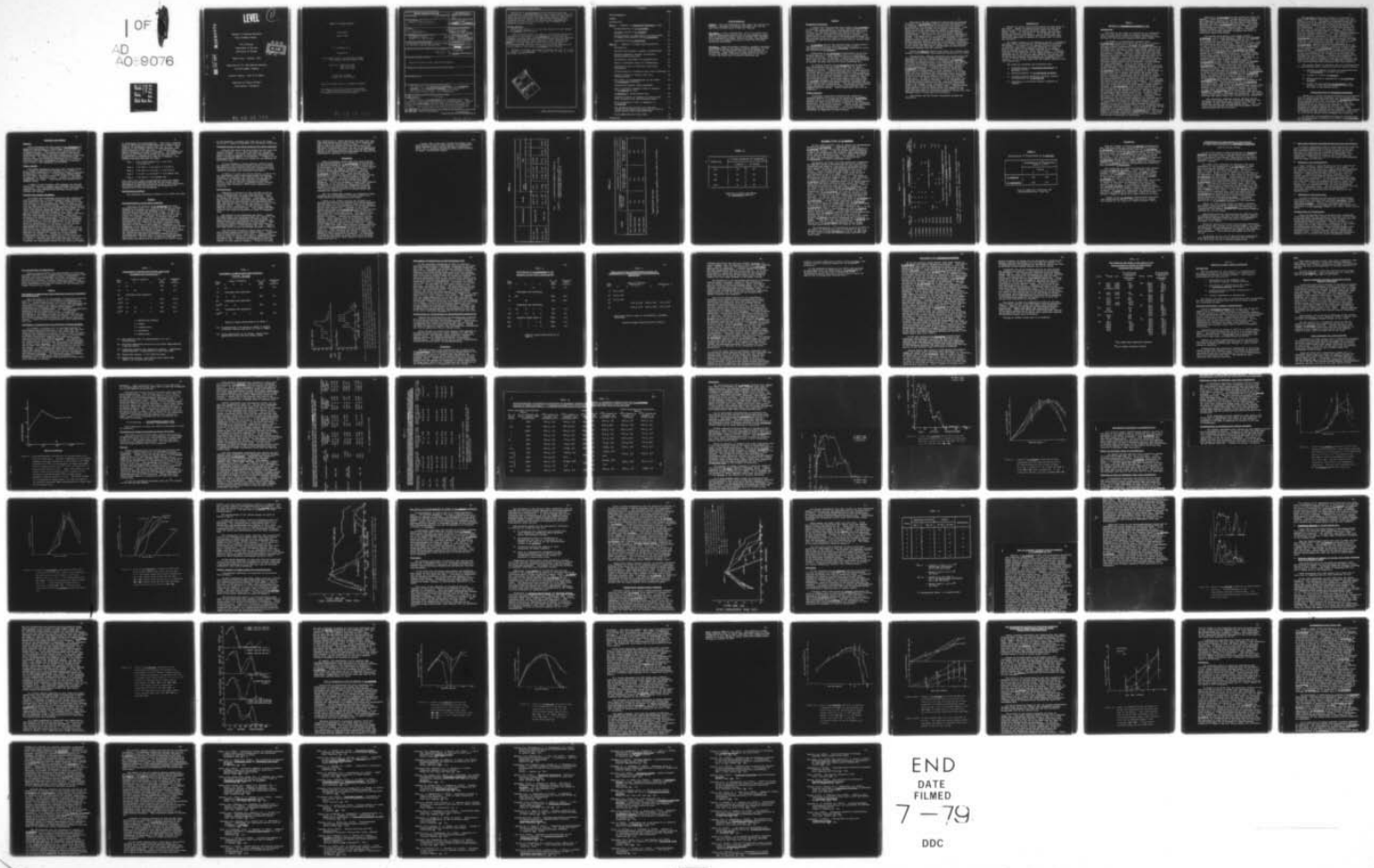

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Immunity to Malaria Parasites

Final Technical Report

R.S. Phillips
Department of Zoology
University of Glasgow

Report Date - January 1979

Supported by U.S. Army Medical Research and Development Command

Contract Number: DAJA 17-76 G9415

Approved for Public Release
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by

R. S. Phillips, Ph. D.

Supported by

US Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

Grants Nos. DAMD 17-76-G-9415
DAMD 17-76-C-9420
DAMD 17-77-G-9434

University of Glasgow
Glasgow, G12 8QQ, Scotland

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER (9)
4. TITLE (and Subtitle) (6) Immunity to Malaria Parasites.		5. TYPE OF REPORT & PERIOD COVERED Final Report. Dec [redacted] 1975 — Jan [redacted] 1978
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) (10) R. S. Phillips, Ph. D.		8. CONTRACT OR GRANT NUMBER(s) (15) DAMD 17-76-G-9415 DAMD 17-76-G-9420 DAMD 17-77-G-9434 (in part)
9. PERFORMING ORGANIZATION NAME AND ADDRESS University of Glasgow Glasgow, G12 8QQ, Scotland Dept. of Zoology	(16)	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62759A 3A762759A831.00.109 62770A 3M762770A802.00.083
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command Fort Detrick, Frederick, Maryland 21701		12. REPORT DATE (11) January 1978 (17)
		13. NUMBER OF PAGES [redacted] 65 pages
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) (12) 79p.		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

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

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

(u) Immunity; (u) Plasmodium falciparum; (u) Plasmodium chabaudi; (u) Plasmodium malariae; (u) Gametocytes; (u) Microtissue culture; (u) Cryopreservation; (u) Asexual blood stages; (u) Irradiation.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Human malaria:

Ring stages of P. falciparum were cryopreserved by snap-freezing in liquid N_2 (nitrogen) using glycerol as cryoprotectant. Gametocytes of P. falciparum differentiated over 8-10 days from ring stages put into culture and also from merozoites released in culture. Gametocytes grew less well in cells containing HbF. P. malariae in culture grew to the schizont stage only.

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Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

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Isolates of P. falciparum from 98 patients have been cryopreserved. From all patients serum samples were collected along with infected blood and from 25% of them 2-3 weeks later after minimal chloroquine treatment. Preliminary results confirmed earlier results that showed higher in vitro anti-parasitic activity in post-treatment sera although a few promoted parasite growth.

Rodent malarias

Increased numbers of K cells were detected in the spleens of P. chabaudi infected mice.

Mice infected within two weeks of irradiation showed some non-specific resistance to P. chabaudi. If infection was delayed to 22 days post-irradiation parasitaemias were enhanced. Enhanced parasitaemias were also seen in mice injected with spleen or bone-marrow cells immediately after irradiation. Immune serum had enhanced activity in irradiated mice. Cyclophosphamide treatment before infection also depressed the early stages of the primary P. chabaudi parasitaemia.

Adoptive transfer experiments confirmed the role of T cells in immunity to P. chabaudi. One role of the T cells is to act as helper cells.

Contents

Acknowledgements

Glasgow: The work described in this report was carried out with Dr V. McDonald as Research Assistant, and with the part-time technical help of Mrs C.D. Pearson.

The Gambia - M.R.C. Laboratories: Dr R.J.M. Wilson and I thank Dr Chris Jones and the late Mr A.K. Rahman for their help in selecting suitable cases of malaria and obtaining blood specimens from them, and Dr R.S. Bray, then Director of the M.R.C. Laboratories, and his staff for providing laboratory facilities.

Personnel: Under the present Contract a graduate zoologist, Miss Kathryn Budge, has replaced Vincent McDonald, who has gained his Ph.D. and joined Professor Irwin Sherman in California. Miss Budge is now fully integrated into the project and making a valuable contribution to the work. I would very much like to continue employing her.

Summary

Plasmodium falciparum

A method for cryopreserving ring stages of P. falciparum is described using glycerol at a final concentration of 17% (v/v). The parasitized blood with glycerol was snap frozen in liquid N_2 and after thawing were washed with sorbitol solution prior to putting the parasites into culture. (The thawed blood is now washed with decreasing concentrations of salt solution (4.5% ⟶ 0.9%) as an alternative to sorbitol.) Trophozoites and schizont-infected cells did not survive snap-freezing.

P. malariae grew to the schizont stage in microcultures but showed little if any reinvasion whereas a small contaminating population of P. falciparum completed $2\frac{1}{2}$ cycles and overgrew the P. malariae.

Gametocytes of P. falciparum differentiated in 14 of 42 microcultures of 4-5 days' duration and 7 of 8 cultures of 10 days' duration. Morphologically mature looking gametocytes took 8-10 days to develop. Gametocytes differentiated from rings put into culture and also from merozoites released in culture and grew less well in cells containing HbF.

One hundred isolates of P. falciparum from 98 patients were cryopreserved and are being tested both for their ability to survive cryopreservation and also to adapt to continuous culture. Eighty-seven per cent of these isolates completed cycle of growth and multiplication when a sample of the blood was put into microculture prior to cryopreservation. About 25% of the patients provided a serum sample at the time the parasitized blood was collected and a second 2-3 weeks later (return serum). Both serum samples are being tested for in vitro antiparasitic activity against homologous and heterologous parasite populations after the parasites had been thawed and put into microculture. Preliminary results showed higher inhibitory activity in return sera although a few promoted parasite growth. (These experiments are still in progress.)

Rodent malarias

Preliminary experiments indicated the presence of increased numbers of K cells in the spleens of P. chabaudi infected mice, i.e. non-phagocytic spleen cells which lysed chicken red cells in the presence of specific antibody. Increased K cells activity was not detected in the peripheral blood cells of malarious children. (These latter experiments were not entirely satisfactory.) A role for antibody-dependent cellular cytotoxicity in the control of malaria infections is suggested by those results.

Mice given 600 rads irradiation showed some non-specific resistance to P. chabaudi compared with non-irradiated mice if infected within two weeks of irradiation but enhanced parasitaemias if challenged 22 days after irradiation. Mice given 600 rads and injected with syngeneic bone-marrow or spleen cells on the following day showed no non-specific resistance to infection 9 days later but showed enhanced parasitaemias. The nature of the non-specific resistance is thought to be indicative of a stimulated reticuloendothelial system. The basis of the enhanced parasitaemias in the irradiated mice is not yet determined. Passively transferred immune serum had enhanced activity in irradiated recipients. Cyclophosphamide also depressed the early primary parasitaemia in mice given 300 mg/Kg or 150 mg/Kg 3 days before infection. In the former mice the patent parasitaemia was subsequently prolonged compared with the controls.

Heated P. chabaudi infected red cells, as a vaccine, gave slightly less protection than irradiated parasitized red cells.

Thymectomized, lethally irradiated and reconstituted NIH mice had more severe relapsing parasitaemias than controls confirming a role for T cells in immunity to P. chabaudi. Treatment of enriched spleen T cell populations from immune mice with antithymocyte serum in one experiment severely, and in another to a lesser extent, diminished their ability to protect syngeneic 600 rad irradiated recipients against a P. chabaudi challenge. Passive transfer of serum from recipients of enrich immune T cells, glass-wool filtered immune cells or normal spleen cells collected 11 or 14 days after the cell recipients were challenged showed protective activity in the sera of immune T and immune cell recipients on day 14 but the higher activity was in the sera of the latter mice. On day 11 only the sera from the immune cell recipients had measurable activity. It is suggested one of the protective activities of the adoptively transferred cells is in leading to the elaboration of protective antibodies, the T cells acting as helper cells. Adherent spleen cells separated from an immune spleen cell population by incubating the cells for 25 hours in petri dishes at 37°C gave good protection to irradiated recipients whereas the cells adhering after 16 hours gave no protection.

The results and the further experiments planned are discussed.

Introduction

Malaria is still a major health problem in many parts of the world. Nearly 500 million people live in areas where there are few or no control measures against the disease (W.H.O. 1977). In some parts of the world, such as India, malaria is showing a resurgence. It is thought that the development of a vaccine against malaria will greatly help reduce the importance of malaria as a major cause of death and morbidity.

The immune response to an antigen is subject to regulatory processes which at any one time can determine its intensity and nature. It appears to be possible in some situations to interfere with these regulatory mechanisms in order to promote one aspect of the immune response to an antigen, such as malaria parasites, rather than another. A detailed knowledge of the character of the immune responses to malaria parasites and of the ways in which immunity acts against the parasites will indicate which aspects of the immune response to the parasites should be preferentially stimulated by a vaccine. It is on this basis that the studies reported below were carried out.

The study has concerned the following areas:

1. cryopreservation of *Plasmodium falciparum* for *in vitro* culture;
2. gametocytogenesis of *P. falciparum* *in vitro*;
3. specific parasite inhibitory antibody response to populations of *P. falciparum*;
4. mechanisms of protective malarial immunity in rodents.

Part I

Immunity to Plasmodium falciparum in man

Introduction

This part of the study was carried out in cooperation with Dr R.J.M. Wilson (Medical Research Council) at the M.R.C. Laboratories, Fajara, The Gambia, West Africa, with the help of the resident technical and medical staff.

The Gambia is a hyperendemic malarious area. P. falciparum accounts for almost all the cases of malaria with the occasional case of P. ovale and P. malariae. Although malaria transmission occurs throughout the year in The Gambia the majority of cases occur during, and for 2 or 3 months after, the annual rainy season which usually lasts from July to October. There is a high mortality rate from the disease in children between 3 months and 3-4 years old. Children who survive these early years gradually build up an effective immunity. Up to adolescence this immunity, an antitoxic immunity, frequently is only sufficient to prevent children showing clinical symptoms in spite of carrying detectable parasitaemias but by adulthood a strong immunity has been established. Even at this stage in their development occasional parasitaemic episodes can be detected in the Gambians. Even in individuals living all their lives within one locality it takes several years of exposure to P. falciparum before an effective immunity to the parasite is attained and even in immune individuals the parasite may persist albeit at a chronic and usually sub-patent level. A number of possible explanations can be proposed to explain why the establishment of a beneficial level of acquired resistance takes so long and even when this has been achieved why parasites may still survive and multiply. Two possibilities will be discussed at this stage. First, it is likely that even within one locality there exists a large number of 'strains' of P. falciparum, each of which necessitates a specific immune response from the host and that it is several years before any one individual has been exposed to most of the existing strains within a locality. It might, however, be anticipated that sensitization to one 'strain' would promote a more rapid response to others. Secondly, during the course of the asexual blood phase of an infection following the bite of a single mosquito, the parasite may be able to change repeatedly its antigenic character in order to escape from the effects of the host's immune response against it, i.e. the parasite might undergo the pattern of antigenic variation so characteristic of the sleeping sickness trypanosomes and spirochaetes. It is quite clear that in the preparation of an antimalarial vaccine it will be very important to establish whether P. falciparum does exhibit the degree of antigenic variability suggested above. The study carried out in The Gambia and reported here was designed to try and give information in this area.

Immunity to P. falciparum in man, in part at least, is humoral. Cohen et al. (1961) pooled serum from a number of adult Gambians, separated out the IgG fraction and showed that this IgG had therapeutic activity when injected into children with acute P. falciparum infections. The immune IgG appeared to be antiparasitic when the parasite in red cells reached the mature (schizont) stage. Such passive transfer studies, however, could clearly for practical reasons not be readily extended to examine the effect of serum from single individuals.

The asexual blood phase of the monkey malaria, P. knowlesi, can be grown through a complete cycle in vitro (see Trigg, 1969). Multiplication rates of 5-8 times can be obtained when cultures are initiated with late trophozoites or early schizonts. Brown et al. (1968, 1970) and later Cohen and Butcher (1970) used this culture system to show that the serum from monkeys immune to P. knowlesi can reduce the rate of reinvasion of fresh red cells and hence the multiplication rate of P. knowlesi in vitro. The growth of P. knowlesi from ring to schizont stages within the red cell was not affected by the immune serum. This culture system therefore provided a sensitive measure of the antiparasitic activity of at least one type of antibody in the serum of immune monkeys. Phillips et al. (1972) extended these studies on simian malaria to P. falciparum in man. These workers devised an in vitro sub-culture technique for the asexual blood stage of P. falciparum. Infected blood was taken from infant Gambians and the asexual blood stage was grown through two and a half cycles. Multiplication rates of 3-8 times were achieved after reinvasion in the first cycle of development. It should be noted that the in vitro culture of P. falciparum poses two major problems not associated with P. knowlesi. First, the former parasite takes 48 hours to complete its asexual growth cycles in the blood compared with 24 hours for P. knowlesi and secondly, P. falciparum but not P. knowlesi withdraws from the peripheral circulation at the late ring stage. This necessitates that P. falciparum infected blood is introduced into in vitro culture at a relatively early stage in the parasite's asexual growth cycle. Phillips et al. (1972) subsequently examined the ability of 15 sera taken from adult Gambians to inhibit reinvasion in vitro by P. falciparum and of these sera 2 had clear inhibitory activity. This was the position when the study (Annual Report DAJA 37-73C-3492) was initiated. The results from this and Annual Report DAJA 37-75C-1620 are summarised.

The sub-culture technique used by Phillips and his co-workers was carried out on a relatively large scale and the amount of each serum sample used in each test for inhibitory activity was correspondingly large. Only a few tests would be carried out with each serum sample because limited quantities were available.

The *in vitro* culture was reduced to a microscale in conventional 96-well microtissue culture plates (see also Diggs et al.,1971). The growth and multiplication rate of the parasites in microcultures was monitored by Giemsa's stained smears made from the cultures, by measuring the incorporation of ^{3}H-isoleucine or production lactate into the supernatant. In excess of 100 sera from Gambians of all ages and 86 Nigerian sera were tested. The majority of sera exhibited a small degree of inhibition but only a few were strongly inhibitory. Some sera inhibited parasites from some children but not others. This variability in activity could be attributed to antigenic diversity in *P. falciparum* coupled with the fact that specific antibody responses to any antigenic type might only remain at a high level for a relatively short period. In order to match antiserum with a particular parasite population of *P. falciparum*, infected blood was cryopreserved while the infected child, after treatment, mounted an immune response to the population of *P. falciparum* of which a sample was cryopreserved. Subsequently, the cryopreserved blood was thawed and in microcultures the ability of the parasites to grow and multiply in the presence of pretreatment and convalescent sera observed. Although the cryopreservation procedure was crude sufficient of the thawed parasites survived and grow in culture to show that convalescent sera showed both specific inhibition of homologous parasites and some cross-reactivity with parasites from other children.

The present report is concerned with the following areas of investigation which followed on from our earlier studies.

1. Attempts to improve the method of cryopreservation of *P. falciparum* for culture purposes.

2. Attempts to culture *P. malariae*.

3. Differentiation of gamotocytes of *P. falciparum in vitro*.

4. Culture of cryopreserved *P. falciparum* in the presence of pre- and post-treatment serum from the donor of parasitized blood.

Cryopreservation of Plasmodium falciparum

Wilson et al. (1977) described preliminary investigations into the optimal freezing conditions for the cryopreservation of rhesus monkey blood infected with *Plasmodium knowlesi*. Their findings showed that the optimal conditions for cryopreservation of the red cell were not the best for preservation of all stages of the parasite. Ring stage parasites in dimethyl sulphoxide survived well after plunging into liquid nitrogen (N_2), a procedure which extensively damaged trophozoites and schizonts. The survival of trophozoites and schizonts was increased by holding them at -31^{o}C for 30 minutes before plunging into liquid N_2.

In this note the preliminary results of extending some of the observations of Wilson et al. (1977) to *P. falciparum* are described.

Materials and Methods

Parasite

Blood containing the ring stages of *P. falciparum* was collected from patients by venepuncture into heparinized Ringer's solution (Geiman et al., 1966) to give 10-17 i.v. of heparin per ml. The donors were mainly infants and children attending the out-patients clinic at the M.R.C. Laboratories, The Gambia, and their parasitaemia varied from 0.5% to 10%. The infected blood was washed twice with modified medium 199 (Wellcome) (Phillips et al., 1972) and the packed red cells resuspended in compatible Caucasian serum to give a 40% suspension of red cells.

Tissue culture

The washed parasitized red cells were cultured either in 2l flasks as described by Phillips et al. (1972) to provide early schizonts or in microtissue trays as described by Phillips et al. (1975). In some cases, ^{3}H-isoleucine (0.5 or 1 μCi - specific activity 26 Ci mmol^{-1}) was added to duplicate or triplicate wells. Samples for microscopic examination were taken. Blood smears were stained with Giemsa's stain.

A sample of each isolate, after washing, was put into culture and allowed to complete one schizogony and undergo reinvasion. The parasitaemia obtained after reinvasion was compared with that obtained with the same isolate after freezing and thawing.

Freezing and thawing procedures

The washed and resuspended red cells were aliquoted in 0.25 ml amounts into 2 ml screw-capped sterilin propylene tubes which were kept on ice. An equal volume of an ice-cold solution of the cryoprotective agent was added to each 0.25 ml sample of parasitized blood. The cryoprotectants used were 34% glycerol (v/v) (38 gm glycerol, 2.9 gm sorbitol and 0.63 gm NaCl in 100 ml distilled water) (Mitchell et al., 1972), and dimethyl sulphoxide (DMSO) at 20%, 24% or 30% (v/v) in Ringer's solution (Geiman et al., 1966). The glycerol solution was added drop-wise with gentle agitation. DMSO was added rapidly to the red cells. Ring stage parasites were plunged into liquid N_2 within 10-15 minutes of addition of the cryoprotectant. Red cells containing early schizonts, in one case (Experiment 30) the parasites in either glycerol at 17% or DMSO at 10, 12 and 15% were frozen at approximately 1°C per minute to -70°C and were then plunged into liquid N_2 and in the other case (Experiment 74) the parasites in either glycerol at 17% or DMSO at 10% were either plunged into methanol at -25°C and were held at this temperature for 15 minutes whereupon the ampoules were plunged into liquid N_2, or were plunged directly into liquid N_2. Thawing was carried out by transferring the tubes from liquid N_2 to a 37°C water-bath as rapidly as possible and gently agitating the tube for one minute. After thawing the tubes were spun at 250g for 3 minutes, the supernatant removed and the cells were washed

by resuspension and centrifugation. There were 5 washing steps as indicated below during which the concentration of sorbitol was gradually reduced from 17.5% to zero. Each addition of the washing solution was made drop-wise with gentle agitation of the red cells. In each step the washing solutions were added in sequence. The sorbitol was made up in phosphate buffer saline, pH 7.2, and sterilized by filtration. Between each step the red cells were deposited by centrifugation at 250g for 3 minutes. The washing solutions were kept ice cold.

Step 1 2 ml 17.5% sorbitol (S) + 2 ml 10% S + 2 ml 7.5% S.

Step 2 1 ml 10% S + 2 ml 7.5% S + 2 ml 5% S.

Step 3 1 ml 7.5% S + 2 ml 5% S + 2 ml 2.5% S.

Step 4 1 ml 5% S + 2 ml 2.5% S + 2 ml medium 199.

Step 5 1 ml 2.5% S + 4 ml medium 199.

After these five steps the parasitized cells were washed once more with modified medium 199 and the packed cells resuspended in compatible Caucasian serum to 40% haematocrit. The optical density (at 412 nm) of the supernatant was measured after each centrifugation.

Scintillation counting

The procedure described by Wilson et al. (1977) was used.

Results

Cryopreservation of ring stage parasites

More than 100 isolates of P. falciparum have been cryopreserved as part of a study on the effects of serum antibody on the growth and reinvasion of the parasite in microcultures (Wilson and Phillips, 1976). To date, 18 isolates (stored in glycerol at 17% or DMSO at 10% - see below) have been thawed and introduced into culture over a period of 12 months. Of these, 13 isolates have given adequate growth and reinvasion rates insofar as the parasitaemias after reinvasion in the microculture were comparable for the samples of the isolate cultured before and after freezing. In these isolates estimates of the viability of the thawed ring stages by examination of the morphology of the parasites (in Giemsa's stained blood smears) immediately after thawing and after a period of growth in culture indicated that 40-50% of rings survived the freezing and thawing process and grew normally. A proportion of the rings were apparently sublethally damaged and were morphologically abnormal after a period of growth in vitro (see Table 3). In 3 of these 13 isolates, immature gametocytes differentiated in cultures of the thawed blood.

In the remaining 5 isolates less than 10% of the rings were able to complete schizogony with subsequent reinvasion.

Cryopreservation of red cells infected with early schizonts

Infected red cells with ring stages were put into bulk culture (Phillips et al., 1972) and grown to the early schizont stages. The blood cells were then washed with medium 199 and resuspended in compatible Caucasian serum prior to freezing samples of the isolate. A sample of the resuspended blood cells was not frozen but was cultured in microtitre trays.

In both attempts to freeze schizont infected cells, the parasites after thawing showed no growth in culture. The parasites when harvested from bulk culture and after washing put directly into microculture continued to grow, completed schizogony and reinvasion took place.

In Table 1 is given the optical density at 412 nm for the supernatants after thawing and during the washing procedure for Experiment 74, together with the red cell count after the washing cells had been resuspended in serum. It can be seen that plunging the schizont-infected blood into liquid N_2 preserved almost 70% more red cells than freezing to -25°C before plunging into liquid N_2.

Cryoprotectant

Comparison of glycerol at 17%, and DMSO at 10% or 12% as cryoprotectant for ring stage parasites has not shown any significant difference between them. DMSO at 15% gave the poorest viability. Measurement of the optical density of the supernatant during washing showed that consistently there was more lysis associated with DMSO than the glycerol although the amount of lysis in all cases was small. Lysis in the DMSO preserved cells appeared to be a consequence of the red cells clumping after the first and second wash. The clumped red cells required more vigorous pipetting in order to resuspend them.

The results of one experiment are given. Isolate 29 (parasitaemia 5.2% mid-ring stages) was collected, the red cells washed and resuspended in Group B serum. A sample was put into microculture and the parasites went through schizogony approximately 36 hours later. After 48 hours of culture reinvasion had taken place and the mean parasitaemia of 6 microcultures was 4.3%. Samples were snap frozen in DMSO or glycerol as shown in Table 2.

Five days after freezing a sample each of 29-1->4 were thawed, washed and put into microculture. Schizogony occurred approximately 36 hours later. Parasitaemias after 71 hours in culture (see Table 2) suggested a higher reinvasion rate in parasites cryopreserved in glycerol and DMSO at 10%. ^{3}H-isoleucine incorporation was also highest in those parasite samples. It has to be noted

that observation on thawed parasites has shown that some ring stage parasites grow only slowly after thawing and fail to reach the mature schizont stage. It is very likely that these parasites do take up ^{3}H-isoleucine and hence ^{3}H-isoleucine uptake can only be an approximate measure of parasite viability after freezing and thawing and has to be combined with an assessment of viability by morphological appearance (see Table 3).

Discussion

These preliminary results show that snap freezing of ring stage parasites of P. falciparum in 17% glycerol or 10% DMSO followed by sorbitol washing to remove the cryoprotectant in about 70% of the samples tested enabled up to 40-50% of the rings to continue growth up to the schizont stages and undergo reinvasion. Similar results were obtained by Wilson et al. (1977) with P. knowlesi in DMSO at 10%. Snap freezing has the advantages of technical simplicity and of causing little lysis of red cells. It is not possible to say why in a small number of isolates freezing and thawing caused the death of 90% or more of the ring stage parasites. It will be necessary to make a more detailed examination of the optimal freezing conditions, rate of addition of cryoprotectant, temperature at which parasites are held when cryoprotectant is added, and the interval between addition of cryoprotectant and plunging into liquid N_2.

Our very preliminary attempts to cryopreserve early schizonts were unsuccessful but further studies are needed before any conclusions can be drawn.

Wilson et al. (1977) reviewed the literature on freezing and thawing intra erythrocytic malaria parasites and they point out that systematic study of the various factors involved in cryopreservation of malaria parasites has not been carried out. Pavanand et al. (1974) washed ring stages of P. falciparum with Tyrodes solution and cryopreserved them directly in liquid N_2 with 8%, 12% or 15% DMSO as cryoprotectant. The parasites were semi-thawed in a 56°C waterbath and then added to 5% glucose in saline. Washing was completed in isotonic saline. These workers, from their published figures, indicate almost 100% survival of the parasites after freezing in 12% DMSO and thawing although they do note finding degenerate parasites in some cultures. Haynes et al. (1976) also used a high glycerol concentration for cryopreserving P. falciparum and froze the parasites to -70°C in a mechanical freezer before transferring to liquid N_2. They estimated that 20-50% of ring stage parasites survived freezing and thawing, a figure more similar to those reported by ourselves. The advantage of plunging into liquid N_2 rather than slower freezing rates is the relatively small degree of red cell lysis associated with it.

Further work is directed towards developing a more rapid washing procedure using sorbitol or alternatively using hypertonic salt solution (Moryman & Hornblower, 1977), and to examining procedures which permit survival of larger intracellular stages.

TABLE 1

	Cryoprotectant	Rbc/ml	O.D. at 412 nm					
			After thawing	Wash				
				1	2	3	4	5
74-1 (P)	Glycerol 17%	2.12×10^9	0.26	0.219	0.06	0.055		
74-1 (-25°C)		1.28×10^9	0.765	0.432	0.225	0.155		
74-2 (P)	DMSO 10%	2.1×10^9	0.298	0.174	0.37	0.18	0.10	0.265
74-2 (-25°C)		1.25×10^9	0.695	0.341	0.31	0.16	0.168	0.164

(P) = Infected blood plunged in liquid N_2

(-25°C) = Infected blood held at -25°C for 15 min before plunging into liquid N_2

TABLE 2

Sample	% Parasitaemia* after 71 hrs in culture	Incorporation of ^{3}H-isoleucine c.p.m. 2 x 10^{-3}**	
		From Day 0 (1845 hrs) to Day 1 (0830 hrs)	From Day 2 (0845 hrs) to Day 3 (1100 hrs)
29-1 (17% glycerol)	7.4	4.7	2.4
29-2 (10% DMSO)	6.7	3.1	2.8
29-3 (12% DMSO	5.8	3.1	1.6
29-4 (15% DMSO)	4.8	2.4	1.1

*Parasites/100 red cells

**Mean of 3 cultures with exception of 29-3 (mean of 2 cultures)

TABLE 3

Isolate No.	% Viable Parasites (by morphology)	
	0 (hours)	18 (hours)
113	58	41
114	43	45
115	33	42
115	55	49

Viability of frozen and thawed
P. falciparum parasites

In vitro culture of P. malariae

In the Gambia clinical cases of malaria are predominantly caused by P. falciparum. A sprinkling of cases of P. malariae are seen and in these cases the parasitaemia is rarely above 0.025%. A case of quartan malaria presented in which the parasitaemia was unusually high, approximately 0.25%, and the opportunity was taken to observe the growth of the parasite in microculture. The patient, an 8 year old girl, was subsequently found to be infected with P. falciparum at a barely detectable level.

The patient was bled, the blood washed and resuspended in compatible serum as described earlier and 5 or 10µl of resuspended red cells put into wells of microtissue culture trays containing 250µl of supplemented medium 199 with 5mM Hepes added. The trays were incubated at 37°C in 5% CO_2 in air. 0.5µCi ^{3}H-isoleucine was added to two wells containing 10µl of blood at 2320h on day 0 and the cells were harvested on day 1 at 1200h. The growth of the parasites in culture is shown in Table 4 . The growth of the parasites followed a similar course in wells containing 5 or 10µl of washed red cells. It can be seen that P. malariae parasites completed growth to the mature schizont stage when individual merozoites were clearly discernible (daisy-head or rosette), over a period of 3 days. None of the mature schizonts seen had more than 8 merozoites. At the start of the culture a single P. falciparum ring stage was seen in 8000 red cells and later on day 0 two early trophozoites of P. falciparum were seen in the tail of the smear. At 2200h on day 1 young rings were present, often more than one ring per red cell, indicating reinvasion had occurred. It was not possible to determine for sure at that time whether the young rings were those of P. malariae or P. falciparum. As the culture continued it became clear that the majority of the young rings were P. falciparum. This brood of parasites subsequently went through schizogony between days 4 and 5 to give a third generation of P. falciparum rings in the culture. On days 4 and 5, of the parasites to be seen, a small proportion were P. malariae, mainly trophozoite stages. It was impossible to say whether these P. malariae parasites were a second generation derived from the schizont-infected cells seen to be maturing earlier in the culture or if they were very slowly growing P. malariae parasites introduced into culture as rings. It was clear that any reinvasion by P. malariae was minimal. From day 6 the cultures declined. By day 10 only a very few P. falciparum parasites of all stages, including gametocytes, were to be found in the smears.

The incorporation of ^{3}H-isoleucine is given in Table 5. The amount of incorporation was very similar to that in a parallel culture of P. falciparum set up at the same time. In this latter culture the infected blood contained 4.4% young rings.

TABLE 4

Growth of *Plasmodium malariae* in microtissue culture trays

Day (time hr)	Hours in culture	*P. malariae*					*P. falciparum*				*P. malariae* or *P. falciparum*	Total parasites/ 1000 red cells
		R	YT	LT	ES	MS	G	R	T	S	R	
0 (1430)	0		12	6	2		1	1				2.75
0 (2300)	8.5		4	9	4	3			2			
1 (2200)	31.5			6	6	5				1	17	5.8
2 (0900)	42.15			2	7	4					16	6.0
3 (1100)	68.5	Accurate count not possible										
4 (1115)	92.75		4(d)		6	1				10		3.2
5 (2200)	127.50		3	1				31	1			3.3
6 (1015)	139.75			1				10	20	1		5.3
7 (1045)	163.25	Few parasites - mainly *P. falciparum* trophozoites										
8 (1130)	188.00	Few parasites - mainly *P. falciparum* trophozoites and rings										
10 (1130)	236.00	Few parasites - *P. falciparum* gametocytes										

R = rings; YT = young trophozoites; LT = late trophozoites; ES = early schizont; MS = mature schizont; G = gametocyte; d = dead

TABLE 5

Incorporation of ^{3}H-isoleucine by *P. malariae*

	Incorporation of ^{3}H-isoleucine* c.p.m. 2 x 10^{-3}	
	Culture 1	Culture 2
P. malariae	2.9	3.2
P. falciparum	3.0	3.3

*0.5 µCi added Day 0 2245 hrs, and cells harvested Day 1 1200 hrs

Discussion

This one attempt to culture *P. malariae* demonstrated that conditions which were satisfactory for the culture of *P. falciparum* (the multiplication rate for *P. falciparum* after its first schizogony *in vitro* was more than ten times) permitted growth of *P. malariae* to the mature schizont stage but few of the merozoites on release were able to invade red cells. It is very likely that the incorporation of ^{3}H-isoleucine was predominantly by *P. malariae* as a parallel *P. falciparum* culture with a higher parasitaemia (4.4% compared with 0.275%) gave a similar degree of incorporation and each *P. malariae* parasite incorporated more radiolabel than individual *P. falciparum*.

The author has not found any record of other attempts to grow *P. malariae* *in vitro*. Trigg (1968) reported attempts to grow *P. inui* in culture. *P. inui*, a simian parasite, like *P. malariae* has a 72-hour asexual growth cycle in the red cells. Trigg found growth of *P. inui* to the schizont stage but little reinvasion took place. *P. malariae* is said to invade mature red cells preferentially. It is unlikely therefore that the failure to invade is the result of unsuitable red cells being available in culture. Two attempts by the author to grow *P. vivax* *in vitro* in petri dishes following Trager and Jensen's procedure (Trager & Jensen, 1976) gave growth of the parasite to the schizont stage but with little invasion. *P. vivax* has a preference for immature red cells and for this reason may be difficult to grow in culture without a supply of blood with a high reticulocyte count.

Samples of the *P. malariae* infected blood used above were cryopreserved and at a later date will be thawed and cultured by the Trager and Jensen procedure (1976).

Differentiation of gametocytes in microcultures of human blood infected with Plasmodium falciparum

(with Dr R.J.M. Wilson)

As the early stages of gametocytogenesis of Plasmodium falciparum are sequestered in the spleen and bone-marrow (Thomson & Robertson, 1935; Garnham, 1966) and are rarely seen in peripheral blood smears, observations on gametocyte development in cultures of infected peripheral blood are of especial interest.

Row (1928) first described the appearance and early development of the gametocytes of P. falciparum in blood cultures using the technique of Bass and Johns (1912). Since then several workers (Trager, 1971; Haynes et al., 1976; Mitchell et al., 1976; Phillips et al., 1976; Smalley, 1976; Trager & Jenson, 1976) have briefly reported the appearance of gametocytes in other forms of cultures of P. falciparum infected blood. Observations on the development of gametocytes in microtissue cultures (Phillips et al., 1975) of peripheral blood from Gambian children infected with P. falciparum were reported by Phillips et al. (1976) and Smalley (1976) and in a previous Annual Report (DAJA 37-73 C3492). Smalley (1976) noted that the development of ring stages to morphologically mature gametocytes took about 10 days in vitro as seems also to be the case in vivo (Garnham, 1976; Hawking et al., 1971). In the following account, experiments are described which show that gametocytes of P. falciparum can develop both from ring stage parasites introduced into culture and also from merozoites released during subsequent schizogonies in vitro. Preliminary observations on the incorporation of ^{3}H-isoleucine by gametocytes of P. falciparum in vitro are also described.

During the rainy season months of August, September and October, blood containing ring-stage parasites but not detectable gametocytes of P. falciparum were collected by venepuncture from Gambian children into heparinized Ringer's solution (17 i.v./ml blood).

Uninfected blood was collected from the umbilical cord of Gambian newborns at the Royal Victoria Hospital, Banjul. These samples contained 90-95% red cells with haemoglobin F (Hb F), as determined by the acid-elution technique of Kleihauer et al. (1957).

Blood was cultured in 96-well flat-bottomed microtissue culture trays (see 1 below) or in 2-litre flasks (see 2 below). The medium was TC-199 supplemented as described by Phillips et al. (1972). Hepes at 5mM was also added to the medium in some experiments. The cultures were gassed with 5% CO_2 in air.

In cultures of 4-5, 6, or 10 days duration, gametocytes were observed at the end of the culture in 14 out of 42, 2 out of 3, and 7 out of 8 cultures respectively.

1) Ring stage parasites transferred directly into microculture

In initial experiments, 10µl of whole heparinized blood was added to 250µl supplemented TC-199 in each well of the tray. Our recommended practice now is first to remove the plasma, wash the red cells twice with Hepes-buffered TC-199, and resuspend the packed washed cells to the approximate blood volume (40% haemotocrit) in Caucasian AB, A or B serum as appropriate. Subsequently, 10µl of the washed and resuspended cells are added to each well.

2) Subculture into microcultures

Larger volumes of infected blood were first cultured for 12-24 hours in 2-litre flasks (Phillips et al., 1972). At the schizont stage the cells were removed and schizont-infected red cells were concentrated using Plasmagel (3% w/v gelatin - Laboratoire Roger Bellon, Neuilly) (Pasvol et al., 1978) as follows. Erythrocytes and schizonts, separated from the culture medium by centrifugation (250g for 5 min), were resuspended to a haematocrit of 40% with supplemented TC-199 in a 15ml centrifuge tube. The resuspended cells were mixed with half their volume of plasmagel and left to stand for 15 min at 37°C. After sedimentation two distinct phases were visible with the schizont-infected red cells concentrated (50-90%) in the upper layer. The concentrated parasitized red cells were washed with medium 199 and resuspended in Caucasian serum as described above before addition to the microcultures.

3) Addition of cord blood cells

In order to demonstrate that gametocytes differentiate from merozoites released during schizogony *in vitro*, washed cord blood cells were added to infected blood shortly before schizogony *in vitro*. Parasites, including gametocytes, subsequently found in Hb F-containing cells must develop from merozoites released *in vitro* (Diggs et al., 1971; Pasvol et al., 1976).

Incorporation of ^{3}H-isoleucine

Small and mid-ring forms of parasites were resuspended in AB serum and 10µl were placed in microtissue culture wells containing 200µl supplemented TC-199, 40µl AB serum, and 10µl TC-199 containing 0.5µCi ^{3}H-isoleucine (26 ci/mmol - Radiochemical Centre, Amersham). Smears were made of the cultured cells at intervals until termination of the culture. The smears were fixed, washed twice in methanol, and autoradiographed by dipping in Ilford Nuclear emulsion (K5) as described by Wilson (1974). The autoradiographs were developed after one week's exposure and were stained with Giemsa's stain. Destaining of the emulsion was assisted by dipping in a 1:3 mixture of ethanol and phosphate buffer.

Size distribution of gametocytes

Giemsa-stained parasites were photographed on 35 mm film (Recordak 'Microfile') at a magnification of approximately X530. Infected cells were photographed at random in successive microscopical fields except that only cells containing a single parasite were selected. Negative images were enlarged ten times and parasite outlines traced onto graph paper. Relative growth measurements were made by cutting out and weighing the images (Wilson et al., 1977).

Results

Development of gametocytes from ring-stage parasites introduced into microculture

The time course of the development of gametocytes from mid-ring forms of parasites in a microculture is summarised in Table 6. Immature gametocytes could be distinguished from late trophozoites after 42 hours of culture, when schizogony and reinvasion was complete and immature gametocytes were easily recognisable as oral-shaped parasites with pigment characteristically splinter-like. The chromatin was apparently dispersed along the longitudinal axis. The subsequent development of the gametocytes was one of elongation, maintaining an oral shape although in some cases one side of the gametocyte was straight rather than bowed. By 113 hours both elongate gametocytes and shorter, less mature ones were present. After 220 hours morphologically mature gametocytes were seen.

Development of gametocytes from merozoites released in vitro

In a number of cultures of 8 or more days duration in which gametocytes differentiated, it was found that they were either first detected at the end of the culture when the parasites had completed two asexual cycles, or that the number of gametocytes increased between days 4 and 8 of the culture. Both these observations suggested that gametocytes could develop from merozoites released in vitro. An experiment that shows this is summarised in Table 7. Gametocytes were not detected until 125 hours when the parasites had undergone two asexual cycles in vitro; the gametocytes were immature and so scanty that they could only be detected in the tail of the smears. As gametocytes that develop in culture from ring stages can be distinguished within 50-60 hours, it is likely that immature gametocytes first seen at 125 hours had developed from merozoites released at the first schizogony and reinvasion in vitro. The increased numbers of immature gametocytes seen at 191 hours could be attributed to differentiation of gametocytes from merozoites released after about 100 hours of culture.

TABLE 6

Development of gametocytes directly from rings introduced into microculture[1]

Time (hr)	Stage of Parasite				No. of red cells	Parasites /100 r.b.c.'s
	R	T	S	G		
0	28				485	5.7
30	6	36			765	5.5
42	Schizogony and reinvasion					
54[2]	54			+	313	17.25
66[3]	43			+	450	9.5
90[4]	18	25		2	425	10.6
113[5]	16	13	7	4	488	8.2

+ = gametocytes present;

R = rings;

T = trophozoites;

S = schizonts;

G = gametocytes.

(1) Each parasite count is representative of 2 or 3 microcultures.

(2) Two early gametocytes detected in 40 fields (approximately 4,000 red cells).

(3) Occasional schizont and trophozoite present. Gametocytes easily detected - 6 gametocytes in 5,100 red cells.

(4) Gametocytes present - 7 in 6,400 red cells.

(5) Gametocytes present - some clearly more mature than others - 30 in 9,400 red cells.

TABLE 7

Development of gametocytes from merozoites released in vitro

Time (hr)	Stage of Parasite R	T	S	G	No. of red cells	Parasites /100 r.b.c.'s
2	24				538	4.4
36	Schizogony and reinvasion					
81	1	27			496	5.6
101	Schizogony and reinvasion					
125(1)	13	2		+	449	3.3
150	Schizogony and reinvasion					
192(2)	9	10		1	320	5.9

Parasite stages abbreviated as in Table 6

(1) No gametocytes in 50 fields in middle of smears: occasional immature gametocyte in tail of smear.

(2) Eleven gametocytes in 50 fields; mainly early gametocytes with a few more mature forms.

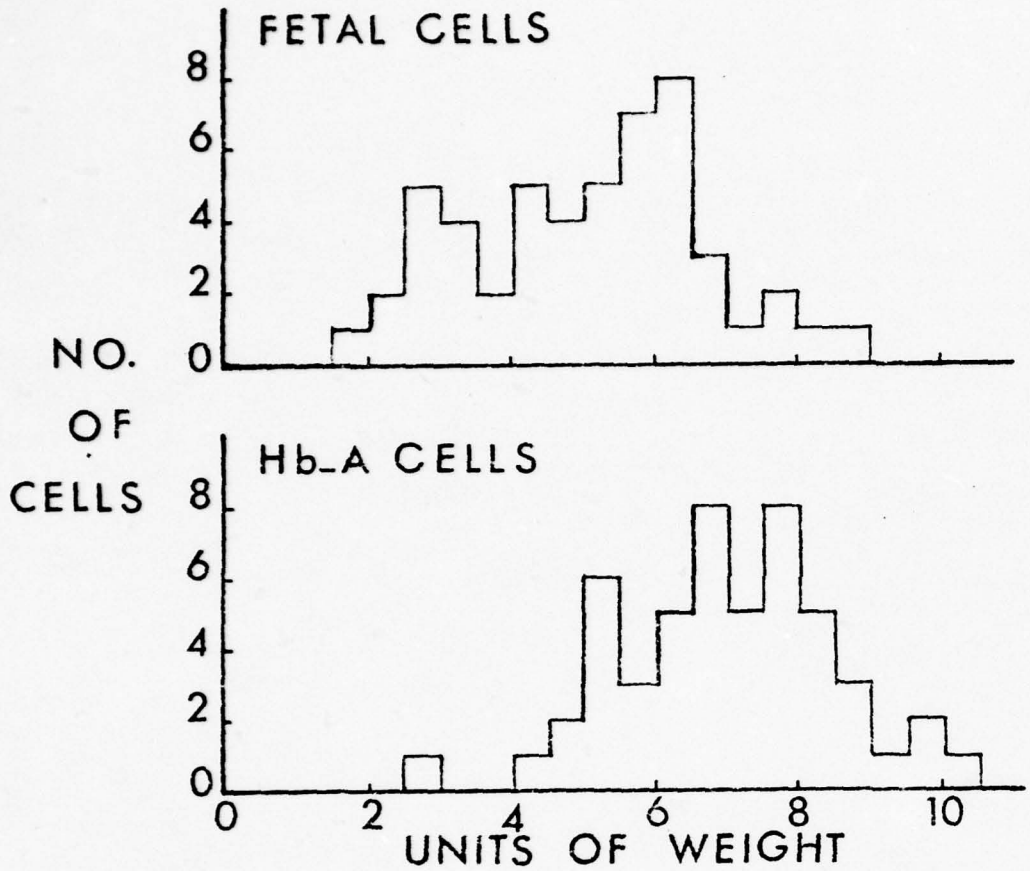


Figure 1. Size distribution of gametocytes in a culture of mixed cord and adult blood. Enlarged photographic images were prepared and weighed for 51 gametocytes in each of the HbF and HbA cell categories. The probability that the gametocytes in HbA and HbF cells were derived from populations of similarly sized parasites was <0.01 by the Wilcoxon Rank test.

Development of gametocytes in HbF-containing cells

In the experiment summarised in Table 8, schizont-infected blood prepared by plasmagel concentration was mixed with cord blood to give a 1:20 ratio of HbA:HbF containing red cells. Schizogony and reinvasion commenced within a few hours of the culture being set up. By 77 hours a second schizogony had occurred and early gametocytes were present, both in red cells containing HbA and in those containing HbF. After 170 hours in culture, 200µl of medium was replaced when the supernatant fluid in the cultures were clearly acid. At 170 hours and 210 hours gametocytes at different stages in development could be distinguished; frequently two gametocytes, and very occasionally three, were present in a single red cell. At this time, all the asexual forms in this particular experiment had died. The gametocytes in the HbA red cells were frequently bigger and further developed than those of HbF cells (see Fig. 1). This provides further evidence that fetal red cells provide a less favourable environment for the growth of the parasite (Diggs et al., 1971; Pasvol et al., 1976; Wilson et al., 1977). The possibility that the larger gametocytes in the HbA cells were derived from the original inoculum and not from the parasites that invaded HbF-containing cells can be excluded for the following two reasons. First, the culture (see Table 8) was inoculated with a plasma gel layer and no immature gametocytes were evident amongst the concentrated schizonts. Secondly, even if gametocytes had been present in the plasma gel layer they would have been morphologically distinguishable by the time of the first schizogony *in vitro* and no such forms were seen.

Incorporation of ^{3}H-isoleucine by differentiating gametocytes

Gametocytes differentiated from mid-ring stage parasites in a culture containing ^{3}H-isoleucine. At intervals up to 81 hours smears were made and autoradiographed. Grain counts at selected times (Table 9) indicated that the isotope was incorporated into both asexual and sexual stages. During the first 60 hours of culture, less isoleucine was incorporated per cell by gametocytes than by asexual forms.

After 81 hours of culture many asexual forms appeared to be dead but grain counts for intact schizonts at this time differed little from maximum values reached by schizonts at earlier times. By contrast, gametocytes and a slowly growing new brood of rings had continued to incorporate ^{3}H-isoleucine. The preparations did not permit morphological distinction between micro- and macrogametocytes.

Discussion

The early stages in the differentiation of gametocytes of *P. falciparum* from merozoites do not usually occur in the peripheral blood but in the spleen and bone marrow and morphologically mature or nearly mature gametocytes are only released into the peripheral circulation after 9 or 10 days (Garnham, 1966). After 9 days in culture, gametocytes were present that had assumed the appearance of mature gametocytes (although their infectivity to mosquitoes and the ability of the microgametocytes to exflagellate were not tested).

TABLE 8

Microculture of P. falciparum in the presence of red cells containing HbF

Time (hr)	R	T	S	G	No. of red cells	Parasites /100 r.b.c.'s
0			18		531	3.4
	Schizogony and reinvasion					
17	128				1064	12.0
56		54			532	10.2
	Schizogony and reinvasion					
77	39	15	6	1	878	6.9
89	8	11	3	9	862	3.6
170	(Asexual stages dead)			16	2000	0.8
216	"	"	"	17	2000	0.9
296	"	"	"	8	1000	0.8

Parasite stages abbreviated as in Table 6.

TABLE 9

Grain counts from autoradiographs showing the incorporation of ^{3}H-isoleucine by differentiating gamotocytes

Time (hr)	Stage of Parasite			
	Generation 1			Generation 2
	R	T S	G	R
9	3.6 ± 2.73			
17	7.5 ± 2.30			
24	8.3 ± 3.14			
67		35.7 ± 6.39	22.9 ± 5.26	3.6 ± 0.70
81		34.5 ± 7.43	30.1 ± 3.80	6.8 ± 1.94

(Each grain count is mean of 10 parasites ± standard deviation)

Parasite stages abbreviated as in Table 6

Although gametocytes may grow more slowly in vitro, our results suggest that the estimate of about 9-12 days (Garnham, 1966; Smalley, 1976) for the maturation of P. falciparum gametocytes is reasonable. A shorter maturation period has been suggested by Mitchell et al. (1976). It is clear from Smalley's (1976) and our observations that the first 24-30 hours of gametocyte growth occurs in the peripheral blood and that at the end of this period sexual forms in common with late rings withdraw to the deeper tissues.

The morphology of the developing gametocytes in Giemsa's stained smears is broadly similar to that described by earlier workers (Thomson & Robertson, 1935), although we have not noted any so-called Garnham bodies, i.e. thick filaments occasionally present as inclusions in the red cell, together with developing gametocytes (Garnham, 1966). Despite their stunted growth in fetal red cells, gametocyte characteristics, such as elongation and splinter-like pigment formation were apparent.

None of the parasite donors for the experiments described above carried detectable gametocytes in their peripheral blood when they were bled (where gametocytes were present in peripheral blood at bleeding further gametocytes differentiated in culture). In some of our cultures, gametocytes differentiated directly from rings put into culture and a further generation of gametocytes subsequently developed from merozoites released during the completion of an asexual cycle in vitro. In other cultures, gametocytes were detected after 7 or 8 days incubation but not earlier. Therefore in the former group of cultures it is likely that the initiation of gametocytogenesis occurred in vivo and once started the process continued in vitro. In the latter cultures, although the stimuli for gametocytes development again may have been received in vivo, it is also possible that it occurred in vitro. We have no information as to the nature of the initiatory process other than to comment that the conditions in some of the long term cultures became adverse to parasites in the asexual cycle; the culture medium became very acid and the asexual stages of the parasite were either dead or their numbers had fallen substantially. Recently, Carter & Beach (1977) reported that gametocytes which developed long term cultures of P. falciparum could be induced to exflagellate. The numbers of the mature gametocytes were particularly high in cultures in which the serum content of the culture medium had been changed from 10% to 50%. They suggest that high serum content and prolonged maintenance of cultures for at least two weeks may be important in the production of mature gametocytes.

It is not clear from our preliminary experiment with ^{3}H-isoleucine whether gametocytes require less isoleucine than asexual forms or if the latter simply grow more quickly. Microcultures, however, provide a means to compare such aspects of the metabolism of both forms as well as to investigate other aspects of the biology of gametocytes, e.g. an analysis of the processes which initiate gametocytogenesis or of the changes in the maturing gametocytes which result in their release into the peripheral circulation.

Further, if mature gametocytes could be grown *in vitro*, they could be used for infecting mosquitoes for sporozoite production.

It was reported by Haynes et al. (1976) and elsewhere in this report that the gametocytes of *P. falciparum* can differentiate in culture from cryopreserved blood. Cryopreserved blood, therefore, could be used to carry out some of the investigations outlined above.

Experiments with Plasmodium falciparum

In the two previous Annual Reports short-term culture of P. falciparum from Gambian children was described. Microtissue culture trays were used for examining the effect of sera from immune adult Gambians on the growth and multiplication of the parasite in vitro. A high proportion of the sera had relatively little inhibitory activity on P. falciparum parasites from various infected infants and children. It was suggested that one possible reason for the lack of inhibitory antibody activity in so many sera was that within the locality in the Gambia where parasites and sera were collected populations of P. falciparum existed which represented a range of antigenic types and that the detectable antibody response of an individual to specific populations of P. falciparum may be short lasting. In order to relate an antibody reponse to a particular population of parasites, P. falciparum infected blood was cryopreserved while the patient's parasitaemia declined following minimal chloroquine administration and the antibody response to the parasites developed. Two or three weeks after chemotherapy a further serum sample was collected from the patient. This latter serum sample and one collected at the time the infected blood was drawn were then compared for their inhibitory activity against the cryopreserved blood after it had been thawed out and put into microcultures. Preliminary results of this experimental approach were described in the previous Annual Report and in Nature (1976, Nature 263, 132). In the present investigation it was planned to extend these observations. First, the procedure for cryopreserving P. falciparum infected blood was improved as described below. Secondly, about 100 samples (isolates) of infected blood were cryopreserved and serum samples obtained at the time the infected blood was collected. The patients were usually given chloroquine (5 mg base per kg) and asked to return 2 or 3 weeks later to provide a further serum sample. Approximately 47% of the patients returned and of these 26% refused to give a second blood sample. The procedure for collecting, washing and resuspending the blood cells in Caucasian serum is described below. For each isolate collected samples were cryopreserved and a sample was put into microculture and the growth of the parasite followed by its morphological appearance in Giemsa's stained blood smears and by the level of incorporation ^{3}H-isoleucine over a 12-hour period before and after reinvasion (Wilson and Phillips, 1976; Annual Report DAJA 37-75 C1620).

Of the 100 isolates of P. falciparum infected blood collected, which included two isolates from each of two individuals on different occasions, 87% grew from ring to schizont stage and underwent reinvasion and made significant incorporation of ^{3}H-isoleucine before and after reinvasion. Of the remaining isolates, 80% made little growth and the rest grew up to the schizont stage but failed to reinvade.

Shortly before this period of investigation was started, Trager and Jensen (1976) described their procedure for the contunous culture of P. falciparum using the candle jar technique. Dr Wilson and I decided, therefore, that it would be useful to gain experience with Trager and Jensen's culture procedure and in due course assess the ability of many of our samples to adapt to continuous culture as well as test the inhibitory activity of sera from children before and after recovery from their malaria infection. After a number of attempts over a

period of months, Dr Wilson has now succeeded in culturing two Gambian isolates continuously for more than two months. It is hoped that now the technique for continuous culture has been established further Gambian samples can now be tested.

Relatively few of the isolates have so far been tested. The procedure was similar to that described in the previous Annual Report. The cryopreserved infected blood was thawed, washed, and the blood cells resuspended in compatible Caucasian serum. Ten microlitres of the resuspended were put into microtissue culture trays in supplemented medium 199. Serum, either 50 µl or 100 µl, was added to the wells in the tray at the start of the culture or when the parasites had grown to trophozoite or early schizont stage. The total volume of each microtissue was 260 µl. Parasites were grown in sera from the donor of the parasites (homologous sera) or sera from other donors (heterologous sera). The sera from patients 2 or 3 weeks after treatment are referred to as return sera. The effect of the various sera on the parasites was determined by measuring the multiplication rate from Giemsa's stained blood smears after schizogony and reinvasion and by measuring the ^{3}H-isoleucine uptake in the cultures over a 12-18 hour period after reinvasion has occurred.

The results to date are summarised in Table 10 and they show a similar picture to the previously reported preliminary results. Many of the return sera showed some inhibitory activity both against homologous and heterologous parasite populations. There were again some unexpected results. With parasite populations 80 and 87 (both thawed and cultured on the same day) four of the return sera tested gave enhanced ^{3}H-isoleucine uptake after reinvasion.

Testing of further return sera is in progress.

TABLE 10

Sera added to the wells at the start of the microcultures or within 19 hours before reinvasion had commenced

Cells	**Serum	(µl)	^{3}H-isoleucine incorporation as % of homologous serum
48	48-1	(100)	100
	48-2	(100)	38.5
	75-2	(100)	38.5
	B control	(100)	77
62	62-1	(100)	100
	48-2	(100)	76.5
41	41-1	(100)	100
	41-1		100
	41-2		77.9
	61-1	(100)	102.1
	61-2	(100)	40.0
*52	52-1		100
	52-2		68
	AB serum		90
*75	75-1		100
	75-2		108
	AB		108
96	96-1		100
	96-2		23.4
	102-1		71.0
	102-2		38.7
	26-1		81.4
	26-2		38.7

Cells	Serum	^{3}H-isoleucine incorporation as % of homologous serum
102	102-1	100
	102-2	69.2
	96-1	176.9
	96-2	88.5
	26-1	115.3
	26-2	100
80	80-1	100
	80-2	180
	87-1	350
	87-2	260
	23-1	360
87	87-1	100
	87-2	198
	74-1	119.2
	74-2	136.5
	23-2	34.6
	82-1	17.3
	82-2	117.3
52	52-1	100
	52-2	62.2
	76-1	40.5
	76-2	40.5
	27-1	125.7
	27-2	85.1
	109-1	71.6
	109-2	78.4
	66-1	75.6
	66-2	43.2

*Sera added when reinvasion underway

**50 µl unless otherwise stated

Part II

Immunity to Rodent Malaria Parasites

Introduction

The work described in this report is a continuation of the study described in the Annual Report for Contract DAJA 37-73-C3492 and Contract DAJA 37-75-C1620. Four lines of investigation have been pursued:

a. investigation of the lymphoid cells involved in immunity to murine malaria;

b. the effects of irradiation on the course of *Plasmodium chabaudi* infections;

c. antibody-mediated cellular cytotoxicity in malarious mice;

d. further experiments on immunization.

The results from each area of investigation will be described, and the conclusions arising from each will be drawn together in a concluding section.

Material and methods - general considerations

Parasite *Plasmodium chabaudi* (A/S strain) was cloned and supplied by Dr D. Walliker, University of Edinburgh. In C57Bl and NIH mice of 6 weeks and older this parasite produces a primary parasitaemia lasting 10-14 days with a peak parasitaemia in the range of 25 to 75%. Following the primary parasitaemia there are occasional patent relapses and 8-10 weeks after infection most of the mice achieve a sterile cure. In our laboratory the asexual blood stage of the parasite grow synchronously, schizogony occurring around midnight. Late trophozoite and schizont stages tend to leave the peripheral circulation.

Parasitized blood is stored at -70°C or at -198°C using glycerol at a final concentration of 17.5% as a cryoprotective agent. Cryopreserved blood was thawed in a 37°C water bath and injected into mice. Subpassages were subsequently made every 3 or 4 days until the parasitaemia was patent.

Heparin at a final concentration of 10-17 i.u./ml blood was used as the anticoagulant and dilutions of infected blood were made in Hanks' balanced salt solution or tissue culture medium as indicated.

Parasitaemias were assessed by examination of tail blood smears stained with Giemsa's stain and recorded as parasitized red cells per 10^4 or 10^5 red cells. Mice were injected with parasitized red cells intraperitoneally (i.p.) or intravenously (i.v.) as indicated in the text. All infections were initiated with infected red cells.

Mice

Three inbred strains of mice were used at different times. C57Bl mice were supplied by Centre for Tropical Medicine, Easter Bush, East Lothian, and NIH mice by Anglia Laboratories. A_2G mice are bred in the Department of Zoology, University of Glasgow.

All mice were fed on Oxoid diet 41B and food and water were given ad libitum. They were maintained at around 22°C with 12 hours light from 0800 to 2000 hours.

Antibody dependent cellular cytotoxicity during malaria infections

A mechanism which may be involved in the death of malaria parasites is antibody-dependent cellular cytotoxicity (ADCC), a non-specific cytotoxic reaction of lymphoid cells on target cells in the presence of antibody directed against the target cell (MacLennan & Harding, 1970; Perlmann & Perlmann, 1970; Perlmann, 1975). The effector cells are apparently non-phagocytic, non-adherent lymphocytic cells (Perlmann & Perlmann, 1970; Calder et al., 1974; Sanderson & Taylor, 1976) and are referred to as K cells. There is evidence that K cells are neither B cells (Wisloff & Freland, 1973; Calder et al., 1974) not T cells (Van Boxel et al., 1972) and may belong to the population of null cells (Greenberg et al., 1973) which exists in the lymph nodes and spleen of normal animals (Stobo, Talal & Paul, 1972). IgG antibody is required for ADCC (Calder et al., 1974). Immune complexes containing IgG may inhibit the reaction (Jewell & MacLennan, 1973).

The activity of K cells may be important in the control of tumour development in mice (Ghaffer, Calder & Irvine, 1976) and also in auto-immune disease (Calder et al., 1973).

We have carried out a preliminary study to look for evidence of altered K cell numbers in the spleens of mice during a P. chabaudi infection. Chicken red blood cells (CRBCS) as the target cells have been used successfully in assaying K cell activity of mouse spleen cells (Ghaffer, Calder & Irvine, 1976) and were used in our study.

Assay procedure for K cell activity

Suspensions of mouse spleen cells in Medium 199 containing 5% foetal calf serum (5% FCS-199) were prepared as described previously (Annual Report DAJA 37-75-C1620), and incubated in 9 cm sterile plastic petri dishes (Sterilin) for 2 hours at 37°C in air. At the end of this period the non-adherent cells were removed from the petri dishes, washed and resuspended in 5% FCS-199.

Blood was drawn into a heparinized syringe (10 i.v. heparin/ml blood) from 6-12 weeks old Leghorn chickens and the blood cells washed three times in phosphate buffered saline (PBS), pH 7.2 A suspension of 2×10^8 CRBCS/ml in PBS was prepared and 0.1 ml of this suspension was added to 0.1 ml of 100 µCi ^{51}Cr labelled sodium chromate (Radiochemicals, Amersham, specific activity in the range 100-350 µCi/mg

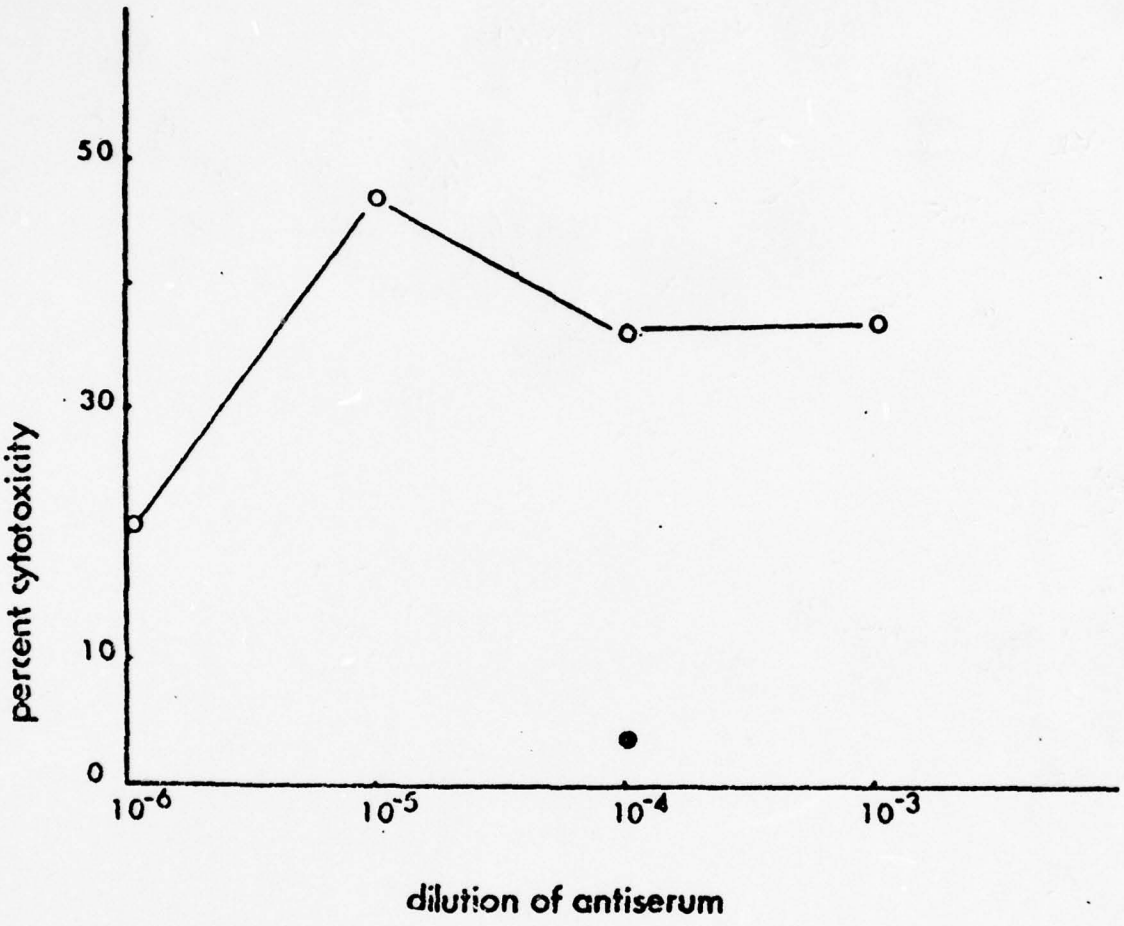


Figure 2. Antibody-dependent cytotoxicity of pooled spleen cells from two C57Bl mice. Cultures containing 2 x 10^6 splenic lymphoid cells were incubated at 37°C with 5 x 10^4 CRBCS labelled with ^{51}Cr, in the presence of rabbit anti-CRBC serum at concentrations of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}, or normal rabbit serum at 10^{-4}. The % cytotoxicity of the spleen cells was calculated as $\%^{51}Cr$ release in the presence of antiserum minus $\%^{51}Cr$ release in the presence of normal serum (o —— o). The $\%^{51}Cr$ release in the presence of normal serum (•) was the same as in controls containing CRBCS and antiserum or normal serum but no spleen cells.

chromium). After incubation for 1 hour at room temperature the labelled CRBCS were washed three times in PBS and resuspended to 5×10^5 CRBCS/ml in 5% FCS-199.

Cultures were carried out in 15 ml glass screw top tubes (Flow Laboratories). Each culture was of a total volume of 0.4 ml which was made up of 5×10^4 CRBCS in 0.1 ml, 0.1 ml 5% FCS-199, 0.1 ml (varying numbers) spleen cells and 0.1 ml of either diluted (see below) rabbit anti-CRBCS serum or diluted normal rabbit serum. Control tubes contained 1 a 10^6 unlabelled CRBCS in 0.2 ml 5% FCS-199, 5×10^4 labelled CRBCS in 0.1 ml, and 0.1 ml diluted antiserum or normal rabbit serum. Unlabelled CRBCS were added to the control tubes (which had no spleen cells) in order to prevent the spontaneous lysis of the labelled CRBCS. The cultures were incubated at 37°C in 5% CO_2 in air for 16 hours. After incubation the cells were centrifuged (250g for 5 minutes) and 200 µl of the supernatant removed. Both supernatant and pellets were counted on a gamma counter (I.C.N. Autogamma 500) and cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{2 \times \text{supernatant count} \times 100}{(\text{pellet} + \text{supernatant}) \text{ count}}$$

Each combination of cells and serum was performed at least in triplicate.

Preparation of rabbit anti-chick red blood cell serum

Six months old New Zealand white rabbits were injected intravenously with 2.0 ml of a 10% suspension of washed CRBCS in PBS on alternate days over 18 days and were exsanguinated on day 21. The control rabbit serum came from the same rabbits before immunization started. All the rabbit sera were heat-inactivated at 56°C for 30 minutes.

Results

Before assessing the K cell activity in malarious mice the optimal working dilution of the anti-CRBC serum was determined. Spleen cells were pooled from two non-infected C57Bl mice. Cultures were set up containing 2×10^6 splenic lymphoid cells, 5×10^4 labelled CRBCS (spleen cell to CRBCS 40:1) and rabbit anti-CRBC serum or normal rabbit serum diluted with 5% FCS-199 to a concentration of either 10^{-3}, 10^{-4}, 10^{-5} or 10^{-6}. The percentage cytotoxicity with antiserum at these concentrations is given in Figure 2. It can be seen that the highest ^{51}Cr release occurred in antiserum diluted to 10^{-5} but at 10^{-6} the cytotoxicity declined considerably. The cytotoxicity associated with antiserum diluted to 10^{-4} and 10^{-3} was similar, and although below that given with 10^{-5} antiserum, in all subsequent experiments an antiserum dilution of 10^{-4} was used. Spleen cells with normal rabbit serum at a dilution of 10^{-4} gave the lowest cytotoxicity.

In all the experiments described below the ^{51}Cr release in the controls was similar.

Three initial experiments are described in which the K cell activity of P. chabaudi infected mice was determined. The details of the experiments and the results are given in Table 11. In all three experiments in which non-adherent spleen cells were prepared from mice either just before or just after peak parasitaemia or when the primary parasitaemia had become subpatent, the percentage cytotoxicity was higher for almost all the spleen cells: CRBS ratios tested in the cultures of spleen cells from infected mice than in the controls. From these it appeared that there was an increase in the non-specific ADCC of spleen cells of infected mice.

Further experiments were subsequently carried out and these are summarised in Table 12. In the first experiment the cytotoxicity of the spleen cells from two mice on the third day of the infection was similar to that of the controls. For the second experiment mice with parasitaemias of 40% on day 6 of the infection were selected. One of the four samples containing spleen cells from the infected mice plus antiserum had a much lower cytotoxicity value than the other three cultures in this group and as a result the observations were only significant at $p = 0.1$. If the unrepresentative culture is omitted the cytotoxicity of the infected cells is significant at the 0.05 level. In the third and fourth experiments, the infected mice being killed on day 5 and 11 of the infection respectively, there was no significant difference in sytotoxicity between infected and control spleen cells. In both these experiments, however, there was an unusually high ^{51}Cr release in control cultures containing normal rabbit serum which suggests that the results of these two experiments should be discounted. In the fourth experiments some cultures were set up in which the mouse red cells in the spleen cell preparation were lysed with 0.83% NH_4Cl before the cultures were set up. No differences were noted between the cytotoxicity observed in the cultures with or without accompanying mouse red cells. In the fifth experiment in which the spleen cells came from two mice with a declining parasitaemia on day 13 after infection there was a significantly higher cytotoxicity level with the infected spleen cells.

Some very preliminary experiments were carried out with lymphoid cells isolated from the peripheral blood of children infected with P. falciparum. Monocytes were isolated by centrifugation of blood on Ficoll/Triosil gradients (standard published procedure used). The optimal lymphoid cell: CRBCS ratio was found to be 2:1 for a rabbit antiserum dilution of 10^{-4}. The results of 11 experiments are presented in Table 13 where it can be seen that there was no significant different between the cytotoxicity values obtained for infected children and Caucasian controls. The net percentage cytotoxicity (% ^{51}Cr release in the presence of antiserum - % ^{51}Cr release in the presence of normal serum) gives values (50-70%) for most of the subjects examined which are within the range of most of 58 control subjects examined by Calder et al. (1974) in their study. A problem encountered with this study on infected children was a tendency for the CRBCS to lyse spontaneously. This could have been the result of using red cells from Shaver rather than Leghorn chickens or more likely because it was not always possible to use freshly drawn chick blood as was the case in the experiments with mice.

TABLE 11

Antibody-dependent cell-mediated cytotoxicity in spleens of P. chabaudi infected C57B1 mice. Spleen cells from two mice were pooled and the ratio of spleen cells:CRBC was varied.

Experiment no.	Parasitaemias	Ratio of spleen cells: CRBC	% cytotoxicity of infected spleens + antiserum (10^{-4})	% cytotoxicity of infected spleens + nor. serum (10^{-4})	% cytotoxicity of normal spleens + antiserum (10^{-4})	% cytotoxicity of normal spleens + nor. serum (10^{-4})
		10:1	30.9 ± 2.52	10.25 ± 0.78	17.45 ± 0.91	8.7 ± 1.98
1 (day 8)	29%, 52%	20:1	a 49.5 ± 10.95	10.2 ± 0.28	32.0 ± 2.68	9.2 ± 1.13
		40:1	60.43± 8.67	8.75 ± 2.61	43.6 ± 2.85	7.9 ± 0.14
		1.25:1	12.3 ± 1.89	5.55 ± 0.49	7.8 ± 1.09	5.25± 0.07
2 (day 11)	35%, 15% (decreasing)	2.5:1	14.8 ± 1.94	7.4 ± 0.99	9.13 ± 1.7	5.85± 1.7
		5:1	23.66± 2.45	5.1	8.8 ± 1.53	6.05± 0.2
		10:1	71.3 ± 10.61	4.7 ± 1.13	31.63 ± 5.16	6.2 ± 7.21
3 (day 15)	0%, 0%	20:1	71.6 ± 3.28	4.75	50.23 ± 13.08	5.35± 0.07
		40:1	a 55.7 ± 4.26	4.35 ± 2.05	53.1 ± 7.4	6.05± 1.2

a = not significant at p = 0.05

TABLE 12

Antibody-dependent cell-mediated cytotoxicity in spleens of P. chabaudi infected C57B1 mice. Spleen cells from two mice were pooled and the ratio of spleen cells:CRBC in culture was 20:1. The cytotoxicity values are given for mice with varying parasitaemias in different experiments.

Parasitaemia of infected mice		Cytotoxicity of inf. spleens + antiserum (10^{-4})	Cytotoxicity of inf. spleens + nor. serum (10^{-4})	Cytotoxicity of nor. spleens + antiserum (10^{-4})	Cytotoxicity of nor. spleens + nor. serum (10^{-4})
6%, 7%	a	26.43 ± 3.06	7.3 ± 2.26	25.03 ± 4.04	9.1 ± 3.11
40%, 40%	a	50.07 ± 9.28	8.0 ± 0.99	36.62 ± 6.83	6.4
	b	(54.23 ± 5.06)			
60%, 60%	a	53.4 ± 2.19	28.05 ± 5.44	61.93 ± 8.6	26.35± 3.88
10%, 10% (decreasing)	a	58.26 ± 9.34	15.85 ± 10.39	57.37 ± 1.85	11.85± 1.67
	x	59.26 ± 2.46	12.35 ± 0.78	60.32 ± 4.49	18.15± 2.47
2%, 2% (decreasing)		46.66 ± 2.85	4.45 ± 0.92	19.75 ± 3.31	5.45± 0.49

a = not significant at p = 0.05

x = splenic red cells lysed prior to culture

b = mean calculated from 3 values instead of 4, one of the 4 values being unusually low. The result then became significant at p = 0.05

TABLE 13

Antibody-dependent cell-mediated cytotoxicity of peripheral lymphoid ce
infected or uninfected donors. A lymphoid cell:CRBC ratio of 2:1 was

Blood leucocytes from infected donors				Blood
Age of donor (years)	No. of parasites per field (approx. 5000 cells/field)	$\%^{51}Cr$ release in presence of antiserum (10^{-4})	$\%^{51}Cr$ release in presence of nor.serum (10^{-4})	$\%^{51}Cr$ pre a
-	25/1	67.3 ± 7.4	12.4 ± 3.3	55.1
1.5	20/1	80.1 ± 2.0	28.6 ± 16.7	78.3
-	100/1	83.5 ± 7.1	41.5 ± 6.1	82.1
-	50/1	60.1 ± 4.2	11.4 ± 0.9	66.0
12	45/1	42.9 ± 4.4	4.1 ± 4.4	50.4
-	80/1	62.8 ± 4.9	2.9 ± 0.4	54.5
1.5	45/1	50.4 ± 4.3	4.65± 2.1	56.3
2.0	10/1	66.8 ± 5.2	2.45 ± 0.7	57.5
0.5	50/1	66.6 ± 11.7	10.0	
2.0	300/1	64.9 ± 8.3	11.95± 5.86	70.4
3.5	40/1	76.9 ± 9.6	9.6	
2.0	200/1	51.6 ± 0.2	13.4 ± 2.5	56.4

2

TABLE 13

xicity of peripheral lymphoid cells from P. falciparum
hoid cell:CRBC ratio of 2:1 was used.

se in of 10^{-4})	$\%^{51}Cr$ release in presence of nor.serum (10^{-4})	Blood leucocytes from uninfected donors: $\%^{51}Cr$ release in presence of antiserum	Blood leucocytes from uninfected donors: $\%^{51}Cr$ release in presence of normal serum
.4	12.4 ± 3.3	55.1 ± 3.3	15.6 ± 5.6
.0	28.6 ± 16.7	78.3 ± 2.12	20.3 ± 3.8
.1	41.5 ± 6.1	82.1 ± 11.6	40.2 ± 7.5
.2	11.4 ± 0.9	66.0 ± 2.9	6.5 ± 2.0
.4	4.1 ± 4.4	50.4 ± 17.5	6.7 ± 5.4
.9	2.9 ± 0.4	54.5 ± 11.6	7.2 ± 10.4
.3	4.65± 2.1	56.3 ± 9.8	8.4 ± 2.1
.2	2.45 ± 0.7	57.5 ± 9.5	13.5 ± 4.5
.7	10.0		
.3	11.95± 5.86	70.4 ± 6.8	16.7 ± 4.5
.6	9.6		
.2	13.4 ± 2.5	56.4 ± 3.2	6.65± 2.3

Discussion

Our investigations with *P. chabaudi* infected mice suggest that there is no decrease in ADCC, although other types of immune responses, e.g. humoral responses to heterologous antigens can be depressed during malaria infection in mice (reviewed by Wedderburn, 1974; McBride et al., 1977). In 6 of the 8 experiments described above, with the spleen cells coming from mice 6-15 days after infection, there was an increase in K cell activity. This increase in cytotoxicity of non-adherent spleen cells from infected mice, to CRBCS in the presence of rabbit anti-CRBC serum could be found in spleen cell:CRBC ratios of between 1.25:1 and 40:1. On the basis of spleen size, there would have been a much greater K cell activity from between days 6 and 15 because the spleens of infected mice became much larger (3-4 times larger at peak parasitaemia) than those of non-infected mice.

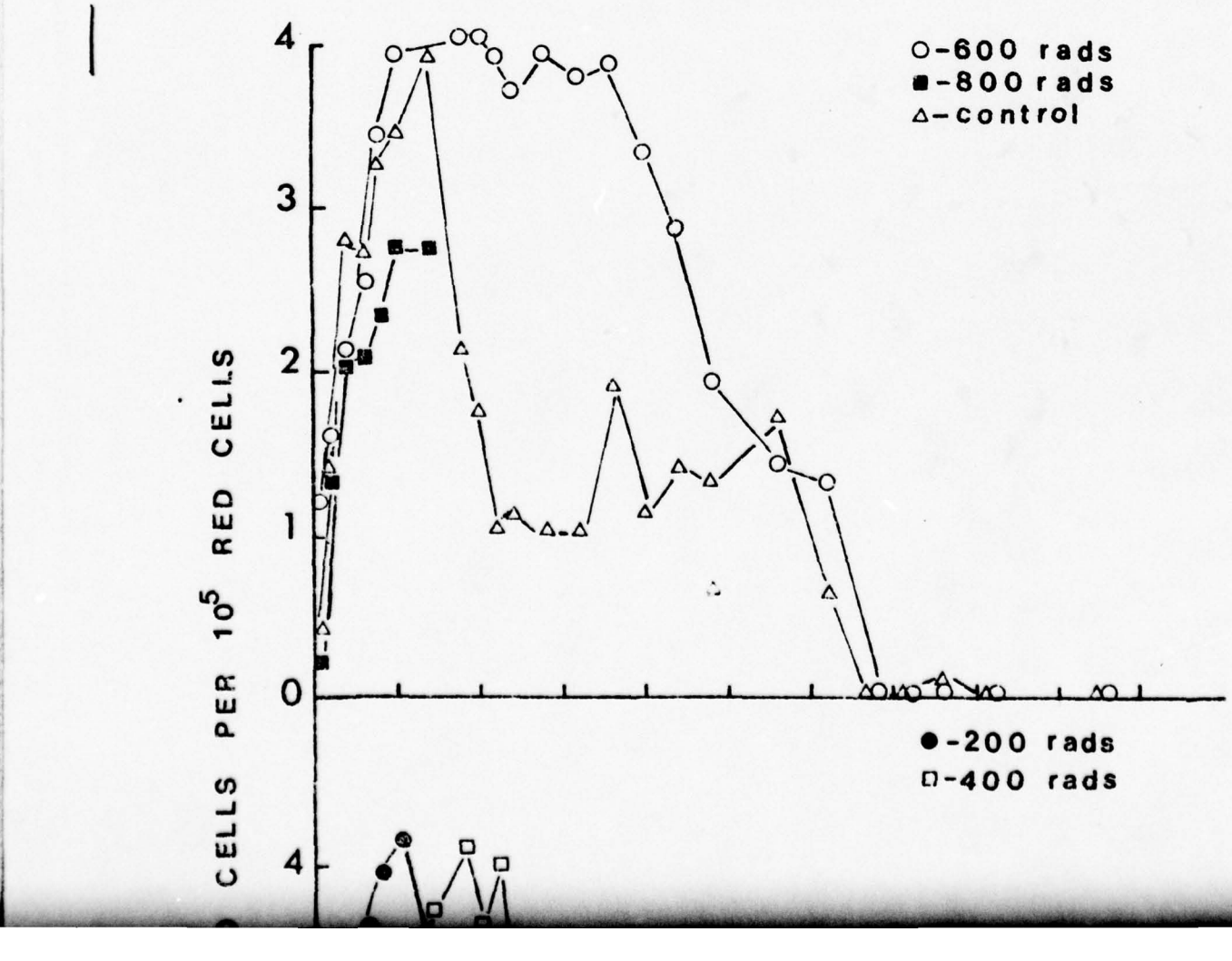
In our preliminary investigations no difference was detected in the K cell activity of blood mononuclear cells from children infected with *P. falciparum* and non-infected controls. The experimental conditions used were not entirely satisfactory. Recently Greenwood, Oduluju & Stratton (1977) have reported an increased K cell activity in the peripheral blood of children infected with *P. falciparum*. Clearly more work on human malarias is indicated.

During a malaria infection there is a decrease in the number of T cells and B cells in lymphoid tissues (Kretti & Nussenzweig, 1974; Gravely, Hamburger & Kreier, 1976) and in the peripheral blood of children infected with *P. falciparum* (Wyler, 1976; Greenwood et al., 1977). This decrease in T and B cells is accompanied by an increase in so-called null cells which category may include K cells (Greenberg, Hudson, Snen & Roitt, 1973).

There is, however, no evidence from these experiments that K cells are effector cells in the lysis of parasitized red cells or free malaria parasites. There is only one report of immune spleen cells being cytotoxic to malaria (*P. berghei*) infected red cells in the presence of antibody (Coleman et al., 1975) and this observation needs to be confirmed in other systems. It has also to be noted that monocytes and polymorphonuclear leukocytes can also act as antibody-mediated cytotoxic cells (Holm et al., 1974; MacDonald et al., 1975). Sanderson, Lopez & Bunn Moreno (1977) report that eosinophils and not K cells are mainly responsible for the lysis of *Trypanosoma cruzi* in the presence of antiserum.

The presence of immune complexes in serum can inhibit K cell activity *in vitro* (Jewell & MacLennan, 1973). Immune complexes in the plasma have been described in the plasma of malarious monkeys (Houba et al., 1976). It is possible that the presence of these complexes could limit the activity of K cells *in vivo* in the control of a malaria infection.

It is also possible that K cells are involved in the excessive anaemia which occurs during acute malarial infection. Auto-antibodies to red cells may be partly responsible for the destruction of non-parasitized red cells (Zuckerman, 1960; McGhee, 1976; Lustig, Nussenzweig & Nussenzweig, 1977) and this destruction may be brought about by K cells in the presence of these antibodies.


4
3
2
1
0
4
CELLS PER 10^5 RED CELLS
○-600 rads
■-800 rads
△-control
●-200 rads
□-400 rads

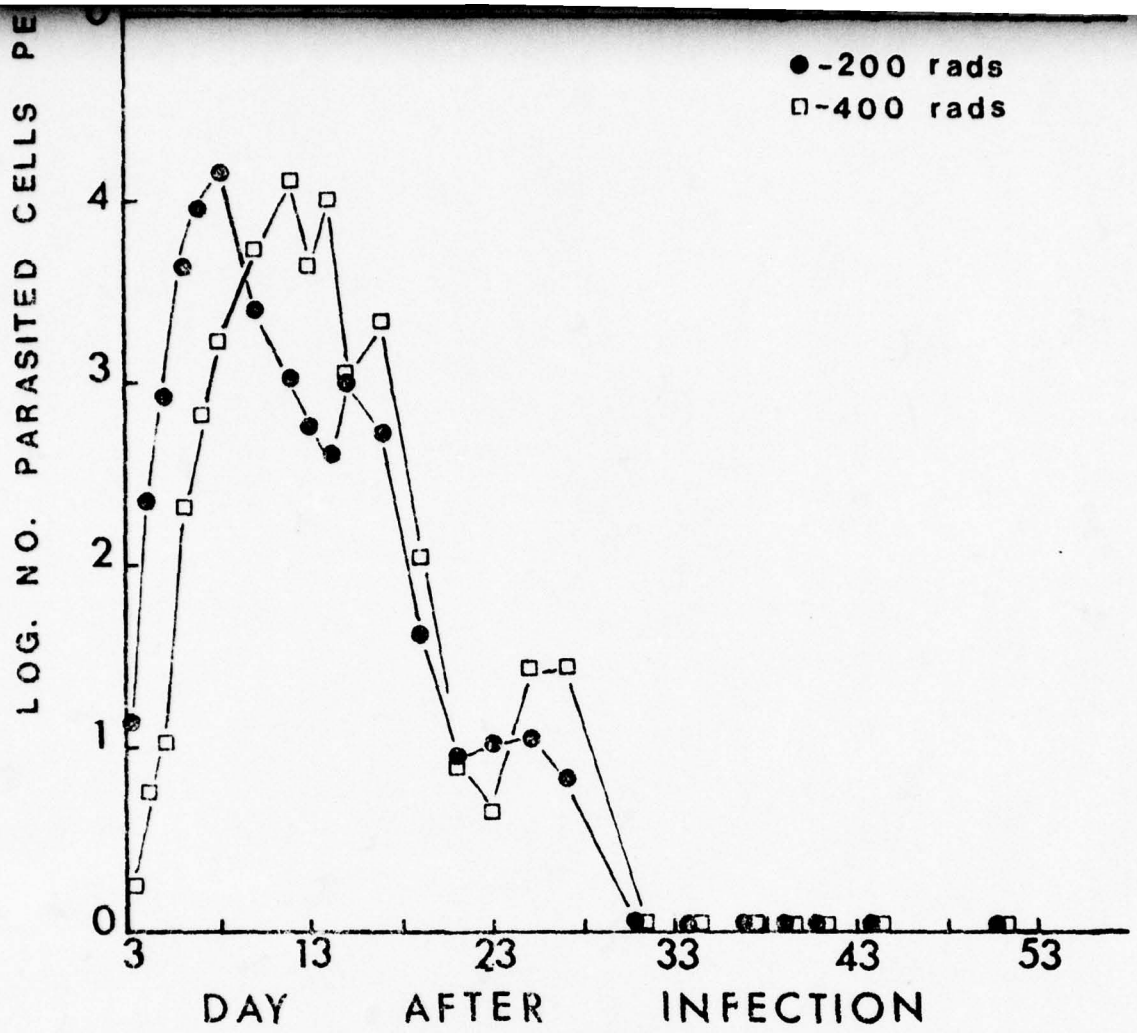


Figure 3. Course of P. chabaudi infections in A_2G mice previously exposed to no irradiation (Δ — Δ), 200 rads (• — •), 400 rads (□ — □), 600 rads (o — o), and 800 rads (■ — ■).

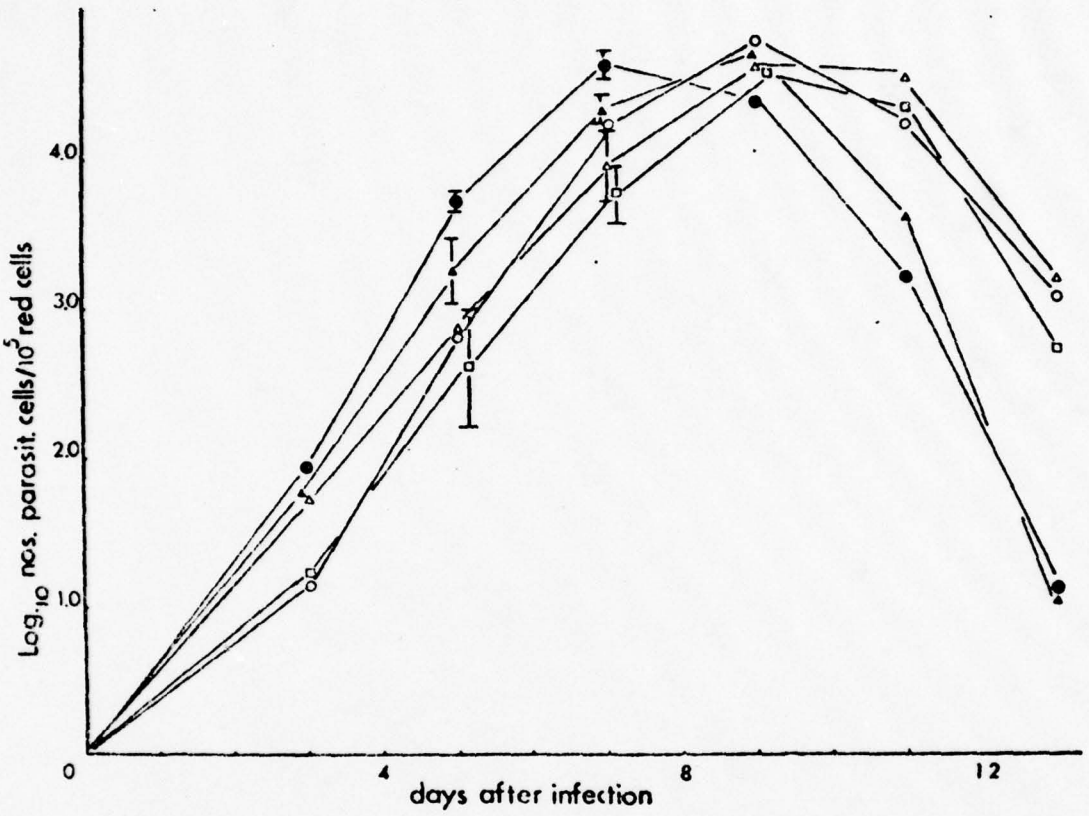


Figure 4. Course of P. chabaudi infections in mice irradiated with 600 rads and injected with 10^5 parasitized red cells either 22 days (● — ●), 15 days (o — o), 8 days (Δ — Δ) or 1 day (□ — □) after irradiation. A non-irradiated control group was included (▲ — ▲).

Non-specific resistance in irradiated mice

In a previous report (Annual Report Contract No. DAJA 37-75C-1620) it was reported that in irradiated mice there was a non-specific resistance to infection with P. chabaudi during the early stages of the infection. This observation has been further investigated. In different experiments mice were either irradiated with different levels of X-irradiation before infection, or were X-irradiated and were then infected at various times afterwards, or were irradiated and reconstituted with varied numbers of spleen or bone-marrow cells and then infected at different times thereafter.

Effect of different doses of X-irradiation

Ten months old male A2G mice were irradiated in groups of 6 mice with either 200, 400, 600 or 800 rads. A non-irradiated group was included. Twenty-two hours later the mice were infected with 5×10^5 P. chabaudi parasitized red cells. The geometrical mean parasitaemias are given in Figure 3. The mice given 800 rads were sacrificed on day 8 to comply with Home Office Regulations. It can be seen that the primary parasitaemia went into significant remission in the control mice 6 or 7 days before that in mice given 200 or 400 rads, and 11 days before the 600 rad mice. The severest parasitaemias were seen in the latter mice in this experiment.

Infection of mice at different times after irradiation

A preliminary experiment with female NIH mice showed that mice infected 16 days after irradiation with 600 rads had significantly higher parasitaemias than non-irradiated mice. In a second experiment 10 weeks old male C57Bl mice in groups of 7 mice were given 600 rads either 22, 15, 8 or 1 day before infection. A non-irradiated group was also included. All the mice were infected with 1×10^5 P. chabaudi infected red cells. The mice received no tetracycline in

400 rads, and 11 days before the 600 rad mice. The severest parasitaemias were seen in the latter mice in this experiment.

Infection of mice at different times after irradiation

A preliminary experiment with female NIH mice showed that mice infected 16 days after irradiation with 600 rads had significantly higher parasitaemias than non-irradiated mice. In a second experiment 10 weeks old male C57B1 mice in groups of 7 mice were given 600 rads either 22, 15, 8 or 1 day before infection. A non-irradiated group was also included. All the mice were infected with 1 x 10^5 P. chabaudi infected red cells. The mice received no terramycin in their drinking water. The course of the infection in five groups is shown in Figure 4. In mice irradiated 22 days before infection, the infection was not delayed in onset but followed a course in advance of the other groups. In the three other irradiated groups there was some initial resistance to infection, especially those mice irradiated 1 and 8 days before infection, and in all three groups the parasitaemia started to decline 2 days after that in the control group and the irradiated mice infected 22 days after irradiation.

These experiments showed, therefore, that after a sub-lethal dose of irradiation, the effect of the irradiation is to depress or enhance the early course of the infection depending on the time of infection in relation to the time of irradiation.

Reconstitution of irradiated mice before challenge

Two similar experiments were carried out with similar results: one is described in detail. Groups of 17 weeks old C57B1 mice, each containing 6-7 mice, were irradiated with 600 rads either 9 or 2 days before infection (on day 0) and reconstituted i.v. with either 1 x 10^5 or 6 x 10^6 bone-marrow cells, or 6 x 10^6 spleen cells on the day after irradiation. In another two groups the mice were irradiated on either day -9 or -2 and in both groups the mice were injected i.p. with 1.0 ml whole blood one day before infection. Another two groups, irradiated

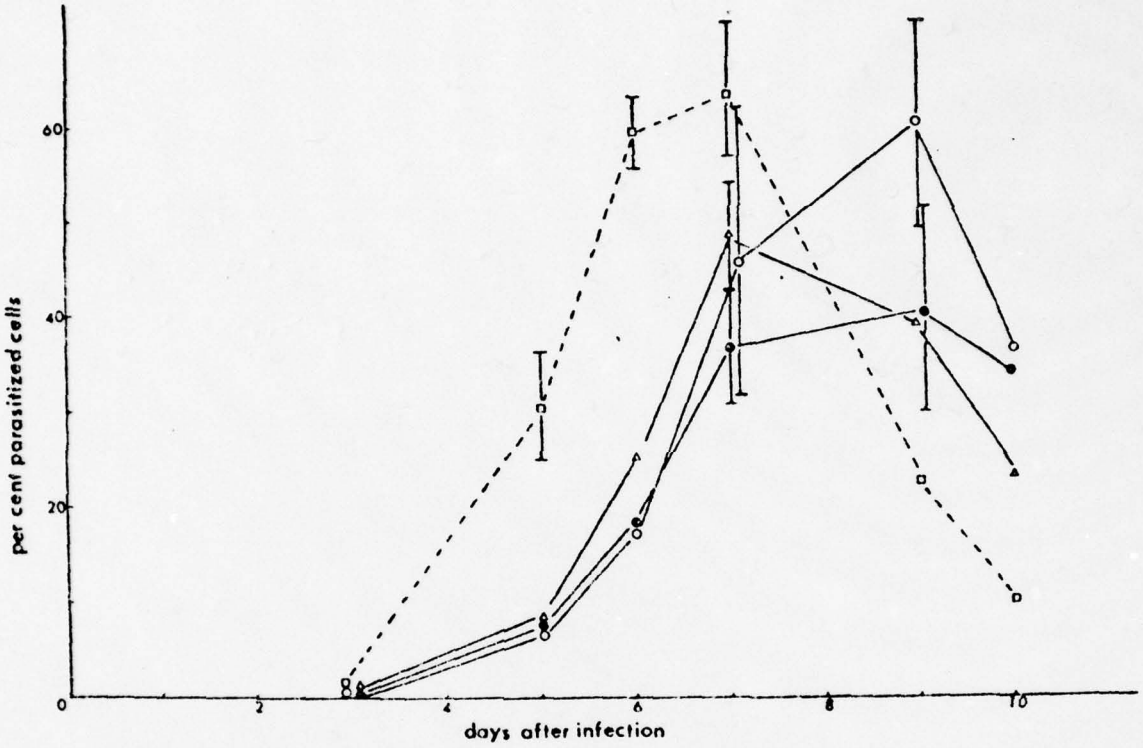


Figure 5. Course of P. chabaudi infection in C57Bl mice irradiated with 600 rads 2 days before infection and injected i.v. with either 6 x 10^6 bone-marrow cells (o — o), 1 x 10^5 bone marrow cells (Δ — Δ), or with no bone-marrow or spleen cells (• — •). Another group of mice received no cells and was not irradiated (-□--□-). The mice were challenged with 10^6 P. chabaudi parasitized cells i.v.

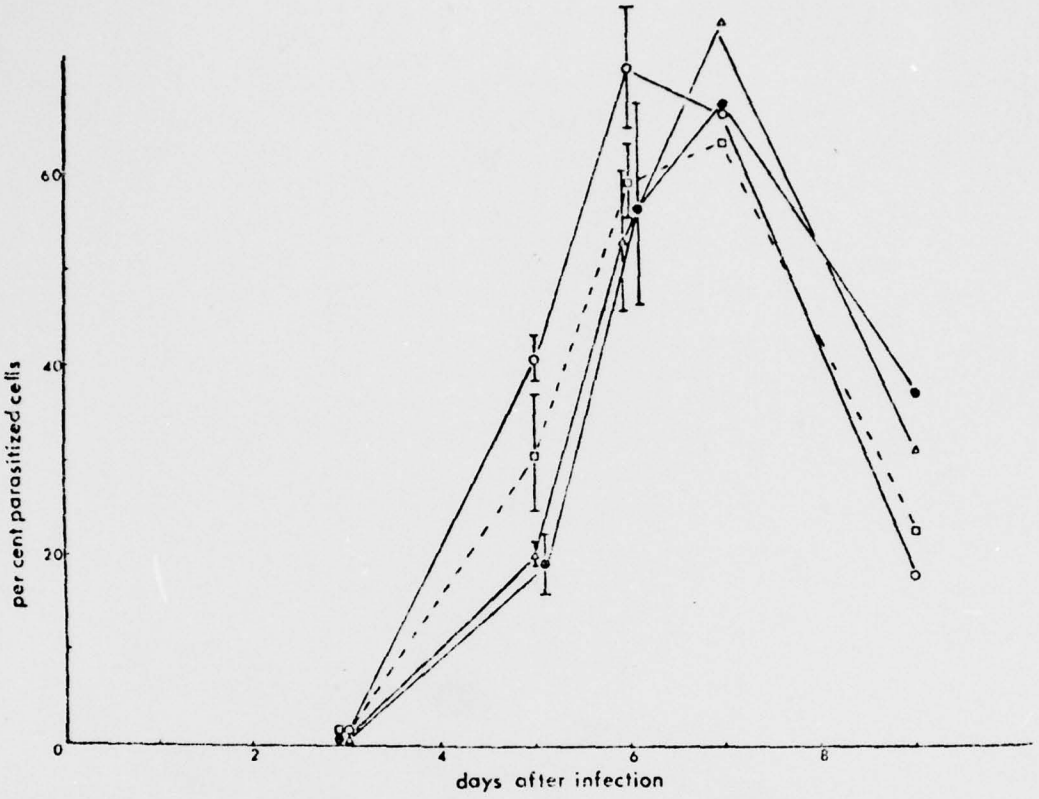


Figure 6. Course of P. chabaudi infections in C57Bl mice irradiated with 600 rads 9 days before infection and injected i.v. with either 6×10^6 bone-marrow cells (o — o), 1×10^5 bone-marrow cells (Δ — Δ), or with no bone-marrow or spleen cells (• — •). A non-irradiated group given no cells was included (□ -- □). The mice were challenged with 10^6 P. chabaudi parasitized cells i.v.

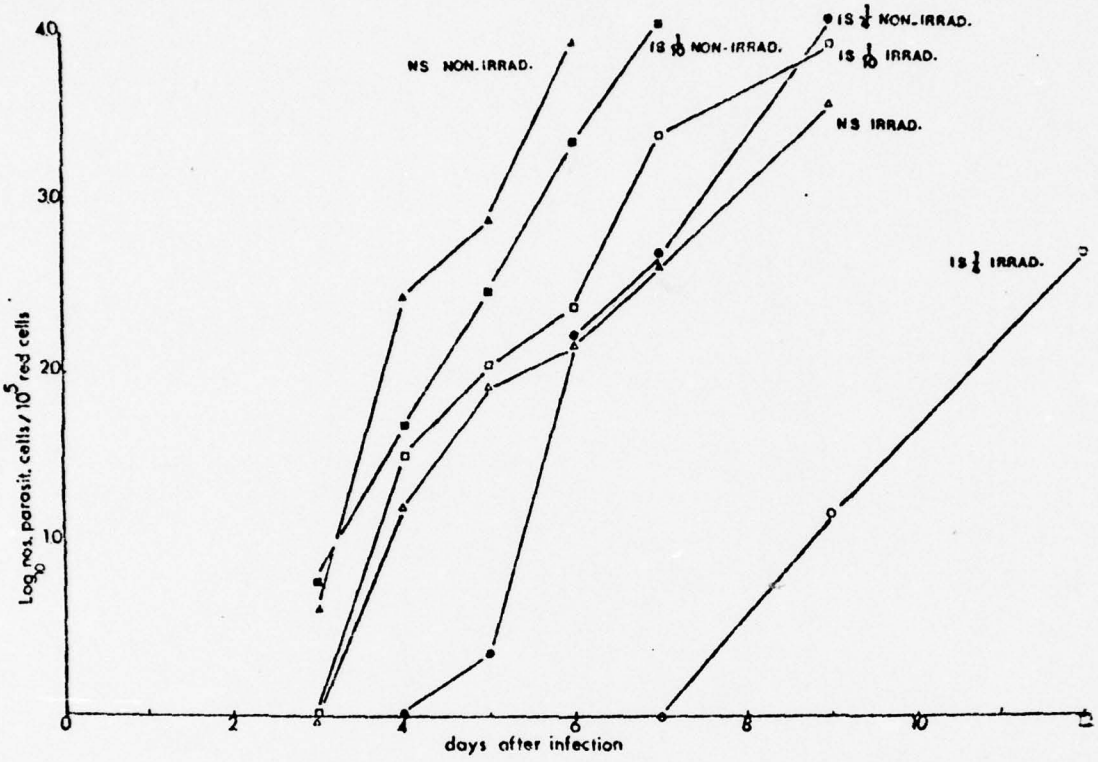


Figure 7. Course of P. chabaudi infection in C57Bl mice given either immune serum (1/4) and 600 rads (o — o); immune serum (1/4) and no irradiation (● — ●); immune serum (1/10) and 600 rads (□ — □); immune serum (1/10) and no irradiation (■ — ■); normal serum and 600 rads (△ — △); normal serum and no irradiation (▲ — ▲).

either 9 or 2 days before infection, received no lymphoid cells and a further group of non-irradiated mice was included. All the mice were injected i.v. with 1 x 10^6 P. chabaudi parasitized red cells on day 0. The mice had no terramycin in their drinking water.

The parasitaemias in the various groups are given in Figures 5 and 6.

In mice infected within 2 days of irradiation there was some resistance: the increase in their parastiaemias was depressed. The irradiated mice reconstituted with 6 x 10^6 spleen cells had parastiaemias very similar to those given 6 x 10^6 bone-marrow cells and are omitted from the graph. Mice irradiated on day -2 and given 6 x 10^6 bone-marrow or spleen cells had higher peak parastiaemias than the other irradiated mice.

Mice irradiated 9 days before infection and reconstituted with 6 x 10^6 bone-marrow or spleen cells (not shown) showed no resistance to infection and, in fact, these mice had enhanced parastiaemias compared with non-irradiated controls during the first 6 days of the infection. In the other two groups of mice irradiated 9 days before infection the parastiaemia was initially depressed although less than their day -2 counterparts. It was noticeable, however, that of these 2 groups the mice given 1 x 10^5 bone-marrow cells had a higher peak parastiaemia than the non-irradiated mice or those given no cells. In the mice irradiated either 9 or 2 days before infection and given whole blood the parastiaemias followed the course of their irradiated counterparts given no cells.

In the second experiment in which the mice were irradiated 1 or 8 days before infection an essentially similar result was obtained other than that the resistance of the mice irradiated 1 day before infection was less than that seen in the mouse irradiated 2 days before infection.

Passive transfer of immune serum into irradiated mice

A preliminary experiment was described in the previous Annual Report.

In this confirmatory experiment immune serum was obtained from C57B1 mice which had been infected 85 days previously. The recipient mice were 18 weeks old C57B1 males. Half the mice were irradiated with 600 rads 1 hour before they were injected i.p. in groups of 3, along with non-irradiated mice, with 1 ml of either a 1 in 4 or a 1 in 10 dilution (in Hanks' balanced salt solution) of immune serum or with neat normal serum. The mice were injected i.v. with 5 x 10^4 P. chabaudi parasitized cells 3 hours later. The parasitaemias for each group of mice after infection are given in Figure 7.

The irradiated mice given 1 in 4 diluted immune serum were the best protected mice, showing a patent parasitaemia at least 3 days after their non-irradiated counterparts. Immune serum diluted 1 in 10 gave no protection in irradiated or non-irradiated hosts. There was a difference of approximately 4 days between the onset of the patent parastiaemias in the irradiated mice given 1 in 4 immune serum and the irradiated controls, and 2 days for the non-irradiated mice given 1 in 4 immune serum and the non-irradiated controls.

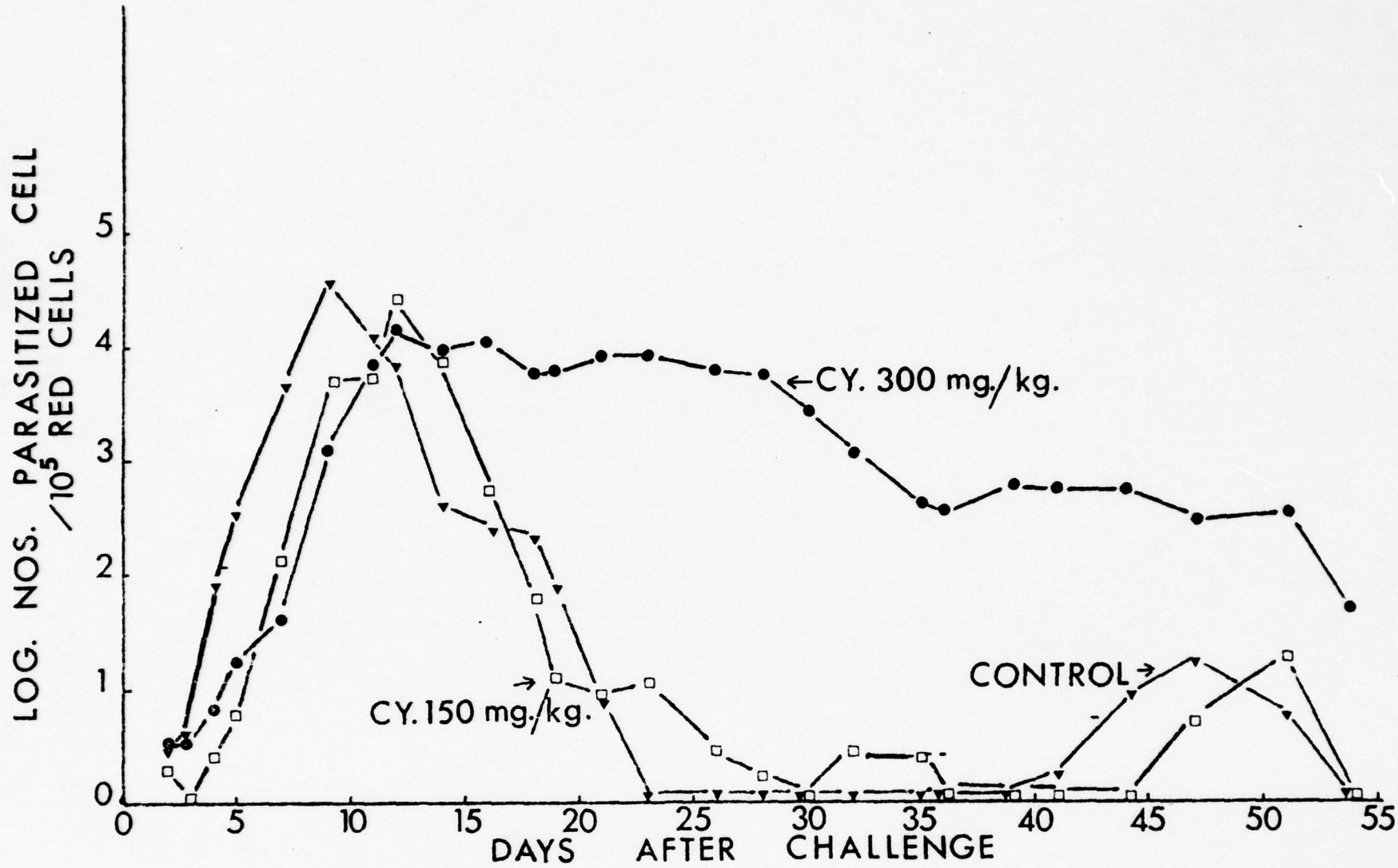


Figure 8. Course of P. chabaudi infection in C57Bl mice given cyclophosphamide at either 300 mg (●—●) or 150 mg/kg (□—□). Control group (▼—▼).

The effect of cyclophosphamide on course of P. chabaudi infection

Cyclophosphamide is widely used as an immunosuppressive agent. Its activity depends on the time of administration in relation to exposure to antigen. Cyclophosphamide given before antigen can depress humoral responses and enhance cell-mediated responses, e.g. those of mice to sheep red cell (Lagrange et al., 1974). Finerty and Krahl (1976) described an increased delayed hypersensitivity response to plasmodial antigen in mice pretreated with cyclophosphamide and this was accompanied by an increased resistance to P. yoelii.

A preliminary experiment was carried out in which cyclophosphamide was given before infection of mice with P. chabaudi. Three months old C57Bl male mice in groups of 6 were inoculated i.p. with cyclophosphamide (Kock Light) at a dose rate of either 300 or 150 mg/Kg 3 days before infection with 1×10^6 P. chabaudi parasitized red cells. A control group received distilled water and the mice were similarly infected. The geometrical mean parasitaemias are given in Figure 8. One effect of the cyclophosphamide was to depress and/or to delay the onset of the early phase of the primary parasitaemia. In the mice given 150 mg/Kg the course of the patent parasitaemia, once established, followed a course similar to the controls. Mice given 300 mg/Kg showed persistent, fluctuating and often acute patent parasitaemias, which had killed 4 of 6 mice 54 days after infection.

Discussion

The principle experiments described in this section were carried out to determine some of the conditions affecting the non-specific resistance to infection in irradiated C57Bl mice. Similar results have been obtained with NIH mice but have not been described.

It was found that the patent parastiaemia was marginally more severe in mice given 200 and 400 rads but was significantly more severe in mice given 600 rads. A low dose of irradiation did not increase resistance to infection and moderate the primary parasitaemia. Suppressor cells are especially radiosensitive and low doses of irradiation have been reported to enhance the immune response to some antigens (Chiorazzi et al., 1976).

The course of the infection in irradiated mice was dependent on the time of infection in relation to the time of exposure to irradiation. Mice challenged three weeks after 600 rads had a primary parasitaemia which increased more quickly than, and reached a peak 2 days before, that of the non-irradiated controls. In mice challenged 2 or 8 days after the same dose of irradiation there was an initial resistance to the parasite insofar as the parasitaemia rose more slowly. Thus with time after irradiation there was a change from a depressed to an enhanced rate of parasite increase in the ascending primary parasitaemia when compared with non-irradiated mice.

The resistance to the parasite after irradiation could be overcome and the parasitaemias enhanced by injecting syngeneic bone-marrow or spleen cells or as noted above by delaying infection after irradiation. It can be assumed that in the irradiated and not reconstituted mice the lymphoid tissues arerepopulated from radioresistant haemopoietic stem cells (Takada, Takada & Ambrus, 1971) and that in the irradiated mice reconstituted with spleen or bone-marrow cells this repopulation occurs earlier.

Some possible reasons for the non-specific resistance to infection in irradiated mice are:

1) in irradiated mice substances are released from cells damaged by irradiation which adversely affect the parasite's growth;

2) the depressive effect of irradiation on haemopoiesis may lead to a reduction in the numbers of red cells of the age preferentially invaded by P. chabaudi;

3) irradiation reduces the numbers of cells regulating the immune response;

4) there is a non-specific stimulation of the reticulo-endothelial system (RES) following irradiation which leads to the removal of parasites or parasitized cells.

Examination of blood smears taken throughout a 24-hour cycle from infected irradiated mice showed no evidence (details not given) that the parasite developed from ring to schizont and reinvaded abnormally. The numbers of merozoites produced in schizonts could have been affected, however.

Resistance to P. berghei in irradiated mice has been ascribed to the reduction in the numbers of reticulocytes in the circulation which follows irradiation (Singer, 1953). P. berghei has a preference for these immature red cells. Our observations and those of Ott (1968) indicate that P. chabaudi has no preference for reticulocytes (as detected by cresyl blue or Giemsa's stain) but it may have a preference for young mature cells. The observation that injection of 1.0 ml whole blood into the irradiated mice did not reduce the non-specific resistance does not support the latter suggestion: injection of whole blood would be expected to provide some of the younger red cells deficient in irradiated mice.

Resistance to Listeria monocytogenes and Brucella abortus infection has been demonstrated in irradiated mice and in athymic nude mice (Campbell, Martens, Cooper & McClatchy, 1974; Cheers & Waller, 1975; Chan, Longshawn & Skamene, 1977). Chan et al. postulated that the loss of regulatory suppressor T cells (Baker, 1975) may be responsible for resistance to bacterial infection since they are radiosensitive and are also depleted in nude mice (Okumara & Tada, 1971; Baker, 1975). A role for suppressor T cells in malarial immunity has yet to be demonstrated.

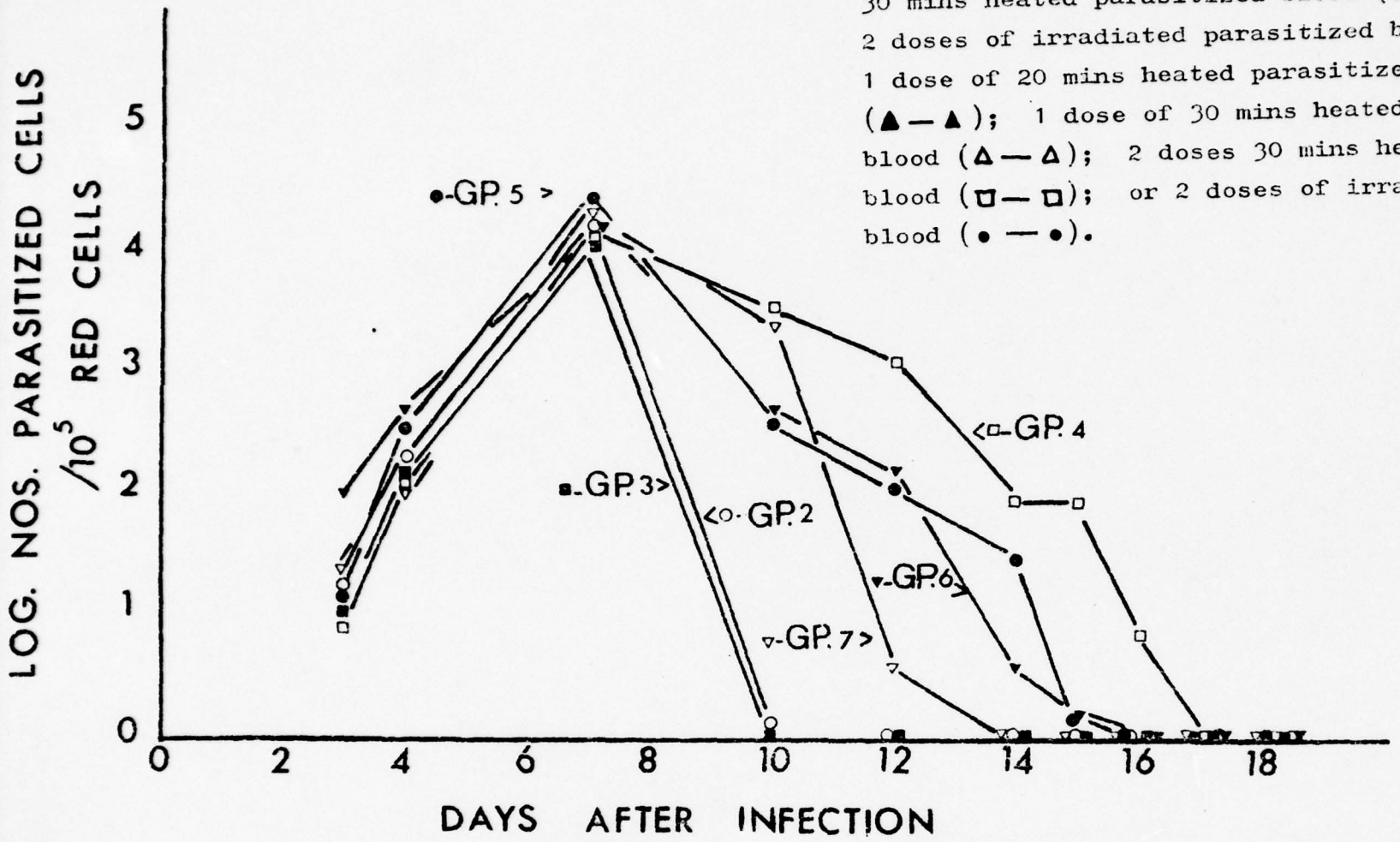
Sljivic (1970a) demonstrated that the phagocytic function of the RES as measured by carbon clearance test, was increased by irradiation of mice. The clearance rate was increased with increased doses of irradiation and could be reduced by injecting lymph node cells or bone-marrow cells into irradiated mice or by introducing antibiotics into the drinking water (Sljivic, 1970b). Since bone-marrow or spleen cells also reduced or abolished the partial resistance to infection with P. chabaudi, it is possible that the mechanism causing an increase in carbon clearance in irradiated mice is similar to that causing resistance to P. chabaudi. Irradiated mice given the antibiotic, terramycin, in this study were still partially resistant, however, to the infection. Cheers and Waller (1975) observed an initial increased resistance to bacterial infection in irradiated mice, and that macrophages from these mice had an enhanced capacity to ingest bacteria in vitro. Both Sljivic and Cheers and Waller suggest that stimulation of the RES was brought about by the leakage of bacteria or bacterial endotoxin from the gut. It is likely that increased mactophage activity following irradiation could provide some non-specific resistance to P. chabaudi since experiments in which mice were injected with RES stimulators, such as Corynebacterium parvum, conferred on the mice some resistance to other rodent plasmodia (Nussenzweig, 1967; Clark, Cox & Allison, 1977; Cottrell, Playfair & de Sousa, 1977). If phagocytosis of parasites is involved in the non-specific resistance to infection, a fall off with time in this resistance followed by enhancement of the parasitaemia may result from a blockade of the RES caused by the eventual uptake of dead cells and cellular debris by fixed macrophages after irradiation. Such a blockage of the RES could result in a reduced phagocytosis or in a suppressed cellular or humoral response to antigen (Sabet, Newlin & Friedman, 1969).

Immune serum showed enhanced activity in recently irradiated mice, probably the result of the combined effects of opsonizing antibody in the serum and the radiation-induced increased macrophage activity. Opsonizing antibody in serum of immune monkeys, rats and mice has been described (Brown, 1971; Criswell, Butler, Rossan & Knight, 1971; Chow & Kreier, 1972). In an earlier report it was noted that small numbers of immune spleen cells conferred a greater level of immunity to P. chabaudi on irradiated than non-irradiated mice. One possible explanation for this observation is that the antimalarial antibodies subsequently produced in the cell recipients would be made more effective because they are being released in an irradiated animal.

Immunization against rodent plasmodia

D'Antonio (1972), in a report lacking important technical details, noted that P. berghei after exposure to temperatures above 37°C, could induce immunity in mice. In the last Annual Report a preliminary experiment was described in which irradiated and heated P. chabaudi infected red cells were compared for their immunizing ability. The result of this experiment, in which irradiated blood was given 40 krad and the heated blood was exposed to 45°C for 45 minutes, and in which the mice were given two immunizing inocula 6 days apart, both irradiated and heated blood protected the mice to some degree. The peak parasitaemias in the mice given irradiated parasitized red cells was lower than in the recipients of heated parasitized blood.

Figure 9. Course of P. chabaudi challenge infections in C57Bl mice immunized with either 2 doses of 30 mins heated parasitized blood (o — o); 2 doses of irradiated parasitized blood (■ — ■); 1 dose of 20 mins heated parasitized blood (▲ — ▲); 1 dose of 30 mins heated parasitized blood (Δ — Δ); 2 doses 30 mins heated control blood (□ — □); or 2 doses of irradiated control blood (• — •).



Two further experiments have been carried out with essentially similar results and only one experiment will be described below in detail. Preliminary experiments showed that placing the parasitized blood (parasitaemia 19-35%) at 45°C for 24 minutes or longer prevented further parasite multiplication. Parasitized blood kept for 20 minutes at 45°C could still contain viable organisms.

Three months old female C57Bl mice were used. Blood was collected from parasitized and control mice into heparin Hank's solution and placed on ice. Whole blood was either heated in a 45°C waterbath for 20 or 30 minutes or irradiated with 40 krads from a gamma ray emitter (^{60}Co). The parasitized blood contained ring stages of P. chabaudi and 19.5% and 17.5% of the red cells were infected for day 0 and day 15 immunizing inocula respectively. Groups of 5 mice were set up and treated as indicated in Table 14. Two mice in Group 1 were found to be infected on day 11 and this group was thereafter excluded from the experiment. All the remaining mice were challenged with 1×10^5 parastized red cells i.v. on day 26. The course of the parasitaemias are shown in Figure 9.

All the groups given irradiated or heated blood were protected to some degree in that the patent parasitaemia declined before that of the controls. Mice given two immunizing inocula were better protected than those given a single inoculum. Irradiated blood was marginally more effective than the heated blood insofar as the (peak) parasitaemia on day 7 was lower in the mice given irradiated blood. A similar finding was noted in the previously reported experiment. The infected blood heated on day 15 was apparently fully inactivated by 20 minutes at 45°C. Infected blood heated for either 20 or 30 minutes was equally immunogenic (Groups 6 and 7 respectively).

Discussion

It was confirmed that P. chabaudi infected blood heated at 45°C for 30 minutes was immunogenic and its immunogenicity compared favourably with that of irradiated blood. Irradiated parasites do continue to metabolize for some time after irradiation (Trigg, Phillips & Gutteridge, 1972) although division is prevented and in the process may release important immunogens. It is likely that following relatively gentle heating some metabolic processes in the malaria parasites may continue. It would be interesting to compare the immunogenicity of heated parasitized red cells with that of parasites killed with formalin, β propriolactone or similar agents.

D'Antonio (1972) found heated P. berghei parasitized red cells to immunize mice as effectively as disintegrated parasites. Spitalny & Nussenzweig (1972), however, found that heat inactivated P. berghei sporozoites protected mice less well than X-irradiated sporozoites. Although our results indicate that heat-inactivated P. chabaudi may be marginally less effective than irradiated parasites the convenience of heat-inactivation recommends its further examination.

TABLE 14

Group	Immunizing inocula*		Heated		Irradiation
	Day 0	Day 15	20 min	30 min	
1	+	+	+P	-	-
2	+	+	-	+P	-
3	+	+	-	-	+P
4	+	+	-	+C	-
5	+	+	-	-	+C
6	-	+	+P	-	-
7	-	+	-	+P	-

*Day 0 - Groups 1, 2 and 3 - 5×10^8 parasitized cells/mouse (2.5×10^9 red cells)

- Groups 4 and 5 - 2.5×10^9 red cells

Day 15 - Groups 1, 2, 3, 6 and 7 - 2.1×10^8 parasitized cells/mouse (12×10^9 red cells)

- Groups 4 and 5 - 1.2×10^9 red cells

P = parasitized blood; C = control blood

Role of different lymphoid cells in immunity to P. chabaudi in mice

Immunity to P. chabaudi in mice can be passively transferred with serum and spleen cells. In last year's report (Annual Report DAJA 37-75 C1620) experiments were described in which immune spleen cell populations were separated into enriched thymus dependent lymphocyte populations (T cells), enriched bursa-dependent lymphocytes (B cells) and strongly adherent cells and their ability to confer immunity in recipient syngeneic mice compared. T and B cell populations were prepared on nylon wool columns. The cell recipients were irradiated with 600 or 800 rads shortly before cell transfer. In brief, these experiments showed as follows. Immunity could be transferred with 1×10^6 or more spleen cells. Enriched immune T cells usually gave poorer protection than spleen cells depleted of macrophages only by filtration on a glass-wool column and this was most clearly seen in the recipients given 800 rads. For a fuller expression of their protective activity therefore the immune T cell population was at least partially dependent on the presence of radiosensitive cells in the recipient mice. Nevertheless, the immune T cell population was usually protective to some degree and this might be attributed to the T cells themselves acting as mediators of cellular immunity, the residual B cells, or the unidentified cells present (such as null cells or theta negative T cells) or combinations of all these cells. The enriched immune B cells gave protection which was never less than that given by the corresponding enriched T cells and more often was significantly better and compared well with the glass-wool filtered immune spleen cells. Where the enriched B cells gave better protection than the enriched T cells it could be concluded that the protection provided by enriched B cell population was not merely emanating from the 13% identifiable T cells present in this population. Although the glass-wool filtered immune spleen cells, which contained approximately 30% identifiable T cells and 30% B cells, consistently gave the best protection, our attempts to show

null cells or theta negative T cells) or combinations of all these cells. The enriched immune B cells gave protection which was never less than that given by the corresponding enriched T cells and more often was significantly better and compared well with the glass-wool filtered immune spleen cells. Where the enriched B cells gave better protection than the enriched T cells it could be concluded that the protection provided by enriched B cell population was not merely emanating from the 13% identifiable T cells present in this population. Although the glass-wool filtered immune spleen cells, which contained approximately 30% identifiable T cells and 30% B cells, consistently gave the best protection, our attempts to show synergy between T and B cells or bone-marrow cells were inconclusive.

Pahgocytic cells of the reticuloendothelial system play an important role in the removal of malaria parasites from the peripheral circulation (Taliaferro & Cannon, 1936). In an earlier report it was shown that parasitized red cells containing late trophozoites or schizonts were phagocytosed in mice which were immune or were becoming immune to P. chabaudi. Phagocytosis occurred in the liver, spleen and occasionally in the peripheral blood. Removal of splenic phagocytes on a glass-wool column did not reduce the ability of the spleen cells to confer immunity on syngeneic recipients: in one experiment 9×10^6 spleen cells depleted of macrophages gave slightly better protection than a similar number of cells of the same spleen cell population not depleted of macrophages. In two experiments (Annual Report 37-73 C3492) splenic phagocytic cells from immune mice were adoptively transferred to irradiated syngeneic recipients in order to examine the activity of these cells in protecting recipients against P. chabaudi. Splenic macrophages were separated from the spleen cell population (as being those cells which adhered to petri dishes after incubation at 37°C for either $1\frac{1}{2}$ hours or 16 hours) and were then injected into irradiated recipients. The adherent cells collected after $1\frac{1}{2}$ hours gave a comparatively high degree of protection to the recipients whereas those collected after 16 hours conferred little protection on the recipients.

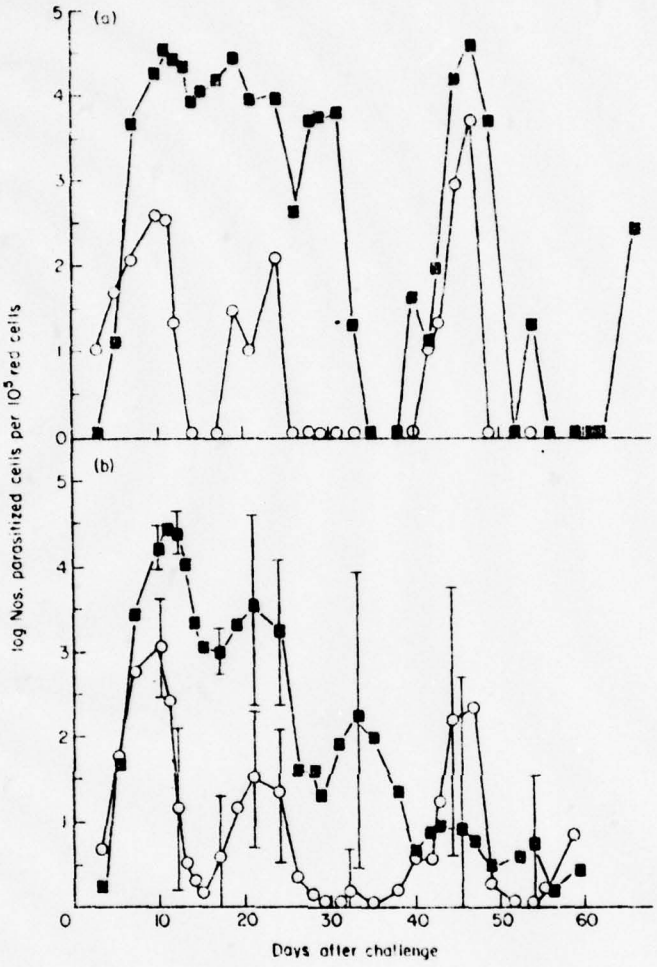


Figure 10. Course of P. chabaudi infection in thymectomized NIH mice. (a) Parasitaemia of one thymectomized and one control mouse. (b) Geometrical mean parasitaemia of thymectomized and control mice.

The current set of experiments have fallen into four parts. First, experiments were carried out to show further the important role of T cells in immunity of mice to P. chabaudi by examining the course of infection in T cell-deprived mice. Secondly, we have started a series of experiments in which the enriched spleen cell populations are being treated with specific antisera which will kill T cells or subpopulations of T cells or B cells prior to adoptive transfer. Thirdly, the ability of splenic macrophages to confer immunity on recipients has been examined further. Fourthly, the protective activity in passive transfer tests of serum for the recipients of immune T cells and glass-wool filtered (g.w.) immune spleen cells has been examined.

1. Plasmodium chabaudi in thymectomized mice

Female NIH mice were thymectomized or sham thymectomized at 12 weeks old, given 800 rads irradiation at 25 weeks, and injected i.v. with 1.25×10^6 syngeneic bone-marrow cells immediately afterwards. The mice were each given 5,000 units of penicillin i.p. and for 2 weeks after irradiation terramycin in the drinking water.

When the thymectomized control mice were 37 weeks old they were infected with 1×10^5 P. chabaudi parasitized red cells from a C57Bl mouse. The geometrical mean parasitaemias in 9 thymectomized and 7 control NIH mice are given in Figure 10, together with the parasitaemia of one mouse selected at random from each group. The primary parasitaemia was considerably more severe and persisted longer in the thymectomized mice. Relapse parasitaemias were also more severe in thymectomized mice.

2. Adoptive transfer of immunity by spleen cells after treatment with anti-thymocyte serum

Anti-thymocyte serum for detecting and killing T cells was prepared in rabbits and used following the method of Rose, Parrott & Bruce (1976). Anti-theta serum was prepared following the procedure of Leif & Allen (1964) by immunizing AKR mice with 7 weekly doses of CBA thymocytes.

Three experiments have been carried out to date, two of which will be summarised and the third described in full.

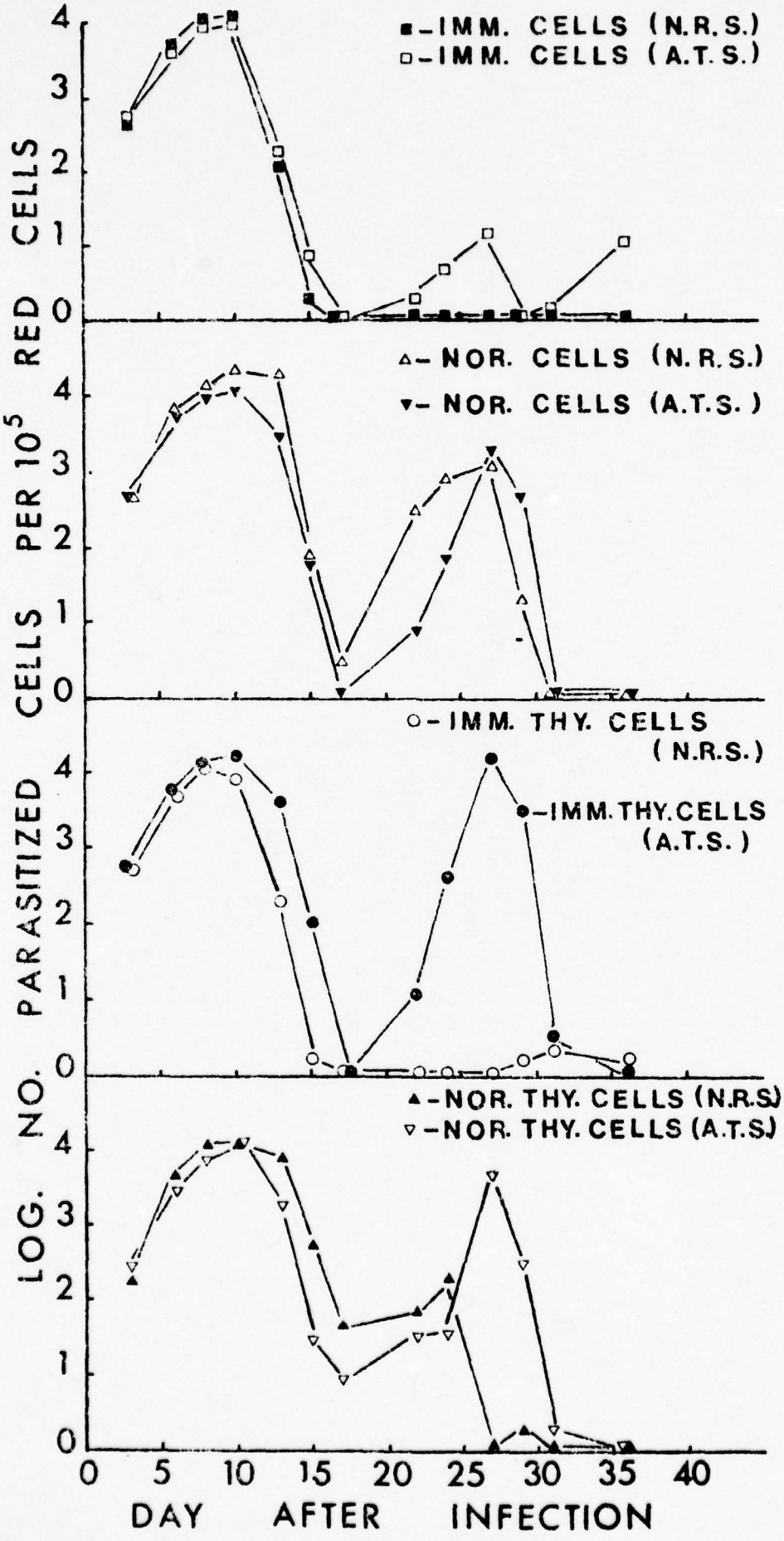
In the first experiment in male C57Bl mice, the immune donors had been infected 275 days and reinfected 148 days before sacrifice. The recipients were 8 weeks old. Enriched immune and normal T cell populations and glass-wool filtered (g.w.) immune and normal spleen cell populations were prepared as described previously (Annual Report DAJA 37-75 C1620). The immunoglobulin bearing cells (B cells) were detected as follows. Cell suspensions were washed once and resuspended to give 5×10^6 - 1×10^7 cells in 0.25 ml phosphate buffered saline (PBS), pH 7.2. To each 0.25 ml of cells was added 25 µl of 1/10 fluorescein conjugated horse anit-mouse immunoglobulin (Progressive Laboratories, Incorporated, Baltimore) diluted in PBS. After 45 minutes incubation on ice, the cells were washed three times in PBS and resuspended to 0.1 ml in PBS. The cells were then viewed under a Leitz Ortholux II microscope set for U.V. illumination. The numbers of B cells in the cell populations were 27%, 23%, 3% and 2.8% in g.w. normal and g.w. immune spleen cells, and normal and immune T cell populations respectively.

The cell populations were treated with anti-thymocyte serum (ATS) diluted to 1/40 in PBS, or normal rabbit serum (1/40) and complement (Rose, Parrott & Bruce, 1976), washed and 2×10^6 of the treated cells injected i.v. into each recipient mouse which had been given 600 rads 2 hours before cell transfer. Treatment with ATS and complement killed 73%, 67%, 27% and 31% of the immune T, normal T, g.w. immune spleen cells respectively. The recipient mice were challenged i.v. with 1×10^6 P. chabaudi parasitized cells within one hour of cell transfer. The course of the parasitaemias is summarised. All the recipients of normal spleen cells had acute relapsing parasitaemias which in the majority of mice became subpatent around day 36 after challenge although most subsequently relapsed: in a small number of mice the parasitaemia was briefly subpatent around day 23. In the recipients of normal spleen cells treated with ATS, the initial parasitaemias were higher than in the recipients of NRS treated cells. In the g.w. immune cell recipients, in both recipients of ATS and NRS treated cells, the parasitaemia was subpatent by day 14 and remained so throughout the 51-day observation period. In the ATS treated g.w. immune cell recipients, however, the mean peak parasitaemia reached was 17.3% whereas in the NRS treated group it was 4.7%. In the immune T cell recipients the primary patent parasitaemias were subpatent by day 16 and in the case of the recipeints of NRS treated T cells remained subpatent throughout the observation period. In the ATS treated group, 3 of 6 mice relapsed between days 27 and 30 and the mean peak parasitaemia was considerably higher than in the NRS treated group (24% vesus 2.1%). It was suspected that the significantly higher parasitaemias in the recipients of cells treated with ATS might be the result of inadequate washing of the treated cells prior to cell transfer and that consequently small amounts of ATS were carried over into the recipients which effectively inactivated residual T cells in the irradiated mice. This experiment showed that treatment of the immune T cell population only impaired the protective activity of these cells insofar as half the mice showed patent relapse parasitaemias.

In a second similar experiment in male C57B1 mice immune spleen cells came from immune donors 112 days after infection and the cells were treated with NRS or ATS diluted to 1/20 and were washed twice with 15 ml of medium 199 before cell transfer. Treatment of immune T, g.w. immune and g.w. normal spleen cells with ATS killed 82%, 48.6% and 43% respectively. Recipient mice were given 600 rads 2 hours before each received 3×10^6 spleen cells, and the mice were challenged with 5×10^5 P. chabaudi parasitized cells. The results showed that the immune T cells treated with ATS conferred no protection on their recipients whereas the NRS treated immune T cells were significantly protected. The recipients of g.w. immune cells gave an anomalous result in that no protection was evident in the recipients of NRS treated cells but 4 of 6 mice given ATS treated cells were protected.

In a third experiment in female NIH mice immune donors were infected 192 days before sacrifice. The procedure was was described in the two previous experiments. The cells were treated with ATS at 1/20 and each 600 rad irradiated mouse received 2.5×10^6 cells i.v. The number of cells killed by the ATS treated was 81%, 71%, 44% and 41% in the immune, T, normal T, g.w. immune and g.w. normal respectively.

Figure 11. Course of P. chabaudi infection in mice irradiated with 600 rads and injected i.v. with either 2.5×10^6 immune T cells treated with either antithymocyte serum (ATS) (● — ●) or normal rabbit serum (NRS) (o — o), 2.5×10^6 glass-wool filtered immune cells treated with either ATS (□ — □) or NRS (■ — ■), 2.5×10^6 normal T cells treated with either ATS (▽ — ▽) or NRS (▲ — ▲), or 2.5×10^6 glass-wool filtered normal spleen cells treated with either ATS (▼ — ▼) or NRS (△ — △).


■-IMM. CELLS (N.R.S.)
□-IMM. CELLS (A.T.S.)
△-NOR. CELLS (N.R.S.)
▼-NOR. CELLS (A.T.S.)
○-IMM. THY. CELLS (N.R.S.)
●-IMM. THY. CELLS (A.T.S.)
▲-NOR. THY. CELLS (N.R.S.)
▽-NOR. THY. CELLS (A.T.S.)
LOG. NO. PARASITIZED CELLS PER 10^5 RED CELLS
0 1 2 3 4
0 5 10 15 20 25 30 35 40
DAY AFTER INFECTION

The cell recipients in groups of 6 mice were challenged with 1 x 10^6 P. chabaudi parasitized cells immediately after cell transfer. The parasitaemias are shown in Figure 11. It can be seen that the immune T cells and the g.w. immune spleen cells treated with NRS conferred some protection on the recipients. This was evident as a suppression of the relapse parasitaemias seen in the control groups: none of the g.w. immune cell recipients relapsed during a 36-day observation period where 2 mice in immune T cells relapsed, on days 29 and 36 respectively. In the recipients of immune T cells treated with ATS the parasitaemias followed those of the control groups. The ability to confer protection on recipients had been abolished by treatment with ATS. In the mice given g.w. immune spleen cells treated with ATS, 2 mice relapsed on days 22 and 24 respectively, at a time when control mice relapsed, 3 mice relapsed on day 36, 10 to 14 days after control mice, and the sixth mouse in the group remained subpatent throughout the observation period.

In summary, the experiments to date indicate that treating immune spleen cells with ATS at 1/20 and 1/40 and complement depressed or even abolished the protective activity of the enriched immune T cell population in irradiated recipients confirming the role of T cells in immunity to P. chabaudi. The effect of ATS treatment in g.w. immune spleen cells was less clear cut but it appeared that it could only reduce the protective activity of this cell population, suggesting a significant role for the surviving cells in this population, perhaps memory of cells.

Role of phagocytic cells in immunity to P. chabaudi

Phagocytosis of parasitized red cells containing late trophozoites or schizonts in the spleen and liver and occasionally in the peripheral blood has been observed in hyperimmune mice and in mice in which the primary parasitaemia was going into remission. Removal of adherent cells from an immune spleen cell population, by passage through glass-wool column, did not impair the ability of these cells to confer immunity in recipient mice: in one experiment it enhanced the protection given. In two other experiments reported earlier, adherent cell populations from immune spleen cells were prepared and transferred to non-immune mice which were subsequently challenged. In one experiment the adherent cells were prepared by collecting cells adhering to petri dishes after 1.5 hours incubation at 37°C and in the other after incubation for 20 hours at 37°C. The protection conferred on the recipients by these populations was quite different. In the former case a strong degree of protection was conferred and in the latter case little protection. Two further experiments examining the ability of adherent cells to confer immunity on recipient mice have been carried out.

Enriched populations of strongly adherent cells were obtained by incubating 1 x 10^7 spleen cells in 10 ml of medium 199 containing 10% foetal calf serum and 20 mM Hepes in 9 cm petri dishes. The cells were incubated for either 1.5 hours or 16 hours (see below) at 37°C in 95% air/5% CO_2 in a desiccator. After incubation, non-adherent cells were removed by repeated washing of the surface of the petri dish with medium and the adherent cells were released by a rubber

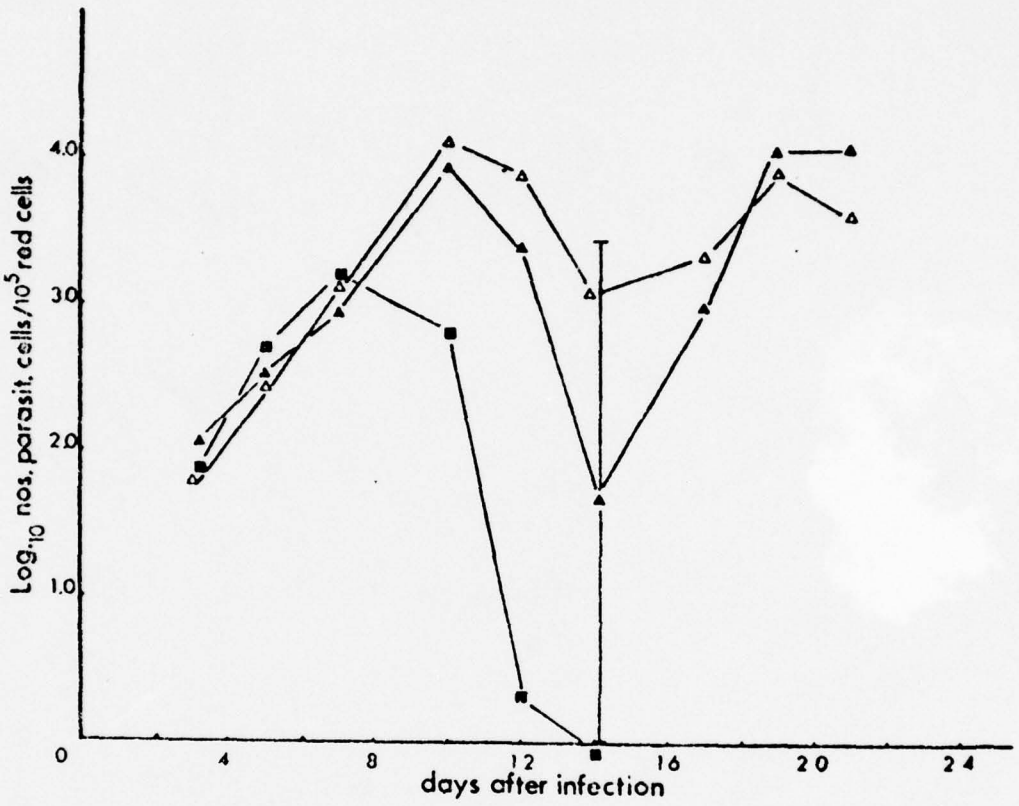


Figure 12. Course of P. chabaudi infection in C57B1 mice irradiated with 600 rads and injected i.v. with either 1 x 10^6 unfractionated immune spleen cells (■ — ■), 1 x 10^6 immune phagocytic cells (▲ — ▲) or 1 x 10^6 normal spleen cells (△ — △).

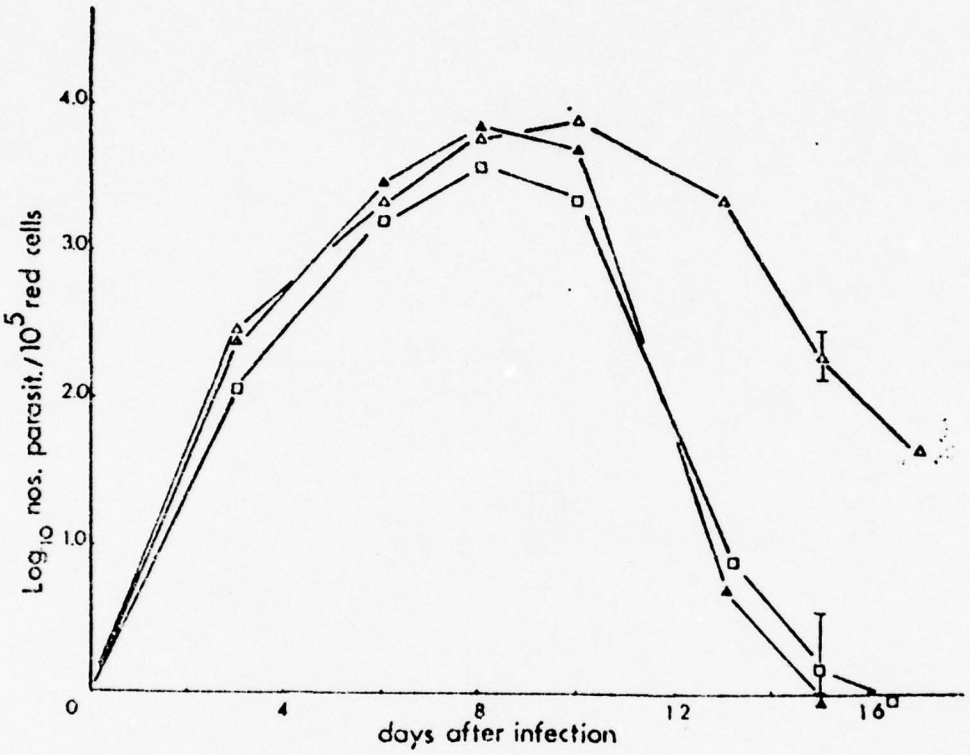


Figure 13. Course of P. chabaudi infection in NIH mice irradiated with 600 rads and injected i.v. with either 1 x 10^6 unfractionated immune spleen cells (□ — □), 1 x 10^6 immune phagocytic cells (▲ — ▲), or 1 x 10^6 normal spleen cells (Δ — Δ).

policeman. The cells were washed three times with medium prior to injection into the recipients. The number of phagocytic cells in the cell populations was determined by the following procedure. A drop of cell suspension containing approximately 5×10^6 cells/ml was mixed with a drop of polystyrene latex particles containing 2×10^9 particles/ml (1.8μ diameter, Dow Chemicals) on a clean glass slide. A coverslip was placed on top of the mixture and the slide incubated for 10 minutes at 37°C. The phagocytic cells which took up latex particles were then counted.

In the first experiment, phagocytic cells were collected after 16 hours incubation from the pooled immune spleens of 2 female C57Bl mice which had been infected on three occasions, the last occasion being 55 days before sacrifice. Eighty per cent of the adherent cells took up latex particles. Recipient female C57Bl mice, 12 weeks old, were irradiated with 600 rads and injected with either 1×10^6 immune phagocytic cells, 1×10^6 unfractionated immune spleen cells or 1×10^6 unfractionated normal spleen cells. The cell recipients were all injected i.p. with 5×10^6 P. chabaudi parasitized red cells 48 hours after irradiation. The course of the parasitaemias are shown in Figure 12 where it can be seen that the phagocytic cells conferred little protection on recipients compared to immune unfractionated spleen cells although around day 14 of infection there appeared to be a transitory protective effect.

In a second experiment with NIH female mice, adherent cells were collected after 2.5 hours incubation and contained 68% cells which took up latex in the immune cell population and 72% in non-immune population. The immune cell donors had been infected twice, the last time being 118 days before sacrifice. The recipient mice were given 600 rads and were injected i.v. with either 1×10^6 immune adherent cells, 1×10^6 unfractionated immune spleen cells or 1×10^6 non-immune adherent cells. Immediately after cell transfer all the mice were challenged with 1×10^6 P. chabaudi parasitized red cells. In this experiment (Figure 13) immune adherent cells clearly conferred some immunity on the recipients.

The different in protective properties of immune adherent cells which were harvested after 2.5 hours and 16 hours may be attributed to a loss of activation of immune macrophages after prolonged incubation in vitro. As immune adherent cells were 80% phagocytic after 16 hours incubation, it is unlikely that a defect in phagocytosis is responsible for such a loss of activation.

The immune adherent cells from the spleens of NIH mice were examined for numbers of immunoglobulin-bearing cells after 2.5 hours incubation at 37°C, and it was found that 25.5% of these cells were immunoglobulin-bearing compared with 28% of the unfractionated spleen cell population. The fluorescence, however, of many of the adherent cells were clearly not as bright as that shown by unfractionated immune cells. Cytophilic antibodies adhering to macrophages may have been at least partially responsible for the fluorescence observed with adherent cells, but it is also possible that B cells were present, and if so, they may have contributed to the protection conferred by immune adherent cells. Phagocytic cells which secrete immunoglobulins have been detected which adhere to

glass surfaces (Löwy et al., 1975). The inability of C57Bl immune adherent cells to confer much protection after 16 hours incubation at 37°C may be due to the fact that lymphocytes die rapidly in culture (Trowell, 1965), 50-70% cells dying within 24 hours in culture at 37°C.

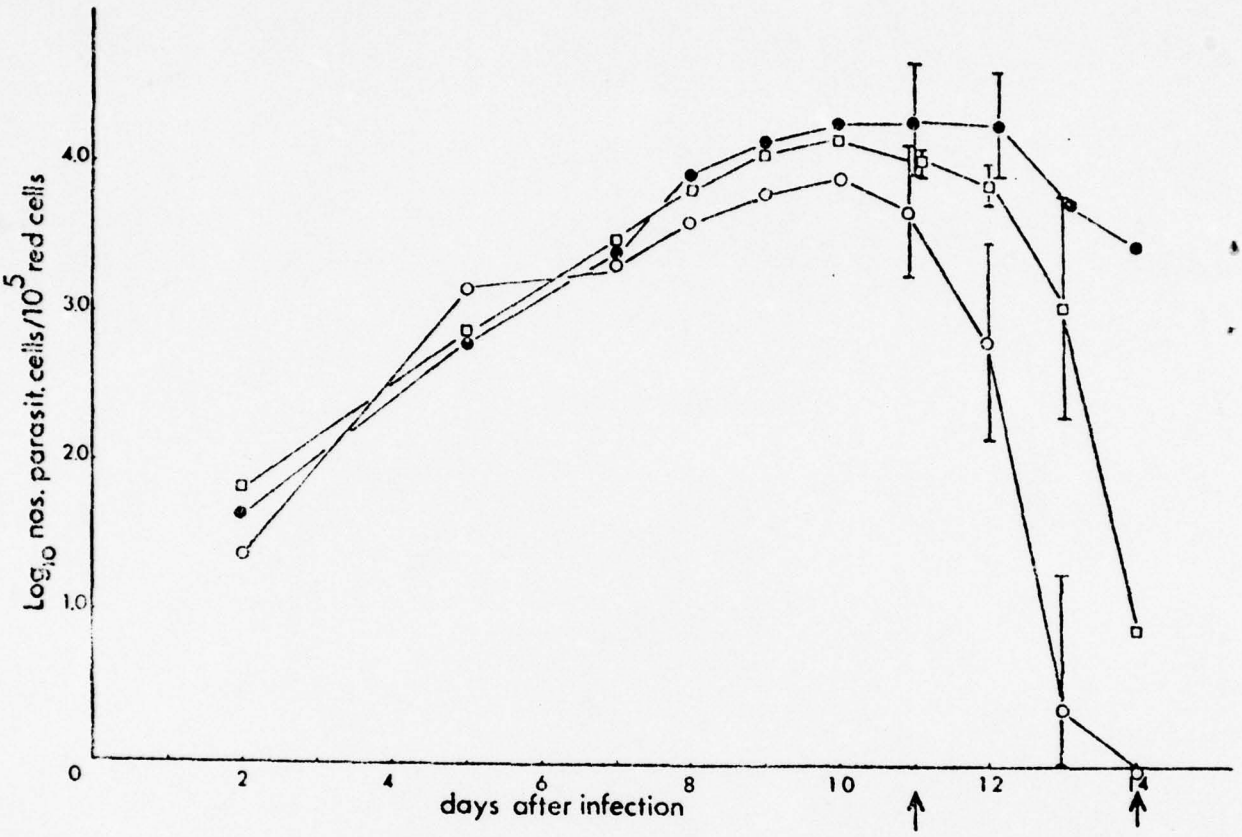


Figure 14. Course of P. chabaudi infection in NIH mice irradiated with 800 rads and injected i.v. with either 2 x 10^6 unfractionated immune spleen cells (o — o), 2 x 10^6 enriched immune T cells (□—□), or 2 x 10^6 normal spleen cells (• — •). Sera were collected on days 11 and 14 (marked with arrows).

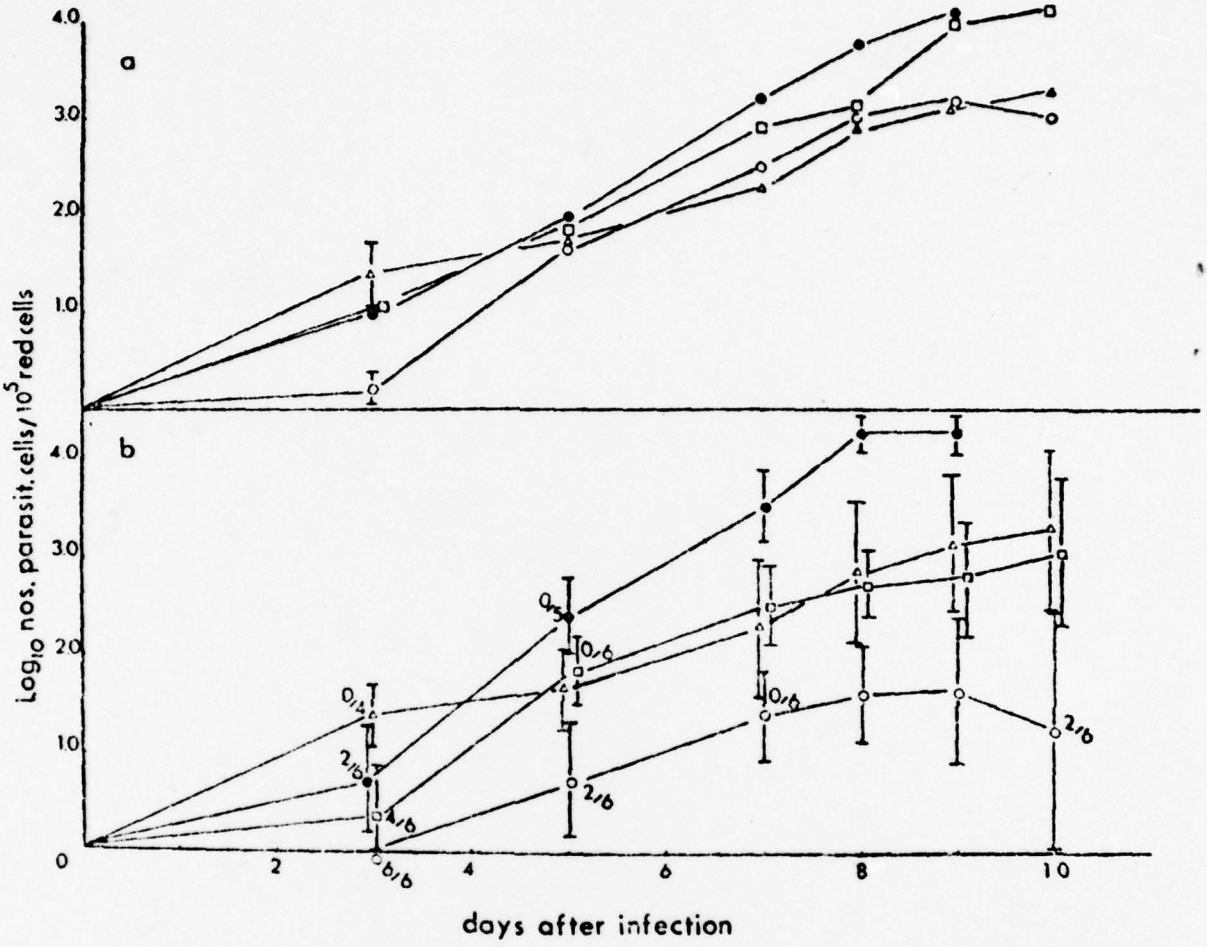


Figure 15(a). Course of P. chabaudi infection in NIH mice irradiated with 600 rads and injected i.v. with 0.5 ml of sera collected on day 11 from recipients of either unfractionated immune spleen cells (o — o), enriched immune T cells (□ — □), or normal spleen cells (● — ●). Control group given normal NIH serum (△ — △).

Figure 15(b). As above except that sera were collected on day 14. The fractions beside certain symbols represent the number of subpatent mice/total number of mice in that group.

The antiparasitic activity of serum from irradiated recipients of immune spleen cells or normal spleen cells during infection

Passive transfer experiments have demonstrated that immune serum provides a degree of protection against malaria infection, in particular, the IgG fraction of immune serum (Cohen et al., 1961; Diggs & Osler, 1969; Butcher et al., 1970; Phillips & Jones, 1972). In the previous report (Annual Report DAJA 37-75 C1620) the passive transfer of immunity with serum was demonstrated with P. chabaudi infections in non-irradiated and more effectively in irradiated mice. Irradiated mice, therefore, were used to examine the protective effect of serum taken at different times during infection from irradiated recipients of either unfractionated immune spleen cells, enriched immune T cells or normal spleen cells.

Groups of 20 eight months old NIH female mice were irradiated with 800 rads and one hour later injected i.v. with g.w. immune spleen cells enriched immune T cells or g.w. normal spleen cells. The number of immunoglobulin bearing cells (B cells) in the enriched immune T cells and the g.w. immune cells was 1% and 34% respectively. The immune cells came from two NIH female donors which had been infected 35 days before spleens had been removed. Normal cells came from age and sex matched mice. The cell recipients were injected i.p. with 2×10^6 P. chabaudi parasitized red cells within two hours of cell transfer.

The course of the parasitaemia in the cell recipients was monitored in five or six mice in each group (see Figure 14). On days 11 and 14 of infection half the mice in each group were sacrificed and bled for serum. Sera from mice in each group were pooled and stored at $-20^{\circ}C$ until the protective activity of each pool was tested as follows. Twelve weeks old NIH mice were given 600 rads and 20 hours later were infected i.v. with 10^4 P. chabaudi parasitized red cells of the same parasite population used for infecting the spleen cell recipients. Within 30 minutes of infection the mice were injected i.v. with 0.5 ml of sera collected on days 11 and 14 from either the g.w. immune cell recipients, the enriched immune T cell recipients or the normal spleen cell recipients. A control group of irradiated mice received NIH serum from non-immune mice.

It can be seen from Figure 14 that the primary parasitaemia declined first in the g.w. immune cell recipients followed by that in the immune T cell mice and the most persistent parasitaemia was in the control mice.

The results of the passive transfers are given in Figure 15. Mice which received serum from day 11 of infection showed little protection although on day 3 four out of five mice which received serum from g.w. immune cells were still subpatent whereas all but one of the mice from the other two groups were showing patent parasitaemias. In the mice given day 14 serum, there was good evidence of protective activity in the serum from the g.w. immune cell recipients: none of these mice showed a patent parasitaemia on day 3 whereas all four normal serum controls were patent and throughout the observation period the course of the parasitaemia was depressed compared with the controls. Serum

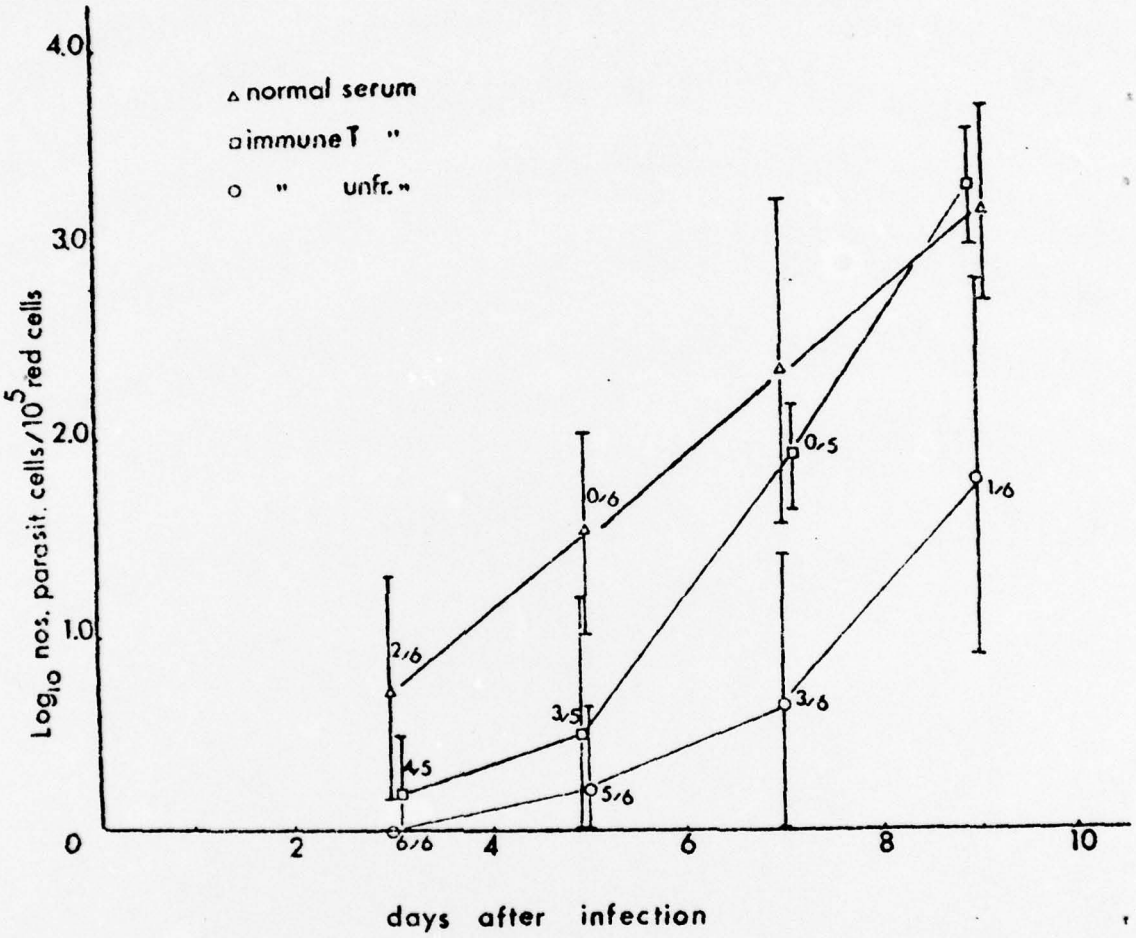


Figure 16. Repeat of experiment shown in Figure 15 except that non-irradiated NIH mice were used as recipients. Mice were injected with 0.5 ml of sera collected on day 14 from unfractionated immune cell recipients (○ — ○), or enriched immune T cell recipients (□ — □). Control group received normal NIH serum (△ — △).

from the immune T cell recipients gave some protection insofar as four of six mice were subpatent on day 3 but thereafter the parasitaemia rose to control levels. Serum from normal spleen cell recipients appeared to enhance the parasitaemia in the irradiated recipients although 2 of the 5 five mice in this group were subpatent on day 3.

A confirmatory experiment was carried out with day 14 serum remaining from the g.w. immune and immune T cell recipients. The serum recipients, 22 week old female NIH mice, were not irradiated. In groups of 5 or 6 mice they were challenged with 1×10^4 P. chabaudi parasitized cells and immediately afterwards by the same route injected with 0.5 ml of normal NIH serum or day 14 serum from either the g.w. immune cell recipients or immune T cell recipients. The resultant parasitaemias are shown in Figure 16. Again the serum from the g.w. immune cells was the most protective and the serum from the immune T recipients gave a measurable although small degree of protection.

Discussion

The protective effect of passively transferred sera isolated from irradiated mice which had been the recipients of g.w. immune spleen cells, enriched immune T cells, or g.w. normal spleen cells reflected the immune status of the serum donors when the serum was collected. Serum collected from the recipients of g.w. normal spleen cells showed little or no protection in the recipients 3 days after challenge and as the infection developed in the serum recipients appeared to enhance the parasitaemia. Serum collected on day 14 was more effective in the passive transfer test than day 11 serum for the g.w. immune cell recipients and to a lesser degree for the immune T cell recipients.

It remains to be shown that the protective activity in the sera from the two groups of immune cell recipients (g.w. immune and immune T) is in one or both cases antibodies. If the low-level of protective activity in the sera from the immune T cell recipients is antibody, it suggests that a role of the T cells is that of helper cells and that there are sufficient B cells in the irradiated mice with which the T memory helper cells may cooperate. The g.w. immune spleen cell population would be expected to include both T and B memory cells which together would have the potential to produce antibodies of various specificities. If we assume that P. chabaudi can undergo antigenic variation, then the more persistent activity of the day 14 g.w. immune cell serum may only reflect the presence in it of antibodies which react or cross-react with a larger number of variant populations which might arise in the serum recipients. Phillips (1970) and Phillips & Jones (1972) clearly showed that in the adoptive transfer of immunity to P. berghei in rats with spleen cells the elimination of the parasite in the recipients after challenge was associated with the appearance of high levels of protective IgG, in the serum. Further experiments are in progress to compare antibody production in immune T and g.w. immune spleen cell recipients.

Recommendations and future work

Trager & Jensen (1976) and Haynes et al. (1976) reported that it was possible to culture the asexual stages of P. falciparum on a continuous basis. This was an important step forward, both with regard to the production of parasites for incorporation into a vaccine and for their use in immunological studies. It remains to be seen if all or at least the majority of wild populations of P. falciparum can be grown in culture on a continuous basis. My colleague, Dr R.J.M. Wilson, after several months frustration, has now succeeded in growing two of our Gambian isolates on a continuous basis and we intend to examine the ability of a large number of samples of infected blood from Gambians to adapt to continuous culture. We have samples of infected blood from 100 Gambians cryopreserved in liquid nitrogen of which 87% grew from ring to schizont stage and underwent reinvasion in microculture when a sample of the freshly collected infected blood was washed, resuspended in serum and introduced into culture. The procedure for cryopreserving and subsequently thawing ring stages of P. falciparum described above gave a satisfactory recovery rate although the washing procedure was drawn out and required care. Snap freezing gave good red cell preservation. At the present time we are examining the use of high concentrations of salt solution (Meryman & Hornblower, 1977) as an alternative to sorbitol for washing the cryopreserved blood. In the procedure under investigation, the thawed blood is first centrifuged and the supernatant removed, the blood cells are resuspended in 0.5 ml of 4.5% saline to which is added 4.5 ml of 3.5% saline. The cells are centrifuged and washed twice in physiological saline. The amount of lysis during the washing process compares well with the sorbitol method and has the advantage of being completed more quickly. The cryopreservation of trophozoites and schizont stages of P. falciparum is more difficult and needs to be examined in detail. The procedure described by Wilson and colleagues (1977) fro cryopreservation of the larger intracellular forms of P. knowlesi may be applicable to P. falciparum although our preliminary attempts were not encouraging.

The development of gametocytes in cultures of P. falciparum from freshly collected and cryopreserved blood is an important observation. This was first reported in Annual Report DAJA 37-73 C3492 and further work is reported above. Since this work was carried out, Carter & Beach (1977) have described the development of gametocytes of P. falciparum in vitro which are able to exflagellate in vitro. Clearly, the in vitro growth of gametocytes to a stage when they are infective to the mosquito may provide the means of producing sporozoites of P. falciparum in the laboratory for vaccination, immunological and biochemical studies without recourse to human volunteers as gametocyte carriers. It is recommended that Carter & Beach's observations be confirmed and that investigations of the factors which initiate gametocytogenesis be undertaken.

It is clear that the immune response to malaria parasites is complex and that both humoral and cell-mediated mechanisms are involved. Our investigations of immunity to P. falciparum in this study have concentrated on humoral immune mechanisms, in particular, on the effect of antibody on reinvasion. Cryopreservation of P. falciparum was used to relate an immune

response in a patient to a specific population of parasites, a procedure which controlled for any antigenic variability within and between strains of P. falciparum. Although it has still to be demonstrated that P. falciparum is able to undergo antigenic variation the indirect evidence suggests that antigenic variability by the parasite should be taken into consideration (Wilson & Phillips, 1976). It is recommended that more research be devoted to devising techniques for determining whether antigenic variation occurs within strains of P. falciparum and that the immune responses, both cellular and humoral, to specific populations of P. falciparum be examined using cryopreserved parasites as described in this report. Lymphocytes can also be cryopreserved (Knight et al., 1977) and retain their immunological activity. It would, therefore, be possible to collect a series of samples of peripheral blood leukocytes from patients during a patent parasitaemia and at intervals thereafter, and subsequently examined the activity of these cells in in vitro correlates of cell-mediated immunity, such as lymphocyte proliferation in the presence of specific malarial antigen, macrophage migration inhibition and K cell activity. Cryopreservation would allow all the cells to be tested on the same occasion thereby making possible a strict comparison of the activity of the leukocytes collected on different occasions.

Although there is currently considerable interest in the immunization of primates with asexual erythrocytic stages of P. knowlesi and P. falciparum, in particular, with merozoites, it seems to the author that the rodent malarias still have an important role in investigation of potential immunization schemes. In the present report it appeared that parasitized red cells, inactivated by minimal heat treatment, are only marginally less effective than irradiated parasitized red cells. The convenience of heat-inactivation recommends its further investigation and comparison with other forms of parasite inactivation, such as formalin (Playfair et al., 1977), or Beta propriolactone treatment. The value of merozoites when compared with other stages in the development of erythrocytic stages could also be evaluated in rodent plasmodia. It is possible to grow both P. chabaudi, P. berghei and P. yoelii to the schizont stage in vitro from which it should be possible to collect merozoites. The rodent system should also be used for evaluating the potential of different adjuvants in malarial vaccines. In the author's view a neglected aspect of malarial immunization is the possibility of using adjuvants to enhance the immunity which follows chemotherapy perhaps by administering an adjuvant at the same time as, or immediately after, chemotherapy.

Our investigation into the mechanisms of immunity to P. chabaudi are at a very interesting stage and should be continued. Our preliminary observations indicating an increase in K cell activity in malarious mice suggests that antibody mediated cytotoxicity may be a mechanism for controlling malaria infections. Our attempts to show increases in K cells in the peripheral blood leukocytes of malarious children were not successful although Greenwood et al. (1977) were able to do so. As noted in the report, we had technical problems with the target cells which made interpretation of the results difficult. Our observations on K cells in malarious mice and those of Greenwood et al. in malarious children indicate a detailed investigation into the role of cellular cytotoxicity, with and without antibody, in the control and elimination of malaria infections.

The adoptive transfer studies involved the use of irradiated recipients. An incidental observation arising from the use of irradiated mice was that irradiation induced resistance to P. chabaudi if infection followed irradiation by less than 7 days or enhanced the course of the infection when infection was delayed for 2-3 weeks. The parasitaemia was enhanced in mice challenged within 7 days of irradiation if the mice were given bone-marrow or spleen cells immediately after irradiation. Asimilar depression of the parasitaemia followed cyclophosphamide administration. It seems worthwhile examining further the nature of the depression and enhancement of the parasitaemia noted above.

The results of the cell transfer studies suggest that this line of investigation should continue and, in particular, P. chabaudi or P. vinckei parasites which have no preference for immature red cells, should be used where the cell recipients are irradiated. It is our intention in trying to identify the active lymphoid cells to examine further the relative role of T abd B cells and, in particular, to try and identify the role of individual subclasses of T cells, i.e. helper, cytotoxic, and suppressor cells. More emphasis is to be given to examining spleen cell and lymph node populations from mice which are recovering from a primary and/or a secondary challenge infection. Recently Clark and colleagues (1977) have emphasised the role of non-antibody cytotoxic factors perhaps lymphokines which are responsible for the appearance of degenerate crisis forms in mice recovering from rodent babesia and plasmodial infections. In view of these observations the incidence of these degenerate forms in immune cell recipients should be determined. Our preliminary results reported above showed that in irradiated mice given immune spleen cells protective activity could be detected in the serum suggesting one role of the transferred cells is in the elaboration of protective antibodies in the recipients although it has to be demonstrated that this protective activity resides in the gamma globulin fraction. This observation should be followed up.

A feature of malaria infections is that in many hosts the infection is persistent if the host survives the acute phase of the disease. In P. knowlesi infections in the rhesus monkeys it is known that the parasite undergoes repeated antigenic variation and this facility probably contributes to the parasite surviving in the semi-immune host. It still remains to be seen if repeated antigenic variation is a feature of most malaria parasites including the human malarias and to ascertain whether the continued survival of parasites in the semi-immune host may also be a consequence of constraints on the immune response of the host to the parasite. This is clearly an area worth investigation.

In last year's Annual Report I said that I felt malaria research at the present time was moving forward at an exciting rate and that not only will this eventually lead to the control of malaria but will reveal many biologically interesting phenomena. This I still feel is the case and I and my staff are grateful to the U.S. Army for allowing us to share in this exciting time in malaria research.

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