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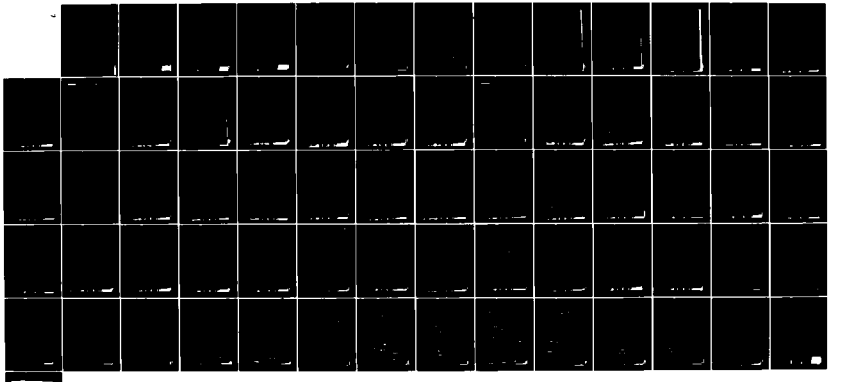

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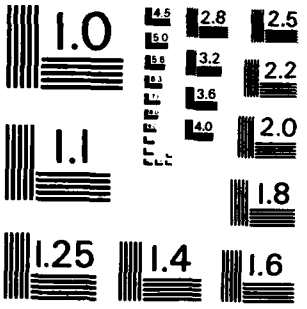

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MECHANISMS INVOLVED IN IMMUNITY TO MALARIAL PARASITES

Final Report

by

R. S. Phillips

July 1980

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FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Summary

Part I - Immunity to Plasmodium falciparum in Man.

The procedure for the continuous cultivation of the erythrocytic stages of P. falciparum following the method of Trager and Jensen (1976) has been established in our laboratory.

Ring stages of P. falciparum in 34% glycerol (v/v) in sorbitol were snap-frozen in liquid N_2. A similar level of parasite viability was obtained after the parasites had thawed out and been washed with either decreasing concentrations of sorbitol/saline or hypertonic saline.

Since only small amounts of blood were available from patients a procedure for culturing P. falciparum on a microscale was established. Growth and multiplication of the parasites in vitro was assessed from Giemsa's stained smears and incorporation of ^{3}H-isoleucine. In microtissue culture trays P. falciparum grew slightly better in RPMI 1640 and Medium 199 containing 10% rather than 5% foetal calf serum, grew better in flat-bottomed trays rather than round-bottomed trays and gave better incorporation of ^{3}H-isoleucine in Medium 199 than in RPMI 1640. The conditions were determined which allowed satisfactory growth and multiplication of P. falciparum in individual cultures of 50µl.

Nineteen sera from non-immunes showed varying abilities to support growth of P. falciparum in microcultures indicating the necessity for adequate controls in testing human immune sera for antiparasitic activity.

Infected blood from 82 patients was cryopreserved. Thirty-four patients returned after recovery and gave usable serum samples to test for antiparasitic activity.

Part II - Immunity to rodent malaria parasites.

Fractionation of immune spleen cells into T-cell and B-cell enriched populations and treatment of these populations with anti-T-cell antisera before transfer into irradiated naive recipients confirmed a role for both T- and B-cells in immunity to P. chabaudi.

Injection of pristane (2, 6, 10, 14-tetramethylpentadecane), emulsified complete Freund's adjuvant or emulsified incomplete Freund's adjuvant intraperitoneally into mice 2-4 days before an intraperitoneal challenge with P. yoelii or P. chabaudi delayed the onset of a patent parasitaemia in the mice. The mice were not so protected if the challenge infection was given intravenously. The protection in the mice was associated with increased phagocytic activity by peritoneal macrophages in the treated mice.

Infection of mice with Mycobacterium lepraemurium, murine leprosy, gave the mice considerable resistance to a later infection with Babesia microti and some resistance to P. chabaudi.

Groups of mice infected with P. chabaudi, were irradiated on a single occasion with 600 rads X-irradiation between days 3 and 37 after infection. Irradiation before or at peak parasitaemia did not extend the primary patent parasitaemia but precipitated a premature recrudescence. Irradiation between days 10 and 17, when the patent parasitaemia was declining, temporarily halted the decline in the parasitaemia and extended the primary patent parasitaemia: subsequently in these mice the recrudescent parasitaemias were delayed. Irradiation after day 21 had no significant effect on the course of the parasitaemia. There is, therefore, a radiosensitive component in the immune mechanisms leading to parasite removal operating between days 10 and 17 after infection approximately.

Late trophozoites, schizonts or merozoites of P. chabaudi are the vulnerable stages in the immune mouse with phagocytosed parasites to be seen in the liver, spleen and occasionally the peripheral blood. Experiments involving removal of the spleen confirms its important role in the suppression of P. chabaudi in mice with patent parasitaemias. In mice with an established immunity which have recently had their immunity boosted by reinfection, removal of the spleen does not prevent the rapid removal of a large challenge infection. The spleen, therefore, may be important in the synthesis of protective antibody but the activity of this antibody can be independent of the spleen.

The cross-immunity between P. chabaudi and P. vinckei brucechwatti is dependent on the spleen. Mice which are hyperimmune to either P. chabaudi or P.v. brucechwatti could rapidly control a large homologous challenge in the absence of the spleen but not a heterologous challenge. Cross-immunity between these two rodent parasites appears to involve the rapid mobilisation of a specific immune response to the challenge parasite in the spleen and that the non-specific element of the cross-immunity appears spleen-dependent also.

Introduction

Although malaria has been effectively controlled in many parts of the world over the last thirty years, it is still the major medical problem of the warmer parts of the world, particularly in the developing countries. Over 400 million people are still living in areas where there are no effective control measures. In Africa alone nearly one million people, mainly babies and infants, are reported to die from malaria each year. 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.

It is well known that the immune response to an antigen is complex, that the character of the immune response changes with time, and that it is subject to regulatory mechanisms. Not all the immune responses to malaria parasites are beneficial to the host: some can be harmful. A detailed knowledge of the form the immune response to malaria parasites takes and of the ways in which immunity acts against the parasites and of its specificity will dictate which aspects of the immune response to the parasites, should be preferentially stimulated by a vaccine. It is with this objective in mind that the following studies were carried out.

The study has concerned the following areas:

1) *In vitro* culture of *Plasmodium falciparum* on a microscale.

2) Specific parasite inhibitory antibody response to populations of *P. falciparum*.

3) Mechanisms of protective immunity to mouse malarias.

Part I

Immunity to Plasmodium falciparum in Man

This part of the study was partly carried out at the Medical Research Council Laboratories, Fajara, The Gambia, West Africa, with the permission of the Director, Dr I.A. McGregor, and with the help of the resident technical and medical staff.

The Gambia is a hyperendemic malarious area, with P. falciparum accounting for most of the cases of malaria. P. ovale and P. malariae occur occasionally. Most malaria in the Gambia is seen in the period from July to December reflecting the increased numbers of mosquitoes found during the rainy season and the three months immediately after it. Although antimalarials can be purchased from traders and are given to patients at village dispensaries, the use of antimalarials for prophylaxis purposes at the present time it rare amongst the majority of the African population. This situation is to change as plans for mass prophylaxis are formulated. Infant and child mortality is high. Most deaths from malaria occur in infants between three months and three to four years. Children who survive these early years gradually build up an effective immunity until by adolescence a strong immunity is established. It is not unusual, however, for adults, during the period of peak transmission, to have patent parasitaemias although rarely showing clinical symptoms of the disease. Even in individuals living all their lives in one locality, therefore, it takes several years of exposure to P. falciparum before an effective immunity to the parasite is built up and even in immune or semi-immune individuals following reinfection a patent parasitaemia may ensue. Two possible explanations are suggested to explain why the establishment of a beneficial level of acquired immunity is a relatively slow process and why when immunity is effective enough to prevent clinical illness the parasites may survive and multiply. First, it is likely that even within one locality a large number of 'strains' of P. falciparum exist, each of which necessitates a specific immune response from the host and thus it may take several years before an individual has been exposed to most of the existing strains within a locality. It would be expected that different strains did share common antigens and that sensitization with one or more strains would promote a more rapid response to further strains the individual may be exposed to. Secondly, P. falciparum, like P. knowlesi in the blood of the rhesus monkey, may be able to undergo antigenic variation (Brown and Brown, 1965) and thereby evade the host's immune response to it. The eventual composition of a vaccine against the erythrocytic stage of P. falciparum may depend upon the occurrence of intra- and interstrain variation in P. falciparum. In the work described below in which immune mechanisms against P. falciparum have been examined, the possibility that inter- and intrastrain variation occurs has both been taken into account and evidence for its occurrence sought.

Immunity to P. falciparum in man is, in part at least, dependent on serum globulins (antibodies). Cohen et al. (1961) pooled serum from a number of adult Gambians, separated out the IgG fraction and showed that this IgG had protective activity when injected into children suffering acute P. falciparum infections. In these children the parasitaemia declined and, as far as could be ascertained, the immune IgG appeared to be antiparasitic when the parasite in the red cells reached the mature schizont stage. In the work of Cohen et al. it is important to note that serum was pooled and that it was transferred into children who were probably already partially sensitized to the

parasite and whose reticuloendothelial system therefore was stimulated. The protective activity and hence specificity of sera from individual donors was not tested. The antiparasitic activity of the serum antibody may be dependent on the cooperation with cellular elements, such as macrophages and K cells present in the infected children, recipients of the immune IgG.

The asexual blood stage of P. falciparum can now be cultured on a continuous basis (Trager and Jensen, 1976), some isolates from patients adapting to continuous culture more easily than others (Jensen and Trager, 1978). The effect of immune serum on the growth and multiplication of P. falciparum in culture has been examined by a number of workers (Phillips et al., 1972; Phillips et al., 1975; Wilson and Phillips, 1976; Mitchell et al., 1976; Reese and Motyl, 1979). Reese and Motyl reported the inhibitory effect of immune Aotus monkey serum on a continuously cultured strain of P. falciparum with which the monkey had been infected. Mitchell et al. (1976) cultured P. falciparum from an infected Aotus monkey with human IgG from immune patients. Our own work in the Gambia has looked at the effect of serum from Gambians of various ages on the growth and multiplication of parasites taken from infected children. Initially individual cultures of 3ml were used (Phillips et al., 1972) but since then the parasites have been cultured in 96-well microtissue culture plates (see Diggs et al., 1971). The growth and multiplication rate of the parasites in microculture through one schizogony and reinvasion was monitored by Giemsa's stained smears, by measuring the incorporation of ^{3}H-isoleucine or the release of lactate into the supernatant. In excess of 100 sera from Gambians of all ages and 86 Nigerian sera were tested for parasite inhibitory activity. In all cases the test sera were compared for their ability to support growth and multiplication of the parasite against serum collected from the parasite donor at the same time as the infected red cells were collected. The majority of sera showed slight inhibitory activity and a few were strongly inhibitory. A few sera appeared to be inhibitory to the parasites of some children but not others. It was suggested that this variability in the antiparasitic activity of sera in these short-term microcultures might be attributed to antigenic diversity in P. falciparum coupled with the fact that specific antibody responses to any antigenic type might remain at a high and detectable level for a relatively short period. It was decided, therefore, that a population of parasites needed to be matched with a specific immune response in an individual. In order to match, for example, antiserum with the particular population of P. falciparum which stimulated its production, infected blood was cryopreserved while the infected parasite donor, after minimal chloroquine therapy, mounted an immune response to that population. Subsequently the recovered patient donated a further serum sample (convalescent or recovered serum) and the cryopreserved blood was thawed and in microcultures the ability of these parasites to grow and multiply in the presence of serum originally collected at the same time as the infected red cells (pretreatment serum) and in convalescent serum, was compared. Preliminary results, reported previously (Wilson et al., 1976; previous Annual Reports), showed that convalescent sera showed both specific inhibitory activity of homologous parasites (i.e. parasites and sera coming from the same donor) and also some activity against heterologous parasites (i.e. parasites from other patients). This first study was considered preliminary because the numbers of patients from whom pretreatment and convalescent serum was obtained was small, and subsequent work has largely been concerned with using the same procedure to increase the number of pretreatment and convalescent sera tested.

The work carried out in the short working visit to the Gambia during the period of this report is described below. On return from the Gambia with a large number of isolates of P. falciparum parasitized blood, cryopreserved in liquid N_2, it was decided that before these isolates and corresponding sera were tested the continuous culture technique for P. falciparum should be established in the laboratory in Glasgow. It was hoped that each isolate would subsequently be tested for its ability to establish in continuous culture. There was a delay in achieving this for two reasons. First, the implementation of the Health and Safety at Work Act (1974) in the Universities of the U.K. necessitated the implementation of handling procedures for dangerous pathogens in which the risk to the worker and other personnel in the vicinity were reduced to a minimum. For the handling of isolates of P. falciparum it was decided to first screen all samples of blood and serum for hepatitis B and destroy all positive samples (12-15% of samples) and to handle all the P. falciparum infected blood using standard dangerous pathogen handling procedures in a recirculating work station. Secondly, the actual establishment of the Trager and Jensen continuous culture procedure took longer than expected. Several months of frustration and failure were experienced. Although it is difficult to identify all the reasons for the parasites failing to grow it is clear that with time technique improved. Batches of human serum and human red blood cells (both from the West of Scotland Blood Transfusion Service) varied in their ability to support growth and multiplication of the parasite. Establishment of a routine in which new batches of serum, medium and red cells are tested for their suitability for culture purposes before being used for all the cultures, removed most sources of failure.

On my arrival at the M.R.C. Laboratories in the Gambia for the period of work described below I was informed that it was no longer possible to obtain convalescent serum samples by venepuncture although the earlier review of the research proposal by the M.R.C./Gambian Government Ethics Committee had not questioned the use of venepuncture in recovered patients where it was thought, by clinician and parent, to be in no way detrimental to the patient. It is now possible to collect convalescent serum by venepuncture. It did mean, however, that convalescent sera had to be obtained by finger prick, and that the quantities obtained were small. Consequently a large part of the six weeks' working period was spent in scaling down the micro-cultures to cultures of 50-100µl which were less demanding on sera and parasites.

Cryopreservation of Plasmodium falciparum

In the previous report a cryopreservation procedure for the ring stage parasites of P. falciparum was described. Parasitized blood was collected into heparin ringer to give a final concentration of 10-17 i.u. heparin/ml of blood. The infected blood was washed twice with medium 199 or RPMI 1640 and resuspended in compatible Caucasian serum to give a 40% suspension of red cells. The infected blood was aliquoted in 0.25-0.30ml amounts in one or 2ml screw-capped Sterilin propylene tubes to which was added, drop-wise, the cryoprotectant which was 34% glycerol (v/v) in sorbitol/saline (38gm glycerol, 2.9gm sorbitol and 0.53gm NaCl in 100ml distilled water). In the original procedure cryoprotectant and parasitized cells were kept on ice. The present procedure is to have parasitized blood and cryoprotectant at room temperature and after mixing to keep the mixture at room temperature for 15 min before placing in ice-water for 5 min

after which the tubes are plunged into liquid N_2. Thawing of cryopreserved blood 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 min, the supernatant removed and the red cells washed in five steps (see below) with decreasing concentrations of sorbitol (17.5% ——> culture medium) in phosphate buffered saline pH 7.2 (PBS). This procedure for recovering cryopreserved infected cells resulted in a good red cell recovery but had the disadvantage of being laborious and time consuming. An alternative recovery method using hypertonic saline has been compared with the sorbitol method. The results of one such experiment are given below. The infected blood from a Blood Group A donor had 2.6% parasitaemia of large rings. It was washed and cryopreserved in sorbitol/glycerol as described above in 0.3ml amounts in liquid N_2. Aliquots were thawed out seven days later and washed with either sorbitol saline or with hypertonic saline following the protocols given below.

Sorbitol wash		Saline wash	
Step 1	2ml 17.5% sorbitol (S) + 2ml 10% S + 2ml 7.5% (S)	Step 1	0.5ml 4.5% saline + 4.5ml 3.5% saline
Step 2	1ml 10% S + 2ml 7.5% (S) + 2ml 5% S	Step 2	5ml 0.9% saline
Step 3	1ml 7.5% S + 2ml 5% (S) + 2ml 2.5% S	Step 3	5 ml medium 199
Step 4	1ml 5% S + 2ml 2.5% (S) + 2ml medium 199		
Step 5	1ml 2.5% S + 4ml medium 199		

After washing, the red cells were resuspended in 150µl Caucasian Group A serum, and put into microtissue culture trays. Five microlitres of sorbitol washed or saline washed infected blood were subsequently put into microcultures containing 50, 100 and 150µl of medium 199 (Phillips et al., 1972) containing 10% foetal calf serum and 10mM Hepes. The cultures were incubated in 5% CO_2 in air.

After resuspending the cells in A serum the sorbitol washed blood had a red cell count of 3.98×10^9/ml and the saline washed blood 3.05×10^9/ml indicating that the sorbitol method gave a slightly better red cell recovery. Growth of the parasites in culture was monitored by examining Giemsa's stained smears and by the uptake of ^{3}H-isoleucine following the procedure described in a previous report and in Wilson et al.(1977). 0.5µCi ^{3}H-isoleucine in 5µl medium 199 were added to the microculture 6hr after the cultures had been initiated and the cells harvested 15hr later.

Results

The level of incorporation of ^{3}H-isoleucine in the cultures is given in Table 1. It can be seen that overall the amount of incorporation was comparable in the saline washed and sorbitol washed blood. The blood smears from the washed and resuspended red cells showed a slightly higher parasitaemia in the sorbitol washed cells. The reinvasion rates were very similar. The amount of incorporation of the tracer in the microcultures reflects the dilution factor of the tracer in the culture of different volumes (see later).

Table 1

Culture of cryopreserved P. falciparum after washing with sorbitol or hypertonic saline

Wash	Volume of culture - µl			c.p.m. x 10^{-3} Mean of duplicate cultures
	50	100	150	
Saline	+			16.6
		+		11.05
			+	6.99
Sorbitol	+			16.08
	+	+		7.91
			+	5.85

Initial parasitaemia - sorbitol wash 1.96%

saline wash 1.53%

Multiplication rate (after reinvasion)

sorbitol wash - x 2.5

saline wash - x 3.5

Conclusion

This and other experiments indicate that both sorbitol and hypertonic saline washing procedures give reasonable and similar parasite survival as judged in short-term cultures. Longer term cultures of cryopreserved infected blood after washing by the two methods are planned.

Variations in the procedure for culturing P. falciparum on a microscale

It was noted in the Introduction that there was a necessity to reduce the size of the microcultures so that the inhibitory activity of the small volumes of sera from patients before and after recovery could be tested. The following experiments were set up largely to find the best culture conditions.

In all the experiments reported below the infected blood was collected from the patient into heparin/ringer to give a final concentration of heparin of 10-20 i.u./ml blood. The blood cells were deposited by centrifugation (250g for 5 min) and the plasma collected. (The plasma was subsequently used for screening the patient for hepatitis B.) The blood cells were subsequently washed twice with medium 199 or RPMI 1640 as appropriate and finally resuspended to 40% haematocrit with compatible serum from a non-immune Caucasian. Unless otherwise stated cultures were carried out in 96-well flat-bottomed microtissue culture trays.

Comparison of growth and multiplication in medium 199 or RPMI 1640 containing 5% or 10% foetal calf serum

Six experiments were carried out. The media used were RPMI 1640 (Flow Laboratories) containing 2.1 g/l $NaHCO_3$, 25mM Hepes and 25µg gentamicin/ml (Trager and Jensen, 1976), and medium 199 (Flow Laboratories containing 1.98 g/l $NaHCO_3$, 10mM Hepes and 25µg gentamicin/ml. Parasites in RPMI 1640 were incubated in a candle jar (Trager and Jensen, 1976) and those in medium 199 were incubated in 5% CO_2 in air. Foetal calf serum (FCS) was added to a final concentration of 5% or 10%. Individual cultures were of a volume of 25µl in microtissue culture trays.

Comparison of the growth and multiplication of the parasite in medium containing 5% or 10% FCS over a period of two to four days revealed that in 199 and RPMI there was usually no significant difference although overall there was a slightly better rate of multiplication and incorporation of ^{3}H-isoleucine in medium containing 10% FCS. One example is given. The parasitised blood contained 4.97% rings. After being washed and resuspended in Caucasian A serum 10µl of infected blood was introduced into each 250µl microculture. Each culture contained approximately 1.6×10^6 parasites and 3.2×10^7 red cells in medium 199 or RPMI 1640 containing 5% or 10% FCS. Twelve cultures of each type were set up at 1700 hr on day 0. After 1.5hr culture 1µCi ^{3}H-isoleucine (specific activity 21 Ci m/mol (Radiochemicals, Amersham) in 10µl of medium was added to four cultures of each type. One culture was for 0hr of incorporation and the remaining three were harvested 15.5hr later. Reinvasion occurred after about 40hr of culture. After 31.5hr of culture 1µCi ^{3}H-isoleucine in 10µl of medium was added to a further four of each

culture and the cultures harvested 14hr later. At intervals during the culture period representative microcultures were discontinued and blood smears made from them. The results are cited in Table 2.

It can be seen that the level of incorporation of the radiotracer was significantly greater in medium 199 reflecting the larger amount of 'cold' isoleucine already present in RPMI 1640. In both RPMI and 199 there was a reduced level of tracer incorporation after reinvasion and that the lowest levels of incorporation were in the media containing 5% FCS. Giemsa's stained smears suggested that there was a higher rate of reinvasion in medium 199 containing 10% FCS. On the basis of these results and those from other experiments it was decided to use medium 199 containing 10% FCS as the culture medium particularly where ^{3}H-isoleucine incorporation was being determined.

Comparison of the growth and multiplication of parasites in flat-bottomed and round-bottomed microtissue culture plates

It was planned to examine the possibility of using a cell harvester (Titertec) to process microcultures to which ^{3}H-isoleucine had been added. A cell harvester would be a more rapid method than the standard method in which individual cultures are spun down in tubes and the red cells washed three times before being bleached with hydrogen peroxide and solubilized in NCS. In the cell harvester the blood cells are collected onto discs of filter paper, washed, bleached and then treated with NCS. The manufacturers of the cell harvester recommended that the cultures be carried out in round-bottomed trays: in flat-bottomed trays there is a danger that some of the cells in the wells would not be completely washed out. Hence it was decided to compare the growth of *P. falciparum* in round-bottomed and flat-bottomed tissue culture trays.

Parasites from eight patients were cultured in flat- and round-bottomed trays in different experiments. The growth of parasites from three are described. For all three donors of parasites the parasites were cultured in 5% CO_2 in air in medium 199 containing 10% foetal calf serum, 10mM Hopes and gentamicin at 25µg/ml. The parasites were grown through one period of schizogony and reinvasion in cultures of 250µl and 50µl. Five microlitres of washed and resuspended red cells were added to each culture. Incorporation of ^{3}H-isoleucine was measured before and after reinvasion. The results are shown in Table 3. After reinvasion comparing 50µl and 250µl cultures the higher parasitaemias were seen in the 250µl cultures. Comparing flat-bottomed and round-bottomed wells it can be seen that after reinvasion the parasitaemias and tracer incorporation were significantly higher in the former wells. Before reinvasion incorporation of the tracer was similar in round- and flat-bottomed wells.

In other experiments similar results were obtained overall. In cultures, therefore, in which reinvasion rates and subsequent growth of the new generation of ring stages are being measured, round-bottomed are less satisfactory than flat-bottomed wells.

Table 2

Comparison of growth of P. falciparum in RPMI 1640 or TC 199 containing 5% or 10% foetal calf serum

Culture medium	Mean ^{3}H-isoleucine incorporation c.p.m. x 10^{-3}		Mean parasites/ 100 red cells after reinvasion
	Before reinvasion	After reinvasion	
5% FCS in RPMI	5.38	2.27	3.48
10% FCS in RPMI	5.47	4.37	3.48
5% FCS in RPMI	18.3	8.62	3.96
10% FCS in RPMI	18.94	14.76	6.65

Table 3

Comparison of the growth of P. falciparum in flat-bottomed and round-bottomed microtissue culture trays

Donor	Volume of culture -µl		Initial parasitaemia -%	R.b.c. /ml culture	Mean incorporation c.p.m. x 10^{-3}		Mean parasitaemia post-reinvasion -%
					Pre-reinvasion[1]	Post-reinvasion[2]	
7839	FB	50	4.2	1.73×10^8	13.4	3.02	4.90
		250	4.2	3.46×10^7	3.61	3.05	23.4
	RB	50	4.2	1.73×10^8	12.97	1.68	1.42
		250	4.2	3.46×10^7	3.97	2.76	8.92
7841	FB	50	0.99	4.14×10^8	11.97	11.61	4.56
		250	0.99	8.28×10^7	3.17	6.7	15.94
	RB	50	0.99	4.14×10^8	10.93	9.39	1.95
		250	0.99	8.28×10^7	3.34	4.46	6.05
7842	FB	50	0.80	4.88×10^8	47.7	1.23	0.57
		250	0.80	9.76×10^7	15.37	9.66	12.78
	RB	50	0.80	4.88×10^8	53.08	1.70	2.31
		250	0.80	9.76×10^7	12.1	4.98	5.47

FB - flat-bottomed; RB - round-bottomed, 1 - from 5-21.5 hr; 2 - from 51.5-66.75 hr.

Variation in size of individual cultures

a. Without medium change

Washed and resuspended parasitized blood from twelve patients was cultured in medium 199 (10% FCS, 25µg/ml gentamicin and 10mM Hepes in 5% CO_2 in air) in flat-bottomed tissue culture trays in cultures of 50µl or 250µl. Five microlitres parasitized blood or 5µl of the blood diluted 1 in 2 or 1 in 5 with normal serum, was added to each well. The growth of the parasites before and after reinvasion was monitored by incorporation of ^{3}H-isoleucine (0.5µCi per well) and by examination of stained blood smears.

The results showed that in ten of the twelve blood samples reinvasion rates were higher in 250µl cultures compared with the 50µl cultures although this difference could be reduced by reducing the number of parasites per well. Growth of the parasites during the first 19hr of culture was similar in 50µl and 250µl cultures. The results from cultures of blood from three patients are given in Table 4. In patient 7831 there was no reinvasion in 50µl cultures initiated with 1.06×10^6 parasites although there was a detectable incorporation of the tracer after 67.75hr of culture; in these latter cultures there were trophozoites and schizonts present at 67hr. In the 250µl cultures initiated with lowest number of infected cells (2.11×10^5) from patient 7831, th reinvasion rate was lower than that in those initiated with 1.06×10^6 parasites. This finding illustrates the frequently made observation that parasite growth and multiplication may be impaired if the number of red cells in individual cultures drops below a critical level. This can be overcome by adding extra uninfected red cells to the cultures which maintains the integrity of infected and uninfected red cells. In general the rate of tracer incorporation reflected the reinvasion rate determined by parasitaemias where reinvasion occurred. As mentioned earlier the amount of tracer incorporation was reduced in the 250µl culture because of the dilution of the tracer.

These and other experiments indicate that it is possible for P. falciparum to be grown through one schizogony in 50µl cultures without changing the medium over the course of three days but it is necessary to ensure that there are sufficient red cells present to prevent spontaneous lysis of red cells and that the number of infected red cells is not too high. Thus where materials, e.g. infected blood, serum or radioisotope, are limited 50µl cultures may be profitably used but it would be advisable to monitor reinvasion by stained blood smears as well as by radioisotope incorporation.

b. Changing the medium

In further investigations into determining the optimal conditions for the growth and multiplication of P. falciparum in cultures of less than 250µl two experiments were carried out in which the medium in individual wells was changed during the course of the culture or in which the volume of medium in the wells was reduced during the course of the culture usually just prior to the radiotracer being added. That is, in order to reduce the possibility of the medium in the microtitre well being exhausted during the culture period by the growing parasite, the parasite initially started growing in excess medium and the culture volume was reduced to that required, just before radiotracer was added.

Table 4

Comparison of the growth of P. falciparum in cultures of either 50µl or 250µl without medium change

Patient	% parasitaemia	Rbcs/ culture x 10^6	Parasites/ culture x 10^5	Volume of culture - µl	Mean c.p.m. x 10^{-3} (2 cultures		% parasitaemia post-reinvasion (67-75hr) - mean of 2 cultures
					Pre-reinvasion (4.75-18.75hr)	Post-reinvasion (53.00-67.75hr)	
7831	7.95%	2;66	2;11	50	2,39	3.27	9,11
				250	0,53	1.09	14,77
		6.65	5.29	50	9,40	5.65	10,29
				250	2.4	3.12	22.17, 9.17*
		13.3	10.6	50	10,81	2.74	no reinvasion
				250	2,68	5.23	22,37
7832	12.8%	3,32	4,24	50	3,63	7.5	21,17
				250	0,94	3.95	29,6**
		8,3	10,6	50	8,4	3.94	10,39
				250	3.1	4.03	21,32
7833	9.9%	3,18	3,17	50	15.09	4.64	15.13
				250	3.2	3.27	28,85
		7.95	7.92	50	42.75	2.58	no reinvasion
				250	9.44	3.39	17.1

*Parasitaemias from 2 individual cultures,

**Parasitaemia of a single culture.

i. In the first experiment cultures were set up containing 50, 100, 150 and 200µl of medium. Some of the 50µl cultures were subsequently left untouched. In others 25µl of medium was replaced after 23.5 or 42.5hr of culture. The 100µl cultures had 50µl medium removed, the 150µl cultures had 100µl medium removed and the 200µl cultures 150µl medium removed in all cases after 23.5 or 42.5hr of culture. That is, in all cases the final volume of each culture after 42.5hr of culture was 50µl. Reinvasion occurred after about 40hr of culture. Duplicate cultures of each treatment received 0.5µCi ^{3}H-isoleucine between either hours 44.75 to 66.00 or 73.00 to 92.50. The parasitaemia after reinvasion was approximately the same in the different cultures, i.e. 5.56-8.14%. The tracer incorporation rates are given in Table 5 from which it can be seen that replacing 25µl of medium or reducing the volume of the culture to 50µl at 23.5 or 42.5hr, particularly after 42.5hr of culture, increased the amount of incorporation of the tracer.

ii. Cultures were set up containing a volume of 50, 100, 150, 200 or 250µl. After 24.75hr of culture some cultures were reduced in volume and others had medium replaced as follows:

50µl cultures – 20µl medium replaced in some cultures.

100µl cultures – 50µl medium replaced in some cultures.

150µl cultures – 50µl medium removed from half the cultures and replaced in others.

200µl cultures – 100µl medium removed from half cultures and to some of these 50µl fresh medium added.

250µl cultures – 200µl removed from half cultures and to some of these 50 or 100µl fresh medium added.

0.5µCi ^{3}H-isoleucine in 10µl was added to wells given the different treatments from 4.5hr to 18.25hr and from 52.50hr to 66.50hr. Reinvasion occurred after 30hr of culture.

The results are given in Table 6. It can be seen that the amount of tracer incorporated between 4.50 and 18.25hr decreased with increasing volume of the cultures. The lowest reinvasion rates, assessed by the stained blood smears were seen in the 50µl cultures: in the 50µl cultures in which 20µl of the medium was replaced the reinvasion rate was increased slightly and the amount of tracer incorporation raised. Although for the cultures of a final volume of 100, 150, 200 and 250µl the reinvasion rates were higher than for the 50µl cultures, there was no indication that any particular volume size gave the best parasite growth and multiplication. Similarly for cultures of a final volume of 100 and 150µl, replacing medium or starting with excess medium and reducing the volume to the final volume, did not enhance parasite growth and multiplication. It is, however, to be noted that in this experiment (ii) the individual cultures each contained 1.84×10^{5} parasites compared with 3.89×10^{5} parasites in experiment (i) and the medium, therefore, was less rapidly exhausted.

Table 5

Growth of P. falciparum in cultures of a final volume of 50µl

Initial volume of culture	Treatment of cultures	Mean c.p.m. x 10^3		Total incorporation
		Period of isotope incorporation		
		44.75-66.00hr	73.00-92.5hr	
50µl	none	18.34	19.33	37.67
50µl	25µl replaced 23.5hr	22.06	17.3	39.36
50µl	25µl replaced 42.5hr	22.95	18.08	41.03
100µl	50µl removed 23.5hr	24.59	20.63	45.22
100µl	50µl removed 42.5hr	28.33	18.96	47.29
150µl	100µl removed 23.5hr	24.06	22.24	46.3
150µl	100µl removed 42.5hr	28.14	20.73	48.87
200µl	150µl removed 23.5hr	22.59	20.42	43.01
200µl	150µl removed 42.5hr	31.16	19.05	50.21

The cultures were initiated with 5µl washed and resuspended infected blood cells containing 3.89 x 10^5 parasites (10.25 x 10^6 rbcs).

Table 6

Growth of P. falciparum in microcultures in which medium was removed and/or replaced

Initial volume of culture - µl	Volume medium removed - µl	Final volume - µl	Volume of medium replaced	Mean % parasitaemia after reinvasion	Mean c.p.m. x 10^{-3}	
					4.5-18.25hr	52.50-66.50hr
50	-	50	-	6.61 ± 0.7	5.79	8.39
50	20	50	20	7.54 ± 0.4		10.46
100	-	100	-	10.33 ± 2.03	3.9	7.38
100	50	100	50	10.8		8.29
150	50	100	-	14.28		7.48
200	100	100	-	10.21		7.46
250	200	100	50	12.14		7.45
150	-	150	-	11.26	2.96	6.26
150	50	150	50	12.27		6.02
200	100	150	50	12.28		6.05
250	200	150	100	10.5		5.57
200	-	200	-	13.02	2.1	4.71
250	-	250	-	12.84	1.88	3.52

Cultures were initiated with 5µl washed and resuspended blood containing 1.84×10^5 parasites (19.15×10^6 red cells). Mean parasitaemias and c.p.m. were calculated from triplicate or

These two experiments confirm that it is possible to have significant growth and multiplication of P. falciparum in 50µl cultures, particularly if medium is changed, although the parasites may do less well than in larger cultures.

Growth of P. falciparum in microcultures in different samples of normal Caucasian serum

As a test of the suitability of small volume microcultures for assessing the ability of different sera to support the growth and multiplication of P. falciparum two experiments were carried out in which the parasite was grown through one schizogony in the presence of serum samples collected from adult females in the U.K. The donors were either women attending ante-natal clinics or a sexually-transmitted diseases (STD) clinic. The sera were stored at -25°C until required.

In the first experiment parasites from a Group O donor were cultured in 50µl medium 199 (10% FCS, 10mM Hepes and gentamicin 25µg/ml) to which 50µl of serum was added. Each serum was tested in three microcultures. Growth before and after reinvasion was assessed by incorporation of ^{3}H-isoleucine (0.5µCi per well) and examination of blood smears. The results are given in Table 7 where it can be seen that substantial reinvasion only occurred in cultures containing culture medium without additional human serum. Human serum at a concentration of 50% supported growth of ring stages with some small degree of variation between different sera, as well as medium alone, as evidenced by the incorporation of the radiotracer between 4.00 and 19.25hr of culture, but reduced schizogony and reinvasion.

In the second experiment 25µl of human serum was added to 50µl of complete medium. Cultures were initiated with 5µl of washed and resuspended infected blood (3.7×10^5 parasites, 17.9×10^6 red blood cells, 2.07% parasitaemia) from a Group O donor. Four cultures with each serum were set up. 0.5µCi ^{3}H-isoleucine in 10µl was added to duplicate cultures of each serum sample after 37.5hr of culture (reinvasion occurred after about 28hr of culture) and the cultures were harvested 16hr later at which time the remaining two cultures of each serum sample were smeared to determine the reinvasion rate. The results are shown in Table 8. It can be seen that on this occasion the reinvasion rate in the cultures containing 33% human serum was as high as that in medium alone. Radiotracer incorporation showed that there was some variation in the uptake of tracer by the new rings in the different sera. A higher level of tracer incorporation was not always reflected in a higher level of parasite reinvasion as seen in the stained blood smears.

These two experiments showed that small microcultures can be used for determining, for example, the ability of sera from immune individuals to support or inhibit the growth and multiplication of P. falciparum. Human sera at a final concentration of 33% supported the parasites very much better than at a concentration of 50% and that with cultures initiated with 3.7×10^5 parasites a total culture volume of 75µl was satisfactory. These experiments clearly showed that there is variation in the ability of sera from non-immunes, even between serum samples taken on different occasions from the same individual, to support P. falciparum.

In experiments, therefore, where anti-parasitic activity of immune sera is being tested for, an adequate number of control sera should be included.

Table 7

Growth of *P. falciparum* in sera from non-immune donors

Serum donor	Blood Group of donor	Ante-natal	STD	Mean c.p.m. x 10^3		Parasitaemia after reinvasion %
				Pre-reinvasion 4.00-19.25hr	Post-reinvasion 48.75-64.75hr	
31	O		+	4.05	2.22	0.77
39	O		+	2.92	1.24	0.20
120 (i)	A	+		2.98	1.54	0.24
120 (ii)	A	+		3.06	1.18	0.20
141 (i)	A	+		3.86	1.37	0.62
141 (ii)	A	+		3.66	1.42	0.25
22 (i)	A	+		3.93	1.90	0.30
22 (ii)	A	+		4.36	2.03	0.44
29 (i)	A	+		4.1	1.58	0.57
29 (ii)	A	+		4.17	2.21	0.53
33	B		+	4.22	3.12	0.75
37	O		+	3.94	2.02	0.12
44	O		+	3.56	2.15	0.05
40	O		+	3.47	1.47	0.07
R.S.P.	A			3.7	1.92	0.76
Medium 199				3.3	4.43	5.4
No addition				5.49	8.80	7.93

Cultures were initiated with 5µl washed and resuspended blood containing 9.6×10^4 parasites (10.45×10^6 red cells - 0.91% parasitaemia).

Table 8

Reinvasion of P. falciparum in different non-immune sera

Serum donor	Blood Group	Mean c.p.m. x 10^3 after reinvasion	Mean % parasitaemia after reinvasion
14 (i)		20.52	18.47
14 (ii)		18.08	16.27
14 (iii)		24.49	19.89
14 (iv)		23.1	20.14
14 (v)		17.42	15.2
9 (i)		18.08	11.31
9 (ii)		17.67	17.58
9 (iii)		21.98	17.6
9 (iv)		22.56	16.30
32		21.19	16.6
35		26.15	15.44
36		24.98	20.24
38		23.37	16.87
41		30.39	15.34
175		25.1	16.75
190		21.12	21.61
229		28.43	20.78
R.S.P. (i)		26.37	18.79
Medium		22.15	17.79

Sera were collected from donors 14 and 9 on four or five occasions, one month between donations.

Testing of return sera for anti-P. falciparum antibodies

Infected blood was collected from 82 patients and samples from each cryopreserved in liquid N_2. Thirty-eight patients returned from whom small serum samples were collected. Four of these patients were subsequently found to be hepatitis B positive and all blood or serum samples from these patients had to be destroyed. As explained in the Introduction it was decided to establish the technique for continuous culture of P. falciparum in the laboratory under acceptable conditions of worker safety before thawing out the cryopreserved infected blood and testing the sera from the patients after recovery. It was hoped that when thawing out the cryopreserved blood samples not only would short-term cultures from each isolate be set up to test the anti-plasmodial activity of the sera but also an attempt would be made to adapt the isolate to continuous culture. P. falciparum is now established in continuous culture and the testing of the sera is in progress. Attempts to adapt the frozen isolates to continuous culture have not been very successful so far. First results continue to confirm the anti-plasmodial activity of return sera. Full details will be held over to the next report.

Part II

Immunity to Rodent Malaria Parasites

Introduction

The work below is a continuation of the study described in the Annual Report DAJA 17-76-99415 January 1979 . The following four lines of investigation have been pursued:

a. lymphoid cells involved in immunity to *Plasmodium chabaudi* in mice;

b. effect of non-specific stimulation of the reticulo-endothelial system on *P. yoelii* and *P. chabaudi* infections;

c. the effect of concurrent infections of *Myoobacterium lepraemurium* on *P. chabaudi* and *Babesia microti* infections;

d. removal of *P. chabaudi* in immune mice
   - effect of irradiation on the removal process
   - role of spleen.

Material and Methods - general considerations

Plasmodium chabaudi (A/S) strain was cloned and supplied by Dr D. Walliker, University of Edinburgh. In C57Bl, CBA and NIH mice aged 6-40 weeks, this parasite produces an acute primary patent parasitaemia usually lasting around 17 days. A period of subpatency follows. In the majority of mice a patent recrudescence emerges around day 28 and lasts 5-7 days. A further recrudescence around day 50 is seen in some mice. In the majority of mice the parasite appears to have been eliminated 10 weeks after infection. In our laboratory the asexual blood stage of *P. chabaudi* grows synchronously, schizogony occurring around midnight. Late trophozoite and schizont stages tend to leave the peripheral circulation. *P. yoelii* (YM strain), *P. vinckei petteri* and *P.v. bruechwatti* all came from Dr D. Walliker, University of Edinburgh.

Parasitized blood is stored at -198°C using glycerol at a final concentration of 17.5% (w/v) as the cryoprotective agent. Cryopreserved blood was thawed rapidly in a 37°C water bath and injected intraperitoneally into mice. Dilutions of freshly thawed blood were made with 17.5% sorbitol in saline. Subpassages of the parasite were made every 3 or 4 days in order to maintain the antigenic character of the parasite.

Heparin at a final concentration of 10-20 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

Four inbred strains of mice CBA, A, NIH and C57Bl were used at different times and they were supplied either by the Centre for Tropical Veterinary Medicine, Easter Bush, East Lothian, or Hacking and Churchill, Huntingdon, or were bred within the Zoology Department.

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

Lymphoid cells involved in immunity to P. chabaudi in mice

It was previously reported (McDonald and Phillips, 1978) that immunity to P. chabaudi could be adoptively transferred to irradiated syngeneic recipients with immune spleen cells, and with populations of immune spleen cells containing predominantly either thymus-derived (T) cells or bursa-derived (B) cells as a result of passing spleen cells through nylon wool columns (Trizio and Cudkowicz, 1974). The best protection was given to lethally irradiated mice with unfractionated spleen cells suggesting that one of the roles of the T-cells was to act as helper cells in the production of protective antibody. Further experiments which have been reported (McDonald and Phillips, 1980) showed that in the irradiated recipients of unfractionated immune spleen cells or enriched immune spleen T-cells the control of the primary patent parasitaemia was associated with a rise in the level of protective antibody in the serum of the mice. This observation also suggests that immune T-cells were acting as helper cells in the production of protective antibody.

In part of a further examination of the relative roles of T- and B-cells in the immune response to P. chabaudi immune cell populations have been treated with anti-thymocyte (Rose et al., 1976) prior to adoptive transfer. In the previous report the results of three such experiments were briefly described in which the effect of anti-thymocyte serum (ATS) on unfractionated immune spleen cells or enriched immune spleen T-cell populations were examined. Although there was one anomalous result, these experiments in general indicated that treatment of the immune spleen cells with ATS before transfer reduced the protection given, particularly that afforded by the immune T-cell population.

Two further experiments are described in this report in which the effect of ATS was extended to enriched immune B-cell populations. The preparation of the spleen cell populations was as described in a previous Annual Report (DAJA 37-75C-1620) and in McDonald and Phillips (1978). In brief, the dissociated spleen cells were first passed through a glass-wool column at room temperature to remove dead cells and some macrophages. The cells which passed through the glass-wool column were subsequently incubated on a nylon wool column at 37°C for 45 min. When more medium was then run through the column the effluent cells were predominantly T-cells. The cells which remained in the nylon wool column could be dislodged by mechanical agitation and these cells were rich in B-cells.

T-cells were identified by their vulnerability to ATS and complement, and B-cells by the immunoglobulin on their surface (McDonald and Phillips, 1978).

In the first experiment with 9 months-old CBA male mice the immune spleen cell donors were sacrificed 58 days after they had been reinfected with P. chabaudi. The normal spleen cell donors were age matched controls. For immune spleen cells, a population subjected to glass-wool filtration only (g.w. immune cells), and enriched T- and B-cell populations were prepared. From the normal donors' spleen cells subjected to glass-wool filtration only were prepared. From each of the four populations a sample was treated with rabbit anti-mouse thymocyte serum (Rose et al., 1976) diluted 1/20 with phosphate buffered saline (PBS) and guinea-pig serum diluted 1/3 with PBS and another sample with normal rabbit serum (NRS) diluted 1/20 and guinea-pig serum diluted 1/3. All the cells were washed three times and injected i.v. into 3½ months-old male CBA mice which had been irradiated with 600 rads X-irradiation 24hr previously. Each mouse received 2.3 x 10^6 cells and for each population of anti-thymocyte and normal rabbit serum treated cells a group of six recipient mice was set up. Two further groups of non-irradiated mice were set up. The first group of mice each received 0.25ml i.v. of the supernatant from the cells treated with diluted ATS and complement and the second group the same volume of diluted normal rabbit serum from the treated cells. All the mice were challenged with 1 x 10^5 P. chabaudi i.v. immediately after serum or cell transfer. The viability of the cells after serum treatment was g.w. NRS treated normal cells - 90%, g.w. ATS treated normal cells - 64%, g.w. NRS treated immune cells - 92%, g.w. ATS treated immune cells 40%, NRS treated enriched immune T-cells - 75%, ATS treated enriched immune T-cells - 4%, NRS treated enriched immune B-cells - 78%, and ATS treated enriched immune B-cells 78%. The mean parasitaemias are given in Figure 1. Surprisingly all the mice given enriched immune T-cells treated with NRS died. Of the mice given ATS treated immune T-cells 3 of 6 died and the survivors suffered fluctuating patent parasitaemias during the 33-day observation period. All the mice given NRS treated normal spleen cells died whereas 3 of the 6 given ATS treated normal spleen cells survived and also showed persistent fluctuating patent parasitaemias. The mean parasitaemia in the mice given NRS treated enriched immune B and NRS treated g.w. immune cells followed a very similar course, the majority of mice becoming subpatent by day 17. ATS treatment reduced the protective activity of both the immune B-cell population and the g.w. immune cells. There was some variation within each group but there was an indication that the effect of the ATS was the greater on the g.w. immune cells. The reduced protective activity of the treated cells was evidenced as an extended primary patent parasitaemia and the emergence of recrudescent parasitaemias. The mean parasitaemias in the mice given supernatant from NRS or ATS treated cells followed similar courses

In a repeat experiment with CBA male mice, the immune spleen cell donors were reinfected 47 days before being sacrificed. The recipient mice received 600 rads X-irradiation 24hr before each mouse received 2.45 x 10^6 cells i.v. Groups of six mice were set up which received the same category of treated cells as in the previous experiment. Groups receiving NRS or ATS were not included. All the recipient mice were challenged with 1 x 10^5 P. chabaudi parasitized cells immediately after cell transfer was completed. The viability of the cell populations after treatment with NRS or ATS was as follows:

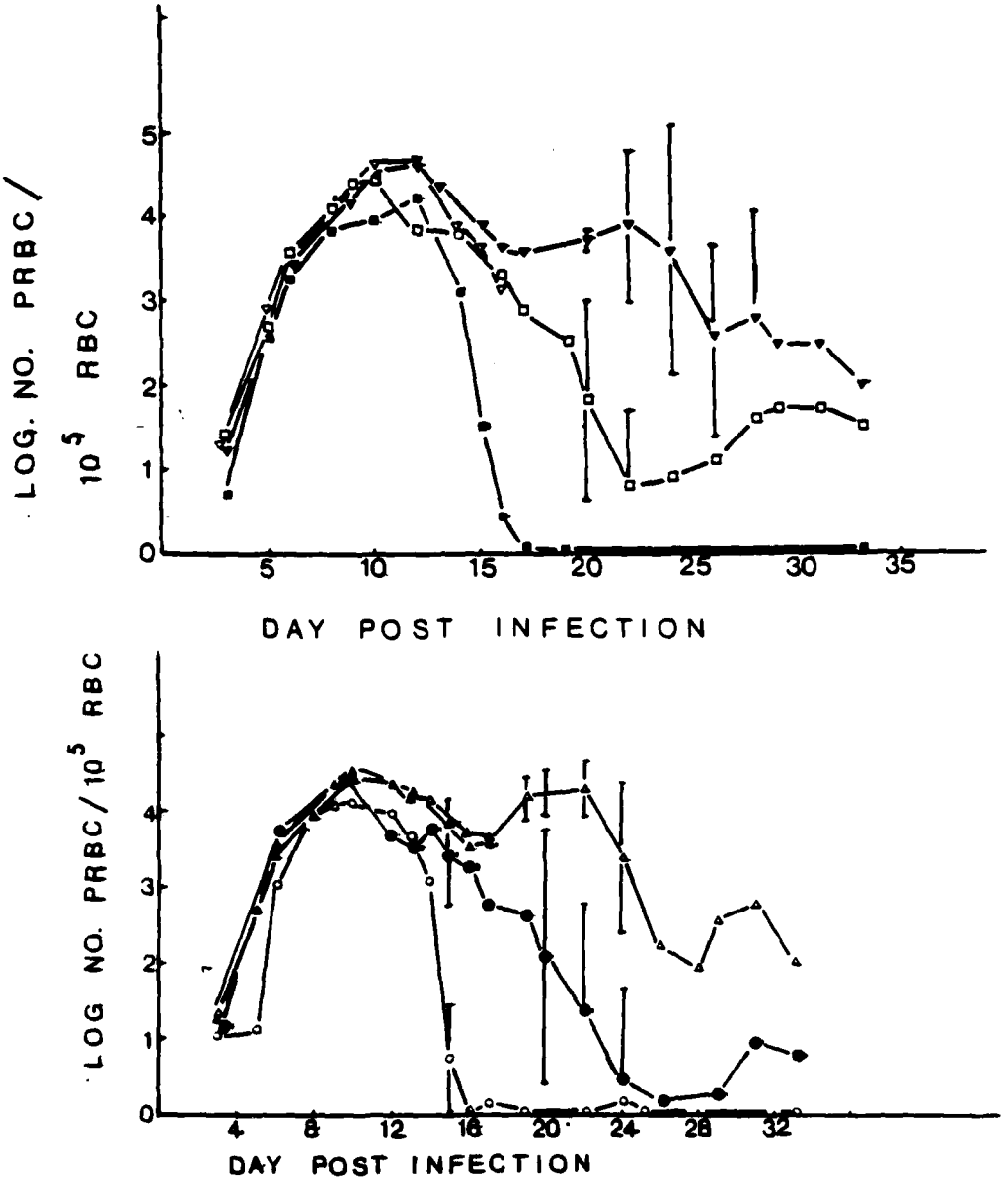


Figure 1. Course of *P. chabaudi* infection in mice irradiated with 600 rads and injected i.v. with either 2.3×10^6 glass-wool filtered immune spleen cells treated with either antithymocyte serum (ATS) (□—□) or normal rabbit serum (NRS) (■—■), 2.3×10^6 immune B-cells treated with either ATS (●—●) or NRS (○—○), 2.3×10^6 immune T-cells treated with either ATS (▲—▲) or NRS (△—△), or 2.3×10^6 glass-wool filtered normal spleen cells treated with either ATS (▼—▼) or NRS (▽—▽).

g.w. NRS treated normal spleen cells - 94%; g.w. ATS treated normal cells - 39%; g.w. NRS treated immune spleen cells - 87%; g.w. ATS treated immune spleen cells - 29%; NRS treated immune T cells - 96%; ATS treated immune T-cells - 6%; NRS treated immune B-cells - 92% and ATS treated immune B-cells - 74%. Fluorescence studies showed that the immune T immune, B and g.w. immune cells contained 2%, 44% and 22.5% immunoglobulin-bearing cells respectively.

The mean parasitaemias for each group after infection are given in Figure 2. First, it is noted that the patent parasitaemia was not first detected until day 6 compared with day 3 in the previous experiment. Thus there was a longer period for the host's immune system to recover from the effect of the X-irradiation before receiving the antigenic stimulus from the parasite. The mice given the normal spleen cells all died with the exception of a single mouse in the recipients of ATS treated spleen cells. Of the mice receiving NRS treated immune T-cells 4 of 6 mice survived, the surviving mice being subpatent by day 20 and thereafter remained so during a 42-day observation period. The mice given g.w. immune cells or immune B-cells treated with NRS all survived and had similar courses of parasitaemias, the infection in all mice being subpatent by day 19 and remaining so: the peak parasitaemias in these mice were lower than those of the NRS treated immune T-cell recipients. Treatment of the immune B-cells with ATS in 5 of 6 mice had no significant effect: in the sixth mouse the primary patent parasitaemia was extended to day 33. The effect of ATS on the g.w. immune cells was more pronounced: the peak parasitaemia was elevated and the primary patent parasitaemia was extended to day 23/24 and in 4 of the 6 mice recrudescent parasitaemias appeared.

The results from these two experiments show some similarities and some differences. First, the g.w. immune and immune B-cell populations treated with NRS protected the 600 rad irradiated mice to a similar degree which followed earlier results (McDonald and Phillips, 1978, and previous Annual Reports), suggesting an important role for both T and B memory cells. The effect of the anti-T-cell serum, in reducing the resistance conferred on the recipients, was more marked on the g.w. immune cell recipients which is to be expected, in view of the higher proportion of T-cells in the g.w. immune cell population when compared with the immune B-cell population. The immune B-cell population, in particular, contained unidentified cells, which may have included erythroid cell precursors (Freeman and Parish, 1978) playing no part in the resistance conferred on the recipients. These unidentified cells may have a role in immunity and need to be investigated. In the first experiment the immune T-cell recipients were not protected. In previous fractionation experiments the protective effect of the immune T-cell population could be reduced by increasing the irradiation dose of the recipients from 600 to 800 or 850 rads. It may be that the recipient mice in this experiment inadvertently received a dose greater than 600 rads or were particularly sensitive to the irradiation. In the second experiment the enriched immune T-cells gave protection marginally less than that associated with the g.w. immune cells and treatment with anti-T-cell serum almost abolished their protective activity. In conclusion these two experiments confirm a role for both T- and B-cells. Presumably T and B memory cells were being transferred because the cell donors were sacrificed 47 and 58 days after their last reinfection. Work under the present grant suggests that at

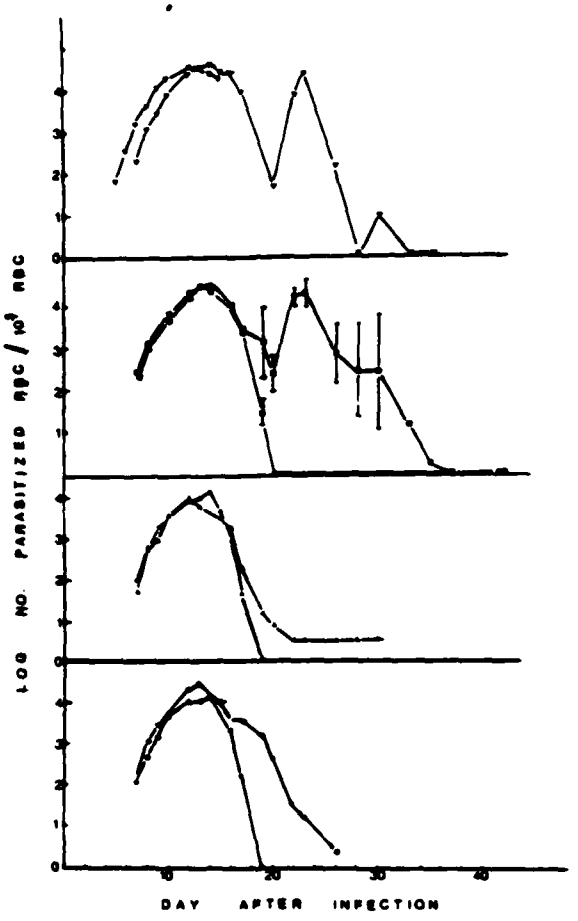


Figure 2. Course of P. chabaudi infection in mice irradiated with 600 rads and injected i.v. with either 2.45×10^6 glass-wool filtered immune spleen cells treated with either antithymocyte serum (ATS) (●—●) or normal rabbit serum (NRS) (○—○), 2.45×10^6 immune B-cells treated with either ATS (Δ—Δ) or NRS (▲—▲), 2.45×10^6 immune T-cells treated with either ATS (□—□) or NRS (■—■), or 2.45×10^6 glass-wool filtered normal spleen cells treated with either ATS (▽—▽) or NRS (▼—▼).

this time the donor mice were not infected and that antibody production, detected as protective antibody or by the indirect fluorescent test (IFAT), was minimal.

Previous work (McDonald and Phillips, 1980) suggests that among the immune T-cells are helper cells which induce protective antibody by the few contaminating B-cells in the immune T-cell population or more likely by those B-cells which survived irradiation and those produced by the recovering immune system in the irradiated mice.

The rate at which the immune system of irradiated CBA mice recovers after 600 rads X-irradiation was examined in one experiment. Four-months old male CBA mice were irradiated with 600 rads 20, 12, 8, 4 and 1 day before being injected with an i.p. injection of 1×10^8 sheep red blood cells (SRBCS). The irradiated mice were not given any spleen cells from non-irradiated mice. A group of non-irradiated mice also received sheep red cells. Ten days after receiving spleen cells the mice were bled out and the level of anti-sheep red cell antibody measured using a haemagglutination test. The results showed that the mice irradiated 1, 4 and 8 days before receiving sheep cells made very little detectable antibody. With an increasing interval, thereafter, the mice showed an increased ability to produce anti-SRBCS antibody but even 20 days after irradiation the mice had antibody levels which were still less than half of those in the control mice. In relation to the cell transfer studies these observations show that the 600 rad irradiated mice have an immune system which is itself capable of production of low levels of antibody but which after 20 days is still performing well below that of the control mice. Thus the protection given by immune cells to irradiated mice, at least that mediated through protective antibody, would be expected to come from, in large measure, the transferred cells rather than the recipients own immune system.

In the current experiments the role of individual subsets of T-cells is being examined, in particular the role of helper cells, cytotoxic T-cells and T-cells mediating cell-mediated immune responses.

Two further transfer experiments were carried out but the detailed results are not given. These experiments were carried out because there was a period when irradiated mice were dying unexpectedly after cell transfer and challenge. In the first of these experiments with CBA mice spleen cells came from mice 99 days after reinfection and populations of g.w. immune spleen cells, enriched immune T spleen cells, enriched immune B spleen cells and g.w. normal spleen cells were injected into syngeneic 600 rad irradiated or non-irradiated mice. Each mouse received 3.5×10^6 cells i.v. and immediately afterwards 1×10^5 P. chabaudi parasitized cells i.v. In all the mice the parasitaemia was slow to develop. In irradiated and non-irradiated mice the different immune cell populations were equally effective. The parasitaemias were extended in the irradiated mice. In the second experiment spleen cells came from CBA female mice 105 days after reinfection. g.w. immune or normal spleen cells were injected i.v. into 600 rad irradiated or non-irradiated syngeneic recipients, each mouse receiving 3×10^6 cells. The mice were challenged with 1×10^5 P. chabaudi parasitized red cells i.v. immediately after cell transfer. Again the parasitaemias in the non-irradiated mice were lower than is usual and the course of the primary patent parasitaemias in the immune and normal spleen cell recipients followed essentially similar courses. In the irradiated mice, however, the immune cell

recipients had patent parasitaemias of a similar duration to that in the non-irradiated but marginally higher. In the irradiated mice the recipients of non-immune cells had extended and significantly more acute patent parasitaemias. These two experiments illustrate the somewhat variable response of mice to irradiation, the variable protection given by immune spleen cells from different groups of recovered mice, but do confirm the value of using irradiated mice for demonstrating the protective activity of immune cells.

The effect of non-specific stimulation of the reticulo-endothelial system on Plasmodium yoelii and P. chabaudi infections

(with A.S. Morton)

Working in our research group during the contract period a Final Honours year student (Miss A.S. Morton) carried out a small project examining the effect of non-specific RES stimulators on rodent malaria infections. The results were of interest and are briefly reported here. The stimulants used were pristane (2, 6, 10, 14-tetramethyl-pentadecane) which is an oil, Freund's complete (FCA) and Freund's incomplete adjuvant (FIA), both being emulsified with an equal volume of phosphate buffered saline before injection, and diethylstilbestrol (DES) which was dissolved in corn oil at a concentration of 4mg/ml.

Pristane

Groups of A2G mice were injected i.p. with 0.5ml pristane, 10, 4 or 2 days or 10 min before being challenged i.p. with 1×10^6 P. yoelii parasitized red cells. A further group were given pristane 4 days before the mice were injected with 1×10^6 parasitized red cells i.v. Control groups were given saline i.p. and challenged i.p or i.v. The mean parasitaemias of each group showed that the early rise in patent parasitaemia was significantly delayed in the mice given pristane 2 days before infection: on days 3 and 4 of infection the parasitaemia was significantly lower ($P>0.05$). Pristane given 10 min before challenge had no effect suggesting that this substance had no significant direct anti-parasitic activity.

Freund's Adjuvant

Freund's complete

Groups of 'A' strain mice were injected with 0.25ml of emulsified FCA i.p. 10, 5 or 3 days before an i.p. challenge with 1×10^6 P. yoelii parasitized erythrocytes. Another group of mice given emulsified FCA were similarly challenged intravenously 3 days after receiving the adjuvant. Control groups were given 0.25ml saline i.p. and challenged i.v. or i.p. The mean parasitaemias are shown in Figure 3 where it can be seen that the onset of the patent parasitaemia was significantly delayed in the mice given the FCA and challenged i.p. The longest delay was in the mice given FCA 3 days before challenge, followed by the day -5 group and least delayed were the day -10 FCA group. The group given FCA 3 days before an i.v. challenge had a parasitaemia which followed that of the control groups.

In a second experiment groups of C57Bl mice were given 0.25ml emulsified FCA or FIA 3 days before i.p. challenge with 1×10^6 P. yoelii parasitized red cells. A further group received 0.25ml emulsified FIA 5 min before an i.p. challenge with P. yoelii.

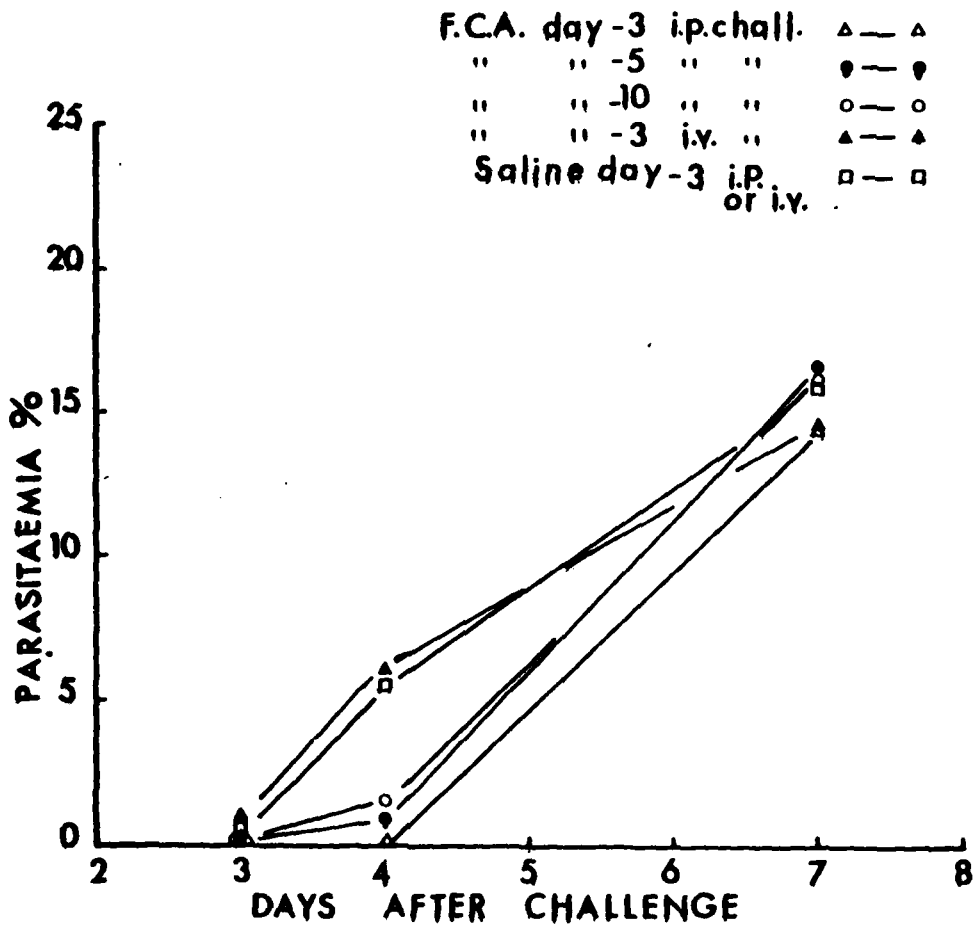


Figure 3, Course of P. yoelii infection in mice treated with emulsified Freund's Complete Adjuvant (FCA) and challenged i.v. or i.p.

Control groups again received saline i.p. and challenged i.p. or i.v. The mean parasitaemias showed that the onset of the patent parasitaemia was again delayed in the mice given emulsified FCA or FIA 3 days before challenge and that the course of the parasitaemia in the two groups was similar. The course of the parasitaemias in the control groups and the mice given FIA 5 min before challenge followed similar courses. It would appear, therefore, that the FIA had no significant directly antiparasitic activity and that FIA and FCA were equally active.

In a third experiment groups of A strain mice were given 0.25ml emulsified FIA 5 days before challenge with 1×10^6 P. chabaudi parasitized red cells. The mice were challenged i.p. with either ring stages or late trophozoite stages of the parasite. A further group of adjuvant treated mice were challenged i.v. with late trophozoite stages. Control groups received 0.25ml saline i.p. and challenges were made i.p. or i.v. with ring or late trophozoite stages. The results showed that the onset of the patent parasitaemia was delayed by 3-4 days in the mice given FIA and challenged i.p. and the length of the delay was the same in the mice infected with ring stages and late trophozoites. In the mice given FIA but challenged by the i.v. route the parasitaemia followed that of the controls. Thus the antiparasitic activity of the FIA appeared to be confined to the peritoneal cavity and that different stages in the asexual growth cycle in the red blood cells were equ lly vulnerable to the antiparasitic effects induced by the adjuvant.

The non-specific stimulatory activity of pristane and emulsified FIA was investigated by examining the in vitro uptake of sheep red cells (SRBCS) by macrophages from the peritoneal cavity of stimulated and control mice. A strain mice were injected i.p. with 0.5ml of pristane, 0.25ml emulsified FCA, or 0.5ml saline. Three days later peritoneal exudate cells were collected by peritoneal lavage with RPMI 1640 medium containing 20mM Hepes and 20 units/ml heparin. The peritoneal cells were washed, resuspended in RPMI 1640 (containing 20mM Hepes and 5% foetal calf serum) to a concentration of 1×10^7/ml and one millilitre of the cell suspension put into Leighton tubes with flying coverslips. The Leighton tubes were incubated at 37°C for 30 min at the end of which time the medium was removed from the tubes, removing the non-adherent cells, and replaced with one millilitre culture medium containing 1×10^8 washed sheep red cells. The tubes were incubated for a further 2hr at 37°C. At the end of this second incubation period the coverslips were washed, air-dried, fixed in methanol and stained with Giemsa's stain. The stained macrophages were then examined and the percentage of macrophages in 15 oil immersion fields containing ingested red cells was calculated. It was found that 48% and 34% of the macrophages from FCA and pristane treated mice respectively contained ingested red cells compared with 14% in the control macrophages indicating that the stimulatory substances had increased macrophage activity.

In view of the fact that it is known that immunity to P. yoelii and P. chabaudi is in part at least mediated through antibody and that phagocytosis of parasites and parasitized red cells is seen to occur in infected animals, probably in part mediated by opsonizing antibody (Hamburger and Kreier, 1976), it was decided to examine the combined effect of antibody and pre-treatment with emulsified FIA on the course of a P. yoelii and P. chabaudi infection. Immune serum was collected from mice which had recovered from infection with one of the two parasites and had been reinfected with the same parasite 17 to 42 days before the serum was collected. In the first of these experiments groups of C57Bl mice were given

0.25ml of emulsified FIA (3 groups) or saline (2 groups) i.p. 3 days before challenge i.p. with 1 x 10^6 P. yoelii parasitized red cells. Immediately after infection a group each of FIA and saline treated mice were given 0.3ml immune serum i.p. A further group each of FIA and saline treated mice received 0.3ml normal serum i.p. The third group of FIA treated mice received the challenge inoculum only. The results showed that from days 3 to 7 inclusive the patent parasitaemias in all the mice receiving FIA and in the mice given immune serum and pretreatment with saline, were significantly lower ($P>0.05$) than that in the mice pretreated with saline and subsequently given normal serum or saline after challenge. On day 8 but not day 9 the parasitaemia in the mice pretreated with FIA and injected with immune serum, was significantly lower than all the other groups. In the second experiment of this pair the same protocol was followed except that A2G mice were used and that 0.7ml immune or normal serum was injected after a challenge inoculum of 1 x 10^6 P. chabaudi parasitized red cells. The results were essentially the same as those of the first of these two experiments. In all mice the pre-treatment with FIA and injections of immune serum in saline pretreated mice delayed the onset of the patent parasitaemia. In mice pre-treated with FIA and injected with immune serum the course of the patent parasitaemia was delayed by a further day compared with the mice given adjuvant or immune serum alone. This pair of experiments suggested that there was an additive antiparasitic effect of the FIA and the immune serum but no good evidence of a synergi tic effect which might result from the presence of activated macrophages and opsonizing antibody.

Diethylstilbestrol

Groups of C57Bl mice were injected i.p. with 1mg DES in 0.25ml corn oil 4 days, 1 day or 10 min before an i.v. challenge with 1 x 10^6 P. chabaudi parasitized red cells. Control mice received 0.25ml corn oil 4 days or 10 min before the i.v. challenge. The results of this single experiment were inconclusive. In all the mice peak parasitaemia occurred on day 8 after which the parasitaemia went into remision. Between days 11 and 18 the mice given corn oil 10 min before challenge showed a significant recrudescence with the parasitaemia reaching a mean of 28%; in none of the other groups, i.e. the DES treated mice and the day -4 corn oil control, did the recrudescent parasitaemia go above 7%, in all groups apart from the DES day -1 group the recrudescent parasitaemia remaining below 2%. In conclusion it can only be tentatively suggested that the DES given one day and 5 min before challenge may have reduced the recrudescent parasitaemias and that corn oil alone given on day -4 may have had an antiparasitic effect even though the challenge infection was given i.v.

Discussion

Pristane, FIA and FCA when injected intraperitoneally all gave mice some protection against an i.p. challenge with P. yoelii or P. chabaudi. The effect of the pristane was shorter lasting than that associated with the other two agents when given in the quantities used. The resulting antiparasitic activity appeared to be confined to the peritoneal cavity because treated mice challenged i.v. were not protected. The three agents in themselves did not appear to have any antiparasitic activity because mice which were challenged i.p. immediately after injection of pristane or FIA had a course of infection which followed that in the controls. The results suggested that the optimal effect was obtained when the stimulatory agents were given 2-3 days before the mice were infected. In mice injected with

either pristane or emulsified FCA a greater proportion of the macrophages collected 3 days later were shown to have non-specific phagocytic activity, suggesting macrophage activation. It is likely, therefore, that an increased phagocytosis of parasitized red cells which initially results in a smaller number of parasites establishing in the circulation, would occur in the pretreated mice. All the mouse strains used were inbred and challenges were made with infected red cells of the same strain of mouse as the recipient mice. Removal of the infected red cells by phagocytosis in the peritoneal cavity of the pretreated mice would, therefore, be expected to involve the recognition of some alteration of the parasitized red cell which would allow it to be distinguished from the non-infected syngeneic red cells. Expression of parasite antigen on or inserted into the red cell membrane is associated with a maturing asexual parasite. For example, the agglutination of P. knowlesi infected red cells by immune serum only occurs when the parasite has reached the late trophozoite/schizont stage (Brown and Brown, 1965). When the effect of pretreatment of mice with FIA on ring stages and late trophozoite stages of P. chabaudi was compared, however, the onset of the patent parasitaemia was similarly delayed in both cases. In repeating this kind of experiment it would be better to include groups challenged with very young stages and schizont-infected red cells. Yoeli (1966) described the protective effects of four injections of FCA i.p. before mice were infected with a lethal dose of P. vinckei by the same route. He attributed the protective effect of the FCA to a non-specific stimulation of the RES in general whereas it is very likely that the protective activity was confined to the peritoneal cavity and was dependent on challenge via the intra-peritoneal route.

Cottrell et al. (1977) described the protective effect of 1mg of diethylstilbestrol given a few hours before an i.v. challenge with either P. vinckei or Babesia microti. In the case of P. vinckei the protective effect of the DES was to delay the onset of the primary patent parasitaemia. It is, therefore, surprising that a similar dose of DES had no obvious protective activity against P. vinckei, a parasite in many respects similar to P. vickei but less virulent.

The effect of concurrent infections of Mycobacterium lepraemurium on P. chabaudi and Babesia microti infections

The following experiment was carried out in collaboration with Dr I.N. Brown (St Mary's Hospital Medical School, London). The induction of non-specific resistance in mice to rodent plasmodia and piroplasms by prior administration of agents, such as BCG (Clark et al., 1976), Corynebacterium parvum (Clark et al., 1977) and Coxiella burnetti (Clark, 1979), has been described. The basis of this resistance has been explained in terms of the production of a soluble non-antibody agent which mediates the killing of the parasitized cells.

In this experiment the effect of prior infection of CBA mice with the intracellular bacterium causing murine leprosy M. lepraemurium (MLM) on P. chabaudi and B. microti infection is described.

Female CBA mice were infected with a standard inoculum of 1×10^8 M. lepraemurium i.v. 79, 107, 123 or 151 days before an i.v. challenge with 1×10^6 P. chabaudi parasitized cells. A control group of mice received the same P. chabaudi infecting inoculum. In another experiment mice were infected with 1×10^6 B. microti infected red cells i.v. 144, 172 or 188 days after infection with M. lepraemurium.

The parasitaemias following challenge with P. chabaudi are given in Figure 4. Prior infection with MLM modified the course of the P. chabaudi infection.

In the controls the primary patent parasitaemia showed two peaks around days 8 and 19 and when smears were discontinued on day 35 a further recrudescence was just appearing. The effect of the MLM was to delay the onset of the first recrudescence, to the greatest extent in the day 107 mice, followed by day 123 and 79 and least protected were the day 151 mice. On day 28 after P. chabaudi infection all the mice, together with non-infected control, were injected i.p. with 0.2ml of a 3% suspension of sheep red cells (SRBCS). The mice were subsequently killed 7 or 10 days later their serum collected and the levels of anti-SRBCS antibody determined. The results showed that there was small elevation in the antibody levels in the days 79, 107 and 123 MLM mice over the P. chabaudi controls, the day 151 MLM mice and the non-infected controls.

After challenge with B. microti all the MLM infected mice demonstrated strong resistance to the parasite. Some mice in all MLM groups showed no parasites and in others there was a low level short-lasting patent parasitaemia. The control mice suffered a typical acute B. microti infection.

The protective effect of the MLM infection was significantly greater against B. microti than against P. chabaudi, and was uniformly effective in all groups against the former but not against P. chabaudi. Against P. chabaudi the protective effect was not to delay the onset of the patent parasitaemia but to reinforce the developing immune response in the infected mice and prevent the first recrudescence seen in the control mice. That is, the control of the malaria infection was a combination of a specific and non-specific antimalarial response. The protective activity of the MLM against P. chabaudi appeared to increase with time after infection reaching a maximum around 107-123 days after infection with MLM and thereafter declined. In the MLM infected mice, after intravenous inoculation of the organisms, the bacterium preferentially invades the lymphoid organs. As the infection develops there is a progressive depletion of lymphoid cells and replacement by macrophages in the thymus and in the lymph nodes a replacement of the immunoblasts in the T dependent areas also with macrophages (Ptak et al., 1970). Cell-mediated immune responses to heterologous antigens are severely impaired (Ptak et al., 1970; Bullock et al., 1977) but antibody responses are not. Although the role of cell-mediated responses in immunity to P. chabaudi are not clear it may be that in the later stages of the MLM infection the non-specific resistance to P. chabaudi resulting from the MLM infection is reinforced to a lesser degree by an impaired specific (cell-mediated) immune response.

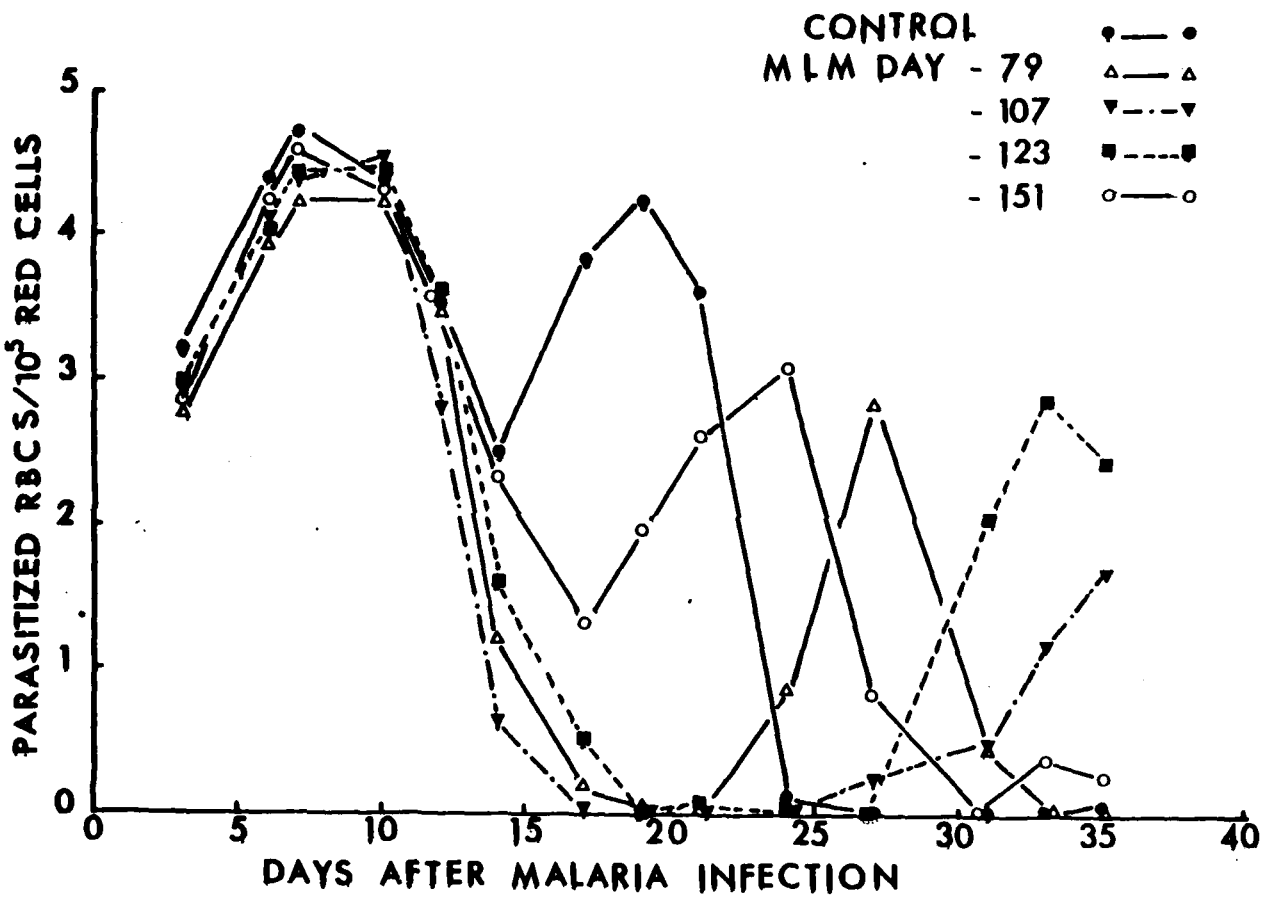


Figure 4. Course of P. chabaudi infection in mice previously infected with Mycobacterium lepraemurium (MLM).

Corynebacterium parvum and BCG, which both gave substantial if not complete resistance to B. microti (Clark et al., 1976, 1977) were found by these workers to be less effective against P. chabaudi or P. vinckei. MLM infection gave the same overall result. The main target cells of the non-specific immune response generated by BCG, C. parvum and other agents (Cox, 1980) is thought to be rapidly dividing parasites within the red cells. It is not generally thought that these agents stimulate or act in a co-operation with the specific immune response but induce the formation of a soluble non-antibody factor which directly or indirectly produces intracellular damage. The experiments with MLM suggest that the effect on B. microti may be mediated through a non-specific mechanism, such as a non-antibody soluble factor. For P. chabaudi, however, this may not be the case because the antimalarial activity was not significant until after peak parasitaemia when the primary specific immune response to P. chabaudi was underway. It could be that that MLM had an adjuvant effect.

The effects of irradiation on the acquired immunity to P. chabaudi

In an investigation of the mechanisms through which the infected mouse removes infected cells the effect of irradiation at different times during the course of an infection on the ability of mice to control their infection or a challenge infection was examined.

In the first experiment female NIH mice, 5 months old, were infected with 1×10^5 P. chabaudi parasitized red cells i.v. and divided into seven groups of six mice. Groups of the mice were irradiated with 600 rads X-irradiation on days 3, 6, 8, 10, 13 or 17 after infection. One group was left non-irradiated. All the irradiated mice were given terramicin in their drinking water for the duration of the experiment. The mean parasitaemias for the seven groups are shown in Figure 5. The parasitaemia became patent by day 2. In the mice irradiated on day 3 (Group 1) two mice died on day 11 and a third mouse on day 16 when the parasitaemia was declining. In this group the acute phase of the parasitaemia was delayed by one or two days compared with that in the controls (Group 2): in the surviving mice the parasitaemia became subpatent before that in the controls and the recrudescent parasitaemia (days 19-27) emerged before that of the controls (days 24-33) and was more severe. Unfortunately all the mice irradiated on day 6 (Group 3), two days before peak parasitaemia in the controls, eventually died. Two of these mice, however, survived to day 17 and 20 and in these two mice the course of the parasitaemia up to death followed that of the controls. None of the mice irradiated on day 8, peak parasitaemia, subsequently died. The primary patent parasitaemia in these mice followed that of the controls but the recrudescent parasitaemia appeared more quickly (days 17-24) than that in Groups 1 and 2. In Group 5 mice, irradiated on day 10, the decline in the primary parasitaemia was arrested, and subsequently the parasitaemia rose again before becoming subpatent on days 19/20, at the same time as the controls. Two of Group 5 mice died when their infections were subpatent and

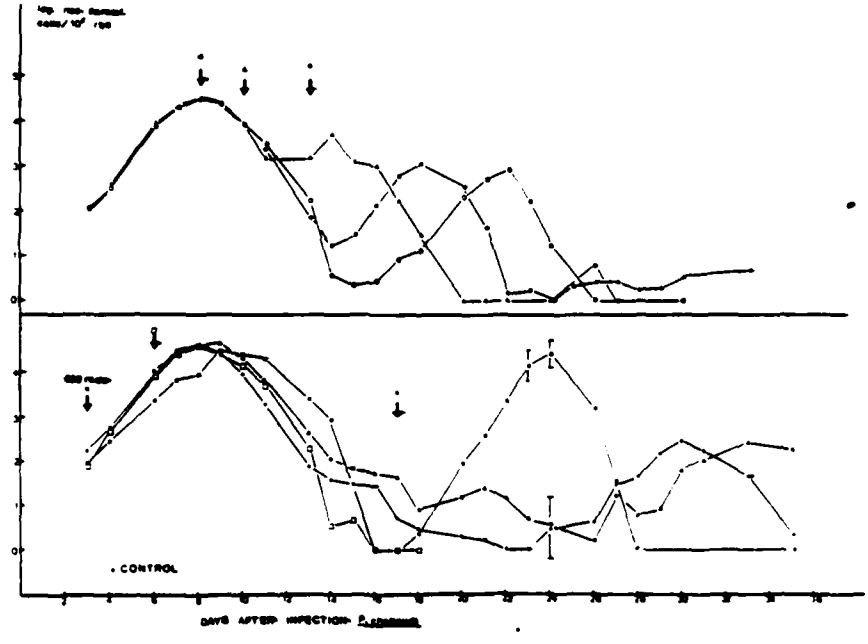


Figure 5. Course of *P. chabaudi* infection in mice irradiated with 600 rads on either day 3 (▪—▪), day 6 (□—□), day 8 (○—○), day 10 (△—△), day 13 (▽—▽) or day 17 (▲—▲) after infection. Non-irradiated mice (●—●).

in the remaining mice the recrudescence was delayed in its appearance when compared with the controls. In the mice irradiated on day 13 (Group 6), when the parasitaemias were only just patent, the patent parasitaemia was extended and rose again before becoming subpatent on day 21. Again in these mice the onset of the main recrudescent parasitaemia was delayed (onset days 29-35). Finally, in Group 7, irradiated on day 17, a low persistent and usually patent parasitaemia followed which developed into more substantial recrudescences around day 33. In summary, therefore, the effect of irradiation was dependent on the time it was given. Irradiation before or on peak parasitaemia did not extent the primary parasitaemia but precipitated a premature recrudescence. Where irradiation was delayed until the primary parasitaemia was going into remission, the decline in the parasitaemia was arrested. Thus between days 10 and 17 the mechanism through which the parasite is removed may include a radiosensitive component although it may be significant that in these mice the arrest in the decline of the parasitaemia was not immediate but was evident on the second day after irradiation. Thus the mechanism through which the parasites are removed may itself not be radiosensitive but some immediate precursor of this process, such as macrophage precursor cells.

In the second experiment not only were the infected mice irradiated with 600 rads at different times during the infection but a number of mice in each group were reinfected with a large intravenous challenge of P. chabaudi infected red cells. Eighteen weeks old female CBA mice were infected with 1×10^5 P. chabaudi infected red cells and divided into seven groups of nine mice. On each occasion within each group of nine mice, three mice were irradiated and reinfected with approximately $5\text{-}7 \times 10^8$ parasitized cells i.v., three mice were irradiated and not reinfected and the remaining three mice were reinfected only. One or two uninfected mice of the same age and sex as the experimental mice received the challenge infection on each occasion. Groups of mice were treated as above on days 11, 13, 17, 21, 27, 31 or 37. A further group of six control mice were infected but were neither irradiated nor reinfected.

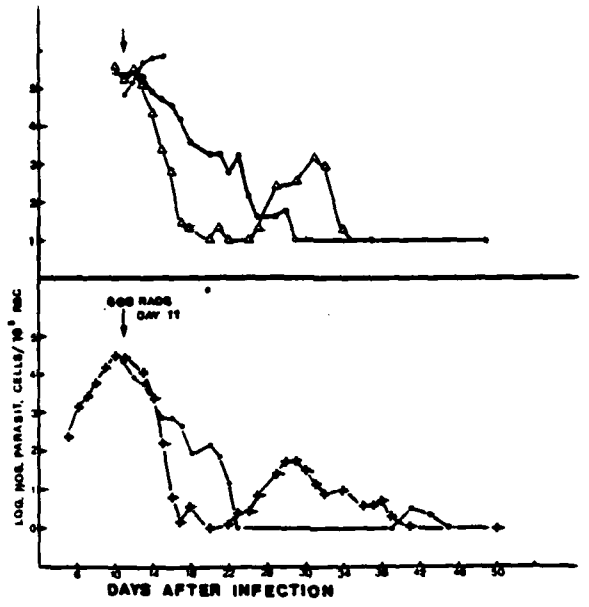
The mean parasitaemias of each group or subgroup of mice are shown in Figure 6.

Control mice (Group 1)

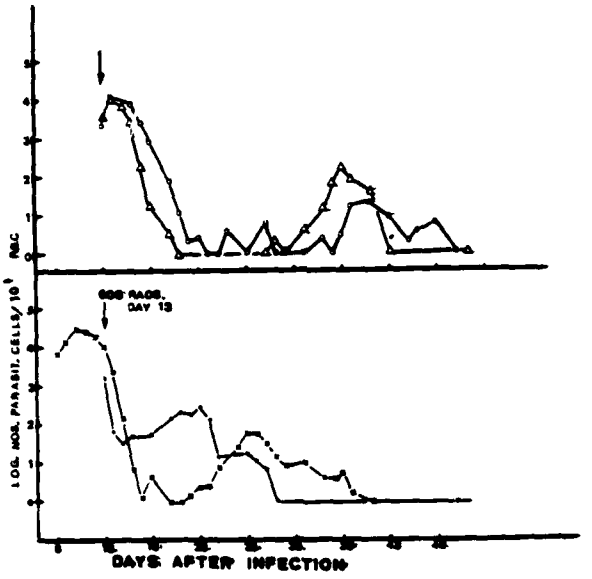
The parasitaemia followed the expected course, an acute patent primary parasitaemia, with a peak parasitaemia on days 10/11, becoming subpatent around days 16/17 and a patent recrudescence occurring between days 24-25.

Reinfection on day 11 (Groups 2)

The non-irradiated mice controlled the challenge and the parasitaemia became subpatent at the same time and the onset of the recrudescence was at the same time as in the controls. In the irradiation mice which were not reinfected, the patent parasitaemia was extended by 4-5 days, and of the two surviving mice only one showed a patent recrudescence and that on day 41. In the three mice which were reinfected and irradiated, the parasitaemia over the succeeding three days matched the declining parasitaemia of the non-irradiated/challenged mice. Thereafter the patent parasitaemia was extended as in the comparable irradiated group which were not reinfected.



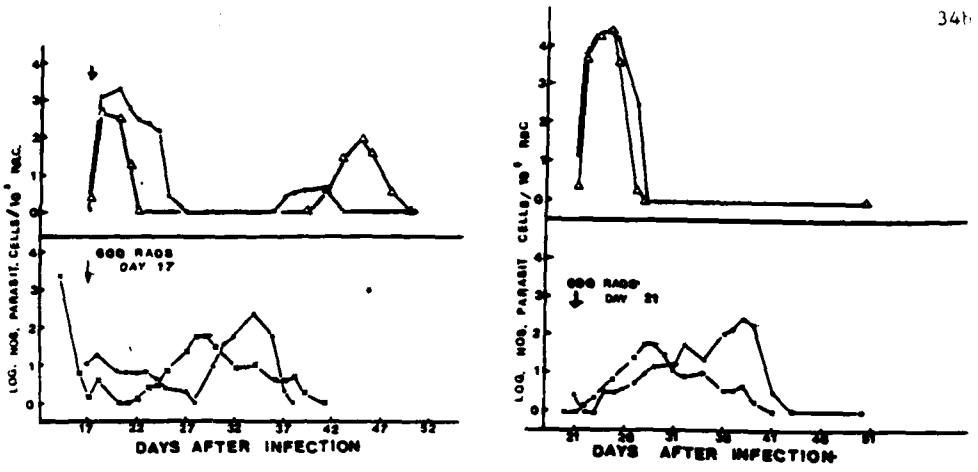
6a - Irradiation and reinfection - day 11.



6b - Irradiation and reinfection - day 13.

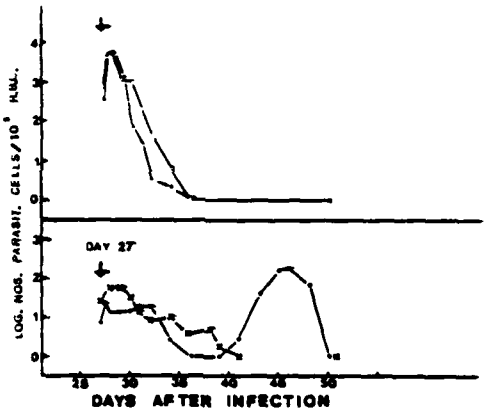
Figure 6. Course of P. chabaudi infection in mice either irradiated after infection or irradiated and challenged after infection.

Key - infected mice irradiated only (●—●). Infected mice irradiated and reinfected (○—○). Infected mice challenged only (△—△). Challenge infection only (▲—▲). Infected mice not irradiated or reinfected (+—+).



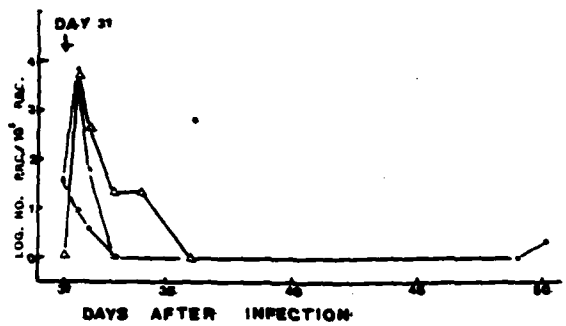
6c - Irradiation and reinfection - day 17.

6d - Irradiation and reinfection - day 21

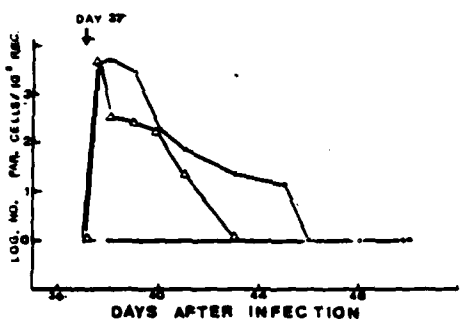


6e - Irradiation and reinfection - day 27.

Figure 6. Course of P. chabaudi infection in mice either irradiated after infection or irradiated and challenged after infection.
Key - infected mice irradiated only (●—●). Infected mice irradiated and reinfected (○—○). Infected mice challenged only (△—△). Challenge infection only (▲—▲). Infected mice not irradiated or reinfected (■—■).



6f - Irradiation and reinfection - day 31.



6g - Irradiation and reinfection - day 37.

Figure 6. Course of P. chabaudi infection in mice either irradiated after infection or irradiated and challenged after infection.
Key - infected mice irradiated only (●—●). Infected mice irradiated and reinfected (○—○). Infected mice challenged only (△—△). Challenge infection only (▲—▲). Infected mice not irradiated or reinfected (■—■).

Reinfection day 13 (Group 3)

By this time the picture was changing. The non-irradiated mice controlled the challenge infection and were subpatent by days 18-20, about the same time as in the controls (Group 1). In the reinfected mice, however, the onset of the recrudescence was delayed to days 34-37, some 7-10 days later than the controls. In the irradiated/reinfected mice the challenge was controlled almost as effectively as in the non-irradiated/challenged mice and again in these mice the recrudescent parasitaemias were delayed, commencing on days 36, 39 and 46 in the three mice. In the irradiated/not reinfected mice the irradiation lengthened the primary patent parasitaemia by 7-10 days over the controls and two mice induced a small rise in parasitaemia. In these mice no recrudescent parasitaemia was detected during a 50-day observation period.

Reinfection day 17 (Group 4)

The non-irradiated mice, two of which were subpatent at challenge, rapidly controlled the parasites and were subpatent by day 22. Significantly the recrudescence in these mice was delayed to days 41-43. In the irradiated/challenged mice, however, all three of which were subpatent at challenge, one mouse controlled the parasites as rapidly as their non-irradiated counterparts, but in the other two mice there was a small drop followed by a small rise in the parasitaemia becoming subpatent by day 25. In these latter two mice no further recrudescence was seen but in the irradiated mouse which rapidly controlled the challenge a recrudescence was seen between days 37 and 41. Two of the three mice which were irradiated but not challenged were just subpatent at irradiation and these mice subsequently recrudesced at about the same time as the controls. The third mouse had a low patent parasitaemia when irradiated and this persisted until day 27, and a patent recrudescence appeared on day 43.

Reinfection day 21 (Group 5)

The ability to control rapidly a challenge infection had diminished by day 21. In the non-irradiated/challenged mice the parasitaemia rose to a peak two to three days later, matching the rise in the non-immune challenge control, finally being reduced to a subpatent level by day 27. No recrudescences were seen in these mice in a 57-day observation period. In two of the three irradiated/challenged mice the parasitaemia was still very high four days after challenge and two of these mice died two to three days later. The third mouse in this trio controlled the challenge to subpatent levels by day 28 but died on day 41. The mice irradiated but not challenged were subpatent or just about subpatent when irradiated and these mice subsequently recrudesced at a time within the range shown in the controls.

Reinfection day 27

At this time the majority of the mice were just beginning their recrudescent parasitaemias following a period of subpatency. Although the challenge infection in the irradiated and non-irradiated mice initially increased the patent parasitaemia the subsequent course in both groups matched that in the controls. In the irradiated/non-infected mice there was no evidence that the irradiation had any effect although surprisingly two of the three mice did not show a recrudescence until days 41/43.

Reinfection day 31 (Group 7)

At this time most of the mice were controlling their recrudescent parasitaemias and irradiation had no discernible effect on the ability of mice to control the challenge or to reduce the recrudescent parasitaemia to subpatent levels.

Reinfection day 37 (Group 8)

By day 37 some of the mice could have reduced their recrudescent parasitaemias to a subpatent level some 2-7 days previously. None of the irradiated/non-reinfected mice were patent when irradiated and remained subpatent. In the reinfected mice although in the majority the challenge was not removed as rapidly as in the day 31 mice there was no good evidence that irradiation reduced the rate at which the challenge was removed.

This second experiment confirmed that irradiation of mice after peak parasitaemia, days 10-17, when the parasitaemia was in remission but was still patent, delayed the parasitaemia going into subpatency (irradiation on day 13 resulted in the parasitaemias becoming higher), and delayed the onset of the recrudescent parasitaemia. Thus irradiation during this period of remission increased the antigenic load and presumably the antigenic stimulus which the mice received which was reflected in turn in a lelay in the onset of the recrudescences. Similarly where the non-irradiated mice were reinfected on days 13, 17 or 21, thereby increasing the antigenic stimulus to the mice when the parasitaemia was going into remission or had just become subpatent, the onset of the recrudescence was delayed by approximately 8, 18 and >34 days respectively. Irradiation of mice on day 21 or thereafter had no obvious effect on the course of the parasitaemia during the 50-57 day observation period. Recent work in our laboratory (McLean and Phillips, 1979) showed that in P. chabaudi infected NIH mice the ability to control a large i.v. challenge of P. chabaudi infected red cells rapidly declined between the onset of subpatency after the primary parasitaemia and the emergence of the recrudescence. Coincident with the decline in the patent recrudescence, a challenge infection was rapidly removed but thereafter the ability to remove a large challenge infection diminished with time. Reinfection of the non-irradiated mice at different times in the second experiment gave similar results. The course of the parasitaemia after reinfection of the irradiated mice on each occasion reflected the parasitaemias in the other two subgroups, that is, the irradiated/non-reinfected and reinfected/non-irradiated mice. By this we mean that the initial control of the challenge infection followed that in the non-irradiated/reinfected mice but the duration of the subsequent patent parasitaemia in some mice would follow that in the irradiated/non-reinfected mice.

In summary the effect of 600 rad irradiation given between days 3 and 8, day 8 being peak parasitaemia, is to precipitate a more rapid appearance of a recrudescent parasitaemia but not to affect the duration of the primary patent parasitaemia. Irradiation on days 10, 13 or 17 extended the patent primary parasitaemia and for irradiation on days 10 or 13 to delay the onset of a patent parasitaemia.

Preliminary experiments using a carbon clearance test (Weir, 1973) on the infected mice did show any reduction in carbon clearance rates after irradiation.

Role of the spleen in removing malaria parasites in the immune host

The importance of the spleen in immunity to malaria parasites has been well documented but details of the precise role(s) of the spleen are still not clear although it is generally assumed to be a source of antiparasitic antibody and a site of phagocytosis of parasites and/or parasitized cells. In experiments described in an earlier report (Annual Report DAJA 37-73-C 3492) phagocytosis of P. chabaudi infected red cells containing late asexual stages by phagocytes in the liver and spleen was reported.

The immune mechanisms through which the parasites are removed are rapidly acting as demonstrated in the following experiment. Three months old male NIH mice were infected with 1×10^6 P. chabaudi infected red cells from which the mice recovered. Between days 30 and 52 and on day 92 the mice were reinfected with 7×10^8 parasitized cells i.v. On day 107 four mice and an age-matched normal control were reinfected for the third time with 7×10^8 parasitized cells i.v. at 0800hr when the parasites were at the young ring stage. At 1715hr P. chabaudi infected donor mice were bled and the parasites (late ring stage) put into in vitro culture following the Trager and Jensen procedure with the exception that the RPMI 1640 containing 10% foetal calf serum and not normal mouse serum. Eight hours later (0245hr) the majority of the parasites in culture were at the late trophozoite or schizont stage, reinvasion had started and there were some free merozoites. At this time, the cultures were discontinued, the blood cells washed twice with and finally resuspended in RPMI 1640. Approximately 3×10^8 of the cultured parasitized cells were injected i.v. into each of four more immune mice and an age-matched non-immune control. The course of the parasitaemias in the two groups of immune and the control mice are given in Figure 7. In the non-immune control mice for the 0800hr challenge the parasitaemia was maintained during the day declining slightly towards midnight as schizogony approached and schizont stages were sequestered in the deep tissues: a few schizonts were seen in the peripheral blood smears in the early hours of the following morning. In the immune mice reinfected at 0800hr the parasites continued to grow normally but as schizogony approached, parasites were lost from the peripheral circulation: reinvasion did not occur. (In an earlier report the phagocytosis of parasitized red cells in the spleen, liver and occasionally in peripheral blood in immune mice was described.) Of interest was the fact that in the immune mice but not the control at 0745hr on the day after challenge a few schizont infected cells were to be seen in the peripheral circulation suggesting that in the immune mice the growth of a small proportion of the parasites was slowed. In the mice injected with cultured parasites smears taken immediately after challenge showed that 4-14% of the parasites in the peripheral circulation were new rings and the rest were late trophozoites or schizont infected cells. Two hours later smears from the immune mice showed that there was a small increase in the number of ring infected cells but a rapid decrease in the mature asexual stages. Five hours after injection the parasitaemias in the immune mice given cultured parasites were below 1% and 85% or more of the parasites were ring stage parasites. At this time the non-immune control mouse was showing a parasitaemia of 5.3%, 99% of the parasites being new rings. This experiment confirms that in the hyperimmune mouse the removal of parasites/parasitized red cells occur at the late trophozoite/schizont stage, growth from the young ring stage in the majority of parasites

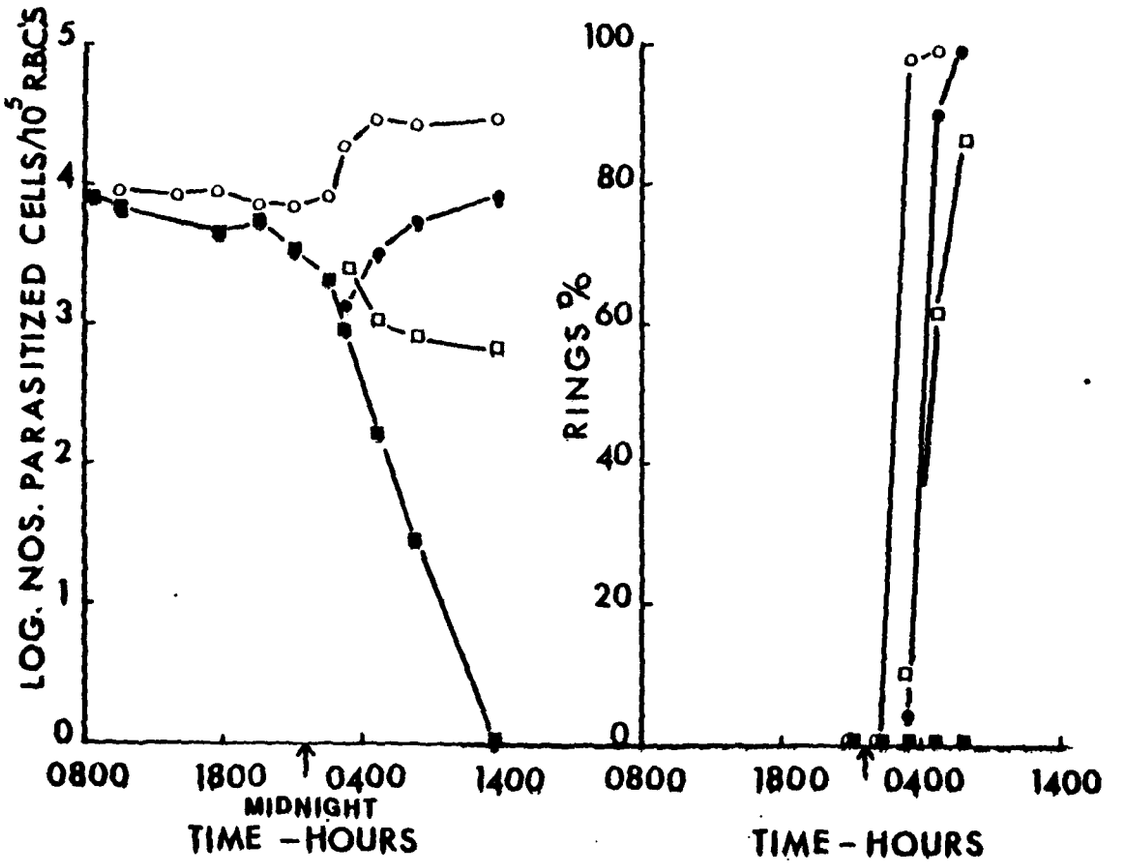


Figure 7. Course of P. chabaudi infection and % ring stage parasites present in either immune mice reinfected at 0800 hours (▪—▪) or 0245 hours the following day (□—□), or in naive controls infected at 0800 hours (○—○) or 0245 hours (●—●) the following day.

occurring normally, and this removal is a rapid process, once the parasite reaches the schizont stage. The majority of recently introduced late trophozoites/schizonts disappearing from the circulation within 2hr.

Previous work has shown that phagocytosis of P. chabaudi/P. chabaudi parasitized cells occurs in the liver and the spleen. The relative importance of the spleen was examined in the following experiments.

In the first experiment, 4 months female CBA mice were infected with 1×10^6 P. chabaudi infected red cells. On day 10 of infection, two days after peak parasitaemia with the parasitaemia declining, two groups of five mice were splenectomized under ether anaesthesia. A third infected group were sham splenectomized. The day after splenectomy the mice in one group, together with a non-immune intact control, were reinfected with 7×10^8 P. chabaudi infected red cells i.v. The parasitaemias are given in Figure 8. In the splenectomized/non-reinfected mice the parasitaemia declined further on the day after splenectomy but thereafter the parasitaemia increased and a fluctuating patent parasitaemia ensued until days 34-38 when the parasitaemia became subpatent for a short period. In the splenectomized/reinfected mice the parasitaemia rose on the two days following reinfection and thereafter continued as a fluctuating and frequently high patent parasitaemia until day 39. In the non-splenectomized infected mice the parasitaemia became subpatent in all mice by day 20. From a previous experiment (page 32) it is known that in infected mice given an additional challenge infection on day 11, the parasitaemia initially rises but thereafter rapidly declines to become subpatent at the same time as in controls. These results confirm the importance of the spleen in the maintenance of immunity in mice in which the primary parasitaemia was going into remission: it is significant, however, that in the splenectomized infected mice the parasitaemia continued to decline in the absence of the spleen on the day after splenectomy.

In the second experiment, immune CBA female mice were reinfected with 10^6 P. chabaudi parasitized cells to boost their immunity and 13 days later when the parasitaemia following challenge had become subpatent two groups of five mice were splenectomized. On day 14, the day following splenectomy, one group of the splenectomized mice (Group C) were challenged with 5×10^8 parasitized cells i.v. A group of the reinfected but sham-splenectomized mice, and non-immune age-matched control mice were similarly challenged. The remaining group of reinfected and splenectomized mice (Group B) were not challenged. The parasitaemias in the various mice from day 14 are shown in Figure 9. The sham-splenectomized (Group A) and splenectomized immune mice (Group C) both controlled the challenge infection as effectively as each other with the exception of a single mouse in Group C which showed a short-lasting recrudescence. Up to day 46 no parasites were seen in either group. One of the mice in the immune splenectomized group (Group B) recrudesced on day 18 and a fatal acute parasitaemia ensued. Thus in the mouse with an established immunity with a recent boosting he spleen was dispensable and the rapid removal of parasitized cells occurred in extra splenic sites.

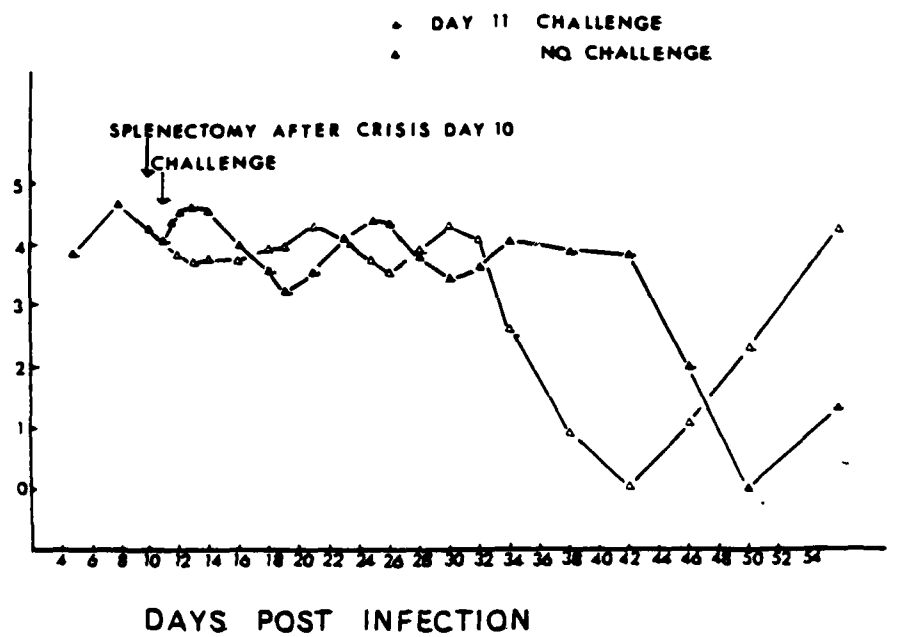


Figure 8. Course of P. chabaudi infection in mice either splenectomized on day 10 (Δ—Δ) or splenectomized on day 10 and reinfected on day 11 (▲—▲).

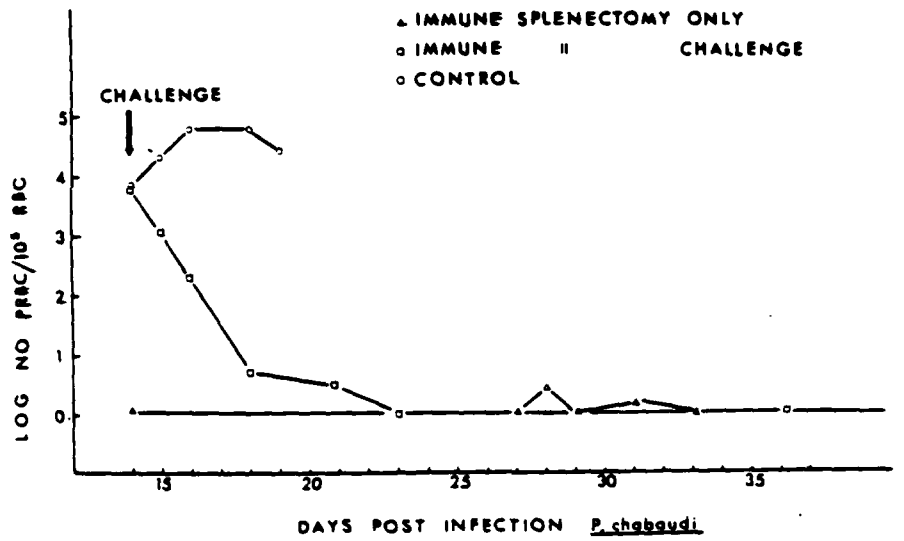
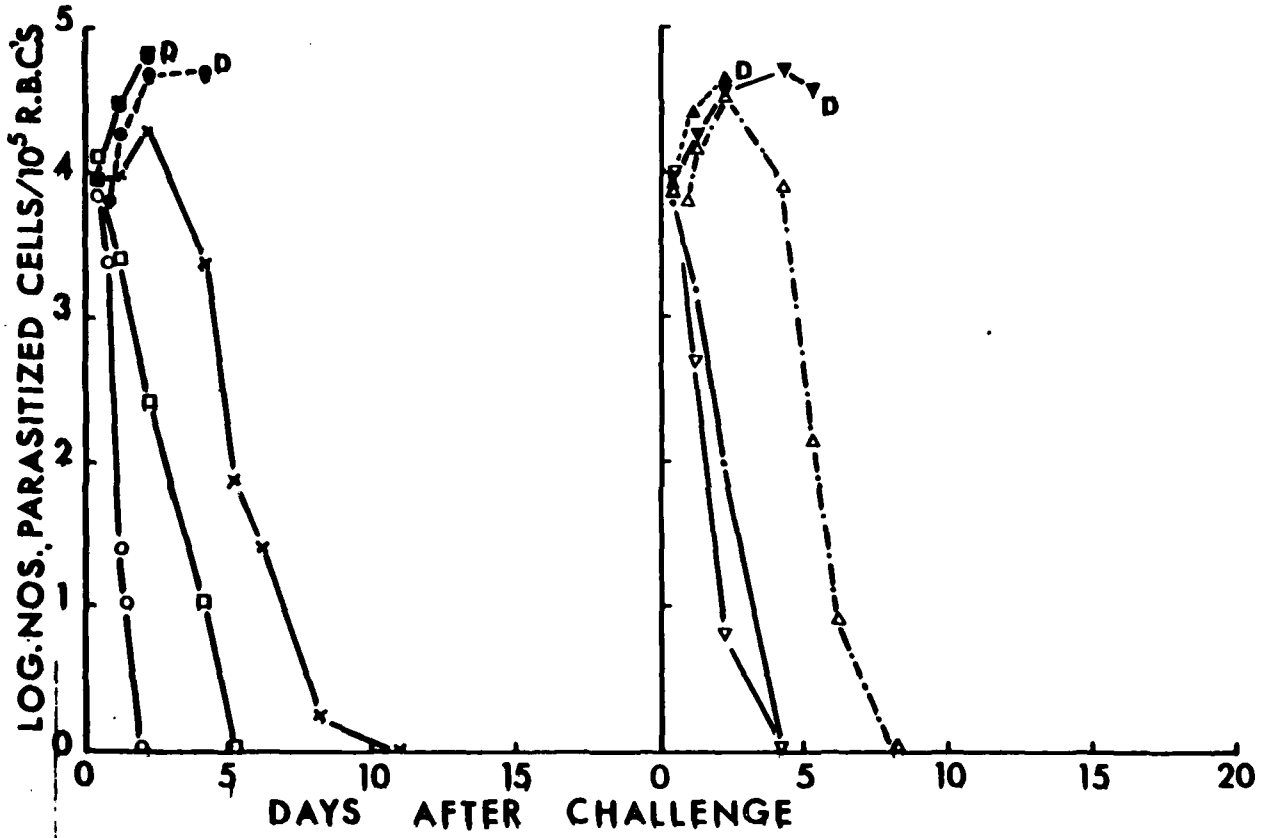


Figure 9. Course of P. chabaudi challenge infection in splenectomized immune mice. Reprimed immune mice. Splenectomized day 13 and challenged day 14 (Group C) (□—□). (Reprimed immune intact mice challenged day 14 (Group A) parasitaemia as Group C.) Reprim ed immune mice, splenectomized day 13 but not challenged day 14 (Group B) (Δ—Δ). Challenge in naive mice (○—○).

In a third experiment 3 months old C57Bl mice were infected with 1 x 10^6 P. chabaudi parasitized cells and on days 14, 20 and 27 four of the mice were splenectomized. In the non-splenectomized mice the parasitaemia followed the expected course, peak parasitaemia being recorded on day 8 or 9 and becoming subpatent by day 20, and a patent recrudescence appearing around day 29. In the mice splenectomized on day 14 the parasitaemia remained patent, and a fluctuating and frequently acute parasitaemia ensued, one of the mice dying. The mice splenectomized on day 20 or day 27 when the mice had just become subpatent, subsequently recrudesced and the parasitaemia was acute and long-lasting. In two of the mice splenectomized on day 20 the recrudescence appeared two days before the majority of the control mice.

The role of the spleen was further explored in homologous and heterologous immunity to P. chabaudi and P. vinckei brucechwatti (P.v.b.c.) in C57Bl mice. Male C57Bl were first infected with P. chabaudi or P.v.b.c. when 2 months old. The P.v.b.c. infected mice had their otherwise fatal infection controlled with chloroquine. Three months later the recovered mice were reinfected with the same parasite with which they were first infected. Twelve days after reinfection five mice from each group of mice were splenectomized. The day following splenectomy subgroups of three or four intact immune mice were challenged homologously or heterologously with 5-7 x 10^8 parasitized red cells intravenously. Two splenectomized P. chabaudi immune mice were challenged, one mouse with P. chabaudi parasitized red cells and the other with P.v.b.c. Two splenectomized P.v.b.c. immune were also challenged either homologously or heterologously. Age-matched non-immune controls were also infected with the challenge inoculum. The parasitaemias are given in Figure 10. The infectivity of the challenge inoculum was confirmed in the non-immune mice which both died. The immune mice including the splenectomized mice challenged homologously rapidly controlled and removed the challenge infection. Immune intact mice challenged heterologously initially showed a small degree of resistance, the parasitaemia rising for 2 to 3 days and thereafter declining to a subpatent level by day 8. In contrast, in the splenectomized immune mice no immunity to the heterologous challenge was discernible and the mice died. Further intact and splenectomized immune mice were challenged i.v. heterologously or homologously 18 days after reinfection and 6 days after splenectomy. In addition, intact mice challenged on day 13 were challenged for a second time on day 18. Non-immune age-matched controls were also challenged. The parasitaemias are given in Figure 11. Both the non-immune controls died of an acute parasitaemia. The splenectomized immune mice challenged heterologously showed no resistance and died at the same time as the non-immune controls. The splenectomized mice challenged homologously both controlled the challenge infection although less quickly than in the splenectomized mice challenged on day 13. In the mice which were challenged for a second time within five days of the last reinfection showed a greater heterologous immunity than in the mice when challenged on day 13 after reinfection. In a repeat experiment essentially the same results were obtained.


LOG. NOS. PARASITIZED CELLS/10^5 R.B.C's
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DAYS AFTER CHALLENGE

Fig. 10

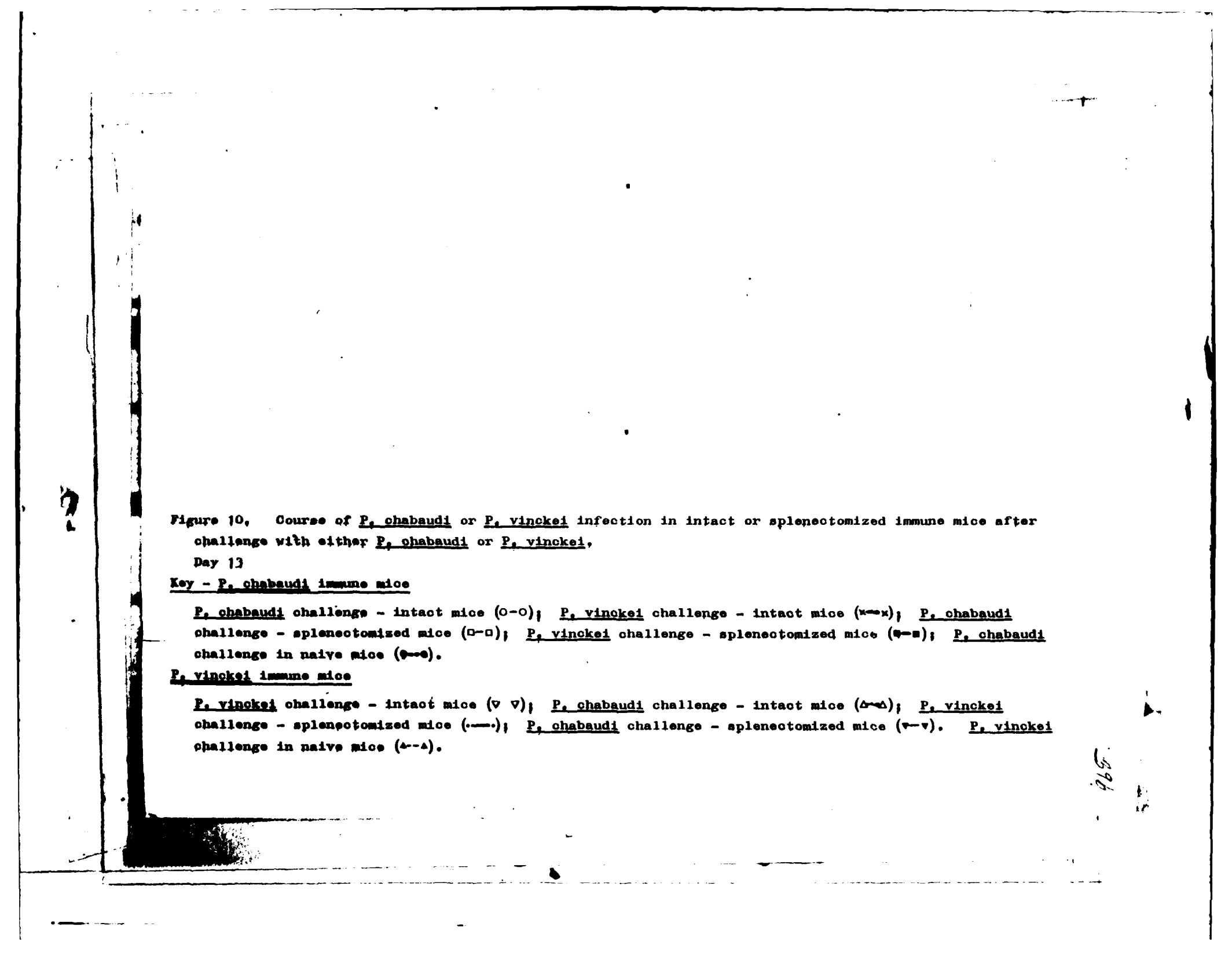


Figure 10. Course of P. chabaudi or P. vinckei infection in intact or splenectomized immune mice after challenge with either P. chabaudi or P. vinckei.

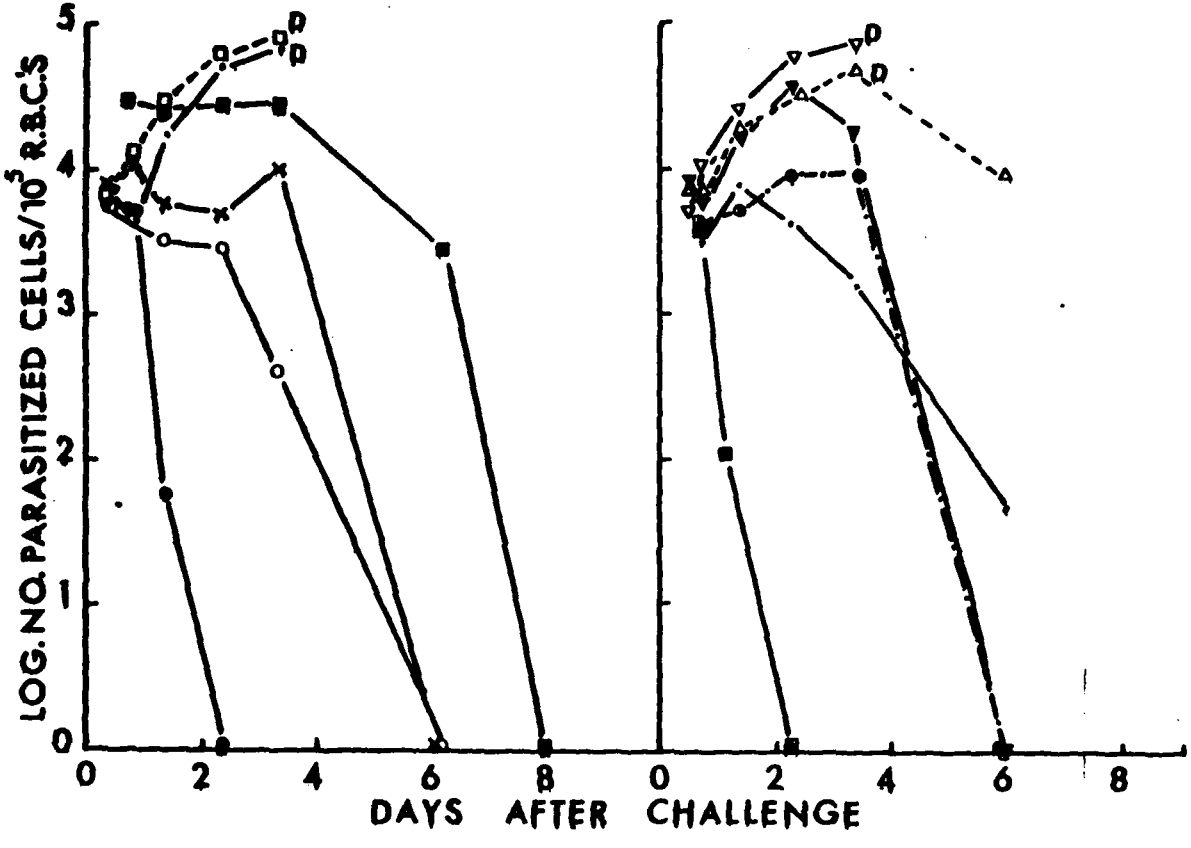
Day 13

Key - P. chabaudi immune mice

P. chabaudi challenge - intact mice (o-o); P. vinckei challenge - intact mice (x-x); P. chabaudi challenge - splenectomized mice (□-□); P. vinckei challenge - splenectomized mice (■-■); P. chabaudi challenge in naive mice (●--●).

P. vinckei immune mice

P. vinckei challenge - intact mice (▽ ▽); P. chabaudi challenge - intact mice (△-△); P. vinckei challenge - splenectomized mice (•—•); P. chabaudi challenge - splenectomized mice (▼-▼). P. vinckei challenge in naive mice (▲--▲).


LOG. NO. PARASITIZED CELLS/10^5 R.B.C.'S
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8
DAYS AFTER CHALLENGE

Fig.11

Figure 11. Course of *P. chabaudi* or *P. vinckei* infection in intact or splenectomized immune mice after challenge with either *P. chabaudi* or *P. vinckei*.
Day 18

Key - *P. chabaudi* immune mice

P. vinckei challenge - intact mice (×—×); intact mice reinfected on day 13 with *P. chabaudi* and on day 18 with *P. vinckei* (○—○); *P. chabaudi* challenge - splenectomized mice (■—■); *P. vinckei* challenge splenectomized mice (□--□); *P. chabaudi* challenge - intact mice (●—●); *P. chabaudi* in naive mice (•—•).

P. vinckei immune mice

P. chabaudi challenge - intact mice (▽—▽); intact mice reinfected on day 13 with *P. vinckei* and on day 18 with *P. chabaudi* (▼—▼); *P. vinckei* challenge - splenectomized mice (•—•); *P. chabaudi* challenge - splenectomized mice (△--△); *P. vinckei* challenge - intact mice (▣—▣); *P. vinckei* challenge in naive mice (▽—▽).

The relative importance of the spleen in immunity to P. chabaudi depends on the stage of the infection. If the spleen is removed on day 10 or day 14 when the primary patent parasitaemia is in decline, although the parasitaemia may decline through one schizogony after splenectomy, subsequently the parasitaemia rises, initially unchecked, to acute levels. Fourteen to 21 days after splenectomy the parasitaemia is reduced, albeit temporarily, to subpatent levels. Irradiation of infected mice as the primary parasitaemia declines has the same effect as splenectomy in extending the patent parasitaemia but it is shorter lasting. The simplest explanation of the effect of splenectomy at this stage in the infection is to assume that the spleen is a major source of protective antibody, and that following splenectomy, the antibody level falls within two days of splenectomy. The effect of irradiation is less well explained on the same basis because antibody producing plasma cells are more radioresistant. Irradiation may be destroying a radiosensitive precursor cell, such as precursors of macrophage or killer cell or depleting the supply of B-cells, the precursors of plasma cells. Splenectomy and irradiation after the primary parasitaemia is into remission has no immediate consequences on the course of the parasitaemia. Any patent recrudescences following splenectomy, however, are of a longer duration.

In mice which have recovered from both the primary parasitaemia and the recrudescent parasitaemia and had also been reinfected to boost the level of immunity, the ability to remove an i.v. challenge given soon after reinfection is unimpaired by splenectomy. Undoubtedly reinfection before challenge will stimulate antibody production and as removal of the parasite is, in part, at least antibody mediated, parasite removal in the absence of the spleen is likely to occur in extra splenic sites, such as the liver.

In contrast, although in hyperimmune mice the spleen is dispensable for the rapid immediate removal of the parasitized cells after a homologous challenge, with a heterologous challenge no immunity is detectable following splenectomy. In intact hyper-immune mice after heterologous challenge the parasitaemia did not initially rise as rapidly as in the non-immune controls and within 6-8 days the parasitaemia was subpatent. Thus both the non-specific immunity which initially controls the heterologous challenge and the specific immune response which develop with the speed of a secondary immune response, to eliminate the heterologous challenge, are dependent on the spleen.

The mechanism of the cross-immunity between different rodent plasmodia has not been elucidated although extensive cross-immunity between rodent plasmodia has been described (for example Cox, 1975). In the published work cross-immunity was detected by infecting mice with one species or sub-species of rodent plasmodium and a few weeks after natural or drug-assisted recovery from that infection the mice were challenged with a relatively small inoculum (1×10^6 parasitized cells) of another species or subspecies of parasite. The level of cross-protection at the time of challenge as detected against a large intravenous challenge was not determined. Indirect fluorescent antibody tests in our laboratory and elsewhere (Cox, 1970) indicate that P. vinckei brucechwatti and P. chabaudi share common antigens. It has not, however, been possible in our laboratory to transfer protection to P.v.b.c. with serum from P. chabaudi immune mice: the same serum gave significant protection to P. chabaudi. The immunity, therefore, which developed over a

period of several days to P. chabaudi and P.v.b.c. after heterologous challenge, would appear to be spleen dependent and likely to involve the production of species specific protective antibody, with the antigens common to both parasites acting as carriers for the species specific antigens (Brown, 1971). Over the past five years a role has been described (reviewed Cox, 1980) for a non-antibody soluble mediator of immunity with a non-specific activity against intracellular parasites, particularly during the primary parasitaemia. Rodent babesias seem to be particularly susceptible to this soluble factor. Cox (1978) reported that mice infected with B. microti exhibit a strong immunity to P. vinckei from day 17 to at least day 22, this immunity being sufficient to remove rapidly an intravenous challenge of 2-8 x 10^8 P. vinckei infected red cells. Cox suggested that heterologous immunity to P. vinckei in B. microti infected mice involved the non-antibody soluble mediator. In the experiments described above the cross-immunity between P. chabaudi and P.v.b.c. was only sufficient to depress the rate at which the patent parasitaemia rose after challenge before a specific immune response was mounted. A soluble non-antibody factor may be responsible for the cross-immunity but if this is the case the spleen must be a major source of this factor and the half-life of the latter be relatively short because splenectomy ablated cross-immunity.

Conclusions and Recommendations

The techniques which are standard in the Blood Transfusion Services for the cryopreservation at low temperatures and subsequent recovery of human red cells have proved suitable for the cryopreservation of P. falciparum infected red cells, albeit the ring stage parasites only. Blood from patients with patent P. falciparum infections contain ring stages and/or gametocytes, the later asexual stages withdrawing into the deeper tissues, such as the spleen, liver, intestine and heart. Thus in the present project snap-freezing small volumes of infected blood containing a final concentration of 19% (w/v) glycerol in a sorbitol/saline solution and recovering the thawed blood by washing with sorbitol/saline or hypertonic saline has proved satisfactory. Cultures of P. falciparum provide the later stages in the asexual cycle. Previous work (Annual Report DAJA 17-76-G9415) showed that trophozoites and schizont-infected cells of P. falciparum did not survive snap-freezing. A technique for the cryopreservation and recovery of viable P. falciparum trophozoites and schizonts would be useful, for example, for the preparation of antigen smears for use in the indirect fluorescent test. As the present time, our attempts to improve the survival rate of these larger intracellular stages of P. falciparum by reducing the temperature of the infected blood in a series of steps (Wilson et al., 1977) have met with only limited success.

The use of cryopreservation to keep P. falciparum parasites from a patient viable while the same patient mounts an immune response to those parasites so that, subsequently, elements of that immune response can be tested against those parasites in vitro is a useful one. This procedure provides a means of controlling for any antigenic variability by the parasites. In this study sera taken at different times after treatment are being tested for homologous and heterologous activity in vitro. The conditions which allow reasonable growth and multiplication of P. falciparum in microcultures of 50-100µl over a few days have been described. Similar sized microcultures have recently been used by Reese and Motyl (1979) in their investigation of anti-malarial activity in the sera of Aotus monkeys immune to P. falciparum. Under the present grant a culture adapted strain of P. falciparum has been maintained for 7 or more days in microcultures by changing 6-70% of the medium on a daily basis. A detailed report on the effect of the 'return' sera on the growth and multiplication of the cryopreserved wild isolates of P. falciparum and also culture adapted strains of P. falciparum of Thailand and Gambian origin will be given in the next report. Microcultures of P. falciparum are currently being used under the present grant to examine the effect of granulocytes and mononuclear cells from the peripheral blood of immune Gambian and non-immune individuals in the presence of Gambian or non-immune Caucasian sera on the growth and multiplication of P. falciparum. Medium was changed daily. Although the results of these latter experiments are somewhat preliminary they have shown that, in vitro, there is an antibody independent uptake of merozoites by granulocytes, particularly by the polymorphonuclear cells, and in a small proportion of the patients tested, their peripheral blood mononuclear cells had an antiparasitic activity in the presence of homologous serum. The phagocytosis of P. falciparum merozoites and polymorphs when infected blood was observed under the microscope directly after being taken from the patient was described by Trubowitz and Masek (1968) and Sinden and Smalley (1976) respectively.

Techniques are now available for the cryopreservation of granulocytes and mononuclear cells which would permit the collection of peripheral blood leukocytes from patients during a patent parasitaemia and at intervals thereafter and subsequently on the same occasion test these samples for activity *in vitro* correlates of cell-mediated immunity and for cytotoxic activity against homologous parasites (also previously cryopreserved) in the presence or absence of specific antibody. A role for cell-mediated immune mechanisms in malaria is indicated although the evidence is in some cases indirect. For example, an increase in cells having a non-specific antibody dependent cellular cytotoxicity (K-cells) in malarious mice (McDonald and Phillips, 1978) or children (Greenwood *et al.*, 1977) suggests that K-cells may be cytotoxic to malaria parasites. In two reports there is more direct evidence of cell-mediated immune mechanisms killing parasites *in vitro* (Coleman *et al.*, 1975; Playfair *et al.*, 1979). It is recommended that the microculture system for growing the asexual stage of *P. falciparum* be used for examining further the specific and non-specific antiparasitic activity of antibody and cells from immune individuals. 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 anticipated (Wilson and Phillips, 1976; Wilson, 1980). The use of monoclonal antibodies prepared against different isolates of *P. falciparum* to compare their antigenic characters should reveal more about the antigenic variability of *P. falciparum* and is clearly an area where considerable effort should be directed. At the present time in our laboratory the monoclonal antibody technique is being established and it is hoped that, in due course, the large number of *P. falciparum* isolated we have cryopreserved in liquid N_2 can be used in experiments with monoclonal antibodies.

The course of a blood-induced *P. chabaudi* infection in mice has the typical malaria characteristic of the acute parasitaemia being followed by a period(s) of subpatency between one or two recrudescent parasitaemias. The cause of the fluctuating parasitaemia is not fully understood. Two possible explanations are being examined in our laboratory at the present time. First, like *P. knowlesi* (Brown and Brown, 1965), *P. chabaudi* during the asexual erythrocytic stage may be able to undergo repeated antigenic variation and thereby evade the immune response, a phenomenon well described in African trypanosomiasis. Secondly, in the infected mice the anti-malarial immune response may be unable to bring about a rapid elimination of the parasite because of constraints upon the immune response, these contraints possibly being of parasite origin. The evidence to date suggests that although *P. chabaudi* may be able to undergo antigenic variation there are fluctuations in the level of antiparasitic activity, at least against the infecting population, which relate to the emergence of the recrudescent parasitaemias. In the experiments described in this report on the effects of irradiation on the course of a *P. chabaudi* infection it was noted that in the non-irradiated mice which were reinfected with a large challenge inoculum during the period when the primary parasitaemia was going into remission, the onset of the patent recrudescence was significantly delayed. If we assume that the recrudescence consists of parasites predominantly of a new variant type this observation suggests that the challenge infection is raising the level of antiparasitic activity which may be active against

P. chabaudi of different antigenic types of that particular strain. The nature of the immune mechanisms active against P. chabaudi when the primary parasitaemia is declining is not fully understood. Serum collected as the primary parasitaemia becomes subpatent can passively protect non-immune mice which suggests that serum factors, probably antibody, are involved. Both irradiation and splenectomy of mice, however, during the declining parasitaemia, did not immediately check the decline in the parasitaemia, but within 2-3 days they did so. Irradiation is thought to have little effect on plasma cells which indicates that the antiparasitic processes which are rapidly impaired by irradiation at this time, are not dependent on the production of antimalarial antibody alone. In the splenectomized hyperimmunized mouse or in mice irradiated after the primary parasitaemia has become subpatent the ability to remove a large challenge infection is the same as in the non-irradiated or non-splenectomized immune mice. It would seem, therefore, that the nature of the immune mechanisms operating against P. chabaudi during the primary parasitaemia and after the primary parasitaemia differ in some respects, the former period perhaps being more dependent on immune mechanisms which did not involve a direct antiparasitic (anti-merozoite) cytotoxic antibody. It is clear that in P. chabaudi infections the ways in which antibody directly or indirectly kills or leads to the removal of parasites or parasitized cells needs careful investigation. A role for opsonizing antibody has been suggested. Activation of macrophages alone has been shown, in this report, to have significant anti-plasmodial activity. The use of in vitro cultures of P. chabaudi might allow more direct observation of the individual and combined roles of antibody and cellular elements of the immune system. In our laboratory in short-term microcultures of P. chabaudi multiplication rates of X2 can be consistently obtained Multiplication occurs more readily in RPMI 1640 medium containing 10% foetal calf serum than in medium containing fresh mouse serum. The inhibitory activity of normal mouse serum in cultures of P. chabaudi can be reduced by heating the serum to 56°C for 30 min. It is possible, therefore, that specific antiparasitic activity of antibody may be detected in short-term cultures of P. chabaudi, providing the antibody activity is independent of these components of complement which are destroyed by heating the serum for 30 min at 56°C.

A role for both T- and B-cells has been confirmed in the adoptive transfer experiments. Of the subpopulations of T-cells, there is evidence that T-helper cells play a role.

Work under the present grant has shown that protection can by adoptively transferred not only with spleen cells but also with peripheral blood mononuclear cells, peritoneal cells and also leukocytes dissociated by shaking from fragments of liver of immune mice. Of particular interest are the observations on the liver leukocytes because in one experiment these cells could be shown to be exerting a protective effect in irradiated recipient mice at a time when there was little detectable antibody present in their circulation.

It would seem, therefore, to be worth closely examining the role of the liver in the immune response to malaria parasites, particularly with regard to cell-mediated immune responses. The recent report of Playfair and colleagues (1979) highlight the role of the liver in mice immunised against P. yoelii. Short-term in vitro cultures of P. chabaudi may allow the identification of cell-mediated antiparasitic responses.

The protection given to mice against Babesia microti and to a lesser extent against P. chabaudi by a concurrent infection of murine leprosy caused by Mycobacterium lepraemurium (MLM) is broadly similar to the protective effect described by Clark and colleagues (1977) following the injection of mice with BCG, and Corynebacterium parvum. Clark and colleagues attribute the protection to a non-antibody soluble factor which causes intracellular death of the parasite. It is not known if a similar substance is produced in the MLM infected mice. The interaction of human leprosy, M. leprae, and malaria infections in the field could be worth investigation.

It is clear that the application of monoclonal antibody technology is going to have a major role in malaria research; particularly with regard to the identification of the antigenic constituents of malaria parasites which are important in the generation of protective immunity, and in the demonstration of antigenic differences between different isolates of the same species of malaria parasites and of antigenic variation within strains of parasites. Identification of the protective immunogens will speed the development of a safe vaccine.

There is an urgent need for an in vitro test which would give an accurate indication of the level of resistance to reinfection/infection particularly to P. falciparum in individuals living in malarious areas, where, for example, control programmes have been initiated, and, in the future, in vaccinated individuals. Resistance to reinfection will involve one or both of (1) the level of antiparasitic activity present at the time of reinfection and (2) the rate at which the immunological memory responds to the parasite and restimulates protective immunity. The growth inhibitory activity in vitro of serum from individuals may provide a measure of (1), although this is open to doubt. Recent work in our laboratory has shown that secondary antibody responses to P. chabaudi by spleen and peripheral blood mononuclear cells can be induced in vitro in Marbrook chambers and that the level of the in vitro antibody response reflects the ability of the donor mice to control a challenge infection. A measure, therefore, of the secondary antibody response to P. falciparum in vitro of peripheral blood leukocytes from patients could provide an indicator of the level of immunological memory.

Malaria research is, at the present time, still making progress. Perhaps the initial optimism, that a vaccine was round the corner which followed the promise of the experimental vaccination of monkeys with merozoites and the successful continuous culture of P. falciparum, has not been fulfilled. Nonetheless, the continued importance of malaria as a killer of babies and infants and of mortality in all age groups in many developing countries is sufficient stimulus to continue work towards methods of ameliorating the disease and its consequences. I and my colleagues are grateful to the U.S. Army for allowing us to be part of that effort.

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