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Reactivity and crossreactivity of mouse helper T cells to malaria parasites

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Summary. The mouse helper T-cell response to four plasmodial and babesial parasites was measured by using them as carriers for a standard hapten (TNP). Helper T cells appeared to recognize all the parasites, but not to be able to distinguish between them.

Helper T-cell responses could be augmented by vaccination with formalin-fixed parasites. However vaccination did not always confer protection against infection. Conversely, mice resistant to infection because of prior recovery from a homologous or heterologous infection had normal or reduced helper T-cell responses.

It is concluded that resistance to infection with these parasites, though dependent on T cells, may not only involve the helper T-cell subpopulation.

INTRODUCTION

Unlike the disease in larger animals, primary malarial and babesial infections in mice either cause rapid death (e.g.: *Plasmodium vinckei, Plasmodium berghei*) or are self-cured, at least as judged by the disappearance of parasites from the blood (e.g.: *Plasmodium yoelii, Babesia microti*). In the latter case, self-cure is dependent on the presence of T cells (Clark & Allison, 1974), and is associated with a massive proliferation of T cells in the spleen, not seen in lethal infections (Jayawardena, Targett, Leuchars, Carter, Doenhoff & Davies, 1975). However, T cells have numerous functions, including B-cell help and suppression, cytotoxicity, and lymphokine production, and it is not known which function is involved in controlling the primary malaria infection.

Following spontaneous or drug-induced cure, mice are usually resistant to re-challenge with the same parasite, and also to challenge with some others; however there is no obvious correlation between the degree of this cross-protection and serological cross-reaction (Cox, 1970). Spleen T cells from immune mice show increased transformation *in vitro* to parasitized red cells or soluble plasmodial antigens (Weinbaum, Evans & Tigelaar, 1976). Cell transfer experiments in rats suggest that T cells play a part in resistance to reinfection (Brown, Jarra & Hills, 1976). The progressive resistance to successive variants of *P. knowlesi* in monkeys has been conjecturally attributed to helper T cells recognizing some invariant antigen (Brown, 1971).

In the present paper we describe studies of the helper T-cell response of mice to *P. vinckei*, *P. yoelii*, *P. berghei* and *B. microti*, using the approach of coupling a standard hapten to the parasite and measuring the anti-hapten antibody response, along lines previously worked out for the helminth parasite *Schistosoma mansoni* (Ramalho-Pinto, De Souza & Playfair, 1976). Our results show extensive cross-reaction between the four parasites as carriers, apparently unrelated to the known serological cross-reactions, but are against the idea that it is exclusively the helper T cells that are responsible for resistance.

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MATERIALS AND METHODS

Mice

(C57BL × BALB/c)F_1 mice were bred in our department from parental strains supplied by the MRC Laboratory Animals Centre, Carshalton. Male mice were used for passaging the parasites and female mice for experiments. Non-inbred 'nude' (thymusless) mice were obtained from the same source.

Parasites

P. yoelii (17 X strain) and *P. berghei* (Anka strain), referred to here as PY and PB respectively, were obtained from the London School of Hygiene and Tropical Medicine and maintained by the transfer of 10^5 parasitized red blood cells (RBC) at 10-day intervals. *P. vinckei* (Katanga strain) and *B. microti* (Kings 56 strain), referred to as PV and BM respectively, were obtained from Professor F. E. Cox of King's College, London, and maintained by the transfer of 10^5 parasitized RBC at 7-day intervals.

Parasites were counted in thin blood smears stained with Giemsa, under oil, with a 100 X objective. Parasitaemias in infected mice followed the course described by Jayawardena *et al.* (1975) and by Cox (1970). Mice recovered from PY and BM infections, but died of PB and PV infections with a mean survival time of 22 and 10 days respectively.

PV and BM infect only adult RBC, while PY and PB infect only reticulocytes. During the course of the study, a variant of PB developed which infected adult RBC as well as reticulocytes, and this line of PB was therefore abandoned. All the experiments quoted were performed before this occurrence.

Production of high parasitaemias

Blood with a high percentage of parasites was obtained as follows. PV: mice were bled 7–8 days after infection, when the parasitaemia was about 70–80 per cent. PY and PB: mice were made anaemic with three injections of phenylhydrazine and then infected. Because of the high resulting reticulocytosis, parasitaemias of about 50 per cent were obtained 5 days later. BM: mice were splenectomized and then infected. Seven days later their parasitaemias averaged 60 per cent.

Formalin-fixed parasites

Parasites were freed from red cells by the saponin method of Spira & Zuckerman (1962). Briefly, blood with a parasitaemia of 50 per cent or more was washed three times in PBS and incubated in 40 vol. of 0·01 per cent saponin for 30 min at 37°, after which the freed parasites were washed once at 15,000 *g*, resuspended in PBS and spun at 150 *g*, when the upper layer (brown in the case of PV, PY and PB; white in the case of BM) could be removed virtually free of unlysed RBC. The parasites were then resuspended in 0·6 per cent formalin for 5 min at room temperature, washed twice in PBS, and resuspended so that the required number of free parasites (easily counted by phase-contrast microscopy) was contained in 0·2 ml, which was injected into the tail vein or the hind foot pad. This procedure will be referred to hereinafter as 'vaccination'.

TNP coating of parasitized blood

Blood with at least 20 per cent parasitaemia was washed twice in PBS and haptenated by the method of Rittenberg & Pratt (1969). Briefly, 2,4,6 benzenesulphonic acid (TNBS) at a concentration of 5 mg/ml in 0·28 M cacodylate buffer (pH 6·9) was added to packed cells and the mixture stirred for 30 min at room temperature, after which the reaction was stopped by the addition of cold barbital buffer and the TNP-coupled cells washed with MEM until no free (yellow) TNP was visible in the supernatant. Very little haemolysis occurred during this procedure. The TNP-coupled parasitized cells, or TNP-coupled normal mouse RBC, were injected i.v. immediately after preparation.

Parasites freed from the erythrocytes by the saponin method described above were TNP-coated by the same process.

Assay for plaque-forming cells

Four days after injection of the TNP-coupled cells, the mice were killed and the direct anti-TNP plaque-forming cells (PFC) in their spleens measured by the method of Cunningham & Szenberg (1968), using sheep RBC coupled with TNP as described above. Each experimental group usually consisted of three to five mice, and most experiments were repeated two to three times to bring the groups up to six to ten. In the figures and tables, PFC are recorded as the geometric mean and the standard error of the log-transformed data.

RESULTS

Parasites as carriers in normal hosts

Both TNP-coated parasitized blood and TNP-coated

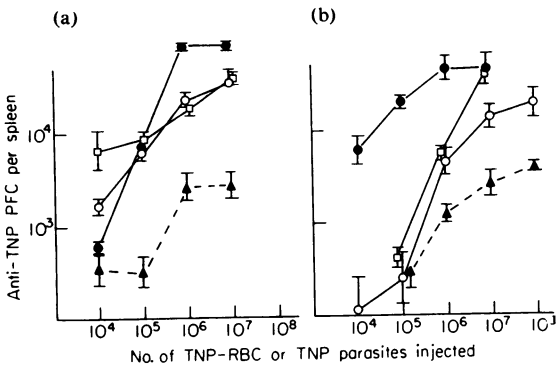

Figure 1. The helper effect of malaria parasites. (a) TNP was coupled to *P. yoelii*-infected (○), *P. berghei*-infected (□), or normal (▲) reticulocytes, or to free *P. yoelii* parasites (●), which were then injected into mice and their direct anti-TNP plaque-forming cells counted 4 days later. (b) TNP was coupled to *P. vinckei*-infected (○), *B. microti*-infected (□), or normal (▲) red cells, or to free *P. vinckei* parasites (●); otherwise as (a). The PFC values shown are the geometric mean ± 1 s.e. of the log-transformed data.

parasites induced a substantial anti-TNP PFC response in normal mice. In Fig. 1 the responses are plotted separately for PY and PB (Fig. 1a) and for PV and BM (Fig. 1b) because of the different stage of the red cell they inhabit. As controls we used TNP-coated normal mouse blood (Fig. 1b) or TNP-coated blood with a phenylhydrazine-induced reticulocytosis of about 50 per cent to correspond with the number of reticulocytes present in the PY and PB infected blood ('TNP-retic', Fig. 1a).

It can be seen that normal reticulocytes are comparable as carriers to normal adult red cells, and that parasitized cells are stronger carriers than unparasitized, this difference being considerably greater with the reticulocyte forms (Fig. 1a) than with the adult red cell forms (Fig. 1b). For example, 10^5 TNP-PY induced 9852 PFC while 10^5 TNP-PV induced only 267.

However when the parasites were first freed from the red cell with saponin and then TNP coated, PV was at least as strong as PY (Fig. 1). The very low responses in nude mice suggest that the helper effect is predominantly T cell-mediated (Table 1). Parasitized blood treated with the cacodylate buffer without TNBS did not induce any significant response (Table 1).

We conclude that all the parasites are strongly immunogenic, but the immunogenicity of PV and BM is expressed less strongly on the (adult) red cell

Table 1. T-cell helper effect of TNP-coated parasitized RBC

(10^7) RBC injected	4-day spleen anti-TNP PFC	
	Normal mice	Nude mice
TNP-PY	33915 (25219–45610)	1088 (978–1210)
PY*	3912 (3400–4500)	
TNP-PB	37274 (32382–42906)	3404 (2695–3907)
PB*	200 (134–298)	
TNP-PV	13203 (10159–17159)	684 (550–850)
PV*	658 (508–852)	
TNP-BM	37916 (31631–45449)	
TNP-MRBC	2641 (2112–3303)	

*Coating procedure carried out without TNBS. The PFC values shown are the geometric mean ± 1 s.e. of the log-transformed data.

than that of PY and PB is on the the reticulocyte. Thus it appears that the younger reticulocyte may provide a generally more immunogenic presentation of antigens derived from intracellular parasites.

Secondary responses in vaccinated mice

When mice were injected with released formalin-fixed PV (FFPV) and challenged with TNP-PV three weeks later, a greatly increased TNP response was obtained. As little as 10^5 FFPV intravenously or 10^6 s.c. gave maximal PFC values, while larger doses tended to cause a lower response. Two injections 3 weeks apart gave slightly higher responses (Fig. 2). This 'carrier priming' showed little or no specificity for the species of parasite used, since vaccinated mice gave approximately equal responses to TNP on any one of the three parasites used for challenge (PV, PY and PB) (Table 2). However some vaccines, such as PY, seemed to be generally more effective in inducing help, while BM was somewhat weaker. Helper effects were not seen in vaccinated mice challenged with TNP-MRBC (Table 2). Thus there is evidence for specificity towards intraerythrocytic parasites, but extensive cross-reaction between the different species, at the level of recognition by the helper T cell.

We cannot rule out the possibility that some of this

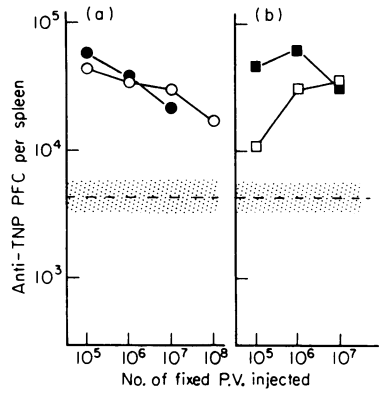

Figure 2. The effect of vaccination with formalin-fixed *P. vinckei* parasites on the anti-TNP PFC response to 10^6 TNP-coated *P. vinckei*-infected red cells 3 weeks later. The shaded area represents the response in non-vaccinated mice (geometric mean ± 1 s.e.). (a) i.v.; (b) s.c. Open symbols $\times 1$; closed symbols $\times 2$.

'cross-priming' is due to non-parasite antigens, such as red cell fragments, present in the saponin-freed parasite preparations and on infected, but not normal, red cells.

In a preliminary experiment, mice vaccinated with formalin-fixed *P. knowlesi* (a parasite of the monkey, kindly provided by Professor S. Cohen), showed considerable priming against a subsequent challenge with TNP-PV and TNP-PY, which suggests that the sharing of immunogenic specificity may not be restricted to parasites of the mouse.

Responses in recovered mice

Mice that had recovered from a PY infection 3 months earlier showed no sign of helper-cell priming; indeed, with the higher challenge doses of TNP-PY their responses were reduced (Table 3). This was equally true when the mice had been TNP (i.e. B cell) primed before infection. Similarly, mice that had recovered from a BM infection had slightly reduced responses to TNP-PV. PY recovered mice showed no significant change in their response to TNP-PV (Table 2), despite the fact that PY and PV cross-react at the helper cell level (Table 1).

In a limited series of experiments *in vitro*, normal and PY-recovered spleen cells gave the same low response to PY-TNP, which agrees with the lack of T-helper cell priming demonstrated to PY in the recovered mice *in vivo*.

Helper-cell priming and resistance to reinfection

PY-recovered mice were resistant to reinfection with PY, eliminating even 10^8 injected parasitized RBC within 24 hr ('early' resistance). They also survived infection with PV, but only after a parasitaemia resembling the normal up to the 7th day, which then rapidly resolved ('late' resistance). BM-recovered mice, on the other hand, showed 'early' resistance to PV infection (Fig. 3). In this, our results agree closely with those of Cox (1970). Comparison with the T-priming results in the section above shows that both early and late resistance are associated, if anything, with reduced rather than increased helper T-cell function.

Mice vaccinated with FFPV, either once or twice, and with evidence of good helper T-cell priming, were not protected against a PV infection (Fig. 3).

Table 2. Specificity of helper effect following vaccination with formalin-fixed parasites

Parasites used for vaccination	Anti-TNP PFC per spleen 4 days after challenge with:				
	10^5 TNP-PY	10^5 TNP-PB	10^6 TNP-PV	10^6 TNP-BM	10^6 TNP-MRBC
—	6077 (5086–7262)	8155 (6667–9976)	4319 (3268–5708)	5644 (5300–6010)	1188 (986–1435)
10^7 FFPY	57525 (49058–67453)	65811 (60154–71999)	43267 (39274–47667)	—	1308 (900–1900)
10^7 FFPB	51417 (40483–65305)	43296 (33158–56531)	21910 (16953–28319)	—	—
10^7 FFPV	44175 (35489–54988)		33277 (29949–36974)	—	607 (491–751)
10^7 FFBM	20908 (16735–26121)	20313 (15799–26118)	13688 (11839–15827)	12726 (8290–19536)	—

Values expressed as in Fig. 1.

Table 3. Helper cell function in normal and recovered mice

Challenge injection	Mice	4-day spleen anti-TNP PFC after injection of: 10^4	10^5	10^6	10^7	10^8
TNP-PY	Normal	1602 (1333–1927)	6077 (5086–7262)	22678 (19317–26624)	33915 (25219–45610)	—
	Normal	490	962	9565	15108	—
	TNP-primed	(400–600)	(900–1029)	(7126–13840)	(13145–17364)	
	PY-recovered	3673 (2932–4604)	6778 (5272–8714)	18470 (15709–21715)	9834 (4743–20390)	—
	PY-recovered	548	783	888	3857	—
	TNP-primed	(500–600)	(675–908)	(516–1527)	(2559–5814)	
TNP-PB	Normal	6981 (4447–10958)	8155 (6667–9976)	18669 (15358–22694)	37274 (32382–42906)	—
	PY-recovered	—	4587 (3765–5589)	—	—	—
TNP-PV	Normal	108 (50–250)	267 (151–472)	4319 (3268–5708)	13203 (10159–17159)	19853 (13800–28560)
	PY-recovered	—	—	5364 (4534–6345)	—	—
	BM-recovered	—	--	1142 (1050–1242)	7933 (6097–10322)	21374 (16200–28200)

Injected with 100 μg TNP-KLH 2 weeks before infection. Values expressed as in Fig. 1.

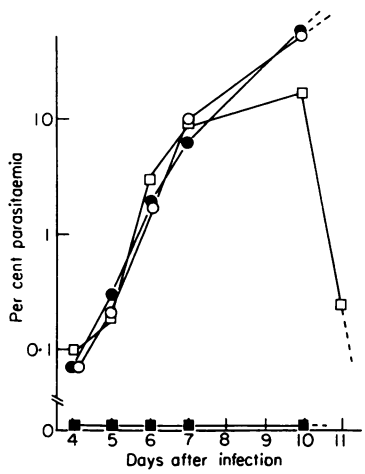

Figure 3. The course of *P. vinckei* infection in normal mice (●), mice vaccinated with 10^7 formalin-fixed *P. vinckei* parasites 3 weeks earlier (○), and mice recovered from infection with *P. yoelii* (□) or *B. microti* (■) 2 months earlier.

However vaccination with FFPY did protect against PY, in the sense of accelerating the disappearance of parasitaemia. But this effect was seen only with the highest doses of vaccine (10^8), whereas the helper effect was greatest with lower doses (10^5–10^6). And the strongest carrier of all in normal mice, PB (Fig. 1) causes a fatal infection. These three observations confirm that the ability to mount a rapid helper response to parasite antigens is probably not directly correlated with the ability to resist the living infection.

DISCUSSION

T-cell specificity

The principal finding reported here is that mouse helper T cells recognize plasmodial and babesial antigens—both on the free parasite and on the infected red cell—but distinguish little or not at all between the different species. This is in contrast to antibody responses (presumably involving both B- and T-cell recognition) in which there is a fair degree of specificity. For example Cox & Turner (1970) found that mice recovered from BM infections had titres of 1/1280 against BM, but only 1/20 against PV, while PY-recovered mice had titres of 1/2560 against PY and 1/40 against PV. Yet our mice vaccinated with FFPY had almost as strong a helper effect against PV as against PY itself (Table 2).

It is always difficult to be sure whether apparent differences between T-cell and B-cell specificity for

antigen are due to real differences in the determinants recognized, and therefore presumably of the receptor involved, or to 'amplification' effects of the T–B co-operation mechanism, the receptors themselves being identical. We have argued for the former idea in the case of the T-cell crossreactivity between heterologous erythrocytes (Playfair & Marshall-Clarke, 1973), but the latter view has also been upheld, for instance on the basis of idiotypic similarity between T and B cells (Binz, Kimura & Wigzell, 1975). The existence of passively acquired Ig on some T cells is a complication (Playfair, 1974), but there is growing evidence for a non-Ig T-cell receptor for antigen that plays a part in T–B co-operation (Taussig & Munro, 1974).

Whatever the mechanism, our results suggest that essentially the same mouse helper T cells can be stimulated by any of the four parasites tested, and we are now investigating whether this is also true for other T-cell functions, such as delayed hypersensitivity.

Helper T cells in recovered mice

During a PY infection there is extensive proliferation of T cells (Jayawardena *et al.*, 1975), of which some are presumably helper cells, since appreciable amounts of specific IgG are made. Therefore it is surprising that the recovered mouse seems to be left with no detectable T helper-cell memory (Table 3). Of course the animal is exposed, during infection, to enormous numbers of parasites, and large doses of antigen are usually less effective at priming for T-cell help. One possible mechanism might be that anti-parasite antibody suppresses the carrier effect of the parasite, as has been shown in many other systems (Playfair, 1974). But one would not expect BM-recovered mice, which have very little anti-PV antibody, to show such a strong reduction of T-cell help towards PV (Table 3). Also spleen cells from PY-recovered mice might have been expected to respond better *in vitro* if the inhibition was due to serum antibody. Serum transfer of the suppression should settle the matter.

A second possibility is cell-mediated suppression, for example by macrophages or other T cells. T-cell suppression of helper T cells has been reported (Tada, Taniguchi & Takemori, 1975; Rubin, 1976). If this is the case, cells ought to adoptively transfer the reduced response.

A third alternative is that no helper T-memory cells are in fact generated. It has been shown that sheep RBC given during a PY infection fail to prime for a secondary response (Greenwood, Playfair & Torrigiani, 1971), and the same might be true for the PY antigens themselves. The low responses in PY recovered mice TNP-primed before infection (Table 3) may even represent an inhibition of pre-existing B memory cells. One could postulate some kind of 'terminal exhaustion' of clones stimulated during infection, conceivably by a parasite-derived factor with polyclonal activating properties. An advantage to the parasite of the prevention of T- and/or B-memory development, affinity maturation etc, would be that only primary type responses were mounted no matter how long the infection lasted. Evidence has been found for a mitogen in human *P. falciparum* (Greenwood & Vick, 1975).

Specificity of protective immunity

The fact remains that recovered mice are resistant to reinfection, and the conclusion seems inescapable that helper T cells are not necessary for this resistance. In any case, the 'early' resistance always seen with the homologous parasite and sometimes with heterologous combinations (i.e. to PV in BM-recovered mice, Fig. 3) is rather rapid for a secondary response involving an initial T-helper cell stage, and seems more likely to be due to either residual antibody or to non-specific elements (see below). On the other hand the 'late' resistance seen to PV in PY-recovered mice (Fig. 3), and to PY in mice vaccinated with large doses of FFPY, has the time course expected of an adaptive immune response (cf. a second-set skin graft rejection) and could well be mediated by T cells, perhaps of the delayed hypersensitivity, cytotoxic, or even suppressor as well as the helper variety. If so, this is encouraging because it suggests that some form of resistance ought to be inducible by vaccination methods particularly aimed at the appropriate type of T cell.

The finding that bursectomized agammaglobulinaemic chickens are immune to reinfection with *P. gallinaceum* following drug cure (Rank & Weidanz, 1976) offers strong supporting evidence that some T-cell function independent of antibody can mediate resistance to malaria. Immunization of mice with the P 815 Y mastocytoma can stimulate preferentially either helper or cytotoxic T cells, depending whether the tumour cells are, respectively, fixed or living (Dennert, 1976); fixed sheep RBC have also been shown to induce good T-cell help but poor delayed hypersensitivity (Ohmici, Nomoto, Yamada &

Takeya, 1976). If there is any analogy with protozoal infections, successful immunisation of the non-helper T-cell population may require a living preparation of the parasite. In keeping with this prediction, protection of mice against *P. berghei* has been estimated to be fifty times more efficient with live than with killed antigen (Jerusalem & Eling, 1969).

This is not to belittle the remarkable degree of resistance produced by non-specific immunostimulation, notably by Clark, Allison & Cox (1976), who have shown that infection of mice with large doses of BCG i.v. confers long-lasting resistance of the 'early' type to BM and PY. The authors concluded that a soluble factor was killing the parasites in the red cells, but admit that the source of this factor is not yet clear. BCG has effects on almost all components of the immune system, and our current attempts to distinguish between T cells, B cells and macrophages as the key cell in this mode of protection will be reported separately.

ACKNOWLEDGMENT

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