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Lymphocyte traffic and lymphocyte destruction in murine malaria

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Summary. Normal lymphocytes labelled with ^{51}Cr were injected into mice at various stages of lethal and non-lethal malaria infections. Marked alterations were seen in the uptake into spleen and liver, which correlated with the outcome of the infection. Non-lethal infections and lethal infections in mice protected by vaccination caused increased uptakes, especially in the liver. In lethal infections, particularly *Plasmodium berghei*, uptakes were below normal values at certain times: this was apparently due to destruction of lymphocytes, probably caused by autoantibody.

INTRODUCTION

We have previously shown that the malaria parasites *Plasmodium berghei* and a variant of *Plasmodium yoelii*, though both lethal in mice, differ greatly in the ease with which vaccination can protect against the blood-stage infection (Playfair, De Souza & Cottrell, 1977). This did not appear to be entirely attributable to differences in antibody response (Playfair & De Souza, 1979). T-cell-mediated responses, however, differed substantially in the two types of infection, whether measured by a classical ear thickness test for delayed hypersensitivity in vaccinated mice (Cottrell, Playfair & De Souza, 1978) or by the tendency for increased accumulation of lymphoid and myeloid cells in the spleen and liver during infection (Playfair, De Souza, Dockrell, Agomo & Taverne, 1979); we have proposed that an impairment of cell traffic may be one factor in the relative resistance of *P. berghei* to host immunity (Playfair *et al.*, 1979).

In the present paper we confirm and extend the correlation between accumulation of lymphocytes in the liver and eventual recovery from infection, and we show that the apparent reduction of cell traffic in *P. berghei* may be due to destruction of lymphocytes in the liver.

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MATERIALS AND METHODS

Mice

(C57Bl × BALB/c)F_1 mice were used in most experiments. These were bred in our laboratory from parental strains originally supplied by the M.R.C. Laboratory Animals Centre, Carshalton. CBA mice were supplied by the National Institute for Medical Research, London.

Parasites

The origin of our *P. yoelii* (referred to hereinafter as PY) and *P. berghei* (PB) has been described previously (Playfair *et al.*, 1977). The lethal variant of PY arose during blood passage of a non-lethal PY, and has remained consistently lethal for over 3 years, killing all mice within 20 days. Our PB kills mice within 28 days. The non-lethal PY gives an infection lasting 20–24

days, followed by permanent disappearance of parasitaemia. *Plasmodium chabaudi* was a gift from Dr G. A. Targett; it gives an infection lasting 15–20 days, followed by a variable period of intermittent low parasitaemia. All parasites were maintained throughout the experiments reported here by blood passage at weekly intervals. All infections were established by an intravenous (i.v.) injection of 10^4 parasitized red cells.

Vaccination

Mice were vaccinated with a crude blood-stage vaccine as described previously (Playfair *et al.*, 1977). Briefly, blood with a parasitaemia of at least 50% is lysed with 0·01% saponin and fixed with 0·06% formalin. This preparation contains parasites of all stages and some collapsed red cell membranes. In the present experiments 10^8 fixed parasites, combined with 10^8 *Bordetella pertussis* organisms (Pertussis vaccine, Lister Institute) were injected intravenously 3 weeks before challenge. Such a vaccine made from lethal PY protects mice of all strains against the homologous infection, recovery occurring regularly on the seventh or eighth day after challenge. However a similar vaccine made against PB only protects about 60% of (C57Bl × BALB/c)F_1 mice, though with CBA the protection is somewhat better while C57Bl mice are very poorly protected (Playfair & De Souza, 1979); recovery seldom occurs before day 14 in any strain.

Cell trapping experiments

The principle was to label normal lymphocytes with ^{51}Cr, inject them at various stages of infection, and measure their organ distribution at intervals thereafter. Lymph node cells were prepared by teasing mesenteric and axillary lymph nodes from normal syngeneic mice in RPMI medium containing 5% foetal calf serum. After two washes through a layer of 100% foetal calf serum, they were made up to 10^8/ml and incubated with 100 μCI of ^{51}Cr/ml for 30 min at 37° (sodium chromate, Radiochemical Centre, Amersham). After labelling the cells were again spun twice through foetal calf serum and their viability checked with ethidium bromide/acridine orange; viability was always between 75% and 90%. Five × 10^6 cells were injected intravenously per mouse. At 4 or 24 hr, the spleen and liver, and sometimes other organs (see text) were removed and counted in a Wallac 1280 Ultrogamma counter (LKB). In one experiment, the whole mice were counted in a modified whirl counter.

Results were expressed as the percentage of the counts given by an aliquot of the same cell suspension as was used for injection. In the case of whole-body counts, the count immediately after injection was taken as 100% for the individual mouse and subsequent counts, corrected for ^{51}Cr decay, expressed as a percentage of this.

Usually there were 3–5 mice per group, and all experiments were repeated at least once. Significance values are based on Student's *t* test.

RESULTS

Lymphocyte homing and the effect of vaccination

Figures 1 and 2 show the changes in 24-hr lymphocyte uptake into the spleen and liver during lethal PY and PB infection in (C57Bl × BALB/c)F_1 mice. The responses were roughly biphasic: during the first week uptake rose in the spleen and fell in the liver, while

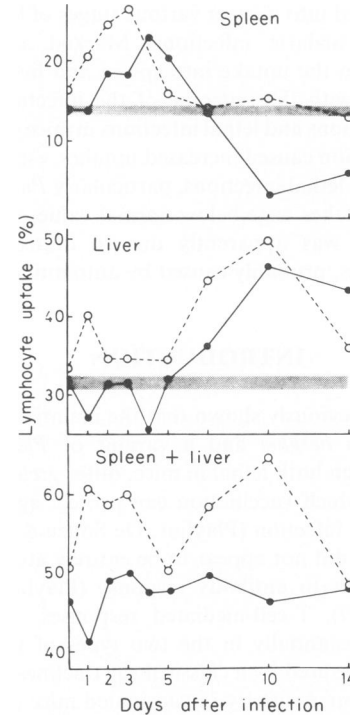

Figure 1. Changes in lymphocyte homing during lethal *P. yoelii* infection. The values shown are the mean 24-hr uptake in groups of 10–20 (C57Bl × BALB/c)F_1 mice. (○) Mice vaccinated 3 weeks before infection; (●) normal unvaccinated mice. The shaded bar represents the mean ± 1 SE of forty control uninfected mice. Standard errors in the infected groups never exceeded 5% and were usually below 1%.

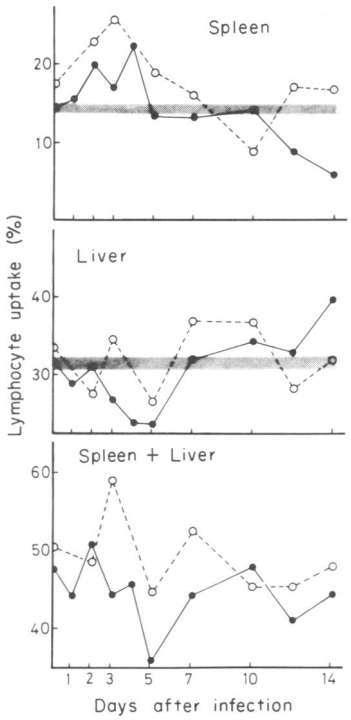

Figure 2. Changes in lymphocyte homing during *P. berghei* infection. Details as in Fig. 1.

during the second week the reverse occurred. Confirming our preliminary report (Playfair *et al.*, 1979) the principal difference between the two parasites was the much reduced uptake in the liver with PB. When the experiments were repeated in vaccinated mice, the difference between PY and PB became even more pronounced (compare Figs 1 and 2). With PY the increase in spleen uptake was earlier and higher as a result of vaccination, the switch to the liver occurred sooner, and the liver uptake was also higher. With PB the effect of vaccination was only prominent during the first week in the spleen.

When the spleen and liver uptakes were pooled, it was seen that the total remained fairly constant during PY infection (Fig. 1). However during PB infection the combined homing to the two organs fell below the control (day 0) values for uninfected mice, this drop being most marked at day 5, when about 5% fewer lymphocytes were taken up, or more than 10% of the normal amount (Fig. 2). In PY-vaccinated mice, the total of spleen and liver homing rose well above control, except for a drop to control values at day 5. Vaccination against PB, however, did not produce this rise except transiently (Fig. 2).

As Fig. 3 shows, these changes of lymphocyte influx occurred quite independently of the size of the spleens and livers. For example the spleen uptake in *P. yoelii* fell below normal after day 7 despite a continuing increase in spleen size, and likewise with the liver at day 5 in *P. berghei*. However it was noteworthy that the greater effect of vaccination in *P. yoelii* than *P. berghei* was reflected in organ size as well as lymphocyte uptake; indeed cell input probably contributed to the increase in size. During the first week of infection there were no significant differences in parasitaemia between the two parasites (data not shown).

Mixed infection

To determine whether the differences noted above were due to an active suppression by PB, the experiment was repeated in mice given PY and PB together (10^4 of each). As Fig. 4 shows, these doubly-infected mice gave the PB pattern of lymphocyte uptake up to day 7, after which they came to resemble PY-infected mice. The responses on day 5 were especially striking: the addition of PB to PY reduced both spleen and liver uptakes and the combined uptake was 10% below the value for PY alone. Mice previously vaccinated against PY and then doubly-infected showed an even more marked reduction of lymphocyte uptake on day 5 (Table 1). We concluded that PB infection exerts an actively suppressive effect on lymphocyte uptake in the spleen and liver which reaches a maximum 5 days after infection.

In a series of more limited experiments with other strains of mice, essentially similar suppression was found in CBA and C57Bl mice, but in the BALB/c strain the responses to PY and PB were consistently the same. This apparent strain difference is under investigation.

Cell homing in non-lethal infections

Because of the apparent correlation in vaccinated mice between increased lymphocyte uptake and protection (Figs 1 and 2), the homing of lymph node cells was also studied in two self-curing malarial infections: the original non-lethal *P. yoelii* (from which the lethal variant arose) and *P. chabaudi*. The results (Fig. 5) show essentially the same early spleen and later liver increase, with a second wave of spleen activity during the third week. The main difference from the lethal

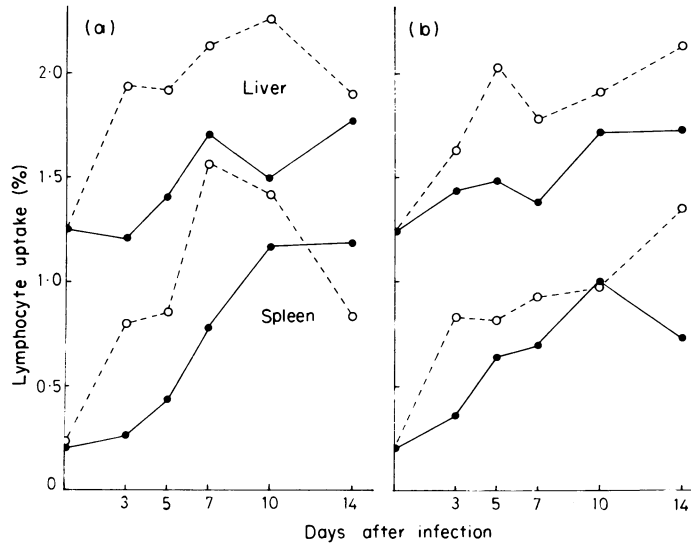

Figure 3. Changes in spleen and liver wet weight during infection with lethal *P. yoelii* (a) and *P. berghei* (b) and the effect of vaccination. Symbols as in Fig. 1. Each point is the mean of four–ten mice.

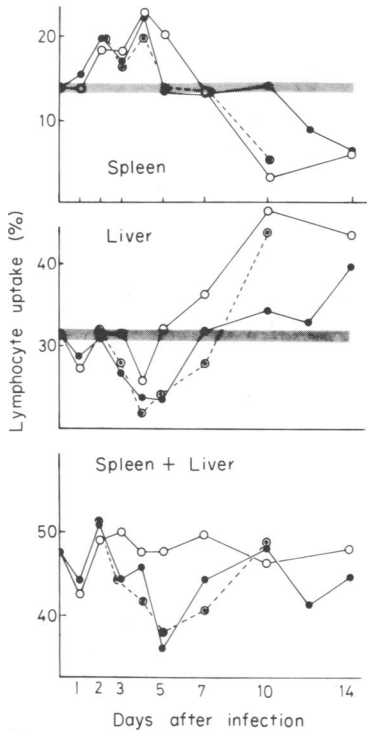

Figure 4. Changes in lymphocyte homing during single and mixed infections. (○) *P. yoelii*; (●) *P. berghei*; (⊙) *P. yoelii* and *P. berghei* together. Other details as in Fig. 1.

infections was that reductions below normal were never seen in the liver and only transiently in the spleen. It was noteworthy that mice began to recover from the non-lethal PY at a time (about day 20) when their aggregate spleen plus liver uptake had reached the same high level as in the lethal PY-vaccinated mice when they recovered (days 7–10). Mice recovered from *P. chabaudi* at a slow rate starting about day 14, and again this seemed to correlate with an increase in lymphocyte uptake.

We concluded that increased lymphocyte uptake in spleen and, particularly, liver correlated with non-lethality, whereas a reduction below normal uptake was associated with a fatal outcome.

Time course of lymphocyte homing

The suppressed 24-hr uptake of injected lymphocytes in the liver on the 5th day of PB infection could have been due either to failure of cells to arrive in the organ or to subsequent loss from it. To distinguish between these possibilities we compared uptakes 1, 2 and 4 hr after injecting the labelled cells. The highest uptakes were at 4 hr, and in PB and both PY infections the 4-hr liver values were normal or above normal (Fig. 6). Thus the low 24-hr values were evidently not caused by non-entry but by later loss or emigration.

Table 1. Reduction of lymphocyte uptake in spleen and liver by P. berghei

Vaccination (−3 weeks)	Infection (−5 days)	Percentage of injected label at 24 hr		
		Spleen	Liver	Spleen plus liver
—	—	14·16±0·23	31·43±0·6	45·59±0·66
—	PB	14·88±0·8	25·64±0·8	40·52±1·21 (−5·07%)
—	PY	19·21±0·7	28·91±0·6	48·12±0·7
—	PY+PB	13·81±0·43	24·13±0·45	37·94±0·7 (−10·18%)
PY	PY	18·1±2·9	31·56±2·6	49·66±1·8
PY	PY+PB	10·6±1·35	24·97±2·7	35·57±4·03 (−14·09%)

The values shown are the means ± 1 SE of groups of 5–20 mice injected with 5×10^6 ^{51}Cr-labelled normal lymph node cells 24 hr earlier on the fifth day of infection with *P. berghei* (PB) and/or lethal *P. yoelii* (PY). The values in brackets show the difference due to the addition of PB.

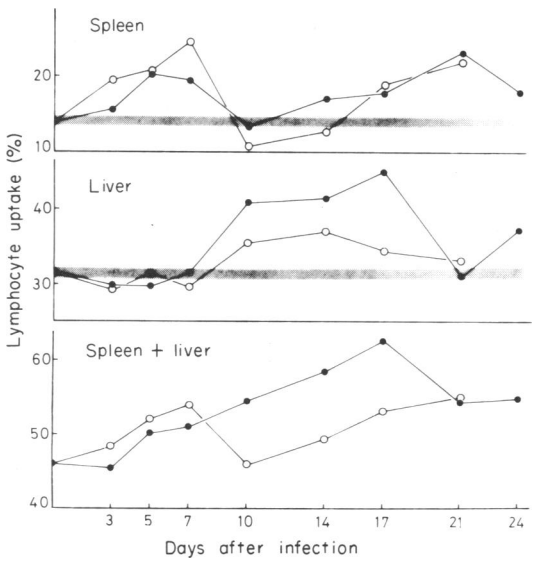

Figure 5. Changes in lymphocyte homing during non-lethal infections. (●) *P. yoelii* (non-lethal variant); (○) *P. chabaudi*. Other details as in Fig. 1.

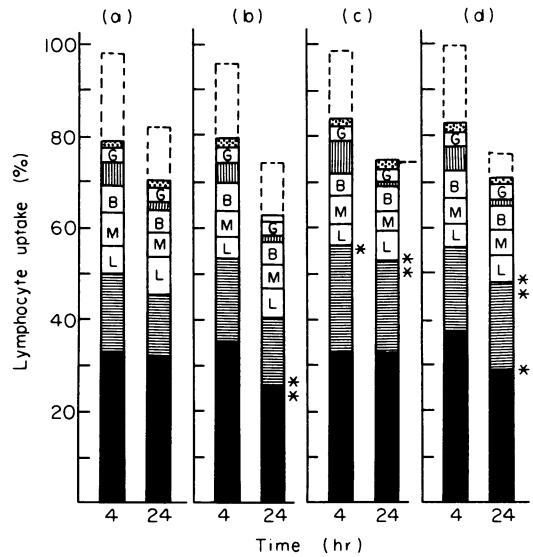

Figure 6. Uptake of lymphocytes in various organs 4 hr and 24 hr after injection, in mice infected 5 days earlier. (a) Uninfected controls; (b) *P. berghei*; (c) non-lethal *P. yoelii*; (d) lethal *P. yoelii* (■) liver; (▤), spleen. L, lymph nodes (a pool of axillary, epitrochlear, cervical, politeal, and mesenteric); M, bone marrow (two femurs × 10); B, blood (0·5 ml × 5). (▦) Two lungs. G, gut (stomach to rectum inclusive). ⊠ two kidneys. The broken lines represent whole-body counts.

* Significantly different from the corresponding control value, $P < 0{\cdot}005$; ** $P < 0{\cdot}0005$.

Table 2. Reduction of uptake of thymocytes and T cells by *P. berghei*

Recipient	Organ	Cell type injected		
		Thymocyte	Lymph node T	Spleen T
Normal	S	14·23±2·3	20·13±1·48	19·11±1·6
	L	27·25±2·8	26·9 ±1·68	22·89±2·8
	S+L	41·48±4·3	47·03±3·1	42·0 ±4·4
P. berghei	S	5·74±4·1	7·0 ±0·54	8·22±1·0
d5	L	28·24±6·2	33·68±2·15	27·88±2·25
	S+L	33·98±3·1 (−7·5)	40·68±2·9 (−6·35)	36·1 ±3·0 (−5·9)
Lethal *P. yoelii*	S	8·34±1·2		
d5	L	33·73±2·6	NT	NT
	S+L	42·07±5·4		
Non-lethal	S	9·9±1·6		
P. yoelii	L	33·02±4·5	NT	NT
d5	S+L	42·92±6·1		

The values shown are the percentage uptake (means ± 1 SE) in groups of 6–10 mice injected with 5×10^6 ^{51}Cr-labelled cells 24 hr earlier.

S, spleen; L, liver.

Figures in brackets show reductions below the values for normal mice.

NT, not tested.

The lymph node and spleen T cells (nylon-wool column passed) contained 2·5% and 3·0% B cells, respectively.

Table 3. Lymphocytotoxic effect of *P. berghei* serum

Serum used for incubation	Percentage of non-viable cells ± 1 S.E.			
	Fresh serum	HI serum	HI serum + GPS	Effect of GPS
Normal mouse	24·8±1·2 (40)	31·4±1·4 (6)	30·7±1 (6)	—
Non-lethal PY day 5	29·3±1·9 (14) NS	32·0±1 (6) NS	35·9±1·3 (6) NS	+3·9
PB day 5	37·5±1·6 (42) $P=0·01$	35·6±2·4 (6) NS	50·7±1·2 (6) $P<0·001$	+15·1

Normal lymphocytes (4×10^5) were incubated at 37° in RPMI with 15% mouse serum and their viability measured after 4 hr by the staining pattern with Ethidium bromide/Acridine orange. The number of samples tested is shown in brackets.

HI, heat-inactivated at 56° for 30 min.

The significance of the difference from normal serum is shown; NS, not significant.

GPS, guinea-pig serum, absorbed with agarose and mouse lymphocytes, used at 1:10.

In a similar study of the homing at day 10 of infection (data not shown) we found that the very low spleen uptake in the lethal PY infection was equally low at 4 and 24 hr. In this case, therefore, a block of lymphocyte entry is a possibility.

Distribution of lymphocytes in other sites in 5-day infected mice

To assess whether lymphocytes might be migrating out of the liver between 4 and 24 hr, we measured the uptake of label in other organs. The results (Fig. 6) showed no detectable compensatory increase in the uptake in any other site in PB-infected mice, there being no significant difference between infected and normal mice in the lymph nodes, bone marrow, blood, gut, lungs, or kidneys. The sum of the counts in all the organs tested was normal at 4 hr but significantly lower in PB-infected mice than controls at 24 hr. With the lethal PY, the slight drop in liver uptake relative to controls at 24 hr was more than compensated by an increase in the spleen, whilst with the non-lethal PY there was an increase in the spleen uptake with no drop in the liver, leading to a higher total-organ value than in controls. Whole-body counting (also shown in Fig. 6) revealed a greater loss of label at 24 hr in all three infections than in control mice, but no difference between the infections. Thus there was a larger amount of label not accounted for in PB (12%) than in lethal PY (5%) or non-lethal PY (none). Insignificant amounts of label were found in brain or thymus; we have not yet been able to satisfactorily estimate the amount in the skin or the urine. We concluded that a feature of lethal infections, and particularly PB, is a loss, and possibly destruction, of lymphocytes occurring mainly or entirely in the liver.

The cell type affected

In an attempt to characterize the type of cell lost, normal or 5-day PB-infected mice were injected with labelled thymocytes or with spleen or lymph node T cells purified by nylon wool filtration (Table 2). In each case the spleen plus liver total uptake at 24 hr was below normal in PB-infected mice, though the reduction was in spleen rather than liver uptake. Thymocytes were also tested in PY infections and no reduction in total uptake was seen (Table 2), though there was a shift from spleen to liver; compared with the PY infections, PB caused a reduction in both organs. However purified T cells did not seem to be enriched for the cell type lost in PB infection (compare Table 1). We concluded that this loss affected both T and B cells. Attempts were not made to use purified B cells, since the manipulations involved would themselves probably have affected their homing pattern.

Effect of malaria serum on normal lymphocytes

One possible explanation for the loss of lymphocytes in PB infection would be a serum factor such as an autoantibody or cytotoxin. To test for this, normal lymph node cells were incubated in normal or 5-day infected serum and their viability measured after 4 hr. The results (Table 3) show that incubation in serum from PB mice significantly reduced the viability of lymphocytes compared with normal serum, whilst serum from non-lethal PY infections did not. When the sera were heat-inactivated and guinea-pig complement was added, the difference between PB and the other sera was increased. However the viability of $(C57Bl \times BALB/c)F_1$ lymphocytes in normal syngeneic serum was invariably rather low (below 80%), and these experiments are being pursued in CBA mice, where lymphocyte survival *in vitro* is greatly superior.

DISCUSSION

Our principal finding is that the uptake of normal lymphocytes into the spleen and liver during blood-stage malaria undergoes periodic changes which correlate with the ability to recover from four different species of the parasite, and with the protective effect of vaccination. Two lethal infections are each characterized by reductions below the normal pattern: in lethal *P. yoelii* there is an apparent blockage of lymphocyte entry into the spleen during the second week of infection, while in *P. berghei* lymphocytes seem to be lost from the liver towards the end of the first week. Neither the increases nor the reductions appear to be related to the size of the spleen or liver which increases progressively during all the infections (Dockrell, De Souza & Playfair, 1980, and see Fig. 3), nor with the levels of parasitaemia, which are roughly comparable in both *P. yoelii* and *P. berghei* during the period under study here.

We have previously suggested that in vaccinated mice, *P. yoelii* parasites may be largely destroyed in the liver (Dockrell *et al.*, 1980), and that the influx of lymphocytes and myeloid cells into this organ may include cells able to kill the parasite, either specifically

or non-specifically (Playfair *et al.*, 1979). The demonstration that *P. berghei* can interfere with this influx and perhaps even cause effector cells to be destroyed, may explain why it is more resistant to vaccination (Playfair *et al.*, 1977). Lymphocyte diversion or destruction might also contribute to the non-specific immunosuppression seen in malaria, which in the case of contact sensitivity is more severe in *P. berghei* than *P. yoelii* infection (Lelchuk & Playfair, 1980). A similar idea has been put forward in relation to murine leprosy (Bullock, 1976).

It should be pointed out that our lymph node preparations contained some 20% of non-viable cells, so that it is theoretically possible that the loss from the liver in *P. berghei* represents exclusively cells which are already dead. However we have shown elsewhere (Dockrell *et al.*, 1980) that fewer viable lymphocytes can be extracted from the liver during *P. berghei* than *P. yoelii* infection, and that the ratio of cells in the liver to cells per millilitre of blood is lower in *P. berghei*, which fits better with the idea that viable lymphocytes do not go to the liver, or do not survive there, as well in *P. berghei* as in *P. yoelii* infection. The 4 hr and 24 hr uptake experiments reported here (Fig. 6) suggest that it is survival rather than arrival in the liver which is abnormal. The experiments with serum *in vitro*, though preliminary, suggest that an autoantibody against lymphocytes may be partly responsible.

One might wonder whether the disappearance from the liver of a relatively small proportion of lymphocytes (about a quarter of those present at 4 hr) would have much effect, especially since it seems to occur only around the fifth day of infection. However it should be considered that the missing cells, which include T cells, may be a critical subpopulation with a particular function, and also that during infection they may include specific cells that have recently been stimulated by parasite antigens; their loss would seriously impair subsequent responsiveness, as has been suggested in the context of tumour immunity (Hutchinson, 1980). This possibility is now being studied, using specifically labelled antigen-stimulated lymphocytes.

Our results agree in principle with those of Brisette, Coleman & Rencricca (1978), who reported a changed pattern of thymocyte homing in *P. berghei* infection, which was later shown to be associated with serum cytotoxic factors, probably including autoantibodies (Chung, Balsam, Gray, Rencricca & Coleman, 1980). However these authors considered the increase in liver homing to be related to cell loss, and consequently to the failure to recover from infection, whereas our study, by the use of several species of parasite and of vaccination, implies on the contrary that increased traffic of cells to the liver is usually associated with effective immunity, and that cell loss is restricted to *P. berghei* infection. There are other unique features of *P. berghei* in mice, such as the generation of non-specific suppressor T cells (Lelchuk & Playfair, 1981), the poor response to vaccination (Playfair *et al.*, 1977), and the resistance to control *in vivo* by non-specific cytotoxic factors (J. Taverne, in preparation). The finding of T-cell deficiency in humans with *Plasmodium falciparum* infection (Wyler 1976; Wells, Pavanand, Zolyomi, Permpanich & Macdermott, 1979) suggests that there may be a link with *P. berghei* in mice, and that studies on cell traffic and on serum (for antilymphocyte factors) will be worthwhile in the human disease.

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