Immunology 1978 **35** 479

Spleen cell changes during fatal and self-limiting malarial infections of mice

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Received 3 *November* 1977; *accepted for publication* 23 *January* 1978

Summary. Changes in the proportions and total numbers of splenic Thy-1·2$^+$ cells, Ig$^+$ cells and normoblasts were analysed during fatal *Plasmodium berghei* and non-fatal *P. yoelii* infections in mice. Thy-1·2$^+$ and Ig$^+$ cells were identified by rosetting techniques, and normoblasts by morphological criteria. The splenomegaly observed during these infections was found to be caused mainly by proliferation of normoblasts. An early increase in the numbers of Thy-1·2$^+$ and Ig$^+$ cells was detected in both infections, but in *P. berghei* infections these responses were subsequently suppressed. In *P. yoelii* infections Thy-1·2$^+$ and Ig$^+$ cell numbers were maintained at four to five-fold above normal levels until the mice had completely recovered. During the acute phase of *P. yoelii* infection it appeared that most splenic T-cells expressed surface immunoglobulin.

INTRODUCTION

The immune mechanism mediating the clearance of primary parasitaemia in malarial infection has not been fully elucidated. In rodents the protective response is T-dependent (Clark & Allison, 1974; Jayawardena, Targett, Carter, Leuchars & Davies, 1977) and probably involves specific antibody (Diggs & Osler, 1969; Weinbaum, Evans & Tigelaar, 1976). However, the role of specific antibody in the protective response is unclear; passive transfer of large amounts of serum from recently recovered mice does not confer protection (Jayawardena, Targett, Davies, Leuchars & Carter, 1975a), and although hyperimmune serum is protective on passive transfer, splenectomy of recipient rats abrogated the protective effect (Brown & Phillips, 1974). These authors suggested that protective immunity to malaria involves the synergistic action of specific antibody and an undefined splenic immune function.

The observations that (1) *Plasmodium yoelii* infections, which are self-limiting in intact mice, are frequently fatal in splenectomized mice (Topley, Bruce-Chwatt & Dorrell, 1970), and (2) splenectomy induces acute recrudescence of *P. vinckei petteri* in recently recovered mice (R. R. Freeman, unpublished) emphasize the central role of the spleen in the development of anti-malarial immunity. This paper describes the changes in Ig$^+$, Thy-1·2$^+$ and erythrocyte precursor cells (normoblasts) in the spleens of mice with fatal or self-limiting malarial blood infections. We found that protective immunity is associated with marked and sustained increases in the numbers of Ig$^+$ and Thy-1·2$^+$ spleen cells, and that, in fatal infections, these proliferative responses are apparently suppressed.

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0019-2805/78/0900-0479 $02.00

MATERIALS AND METHODS

Mice

Female BALB/c mice 6–8 weeks old were used in this study.

Parasites

Blood-passaged *Plasmodium berghei* K173 and stabilated *P. yoelii* 17X were used. The infecting dose was 10^4 parasitized red cells, given intravenously. Parasitaemia was determined by examination of Giemsa-stained smears of tail blood at ×1000 magnification.

Spleen preparations

Spleens were disrupted by forcing them through wire mesh sieves into 10 ml of cold phosphate-buffered saline supplemented with 10% (v/v) foetal calf serum (PBS/FCS), using a rubber-tipped syringe plunger. The cell suspensions were then centrifuged at 300 *g* for 5 min and the supernatants were discarded. The cells were resuspended in 10 ml PBS/FCS and the debris was sedimented by centrifuging at 60 *g* for 20 sec. Cell concentrations were determined by haemocytometer counting, and were adjusted to 4×10^7 cells/ml. Viability, measured by trypan blue dye exclusion, was usually about 75% in these preparations.

Differential spleen cell counts

Smears of each spleen cell preparation were made using a Shandon cytocentrifuge. The smears were Leishman-stained (pH 7·2) and were examined at ×1000 magnification. For each smear 100–500 nucleated cells were scored as being: (1) lymphocytes, (2) normoblasts or (3) other cell types, on the basis of their morphology.

Determination of total Thy-1·2$^+$ cells

The proportion of spleen cells bearing the Thy-1 alloantigen was determined using a rosetting technique described fully by Parish & McKenzie (1978). For each sample, 10^7 cells were resuspended in Eagle's medium/10% FCS. To 100 μl of this suspension was added 5 μl of an immunoabsorbant-purified sheep F(ab′)$_2$ antiserum specific for mouse Ig, and the mixture was incubated for 75 min at 37° in order to clear the spleen cells of surface Ig. The cells were then washed twice in PBS/FCS at 4°, and the pellet was finally resuspended in 250 μl PBS/FCS. To 50 μl of this suspension was added 50 μl of a 1 in 20 dilution of congenic anti-Thy-1·2 antiserum. After incubation on ice for 30 min the cells were washed twice in PBS/FCS. Finally the cell pellet was resuspended in 50 μl of PBS/FCS, and 50 μl of a 2% (v/v) suspension of sheep erythrocytes coated with sheep anti-mouse Ig (Parish & McKenzie, 1978) was added. The cells were sedimented at 200 *g* for 5 min at 4° to form rosettes. The samples were gently resuspended in their supernatants and were stored on ice until they were examined microscopically for rosette-forming cells (RFC) as described by Parish & McKenzie (1978). The total number of Thy-1·2$^+$ cells per spleen was computed with reference to the total nucleated cell count.

Determination of total Ig$^+$ cells

The proportion of spleen cells bearing surface immunoglobulin was determined by a direct rosetting technique. Spleen cells were made to 4×10^6/ml of the 50 μl of this cell suspension was added 50 μl of a 2% suspension of sheep erythrocytes coated with sheep anti-mouse Ig. Rosettes were formed and RFC were scored as described above. Calculation of total Ig$^+$ cells per spleen was then made.

Determination of spleen cells simultaneously bearing surface Ig and Thy-1·2 alloantigen

Spleen cell samples (4×10^7/ml in PBS/FCS) were prepared from uninfected mice and from mice at various stages of *P. yoelii* infection. Each sample was divided into two aliquots of 2×10^7 cells in 0·5 ml. To one aliquot was added 0·5 ml of PBS/FCS. To the other aliquot was added 0·5 ml of a 20% suspension of sheep erythrocytes coated with sheep anti-mouse Ig, and Ig rosettes were formed by centrifuging at 300 *g* for 5 min at 4°, after addition of 5 μl of 25% (w/v) NaN_3, the pellet was gently resuspended in the supernatant. Both aliquots were layered over 4·0 ml Isopaque/Ficoll in polycarbonate centrifuge tubes (Parish, Kirov, Bowern & Blanden, 1974) and centrifuged at 2000 *g* for 15 min at 20°. In the unrosetted aliquot all viable, nucleated cells remained at the Isopaque/Ficoll interface, whereas in the Ig-rosetted aliquot, rosetted Ig$^+$ cells sedimented to the bottom of the tube, and only Ig$^-$ cells remained at the interface. For both aliquots, cells remaining at the interface were recovered, washed twice in PBS/FCS, and made to 4×10^6/ml. The proportions of Thy-1·2$^+$ cells and Ig$^+$ cells in each aliquot were then determined by the rosetting techniques described. These results were used to

calculate the proportion of Thy-1·2$^+$ cells which had been depleted after Ig rosetting.

For example, if:

a = % Ig$^+$ cells in the unfractionated aliquot,

b = % Thy-1·2$^+$ cells in the unfractionated aliquot,

and c = % Thy-1·2$^+$ cells in the Ig-depleted aliquot, then x, the percentage of Thy-1·2$^+$, Ig$^+$ cells in the unfractionated sample was calculated from the equation

$$\frac{b-x}{100-a}=\frac{c}{100}$$

$$\text{i.e.}\quad x=b-c+\frac{a.c.}{100}$$

Thus the percentage of Thy-1·2$^+$ cells rosetting for surface Ig was equal to $\frac{x}{b}\times 100$, and the percentage of Ig$^+$ cells which rosetted for Thy-1·2 was equal to $\frac{x}{a}\times 100$.

RESULTS

Parasitaemia and splenomegaly

Infections of BALB/c mice with *P. berghei* were invariably fatal, the mean survival time being 15 days. *P. yoelii* infections were self-limiting with maximum parasitaemias of 10–20%. Between days 12 and 20 parasites were cleared from the blood (Fig. 1a).

Spleen enlargement was noticeable 4 days after infection, and maximum spleen weights were observed on day 12 in both infections (Fig. 1b). In *P. yoelii* infections enlargement of the spleen occurred more rapidly and was more pronounced at its peak than was the case in *P. berghei* infections.

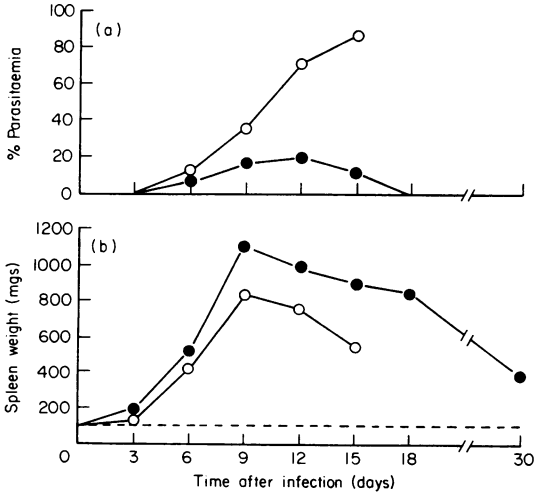

Figure 1. Kinetics of (a) parasitaemia and (b) spleen weight in mice infected *P. yoelii* (●) or *P. berghei* (○).

Contribution of erythropoiesis to splenomegaly

Differential cell counts on stained cytocentrifuge smears showed that marked changes occurred in the relative proportions of lymphocytes and normoblasts during *P. berghei* and *P. yoelii* infections (Fig. 2). In the spleens of uninfected BALB/c mice about 70% of free cells are lymphocytes and about 20% are normoblasts, but during the acute phase of malarial infection the situation was essentially reversed. Normoblasts at all stages of maturation were seen, and mitotic activity was pronounced in this population. Late normoblasts often contained parasites.

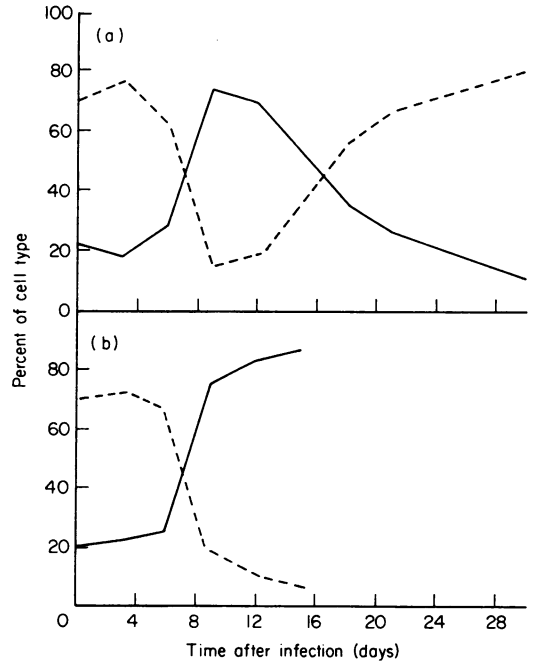

Figure 2. Percentage changes in the lymphocyte (– – –) and normoblast (——) populations in the spleens of mice infected with (a) *P. yoelii* or (b) *P. berghei*.

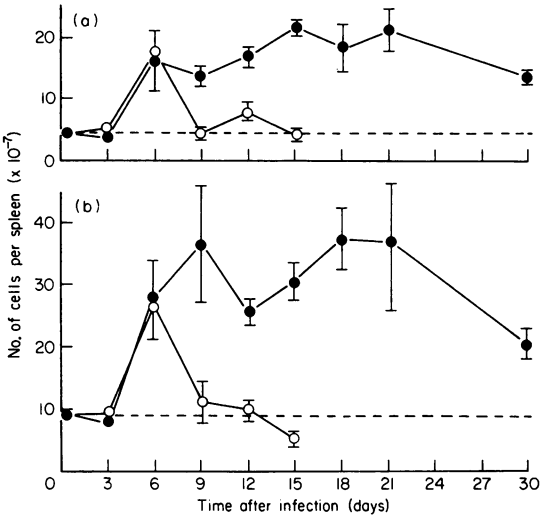

Figure 3. Changes in the total numbers of (a) nucleated cells and (b) normoblasts in the spleens of mice during *P. yoelii* (●) and *P. berghei* (○) infection.

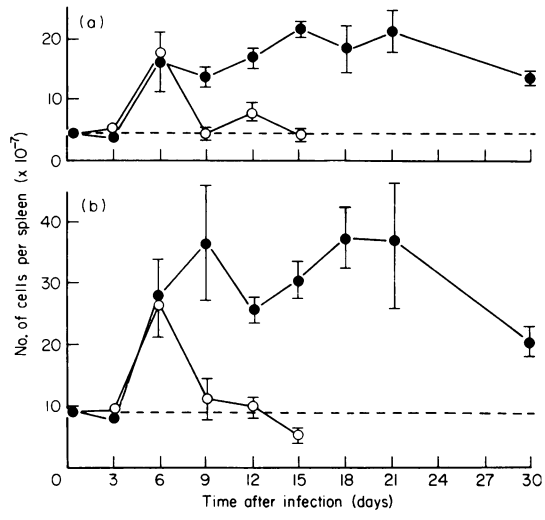

Figure 4. Changes in the total numbers of (a) Thy-1·2⁺ cells and (b) Ig⁺ cells in the spleens of mice during *P. yoelii* (●) and *P. berghei* (○) infection. Vertical bars indicate the standard errors.

When the total number of normoblasts per spleen was computed and compared with the total number of nucleated cells per spleen it became evident that the bulk of the splenic enlargement observed during these infections was due to compensatory erythropoiesis (Fig. 3).

Changes in total Thy-1·2⁺ cells

The number of Thy-1·2⁺ cells in the spleens of uninfected mice showed a four-fold increase between days 3 and 6 (Fig. 4a). In *P. berghei* infections the number declined sharply after day 6 and remained at low levels until the mice died. In *P. yoelii* infections, however, the number of Thy-1·2⁺ cells continued to rise after day 2 and was maintained at a level about five-fold above normal until the mice had fully recovered.

Changes in total Ig⁺ cells

The kinetics of proliferation of Ig⁺ cells in the spleens of *P. berghei* and *P. yoelii*-infected mice were similar to those of Thy-1·2⁺ cells (Fig. 4b). In *P. berghei* infections an early increase in Ig⁺ cell numbers was followed by a sharp decline after day 6. By contrast, in *P. yoelii* infections the Ig⁺ cell response was maintained strongly throughout the phase of parasite clearance (days 12 to 20), when about 35×10^7 Ig⁺ cells per spleen were detected, an increase of about four-fold over the number observed in uninfected mice.

Incidence of Thy-1·2⁺ cells bearing surface Ig

By morphological criteria, it was found that, at day 12 of *P. yoelii* infection, 18% of nucleated spleen cells were lymphocytes. However, the sum of the percentages of Thy-1·2⁺ cells and Ig⁺ cells, detected by rosetting, was 30%, suggesting an overlap between the Thy-1·2⁺ and Ig⁺ populations.

Table 1. Incidence of Ig-bearing Thy-1·2⁺ cells in the spleen during *P. yoelii* infection

Day of *P. yoelii* infection	% of Thy-1·2⁺ cells rosetting for surface Ig*	% of Ig rosetting cells which were Thy-1·2⁺ cells*
0	6·5†	3·7
6	64·6	29·2
12	96·7	75·2
16	43·2	27·9
23	8·6	2·9

* See Materials and Methods section of text.
† One spleen only for each time point.

By comparing the proportions of Thy-1·2$^+$ cells and Ig$^+$ cells in unfractionated and Ig-depleted aliquots of spleen cell samples, the percentage of Thy-1·2$^+$ cells bearing surface Ig was calculated as described in the Materials and Methods section. Table 1 shows the extent of overlap between the Thy-1·2$^+$ and Ig$^+$ populations during *P. yoelii* infection. In the uninfected mouse spleen, 6·5% of Thy-1·2$^+$ cells rosetted for surface Ig. However, during acute *P. yoelii* infection the percentage of Thy-1·2$^+$ Ig$^+$ cells was found to be high: at the peak of infection >90% of Thy-1·2$^+$ cells appeared to bear surface Ig. During the phase of parasite clearance, Thy 1·2$^+$ Ig$^+$ cells accounted for only a small proportion of the total number of Ig$^+$ cells.

DISCUSSION

Jayawardena, Targett, Davies, Leuchars & Carter (1975b) compared the splenic T-cell responses of chimaeric mice during *P. yoelii* and *P. berghei* infections. They found that a sustained T-cell mitotic response was associated with the non-fatal infection but was less marked during the fatal infection. The results of the present study also show that a marked proliferation of T cells, identified by the Thy-1 alloantigen, occurs prior to parasite clearance, and that in the absence of such a response no protective immunity ensues. Furthermore, this study also shows that the population of splenic Ig$^+$ cells, mainly B cells and plasma cells, undergoes a marked expansion in parallel with the observation for T cells. Again, when no protective response occurs, only transient B-cell proliferation is observed.

The degree to which compensatory erythropoiesis contributes to splenomegaly in *P. berghei* and *P. yoelii* infection has been quantitatively described in this paper. Although this response is not strictly an immune response, it is relevant to our understanding of immunity to rodent malaria: when comparing the *in vitro* mitogen responsiveness (Weinbaum, Evans, Baker & Tigelaar, 1976) or the *in vitro* specific immune responsiveness (Warren & Weidanz, 1976) of spleen cells from malarious and uninfected mice it is a factor which must be taken into account.

We found that normoblasts account for 20% of the free, nucleated cells in the spleens of uninfected BALB/c mice. This non-lymphoid population appears to account for the bulk of the previously described 'null cell' population (Parish, 1975). In the present study we found, comparing the morphological and rosetting results, that we could account for 95–100% of splenic lymphocytes in terms of their Ig and Thy-1 surface markers.

Wyler & Gallin (1977) described an early increase in the numbers of Fc receptor-bearing (FcR$^+$) cells in the spleens of *P. berghei* infected mice. They interpreted this as indicating an accumulation of macrophages, but we consider that the FcR$^+$ criterion is not sufficient to identify a macrophage, as subpopulations of B lymphocytes (Basten, Miller, Warner, Abraham, Chia & Gamble, 1975; Möller, 1974) and T lymphocytes (Soteriades-Vlachos, Gyöngyössy & Playfair, 1974; Stout & Herzenberg, 1975) as well as K cells (Ramshaw & Parish, 1976) also possess Fc receptors. It is possible, therefore, that the effect observed by Wyler & Gallin (1977) was a transient lymphocyte proliferation similar to that observed during *P. berghei* infection in the present study.

During the course of *P. yoelii* infection it appeared that a progressively greater proportion of T cells expressed surface Ig. It is most likely that the bound Ig was complexed with antigen and passively absorbed to the T cell surface via Fc receptors. The functional significance of FcR$^+$ T cells is uncertain, but although they do not appear to be helper cells, they may indeed have a suppressor role in the immune response (Playfair, 1974; Stout & Herzenberg, 1975; Rubin, 1977). It is of interest, therefore, to note that at the time of maximum Ig (complex) expression by T cells during *P. yoelii* infection, splenic humoral responses to such antigens as sheep erythrocytes and SIII are maximally depressed (Salaman, Wedderburn & Bruce-Chwatt, 1969; Wedderburn & Dracott, 1977).

In *P. berghei* infection the erythropoietic response was sustained until the terminal stage of the disease. However, lymphocyte proliferation was apparently suppressed after day 6, and there followed a depletion of lymphocytes from the spleen. Gravely, Hamburger & Kreier (1976) found total cell death in the thymus in *P. berghei* infections of young rats, and Jerusalem & Bruckhausen (1967) have reported the apparent toxic reaction of mice to *P. berghei* infection. It is possible, therefore, that the suppression of the lymphoproliferative response and the absence of the protective immune response to *P. berghei* is due to the selective cytotoxic action of a parasite product.

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