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Activation of liver macrophages in murine malaria is enhanced by vaccination

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SUMMARY

During blood-stage infection of mice with a lethal variant of *Plasmodium yoelii*, cells in both spleen and liver became activated to reach a peak at day 5. In mice protected by vaccination, activation was accelerated after infection. The most striking difference observed was in the 10-fold greater yield of infiltrating cells, including macrophages, obtained from the liver just before the mice recovered. Their capacity to give an oxidative burst and their cytotoxic activity against tumour cells was also more than 10 times normal. This suggests that the recruitment of inflammatory cells to the liver plays an important role in the protection of vaccinated mice against malaria.

Keywords malaria macrophage liver spleen vaccination

INTRODUCTION

Protective immunity in malaria is now well recognized to depend on both humoral and cell-mediated components of the immune response. Macrophages are important effector cells involved in the elimination of malarial parasites, partly through the action of reactive oxygen metabolites released during the respiratory burst (Clark & Hunt, 1983; Ockenhouse & Shear, 1984; Dockrell & Playfair, 1984). In murine malaria, macrophages in the spleen become activated in terms of their oxidative and tumoricidal capacity (Brinkmann *et al.*, 1984; Dockrell, Alavi & Playfair, 1986a, Taverne, Treagust & Playfair, 1986).

Although the spleen becomes greatly enlarged and is necessary for the development of immunity against some malarial parasites, in other instances splenectomized mice are still capable of recovery from infection. Hepatomegaly is also a feature of the disease and migration of mononuclear cells into the liver occurs during infection (Dockrell, de Souza & Playfair, 1980; Lee, Crocker & Gordon, 1986). In mice with a self-limiting infection caused by *Plasmodium yoelii* 17X, macrophages in the liver, like those in the spleen, develop an enhanced oxidative capacity (Dockrell *et al.*, 1986a; Lee *et al.*, 1986) and also show a change in phenotype (Lee *et al.*, 1986).

We showed previously that in vaccinated mice spleen macrophages were activated sooner after challenge with *P. yoelii* than in controls (Dockrell *et al.*, 1986a; Taverne *et al.*, 1986). In addition, liver macrophages from vaccinated mice displayed a significantly greater respiratory burst 5 days after challenge (the one time-point tested) than those from unvaccinated mice. The cytotoxic activity of spleen macrophages from mice infected with two non-lethal species of parasite was also found to be greater than that in two lethal infections.

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To compare the time course of macrophage activation in the liver with that in the spleen and to see if, as was observed with the spleen, liver macrophages are less activated in lethal than in non-lethal infections, we measured their oxidative capacity and their ability to kill tumour cells during infection with a lethal variant of *P. yoelii*. As induction of immunity against this variant involves a cell-mediated component (Freeman & Holder, 1983) we also measured these activities in liver macrophages in mice protected by vaccination.

MATERIALS AND METHODS

Mice. Female outbred mice (Tuck No 1 from A. Tuck and Sons, Battlesbridge, Essex) were used at 8–10 weeks of age.

Malaria parasites. Non-lethal *P. yoelii* 17X and a lethal parasite, *P. yoelii* YM, (obtained from Dr Walliker, University of Edinburgh as described by Freeman & Holder, 1983), which kills the mice within 10 days, were used. Infections were initiated by injection of 10^4 parasitized erythrocytes i.v. Parasitaemia was determined by enumeration of parasitized erythrocytes in blood films stained with Giemsa.

Vaccination and challenge. Mice were vaccinated by injection i.v. of a crude blood-stage vaccine (Playfair, de Souza & Cottrell, 1977) made from saponin-lysed, formalin-fixed parasites. The vaccine consisted of 10^8 parasites and 10^8 *Bordetella pertussis* organisms (Wellcome Pertussis Vaccine BP, Wellcome Foundation Ltd, London). Three weeks after vaccination the mice were challenged i.v. with 10^4 parasitized erythrocytes. The parasitaemia became patent 3 days after infection, reached a peak of 8–10% on day 5 and declined by days 7–10, when the animals recovered.

Isolation of liver macrophages. Livers were perfused via the portal vein with 10 ml of warmed Ca^{2+} -free and Mg^{2+} -free Hanks Buffered Salts Solution (Flow Laboratories Ltd) containing 10 mM HEPES, pH 7·3 (BSS), followed by 5 ml of phenol red-free BSS containing 0·04% collagenase (Boehringer Mannheim (GmbH) pH 7·4. Each liver was then excised, chopped and digested for 45 min in 20 ml phenol red-free BSS containing 0·04% collagenase at 37°C with stirring. After 30 min, 1 mg DNase (Sigma, deoxyribonuclease I) was added. The cell suspension was then washed three times and the liver macrophages were isolated on a Percoll gradient, density 1·037 g/ml (Crocker, Blackwell & Bradley, 1984) by centrifugation for 30 min at 650 ***g***. The cells in the pellet were collected, washed twice, counted using ethidium bromide and acridine orange and resuspended in RPMI 1640 medium (Flow Laboratories Ltd) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% fetal calf serum (FCS) to give a final concentration of 3×10^6 viable cells per ml. The cell suspension was dispensed at 3×10^5 cells per well in 96-well microtitre plates (Nunc) and incubated at 37°C in an atmosphere of 5% CO_2 in air. After 2 h, non-adherent cells were removed by washing the wells with warm phenol red-free BSS.

Differential counts on cytocentrifuge preparations before adherence revealed that about half the cells were macrophages and half were lymphocytes; the mean contamination with polymorphonuclear leucocytes was 7·0% ± 1·0.

Isolation of spleen macrophages. Spleens were teased apart in ice-cold BSS and erythrocytes lysed by treatment with 0·16 M NH_4Cl in 0·17 M in Tris-HCl, pH 7·2, at 37°C. The spleen cells were washed twice, counted and adjusted to 1×10^7 cells per ml in 10% FCS in RPMI 1640. The cells were plated at 1×10^7 viable cells/well in 24-well plates (Nunc) and 2 h later non-adherent cells were removed as described above.

Superoxide anion production. This was measured by the reduction of nitro-blue tetrazolium as previously described (Dockrell *et al.*, 1986a). Phorbol myristate acetate (PMA, Sigma) at a final concentration of 125 ng/ml was used to trigger the respiratory burst. Liver cell monolayers were tested in sextuplicate, spleen cell monolayers in triplicate. The optical density of solubilized formazan was measured at 630 nm in a Dynatech Microelisa Autoreader and the readings were converted into nmoles NBT reduced in 1 h, using the conversion factor 0·1 OD630 = 1·25 nmoles NBT (Rook *et al.* 1985).

To confirm that the NBT reduction was caused by superoxide anion, superoxide dismutase (SOD; Sigma) was incorporated in some assays. The inhibition of NBT reduction caused by 100 μg/ml SOD was approximately 50% in all samples tested.

Assay of macrophage cytotoxicity. This method was adapted from that used to assay tumour necrosis factor (TNF) (Taverne *et al.*, 1986). Doubling dilutions of liver or spleen cell suspensions were added to monolayers of L929 mouse tumour cells and after 4 h incubation at 37°C in an atmosphere of 5% CO_2 in air, an equal volume of medium containing 2 μg per ml of actinomycin D (Sigma) was added. Next day, cultures were fixed, stained with crystal violet and the dye eluted and its optical density measured in a Dynatech Microelisa Autoreader. Percentage cell survival was calculated by reference to control monolayers incubated in medium only. One cytotoxic unit is defined as the number of cells that killed 50% of the tumour cells. Results are expressed as cytotoxic units per 10^6 cells plated or per organ.

Statistics. Groups of 4–6 mice were tested at each time except that groups of three were used in experiments with spleens from vaccinated mice; results are given as mean ± s.e. Vaccinated mice were always compared with unvaccinated controls infected on the same day. The results were compared by Student's t-test.

RESULTS

In preliminary experiments on liver macrophages from mice infected with the non-lethal parent *P. yoelii* 17X, increased activation was observed during the first week after infection and again about the time of recovery, both in terms of the oxidative burst and of cytotoxicity for tumour cells. In both cases the first peak of activation occurred a few days later than in the spleen.

In these early experiments only two livers were tested at most of the time points and since less variability was generally encountered in cytotoxicity assays than in NBT tests, only the results of the

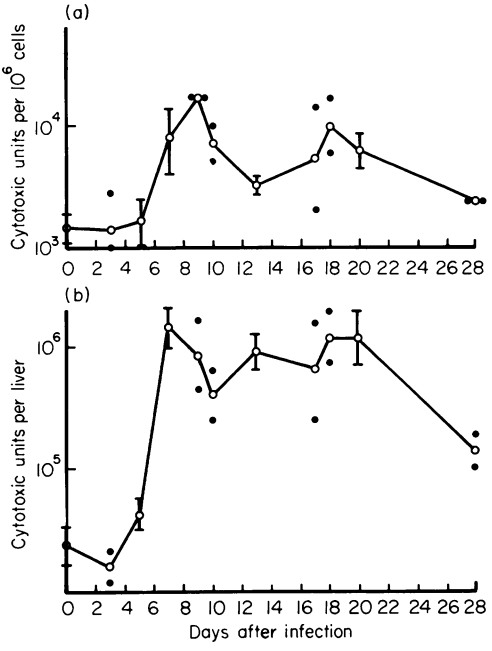

Fig. 1. Cytotoxic activity of non-parenchymal cells obtained from livers of mice infected with non-lethal *P. yoelii* 17X. (a) cytotoxic units per 10^6 cells plated; (b) cytotoxic units per liver. Results obtained from individual mice (●) are shown with their means (○); with groups of more than four mice means ± s.e. are shown.

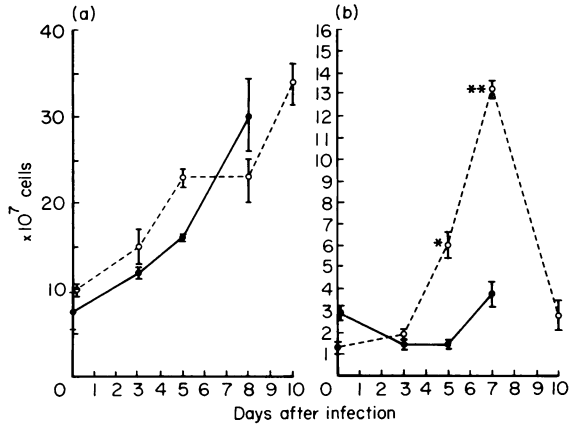

Fig. 2. Cells recovered from the spleens and livers of mice infected with *P. yoelii* YM. (a) Total viable cells per spleen; (b) Non-parenchymal cells recovered per liver. (●) Unvaccinated mice, (○) vaccinated mice. Figures are means ± s.e. Significantly different from unvaccinated mice: $*P<0{\cdot}01$; $**P<0{\cdot}0005$.

former are illustrated here (Fig. 1). During this infection the yield of non-parenchymal cells per liver increased more than 10-fold, from a mean of $1{\cdot}6\times10^7$ (s.e. = 0·3) for normal mice, to $2{\cdot}2\times10^8$ (s.e. = 4·0) on day 20. When the results expressed as cytotoxic units per liver are compared with the activity per 10^6 cells, it was apparent that although both showed an increase at the end of the first week a large part of the enhancement of the activity of the whole organ was due to this increase in the yield of cells.

Previously, vaccination of mice against this parasite was observed to enhance both the oxidative burst capacity and cytotoxic activity of liver macrophages at day 5 (Dockrell *et al.*, 1986a; Dockrell *et al.*, 1986b). For a more thorough investigation of the effect of vaccination on macrophage function in the liver, groups of normal and vaccinated mice were infected with a lethal variant of *P. yoelii* and at intervals adherent cells obtained from their livers were tested for their oxidative capacity, and the cell suspensions were also tested for their cytotoxicity against tumour cells. The results were compared with those obtained with spleen macrophages taken at the same times. Infections caused by this parasite have the advantage that they cover a shorter time span than those of the non-lethal parasite and the effect of vaccination is more dramatic.

Both the spleen and liver enlarge during infection and this is reflected in the increase in the number of cells recovered; in both organs this was enhanced by vaccination (Fig. 2). The effect of vaccination was particularly marked in the liver: the yield of non-parenchymal cells from vaccinated mice was nearly 10 times normal on day 7 after challenge. It was significantly greater than the yield from unvaccinated infected mice on both day 5 and day 7 ($P<0{\cdot}005$ and $P<0{\cdot}01$ respectively).

Spleen macrophages. Both the oxidative capacity and the cytotoxic activity of spleen macrophages from mice infected with this lethal variant of *P. yoelii* increased after infection to reach a peak at day 5 (Fig. 3). In the case of the oxidative burst, the size of the peak expressed per 10^7 cells or per spleen was no different from that obtained with non-lethal *P. yoelii* (Dockrell *et al.*, 1986a). Cytotoxic activity, however, was lower and only reached a peak of $9{\cdot}04\times10^5\pm1{\cdot}7$ U per spleen, compared with $1{\cdot}9\times10^6\pm0{\cdot}49$ for the non-lethal parasite (Taverne *et al.*, 1986). By both measures, vaccination accelerated activation; expressed per spleen, activation was significantly enhanced on day 3 ($P<0{\cdot}005$ for cytotoxicity).

Liver macrophages. Like those in the spleen, cells in the liver became activated after infection reaching a peak at day 5 as measured per cell in both assays (Fig. 4). Vaccination did not alter the time course of this activation significantly. But by both measures, vaccination again greatly accelerated activation when results were expressed per liver, that is, in terms of the total yield of non-parenchymal cells obtained. Thus, by the NBT test, activation at day 5 was significantly greater than

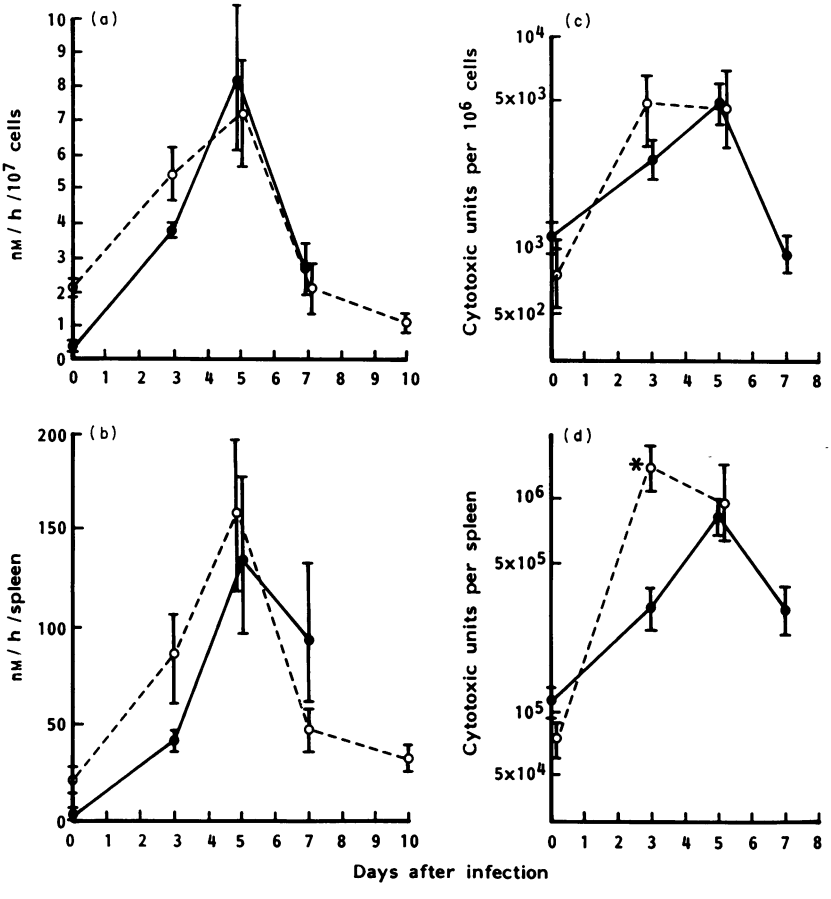

Fig. 3. Macrophage activation in the spleens of mice infected with *P. yoelii* YM. Oxidative burst capacity measured as nM of NBT reduced per h by adherent cells (a) from 10^7 cells plated and (b) per spleen; cytotoxicity for tumour cells measured (c) per 10^6 cells plated and (d) per spleen. (●) Unvaccinated mice; (○) vaccinated mice. Figures are means ± s.e. *Significantly different from unvaccinated mice, $P < 0{\cdot}005$.

that observed with cells from unvaccinated mice ($P < 0{\cdot}025$, see Fig. 4b) and in the cytotoxic assay it was significantly greater on both day 3 and day 5 ($P < 0{\cdot}025$, see Fig. 4d).

DISCUSSION

Our results demonstrate that cells in the liver become activated, in terms both of their capacity to produce an enhanced oxidative burst and in their increased cytotoxicity for tumour cells, in mice infected with this lethal variant of *P. yoelii*. Expressed per cell, both activities coincided at a peak on day 5, in contrast to spleen cells from mice infected with the non-lethal *P. yoelii* which showed a peak in oxidative burst capacity 2–3 days before peak cytotoxicity (Dockrell *et al.*, 1986a; Taverne *et al.*, 1986).

Liver preparations appear more active than spleen cell suspensions because they contain more macrophages. It should be noted that whereas measurements of the oxidative burst were made on adherent cells (i.e. macrophages), unfractionated cell populations were used in the cytotoxicity assays, so that cellular interactions could have contributed to the death of the tumour cell targets.

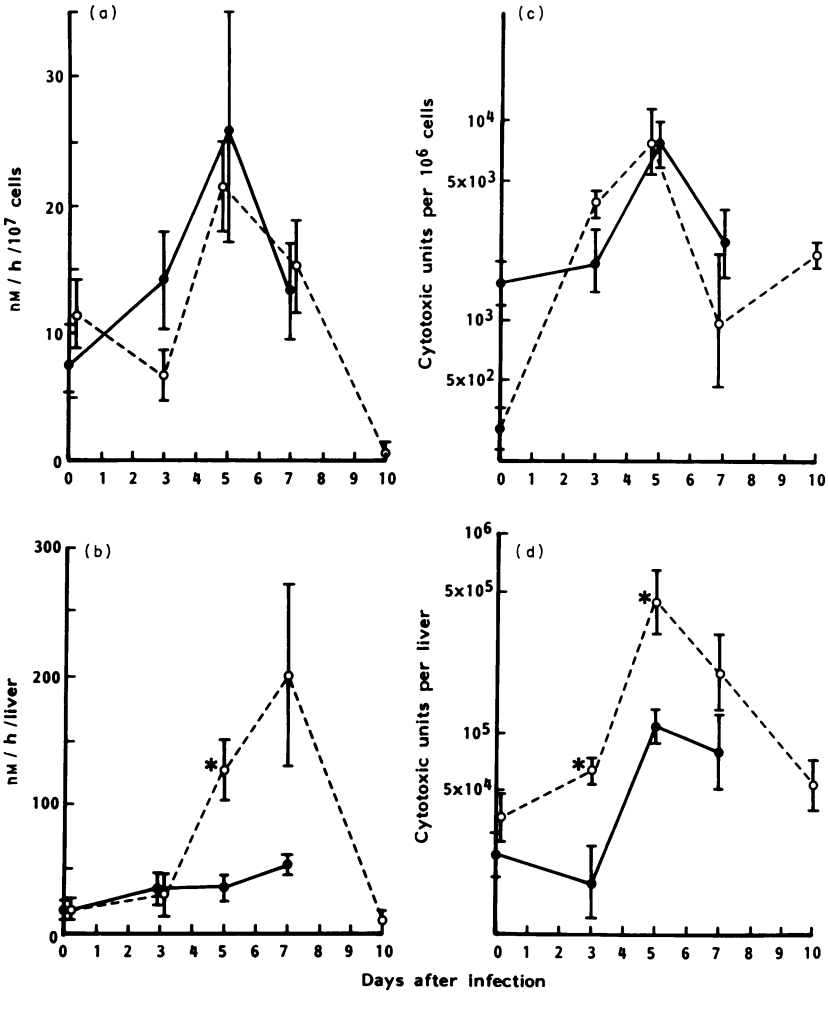

Fig. 4. Macrophage activation in the livers of mice infected with *P. yoelii* YM. Oxidative burst capacity measured as nM of NBT reduced per h by adherent cells (a) from 10^7 cells plated and (b) per liver; cytotoxicity for tumour cells measured (c) per 10^6 cells plated and (d) per liver. Figures are means ± s.e. (●) Unvaccinated mice; (○) vaccinated mice. * Significantly different from unvaccinated mice $P < 0{\cdot}025$.

Our findings support the view (Playfair *et al.*, 1979) that cell-mediated immunity in the liver is associated with recovery from infection, whether recovery is spontaneous or induced by vaccination, and it appears the protection may be related to the influx of newly recruited macrophages to the liver. First, in the non-lethal infection, macrophage activation expressed as cytotoxicity per organ was greater in the liver during the second 2 weeks of infection than in the spleen (Taverne *et al.*, 1986) and, expressed per cell, the second peak of activity occurred in the liver just before recovery while that in the spleen occurred just after. Secondly, when the infection was lethal, activation in the liver was less than in the spleen.

By contrast, vaccination against the lethal infection induced dramatic changes in the liver. A large increase in the total number of non-parenchymal cells occurred, associated with activation of macrophages, as determined by both tests. Following current views on the ontogeny of liver macrophages, we would interpret our results to indicate that more blood monocytes are attracted to the liver in vaccinated animals. Since it appears that resident macrophages cannot be stimulated to

exhibit a respiratory burst (Lepay *et al.*, 1985a) and secrete only low levels of superoxide (Arthur, Kowalski-Saunders & Wright, 1986), the increased activity observed in the livers of vaccinated mice suggests that newly recruited cells are activated, as proposed for Listeriosis (Lepay *et al.*, 1985b, Campbell, 1986). They may be activated before they migrate to the liver or after arrival, though the presence there of lymphocytes possibly secreting macrophage activating factors such as γ-interferon makes the latter more likely. Identification of the factors responsible for recruitment of inflammatory cells to the liver and spleen as well as those inducing macrophage activation would contribute to our understanding of the cellular interactions involved in immunity.

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