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Reassessment of the Role of Splenic Leukocyte Oxidative Activity and Macrophage Activation in Expression of Immunity to Malaria

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The role of splenic leukocyte oxidative activity and macrophage activation in the development of protective immunity was examined during acute *Plasmodium chabaudi adami* malaria. Splenic leukocyte oxidative activity was compared in infected BALB/c and P/J mice; the latter are known to suffer from defects in macrophage function. Phorbol myristate acetate-stimulated chemiluminescence and superoxide anion production by splenic leukocytes from infected BALB/c mice were found to be increased dramatically, while the response of splenic leukocytes from infected P/J mice was elevated only minimally. Hydrogen peroxide release was slightly increased in splenic leukocytes from infected BALB/c mice but remained essentially unchanged in those from infected P/J mice. Macrophage function was assessed on the basis of measurements of tumoricidal activity. Splenic macrophages from uninfected BALB/c mice displayed significant tumoricidal activity against L929 target cells. As a result of splenomegaly during infection, tumoricidal activity, when calculated on a per-spleen basis, was increased further in infected BALB/c mice. In contrast, the tumoricidal activity of splenic macrophages from P/J mice was minimal, regardless of infection. Despite these differences, both strains of mice developed malarial infections that resolved within 16 days. Thus, while the production of reactive oxygen radicals by splenic leukocytes and the phenomenon of macrophage activation have traditionally been associated with the resolution of malarial infection, this study failed to establish a correlation between these parameters and the development of protective immunity to blood-stage infection with *P. chabaudi adami*.

Immunity to malaria occurs after repeated infections over several years. Such immunity is not absolute since low levels of parasitemia persist in the absence of clinical disease. It is not known how the host kills blood-stage parasites or inhibits their growth. The use of murine models may facilitate the identification and characterization of the immune mechanism(s) involved in resistance to infection.

Using immunodeficient mice, we determined previously that immunity to the rodent malarial parasite *Plasmodium chabaudi adami* was mediated by T-cell-dependent, nonantibody immune mechanisms (12). B-cell-deficient mice and immunologically intact mice resolved acute infection with the parasite, while athymic nude mice developed unremitting parasitemia leading to death. Immunity was adoptively transferred to these mice with splenic T cells but not with B cells (6). In addition, the adoptive transfer of immunity was achieved with parasite-specific T-cell lines and clones (3, 4). More recently, we reported that B-cell-deficient mice also resolved acute infections caused by *P. vinckei petteri*, *P. chabaudi chabaudi*, and *Babesia microti* in the absence of chemotherapy (L. Cavacini, L. Parke, J. Sundy, T. Daly, and W. Weidanz, FASEB J., **4**:A453, 1988). Together, these findings suggest that T-cell-dependent, cell-mediated immune mechanisms play an essential role in resolving malarial infections caused by *P. chabaudi adami* and related hemosporidia.

While the effector mechanism(s) responsible for the expression of protective immunity against *P. chabaudi adami* remain(s) to be determined, the activation of macrophages by the products of parasite-specific T cells (e.g., γ-interferon) might play a key role. It has been proposed that macrophages activated during malarial infection function as effector cells in parasite destruction (24, 26) through the production of toxic oxygen radicals (8, 10, 11, 20; for a review, see reference 1), cytokines (including tumor necrosis factor) (9, 13, 27), or lymphocyte-activating factors (9). To investigate the role of macrophage activation in resistance to malaria, we examined the ability of a macrophage-defective mouse strain, P/J, to clear acute *P. chabaudi adami* infection. The oxidative activity of their splenic leukocytes, as well as the tumoricidal activity of splenic macrophages, was measured during the course of disease. The results show that P/J mice clear *P. chabaudi adami* from their blood despite the fact that their splenic leukocytes display defective oxidative activity and their splenic macrophages lack significant tumoricidal activity.

MATERIALS AND METHODS

Experimental animals. BALB/c mice of both sexes were raised in our animal colony, while male and female P/J mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). All mice were housed and maintained in our American Association for Laboratory Animal Care (AALAC)-accredited animal facility under standard conditions. Age- and sex-matched animals 7 to 12 weeks of age were used in all experiments. Mice in the animal colony were routinely tested for, and found to be free of, mouse hepatitis virus.

Malarial parasites. *P. chabaudi adami* 556KA was originally obtained from David Wyler of the National Institutes of Health. Parasites were cloned as previously described (14) and maintained as frozen stabilates in the vapor phase of a liquid nitrogen freezer. Parasite material was tested for, and found to be free of, lactate dehydrogenase-elevating agents (22). All experimental infections were initiated with either

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10^5 or 10^6 parasitized erythrocytes obtained from donor mice infected with stabilate material. Parasitemia was estimated by enumerating parasitized erythrocytes on Giemsa-stained films of tail blood as previously described (14). The percent parasitemia was calculated as the number of parasitized erythrocytes per 200 erythrocytes.

Preparation of splenic leukocytes. Spleens were removed from infected and uninfected animals and dissociated into single-cell suspensions in Hanks balanced salt solution. Erythrocytes were lysed by hypotonic lysis, and the remaining cells (designated splenic leukocytes) consisted of a mixed population of cells, including polymorphonuclear leukocytes and macrophages, known to have oxidative activity.

Measurement of chemiluminescence. Luminol-enhanced chemiluminescence was determined as described previously (5). Briefly, 10^7 splenic leukocytes in 1.0 ml of Hanks balanced salt solution without phenol red were added to 20-ml polyethylene scintillation vials. Luminol (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 10^{-5} M. Phorbol myristate acetate (PMA) (200 ng/ml) was used as an inducing stimulus and was added immediately prior to counting in a liquid scintillation counter in the tritium mode. Samples were counted for 6 s at 3- to 4-min intervals for 30 to 45 min to determine peak chemiluminescence. After adjusting for background counts, results were expressed as the number of counts per minute per spleen.

Hydrogen peroxide assay. Hydrogen peroxide concentrations were determined as described previously (23). A volume of 10^6 splenic leukocytes was added to 1.0 ml of phenol red solution containing 0.028 M phenol red (Sigma), 5 mg of horseradish peroxidase (type II; Sigma), 82 mg of sodium chloride, 10 mg of glucose, and 0.010 M potassium phosphate buffer (pH 7.4). PMA (200 ng/ml) was added, and the resulting mixture was incubated at 37°C for 90 min, after which time it was transferred to glass vials. The pH was raised to 12.5 by the addition of 10 μl of sodium hydroxide (1 N), and the A_{610} of the samples was determined by using 1.0 ml of phenol red solution, pH 12.5, as a blank. Hydrogen peroxide concentrations were determined from a standard curve run concurrently with each experiment.

Superoxide anion assay. The concentration of superoxide anion was determined in the cytochrome *c* assay (25). Splenic leukocytes (10^6) were incubated with 80 μM cytochrome *c* (type III, from horse heart; Sigma) and 200 ng of PMA for 90 min at 37°C. Superoxide dismutase (40 μg/ml, from bovine erythrocytes; Sigma) was added to duplicate samples as a control. After 90 min, the tubes were centrifuged and the A_{550} of the supernatant fluids was determined. The concentration of superoxide anion was determined by using the extinction coefficient $2.1 \times 10^4\ M^{-1}\ cm^{-1}$.

Macrophage-induced cytotoxicity. Mononuclear cells from splenic leukocytes were collected from the interface of a Ficoll-Hypaque gradient (density, 1.077 g/ml). The mononuclear cells were washed and adjusted to 7×10^6 cells per ml in Dulbecco modified essential medium (GIBCO, Grand Island, N.Y.) containing 10% (vol/vol) heat-inactivated fetal calf serum (Hyclone, Logan, Utah) and 50 μg of gentamicin sulfate per ml. One hundred microliters of this cell suspension was added to each well of a 96-well flat-bottomed plate, and the macrophages were allowed to adhere for 2 to 3 h at 37°C in an atmosphere of 5% CO_2. The plates were then washed four times with Dulbecco phosphate-buffered saline (PBS) to remove nonadherent cells. Microscopic examination revealed that similar numbers of mononucleated cells, hereafter designated splenic macrophages, had adhered to each well. Tumor cytotoxicity was determined as described previously, with slight modifications (2). L929 target cells (ATCC CCL1) were adjusted to 1.5×10^5 cells per ml, and 100 μl was added to each well of a 96-well plate containing splenic macrophages. The plates were incubated for 48 h at 37°C in 5% CO_2 in air. Duplicate wells containing splenic macrophages were incubated without L929 cells as controls. Viability of the L929 cells was then determined by using a colorimetric assay. Briefly, plates were emptied by inversion and washed once with PBS. The plates were emptied again, and the adherent cells were fixed with 100 μl of methanol per well for 15 min at room temperature. After the methanol was removed, the dishes were air dried and stained with 0.1% (wt/vol) crystal violet in distilled water (100 μl per well) for 5 min at room temperature. The wells were emptied by inversion and washed with 200 μl of distilled water per well. Sodium deoxycholate (100 μg of a 2% [wt/vol] solution in water) was then added to each well, and the plate was heated in a microwave oven at a medium setting for 1 min to solubilize the dye. The A_{570} was read by using an automatic plate reader. L929 cells incubated with medium alone served as a control for spontaneous, or 0%, lysis, while 100% lysis was determined by incubating L929 cells with 5% sodium dodecyl sulfate. As an additional control, splenic macrophages were incubated without L929 cells and the absorbance value obtained, which in each experiment was less than 0.205, was subtracted from the experimental values obtained from wells containing splenic macrophages and L929 cells. One cytotoxicity unit was defined as lysis of 50% of the L929 cells. The number of cytotoxicity units per portion of 7×10^5 splenic leukocytes was adjusted for total spleen cell number to determine the number of cytotoxicity units per spleen.

RESULTS

Kinetics of *P. chabaudi adami* infection in P/J mice. When infected intraperitoneally with 10^6 *P. chabaudi adami*-parasitized erythrocytes, P/J mice developed acute infections similar to those seen in BALB/c mice (Fig. 1). Parasites were detected in blood films 5 days after infection, with a peak parasitemia of approximately 18% occurring on day 10. Acute infections in both P/J mice and BALB/c mice were resolved by 16 days postinfection. Subsequently, both strains of mice resisted challenge infection with homologous parasites (data not shown). *P. chabaudi adami* infection in both BALB/c and P/J mice was accompanied by significant splenomegaly (Fig. 1).

Chemiluminescence responses during acute *P. chabaudi adami* infection. Since it has been reported that macrophages of P/J mice are defective in their response to activating stimuli, we examined the generation of oxidative metabolites during malarial infection. With PMA as an inducing stimulus, the chemiluminescence response of splenic leukocytes from uninfected P/J mice was found to be significantly lower than that of uninfected BALB/c mice (data not shown). This difference became even more pronounced when mice were infected with *P. chabaudi adami*. As shown in Fig. 2, the response of splenic leukocytes from infected P/J mice was approximately 10% of that observed with splenic leukocytes obtained from infected BALB/c mice.

Oxidative metabolite production during malarial infection. Chemiluminescence permits measurement of many products of the respiratory burst. To determine whether production of a particular metabolite was affected during infection, we measured the levels of two major oxidative metabolites: hydrogen peroxide and superoxide anion. As shown in Fig.

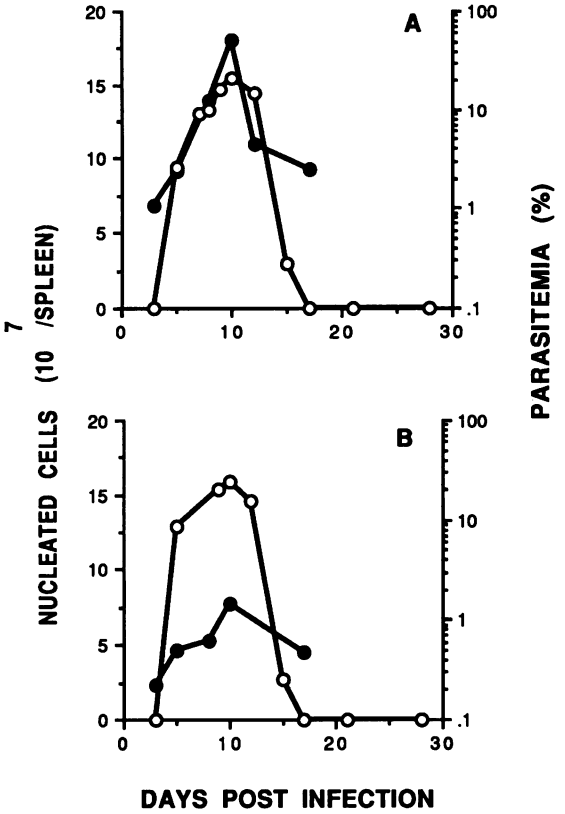

FIG. 1. Kinetics of *P. chabaudi adami* infection in BALB/c and P/J mice. Parasitemia (○) and the number of cells per spleen (●) in BALB/c mice (A) and P/J mice (B) infected intraperitoneally with 10^6 *P. chabaudi adami*-parasitized erythrocytes were determined. Five animals were used per group.

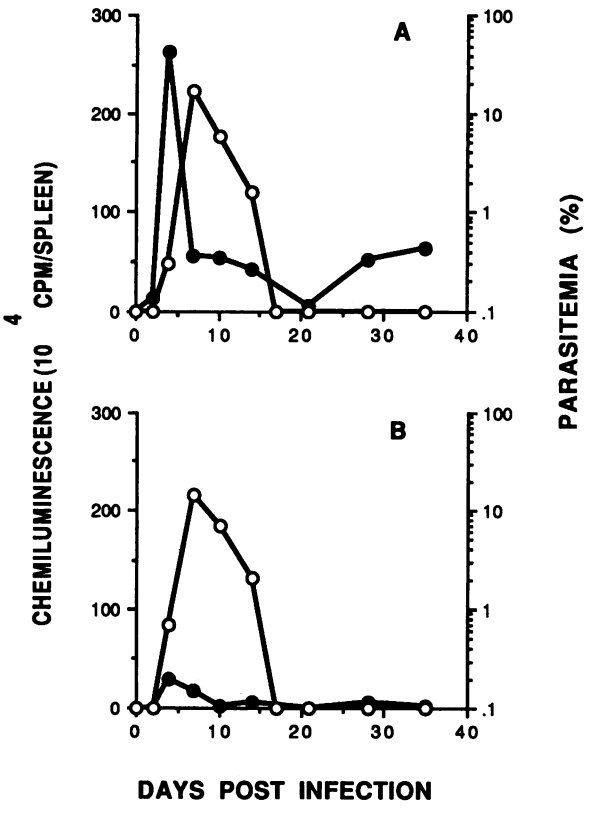

FIG. 2. Measurement of luminol-dependent chemiluminescence responses during *P. chabaudi adami* infection. Spleen cells derived from BALB/c mice (A) or P/J mice (B) were examined for PMA (200 ng/ml)-induced luminol-dependent chemiluminescence responses (●) following infection with 10^5 *P. chabaudi adami*-parasitized erythrocytes. Concurrent parasitemia is also shown (○). For each time point, background counts for the splenocytes of uninfected mice were subtracted from experimental values. Results are the means of two experiments using two to four mice per group.

3, there was an increase in the production of hydrogen peroxide by splenic leukocytes from both infected BALB/c and P/J mice when stimulated with PMA. However, splenic leukocytes from infected BALB/c mice produced at least 50% more hydrogen peroxide than did splenic leukocytes from infected P/J mice.

A significant increase in the production of superoxide anion was also observed when splenic leukocytes were obtained from infected BALB/c mice shortly after peak parasitemia (Fig. 4). Such an increase in superoxide anion production was not seen with splenic leukocytes from infected P/J mice. The levels of superoxide anion produced by cells from these animals resembled those produced by splenic leukocytes of uninfected BALB/c and P/J control mice.

Macrophage tumoricidal activity in *P. chabaudi adami*-infected mice. Using L929 cells as targets, we examined the tumoricidal activity of splenic macrophages to determine the level of macrophage activation during acute *P. chabaudi adami* infection. As shown in Fig. 5, the macrophages of infected as well as uninfected BALB/c mice were cytotoxic for L929 cells. The enhanced cytotoxicity of macrophages observed in infected mice can be attributed in part to the increase in splenic cellularity resulting from infection. Essentially no tumoricidal activity was seen with splenic macrophages from P/J mice, regardless of their infection status.

DISCUSSION

Accumulating evidence from in vitro studies indicates that cells of myeloid origin play a significant role in the destruction of blood-stage plasmodia. While the macrophage has been the focus of attention, other cells, including eosinophils, neutrophils, and monocytes, have been reported to either kill blood-stage parasites or inhibit their growth (for a review, see J. H. L. Playfair, K. R. Jones, and J. Taverne, *in* M. M. Stevenson (ed.), *Malaria: Host Response to Infection,* in press). How this is accomplished has been the subject of numerous studies conducted over the past 10 years. At present, the consensus is that reactive oxygen intermediates released by macrophages, or possibly, polymorphonuclear leukocytes following activation by T-cell products, play a major but not exclusive role in killing intraerythrocytic plasmodia. Notable exceptions include the observation that monocytes (21) and polymorphonuclear leukocytes (16) from patients with chronic granulomatous disease have been shown to inhibit the growth of *P. falciparum* despite the inability of these cells to produce reactive oxygen intermediates. Also, a variety of factors from assorted cell types kill or inhibit the growth of *P. falciparum* in vitro (for a review, see Playfair et al., in press).

In the present study, P/J mice were used to determine whether these mice with known genetic defects in macrophage activation were capable of resolving acute blood-stage infection with *P. chabaudi adami*. P/J mice have been shown previously to be defective in their ability to develop vaccine-induced immunity to *Schistosoma mansoni* (15). Their mac-

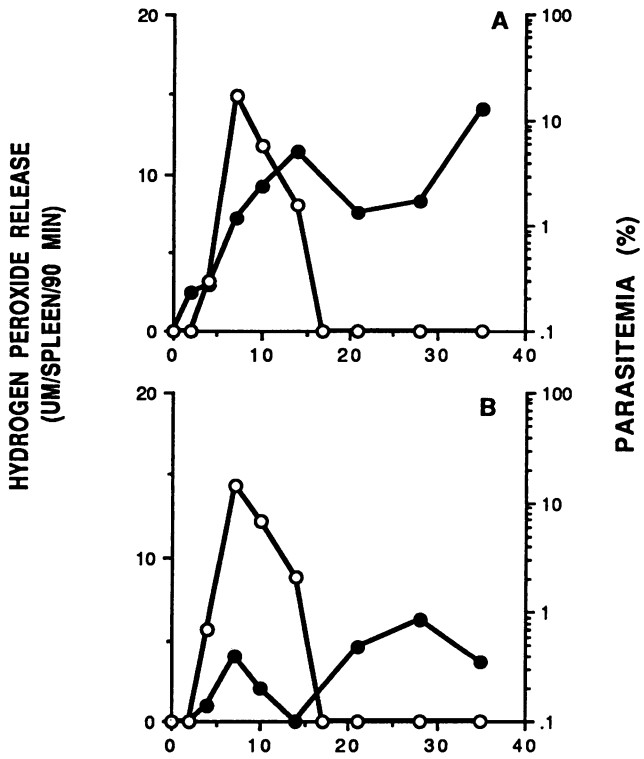

FIG. 3. Hydrogen peroxide release during *P. chabaudi adami* infection. The release of hydrogen peroxide (●) by spleen cells from BALB/c mice (A) and P/J mice (B) was determined following infection with 10^5 *P. chabaudi adami*-parasitized erythrocytes. Kinetics of parasitemia are also shown (○). For each time point, background values for the splenocytes of uninfected mice were subtracted from experimental values. Results are the mean values from two experiments using two to four mice per group.

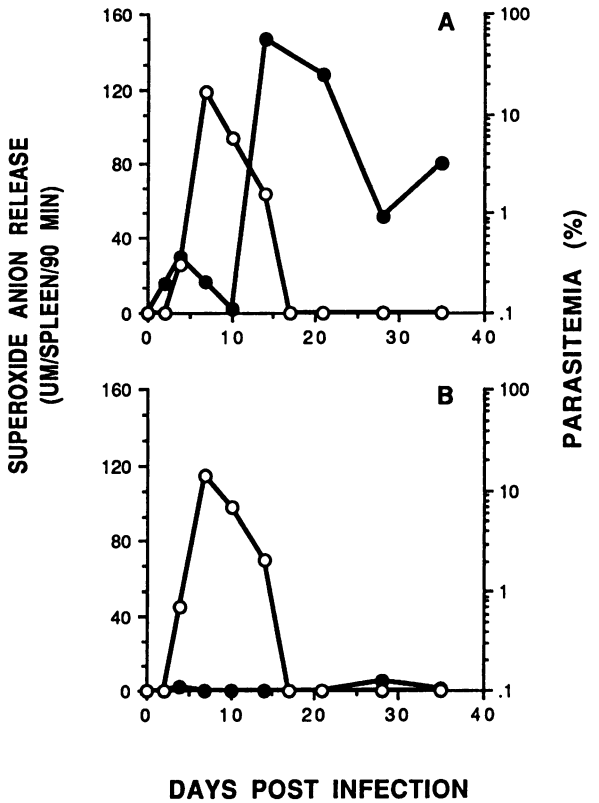

FIG. 4. Release of superoxide anion during *P. chabaudi adami* infection. Superoxide anion release (●) by spleen cells from BALB/c (A) and P/J mice (B) was examined following infection with 10^5 *P. chabaudi adami*-parasitized erythrocytes. Parasitemia is also shown (○). For each time point, background values for the splenocytes of uninfected mice were subtracted from experimental values. Results are the means of two experiments using two to four mice per group.

rophages also failed to kill intracellular *Leishmania tropica* (C. A. Nacy, A. L. Haverty, and P. L. Russell, Fed. Proc. **40**:1114, 1981; C. A. Nacy and M. S. Meltzer, Fed. Proc. **41**:730, 1982). Moreover, splenic macrophages from P/J mice were shown to be defective in tumoricidal activity (2). These defects in macrophage function were attributed to an inability of the cells to respond to activation stimuli as well as an impaired ability of the spleen cells of P/J mice to produce activating stimuli. Interestingly, an avirulent strain of *Salmonella typhimurium*, but not *Mycobacterium bovis* BCG, was reported to activate macrophages from P/J mice for tumor cytotoxicity and leishmaniacidal capacity, indicating that defective macrophage function can be overcome through the use of selective modulating agents (R. Shafer, M. T. Largon, C. A. Nacy, and T. K. Eisenstein, Proc. 6th Int. Congr. Immunol., abstr. no. 5.27.21, 1986). In the present study, P/J mice resolved acute *P. chabaudi adami* infections with parasitemia kinetics similar to those observed in BALB/c mice infected with the same parasite. To determine whether infection provided a stimulus capable of overcoming the macrophage activation defect of the P/J mouse, we compared the oxidative activity of splenic leukocytes derived from infected BALB/c and P/J mice with that of splenic leukocytes from uninfected BALB/c and P/J mice.

When luminol-dependent chemiluminescence responses were measured during acute *P. chabaudi adami* infection, splenic leukocytes from BALB/c mice, in contrast to those from P/J mice, generated significantly greater responses (approximately 100-fold) when stimulated with PMA. Peak chemiluminescence was observed prior to peak parasitemia. Others have noted an increase in chemiluminescence responses from splenic adherent cells (5) and peritoneal exudate cells (17, 18) during infection with *P. yoelii* and *P. berghei*, respectively. In these instances, enhanced chemiluminescence was associated with decreasing parasitemia. Although chemiluminescence is a result of light emission when oxidizing agents such as superoxide anion and hydrogen peroxide interact with ingested particles (7), the use of chemiluminescence as a measure of oxidative metabolism remains controversial (19). Therefore, to assess further the oxidative activity of splenic leukocytes derived from P/J mice infected with *P. chabaudi adami*, we measured the ability of these leukocytes to produce superoxide anion and hydrogen peroxide following stimulation with PMA at different times during the course of infection. Splenic leukocytes obtained from both infected and uninfected BALB/c mice served as controls. Whereas the release of superoxide anion by splenic leukocytes from BALB/c mice was markedly enhanced by infection, a finding consistent with the findings of others, PMA-induced superoxide anion release from P/J splenic leukocytes remained essentially unchanged by infection. PMA-induced release of superoxide anion by splenic leukocytes was similar in uninfected BALB/c and P/J mice. There was therefore no correlation between superox-

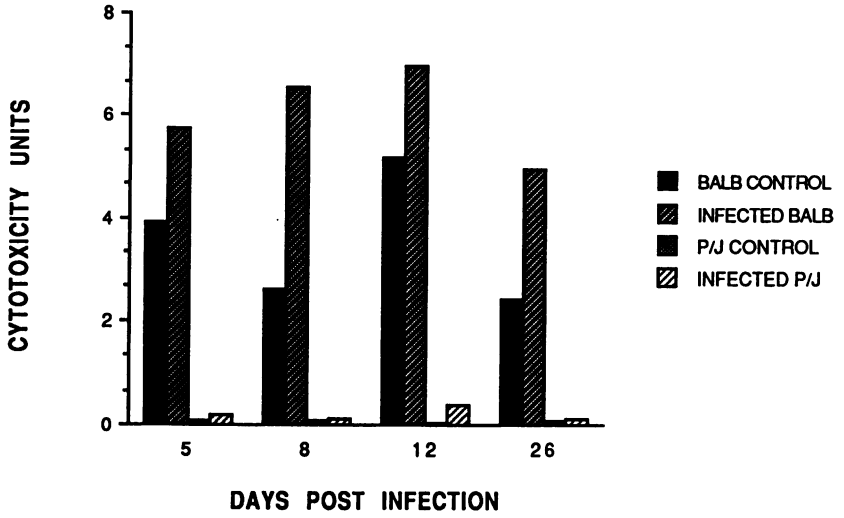

FIG. 5. Macrophage-induced tumor cytotoxicity during malarial infection. L929 cells were added to splenic macrophages isolated from infected BALB/c or P/J mice. Results are expressed as cytotoxic units per spleen. The responses of four mice were determined for each time point. Results are the means of two experiments.

ide anion production and the ability of the mice to clear parasite infection.

Splenic leukocytes obtained from uninfected BALB/c or P/J mice released small amounts of hydrogen peroxide when induced with PMA (<2 μM per spleen per 90 min). In contrast, hydrogen peroxide release by the splenic leukocytes from infected BALB/c mice (but not by those from P/J mice) was significantly enhanced during *P. chabaudi adami* infection (Fig. 3). These results indicate that, while release of oxidative metabolites known to inhibit parasite growth in vitro (27) can be observed during malarial infections, the generation of oxygen radicals by splenic leukocytes does not correlate directly with the development of protective immunity.

The experiments described up to this point utilized splenic leukocytes consisting of a mixture of polymorphonuclear leukocytes, macrophages (or possibly monocytes), null cells, and lymphocytes. While there is no doubt that the oxidative activity of splenic leukocytes from malaria-infected P/J mice is substantially less than that of splenic leukocytes from infected BALB/c mice, the question remained as to whether *P. chabaudi adami* infection can activate splenic macrophages of P/J mice. Whereas splenic macrophages from infected BALB/c mice showed enhanced cytotoxicity for L929 cells when compared with splenic macrophages from uninfected controls, the splenic macrophages of P/J mice displayed little tumor cytotoxicity, regardless of their infection status. Thus, while infection with *P. chabaudi adami* failed to activate splenic macrophages of P/J mice as measured by this parameter, the mice were clearly capable of resolving acute infection. How they accomplish this remains to be determined. The use of P/J mice in future experiments may serve to uncover novel as well as important mechanisms of resistance which, in other infection models, may be obscured by the oxidative and cytotoxic activities of macrophages.

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