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Chemiluminescence Response of Peritoneal Macrophages to Parasitized Erythrocytes and Lysed Erythrocytes from *Plasmodium berghei*-Infected Mice

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The chemiluminescence response of normal mouse peritoneal macrophages to parasitized erythrocytes isolated from mice 3 weeks after infection with *Plasmodium berghei* was examined. Only 4 of 12 animals showed positive responses, whereas 8 showed negative responses. Photomicrographs revealed that only in chemiluminescence-positive animals were parasitized erythrocytes attached to or phagocytized by macrophages. When lysed parasitized-erythrocyte cell suspensions were added to the peritoneal macrophages, chemiluminescence could be induced in all cases. The response was enhanced remarkably by the addition of very small amounts of immune serum. Normal macrophages activated in vitro by supernatant from antigen-stimulated spleen cells from immune mice showed much higher parasite-induced chemiluminescence responses than did nonactivated macrophages, especially in the presence of immune serum.

Mice which have recovered from plasmodial infection show a strong resistance to reinfection for some time. The mechanism of this acquired immunity has not, however, been completely elucidated, because plasmodial infection induces very complicated immune responses. Phagocytosis of free parasites or parasitized erythrocytes by macrophages has been recognized as playing a crucial role in the defense of the host during malarial infection, particularly when associated with acquired immunity (5). Peritoneal macrophages from immune mice show a more efficient increase in phagocytosis of parasitized erythrocytes, which is enhanced in the presence of immune serum (11).

In the past decade, it has been revealed that the production of activated-oxygen species of macrophages is associated with their phagocytic and cytotoxic activities (6, 13). Luminol-aided chemiluminescence (CL) measurement is a very sensitive and simple method for the detection of activated-oxygen species generated by phagocytes (1).

In the experiments described here, we examined the CL response of macrophages to parasitized erythrocytes or free parasites in the presence and absence of antiplasmodial immune serum. In addition, macrophages activated in vitro with antigen-stimulated immune spleen cell supernatant were tested for their potential to induce CL.

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MATERIALS AND METHODS

Animals. Male C57BL/10 mice, 6 to 10 weeks of age, were used. They were bred in our own specific pathogen-free colony.

Parasites. The *Plasmodium berghei* strain was obtained from Behringwerke AG, Marburg, West Germany. This strain kills adult C57BL/10 mice 3 to 4 weeks after intraperitoneal injection of 10^6 parasitized erythrocytes.

Malaria-immune mice. Immunity in mice was induced by using the drug-cure method previously described (11). In this experiment, pyrimethamine (Daraprim; Burroughs Wellcome Co., Research Triangle Park, N.C.) was used as the curing drug.

Immune serum. Immune serum was obtained from immune mice 2 weeks after challenge with 10^6 parasitized erythrocytes, inactivated at 56°C for 30 min, and stored at −70°C. Before use, a portion of the serum was diluted 1:10 with phosphate-buffered saline (PBS) and centrifuged at $10^5 \times g$ for 60 min.

Parasitized erythrocytes. Infected blood was obtained from mice 3 weeks after intraperitoneal infection of 10^6 parasitized erythrocytes. The blood was collected in PBS-heparin (5 μg/ml), washed once with cold PBS, and then passed through a cellulose powder column with Eagle medium buffered with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4) to remove leukocytes and platelets (3). The eluted erythrocyte suspension (5 ml) was overlaid on 2 ml of Percoll at a density of 1.076 g/ml (15). After centrifugation at room temperature (1,300 × *g*, 20 min), the top layer was collected. After washing, the cell concentration was adjusted to make a 5% suspension of parasitized erythrocytes.

Lysed parasitized-erythrocyte suspension (free parasites). A 20-μl portion of the separated parasitized-erythrocyte pellet was mixed with 0.25 ml of chilled

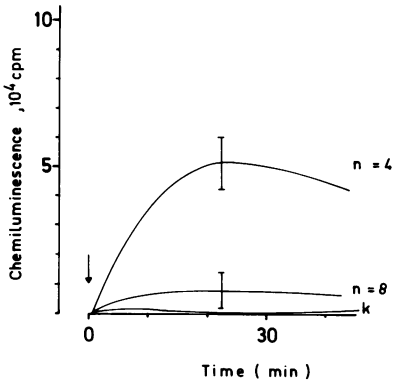

FIG. 1. CL response of normal peritoneal macrophages after the addition of parasitized erythrocytes. A 10-μl amount of 5% parasitized erythrocytes was added to each vial containing 4×10^5 macrophages and 20 μg of luminol on 0.5 ml of Eagle medium buffered with HEPES (pH 7.4) at 37°C. Parasitized erythrocytes from four animals induced a high CL response, and parasitized erythrocytes from another eight animals induced only a weak CL response. k, Control.

0.2% NaCl solution for 20 s, and then an equal volume of 1.6% NaCl solution was added.

Supernatant of antigen-stimulated spleen cell culture. Spleen lymphocytes from immune or normal mice were separated from disrupted spleens by a method using Ficoll-Hypaque (4). The cells were suspended in RPMI 1640 with 5% inactivated fetal calf serum and adjusted to a concentration of 10^7 cells per ml. The cell suspension was incubated with lysed parasitized-erythrocyte suspension (10 μl/ml) for 24 h at 37°C in 10% CO_2.

Macrophages. Peritoneal macrophages were collected from unstimulated normal mice by washing the peritoneal cavity with cold PBS-heparin. The cells were used for CL assays and morphological observations of phagocytosis.

CL measurement. Peritoneal macrophages (4×10^5) were cultivated for 24 h in 0.5 ml of RPMI 1640 containing 15% fetal calf serum and 5% supernatant from immune or normal spleen cell culture in plastic vials in a CO_2 incubator. After 24 h of cultivation, the culture medium was exchanged with 0.5 ml of Eagle medium buffered with HEPES and preincubated for 30 min at 37°C. Then, 10 μl of luminol solution (2 mg/ml) was added to each vial, and background CL was recorded. Ten minutes later, 10 μl of parasitized erythrocytes or lysed parasitized-erythrocyte suspension was added to each vial, and the CL of six samples was measured simultaneously and continuously in a Biolumat (LB 9505; Berthold Co., Wildbad, West Germany) according to a method described previously (2, 7). In some experiments, 5 μl of diluted immune or normal serum was added 10 min before the addition of lysed parasitized-erythrocyte suspension.

Morphological observation of phagocytosis. Peritoneal macrophages (10^6) were plated in multidish trays (3041; Falcon Plastics, Oxnard, Calif.) each containing a round slip and were cultivated for 24 h in a 10% CO_2 incubator. Then, 50 μl of parasitized erythrocytes was added to each dish containing a macrophage monolayer. After incubation for 45 min, the cover slips were rinsed in PBS and stained with a Giemsa stain solution for microscopic observation.

RESULTS

Normal resident macrophages exposed to parasitized erythrocytes showed positive CL responses increasing to a peak at 20 min and then declining gradually (Fig. 1). In eight cases the responses were weak and not convincing. Normal erythrocytes used in control experiments induced no response.

These CL responses were confirmed by a photomicrograph which showed many parasitized erythrocytes attached to or phagocytized by macrophages (Fig. 2a) in the case of appreciable CL. In contrast, animals showing weak CL, parasitized erythrocytes were practically not attached to or phagocytized by macrophages (Fig. 2b).

When lysed parasitized-erythrocyte suspension was added to peritoneal macrophages, a high CL response was always observed. The response was enhanced remarkably by the addition of immune serum. Figure 3 shows the effects of the addition of various dilutions of heat-inactivated immune serum on the CL response induced by lysed parasitized-erythrocyte suspension. The CL response was optimal with a 1:4,000 dilution and lower with either a higher or lower dilution of serum. The plasmodial immunoglobulin G antibody titer of the immune serum was 1:5,000, as measured by the indirect immunofluorescence assay (16).

Macrophages activated in vitro by antigen-stimulated immune spleen cell supernatant showed a much higher free parasite-induced CL response in the presence of immune serum than did nonactivated macrophages or macrophages treated with control spleen cell supernatant from normal mice (Fig. 4).

DISCUSSION

In the first series of experiments, some parasitized erythrocyte preparations taken from mice 3 weeks after infection induced appreciable CL from normal macrophages, but other preparations failed to do so; free parasites induced CL in any case. Since normal erythrocytes did not induce any response, it is reasonable to assume that CL induced by parasitized erythrocytes might be dependent on the presence of parasite-derived antigen or immune complexes on the surfaces of parasitized erythrocytes. In the first stage of an infection, parasitized erythrocytes bearing antigen or immune complexes on their surfaces might be rapidly removed by the reticuloendothelial system of the spleen and liver, whereas 10 to 20% of the infected reticulocytes

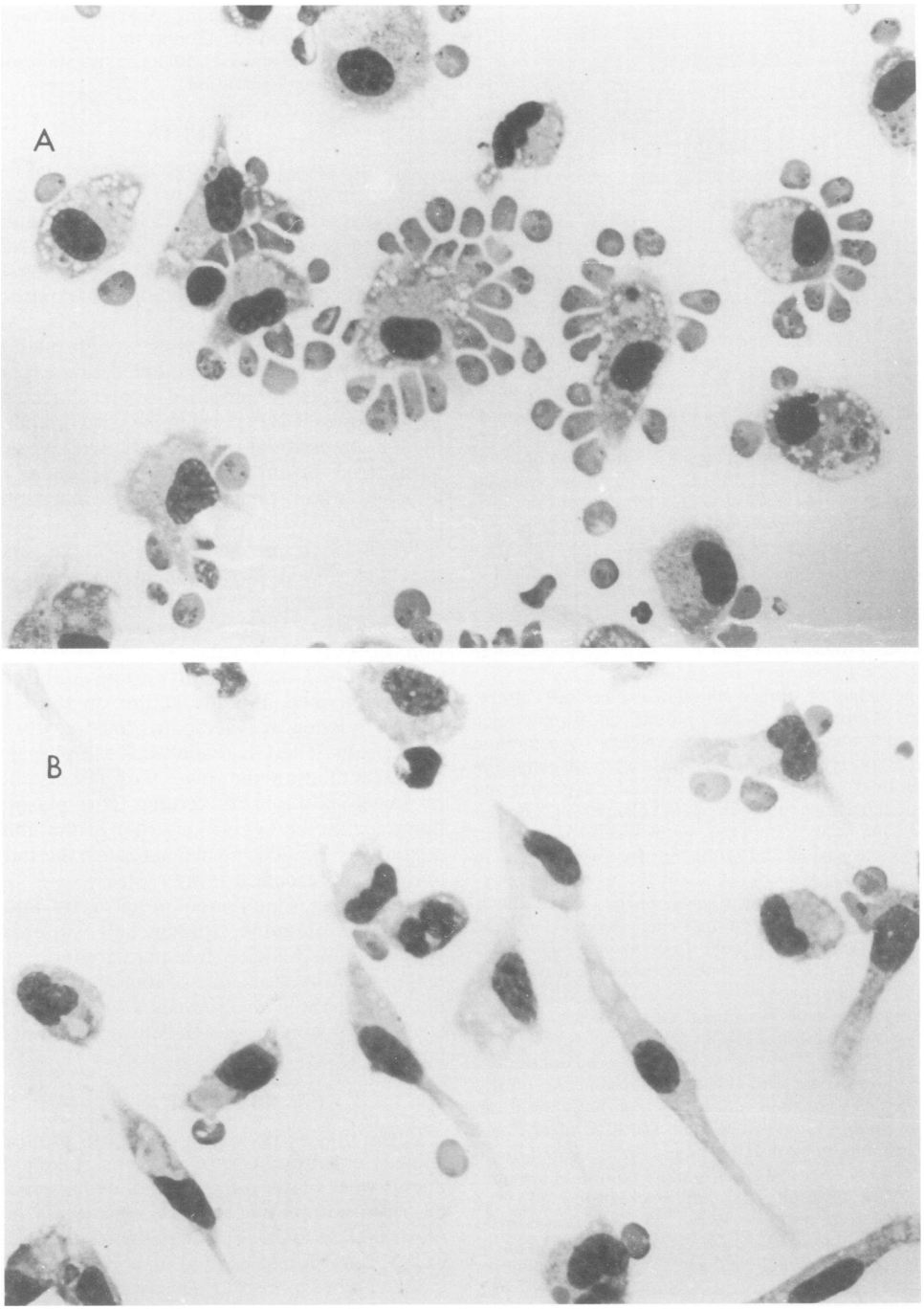

FIG. 2. (A) Macrophage cultures which developed appreciable CL after the addition of parasitized erythrocytes. After incubation for 45 min, the cover slips were stained with Giemsa stain. Many parasitized erythrocytes were attached to or phagocytized by macrophages. ×486. (B) Macrophage cultures which developed weak CL. Only a few parasitized erythrocytes can be observed attached to the macrophages.

from mice in the last stage of *P. berghei* infection possess plasmodial antigens on the erythrocyte membrane (14). The decrease in the clearance of foreign particles from circulating blood during the last stage of infection has been described elsewhere (9). The deficiency in reticulo-

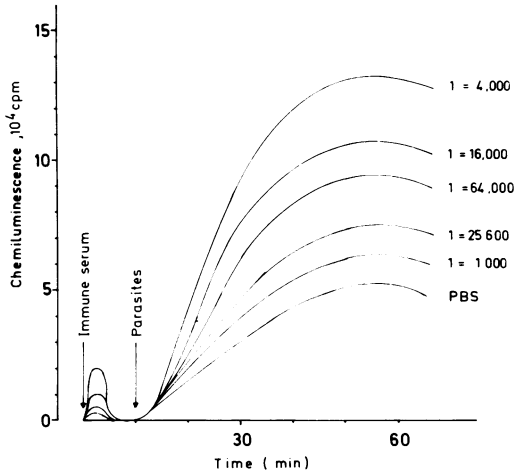

FIG. 3. Effects of the addition of various concentrations of heat-inactivated immune serum on the CL responses of normal peritoneal macrophages to lysed parasitized-erythrocyte suspension (free parasites). Diluted immune serum (5 μl) was added 10 min before the addition of lysed parasitized-erythrocyte suspension.

endothelial system phagocytic function at this stage might be associated with our recent observation that, contrary to normal spleen cells, malaria-infected spleen cells practically do not respond to zymosan in the CL test (8). Thus, antigen- or immune complex-possessing parasitized erythrocytes might appear in the circulating blood of some mice only at the last stage of infection.

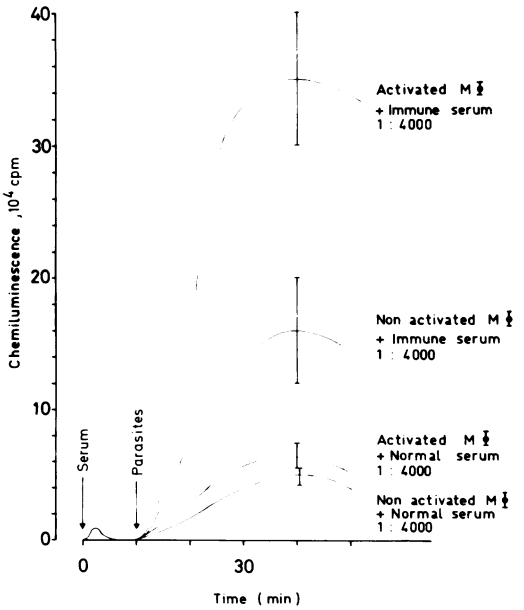

FIG. 4. Free-parasite-induced CL of activated and nonactivated macrophages (Mϕ) in the presence of immune or normal serum.

It has to be considered that different kinds of parasite stages occur in suspensions prepared by hypotonic treatment of parasitized erythrocytes and that the immune serum used might contain antibodies against these different stages of parasites. It has been reported that trophozoites in free-parasite preparations are adherent to and phagocytized by macrophages in the presence of either normal or immune serum. In contrast, merozoites are adherent to and phagocytized by macrophages exclusively in the presence of immune serum (10). In our experiment, the CL induced with free parasites and normal serum might be due to trophozoites or schizonts in the preparation.

The fact that a very small amount of immune serum was found to remarkably enhance free-parasite-induced CL suggests that specific antibodies play an important role in malaria protective immunity, not only by their inhibition of the penetration of merozoites into erythrocytes (12) but also by their opsonizing capacity as a prerequisite for phagocytosis.

It has been shown that peritoneal macrophages from malaria-immune mice are in some way activated for increased phagocytosis of parasitized erythrocytes in the presence of immune serum (11). Furthermore, these cells have increased myristate-induced superoxide anion-generating capacity (S. Makimura and N. Suzuki, Jpn. J. Vet. Sci., in press). We extended these observations. Normal resident macrophages were activated by the supernatant of immune, but not normal, spleen cells cultured in the presence of parasitic antigens, so that an enormous increase in the CL response to the parasites occurred. This finding suggests the production of lymphokines by immune spleen cells which stimulate macrophages to a higher CL response in the presence of immune serum. This response may be correlated to the high phagocytic and cytotoxic capacities of the activated cells (13).

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