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Role of Lymphocytes in Macrophage-Induced Killing of *Coccidioides immitis* In Vitro

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Peritoneal macrophages from normal mice phagocytized arthroconidia and endospores of *Coccidioides immitis* without affecting the viability of the spores within 4 h after infection. In contrast, macrophages, when infected in the presence of lymphocytes from immune mice, significantly reduced the viability of phagocytized endospores and arthroconidia. The inability of macrophages from normal mice to kill *C. immitis* may in part be explained by the observation that *C. immitis* appeared to inhibit fusion of the phagosomes containing fungal spores with the lysosomes within the macrophages. However, fusion of phagosomes containing spores and lysosomes was observed in macrophages infected in the presence of lymphocytes from immune mice.

After inhalation into the lung, the arthroconidia of *Coccidioides immitis* develop into multinucleate spherical sporangia (spherules) which later release numerous uninucleate endospores. Phagocytized arthroconidia which develop into spherules have been observed within macrophages from infected mice (21). Fewer spherules were observed in the alveolar macrophages found in lung washings from immunized mice than from nonimmunized mice after challenge with arthroconidia (21), indicating that possible killing of arthroconidia or immature spherules may occur in macrophages from immune animals. It is not clear how the spores of *C. immitis* survive in the macrophages in a normal animal. However, in a quantitative evaluation of the survival of both arthroconidia and endospores in alveolar macrophages from normal rhesus macaques (*Macaca mulatta*), Beaman and Holmberg found that both forms of *C. immitis* inhibited phagosome-lysosome fusion. The surviving spores developed into spherules (4).

Peritoneal macrophages from guinea pigs, when infected in the presence of lymphocytes from immune animals, phagocytized arthroconidia, and it was thought that the lymphocytes elaborated fungicidal factors which decreased the viability of the phagocytized spores (15). Beaman et al. demonstrated that T lymphocytes from immune mice were essential for the transfer of resistance to a lethal infection to a normal host (5); however, the cells which actually kill *C. immitis* in immune mice were not defined. The present communication describes the interaction of murine macrophages, lymphocytes, and *C. immitis* in vitro. The results presented herein demonstrate that only in the presence of lymphocytes from immune mice were macrophages able to significantly reduce the viability of phagocytized *C. immitis* endospores and arthroconidia.

MATERIALS AND METHODS

Cultures and vaccine. The spherule-endospore phase of *C. immitis* (strain Silveria) was grown in modified Converse medium (17), and the endospores were harvested after 60 h of incubation. Arthroconidia were harvested from slants of glucose-yeast extract agar and were stored in distilled water until used.

Mature endosporulating spherules were harvested after 40 h of incubation in modified Converse medium. For the preparation of vaccines, formaldehyde was added to the spherule suspensions to a final concentration of 0.18%. The suspensions were kept at 4°C for 8 days. The spherules were then washed and suspended at 10 mg (dry weight) per ml of phosphate-buffered saline, pH 7.2, for use as a vaccine. Merthiolate was added to a final concentration of 1:10,000 (wt/vol).

Candida krusei was maintained on glucose-yeast extract slants. The experimental inoculum was grown in tryptose soy broth, centrifuged, and suspended in phosphate-buffered saline.

Mice. Female DBA/2 mice (Timco Laboratories, Houston, Tex.) weighing 18 to 22 g were used as a source of peritoneal macrophages.

Mice were immunized with three intramuscular injections of 1 mg (in 0.1 ml) of the Formalin-killed spherule suspension at days 1, 10, and 14. At 30 days after the last injection, the mice were given a booster injection of 1 mg of spherules, and 7 to 10 days later these mice were used as donors of immune peritoneal macrophages or lymphocytes.

Preparation of lymphocytes and macrophages. Peritoneal cells were obtained by injecting

mice intraperitoneally with 2.5 ml of phosphate-buffered saline and aseptically removing the exudate. The cells were centrifuged and suspended to a cell concentration of 2×10^6 per ml in growth medium consisting of medium 199 with 25% fetal calf serum, 100 U of penicillin per ml, and 100 mg of streptomycin per ml. One milliliter of the cell suspension was cultured on 22-mm^2 cover slips in 35-mm petri dishes for 60 min at 37°C in an atmosphere of 5% CO_2. The cells that did not adhere to glass were removed, centrifuged, and suspended in growth medium as a source of peritoneal lymphocytes. The adherent cells were washed three times before infection to remove all nonadherent cells. Spleen cell suspensions were prepared from individual organs by mincing and pressing the tissue through a 40-gauge stainless-steel screen. The cells were centrifuged, suspended in 0.87% NH_4Cl for 10 min to lyse the erythrocytes, and diluted in growth medium for use as splenic lymphocytes.

Infection of macrophages. A total of 0.9 ml of growth medium and 0.1 ml of spore suspension were added to each macrophage monolayer and separately to sterile petri dishes. The latter served as an untreated control for the viability of the spore inoculum. In some cases 0.8 ml of growth medium plus 0.1 ml of 10^8 splenic or of 10^7 peritoneal lymphocytes and 0.1 ml of diluted spores were added. The dishes were rocked gently on a rocker platform (Bellco Glass, Inc., Vineland, N.J.) for 4 h at 37°C in an atmosphere of 5% CO_2. The culture supernatants with unphagocytized spores were then removed to a tube, and the macrophage monolayer was washed twice in 1 ml of distilled water, which was added to the culture supernatant. The macrophage monolayers were overlaid with 0.8 ml of distilled water–0.2 ml of 2.5% sodium deoxycholate for 10 min to lyse the macrophages and to liberate phagocytized spores. The lysate from each plate was diluted in 9 ml of distilled water. The dilutions were mixed in a Vortex mixer, and 0.1 ml was streaked on the surface of tryptic soy agar plates (Difco Laboratories, Detroit, Mich.). The control spore suspension was also diluted in 9 ml of distilled water, mixed in a Vortex mixer, and plated on tryptic soy agar. The plates were incubated at 37°C for 72 h, and the colony-forming units (CFU) were counted. The CFU found in the culture supernatant were subtracted from the CFU found in the control spore suspension, divided by the total CFU in the inoculum, and multiplied by 100 to determine the percent inoculum phagocytized by the macrophages. The proportion of spores killed by the macrophages was determined by comparing the loss of viable CFU in the macrophage cultures with the CFU in the control spore suspension. The mean ± the standard error of the mean was determined from two samples in three different experiments.

Lysosome straining with acridine orange. The macrophage monolayers were washed in balanced salt solution to remove serum components. Acridine orange (5 μg/ml) was added, and the cells were incubated for 10 to 15 min. The cover slips were washed free of acridine orange, and fresh medium was added before infection with *C. immitis* or *C. krusei* cells. At 4 h after infection, the cover slips were washed, air dried, mounted on slides, and examined for evidence of fusion of the orange-colored lysosomes with the phagocytized coccidioides or candida as previously described (1, 4). The number of infected macrophages, the number of intracellular organisms, and the number of intracellular organisms stained orange were counted. The number of intracellular organisms stained orange was divided by the total number of intracellular organisms observed and multiplied by 100 to give the percent phagocytized organisms showing evidence of phagosome-lysosome fusion. The mean ± the standard error of the mean was obtained by counting a minimum of 100 macrophages on two cover slips from three experiments.

RESULTS

Infection of macrophage cultures. The peritoneal exudate cells obtained from normal or immune mice were allowed to adhere to glass cover slips for 1 h before the non-glass-adherent cells were washed off and *C. immitis* was added. In some experiments, splenic lymphocytes or peritoneal lymphocytes (non-glass-adherent cells) were added to the macrophage cultures. The percent inoculum phagocytized in three different experiments is summarized in Table 1. On the basis of the proportion of the inoculum recovered from the culture supernatant, 70 to 80% of the spores had been phagocytized. There was no significant increase in phagocytosis of arthroconidia when serum from immunized mice was present. The addition of lymphocytes from immune mice to the macrophage cultures did not enhance the uptake of endospores or arthroconidia.

Quantitation of infection. In three different experiments, the percent phagocytized arthroconidia or endospores that were killed in 4 h was determined from six samples. When arthroconidia or endospores were incubated with splenic lymphocytes, 93 to 98% of the inoculum was recovered (Fig. 1 and 2, bars A and B). The addition of normal macrophages to normal splenic lymphocytes did not result in significant killing of arthroconidia since 98% of the spores were recovered.

Macrophages from immune mice killed only 10 ± 6% of the phagocytized arthroconidia (Fig. 1, bar G). The presence of normal splenic lymphocytes did not enhance the killing of arthroconidia (10 ± 8%). After the addition of splenic lymphocytes from immune mice, 37 ± 3% of the phagocytized arthroconidia were killed (Fig. 1, bar I).

Macrophages from immune mice killed 25 ± 5% of the phagocytized endospores (Fig. 2). However, in the presence of splenic lymphocytes from immune mice, more of the phagocytized endospores were killed (41 ± 4%). Thus, whereas macrophages from normal or immune mice were

comparable in their phagocytic activity, the killing of *C. immitis* was facilitated markedly by the presence of immune lymphocytes.

In some experiments, peritoneal lymphocytes were used instead of splenic lymphocytes. A mixture of peritoneal lymphocytes and macrophages from immune mice resulted in the killing of 41 ± 4% of the phagocytized arthroconidia (Fig. 3, bar A). When peritoneal lymphocytes from immune mice were added to macrophages from normal mice, 46 ± 7% of the phagocytized arthroconidia were killed (bar E); in contrast, only 2% of the phagocytized arthroconidia were killed by normal macrophages in the presence of normal lymphocytes (bar C). Macrophages from immune mice when cultured with peritoneal lymphocytes from immune mice killed 50 ± 5% of the phagocytized endospores (bar B). The presence of peritoneal lymphocytes from immune mice in normal macrophage cultures resulted in the killing of 50 ± 9% of the phagocytized endospores (bar F). Normal macrophages with normal lymphocytes killed 15 ± 10% of the phagocytized endospores (bar D).

TABLE 1. *Phagocytosis of C. immitis by murine peritoneal macrophages in the presence of immune or normal lymphocytes*

Inoculum	Source of:		% Inoculum phagocytized[a]
	Lymphocytes	Macrophages	
Arthroconidia	Normal spleen	Normal PEC[b]	74 ± 3
Arthroconidia	Not present	Immune PEC	76 ± 6
Arthroconidia	Immune spleen	Immune PEC	77 ± 3
Arthroconidia	Immune PEC	Immune PEC	79 ± 1
Arthroconidia	Immune PEC	Normal PEC	79 ± 4
Endospores	Normal spleen	Normal PEC	71 ± 4
Endospores	Not present	Immune PEC	71 ± 2
Endospores	Immune spleen	Immune PEC	72 ± 2
Endospores	Immune PEC	Immune PEC	67 ± 2
Endospores	Immune PEC	Normal PEC	74 ± 4
Endospores	Immune spleen	Normal PEC	72 ± 4
Arthroconidia	Immune spleen with serum from immune mice	Immune PEC	76 ± 2

[a] Peritoneal exudate cells (PEC) were obtained from normal or immune DBA/2 mice. The mice were immunized with 3 mg of Formalin-killed spherules, held for 30 days, and given a final injection of 1 mg of killed spherules 7 to 10 days before use. The non-glass-adherent cells were termed lymphocytes.

[b] The number of CFU recovered from the macrophage culture medium was subtracted from the number of CFU recovered from the inoculum (diluted in medium and incubated for 4 h) to determine the number of spores phagocytized by the macrophages. The mean obtained from two samples in three different experiments is presented.

Phagosome-lysosome fusion. In three different experiments, the percent phagocytized spores showing evidence that phagosome-lysosome fusion had occurred was determined in six samples of a minimum of 100 macrophages counted per sample. *C. krusei* was used as a control. After macrophages were incubated with acridine orange for 15 min, washed in phosphate-buffered saline, and infected with *C. krusei*, 72 to 83% of the observed phagocytized yeast cells were stained with acridine orange, indicating the occurrence of fusion between the phagosomes containing yeast cells and the lysosomes containing acridine orange (Fig. 1 and 2). When

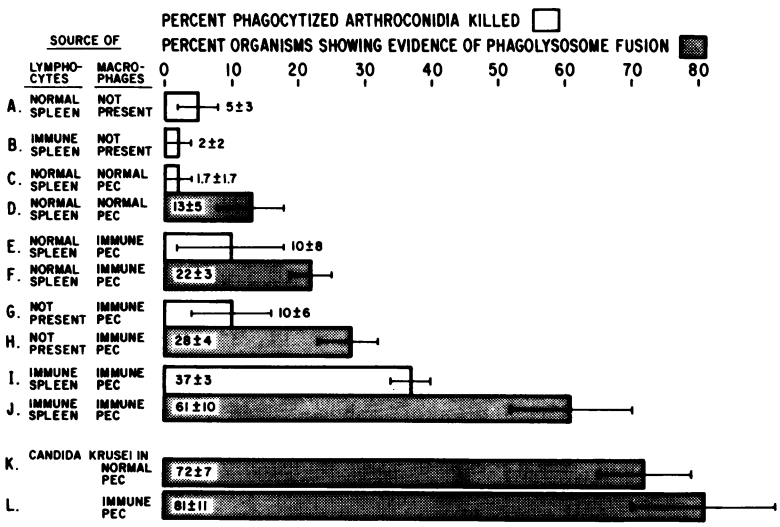

FIG. 1. *Percent phagocytized arthroconidia killed and percent organisms showing evidence of phagolysosome fusion 4 h after infection of macrophages from normal or immune mice in the presence of normal or immune lymphocytes. PEC, Peritoneal exudate cells.*

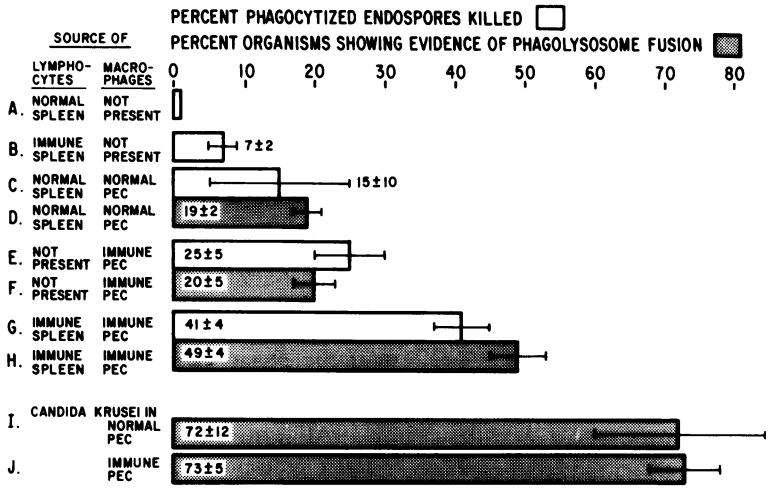

FIG. 2. *Percent phagocytized endospores killed and percent organisms showing evidence of phagolysosome fusion 4 h after infection of macrophages from normal or immune mice in the presence of normal or immune lymphocytes. PEC, Peritoneal exudate cells.*

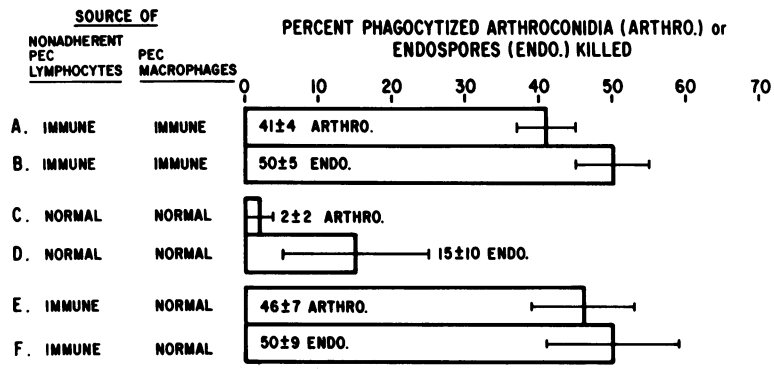

FIG. 3. *Percent phagocytized arthroconidia or endospores killed 4 h after infection of macrophages from normal mice in the presence of lymphocytes from immune mice. PEC, Peritoneal exudate cells.*

normal macrophages were infected with arthroconidia, fusion of the phagosomes and lysosomes could be seen with only 13 ± 5% of the phagocytized spores. With immune macrophages alone, phagosome-lysosome fusion was observed with 28 ± 4% of the phagocytized arthroconidia. A significant increase in phagosome-lysosome fusion to 61 ± 10% was observed when splenic lymphocytes from immune mice were added to immune macrophages (Fig. 1, bar J). Similar observations were made when macrophages were infected with endospores. The addition of splenic lymphocytes from immune mice to immune macrophages resulted in a significant increase in the number of endospores, showing that phagosome-lysosome fusion had increased from 20 ± 3% with immune macrophages alone to 49 ± 4% with immune macrophages and immune lymphocytes (Fig. 1, bars F and H).

With 52 ± 10% of the arthroconidia phagocytized by immune macrophages in the presence of immune peritoneal lymphocytes, evidence that phagosome-lysosome fusion had occurred was observed (Fig. 4). When the immune peritoneal lymphocytes were first incubated with monoclonal anti-mouse Thy 1.2 serum (New England Nuclear Corp., Boston, Mass.) and guinea pig complement and then added to the immune macrophages before injection with arthroconidia, only 26 ± 3% (Fig. 4, bar C) of the phagocytized spores showed evidence of phagosome-lysosome fusion. The presence of either immune peritoneal (Fig. 4, bar E) or splenic (Fig. 4, bar H) lymphocytes in normal macrophage cultures at the time of infection with arthroconidia resulted in a significant increase in phagosome-lysosome fusion as compared with that seen when macrophages and normal lympho-

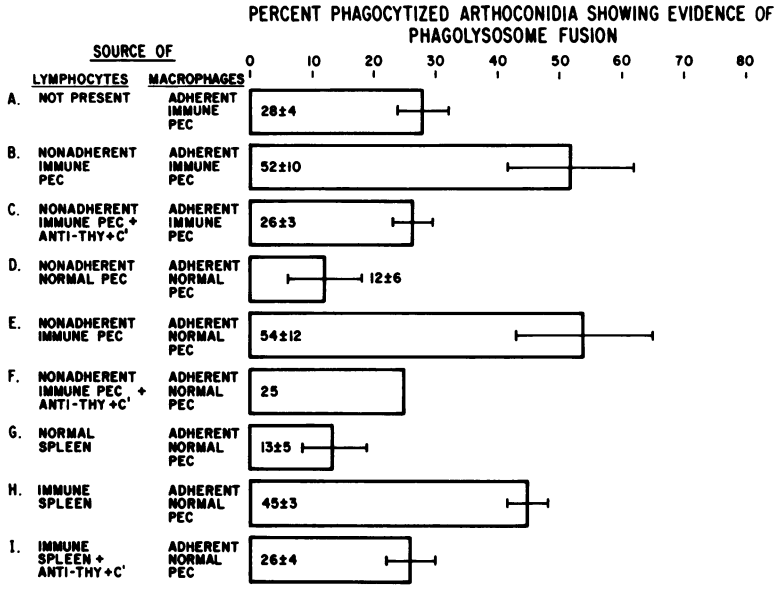

FIG. 4. *Percent phagocytized arthroconidia showing evidence of phagolysosome fusion 4 h after infection of macrophages in the presence of lymphocytes and T-cell-depleted lymphocyte populations from immune mice. PEC, Peritoneal exudate cells; C', Complement.*

cytes were infected (Fig. 4, bars D and G). The effect of the immune lymphocytes was abrogated by treatment with anti-mouse Thy 1.2 plus guinea pig complement (Fig. 4, bars F and I).

DISCUSSION

The present results affirm the importance of immune lymphocytes in the favorable response of mice to infection with *C. immitis*. The lymphocytes accomplish this by promoting an antifungal action of macrophages. Although macrophages appear to be capable of efficiently phagocytizing *C. immitis* arthroconidia and endospores, only in the presence of immune lymphocytes was the viability of the fungal cells impaired.

Within macrophages from normal mice, both arthroconidia and endospores appear to inhibit phagosome-lysosome fusion, which may in part explain the inability of macrophages to kill *C. immitis*. A significant increase in phagosome-lysosome fusion was observed in macrophages in the presence of lymphocytes from immune mice, which correlates with the ability of these cells to kill *C. immitis*. Since the lymphocyte response was significantly reduced by treatment with anti-Thy 1.2 plus guinea pig complement, apparently T lymphocytes are required to kill *C. immitis*. However, evidence that the T lymphocytes directly affected the spores was not obtained. This inability to demonstrate killing of *C. immitis* by lymphocytes conflicts with the earlier conclusions of Kashkin et al. (15), which suggested that factors fungicidal for *C. immitis* were present in supernatants from immune guinea pig lymphocytes. However, although there may be such a difference between the mouse and guinea pig systems, it is difficult to ascribe the effect observed by Kashkin et al. (15) simply to the immune guinea pig lymphocytes, since the buffy coat used by these workers likely contained other leukocytes present in the peripheral blood. There was no description of a specific separation of the leukocyte types. The presence of components of granulocytes or monocytes could have provided antifungal effects that led to the conclusion that killing of *C. immitis* was mediated by lymphocytic factors.

The present studies indicate that murine lymphocytes appear to affect *C. immitis* only through their effects on macrophages. Howard et al. (13) had similarly found that immune lymphocytes alone did not affect the growth of *Histoplasma capsulatum* in vitro, but when immune lymphocytes were combined with macrophages, inhibition of *H. capsulatum* was achieved. Previously sensitized lymphocytes subsequently exposed to the same sensitizing antigen have been shown to induce the activation of normal macrophages to inhibit multiplication of several different microorganisms (2, 12, 16, 18–20, 22). Borges and Johnson found that lymphocytes from toxoplasma-immune individ-

uals, when incubated with a specific antigen for as short a time as 15 min, resulted in the release of soluble factors capable of activating monocytes within 2 h (6). This is in agreement with the present study that immune lymphocytes incubated with fungal antigens activate normal macrophages within 4 h to kill *C. immitis*.

The effect of antibody on phagocytized *C. immitis* is not clear, since the mice do not produce high-titer antibody specific for coccidioidin. *Mycobacterium tuberculosis*, chlamydiae, *Rickettsia prowazekii*, *Toxoplasma gondii*, and *Nocardia asteroides* inhibit phagosome-lysosome fusion, but prior coating of the organisms with a specific antibody results in enhanced phagosome-lysosome fusion (3, 9, 10, 14; W. A. Meyer and C. L. Wisseman, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, D10, p. 39). However, in previous experiments, antibody reactive with coccidioidin in the presence of complement did not affect phagosome-lysosome fusion in macrophages infected with endospores (4).

It has been postulated that the ability of some organisms to inhibit phagosome-lysosome fusion either is the result of secretion of a product by the organism or is a reflection of some structural feature unique to the parasite (11). At least four classes of substances have shown an effect on the fusion of lysosomes and phagocytic vacuoles: cyclic nucleotides, concanavalin A, polyanionic agents, and weak acridine bases (11). In the case of *M. tuberculosis*, sulfatides or possibly other acidic lipids present in the cell wall may play such a role (11). Evidently there is no definition as yet of what fungal components may prevent phagosome-lysosome fusion. How the spores interact with phagosomes to prevent the subsequent fusion with lysosomes is now being studied in attempts to define substances which promote or deter fusion.

The present investigation confirms previous observations that phagocytic vacuoles containing the endospores or arthrospores of *C. immitis* do not readily fuse with lysosomes within macrophages. The presence of lymphocytes from immune animals in the macrophage culture resulted in enhanced fusion, as has been previously observed with *N. asteroides* (9), and in our investigation, fusion of the phagosomes and lysosomes correlated with the extent of fungicidal activity. Collins and Pappagianis found that lysozyme has a lethal effect in vitro on spherules (8); however, lysozyme secretion by macrophages apparently is not altered by activation of macrophages (7). Thus, future experimentation is required to determine how *C. immitis* is killed and to define the mechanism whereby lymphocytes activate macrophages.

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