Macrophage-Related Fibrinolysis in Experimental Disseminated Histoplasmosis

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A model of disseminated histoplasmosis in CBA/J mice was developed. Cultures of Histoplasma capsulatum from the spleens of infected mice suggested almost complete clearance of fungi by week 3. The adherent spleen cells from infected mice showed a 2- to 20-fold increase in fibrinolysis. The increase in activity was maximal around 1 to 2 weeks and disappeared after week 3 of infection, and this paralleled the progressively decreasing number of culturable fungi from the spleen. In vitro coculture of infected spleen cells or nylon wool-purified immune T cells and protease peptone-induced macrophages resulted in increased fibrinolysis. Peritoneal exudate cells from infected mice also showed increased fibrinolysis. The addition of soluble antigen to an in vitro culture system resulted not only in an increase in fibrinolytic activity of peritoneal exudate cells derived from infected mice but also of protease peptone-induced macrophages. These observations suggest that spleen and peritoneal macrophages from H. capsulatum-infected mice exhibit increased fibrinolysis which is a result of macrophage activation. The mechanism of activation occurs as a result of immunologically specific T cell-macrophage interaction and by the action of histoplasma products on the macrophages. The significance of these findings and the role of the plasminogen activator assay in studies of disseminated fungal infection are discussed.

Histoplasmosis varies clinically from asymptomatic infection to disseminated life-threatening disease. A major factor which determines the extent of infection is the immunologic status of the host (5, 6). Thus, patients who are immunosuppressed owing to drugs or disease are more prone to develop disseminated histoplasmosis than are normal hosts (6). Conversely, patients with disseminated histoplasmosis exhibit markedly impaired cell-mediated responses (16, 22). Data from animal studies have shown the critical importance of cell-mediated immunity in the eradication of Histoplasma capsulatum infection (11, 16).

An experimental model of histoplasmosis has shown disturbances in helper and suppressor cell activity within the splenic microenvironment of infected mice (2). These changes correspond to massive splenic infiltration by hystocytes and macrophages which suppress growth of H. capsulatum and result in fungicidal activity (1). Macrophage activation is obligatory in this sequence. Previous studies showed that immune lymphocytes from H. capsulatum-infected mice can mediate suppression of intracellular growth of the fungi in normal macrophages (11).

In the present study, we show that macrophages obtained from the spleen or peritoneum of mice which are effectively eradicating fungal infection exhibit increased fibrinolysis, which is an index of activation of these cells. Further in vitro studies showed that this is a result of immune T cell-macrophage interaction. The significance of these findings is discussed.

MATERIALS AND METHODS

Animals. CBA/J female mice, 6 to 8 weeks of age, were purchased from Jackson Laboratory, Bar Harbor, Maine. They were housed at the Mayo Clinic animal facilities and provided with Purina mouse chow, chlorinated water, and standard care.

Materials. Dulbecco modified Eagle medium (DMEM), Hanks balanced salt solution (HBSS), penicillin, streptomycin, amphotericin B (Fungizone), and trypsin blue were purchased from Gibco Laboratories, Grand Island, N.Y. Proteose peptone (PP) was obtained from Difco Laboratories, Detroit, Mich. Fetal bovine serum (FBS) was purchased from Microbiological Associates, Bethesda, Md. Trypsin and soybean trypsin inhibitor (SBTI) were from Worthington Diagnostics, Freehold, N.J. Concana valin A was from ICN Nutritional Biochemicals, Cleveland, Ohio. Fibrinogen was purchased from Calbiochem-Behring, La Jolla, Calif. Radioactive iodine (125I) was obtained from New England Nuclear Corp., Boston, Mass. All other agents were of the highest laboratory grade.

H. capsulatum and antigen preparation. The Scritchfield strain of H. capsulatum was maintained in yeast phase at 37°C on brain heart infusion agar slants supplemented with 5% sheep erythrocytes. The cultures were maintained by weekly transfer, and yeast morphology was confirmed by staining with lactophenol cotton blue.

Yeast-phase H. capsulatum cells grown in brain heart infusion agar supplemented with 1% penicillin and 1% streptomycin at 37°C were harvested after 48 h during log growth. The organisms were carefully removed with a sterile wire loop and suspended in cold phosphate-buffered saline (PBS). The clumped yeasts were dispersed by vortex agitation for 30 s in a test tube containing 1-mm sterile glass beads. To obtain a uniform suspension, the organisms were resuspended in PBS and sonicated for 10 s in a Branson S75 sonifier. Uniformly suspended H. capsulatum organisms were counted in a properly calibrated Coulter Counter.
Inoculum viability was checked by placing the suspension onto Sabouraud culture media with 5% dextrose. Soluble *H. capsulatum* antigen (histoplasmin) was obtained from the Centers for Disease Control (Atlanta, Ga.) and was prepared from stains 6623 and 6624 on Smith asparagine medium (4). The antigen was used in routine complement fixation and precipitating tests.

**Animal infection and cell preparation.** Female mice, 6 to 8 weeks of age, were injected via the tail vein with 10 to 15 ml of HBSS. The spleens were dissected, and single-cell suspensions were prepared by using sterile wire mesh. The cells from each spleen were suspended in 5 ml of sterile saline, and 1 ml of this suspension was layered onto Sabouraud culture medium with 5% dextrose agar. The cultures were incubated at room temperature in a dark closet, and the fungal colonies were counted after 2 weeks of incubation.

Spleen cells were washed and suspended in DMEM, and cell viability was determined by trypan blue exclusion; more than 95% of the spleen cells were viable in each experiment. A known number of cells were distributed into each 125I-labeled fibrin-coated Linbro well as described in the figure and table legends in this report.

**T cell purification.** Splicic T cells were isolated and purified by the method of Julius et al. (12). Briefly, 8 x 10^7 to 10 x 10^7 spleen cells were incubated for 45 min at 37°C in plastic syringes (10 cc) containing prewashed nylon wool in DMEM containing 10% FBS. The nonadherent cells were eluted with warm medium, washed twice, and used in the plasminogen activator assay.

**Peritoneal exudate cells.** CBA/J female mice were injected i.p. with 2 ml of 10% PP. Induced peritoneal exudate cells were obtained 3 days later by washing with HBSS. Peritoneal washings were also obtained from control normal mice.

**Culture and assay of fibrinolytic activity.** 125I-labeled fibrin-coated Linbro plates were used to measure fibrinolysis by peritoneal exudate and adherent spleen cells (7). Bovine fibrinogen was purified by the method of Laki (14) and radioiodinated with 125I by the chloramine-T method. Each of the 24 wells of the plates was coated with 20 µg of unlabeled fibrinogen and 5 x 10^4 to 10 x 10^4 cpm of trichloroacetic acid-precipitable radioiodinated fibrinogen. The fibrinogen was converted to fibrin by treatment with 1 ml of 10% FBS in HBSS, which served as a source of thrombin, for 1 h at 37°C. The plates were washed twice with HBSS. No more than 10 to 15% of the radioactivity was lost in this process. The cells were added in a volume of 0.1 to 0.2 ml to each well. Heat-inactivated FBS (56°C for 45 min) and SBTI were added to give a final concentration of 2 to 10% and 100 µg/ml, respectively. The cells were cultured in 1 to 2 ml of DMEM containing penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (2.5 µg/ml) at 37°C in a 5% CO2-humidified incubator.

Mitogens and soluble histoplasmin were added at the start of cell incubation. The adherent cells were monitored daily by observation under an inverted phase-contrast microscope. The medium was aspirated after 24 to 48 h, and the adherent cells were washed three times with HBSS. The culture fluid and each washing were monitored for released radioactivity. Actual fibrinolysis was initiated by incubation of the cells in 5% acid-treated FBS (ATFBS) in DMEM. The ATFBS was the source of plasminogen. Plasminogen activator released extracellularly from macrophages converted plasminogen to plasmin which split plastic-bound fibrin into fibrin split products. At specific periods thereafter, 100-µl samples were removed and the amount of radioactivity released was counted in a Packard gamma spectrometer. Assays were performed in duplicate, and appropriate controls were included in all experiments. The average counts of duplicate samples were within 10% of one another and were expressed as total counts per minute released minus background per 100 µl or as percentage of total trypsin-releasable radioactivity minus background.

**RESULTS**

**Description of animal model.** Sublethal disseminated infection in mice resulted in no mortality up to postinfection week 8. *H. capsulatum* was recovered from the lungs, liver, and spleen. Fig. 1 shows the number of fungal CFUs per milliliter of spleen suspension from day 2 to postinfection week 8. Within week 1, there was a decrease from 300 to 100 CFU/ml of spleen suspension. The colonies further decreased to 30 on day 14 and to fewer than 10 on day 21 and subsequent days of infection.

**Fibrinolytic activity of splenic adherent cells.** Spleen cells from control noninfected mice and mice infected with two different inoculum sizes of *H. capsulatum*, i.e., an HD (1.25 x 10^8) and an LD (0.63 x 10^6), were cultured in 10% FBS, DMEM, and SBTI (100 µg/ml) for 24 to 48 h on 125I-labeled fibrin-coated Linbro plates. The nonadherent cells were washed off, and 5% ATFBS in DMEM was added. Under these conditions, specific release of plastic-bound radioactivity into the supernatant served as an indicator of the enzymatic conversion of plasminogen to plasmin by plasminogen activator secreted by adherent phagocytic cells (macrophages).

![FIG. 1. Recovery of viable *H. capsulatum* from the spleens of CBA/J mice i.v. infected with 1.25 x 10^8 yeast cells. Bars represent the mean CFUs from three animals.](http://iai.asm.org/)
FIG. 2. (A) Spleen cells from mice at week 1 of HD (○) or LD (△) infection with *H. capsulatum* and noninfected controls (●) were cultured at a concentration of \(4 \times 10^6\) cells per well in 10% FBS, DMEM, and SBTI (100 μg/ml) on \(^{125}\)I-labeled fibrin-coated plastic wells for 48 h. The adherent cells were washed, and 5% ATFFS-DMEM was added. The percentage of total released activity minus background is shown on the vertical line, and the time at which samples were obtained is shown on the horizontal line. (B) Same as above, except that spleen cells (\(8 \times 10^6\) per well) were cultured for 90 min. The nonadherent cells were washed off, and adherent cells were cultured for 24 h before the start of the fibrinolysis assay.

After 1 week of infection, adherent spleen cells from HD and LD *H. capsulatum*-infected mice showed enhanced fibrinolysis (Fig. 2A). The increased fibrinolysis progressed with time, suggesting the secretory nature of plasminogen activator released by the adherent cells. No difference in fibrinolysis was observed between adherent cells from groups receiving different inoculum sizes at cell concentrations of \(4 \times 10^6\)/ml. Culturing cells (\(4 \times 10^6\) per well) for less than 48 h resulted in minimal noticeable differences. However, spleen cells (\(8 \times 10^6\) per well) incubated for 90 min to allow phagocytic mononuclear cells to adhere and then cultured for 24 h before the start of the assay also showed noticeable increased fibrinolytic activity by splenic adherent cells from HD-infected mice (Fig. 2B). During week 2 of infection, similar experiments showed (Fig. 3) that adherent cells from *H. capsulatum*-infected mice exhibited a 2- to 10-fold increase in fibrinolysis. However, during week 3 of infection (data not shown), no such difference was evident, even after using several lots of FBS, altering cell concentration, and modifying the duration of the culture period. These data suggest that during the time when infected mice are effectively eradicating *H. capsulatum* from the spleen, splenic adherent cells (macrophages) exhibit enhanced fibrinolytic activity. Around week 3 of infection, the fungal colony counts in the spleen were effectively reduced, and at the same time no increased fibrinolysis was shown by the splenic macrophages. It should be pointed out that the fibrinolysis in this assay was a result of phagocytic mononuclear cells and was not owing to lymphocytes. Polymorphonuclear leukocytes were not the source of plasminogen activator, because they die in the first 12 to 18 h and are washed off before the final assay period (10).

**Role of phagocytic mononuclear cells.** Several factors may account for the aforementioned observations. First, there may be an increase in the absolute number of phagocytic mononuclear cells in the spleens of infected animals. Histopathologic examination of spleens from infected mice has revealed an enormous amount of infiltration of macrophages and histiocytic cells (1). Secondly, the phagocytic cells may be activated and therefore show enhanced fibrinolytic activity (24). Thirdly, young monocytes/macrophages which have been recruited from the infected spleen may exhibit greater fibrinolytic activity compared with fixed-tissue macrophages (15). To investigate these possibilities, the following experimental protocol was followed. Spleen cells from infected and noninfected mice were cultured at concentrations of \(2 \times 10^5\)/ml. The adherent cells obtained from this concentration of spleen cells showed no appreciable difference in the degree of fibrinolysis (data not shown). In other sets of culture, \(0.5 \times 10^6\) PP-induced peritoneal exudate cells,

FIG. 3. Mice were infected with two different inoculum sizes of *H. capsulatum*, \(1.25 \times 10^6\) (○) and \(0.625 \times 10^6\) (△), plus age-matched noninfected controls (●). Assays were performed as described in panel A, except that spleen cells were obtained from animals at week 2 of infection.
which are fibrinolytically inactive, were cocultured with 2 × 10⁶ spleen cells for 48 h (Fig. 4). After initiation of the fibrinolysis assay, only macrophages which were cultured with spleen cells from HD or LD *H. capsulatum*-infected mice exhibited enhanced fibrinolytic activity. As a positive control, PP-induced peritoneal exudate cells were cultured with concanavalin A (20 μg/ml), and these cells showed 19.8 and 25.4% of the total radioactivity released at 18 and 24 h, respectively. This experiment showed that significantly reducing the contribution of macrophages in the spleen cell preparation and adding exogenous inflammatory syngenic macrophages results in enhanced fibrinolysis after a period of in vitro incubation. Because the readout system was fibrinolytic in nature, which is a function of macrophages, enhancement of fibrinolytic activity by macrophages must have occurred as a result of lymphocyte products. These experiments further suggest that enhancement of fibrinolysis can be initiated by in vitro conditions. This is a representation of lymphocyte-macrophage interaction; to further characterize the population of lymphocytes responsible for this phenomenon, the following experiments were done.

**Role of T lymphocytes in induction of macrophage-related fibrinolysis.** The comparative effect of control noninfected spleen cells or nylon wool-purified T cells versus *H. capsulatum*-infected spleen cells or T cells on syngenic macrophages was studied as described in the legend to Table 1. As shown, the addition of infected spleen cells resulted in a twofold increase in fibrinolysis compared with control spleen cells, whereas the addition of purified T cells from infected spleens resulted in a 16-fold increase compared with similarly purified noninfected T cells. These data suggest that sensitized cells obtained from *H. capsulatum*-infected mice induced plasminogen activator activity in syngenic inflammatory macrophages. Enrichment of the immune T cells resulted in marked enhancement of this activity. However, the possibility of cells other than T cells inducing fibrinolytic activity in macrophages has not been excluded.

**Effect of route of infection.** Two groups of mice were injected intravenously (i.v.) or i.p. with the same inoculum (1.25 × 10⁹) of *H. capsulatum*. One week later, various numbers of spleen cells were cultured (Fig. 5). No fibrinolytic activity was shown in cells from noninfected mice. The adherent cells from i.v.-infected mice exhibited a 5- to 10-fold increase in plasminogen activator activity over controls, whereas i.p.-infected cells exhibited 5 to 20 times greater activity than controls. These data indicate that adherent spleen cells from i.p.-infected animals possess greater macrophage activation than do cells from animals infected by the i.v. route.

**Activation of peritoneal exudate cells.** Previous studies were conducted with cells obtained from the spleens of infected mice. Because increased fibrinolysis was shown in adherent spleen cells, i.e., macrophages, we reasoned that similarly increased activity might be detectable in peritoneal exudate cells. Therefore, we collected cells from the peritoneal washings of noninfected control mice, PP-injected mice, i.v.- and i.p.-infected mice, i.p.-infected mice which had received PP boosters, and finally mice infected i.p. and given a booster 48 h previously with 1 cc of histoplasmin (diluted 1:200). Equal numbers of peritoneal exudate cells (10⁶) were

![Graph showing fibrinolytic activity](image)

**TABLE 1. Effect of spleen and T cells from control and *H. capsulatum*-infected mice on macrophage-induced fibrinolysis**

<table>
<thead>
<tr>
<th>Cells added</th>
<th>(³²P cpm [10⁶] - background)/100 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>0.5</td>
</tr>
<tr>
<td>Control spleen cells</td>
<td>1.4</td>
</tr>
<tr>
<td>Control T cells</td>
<td>1.5</td>
</tr>
<tr>
<td><em>H. capsulatum</em>-infected spleen cells</td>
<td>3.5</td>
</tr>
<tr>
<td><em>H. capsulatum</em>-infected splenic T cells</td>
<td>24.1</td>
</tr>
</tbody>
</table>

* PP-induced peritoneal exudate cells were cultured on ³²P-labeled fibrin-coated plates at concentrations of 0.3 × 10⁶ per well for 1 h in 10% FBS, DMEM, and SBTI. Unfractionated and nylon wool-passed splenic T cells (2 × 10⁶ per well) from noninfected control and *H. capsulatum*-infected mice were added. Plates were incubated for 48 h and washed, and fibrinolysis was assayed at 24 h.

![Graph showing cell number per well](image)
cultured for 48 h, and fibrinolysis was studied 24 h later. Peritoneal exudate cells from both the i.v.- and i.p.-infected groups showed increased fibrinolysis (Table 2). Injection of PP or soluble histoplasmin antigen 3 and 2 days before harvesting did not significantly increase activity. These data also show that enhanced fibrinolysis of adherent cells is not shown only in spleen cells but also in the phagocytic mononuclear cells of the peritoneum.

Effect of in vitro addition of soluble H. capsulatum antigen.

The addition of soluble H. capsulatum antigen to in vitro cultures of infected and noninfected spleen cells often resulted in fibrinolysis of less than background activity. This was perhaps the result of a toxic effect on the lymphocytes or macrophages or both. This was confirmed by a substantial decrease in the number of adherent cells attached as seen under the phase-contrast microscope. Reducing the dose of soluble antigen did not seem to eliminate this effect. However, increasing the ratio of macrophages to lymphocytes either by the addition of exogenous inflammatory macrophages or by obtaining peritoneal exudate cells from infected mice and then adding soluble H. capsulatum antigen seemed to neutralize the deleterious effect of the antigen. In the experiment shown in Fig. 6, peritoneal exudate cells obtained from PP-injected and i.v.-infected mice showed further enhancement of fibrinolysis when incubated in the presence of soluble histoplasmin antigen. This was dose related but not antigen specific, because PP-induced cells also showed enhancement. Resident peritoneal macrophages were also stimulated with this antigen preparation (data not shown).

Observation of in vitro-activated macrophages. Microscopic observation of the macrophages obtained from the spleen or peritoneum showed that the cells appeared to be diffusely spread, and membranes were ruffled and bore the cellular characteristics of activated macrophages.

**DISCUSSION**

The present studies showed that disseminated histoplasmosis in mice results in marked increases in the fibrinolytic activity of splenic macrophages and peritoneal exudate cells. Previous published studies have shown that macrophage-induced fibrinolysis occurs as a result of increased plasminogen activator synthesis (24). Resident peritoneal macrophages secrete very little of this enzyme in the resting state, but it has been shown that upon in vivo or in vitro activation, the synthesis of plasminogen activator increases by several hundredfold (3, 19). Thus, the production of plasminogen activator serves as an excellent indicator of macrophage activation (19). The existence of considerable fibrinolytic activity during week 1 and 2 of disseminated H. capsulatum infection and the absence of similar changes in the third and subsequent weeks of infection correlated with the almost complete disappearance of culturable fungi from the spleen. Admittedly, some of the differences in fibrinolysis between spleen cells of infected mice and controls could have resulted from increased numbers of adherent cells in the wells rather than from activation per se. However, this seems unlikely because unactivated macrophages generally are fibrinolytically nonactive. Thus, macrophage activation directly correlates with the ability of mice to eradicate infection. Moreover, macrophage activation occurs at a time when the generation of suppressor T cells within the spleen is maximal (P. C. McNabb, M. L. Tiku, and T. B. Tomasi, Jr., unpublished observations).

The mechanism of macrophage activation in the spleen, peritoneum, and other organs probably reflects lymphocyte-macrophage interactions (21). The development of cellular immunity to H. capsulatum results in the proliferation and activation of clones of lymphocytes which secrete soluble lymphokines that have the capacity to induce macrophage activation (8, 13, 25). Apparently, even close physical association between lymphocyte and macrophage, without soluble lymphokine production, is able to yield macrophage activation. In our experimental system, the coculture of immune splenic lymphocytes with PP-induced, inflammatory macrophages resulted in enhanced fibrinolysis. Moreover, the addition of splenic lymphocytes or nylon wool-purified T cells resulted in macrophage activation without the addition of specific antigen. The most likely explanation for this observation is that the immune lymphocytes were activated in vivo and continued to produce lymphokines in vitro, which resulted in macrophage activation. The other possibility is that the antigen carry-over from the infected spleen

**TABLE 2. Fibrinolytic activity of peritoneal exudate cells from H. capsulatum-infected and noninfected mice**

<table>
<thead>
<tr>
<th>Source of peritoneal exudate cells (10^6 cells per well)</th>
<th>Without addition of histoplasmin</th>
<th>With addition of histoplasmin (1:200 dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noninfected</td>
<td>1.5</td>
<td>ND*</td>
</tr>
<tr>
<td>PP induced</td>
<td>2.1</td>
<td>7.0</td>
</tr>
<tr>
<td>i.v. infected</td>
<td>18.8</td>
<td>30.5</td>
</tr>
<tr>
<td>i.p. infected</td>
<td>12.0</td>
<td>20.2</td>
</tr>
<tr>
<td>i.p. infected and PP boosted</td>
<td>10.9</td>
<td>19.8</td>
</tr>
<tr>
<td>i.p. infected and in vivo boosted with histoplasmin</td>
<td>13.8</td>
<td>ND*</td>
</tr>
</tbody>
</table>

* Conditions were the same as described in the legend to Table 1, except that peritoneal exudate cells were obtained from 10-day-old infected mice.
  * ND, Not done.
led to the lymphocyte activation being carried over under in vitro conditions and the release of lymphokines.

The addition of soluble histoplasmin to resident, inflammatory, or infected mice macrophages resulted in an increase in macrophage fibrinolysis in a dose-response manner. As expected, the peritoneal exudate cells obtained from infected mice showed enhanced macrophage activation upon in vitro antigen exposure (8). However, similar observations on nonimmune PPD-induced and resident macrophages were unexpected. One possible explanation is that the histoplasmin contained particulate antigen, and this seems unlikely because the antigen preparation was passed through a Millipore filter. Secondly, it is possible that contaminating endotoxins were present in the antigen preparation, but this would not be expected to result in enhanced fibrinolysis of resident macrophages in vitro because this process requires two-stage stimuli (9). It was therefore concluded that histoplasmin antigen either produces or contains products capable of inducing macrophage activation.

It should be pointed out that macrophage activation as assessed by fibrinolysis (plasminogen activator synthesis) or other tests may not always correlate with enhanced killing activity against pathogens. For example, Nogueira et al. showed that thioglycolate-induced macrophages lack trypanosomidal activity yet demonstrate a marked increase in plasminogen activator synthesis (18). At the same time, immunologically activated macrophages (induced by Trypanosoma cruzi infection) showed concomitant increases in trypanosomidal activity and increased plasminogen activator production (17, 18). It is conceivable that during massive disseminated infections, macrophages may appear to be activated by morphological and other criteria such as plasminogen activator production but actually lack fungicidal activity. Under these circumstances, phagocytic load by fungal proliferation and elaboration of soluble fungal products could provide additional stimuli for macrophage activation. Studies of other experimental models of intracellular infections including toxoplasmosis and trypanosomiasis have revealed that immune activation of macrophages is perhaps the single most important factor in stimulating killing activity against the pathogen, thereby eradicating the infection (17, 20). This is probably the case during the initial weeks of infection in the animal model of disseminated histoplasmosis as well.

Immunoregulatory abnormalities have been shown in the animal model of disseminated histoplasmosis (2) and in various human fungal infections as well (23). Although some of these observations are indeed due to migration, proliferation, and recruitment of lymphocytes, it is possible that the presence of activated macrophages resulted in some of the immune disturbances (2). Additional studies are needed to resolve this question.

LITERATURE CITED