Cytolytic Plasma Factor in Experimental Coccidioidomycosis

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Peripheral blood leukocytes from guinea pigs infected with Coccidioides immitis 5 weeks previously were lysed significantly when incubated at 37 C for 3.5 hr with a concentrated C. immitis culture filtrate, but not with a concentrated Aspergillus fumigatus or Blastomyces dermatitidis culture filtrate. Mononuclear leukocytes and presumably granulocytes were both lysed. Characterization of the factor responsible for this leukocytolysis reaction revealed that it was contained in plasma and serum but not in leukocytes from infected guinea pigs. It was complement-dependent, active after heating at 56 C, and stable after storage at −20 C for as long as 14 months.

Infection with Coccidioides immitis generally engenders humoral antibodies detectable in tube precipitin, complement fixation, and immunodiffusion tests, and also engenders cellular hypersensitivity manifested by "delayed" skin reaction to coccidioidin (5, 10, 11). A preliminary study demonstrated lysis of peripheral blood leukocytes in fresh whole blood from guinea pigs infected with C. immitis after incubation in vitro with a culture filtrate of this fungus (7). Absence of lysis of these cells after incubation with physiological saline or uninoculated Sabouraud broth and absence of lysis of peripheral blood leukocytes from uninfected guinea pigs after incubation with the culture filtrate of C. immitis indicated that the reaction was not a result of a nonspecific increase in cell fragility or toxicity of the culture filtrate. Relative importance of humoral and cellular factors and degree of specificity in this leukocytolysis reaction were not determined.

Observations of specific in vitro toxicity of microorganism products to leukocytes from infected animals include cytotoxicity in tissue culture, inhibition of migration in a coagulated plasma medium, and lysis in whole blood or leukocyte suspensions (4). These have been considered to represent in vitro examples of delayed hypersensitivity and to depend on sensitized leukocytes. Lysis of leukocytes has been studied in most detail in tuberculosis (1, 8, 9, 12) and brucellosis (2, 3), but in these instances humoral and not specific cellular factors have been found to be principally responsible for the leukocytolysis reaction (3, 8, 9).

This paper presents a preliminary evaluation of the specificity of the culture filtrate and an analysis of the role of humoral factors in leukocytolysis in experimental coccidioidomycosis.

MATERIALS AND METHODS

Fungus. C. immitis, Silveira strain, was cultured in Sabouraud dextrose broth at 37 C on a rotary shaker for 5 days. The fungus was concentrated by centrifugation, washed three times with sterile 0.85% NaCl, and ground thoroughly in a Ten Broeck tissue grinder to obtain a uniform suspension. The final suspension in saline was adjusted to 50% by volume.

Animals. Twenty-two female albino guinea pigs weighing from 421 to 614 g were divided into two equal groups with approximately the same distribution of weights. Each animal was housed in a separate cage. One group was inoculated intraperitoneally with 0.25 ml of the suspension of C. immitis, and the other group was inoculated intraperitoneally with 0.25 ml of sterile saline and retained as a control.

Cardiac punctures were performed at 5 and 6 weeks after inoculation. A portion of each "5th-week" blood sample and all of the "6th-week" samples were heparinized to a final concentration of 10 USP units/ml of blood (Heparin, sodium; Nutritional Biochemicals Corp., Cleveland, Ohio) for immediate testing. Another portion of each 5th-week sample was allowed to clot and then separated by centrifugation, and the serum was stored at −20 C.

All animals were autopsied at the time of death or after sacrifice at the end of the 7th week after inocu-

lation. Samples of the lung, liver, and spleen were cultured on Sabouraud dextrose agar.

Antigens. The same strain of *C. immitis* was also cultured in Sabouraud dextrose broth at 37°C on a rotary shaker for 3 weeks. Actively growing strains of *Aspergillus fumigatus* and *Blastomyces dermatitidis* were similarly cultured at room temperature and 37°C, respectively. Each culture was examined for contamination, autoclaved at 121°C for 15 min at 15 lb/in² of pressure, and then filtered through Seitz sterilizing pads. The filtrate was lyophilized and reconstituted in various concentrations with a saline solution of distilled water, the pH was adjusted to 7.0 with 1 M NaOH and was stored at −20°C. This preparation was termed the "concentrated" antigen. Sterile buffered saline with a pH of 7.0 was prepared by adding 9 g of NaCl, 0.19 g of Na₂HPO₄·H₂O, and 0.88 g of Na₂HPO₄·7H₂O to 1 liter of distilled water.

Leukocytolysis tests. A 0.4-ml amount of a fresh heparinized 5th-week blood sample or a suspension of cells in plasma or serum was placed into a sterile siliconized 12 by 70 mm tube containing 0.1 ml of a concentrated antigen or buffered saline. The tubes were shaken gently, and base line total leukocyte counts were made. The tubes were then tightly stoppered and incubated at 37°C with gentle manual agitation every 15 min. After 3.5 hr of incubation, leukocyte counts were repeated. Standard white blood cell counting pipettes and procedures were used. Only cells with intact cytoplasmic membranes were counted. All dilutions in pipettes were performed in duplicate, and the mean was recorded as the result. The percentage change in each tube was determined. A negative value denotes net lysis produced by the culture filtrate. Differential cell counts of the 5th-week samples were made from Wright stained slides; 200 cells were classified as either granulocytes or mononuclear cells. The leukocytolysis index for each cell type was computed. Base line counts obtained from the saline control tubes after appropriate adjustment for dilution were recorded as the total leukocyte count and differential count of each guinea pig.

Cells of fresh heparinized 6th-week samples were separated by centrifugation at 4°C at 250 × g for five min. The cells were washed three times in buffered saline. Equal volumes of plasma and packed cells from infected and control guinea pigs were recombined in various manners and tested with the concentrated *C. immitis* antigen.

Several 5th-week serum samples were thawed after 6 weeks of storage. Washed packed cells were prepared from a pool of fresh heparinized blood obtained from several uninjected stock adult guinea pigs. Equal volumes of serum and packed cells were recombined and tested with the concentrated *C. immitis* antigen.

Several pooled sera were divided into two parts, and one part was heated at 56°C for 30 min. These sera had originally been obtained 4 weeks after inoculation from another similarly inoculated group of guinea pigs and had been stored at −20°C for 14 months. Each pool consisted of sera from three or four control or infected guinea pigs. Suspensions were prepared for testing with the concentrated *C. immitis* antigen by combining 4 volumes of heated or unheated serum, 1 volume of buffered saline or reconstituted lyophilized guinea pig complement (Hyland Laboratories, Los Angeles, Calif.), and 5 volumes of fresh packed cells from uninfected guinea pigs. Heparin was added to a final volume of 10 units/ml.

Immunodiffusion test. This test in agar gel was performed as previously described (10) with undiluted serum and concentrated antigens, and 10-fold, 100-fold, and 1,000-fold dilutions in buffered saline of concentrated antigens. An additional concentrated *C. immitis* antigen was used only in this test. It was prepared identically to the method of the other, with the single exception that the culture was not autoclaved prior to filtering.

Skin tests. These tests were performed during the 7th week after inoculation with a 10-fold dilution of concentrated antigens. A 0.1-ml amount of each was injected intradermally, and readings were made at 24, 48, and 72 hr later. The maximal intensity of the reaction was scored 1+, 2+, or 3+ depending on whether induration was present in an area with a diameter of 6 to 10 mm, 11 to 20 mm, or over 20 mm, respectively.

Statistics. Means, standard errors of means, and medians were determined. The t test was used to compare the means and the chi-square test was used to compare the medians. A probability of occurrence of *P* < 0.05 was considered statistically significant.

RESULTS

Several observations distinguished the infected from the control guinea pigs. Statistically significant findings in infected guinea pigs included a mean weight loss after 7 weeks of 6 g, positive gel-diffusion precipitin tests through use of a concentrated nonautoclaved *C. immitis* antigen in all 11 guinea pigs, positive delayed skin tests to the autoclaved *C. immitis* antigen in 8 of 11 guinea pigs, and gross and cultural autopsy evidence of infection with *C. immitis* in all 11 guinea pigs. Control guinea pigs had a mean weight gain of 109 g, and none exhibited positive precipitin tests, positive delayed skin tests to the *C. immitis* antigen, or autopsy evidence of *C. immitis*. One control guinea pig developed paralysis of the hind legs during the 1st week and was sacrificed. An autopsy was unrevealing. One control and one infected guinea pig died after the first cardiac puncture from massive thoracic hemorrhages.

Sera from one infected guinea pig formed three precipitin lines, and sera from four others formed two lines when tested against the concentrated nonautoclaved *C. immitis* antigen. No precipitin lines developed with any of the other antigens.
including the concentrated and diluted autoclaved C. immitis antigen.

The positive skin test reactions to the C. immitis antigen were 3+ in one infected guinea pig and 2+ in five others. The two infected guinea pigs with negative C. immitis skin tests differed from the others in the group only in that they had the two largest weight losses. Their reactions in the leukocytolysis tests were not different from those of the other infected guinea pigs. A minimally positive skin test reaction to the A. fumigatus antigen was present in two control guinea pigs, and a minimally positive reaction to the B. dermatitidis antigen was present in one control guinea pig.

Mean total leukocyte counts of infected and control guinea pigs 5 weeks after inoculation were 10,000 ± 1,400 and 11,000 ± 800 cells/ml, respectively. Mean absolute granulocyte counts of the two groups were 2,700 ± 300 and 2,800 ± 400 cells/ml, respectively. The differences are not significant.

Percentage changes in leukocytes and leukocytolysis indices of fresh heparinized 5th-week blood samples are presented in Fig. 1 and 2, respectively. After incubation with the concentrated C. immitis antigen, mean percentage changes of the blood samples from infected and control guinea pigs were −12 ± 1.7 and +3 ± 1.7, respectively, and mean leukocytolysis indices were −11 ± 1.7 and +5 ± 1.9, respectively.

These differences are significant. Mean leukocytolysis indices of mononuclear cells in samples from infected and control guinea pigs were −13 ± 3.0 and +5 ± 3.0, respectively, and mean indices of granulocytes were −4 ± 6.1 and +12 ± 5.5, respectively. Indices of the mononuclear cells are significant. No significant effect was observed with either the A. fumigatus or B. dermatitidis antigens.

Results of recombinations of plasma and cells from fresh heparinized 6th-week blood samples are presented in Fig. 3. In the presence of plasma from control guinea pigs, mean leukocytolysis indices of leukocytes from infected and control guinea pigs were +7 ± 0.3 and +8 ± 2.7, respectively. In the presence of plasma from infected guinea pigs, mean leukocytolysis indices of leukocytes from infected and control guinea pigs, however, were −10 ± 2.8 and −11 ± 4.0, respectively. Mean indices in the presence of plasma from infected guinea pigs compared with those in the presence of plasma from control guinea pigs are significantly different regardless of the source of the cells.

Mean leukocytolysis indices determined with
frozen 5th-week sera were $-9 \pm 2.2$ based on nine sera samples from infected guinea pigs and $+7 \pm 4.1$ based on seven sera samples from control guinea pigs. These differences are significant. Mean leukocytolysis indices of mononuclear cells in samples from infected and control guinea pigs were $-10 \pm 3.1$ and $+7 \pm 5.1$, respectively, and mean indices of granulocytes were $+2 \pm 11.9$ and $+8 \pm 21.7$, respectively. Indices of the mononuclear cells are significantly different.

Results of representative studies of a pool of sera from infected guinea pigs and one from control guinea pigs are presented in Table 1. Two other pools from each source were examined with similar findings. The findings indicate that a heat-labile component in the cytolytic serum from infected guinea pigs was essential for leukocytolysis. Leukocytolysis occurred in tubes containing unheated cytolytic serum with and without added complement but occurred only in the tube containing heated cytolytic serum with added complement. Leukocytolysis did not occur in any tube containing control serum.

Statistical results with medians in all instances paralleled those with means.

**DISCUSSION**

These studies characterize in part the factor in guinea pig whole blood responsible for the leukocytolysis reaction in experimental coccidioidomycosis. It is contained in plasma and serum but not in leukocytes obtained from guinea pigs 5 weeks after infection with *C. immitis*. It is complement-dependent, active after heating at 56 C for 30 min when complement is added again, and stable after storage at $-20$ C for 6 weeks and 14 months. The factor is presumably a humoral antibody since it appears after an antigenic stimulus and is relatively specific in response to the eliciting antigen.

Analysis of leukocytolysis indices for cell types suggests that both mononuclear cells, mostly lymphocytes, and granulocytes are lysed. Significantly more mononuclear cells were lysed in the presence of plasma or frozen serum from infected animals than from controls. The same trend, although not statistically significant, was observed for granulocytes in the two instances in this study and in a previous one (7).

The findings are essentially consistent with those in humans and guinea pigs with tuberculosis and in guinea pigs with brucellosis. The cytolytic factor in each instance was contained in plasma, was complement-dependent, and lysed mononuclear cells and granulocytes (2, 3, 8, 9). The cytolytic factor in plasma from tuberculous patients was destroyed by heating at 56 C for 15 min, and its complement dependency was demonstrated after removal of complement with an unrelated antigen-antibody system (9). Species differences in the leukocytolysis reaction in experimental tuberculosis have included thermostability of the cytolytic factor in rabbit serum (12) and lysis of lymphocytes, but not granulocytes, in blood from mice (1).

The antigen yielding an immunodiffusion reaction which correlates with the standard complement fixation test in coccidioidomycosis has been found to be heat-labile, whereas the antigen yielding an immunodiffusion reaction which correlates with the standard tube precipitin test has been found to be heat-stable (5). Absence of activity in the immunodiffusion test of the antigen

**FIG. 3.** Distribution of leukocytolysis indices of four combinations of plasma and cells from blood samples obtained from guinea pigs 6 weeks after infection with *Coccidioides immitis* and control guinea pigs. Leukocytolysis index equals percentage change in a tube containing a *C. immitis* antigen minus percentage change in a saline control tube. A negative value denotes net lysis.

**TABLE 1. Effect of heat inactivation on leukocytolysis**

<table>
<thead>
<tr>
<th>Suspension of control cells in</th>
<th>Leukocytolysis index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytolytic serum</td>
<td>$-19$</td>
</tr>
<tr>
<td>Cytolytic serum + complement</td>
<td>$-15$</td>
</tr>
<tr>
<td>Heated cytolytic serum</td>
<td>$+6$</td>
</tr>
<tr>
<td>Heated cytolytic serum + comple-</td>
<td>$-26$</td>
</tr>
<tr>
<td>ment</td>
<td></td>
</tr>
<tr>
<td>Control serum</td>
<td>$+3$</td>
</tr>
<tr>
<td>Control serum + complement</td>
<td>$+3$</td>
</tr>
</tbody>
</table>

* Leukocytolysis index equals percentage change in a tube containing a *Coccidioides immitis* culture filtrate minus percentage change in a saline control tube.

* Pooled serum obtained from several guinea pigs 4 weeks after infection with *C. immitis*.

* Serum heated to 56 C for 30 min.
used in the leukocytolysis reaction in this study cannot be interpreted as indicating that the precipitin antigen in this instance is heat-labile, since a preparation from an autoclaved culture filtrate for a previous study possessed this antigenic activity. The most probable explanation is that this represents the well-recognized variability in crude preparations of antigens for coccidioidomycosis studies (11).

The ultimate value of the leukocytolysis reaction as a diagnostic or prognostic aid in human coccidioidomycosis will depend upon its correlation with the clinical course of infection and with standard precipitin and complement fixation tests, upon more extensive studies of its specificity, and upon some technical modifications of the procedure. Improvement in the accuracy and technical simplification of the procedure may be accomplished by determination of release of an intracellular lysozyme-like enzyme as an indicator of cell lysis (6), or by utilization of an electronic counting apparatus. Although the leukocytolysis reaction involves fixation of complement, the antigen-antibody reaction measured may not be the same as that in the standard complement fixation test. The complement fixing antigen used in the standard complement fixation test in coccidioidomycosis is generally considered to be destroyed by autoclaving (5, 11), whereas the antigen used in the leukocytolysis reaction was an autoclaved culture filtrate. In experimental tuberculosis in rabbits, the leukocytolysis reaction did not correlate with the complement fixation test but did with a tuberculin-coated erythrocyte agglutination test (12). Additional studies designed to elucidate leukocytolysis in experimental coccidioidomycosis are currently in progress.

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