Effects of Stimulation and Suppression of Cell-Mediated Immunity on Experimental Cryptococcosis

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A model of cryptococcosis was developed using intraperitoneal infections of guinea pigs. This model shared characteristics with cryptococcosis in humans and was used to study the effects of immunosuppression and immunostimulation on cryptococcosis. Female guinea pigs survived longer than males; perhaps this was related to a greater capacity of their monocytes to kill cryptococci. A brief course of cortisone shortened survival of females and resulted in depressed immune and inflammatory responses, which persisted long after cortisone was stopped. Stimulation of the immune response by treatment with cryptococci in Freund complete adjuvant improved survival in males. Preliminary studies indicated the usefulness of this model for the study of other potential immunostimulants, including immune lymphocytes, transfer factor prepared from immune lymphocytes, and levamisole. Overall, long-term survivors appeared to clear disseminated cryptococci from extraperitoneal sites including brain, rather than prevent dissemination of cryptococci from the peritoneal cavity. The quantity of the inflammatory response in infectious foci, rather than the ability of individual leukocytes to kill cryptococci, may have determined the outcome of most infections.

Circumstantial evidence strongly suggests that human exposure to Cryptococcus neoformans is common, but that failure of immunity and development of extrapulmonary cryptococcosis is rare (27). Development of disseminated cryptococcosis in man has been associated with abnormalities in in vitro tests of cell-mediated immunity (8, 16, 28). Furthermore, C. neoformans can be killed in vitro by phagocytic neutrophils (7, 32), monocytes (7, 9), and macrophages (21, 29), as well as by nonphagocytic mechanisms involving monocytes or macrophages (18) or several types of leukocytes with specific anticytotoxic antibodies (11). To define the relative importance of these mechanisms in the host, an experimental model was developed that would permit studies of the effects of stimulation and suppression of cell-mediated immunity on cryptococcosis. The guinea pig was chosen because this species is resistant to corticosteroids, like man (6). In addition, guinea pigs may develop a relatively chronic form of experimental cryptococcosis, thereby facilitating studies of factors that shorten or prolong survival after infection. The intraperitoneal (i.p.) route of infection provided a route for delivery of a reproducible inoculum to a local site, which could later act as a focus for dissemination.

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MATERIALS AND METHODS

Infection and treatment of animals. Hartley or strain 13 guinea pigs were injected i.p. with $1 \times 10^6$ to $2 \times 10^6$ C. neoformans (National Institutes of Health isolate 26, an isolate with an intermediate capsule size, kindly supplied by John E. Bennett). At intervals after infection, animals from each group were selected at random for sacrifice to obtain organs for quantitative culture and histology and leukocytes for in vitro studies. The peritoneal cavity was lavaged repeatedly with Hanks balanced salt solution. Peritoneal washings and homogenized organs were serially diluted in distilled water, and pour plates were prepared with Sabouraud agar.

Some animals were treated with 100 mg of cortisone acetate per kg per day or with its vehicle for injection (gift from Upjohn, Kalamazoo, Mich.) given subcutaneously for 7 days before infection, on the day of infection, and on the 3 days after infection. Other groups of animals received approximately $10^6$ killed C. neoformans in Freund complete adjuvant, or Freund complete adjuvant alone. For transfer studies, lymphocytes were obtained after mincing of lymph nodes or spleens, passed twice through cheese cloth, and washed in Eagle minimum essential medium. Peripheral blood mononu-
clear cells were obtained by separation on a Hypaque-Ficoll gradient (4). Total lymphocytes were calculated after exclusion of phagocytic cells by examination of Giemsa-stained smears made after exposure to latex particles and counts of viability by trypan blue exclusion. Transfer factor was prepared from lymphocytes by the method of Kirkpatrick and Smith (20). After repeated freeze-thawing, cell lysates were dialyzed against 0.02 M NH₄HCO₃ (pH 7.4), lyophilized, reconstituted with saline, and sterilized by membrane filtration (Millipore Corp.) just before use. Levamisole was obtained from Janssen R&D (New Brunswick, N.J.), diluted in saline, and injected subcutaneously.

In vitro studies of leukocytes. Heparinized peripheral blood was obtained by cardiac puncture. Mononuclear cells were then separated on a Hypaque-Ficoll gradient, and granulocytes were obtained by a subsequent dextran sedimentation procedure (4). Peritoneal macrophages were obtained by lavage with heparinized Hanks balanced salt solution (calcium and magnesium free). In some cases, animals were stimulated with sodium caseinate (26), heat-killed cryptococci, or cryptococcin (see below) given i.p. 1 to 3 days before harvesting macrophages. Quantitative studies of phagocytosis and killing of C. neoformans by leukocytes were performed as previously described (7, 9).

Delayed cutaneous hypersensitivity. Cryptococcin was prepared from the isolate used to infect animals in these studies, according to the procedure of Atkinson and Bennett (1). Skin tests were examined at 6, 24, and 48 h. Using this material, 14 uninfected control guinea pigs did not react, and 9 of 10 guinea pigs previously immunized with cryptococci in Freund complete adjuvant had ≥6-mm induration.

Serological tests. Sera were tested for cryptococcal capsular polysaccharide by latex agglutination (2). Anticryptococcal antibodies were detected by the indirect fluorescent-antibody procedure described by Bindschadler and Bennett (3), modified so as to use goat anti-guinea pig immunoglobulin G (IgG) or IgM conjugates (Hyland Laboratories, Los Angeles, Calif.).

Statistical analysis. Means and standard errors of sets of observations were compared by a two-sample t test, frequency of observations was calculated by the chi-square test (or Fisher exact test, where appropriate), and distribution of series of values was computed by the Wilcoxon rank sum test (12).

RESULTS

Survival of animals after infection. In initial studies, there was great variability in the duration of survival of guinea pigs, some surviving less than 1 month after infection, and others apparently clearing infections and surviving indefinitely. This proved to be due in large part to sex differences in response to infection (Fig. 1). Females survived cryptococcosis significantly longer than males (P = 0.015 by the Wilcoxon rank sum test). Once this became apparent, males were used for studies of immunostimulation, and females were used for experiments on immunosuppression (Fig. 1).

Survival of male guinea pigs was prolonged by immunization with cryptococci in Freund complete adjuvant (P < 0.001), but not by Freund complete adjuvant alone (P = 0.118). Survival of females was shortened by a brief course of cortisone acetate (P = 0.004). These findings were duplicated in one or more other experiments.

Killing of C. neoformans by leukocytes. Explanations for differences in survival were sought in leukocyte function studies. In studies using leukocytes from uninfected animals with a 2:1 ratio of leukocytes to cryptococci, the absolute percentages of cryptococci killed were low (Fig. 2). Nevertheless, neutrophils killed cryptococci more efficiently than did monocytes (P < 0.005), and monocytes from females killed cryptococci more efficiently than did monocytes from males (P < 0.005). In these experiments, no differences were seen when monocytes from uninfected animals were compared with those from animals treated with cortisone or with cryptococci in Freund complete adjuvant. Comparable data were obtained using leukocytes from infected animals.

Peritoneal macrophages killed cryptococci poorly or not at all (range, 0 to 11.7% in 11 experiments). This included macrophages obtained from "immune" animals who had cleared their infections and had strong delayed skin reactions to cryptococcin. Macrophages from such animals were stimulated before har-
vesting by i.p. injection of cryptococcin or killed C. neoformans, or after harvesting by culture in vitro with these agents. Despite this, no augmentation of killing occurred, though these macrophages appeared larger and spread on surfaces more extensively than macrophages from control animals.

Antibody-dependent killing of C. neoformans proved difficult to study in this model. Levels of cryptococcal polysaccharide were high enough to block activity for several months after infection. Anticryptococcal antibody was barely detectable by the indirect fluorescent-antibody technique in only 7 of 63 sera tested. All seven reacted with the anti-IgG conjugate, and none reacted with the anti-IgM. Using these sera, only low levels of killing (0 to 28.2%) of cryptococci were observed with leukocytes from some animals.

Effects of cortisone. Cortisone treatment resulted in more severe infections, with prolonged fungemia, and proliferation of organisms inside and outside the central nervous system (Fig. 3). In contrast, control animals cleared cryptococci from blood and appeared to do so from other sites as well, including brain. Furthermore, cortisone-treated animals did not clear fungi from the initial site of infection, as shown by 10^4 more colony-forming units in peritoneal washings from cortisone-treated animals than in washings from control females, at 3 and 7 days after infection. At 30 days after infection, i.e., 27 days after the last cortisone injection, cortisone-treated animals had 10^5 more cryptococci in brain than control animals, and serum cryptococcal polysaccharide (antigen) titer was 32-fold higher (1:32,768 versus 1:1,024). In addition, cryptococcin skin tests remained depressed (P < 0.005) long after the last dose of cortisone (3.3 ± 1.3 mm of induration at 48 h), when compared with control animals (8.5 ± 0.9 mm). Histological examination of brain from cortisone-treated animals sacrificed at 30 days showed frequent cystic clusters of fungi with no surrounding inflammatory reaction, identical to lesions seen in brains from most human patients (Fig. 4). In contrast, brain lesions in control animals were difficult to find. When located, the cellular inflammatory reaction was striking (Fig. 5).

Clearance of disseminated fungi. This vigorous inflammatory reaction with organisms in brain was observed in other groups of animals

Fig. 2. Killing of C. neoformans by peripheral blood monocytes (MONO) and granulocytes (PMN) from uninfected male and female animals. Each point denotes a single study. The wide lines represent the mean percentages of the original inocula killed in a 3-h experiment, and the extending perpendicular lines represent ± 2 standard errors.
Fig. 4. Section of brain from cortisone-treated female guinea pig sacrificed 30 days after infection: (A) hematoxylin and eosin (×80), (B) periodic acid-Schiff (×400), showing many yeasts but no inflammatory reaction.
Fig. 5. Section of brain from control (vehicle-treated) female guinea pig sacrificed 30 days after infection: (A) hematoxylin and eosin (×80), (B) periodic acid-Schiff (×400), showing yeasts with striking cellular inflammatory reaction.
that commonly survived infections, e.g., male animals immunized with cryptococci in Freund complete adjuvant. Sequential counts of organisms in tissue from infected immunized and untreated males were comparable to those noted above for females (Fig. 3). To examine further the possibility that fungi could be cleared from sites of dissemination, including brain, results of cultures of brains were reviewed from the two groups which often clear infections completely, females and immunized males (Table 1). Cryptococci were cultured from brains of a high proportion of animals up to 50 days after infection, but not from any of the brains from animals surviving 120 days or more.

**Immunostimulation.** Preliminary studies involving lymphocyte transfer were performed using lymphocytes from male inbred strain 13 guinea pigs. Cells and sera were harvested from animals 14 days after immunization with cryptococci in Freund complete adjuvant or saline. Three days before infection, groups of four animals received 10⁸ lymphocytes, 8 ml of saline, or 8 ml of serum i.p. Cryptococcin skin tests converted to positive in two of four animals in the group that received "immune" lymphocytes, but not in any of the other groups of animals. Though the observed effect was extremely small, guinea pigs that received "immune" lymphocytes survived longer than those that received nonimmune lymphocytes (P = 0.029) or saline (P = 0.029), but "immune" serum was not protective. Dialyzable transfer factor was then prepared from pooled lymphocytes from Hartley guinea pigs that had survived C. neoformans infections ≥120 days and from uninjected control animals of comparable ages. Extracts equivalent to 10⁸ lymphocytes, or an equivalent volume of saline, were given i.p. to groups of five male animals 3 days before infection with C. neoformans. Cryptococcin skin tests did not convert to positive in any of the animals, and effects on survival were marginal. However, transfer factor prepared from pooled peripheral blood and lymph node lymphocytes did prolong survival slightly when compared with saline-treated animals (P = 0.048), whereas transfer factor prepared from lymphocytes from nonimmune animals did not. In addition, transfer factor derived from splenic lymphocytes from immune animals did not prolong survival of animals, even when large numbers of lymphocytes (up to 5 x 10⁹ per animal) were used. The effects of several dose levels of levamisole on cryptococcosis were also studied. Levamisole, 1 to 2.5 mg/kg given twice weekly on consecutive days to groups of 5 to 10 animals, had no significant effect on survival of untreated male or cortisone-treated female animals. Higher doses of levamisole appeared to be detrimental.

**DISCUSSION**

This experimental model of cryptococcosis in guinea pigs shares several features with cryptococcosis in man, e.g., an initially local infection that later disseminates, increased susceptibility of males (10), effects of corticosteroids, and histology of lesions in brains of cortisone-treated female guinea pigs (14). Once sex-related differences in response to experimental cryptococcosis were recognized, the model proved useful for studies of the effects of immunosuppression and immunostimulation on cryptococcosis. The better survival of female guinea pigs compared with males may have been partially related to the greater capacity of their monocytes to kill cryptococci. However, the absolute percentages of cryptococci killed in these studies were low, and other factors may be involved. For example, concentrations of estradiol as low as 1 µg/ml have been reported to inhibit growth of some isolates of C. neoformans in vitro (22). In addition, leukocytes from patients with cryptococcal meningitis were observed to have depressed phagocytosis of C. neoformans in vitro, which was corrected after 7 to 14 daily doses of 5 mg of diethylstilbestrol (23). Physiological levels of estrogens also have nonspecific stimulatory effects on the reticuloendothelial system (24) and on mitosis of immunocompetent cells (19).

Because of their better survival, female guinea pigs proved useful in studies of suppression of the immune response to cryptococcosis. Corticosteroid therapy is known to predispose to cryptococcosis in man (10), and more severe infections with a markedly reduced inflammatory reaction have been noted in corticosteroid-treated mice (30). In the studies reported here, female guinea pigs, which are cortisone-resistant animals, like humans (6), were given brief courses of cortisone acetate just overlapping the

**Table 1. Presence of Cryptococcus neoformans in brain by culture at intervals after i.p. infection**

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>Immune males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-7</td>
<td>6/6⁸</td>
<td>9/12</td>
</tr>
<tr>
<td>30</td>
<td>3/3</td>
<td>8/9</td>
</tr>
<tr>
<td>60</td>
<td>Not done</td>
<td>5/6</td>
</tr>
<tr>
<td>&gt;120</td>
<td>0/7</td>
<td>0/5</td>
</tr>
</tbody>
</table>

* Received killed C. neoformans in Freund complete adjuvant 10 days before infection.

* Number of brains culture positive/total number cultured.
time of infection with *C. neoformans*. These animals developed severe cryptococcosis with depressed immune and inflammatory responses, which persisted long after cortisone was stopped. The prolonged state of immunosuppression observed in these animals may be attributable to several factors. However, it is likely that the overriding factor was the initial inability to form an inflammatory response because of the cortisone therapy (5, 15, 25), resulting in a rapidly increasing antigenic load of organisms. For example, it is known that anergy in guinea pigs may be produced by repeated doses of moderate amounts of antigen (13). Further studies are in progress to determine whether the cortisone-induced suppression of the immune response to cryptococcosis is due to passive suppression or tolerance induced by high levels of antigen, or to an active process such as a suppressor cell.

In vitro studies of killing of cryptococci by neutrophils, monocytes, and macrophages from cortisone-treated, immunized, and control animals revealed only low levels of killing, and no significant differences in leukocyte function were seen when animals of the same sex were compared. Sera containing anticytotoxic antibodies did not consistently augment killing of cryptococci by leukocytes. Nevertheless, animals that had long-term survival appeared to clear disseminated cryptococci from extrapitoneal sites, including brain, rather than prevent dissemination of cryptococci from the peritoneal cavity. Studies of experimental cryptococcosis in mice by others have suggested that the inflammatory response in brain is delayed, but that organisms can be cleared from brain lesions (7, 31). Several different mechanisms for killing of *C. neoformans* by leukocytes in vitro have been described (7, 9, 11, 18, 21, 29, 32). All are relatively inefficient when compared with mechanisms for killing of most bacteria by leukocytes, and none have been clearly and consistently related to susceptibility to or severity of cryptococcosis in man. In this model of experimental cryptococcosis in the guinea pig, histological observations suggested that the quantity of the cellular inflammatory response in infectious foci, rather than the ability or efficiency of individual cells to kill cryptococci, determined the outcome of infections in most cases.

Finally, male guinea pigs proved useful in preliminary studies of stimulation of the immune response to cryptococcosis. Survival was prolonged after treatment with cryptococci in Freund complete adjuvant. Initial studies using immune lymphocytes or transfer factor derived from immune lymphocytes suggested the usefulness of this model for evaluation of these modes of immunostimulation. However, more extensive experiments with larger groups of animals will be required to verify any protective or therapeutic effects and to determine the specificity of immunity transferred, the optimum dosages, and dosage schedules. Levamisole, from the results in this model, appears much less promising, since several dosage regimens failed to prolong survival of animals after infection or to augment skin test reactivity. In any event, use of male guinea pigs, or cortisone-treated female guinea pigs, in this model appears to offer an adaptable system for the study of the effects of the immunostimulation on a chronic infection.

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LITERATURE CITED