Killing of *Cryptococcus neoformans* Strains by Human Neutrophils and Monocytes

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The susceptibility of various strains of *Cryptococcus neoformans* to killing by human polymorphonuclear leukocytes (PMNs) and monocytes was investigated. Five previously characterized strains of *C. neoformans* serotype A, a capsule-free mutant, and six recent clinical isolates were compared. PMNs and monocytes were isolated from normal peripheral blood and allowed to adhere to the flat-bottom wells of microtiter plates. Yeast cells of *C. neoformans* were added in the presence of normal human serum, and the plates were incubated at 37°C. After 4 h, killing was determined by comparing the quantitative plate counts of viable yeast cells in experimental wells with counts in control wells containing yeast cells in the absence of leukocytes. No appreciable growth of yeast cells occurred in the wells during the incubation period. Both PMNs and monocytes effectively killed yeast cells at effector-to-target ratios as low as 1:1, although monocytes failed to kill the capsule-free strain 602 at a 1:1 ratio. With 9 of 12 strains, PMNs killed *C. neoformans* more effectively than did monocytes. Significant interstrain variation in killing occurred for both monocytes and PMNs, and the recent, clinical isolates were more resistant to killing by monocytes and PMNs than were the previously characterized strains. The extent to which different strains were killed by monocytes and PMNs was not consistently related to the size of the capsule or the entire cell. Normal PMNs and monocytes are remarkably effective in killing strains of *C. neoformans* in the absence of specific antibody and appear to constitute a significant defense mechanism in the peripheral circulation.

*Cryptococcus neoformans* is a significant cause of opportunistic infection in patients with impaired cellular immunity (26, 28). Cryptococcal meningitis occurs more frequently in patients who have received corticosteroids, patients with hematological malignancies, and patients with AIDS, in whom the incidence of cryptococcosis is estimated at 10 to 15% (14, 17, 26).

Clinical and experimental evidence indicates that the initial pulmonary defenses against cryptococcosis involve the responses of T cells and the activities of alveolar macrophages (4, 5, 10, 11, 25, 26, 38). Recent murine studies suggest that natural killer cells may contribute to natural immunity against yeast cells (31, 32), but human natural killer cells are ineffective against *C. neoformans* in the absence of antibody (27). Therefore, in the peripheral blood, polymorphonuclear leukocytes or neutrophils (PMNs) and monocytes are the likely crucial defenses against cryptococcosis.

Phagocytosis of *C. neoformans* by PMNs and monocytes is promoted by complement. Activation of complement and deposition of C3 usually occurs by the alternative complement pathway, since specific anticytotoxic antibody is rarely present in patients with cryptococcosis (13, 15, 16, 23). Killing of *C. neoformans* by phagocytes may occur intracellularly (13) or extracellularly (38). Several studies have explored the mechanism of phagocytosis of *C. neoformans* by granulocytes and monocytes (9, 20, 21, 23), but only one study has carefully examined the killing of these yeast cells by peripheral leukocytes in the absence of specific antibody (13).

The purpose of this study was to compare the abilities of human PMNs and monocytes, under uniform conditions and in the absence of specific antibody, to kill strains of *C. neoformans* that differed in origin and in sizes of the cell and capsule. Strains from patients with AIDS or other underlying diseases and differing in diameter of the cell and capsular thickness were evaluated.

**MATERIALS AND METHODS**

*C. neoformans* strains. Six previously characterized strains of serotype A and six recent clinical isolates of *C. neoformans* were studied. All yeast strains were maintained at 37°C on slants composed of 2% glucose, 1% yeast extract (Difco Laboratories, Detroit, Mich.) (GYE), and 2% agar and were transferred at biweekly intervals. Strains designated 6, 15, 98, 110, and 145 are stable variants of serotype A that differ in (i) the size of the capsule, (ii) the degree to which they are phagocytized and killed by alveolar macrophages (4, 5), and (iii) the relative molecular sizes, compositions, and binding capacities of the capsular polysaccharides (34, 36). Strain 602, a capsule-free mutant, was a gift from Thomas R. Kozel (18). As described in a previous report (27), six recent isolates were obtained from the Clinical Mycology and Mycobacteriology Section of the Clinical Microbiology Laboratory at Duke Hospital and designated with the accession numbers 1188, 1218, 1458, 1508, 1948, and 1988; these strains were evaluated within 6 months or less from the time of recovery from patient specimens. The underlying diseases of the patients and the specimens from which these strains were isolated, the dimensions of the yeast cells and capsules, and the growth rates of these strains have been described elsewhere (27).

Isolation of human PMNs and monocytes. Venous blood from healthy human donors was collected in heparin (20 U/ml) and subsequently mixed with an equal volume of 3% dextran (Sigma, St. Louis, Mo.) in 0.85% NaCl. The cells

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were allowed to sediment by gravity for 45 min; the supernatant was layered onto a discontinuous gradient of Ficoll-Hypaque (LSM; Organon Teknica, Durham, N.C.) and centrifuged at 400 x g for 30 min. The cells at the resulting interface were removed, washed, and resuspended in medium composed of RPMI 1640 (GIBCO, Grand Island, N.Y.) supplemented with 1-glutamine, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, penicillin (100 U/ml), and streptomycin (100 µg/ml) (RPMI). To recover monocytes, the cell suspension was incubated in petri dishes at 37°C under 5% CO2–95% air for 60 min. The nonadherent cells were discarded, and the plates were rinsed several times with warm phosphate-buffered saline, pH 7.4. The adherent cells were mechanically removed with a rubber policeman. Viability, as determined with trypan blue, ranged between 80 and 90%, and >95% of the cells were mononuclear in appearance. The cells were resuspended at 3 x 106 viable monocytes per ml in RPMI with 1% (vol/vol) heat-inactivated fetal calf serum (GIBCO) (RPMI-FCS). The remaining supernatant from the Ficoll-Hypaque separation was discarded, the pellet was resuspended, and the erythrocytes were lysed by trituration with 0.1% NaCl, after which 1.7% NaCl was added to restore isotonicity. This suspension was centrifuged for 15 min at 250 x g, the supernatant was discarded, and the PMNs were suspended in RPMI-FCS. Both purity and viability were >98%, as determined by trypan blue, and the PMNs were adjusted to 3 x 106 viable cells per ml.

**Killing of C. neoformans by PMNs and monocytes.** Killing of *C. neoformans* was evaluated by comparing the percentage of viable yeast cells incubated in the presence of effector cells (PMNs or monocytes) with control growth in the absence of effectors. The viable yeast census was determined with a quantitative pour plate assay as described previously (27). After growth for 3 days on GYE slants at 37°C, yeast cells of *C. neoformans* were harvested, counted in a hemacytometer chamber, and diluted in RPMI. One hundred microliters of effector cells at 3 x 106 viable cells per ml was added to the flat-bottom wells of microtiter plates (no. 3596; Costar, Cambridge, Mass.). The plates were incubated at 37°C, 5% CO2 for 7.0 h. The cells at the resulting suspension were assayed for viability by trypan blue, and the PMNs were adjusted to 3 x 106 viable cells per ml.

**RESULTS**

Since variations in the growth rates of the different strains of *C. neoformans* could affect the assays of killing, growth curves were generated for most of the strains, and the doubling times were calculated during logarithmic growth. In either buffered glutamine-glycine-asparagine (36) medium or RPMI-FCS, the mean doubling time and standard deviation was 5.93 ± 0.68 h and ranged from 5.2 to 7.0 h (27). Since growth was negligible during the 4-h incubation time, a reduction in viable census was assumed to represent killing.

The effects of human PMNs and monocytes on *C. neoformans* were tested under experimental conditions that were similar to those used in previous reports of the anticytopycocal activities of these effector cells. Five encapsulated strains of *C. neoformans* serotype A (strains 145, 110, 98, 15, and 6) that differ in their susceptibilities to phagocytosis and killing by alveolar macrophages (4, 5), as well as in the sizes and chemical properties of their capsules (34, 36), and the capsule-free strain 602 (18, 25) were compared to evaluate any strain differences in resistance to killing by PMNs and monocytes. Yeast cells (T) of each strain were tested against PMN or monocyte effector (E) cells at E:T ratios of 100:1, 50:1, and 1:1, and growth was quantified in the presence and absence of effectors after incubation for 4 h.

The percentages of killing of these strains by human PMNs and monocytes are shown in Tables 1 and 2, respectively. Both PMNs and monocytes significantly killed (*P < 0.005*) all of the encapsulated strains of *C. neoformans* at all three E:T ratios. The percentages of killing of the encapsulated strains were consistently >75% for both effector leukocytes at E:T ratios of 100:1 and 50:1. At the ratio of 1:1, killing by PMNs varied from 33.5 to 88% (Table 1), and monocytes killed 14.5 to 63.5% of the yeast cells (Table 2). The capsule-free strain 602 was more susceptible than the
encapsulated strains to killing by PMNs (Table 1) and more resistant to monocytes (Table 2). PMNs were generally more effective than monocytes in killing the encapsulated strains. Since none of the strains multiplied appreciably during the 4 h of incubation in control wells containing medium without leukocytes, any difference between experimental and control wells could be attributed to killing of the yeast cells by the leukocytes rather than inhibition of yeast cell growth. (Triton X-100, which was added to lyse the leukocytes and release phagocytized yeast cells, did not affect the viability of the yeast cells, and no clumping of yeast cells occurred in experimental dilutions.)

Killing of these encapsulated strains by PMNs at the E:T ratio of 1:1 (Table 1) was inversely related to both capsule width and particle diameter (i.e., cell diameter plus twice the capsule width) (27). The respective linear regression coefficients for these relationships were 0.97 and 0.95. A similar but less striking correlation existed between killing by monocytes at the 1:1 ratio and dimensions of the capsule and particle.

Six recent clinical isolates were also tested against PMNs and monocytes. These strains varied according to the specimen from which they were isolated, the underlying disease of the patient, and the capsule and cell sizes (27). To discern any differences in susceptibility to PMNs or monocytes, only E:T ratios of 1:1 were tested since higher ratios were effective against C. neoformans. Although the strains varied in susceptibility (Table 3), both PMNs and monocytes killed these strains quite effectively (P < 0.001, compared with controls). Strain variation in susceptibility to killing was apparent. At the same ratio, the recently isolated strains were significantly more resistant (P < 0.01) to PMNs and monocytes than were the previously characterized strains of comparable capsule and particle size (Table 3 versus Tables 1 and 2). Reproducible strain differences in killing by monocytes were observed. PMNs were more effective than monocytes in killing three of the strains (1458, 1948, and 1508), but killing was comparable against the other three strains (1218, 1188, and 1948). As found previously, no appreciable growth of these strains occurred during the period of incubation. Strains 1508, 1188, and 1948 were obtained from patients with AIDS (27). No correlation was found between PMN or monocyte killing and capsule or particle size of these clinical isolates.

**DISCUSSION**

Human PMNs and monocytes, in the presence of normal human serum, efficiently killed strains of C. neoformans at E:T ratios as low as 1:1. Killing of C. neoformans by phagocytes occurs both intracellularly and extracellularly (13). This investigation did not distinguish between intracellular and extracellular killing. Phagocytosis is mediated by activation by the alternative complement pathway and opsonization of the yeast cell (9, 20, 23). C3b and iC3b are concentrated at the periphery of the capsule, facilitating interaction with phagocytic complement receptors (21, 22). Anticytotoxic antibody was not used in this study as an opsonin since antcapsular immunoglobulin G does not increase granulocyte or monocyte phagocytosis or killing capacity (9, 13, 20).

Considerable strain variation in susceptibility to killing was observed among the 12 strains of C. neoformans that were tested against PMNs and monocytes. The range of percent killing by both PMNs and monocytes was consistent with reports of killing by human peripheral leukocytes (13). Nine of the strains were killed more efficiently by PMNs than monocytes. Diamond et al. (13) reported that PMNs were more effective than monocytes in killing both strains of C. neoformans that they studied. Kozeł et al. (20) showed that PMNs were superior to monocytes in phagocytizing the yeasts in the presence of C3 or rabbit antcapsular immunoglobulin G.

Comparison of strains of similar capsule and particle sizes showed that the recent isolates were more resistant to killing by both PMNs and monocytes than were the previously studied laboratory strains. This increased resistance may indicate that maintenance for long periods selects less resistant cells. To test this hypothesis, inbred animals will be used to compare the virulence and phagocytosis of these strains. Isolates from patients with AIDS were reported to

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**TABLE 1. Killing of various strains of C. neoformans by human PMNs**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Capsule width (µm)</th>
<th>Particle size (µm)</th>
<th>% Killing at E: T ratio of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>100:1</td>
</tr>
<tr>
<td>15</td>
<td>&lt;1.0</td>
<td>&lt;6.0</td>
<td>96.5 ± 1.5</td>
</tr>
<tr>
<td>145</td>
<td>1.7</td>
<td></td>
<td>8.5</td>
</tr>
<tr>
<td>98</td>
<td>2.0</td>
<td></td>
<td>8.0</td>
</tr>
<tr>
<td>110</td>
<td>2.4</td>
<td></td>
<td>2.4</td>
</tr>
<tr>
<td>6</td>
<td>&gt;3.5</td>
<td></td>
<td>&gt;14.1</td>
</tr>
<tr>
<td>602</td>
<td>None</td>
<td></td>
<td>3.8</td>
</tr>
</tbody>
</table>

* a Average capsule width or particle (cell diameter plus twice the capsule width) size from data reported previously (27).

b Determined as (1 mean experimental CFU per well mean control CFU per well) 100 ± standard error of the mean. Each value was determined from two or more experiments and at least 12 plates. P < 0.001 for all values, comparing mean experimental with control CFU.

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**TABLE 2. Killing of various strains of C. neoformans by human monocytes**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Capsule width (µm)</th>
<th>Particle size (µm)</th>
<th>% Killing at E: T ratio of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>100:1</td>
</tr>
<tr>
<td>15</td>
<td>93.0 ± 1.0</td>
<td>93.5 ± 1.5</td>
<td>63.5 ± 0.5</td>
</tr>
<tr>
<td>145</td>
<td>96.0 ± 2.5</td>
<td>91.0 ± 1.5</td>
<td>63.0 ± 3.5</td>
</tr>
<tr>
<td>98</td>
<td>91.5 ± 3.5</td>
<td>87.0 ± 2.0</td>
<td>48.5 ± 1.5</td>
</tr>
<tr>
<td>110</td>
<td>87.5 ± 0.5</td>
<td>81.0 ± 0.1</td>
<td>45.0 ± 1.0</td>
</tr>
<tr>
<td>6</td>
<td>95.5 ± 0.5</td>
<td>92.0 ± 1.0</td>
<td>14.5 ± 0.5</td>
</tr>
<tr>
<td>602</td>
<td>46.5 ± 2.5</td>
<td>17.0 ± 1.0</td>
<td>0</td>
</tr>
</tbody>
</table>

* a See Table 1, footnote b. P < 0.005 for all values comparing mean experimental with control CFU with the exception of strain 602 at the ratio of 1:1 (P > 0.1).

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**TABLE 3. Killing of recent clinical isolates of C. neoformans by human PMNs and monocytes**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Capsule width (µm)</th>
<th>Particle size (µm)</th>
<th>% Killing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PMN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Monocyte</td>
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<tr>
<td>1218</td>
<td>&lt;1.0</td>
<td>&lt;5.2</td>
<td>48.5 ± 2.5</td>
</tr>
<tr>
<td>1458</td>
<td>1.6</td>
<td>8.4</td>
<td>46.5 ± 2.5</td>
</tr>
<tr>
<td>1948</td>
<td>2.0</td>
<td>9.0</td>
<td>44.5 ± 2.5</td>
</tr>
<tr>
<td>1508</td>
<td>&lt;1.0</td>
<td>&lt;6.2</td>
<td>44.0 ± 2.0</td>
</tr>
<tr>
<td>1188</td>
<td>&lt;1.0</td>
<td>&lt;5.9</td>
<td>46.0 ± 4.0</td>
</tr>
<tr>
<td>1948</td>
<td>2.3</td>
<td>8.8</td>
<td>28.0 ± 3.0</td>
</tr>
</tbody>
</table>

* b See footnotes to Table 1 for explanation of capsule widths, particle sizes, and percent killing by PMNs and monocytes. E:T ratio is 1:1; P < 0.001 for all values, comparing mean experimental with control CFU.
have smaller capsule and cell sizes (6, 7), but in this study, the three strains from AIDS patients (Table 3, strains 1508, 1188, and 1948) were neither significantly smaller than the other recent clinical isolates (strains 1218, 1458, and 1988) nor more susceptible to killing by PMNs or monocytes.

The capsular polysaccharide of C. neoformans is associated with virulence and possesses a number of pathobiological properties, including specific inhibition of phagocytosis (8, 22, 35) and suppression of both antibody production and cell-mediated immune responses (2, 3, 19, 29, 30).

The capsule may impede phagocytosis by preventing adequate contact between phagocytic receptors and opsonins or ligands on the cell surface (24). To determine whether capsule width or whole particle size (cell size plus twice the capsule width) correlated with killing by PMNs or monocytes, both the capsule width and cell size were measured (Tables 1 and 3). Killing of the five previously characterized, encapsulated strains by either PMNs or monocytes was somewhat inversely correlated with both capsule and particle sizes. This relationship was more apparent at the E:T ratio of 1:1. Strains 145, 98, and 110 were relatively similar in size and comparably killed by PMNs, whereas the largest strain, strain 6, was more resistant and the smallest, strain 15, was killed most effectively (Table 1). This pattern was similar with monocytes (Table 2). The relative influence of capsule size versus particle size could not be differentiated.

With rat alveolar macrophages, strain variation in susceptibility to phagocytosis is inversely related to capsule size, but the amount of killing is unrelated to either capsule size or the extent of phagocytosis (4, 5). The patterns of killing of these strains were not entirely consistent. At 1:1 ratios, strains 98 and 145 were relatively susceptible to killing by both human monocytes and rat alveolar macrophages. However, strain 110 was the most resistant to rat alveolar macrophages (5), and strain 6 was the most resistant to human PMNs and monocytes (Tables 1 and 2). The capsule-free strain 602 was killed most effectively by PMNs but less efficiently by monocytes than were the encapsulated strains, which is consistent with the recently reported resistance of capsule-free cells to alveolar macrophages (25).

Among the recent clinical isolates, killing by both PMNs and monocytes was pronounced (Table 3). However, the variation among strains in susceptibility to killing did not correlate with capsule or particle size. Strain variation in killing may also provide a tool to study the differing mechanisms of killing among PMNs, monocytes, and macrophages. Resistance to killing is a likely determinant of the pathogenicity of a strain.

PMNs and monocytes represent effective host defenses against both poorly and highly encapsulated yeast cell strains of C. neoformans. Yeasts that are not killed within the lung, or elsewhere, may enter the circulation. (A frequent risk factor associated with cryptococcosis is the administration of corticosteroids [33].) These nonspecific effector cells undoubtedly provide superior defense against invasion of the bloodstream, as natural killer cells were ineffective in vitro (27). Although PMNs are not a common feature in cryptococcal lesions, and patients with neutropenia are not at particular risk for cryptococcosis, patients receiving immunosuppressive therapy and patients with AIDS are more susceptible to cryptococcosis and often have phagocytic and chemotactic defects of their neutrophils and monocytes (1, 12). However, in autologous serum, monocytes from patients with AIDS were able to kill a strain of C. neoformans (37). Several novel observations are presented in this report.

Human PMNs and monocytes were effective against a relatively large number of strains of C. neoformans, and PMNs were usually more effective than monocytes. The strains exhibited significant variation in the extent to which they were killed by PMNs or monocytes. This variation was not consistently related to size of the yeast cell and capsule. Strains from AIDS patients were comparable in susceptibility, as well as in cell and capsule dimensions, to other strains. Cells of the capsule-free strain were more resistant to monocytes than were encapsulated yeast cells. The basis for strain variation in killing by PMNs and monocytes is under investigation.

ACKNOWLEDGMENTS

The technical assistance of Elizabeth Z. Freedman is greatly appreciated. This research was supported by Public Health Service grant AI 25783 from the National Institute of Allergy and Infectious Diseases.

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