Effects of the Two Varieties of *Cryptococcus neoformans* Cells and Culture Filtrate Antigens on Neutrophil Locomotion

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Cryptococcus neoformans var. *gattii* (serotype B and C) isolates have a relative predilection for immunocompetent hosts, and *C. neoformans* var. *neoformans* (serotype A and D) isolates have a relative predilection for immunocompromised hosts, suggesting that normal host resistance to the former may be relatively inefficient compared with that to the latter variety. In order to assess the possibility that normal cellular host defense is inadequate in protecting against *C. neoformans* var. *gattii*, we compared the two varieties of *C. neoformans* cells and their culture filtrate antigens (CneF) with respect to effects on neutrophil (polymorphonuclear leukocyte [PMN]) locomotion. In a 48-well modified Boyden chamber, the cells and CneF of *C. neoformans* var. *neoformans* (serotype A and D) isolates stimulated chemotaxis and chemokinesis of human PMN and activated a complement component(s) in pooled human serum to become a chemoattractant(s) for human PMN. In contrast, the cells and CneF of *C. neoformans* var. *gattii* (serotype B and C) isolates did not stimulate chemotaxis or chemokinesis in human PMN but rather inhibited chemokinesis and chemotactic responses of PMN to pooled human serum and formylmethionyl leucyl phenylalanine. Neither of the CneF from the *C. neoformans* var. *gattii* isolates was cytotoxic to PMN. Furthermore, with the mouse model, we found that CneF from *C. neoformans* var. *neoformans* caused migration of PMN into gelatin sponges implanted in naive and immunized mice, whereas CneF from *C. neoformans* var. *gattii* inhibited PMN migration into sponges. Our results, combined with findings of others showing reduced PMN infiltration in lungs of mice infected with *C. neoformans* var. *gattii* compared with PMN infiltration in lungs of mice infected with *C. neoformans* var. *neoformans*, indicate that the relative inadequacy of normal host resistance mechanisms to prevent infection with *C. neoformans* var. *gattii* results, in part, from inhibition of PMN migration to the site of the organism.

Cryptococcus neoformans, an encapsulated yeast-like organism, has two varieties, *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*. There are two serotypes in each variety, serotypes A and D for *C. neoformans* var. *neoformans* and serotypes B and C for *C. neoformans* var. *gattii*. *C. neoformans* var. *neoformans* differs markedly from *C. neoformans* var. *gattii* with regard to its natural habitat (9), geographic distribution (14), and occurrence in patients with AIDS (10, 15, 32). *C. neoformans* var. *neoformans* has been found in nature worldwide, primarily in association with bird droppings (14). *C. neoformans* var. *gattii* has recently been isolated from nature in association with eucalyptus trees (9). Infections caused by the latter variety occur mainly in tropical and subtropical regions. The isolates from AIDS patients, with few exceptions, have been *C. neoformans* var. *neoformans* (15). Moreover, other differences in the two varieties of *C. neoformans* have also been reported. *C. neoformans* var. *gattii* has a relative predilection for immunocompetent hosts, and *C. neoformans* var. *neoformans* has a relative predilection for immunocompromised hosts (29, 31). A retrospective analysis by Henderson et al. (12) suggests that infections with *C. neoformans* var. *gattii* are more difficult to treat successfully than infections caused by *C. neoformans* var. *neoformans*.

The mechanisms underlying these pathogenetic differences between the two varieties of *C. neoformans* are not clear, but it is reasonable to suspect that virulence and/or the mechanisms of host resistance are different for the two varieties. Washburn et al. (34) suggested that inefficient binding of alternative com-

plement pathway components to *C. neoformans* var. *gattii* contributes to the relative difficulty in successfully treating infections caused by this organism.

Effective host defense against *C. neoformans* depends upon a combination of multiple mechanisms that include both natural and immune defensive measures (21). Early after exposure to the organism, natural cellular defense mechanisms are the primary means by which the organism is eliminated from the host, whereas later, i.e., 1 to 2 weeks after infection in the mouse model, cryptococci are cleared from tissues predominantly by cell-mediated immune (CMI) mechanisms (13, 21). Because exposure to *C. neoformans* var. *neoformans* is probably common and clinically apparent cases of cryptococcosis in immunocompetent individuals are rare, it is presumed that most immunocompetent people can mount adequate host defenses upon exposure to *C. neoformans* var. *neoformans*. On the other hand, *C. neoformans* var. *gattii* causes clinical disease in immunocompetent individuals. Consequently, it is reasonable to postulate that host resistance of immunocompetent humans to *C. neoformans* var. *gattii* is relatively inefficient.

Polymorphonuclear leukocytes (PMN) are regarded as one of the effective components of the host's natural cellular defenses, and PMN can aid in early clearance of *C. neoformans* from the host (13, 18, 20). Recently, Buchanan and Murphy (3) found that PMN were one of the infiltrating cell types in the anticryptococcal CMI reaction, suggesting that PMN participate in the CMI response against *C. neoformans*. If PMN act as effector cells against *C. neoformans*, irrespective of whether their action is direct or indirect, PMN must first migrate to the site of infection. If, for example, migration of PMN to *C. neoformans* var. *gattii* is weak or decreased compared with migration toward *C. neoformans* var. *neoformans*, then rela-

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tively inefficient normal host resistance to *C. neoformans* var. *gattii* would be the result.

Our previous studies have shown that a serotype A isolate of *C. neoformans* and the culture filtrate antigen (CneF) derived from the isolate stimulated directed migration of normal human PMN in the absence of serum and activated a heat-labile component(s) in fresh human serum to become a chemoattractant(s) for normal human PMN (8). Also as mentioned above, infection or immunization of mice with *C. neoformans* serotype A results in significantly increased numbers of infiltrating PMN into delayed-type hypersensitivity (DTH)-reactive sponges compared with saline-injected sponges in the same animals (3).

The main purpose of the present study was to determine whether isolates of *C. neoformans* var. *gattii* have abilities similar to or different from those of isolates of *C. neoformans* var. *neoformans* to induce migration of PMN. To gain information concerning this question, we first assessed whether or not the two varieties of *C. neoformans* cells and CneF prepared from the different-serotype isolates have different abilities to directly stimulate migration of normal human PMN in the absence of serum. In addition, we determined the abilities of the organisms and their CneF to activate a complement component(s) in pooled human serum (PHS) to become a chemotactic factor(s) for normal human PMN. Finally, we compared the ability of CneF from the two varieties of *C. neoformans* to attract PMN into gelatin sponges implanted in naive and immunized mice.

MATERIALS AND METHODS

Mice. Female, inbred CBA/J mice purchased from the Jackson Laboratory, Bar Harbor, Maine, were used at 7 to 10 weeks of age.

Maintenance of endotoxin-free conditions. To prevent endotoxin from influencing the experimental results, all experiments were performed under conditions which minimize endotoxin contamination. This included the use of plasticware that was endotoxin free, glassware that had been heated for 3 h at 180°C, and reagents that had been found to contain less than 8 pg of endotoxin per ml (minimal detectable level) with the *Limulus* assay (Whittaker Bioproducts, Inc., Walkersville, Md.).

Organisms. Two isolates of each variety, serotype A and D isolates of *C. neoformans* var. *neoformans* and serotype B and C isolates of *C. neoformans* var. *gattii*, were used throughout the study. The serotype A isolate was 184A, the serotype B isolate was B435B, the serotype C isolate was B13C, and the serotype D isolate was B3501D. Isolate 184A has been previously described (22), and when grown on Sabouraud agar it has a mean capsule thickness of 0.58 ± 0.19 (standard error of the mean) μm . Isolates B435B and B13C were obtained from J. E. Bennett, National Institutes of Health, and on Sabouraud agar they have mean capsule thicknesses of 1.43 ± 0.65 and 0.65 ± 0.17 μm , respectively. Isolate B3501D was obtained from K. J. Kwon-Chung, National Institutes of Health, and its mean capsule thickness on Sabouraud agar is 0.90 ± 0.33 μm . All of the organisms were maintained on modified Sabouraud agar slants. Heat-killed cryptococci were prepared by harvesting the blastoconidia in endotoxin-free, sterile physiologic saline solution (SPSS) after 3 days of growth at room temperature on modified Sabouraud agar slants and incubating the organisms at 60°C for 1 h. After heat treatment, sterility of the culture was demonstrated by lack of growth on modified Sabouraud agar slants. The heat-killed cryptococci were washed three times and resuspended in Hanks' balanced salt solution (HBSS).

Human PMN. Human PMN were obtained by the method previously described (8, 36). The PMN-enriched cell populations were composed of >95% neutrophils as determined by differential counts on Diff-Quick (Baxter Healthcare Co., Miami, Fla.)-stained smears. The viability of the cells in the PMN-enriched fraction was >95% by trypan blue dye exclusion.

Chemotactic reagents. Formylmethionyl leucyl phenylalanine (FMLP) was obtained from Sigma. PHS was sera pooled from three volunteers and stored at -130°C . The main chemoattractant in PHS is C5a or a C5a derivative (25).

CneF. CneF-184A, CneF-B435B, CneF-B13C, and CneF-B3501D were prepared by the procedure described by Buchanan and Murphy (3) from the corresponding *C. neoformans* isolates. Briefly, *C. neoformans* was cultured in a liquid defined medium for 5 days, after which time the *C. neoformans* cells were removed with a Millipore OM-141 Pellicon Tangential Flow System and a 0.45- μm -pore-size filter. The culture filtrates were washed with 10 volumes of sterile physiologic saline solution with a 30,000-molecular-weight cutoff cassette in the Pellicon System, and then the material retained was concentrated to 1/10 of the original volume. The protein concentrations were 134 $\mu\text{g}/\text{ml}$ for CneF-184A, 128 $\mu\text{g}/\text{ml}$ for CneF-B435B, 131 $\mu\text{g}/\text{ml}$ for CneF-B13C, and 108 $\mu\text{g}/\text{ml}$ for CneF-

B3501D on the basis of the bicinchoninic acid assay (BCA Protein Assay; Pierce Chemical Co., Rockford, Ill.). The carbohydrate (CHO) concentrations were 2 mg/ml for CneF-184A, 1.65 mg/ml for CneF-B435B, 1.85 mg/ml for CneF-B13C, and 1.60 mg/ml for CneF-B3501D as determined by the phenol-sulfuric acid assay (8). Each CneF was analyzed by anodic polyacrylamide slab gel electrophoresis and concanavalin A blotting as described previously by Murphy et al. (24). All CneF preparations had similar banding and staining patterns on the electrophoretic gels, as previously reported for CneF-184A (24). Furthermore, there was a concanavalin A-binding component (mannoprotein) in each of the CneF preparations.

Chemotaxis and chemokinesis assay. PMN chemotaxis and chemokinesis assays were performed as previously described (8). Briefly, we used a 48-well modified Boyden chamber (NeuroProbe, Inc., Bethesda, Md.) with a 10- μm -thick, polyvinylpyrrolidone-free polycarbonate membrane (Nuclepore Corp., Pleasanton, Calif.) having a 3- μm pore size. Polyvinylpyrrolidone-free membranes were used because PMN remain on the bottom of the membranes and do not drop off into the medium below (11). The chambers, with PMN at a final number of 2×10^5 per well in 50 μl of HBSS in the top compartment and chemoattractants in the lower compartment, were incubated for 60 min at 37°C. In all experiments, FMLP at 10^{-6} M in 25 μl of HBSS was loaded in the lower wells as a positive chemotaxis control. PHS was used at a final concentration of 25% in HBSS. In certain experiments, PMN and various dilutions of the designated CneF were loaded in the upper wells. The numbers of PMN which migrated to the reverse side of the membrane were counted in five high-power microscopic fields. The results are reported as the mean of triplicate determinations of the numbers of PMN which migrated across the membrane $\pm$ the standard error of the mean.

In some experiments, a single preparation of PMN from one donor was used with CneF prepared with one *C. neoformans* serotype. Therefore, it is possible that the differences in PMN migration stimulated by CneF made from two different varieties of *C. neoformans* resulted from the use of PMN from different individual donors. To rule out this possibility, all such experiments were repeated by using PMN from one donor to test individually but simultaneously CneF from the two varieties of *C. neoformans*. We found that CneF prepared with *C. neoformans* var. *neoformans* isolates gave similar results with PMN from all of the donors tested and CneF prepared with *C. neoformans* var. *gattii* isolates had similar effects on all of the populations of PMN tested.

Footpad testing. Induction of the anticytotoxic cell (CMI) response by immunization with CneF-184A emulsified in CFA (complete Freund's adjuvant) was achieved by previously described methods (3). Briefly, 0.1 ml of a 1:1 emulsion of CneF-184A in CFA was injected subcutaneously at each of two sites at the base of the tail. At 6 days after immunization, the hind footpads of mice were measured; the left footpads were then injected with 30 μl of SPSS, and the right footpads were injected with 30 μl of CneF-184A, CneF-B435B, or CneF-B13C. The footpads were measured 24 h after the challenge injection. The increase in footpad thickness was calculated by subtracting the difference in swelling in SPSS-injected footpads from the difference in swelling in CneF-injected footpads (23). Control mice were injected with SPSS-CFA and were otherwise treated in the same way as immunized mice.

Induction and detection of leukocyte infiltration into gelatin sponges implanted in mice. The procedures for sponge implantation, retrieval, disaggregation, cell counting, and differential analysis of cells infiltrating sponges were the same as those previously described by Buchanan and Murphy (3). Acute leukocyte infiltrations induced by CneF prepared from the two different varieties of *C. neoformans* were compared in naive mice. At 4 days after two sponges were implanted into each side of naive mice, one sponge was injected with 0.1 ml of SPSS and the other was injected with 0.1 ml of CneF-184A, CneF-B435B, or CneF-B13C. At 6 h after the injection, the sponges were removed and cells were collected and counted in a hemacytometer. Cytospins of the cell suspensions were stained with Diff-Quick, and differential counts were determined by counting a total of 200 cells per sample. The numbers of the specific leukocyte types infiltrating the sponges were determined by multiplying the percentage of the specific leukocyte type by the total number of leukocytes in the sponge.

For comparison of leukocyte infiltration into the site of a DTH reaction, the different CneF were independently tested by injecting the CneF into sponges in immunized and control mice. For immunization, test mice were injected with CneF-184A-CFA and control mice were treated with SPSS-CFA by using the procedure described for footpad testing. At 6 days after treatment or 4 days after sponge implantation, one sponge in each mouse was injected with 0.1 ml of SPSS and the other was injected with 0.1 ml of CneF-184A, CneF-B435B, or CneF-B13C. At 24 h after injection, the sponges were removed and processed as described above.

Statistical analyses. The mean, the standard error of the mean, and Student's *t* test results were used to analyze the data. When two groups were compared, a *P* of ≤ 0.05 was considered to indicate a significant difference between the groups.

RESULTS

PMN chemotactic responses to the two varieties of *C. neoformans* cells and to PHS activated with *C. neoformans* cells. As previously shown (8), *C. neoformans* var. *neoformans* serotype

FIG. 1. (A) Chemotactic responses of human PMN in HBSS to 1.25×10^7 to 5×10^7 blastoconidia of *C. neoformans* isolate 184A (serotype A), B435B (serotype B), B13C (serotype C), or B3501D (serotype D) per ml. (B) Chemotactic responses of human PMN to PHS before and after incubation of the PHS with 1.25×10^7 to 5×10^7 cells of *C. neoformans* isolate 184A, B435B, B13C, or B3501D per ml. PHS was incubated with heat-killed *C. neoformans* cells for 30 min at 37°C , and then the cryptococcal cells were removed by centrifugation before the PHS was used in the PMN chemotactic assay. Data are from a representative experiment. The experiment was repeated two times with similar results. SEM, standard error of the mean.

A cells stimulated migration of PMN across the membrane of a chemotactic chamber. Likewise, a serotype D isolate of *C. neoformans* var. *neoformans* also induced migration of PMN but to a lesser degree than did the serotype A cells (Fig. 1A; $P < 0.01$). In contrast, *C. neoformans* var. *gattii* serotype B and C cells did not stimulate PMN to migrate across the membrane compared with the migration of the HBSS control (Fig. 1A; $P > 0.05$).

In our previous studies (8), we had found that PHS activated with 2.5×10^7 to 5.0×10^7 serotype A cryptococcal cells per ml displayed significantly greater chemotactic activity (indirect chemotactic activity) than did unactivated PHS. In the present study, we found that *C. neoformans* var. *neoformans* cells of serotype D also had the ability to activate PHS to generate a chemotactic factor(s) (Fig. 1B; $P < 0.01$; PHS activated by 5×10^7 serotype D cryptococcal cells per ml compared with unactivated PHS); however, the indirect chemotactic activity of serotype D cryptococcal cells was less than that of serotype A cryptococcal cells (Fig. 1B; activation with 2.5×10^7 to 5×10^7

serotype A cryptococcal cells per ml compared with activation by the respective numbers of serotype D cells; $P < 0.01$). In contrast, pretreatment of PHS with *C. neoformans* var. *gattii* serotype B or C cells not only did not activate PHS chemotactic activity for PMN (Fig. 1B) but reduced the activity of PHS below levels observed with untreated PHS (Fig. 1B; $P < 0.01$).

PMN chemotactic responses to CneF prepared from different varieties of *C. neoformans* with or without PHS. In our previous studies, we had found that CneF prepared with a *C. neoformans* var. *neoformans* serotype A isolate stimulated directed migration of PMN in the absence of serum and activated a heat-labile component(s) in fresh PHS to become a chemoattractant(s) for PMN (8). On the basis of our earlier results, we were interested in comparing the chemotactic activities of CneF prepared from another *C. neoformans* var. *neoformans* of the D serotype and from *C. neoformans* var. *gattii* to that of the serotype A CneF with or without PHS. At a dilution of 1:2 (CHO, 0.8 mg/ml) or 1:4 (CHO, 0.4 mg/ml), serotype D CneF in the absence of PHS had positive chemotactic activity for PMN compared with the HBSS control ($P < 0.01$ compared with the HBSS control) and had chemotactic activity for human PMN similar to that of a 1:2 (CHO, 1 mg/ml) or 1:4 (CHO, 0.5 mg/ml) dilution of serotype A CneF (Fig. 2A and D). In contrast, CneF made from *C. neoformans* var. *gattii* at all of the dilutions tested lacked chemotactic activity for PMN. In fact, a 1:2 or 1:4 dilution of CneF prepared from the *C. neoformans* var. *gattii* isolates consistently caused a reduction in PMN migration compared with the HBSS control (Fig. 2B and C; $P < 0.01$).

When PHS was mixed with CneF from any one of the four *C. neoformans* isolates, results similar to those obtained with the respective whole cryptococcal cells were obtained. At a 1:2 or 1:4 dilution, both serotype A and D CneF displayed indirect chemotactic activity (Fig. 3A and D; $P < 0.01$ compared with PHS alone). In contrast, when PHS was mixed with a 1:2 or 1:4 dilution of serotype B or C CneF in lower wells of the chemotactic chamber, PMN migration toward the lower chamber was significantly decreased (Fig. 3B and C; $P < 0.01$ compared with PHS alone). These results demonstrate that the CneF made from *C. neoformans* var. *gattii* do not increase chemotactic activity of PHS but rather inhibit the chemotactic response of PMN to PHS.

Effects of CneF from the two varieties of *C. neoformans* on chemotactic responses of PMN to FMLP. In order to determine whether the inhibitory effect of the serotype B and C CneF on the chemotactic response of PMN to PHS is unique to PHS as a chemoattractant, we tested the chemotactic responses of PMN to FMLP in the presence of various concentrations of CneF prepared with the various *C. neoformans* isolates. As shown in Fig. 4, when FMLP was mixed with a 1:2 or 1:4 dilution of serotype A or D CneF, more PMN migration was stimulated than when FMLP was used alone (Fig. 4A and D; $P < 0.01$). This increased chemotactic activity to FMLP with serotype A or D CneF could be attributed to the combined chemotactic activities of FMLP and CneF. In contrast, FMLP mixed with a 1:2 or 1:4 dilution of serotype B or C CneF induced fewer PMN to migrate than did FMLP alone (Fig. 4B and C; $P < 0.01$).

Effects of CneF from the two varieties of *C. neoformans* on PHS- and FMLP-induced PMN migration when CneF is mixed with PMN in the upper wells of chemotactic chambers. The previous experiment showed that serotype B or C CneF was not a chemoattractant for human PMN but rather inhibited PMN migration in HBSS and in response to PHS and FMLP. To obtain more information about the inhibitory effects mediated by the serotype B and C CneF, PMN were mixed with

FIG. 2. Chemotactic responses of human PMN to sequential twofold dilutions of CneF from two isolates of *C. neoformans* var. *neoformans* and two isolates of *C. neoformans* var. *gattii* in HBSS. FMLP at 10^{-6} M in HBSS was used as a positive chemotaxis control. For each panel, a single preparation of PMN from one donor was used. Experiments for each CneF were repeated with PMN from five different donors with similar results. SEM, standard error of the mean.

serotype A, B, C, or D CneF and placed in the upper wells of the chemotactic chamber and HBSS, PHS, or FMLP was put into the lower wells. In the presence of serotype A or D CneF, irrespective of the concentration, PMN migration in HBSS and in response to PHS was the same as in the absence of the CneF in the upper wells (Fig. 5A and D; $P > 0.05$). In contrast, both serotype A and D CneF, at the highest concentration tested (1:2 dilution), inhibited PMN migration in response to FMLP. As shown by the data in Fig. 4, FMLP and serotype A or D CneF have additive effects on PMN migration. Thus, when serotype A or D CneF at a 1:2 dilution was added to PMN in the upper wells, the CneF appeared to desensitize PMN migration in response to FMLP. Results such as this are similar to results observed when FMLP itself is added to the upper wells (19).

In similar experiments, when PMN were mixed with serotype B or C CneF, their migration in HBSS or in response to PHS or FMLP was significantly inhibited (Fig. 5B and C; when a CneF dilution of 1:2, 1:4, or 1:8 was compared with the HBSS control, $P < 0.01$). Inhibitory effects of serotype B or C CneF when mixed with PMN in upper wells seemed to be stronger than those observed when the B or C CneF was mixed with HBSS, PHS, or FMLP in lower wells. PMN migration in HBSS or in response to PHS or FMLP was completely inhibited (no PMN were found on the reverse side of the membrane and in lower wells of the chamber) with a 1:2 dilution of serotype B or C CneF in PMN wells. A 1:8 dilution of serotype B or C CneF that did not show inhibitory effects on PMN migration when put into lower wells with HBSS, PHS, or

FMLP displayed inhibitory activities when mixed with PMN in upper wells (Fig. 5B and C).

Comparison of PMN chemokinesis in response to CneF prepared with the two different varieties of *C. neoformans*. Our previous studies have shown that when a 1:8 dilution, but not a 1:2 or 1:4 dilution, of serotype A CneF was placed in both the upper wells with PMN and lower wells (no concentration gradient), an increase in random locomotion of PMN was observed (8). Thus, at a 1:8 dilution, serotype A CneF displayed chemokinetic activity. Similar results were obtained in this study (Fig. 6A). Moreover, serotype A CneF at an even lower concentration (1:16 dilution) induced chemokinesis of PMN (Fig. 6A; when a 1:16 dilution of serotype A CneF was placed in both upper and lower wells, $P < 0.01$ compared with the HBSS control). Like serotype A CneF, serotype D CneF also stimulated chemokinetic activity in human PMN (Fig. 6D). In contrast, neither serotype B nor C CneF at any dilution tested displayed chemokinetic activity. In fact, both serotype B and C CneF at a 1:2, 1:4, or 1:8 dilution had inhibitory effects on chemokinesis of PMN (Fig. 6B and C; when a 1:2 or 1:4 dilution was used, no PMN were found on the reverse side of the membrane and no PMN were observed in the fluid in the lower wells of the chambers).

Effects of CneF prepared with the two different varieties of *C. neoformans* on PMN viability. The above experiments showed that CneF prepared with either of the *C. neoformans* var. *gattii* isolates could inhibit chemokinesis and chemotaxis of human PMN, and the inhibitory effects were concentration dependent. If the *C. neoformans* var. *gattii* CneF were toxic for

FIG. 3. Migration of human PMN in HBSS and in response to FMLP or PHS or to PHS with sequential twofold dilutions of CneF from one of four isolates of *C. neoformans*. For each panel, a single preparation of PMN from one donor was used. Experiments with each CneF were repeated with PMN from five different donors with similar results. SEM, standard error of the mean.

the PMN, we would have obtained the results presented in Fig. 2, 5, and 6. To assess whether or not the CneF prepared with *C. neoformans* var. *gattii* isolates were cytotoxic for PMN, PMN were incubated with each of the four CneF at the highest concentration used in earlier experiments (1:2 dilution) for 60 min before removal of the CneF from the PMN and assessment of PMN viability. With the trypan blue dye exclusion assay, we found that the viability of the PMN after incubation with any one of the CneF was the same as when the PMN were incubated in HBSS (>90% viability). When CneF-pretreated PMN were placed in upper wells and HBSS or PHS was added to the lower wells of Boyden chambers, we observed that PMN pretreated with either serotype A or D CneF displayed greater mobility to HBSS or PHS than did HBSS-pretreated PMN (Fig. 7; $P < 0.05$). On the other hand, PMN pretreated with serotype B or C CneF did not display increased migration in HBSS than did HBSS-pretreated PMN (Fig. 7; $P > 0.05$) but did display increased migration in response to PHS (Fig. 7; $P < 0.05$). These results confirmed that neither serotype B nor C CneF was cytotoxic to PMN, and the inhibitory effects of CneF on PMN migration in HBSS or in response to PHS occurs only when CneF is in the chemotactic chamber.

Abilities of the various CneF to induce acute leukocyte infiltration into gelatin sponges implanted in naive mice. Buchanan and Murphy (3) have established a gelatin sponge implant model in mice for studying the cellular infiltrates in response to *C. neoformans* or CneF. In the present study, a similar model was used to determine whether or not CneF prepared with the two different varieties of *C. neoformans* have

effects on PMN migration in vivo similar to those which had been observed in vitro. Sponges were implanted into naive mice 4 days before injection of the sponges with SPSS or one of the CneF preparations. When sponges were retrieved from naive mice 6 h after sponge injection with SPSS or CneF, the mean total leukocyte number obtained from the serotype A CneF-injected sponges was significantly greater than the mean number of leukocytes obtained from the SPSS-injected sponges (Fig. 8A; $P < 0.05$). From differential analysis of the sponge-infiltrating cells, it was found that serotype A CneF attracted more PMN (Fig. 8B; $P < 0.01$), lymphocytes (Fig. 8C; $P < 0.05$), and monocytes-macrophages (Fig. 8D; $P < 0.05$) into sponges than did SPSS. In contrast, the mean total leukocyte number obtained from the sponges injected with serotype B CneF was less than the mean number of leukocytes in SPSS-injected sponges (Fig. 8A; $P < 0.05$). The reduction of total leukocyte numbers in serotype B CneF-injected sponges was due primarily to reduced numbers of PMN (Fig. 8B; $P < 0.01$) and possibly to reduced numbers of monocytes-macrophages (Fig. 8D; $0.05 < P < 0.1$) but not to diminished lymphocyte numbers (Fig. 8C; $P > 0.1$). When serotype C CneF was injected into sponges, the numbers of leukocytes infiltrating the sponges were similar to those from sponges injected with serotype B CneF (data not shown).

Comparison of leukocyte infiltration into the sites of a DTH reaction elicited by injecting sponges in immune mice with CneF prepared with the two different varieties of *C. neoformans*. With the mouse model, it has been demonstrated that immunization with serotype A CneF emulsified in CFA in-

FIG. 4. Migration of human PMN in HBSS and in response to FMLP or to FMLP with sequential twofold dilutions of CneF from one of four isolates of *C. neoformans*. For each panel, a single preparation of PMN from one donor was used. Experiments with each CneF were repeated with PMN from five different donors with similar results. SEM, standard error of the mean.

duces a CMI response in mice detectable by DTH reactivity to serotype A CneF (3). The DTH reactivity can be demonstrated as an increase in delayed footpad swelling at 24 h after injection of CneF into the footpad or by increased numbers of leukocytes infiltrating gelatin sponges at 24 h after injection of the sponges with CneF (3). For this study, mice were injected with serotype A CneF-CFA or SPSS-CFA 2 days before sponges were implanted. Four days after sponge implantation, one sponge or footpad of each mouse was injected with SPSS and the other was injected with a CneF preparation. The sponges were removed from the mice or the footpads were measured at 24 h after injection of SPSS or CneF. Mice immunized with CneF-184A-CFA and footpad tested with CneF-B435B displayed positive footpad swelling which was slightly but not significantly less than the swelling observed in immunized mice footpad challenged with CneF-184A (Fig. 9; $P > 0.05$). These observations indicate that the mice were sensitized to both serotype A and B CneF. Moreover, significantly more leukocytes infiltrated serotype A or B CneF-injected sponges than SPSS-injected sponges in immunized mice (Fig. 10A; $P < 0.01$). The leukocyte types responsible for the increased numbers of cells in the CneF-injected sponges were PMN (Fig. 10B; $P < 0.01$), lymphocytes (Fig. 10C; $P < 0.01$), and monocytes-macrophages (Fig. 10D; $P < 0.01$). The total leukocyte numbers in serotype A CneF-injected sponges were similar to the leukocyte numbers in serotype B CneF-injected sponges (Fig. 10A; $P > 0.05$); however, PMN influx was considerably less in the sponges injected with serotype B CneF

than in the serotype A-injected sponges (Fig. 10B; $P < 0.01$). Lymphocyte numbers in serotype B CneF-injected sponges were higher than lymphocyte numbers in serotype A CneF-injected sponges (Fig. 10C; $P < 0.05$). There were no significant differences in the numbers of monocytes-macrophages infiltrating serotype A CneF-injected sponges compared with serotype B CneF-injected sponges (Fig. 10D; $P > 0.05$).

Buchanan and Murphy (3) have reported that serotype A CneF injected into sponges implanted in SPSS-CFA-treated mice does not induce any more leukocyte infiltration of sponges at 24 h after sponge injection than is observed in SPSS-injected sponges. Furthermore, at 24 h after antigen injection, the composition of leukocyte types in serotype A CneF-injected sponges is the same as that in SPSS-injected sponges in SPSS-CFA-injected control mice. The same observation was made in these studies (Fig. 11; $P > 0.05$). In contrast, the mean total leukocyte number in serotype B CneF-injected sponges was significantly less than the mean number of leukocytes in SPSS-injected sponges in SPSS-CFA-injected mice (Fig. 11A; $P < 0.05$). The reduction of total leukocyte numbers in serotype B CneF-injected sponges was primarily due to reduced numbers of PMN (Fig. 11B; $P < 0.001$). Reduced, but not significantly reduced (Fig. 11C and D; $0.05 < P < 0.1$), numbers of lymphocytes and monocytes-macrophages were observed in serotype B CneF-injected sponges compared with SPSS-injected sponges at 24 h after sponge injection.

A second set of experiments was done in which serotype C CneF was used in place of serotype B CneF for injection of

FIG. 5. Migration of PMN in response to HBSS, PHS, or FMLP in HBSS or in sequential twofold dilutions of CneF from each of the four isolates of *C. neoformans*. For each panel, a single preparation of PMN from one donor was used. Experiments with each CneF were repeated with PMN from five different donors with similar results. SEM, standard error of the mean.

footpads and sponges in control and immunized mice. The results were similar to those obtained with the serotype B CneF (data not shown). The results from all of the in vivo experiments indicate that the CneF prepared from the two different varieties of *C. neoformans* affect PMN migration differently in vivo, just as they do in vitro.

DISCUSSION

Blastoconidia of a *C. neoformans* serotype A isolate, 184A, or a soluble culture filtrate prepared with isolate 184A (CneF-184A) can induce directed migration of human PMN in the absence of serum (chemotactic activity) (8). In addition, the serotype A cells or the soluble antigen can activate human serum to generate a chemotactic factor (possibly C5a) for PMN (chemotaxigenic activity) (8). In the present study, we confirmed and extended those findings. Because CneF-184A has chemotactic and chemotaxigenic activities for PMN as shown in vitro, one might predict that CneF-184A would induce PMN infiltration in vivo. With the gelatin sponge model, we confirmed that prediction and demonstrated that within 6 h monocytes and lymphocytes infiltrate CneF-184A-injected sponges implanted in naive mice. By 24 h after injection of CneF-184A into sponges in nonimmune mice, PMN infiltration of the sponges is similar to PMN infiltration of saline-injected control sponges. The early (6-h) PMN influx in re-

sponse to CneF is referred to as acute PMN infiltration and can be attributed, in part, to the chemotactic and chemotaxigenic activities of CneF-184A. Other mechanisms, such as induction of the proinflammatory cytokine tumor necrosis factor by CneF, may also contribute to the 6-h infiltration of CneF-injected sponges by PMN in naive mice (4, 30).

As mentioned earlier, serotype D isolates belong to the same variety of *C. neoformans* as serotype A isolates and have genetic and biochemical characteristics similar to those of serotype A isolates (1, 16, 35). In this study, the serotype D isolate was shown to be similar to the serotype A isolate with respect to chemotactic and chemotaxigenic activities for human PMN; however, the serotype D isolate had weaker direct chemotactic activity and induced less chemotactic factor from PHS than did the serotype A isolate. If these characteristics of weaker chemotactic and chemotaxigenic activities hold true for serotype D isolates in general, then these characteristics might explain why in earlier investigations a serotype D isolate, 613, did not display direct chemotactic activity but was able to activate the alternative complement pathway in serum to produce a chemotactic factor for rabbit PMN (17). Although we did not determine if the serotype D CneF can induce PMN migration into the sponges, on the basis of our in vitro studies and results of Rhodes (26), we predict that it would. Rhodes (26) has shown that serotype D isolate 3502 induces PMN migration into lungs and peritoneal cavities of mice at 1 h after injection.

FIG. 6. Chemokinesis of human PMN in response to CneF prepared from each of four isolates of *C. neoformans*. The same dilutions of CneF (1:16 and 1:16, 1:8 and 1:8, 1:4 and 1:4, and 1:2 and 1:2) were placed in the lower and upper wells with PMN. In samples designated FMLP, PMN with HBSS were placed in the upper wells and FMLP was placed in the lower wells as a positive control. For each panel, a single preparation of PMN from one donor was used. Experiments with each CneF were repeated with PMN from two additional donors with similar results. SEM, standard error of the mean.

C. neoformans var. *neoformans* blastoconidia also induce directed migration of PMN in a manner similar to that stimulated by the respective soluble antigen CneF. It is known that the surface of encapsulated *C. neoformans* blastoconidia is composed predominantly of a high-molecular-weight polysaccharide described as a glucuronoxylomannan (GXM) (5). Our previous studies have shown that GXM is a major component of CneF and is responsible for direct chemotactic activity of CneF (8). In this study, heat-inactivated cryptococcal cells were placed in the lower wells of the chemotactic chamber for only 1 h, a time at which no detectable GXM could be detected in the supernatants by gel electrophoresis. Consequently, it is unlikely that the chemotactic activity of cryptococcal cells was due to GXM released by the heat-killed cryptococcal cells. A more plausible explanation for this result is that the directed migration of PMN stimulated by cryptococcal cells is due to haptotactic activity of surface GXM on cryptococcal cells. It is known that PMN can migrate in response to soluble (chemotaxis) or substrate-bound gradients of chemoattractants (haptotaxis) (27). Therefore, strictly speaking, the chemotaxis induced by intact *C. neoformans* cells should be referred to as haptotaxis of PMN. Recently, it was shown that PMN migration in response to interleukin 8 is a result not of chemotaxis but of haptotaxis, direct cell migration along the gradient of interleukin 8 molecules bound to the substrate (a polycarbonate chemotactic filter) (27, 28).

One prime objective of this study was to determine whether

FIG. 7. Migration of human PMN pretreated with one of four CneF preparations in response to HBSS or PHS. Pretreated PMN were prepared by incubation of PMN with HBSS or one of four CneF diluted 1:2 in HBSS for 60 min at 37°C, and then the PMN were washed three times with HBSS before measurement of migration to HBSS or PHS. The data shown are from a representative experiment. The experiment was repeated two times with similar results. SEM, standard error of the mean.

FIG. 8. Leukocyte migration into sponges implanted in naive mice in response to intrasponge injection of CneF-184A or CneF-B435B. One sponge in each mouse was injected with SPSS, and the other sponge was injected with CneF-184A or CneF-B435B (0.1 ml per sponge) at 6 h before removal of sponges. (A) Mean total numbers of leukocytes harvested per sponge. (B) Mean numbers of PMN harvested per sponge. (C) Mean numbers of lymphocytes harvested per sponge. (D) Mean numbers of monocytes-macrophages harvested per sponge. The data shown are from a representative experiment. The experiment was repeated two times with similar results. Four mice were used per group in each experiment. SEM, standard error of the mean.

or not *C. neoformans* var. *gattii* (serotype B and C) isolates have similar or different abilities to induce human PMN migration, as was observed for *C. neoformans* var. *neoformans* (serotype A and D) isolates. Our data show that *C. neoformans* var. *gattii* cells and CneF lack chemotactic and chemotaxigenic activities, demonstrating that *C. neoformans* var. *gattii* differs from *C. neoformans* var. *neoformans*, which displays chemotactic, haptotactic, and chemotaxigenic activities. Furthermore, consistent with our in vitro observations, injection of CneF-B435B or CneF-B13C into sponges implanted into naive mice did not induce PMN, monocyte, or lymphocyte infiltration.

C. neoformans var. *gattii* cells and CneF not only did not have chemotactic, haptotactic, or chemotaxigenic activity but also inhibited chemotactic responses of human PMN to known chemotactic factors, such as PHS and FMLP. There are several possible mechanisms by which serotype B or C cells or CneF could inhibit migration of PMN in response to PHS and FMLP. These mechanisms include the possibilities that *C. neoformans* var. *gattii* cells or the CneF derived therefrom (i) might interact with the chemotactic factor in PHS or directly with FMLP to block or destroy the activity of the chemotactic factors or (ii) might diminish the ability of PMN to respond to the chemotactic factors. The mechanism(s) by which serotype B or C cells or CneF inhibit PMN migration toward known chemoattractants is not known.

FIG. 9. DTH responses detected by footpad swelling after elicitation of the response with CneF-184A or CneF-B435B in mice injected with SPSS-CFA (control) or CneF-184A-CFA (immunized). The data shown are from a representative experiment. The experiment was repeated two times with similar results. Four mice were used per group in each experiment. SEM, standard error of the mean.

FIG. 10. Leukocyte migration to the intrasponge sites of a DTH response to CneF-184A or CneF-B435B in mice immunized with CneF-184A-CFA. Sponges were injected with SPSS or CneF-184A in one group of immunized mice and with SPSS or CneF-B435B in another group of immunized mice at 24 h before removal of sponges. Mean numbers of total leukocytes (A), PMN (B), lymphocytes (C), and monocytes-macrophages (D) harvested per sponge are shown. The data are from one representative experiment. The experiment was repeated two times with similar results. Four mice were used per group in each experiment. SEM, standard error of the mean.

In addition to directed migration induced by chemoattractants, PMN also have the ability to randomly migrate in the absence of a concentration gradient of chemoattractants. It has been reported that most of the chemotactic factors also can stimulate random movement or chemokinesis of PMN (33). Similarly, CneF-184A and CneF-B3501D, which are chemotactic factors as demonstrated in this study and our earlier study (8), also display chemokinetic activities with human PMN. In contrast, *C. neoformans* var. *gattii* CneF not only do not display chemokinetic activities but inhibit random movement of human PMN.

We do not know the exact mechanism(s) by which the two *C. neoformans* var. *gattii* CneF inhibit random movement of PMN. However, our results indicate that the *C. neoformans* var. *gattii* isolates display inhibitory effects on random movement of PMN only under certain circumstances. First, soluble molecules in the *C. neoformans* var. *gattii* CneF are the major mediators of inhibition of PMN. This point is made on the basis of the demonstration that intact *C. neoformans* var. *gattii* cells did not stimulate migration of PMN or display inhibitory effects, whereas the *C. neoformans* var. *gattii* CneF were inhibitory to human PMN. Finally, the *C. neoformans* var. *gattii* CneF must be present during the entire time of the in vitro chemotactic assay for the inhibition to occur. The *C. neoformans*

var. *gattii* CneF were not cytotoxic to PMN, as shown by (i) the high (90%) viability of PMN after treatment with *C. neoformans* var. *gattii* CneF and (ii) the ability of PMN pretreated with one of the *C. neoformans* var. *gattii* CneF to display random movement in HBSS medium (Fig. 7). Thus, lack of viability of PMN after treatment with *C. neoformans* var. *gattii* CneF could not be the explanation for the inhibitory effects of *C. neoformans* var. *gattii* CneF on PMN migration. CneF preparations, especially at the higher concentrations, are more viscous than HBSS. Therefore, increased viscosity of CneF-containing medium in which PMN migration occurs could be responsible for the retardation of PMN movement. We still cannot completely rule out this possibility; however, similar concentrations of serotype A or D CneF which have viscosities similar to those of the B and C CneF did not inhibit random movement of PMN to the same extent as was observed with serotype B or C CneF, suggesting that retardation due to viscosity is a minor concern. If the *C. neoformans* var. *gattii* CneF could block PMN adherence to the reverse side of the membrane, then the PMN would tend to fall down into the lower well of the chemotactic chamber, resulting in what would appear to be inhibition of migration. This possibility can be excluded, however, by the fact that no detectable PMN could be found in the lower wells of the chambers at the end of the

FIG. 11. Leukocyte migration into sponges implanted in SPSS-CFA-injected (control) mice in response to intrasponge injection of CneF-184A or CneF-B435B. SPSS or CneF-184A was injected into sponges in one group of mice, and SPSS or CneF-B435B was injected into sponges in another group of control mice at 24 h before removal of sponges. Mean numbers of total leukocytes (A), PMN (B), lymphocytes (C), and monocytes-macrophages (D) harvested per sponge are shown. The data are from one representative experiment. The experiment was repeated two times with similar results. Four mice were used per group in each experiment. SEM, standard error of the mean.

assays. It is still possible, however, that the *C. neoformans* var. *gattii* CneF blocks PMN adhesion to the upper side of the membrane, resulting in inability of the PMN to move through the 3- μ m pores of the membrane to the reverse side. We have not experimentally evaluated this possibility and thus cannot rule it out.

Our *in vitro* findings obtained with *C. neoformans* var. *gattii* CneF were confirmed in *in vivo* studies. For instance, PMN did not accumulate in CneF-B435B- or CneF-B13C-injected sponges to the same extent that they infiltrated saline-injected sponges. This result may be attributed partly to the inhibitory effects on PMN chemokinesis displayed by CneF-B435B and CneF-B13C. However, additional mechanisms may also contribute to the lack of PMN infiltration of CneF-B435B- or CneF-B13C-injected sponges. For example, it is possible that *C. neoformans* var. *gattii* CneF blocks the activity of host cell-derived chemotactic factors induced by sponges as a foreign body and thus there is less PMN infiltration of *C. neoformans* var. *gattii* CneF-injected sponges. Our results demonstrate that soluble components of the *C. neoformans* var. *gattii* isolates are inhibitory to PMN migration both *in vitro* and *in vivo*; however, considerably more research is required to elucidate the mechanism(s) by which this inhibition occurs.

CneF-184A has been reported to be composed of three

components (8, 24). The predominant component is a high-molecular-weight GXM (88%). The other two, minor constituents are galactoxylomannan and mannoprotein (MP). When analyzed by gel electrophoresis and concanavalin A blotting, all of the CneF preparations used in this study were found to contain GXM, galactoxylomannan, and MP. GXM determines the serotype, and the structural complexity of the GXM differs among the serotypes. The structural complexity of GXM increases from serotype D to A to B to C (2, 5). We have reported that serotype A GXM is a chemoattractant for PMN, with activity similar to that of the CneF from which it was isolated (8). It is reasonable to suspect that the structural complexity of GXM contributes to differential effects of the CneF from the two different varieties of *C. neoformans* on PMN migration. The relationship between the structures of the four serotype GXMs and their effects on PMN migration merits further investigation.

The concentrations of CneF used for our *in vitro* studies are within the range of cryptococcal polysaccharide concentrations reported to be in serum and spinal fluid from many AIDS patients with cryptococcosis and possibly similar to the concentrations of cryptococcal polysaccharide found in non-AIDS cryptococcosis patients with the highest titers (7, 10). For example, titers of cryptococcal polysaccharide in spinal fluid and

serum from AIDS patients with cryptococcosis frequently exceed 1:100,000 (equivalent to approximately 1 mg of cryptococcal polysaccharide per ml) and go as high as 1:2,000,000 (10). In contrast, cryptococcal polysaccharide titers in spinal fluids from non-AIDS patients with cryptococcosis are lower and typically range from 1:64 to 1:32,896 (7). In this study, we found that the minimal concentrations of serotype A and D CneF that displayed chemotactic activity were 0.4 to 0.5 mg of CHO per ml, which are equivalent to titers below 1:100,000. At concentrations of 0.1 to 0.125 mg of CHO per ml, serotype A and D CneF have chemokinetic activity. The minimal concentrations of serotype B and C CneF for inhibition of PMN chemokinesis and chemotaxis are 0.2 to 0.225 mg of CHO per ml. Although the concentrations of CneF we used in this study are in the range of concentrations of cryptococcal antigen in serum or spinal fluid of some cryptococcosis patients, the concentrations of cryptococcal polysaccharide in the tissues where there are many cryptococcal cells may be considerably higher and thus would be within the range of the concentrations used here. On the basis of our findings, one would expect the concentrations of cryptococcal polysaccharide in infected tissues and the numbers of cryptococcal cells to influence, to some degree, the influx of PMN.

Effects of the two varieties of *C. neoformans* on lymphocyte and monocyte migration were not the major focus of this study; however, we did observe in vivo effects of CneF on migration of mouse lymphocytes and monocytes. With the sponge model in naive mice, we found that CneF-184A induced infiltration of lymphocytes and monocytes into the sponges, whereas neither CneF-B435B nor CneF-B13C attracted lymphocytes or monocytes into sponges. Therefore, it is possible that lymphocytes and monocytes do not migrate into tissues infected with *C. neoformans* var. *gattii* isolates early before the CMI response has developed. Such a situation would be expected to decrease the ability of the host to clear the *C. neoformans* var. *gattii* organisms and control the disease at an early stage.

The anticryptococcal CMI response is another mechanism by which leukocytes are called into *C. neoformans*-infected tissues (3). The CMI response is regarded as a very effective defense mechanism for mediating clearance of *C. neoformans* from tissue, but this mechanism must be induced and therefore only begins to take effect at 1 to 2 weeks into an infection with *C. neoformans* (13, 21). With the mouse model, it has been demonstrated that immunization with serotype A CneF emulsified in CFA induces a CMI response detectable by a footpad swelling reaction to serotype A CneF. In addition, an anticryptococcal CMI response in mice can be demonstrated by increased numbers of leukocytes infiltrating gelatin sponges which were injected 24 h earlier with CneF (3). In this study, we confirmed these findings and showed that CneF made with either of the *C. neoformans* var. *gattii* isolates elicited a DTH reaction in mice immunized with serotype A CneF. This finding suggests that serotype A and serotype B or C organisms have an immunodominant antigen(s) in common which elicits a CMI response. This immunodominant antigen is most likely the MP component of CneF because MP from CneF-184A has been identified as the component recognized by the anticryptococcal CMI response (24). MP is found in all CneF preparations regardless of the serotype used to prepare the CneF. These findings suggest that MP from any *C. neoformans* isolate has potential as a vaccine to induce a protective CMI response against all serotype isolates. Very interestingly, when we compared DTH responses elicited with serotype A with responses elicited by serotype B or C CneF in footpad swelling assays or in determination of the total leukocyte numbers in DTH-reactive sponges, there were only slight differences among the

footpad swelling responses, but the compositions of the leukocytes in DTH-reactive sponges differed between DTH responses to serotype A antigen and responses to serotype B or C antigen. Fewer PMN and more lymphocytes are found in serotype B or C CneF-injected sponges undergoing a DTH reaction than are found in serotype A CneF-injected sponges in immunized mice. Moreover, at 24 h after injection of serotype B or C CneF into sponges implanted in saline-CFA-treated mice, the numbers of PMN in sponges are less than in saline- or serotype A CneF-injected sponges in the same mice. Thus, it seems reasonable to predict that the decrease in PMN infiltrates in the serotype B or C CneF-injected sponges is due to the inhibitory effects of the *C. neoformans* var. *gattii* antigens. Whether or not increased lymphocyte infiltration into DTH-reactive sponges which were injected with the *C. neoformans* var. *gattii* CneF is a result of the reduced PMN numbers or is due to other mechanisms is not clear. Having made these observations concerning the different cellular constituents in the DTH-reactive sponges elicited by serotype A CneF compared with serotype B or C CneF, we are curious how this might affect the capacities of the DTH reactions having the different leukocyte compositions to clear *C. neoformans* from tissues.

In conclusion, *C. neoformans* var. *neoformans* (serotypes A and D) and *C. neoformans* var. *gattii* (serotypes B and C) appear to differ markedly in the ability to induce migration of leukocytes, especially PMN. Our findings are in agreement with the findings of Church and Washburn (6), who showed by histological examination that lungs of mice infected with serotype A or D displayed a higher degree of PMN infiltration than did lungs of mice infected with serotype B or C. Decreased leukocyte migration early after exposure to *C. neoformans* var. *gattii* may lead to inefficient clearance of *C. neoformans* var. *gattii* from infected tissues. Our findings may partly explain why serotype B and C isolates are considered to have a greater predilection for immunocompetent hosts than do *C. neoformans* var. *neoformans* isolates.

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