The pathogenesis of Cryptococcus neoformans pulmonary infection in the rat was studied after intratracheal inoculation. Lungs were examined at various times following infection for histopathology in conjunction with macrophage markers, proliferating cell nuclear antigen (PCNA), and capsular glucuronoxylomannan (GXM) antigen. Serum GXM, immunoglobulin M (IgM) and IgG titers and organ fungal burden were compared with pathological findings. C. neoformans organisms were in the lung parenchyma 2 h postinoculation, and GXM antigen was present in surrounding tissues shortly thereafter. Extrapulmonary dissemination occurred early in infection. Two phases of host cellular inflammatory response were discernible: early local macrophage recruitment at 2 to 4 days followed by granulomatous inflammation, which reached maximum intensity 14 days after infection. The granulomatous phase was preceded by lymphocyte influx with macrophage proliferation and maturation into epithelioid histiocytes; this was paralleled by a shift of yeasts from extracellular to intracellular spaces. Tissue IgG deposits, serum IgG to GXM, and localization of tissue GXM immunoreactivity to epithelioid cells were noted at 2 to 4 weeks. A 10-fold decrease in lung fungal burden occurred 25 days postinfection and was associated with resolving granulomas, fewer proliferating cells, and decreased tissue GXM. The present study demonstrates that (i) C. neoformans penetrates the lung parenchyma shortly after infection; (ii) immunocompetent rats control pulmonary cryptococcosis efficiently, with minimal extrapulmonary dissemination and low levels of serum GXM; and (iii) macrophage activation is likely to play a crucial role in limiting C. neoformans infection in the rat lung.
blunt-tip 23-gauge spinal needle. Following inoculation, 0.3 ml of air was injected at the same site to aid in the dispersion of organisms. The rats tolerated the procedure well. i.p. infection was done by injecting 1.8 × 10⁷ yeast cells in PBS into the peritoneum.

Organ fungal burden. Rats were killed by prolonged exposure to methoxyflurane. Organs were removed, weighed in an analytical balance, and homogenized by mechanical grinding in PBS, and dilutions of the tissue homogenate were plated in Sabouraud dextrose agar for CFU determination. Colonies were counted after 48 h of incubation at 30°C.

Serum GXM level. Serum glucuronoxylomannan (GXM) levels were measured by latex agglutination and enzyme-linked immunosorbent assay (ELISA). Latex agglutination was done with the CALAS kit (Meridian, Cincinnati, Ohio) according to the manufacturer's instructions. The ELISA for GXM has been described and used to study GXM levels. Antigen levels were determined relative for fungi.

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GXM determinations by latex antigen agglutination were <1:2 with the exception of one. By day 25 all rats tested (five of five) had low positive latex endpoints (≥1:4). GXM capture ELISA confirmed that the vast majority of rats had antigen levels <1 µg/ml. The low level of serum GXM in pulmonary infection was surprising and led us to study GXM levels in systemic infection. Three rats given 1.8 X 10^7 C. neoformans organisms i.p. had disseminated infection, with average lung, brain, spleen, and kidney log_{10} CFU of 8.4, 6.5, 6.6, and 6.0, respectively, and serum GXM levels >1,000 µg/ml 2 weeks postinfection. Detection of high levels of GXM by latex agglutination and capture ELISA in i.p. infection indicate that these assays work well with rat sera.

Serum antibody response to GXM in infection. Three of six rats had an IgM titer to GXM of >1:243 detected on day 7. However the IgM response was transient and, no significant IgM was detected subsequently. C. neoformans infection elicited IgG to GXM in most rats, such that at day 7 five of six rats had a greater than threefold increase in IgG titer. In contrast to IgM, serum IgG persisted and there was an upward trend in IgG titer, with three of five rats having ninefold increases in IgG to GXM at day 25 relative to day 2. Sera from infected rats were more opsonic than sera from uninfected rats. Average phagocytic indexes for sera from uninfected and infected rats were 0.03 ± 0.04 and 0.13 ± 0.08, respectively (P < 0.01).

Histopathology of pulmonary infection. Histopathological findings are summarized in Table 1. At 2 h postinoculation, several foci of C. neoformans deposition were apparent. C. neoformans organisms were localized to the alveolar and bronchiolar air spaces as well as to tissue parenchyma (Fig. 2a). Parenchymal organisms were predominantly extracellular and not associated with tissue necrosis or inflammation. A few macrophages with intracellular C. neoformans were seen in occasional bronchial lumens (not shown). The organisms showed little variation in size and shape (Fig. 2a). Since the C. neoformans doubling time in vitro is ~2.5 h, the homogeneity of yeast forms is consistent with origin from the infecting inoculum. GXM immunoreactivity was localized to the capsule (Fig. 2a), with minimal diffusion to surrounding tissues. GSA lectin staining revealed macrophages in alveolar and interstitial spaces, with a predilection for subpleural spaces (Fig. 2i). Similar macrophage distribution was noted in control rats. At 12 h postinoculation, immunohistochemical staining revealed diffusion of GXM into surrounding tissues as well as adjacent alveolar epithelial surfaces (Fig. 2b). There was little change in the number and morphology of organisms or in host inflammatory reaction, but tissue penetration by C. neoformans was greater. Similarly, 24 h postinoculation there was little tissue inflammatory response or appreciable increase in the number of C. neoformans organisms. However, several bronchi demonstrated increased secretions containing C. neoformans organisms and desquamated epithelial cells, consistent with upward clearance of fungi from alveoli. At 48 h, GXM immunoreactivity and yeast forms were detected in the peri-bronchial lymphoid tissue (not shown). There was a steady increase in the bronchial secretion in association with peri-bronchial inflammation. Also noted at this time was variation in the size of C. neoformans cells, with emergence of small forms indicative of replication in vivo (not shown). At 72 h, GSA lectin histochemistry revealed recruitment of local macrophages to foci of C. neoformans, with GSA+ cells containing several ingested yeast forms (Fig. 2j). In one rat, early perivascular lymphocyte cuffing was noted. At 120 h, perivascular lymphocyte cuffing was more pronounced (Fig. 2e). Adjacent parenchymal lung tissue showed a polymorphous inflammatory infiltrate composed of lymphocytes, macrophages, neutrophils, and rare eosinophils. Diffuse, widespread tissue immunoreactivity for GXM persisted, and C. neoformans was found predominantly extracellularly (Fig. 2f).

At 7 days, early granuloma formation was noted; changes at

![Diagram](image)

**FIG. 1.** (A) Lung CFU per gram during the first 5 days of infection. (B) Lung CFU per gram at days 7, 14, and 25 of infection. In panels A and B each point represents one rat. The line is drawn through the average CFU per gram at each time point. (C) Lung weights obtained immediately after removal from rats at days 7, 14, and 25. The line is drawn through the average lung weight, and dashes indicate the standard deviation. There was no significant increase in the average weight of the lungs removed from rats during the first five days of infection (data not shown).
14 days were qualitatively similar to those occurring at 7 days, but granuloma formation was the predominant finding, involving 50 to 70% of lung areas, with loss of air spaces. Granulomas were characterized by sheets of epithelioid cells (Fig. 2g), occurring predominantly in the area of terminal bronchioles (Fig. 2h) and often forming nodular protrusions into bronchial and bronchial lumens. Lymphocytes were no longer limited to perivascular space but infiltrated through parenchyma at the periphery of granulomas. Occasional neutrophils, eosinophils, and plasma cells were mixed with lymphohistiocytes. Numerous mitotic figures were present in macrophages (not shown). PCNA immunocytochemistry demonstrated that up to 30% of the cells in inflammatory areas were proliferating (Fig. 2i and m). PCNA+ cells were identified as macrophages (Fig. 2m), lymphocytes (Fig. 2l) (both in lesional and in peribronchial lymphoid tissue), and damaged bronchial epithelium (not shown). Control rats (n = 3) had few or no PCNA+ cells. Coinciding with granuloma formation was a shift of yeast cells from extracellular to intracellular spaces (Fig. 2f), which occurred mostly in epithelioid histiocytes (Fig. 2g). Many yeast cells were sickle shaped, suggestive of dead organisms. Epithelioid histiocytes expressed very low levels of diffuse cytoplasmic staining for GSA lectin (Fig. 2k). Several foci of confluent granulomas showed central necrosis with PAS+ debris and karyorrhectic figures. In contrast to the diffuse tissue staining noted earlier in infection, GXM immunoreactivity was localized to granulomas within epithelioid histiocytes (Fig. 2c). The peribronchial lymphoid tissue showed hyperplasia, granulomatous inflammation, and C. neoformans penetration into the parenchyma of the lung. Immunocytochemistry for tissue IgG revealed staining of serum in vascular spaces (in both control and infected rats) and diffuse staining of perivascular inflammatory cells, including macrophages. Clusters of cells with dot-like discrete paranuclear staining (plasma cells) were detected in infected rats, consistent with local immunoglobulin production (not shown).

At 25 days, granulomatous inflammation subsided considerably but many vessels showed persistent lymphocyte cuffing and many histiocytes were large foamy macrophages exhibiting GXM immunoreactivity (Fig. 2d). PCNA+ cells were much less frequent than at 14 days, consistent with resolution of the inflammatory process (Fig. 2n). The histologic pattern at 25 days was consistent with a resolving inflammatory process.

**DISCUSSION**

i.t. inoculation of C. neoformans reliably produced pulmonary infection in rats without the need for surgical techniques to expose the trachea. Our model differs from those described earlier (14, 16) in that it involves no disruption of tissue planes or damage to pulmonary structures. i.t. inoculation results in rapid deposition of C. neoformans at the alveoli. At 2 h postinfection C. neoformans organisms were seen within the parenchyma of the lung, indicative of rapid penetration at the level of alveoli. In vitro, C. neoformans has been shown to be internalized by primary rat lung cultures (25) and that alveolar macrophages (2) even in the absence of opsonins. Despite initial deposition of yeasts in the alveoli, organisms and inflammation were subsequently localized to the peribronchial regions such that at 7 days the histologic picture was that of a bronchiolar pneumonia. This observation contrasts with primary alveolar involvement in steroid-suppressed rats (14).

In immunocompetent rats efficient alveolar clearance could result from alveolar macrophages and/or upward mucociliary transport.

Extrapulmonary dissemination occurred early in the course of infection. However, the extrapulmonary burden was low despite extensive pulmonary involvement. Containment and reduction of C. neoformans infection in the lungs appear to depend largely on an effective cellular immune response. The course of pulmonary infection in the rat is similar to that described for mice (17). The rat immune response reduced the C. neoformans burden in the lung by day 25 of infection.

Reduction in lung CFU was temporally correlated with granuloma formation following lymphocyte and macrophage infiltration of the lung parenchyma. Granuloma formation was accompanied by tremendous cellular proliferation by day 14 of infection. The sequence of cellular changes suggests that effective limitation of infection requires expansion and activation of macrophages. Lymphocyte recruitment occurred 4 days after infection, and an appreciable increase in macrophage number was not apparent before this time. Macrophage expansion would have increased the number of phagocytic cells, which, in turn, may have cleared the yeast burden. The influx of lymphocytes may have activated macrophage function.

The signals responsible for the lymphocyte influx and macrophage expansion and activation in the rat are unknown. Early in infection there were isolated pulmonary macrophages
containing phagocytosed yeast forms which appeared to be derived from resident pulmonary macrophages rather than from circulation (Fig. 2). Although few in number, local macrophages could have initiated a signalling cascade leading to T-cell-mediated recruitment and activation of macrophages in the lung. The expansion and apparent activation of lung macrophages are consistent with and support a role for cytokines in the production of an effective response in rat pulmonary cryptococcosis. For instance, phagocytosis of Mycobacterium tuberculosis by monocytes leads to rapid secretion of tumor necrosis factor alpha, as well as monocyte chemoattractant peptide 1 and interleukin-8 (13). Rat bronchoalveolar macrophages do not efficiently limit proliferation of Cryptococcus neoformans unless stimulated with granulocyte-macrophage colony-stimulating factor (8). Direct evidence for cytokine involvement awaits studies using immunochemo- or molecular probes.

The rat antibody response to GXM in i.t. C. neoformans infection included IgM and IgG. IgM was transient and measurable only at day 7 for only 50% of the rats. For most rats an increase in IgG was noted with time. When interpreting the serum titers it is important to note that the ELISA immobi- lized serum immunoglobulin and assayed its capacity to bind GXM. This ELISA circumvents the problem of rat antibody cross-reacting with the ELISA blocking proteins and has the theoretical advantage that it could detect antibody in antigen-antibody complexes. However, since specific antibody to GXM composes only a small fraction of the total antibody pool, the titers measured are relatively low and not comparable to those obtained with direct GXM binding ELISAs (5, 7). Similar ELISAs have been used to measure IgA in C. neoformans-infected mice (5). The source of the serum IgG was not determined, but some may have originated from the pulmo- nary plasma cells noted by immunohistochemistry.

Reduction in lung CFU was accompanied by a shift in the ratio of extracellular to intracellular C. neoformans organisms. In the absence of opsonins, phagocytosis of C. neoformans is inefficient (3). The shift to intracellular predominance suggests effective opsonization and may have been associated with local antibody production. For some rats there was a reduction in lung CFU without an appreciable increase in serum IgG titer, suggesting phagocytosis without opsonins (2) or alternative opsonins such as complement proteins (3, 4) or IgA.

Immunohistochemistry revealed GXM release into tissues by C. neoformans 12 h after infection. GXM immunoreactivity was present diffusely through infected tissues, and yeast cells stained less distinctly. Capsular polysaccharide synthesis is known to be affected by a variety of stimuli in vitro including CO₂ tension (15), osmolality (11), and sugar concentration (11). Release of GXM into lung tissues may contribute to successful establishment of pulmonary infection, since capsular polysaccharide can inhibit phagocytosis (21). Serum GXM levels were low despite extensive pulmonary deposits of GXM. The low serum GXM level accompanying pulmonary infection was in sharp contrast to the high serum GXM levels in rats i.p. infected. GXM shed into pulmonary tissues either does not efficiently disseminate to the serum compartment or is re- moved from serum. In mice MAb administration reduces GXM levels, and serum IgG could have contributed to low serum GXM levels (26). Human primary pulmonary cryptoco ccosis has been reported to be serum antigen negative (31). The rat observations are consistent with this finding.

C. neoformans pulmonary infection in rats is a dynamically evolving process on the part of the host and the pathogen. The rat responds to pulmonary infection with a complex array of effector cells including macrophages, neutrophils, lymphocytes, and occasional eosinophils, which effectively reduce fungal burden. Despite massive infection the rat pulmonary defenses are effective at minimizing dissemination and controlling infection. The rat i.t. infection model may have some unique advantages as an animal system for the study of C. neoformans pathogenesis. In contrast to rabbits, rats can be infected without the need of immunosuppression. Because rats are approximately 10 times the size of mice, it is possible to do certain experimental procedures such as direct tracheal visual- ization, bronchoalveolar lavage, and repeated cerebrospinal fluid sampling that are difficult if not presently impossible in mice. However rats are more expensive than mice to obtain and maintain, and cost is a significant factor in the design of large studies. The rat provides new insights into C. neoformans pathogenesis, and this animal system is fertile ground for the dissection of the host-pathogen interaction.

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