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NOTES

Antibodies Produced in Response to *Cryptococcus neoformans* Pulmonary Infection in Mice Have Characteristics of Nonprotective Antibodies

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Murine cryptococcal pulmonary infection elicited serum immunoglobulin M (IgM) and IgG to the capsular polysaccharide, but only IgG stained yeast cells in alveoli. Both isotypes produced punctuate immunofluorescence patterns on yeast cells like those of nonprotective antibodies. The difficulties involved in associating humoral immunity with protection in murine cryptococcal infection could reflect nonprotective antibody responses.

Host defense against the pathogenic fungus *Cryptococcus neoformans* is believed to depend primarily on the cellular response (see review in reference 6), although a large body of evidence indicates that certain antibodies (Abs) can mediate protection in experimental infection (2, 13, 15, 25, 27). For *C. neoformans* infection, granuloma formation and cellular recruitment to the areas of infection appear to be the most effective mechanisms of defense. In fact, it has been difficult to conclusively demonstrate that the natural Ab response to *C. neoformans* infection contributes to host defense (3). The reliance of host defense on cellular immunity despite a preponderance of evidence that certain antibody responses can be effective is peculiar and poses a paradox that remains unexplained.

C. neoformans possesses a thick polysaccharide capsule surrounding the cell body that is composed primarily of glucuronoxylomannan (GXM). The presence of the capsule is required for virulence and GXM can mediate a large number of deleterious effects in the host (12, 20, 28, 35). The capsular polysaccharide confers antiphagocytic properties to the organism (1, 17, 36), and injection of polysaccharide into mice can produce Ab unresponsiveness in the host (16, 28). Ab titers in both animal experimental infection and human cryptococcosis are usually low (7, 9, 10), a finding attributed to poor immunogenicity of the capsular polysaccharide (16, 28) and/or sequestration of Ab by tissue polysaccharide (10). For this reason, the extent to which natural Ab responses play a prominent role in the mechanism of defense against the pathogen is uncertain. One potential explanation for the difficulty in demonstrating the importance of Ab-mediated immunity is that insufficient Ab is made in response to infection. In contrast, several monoclonal Abs (MAbs) to *C. neoformans* have been isolated and shown to mediate protection in naïve hosts when the MAbs are exogenously injected into the mouse (12, 15, 27, 31). However, other MAbs have been shown to be nonprotective or disease enhancing (24). Although the mechanism of Ab action in vivo is not well characterized, a correlation between the effectiveness of the Ab and the fluorescence pattern produced in vitro has been made when the Ab is bound to the capsule, with protective and nonprotective Abs producing annular and punctuate patterns, respectively (8, 23, 29; W. Cleare, M. E. Brandt, and A. Casaderall, Letter, J. Clin. Microbiol. **37:**3080, 1999). These differences in binding for protective and nonprotective Abs also manifest themselves as qualitatively different capsular reactions (18,19). Further complicating the interpretation of these observations is the fact that a single MAb can be disease enhancing or protective depending on the concentration of the Ab and on the dose of the infecting organism (32,33). Given the uncertainty of the role that humoral immunity plays in murine defense against cryptococcal infection, we have analyzed the amount and type of Ab bound to *C. neoformans* cells during murine infection in an attempt to gain insight into the problem.

C. neoformans strain H99 was obtained from John Perfect (Durham, N.C.) and grown in Sabouraud dextrose medium overnight. Cells were collected during logarithmic phase by centrifugation, washed twice with phosphate-buffered saline (PBS), and suspended at 2×10^8 cells/ml. Fifty microliters of this suspension (10^7 yeast cells) was inoculated intratracheally into female C57BL/6J mice (8 to 10 weeks old, 18 to 20 g; Jackson Laboratories) anesthetized with ketamine. In some experiments, the mice were treated with 1 mg of 18B7 (4) or 12A1 (21), which is an immunoglobulin G1 (IgG1) or IgM Ab, respectively, to the cryptococcal capsule, to ascertain the extent to which a protective MAb can penetrate into the lung. Mice were killed at the times indicated below, and the lungs were removed and homogenized in 10 ml of PBS. To isolate the *C. neoformans* cells, the lung homogenate was treated with 1 mg of collagenase A (Roche, Indianapolis, Ind.) per ml for 1 h at 37°C with occasional shaking by vortexing. The homogenate was then washed four or five times with water to lyse host

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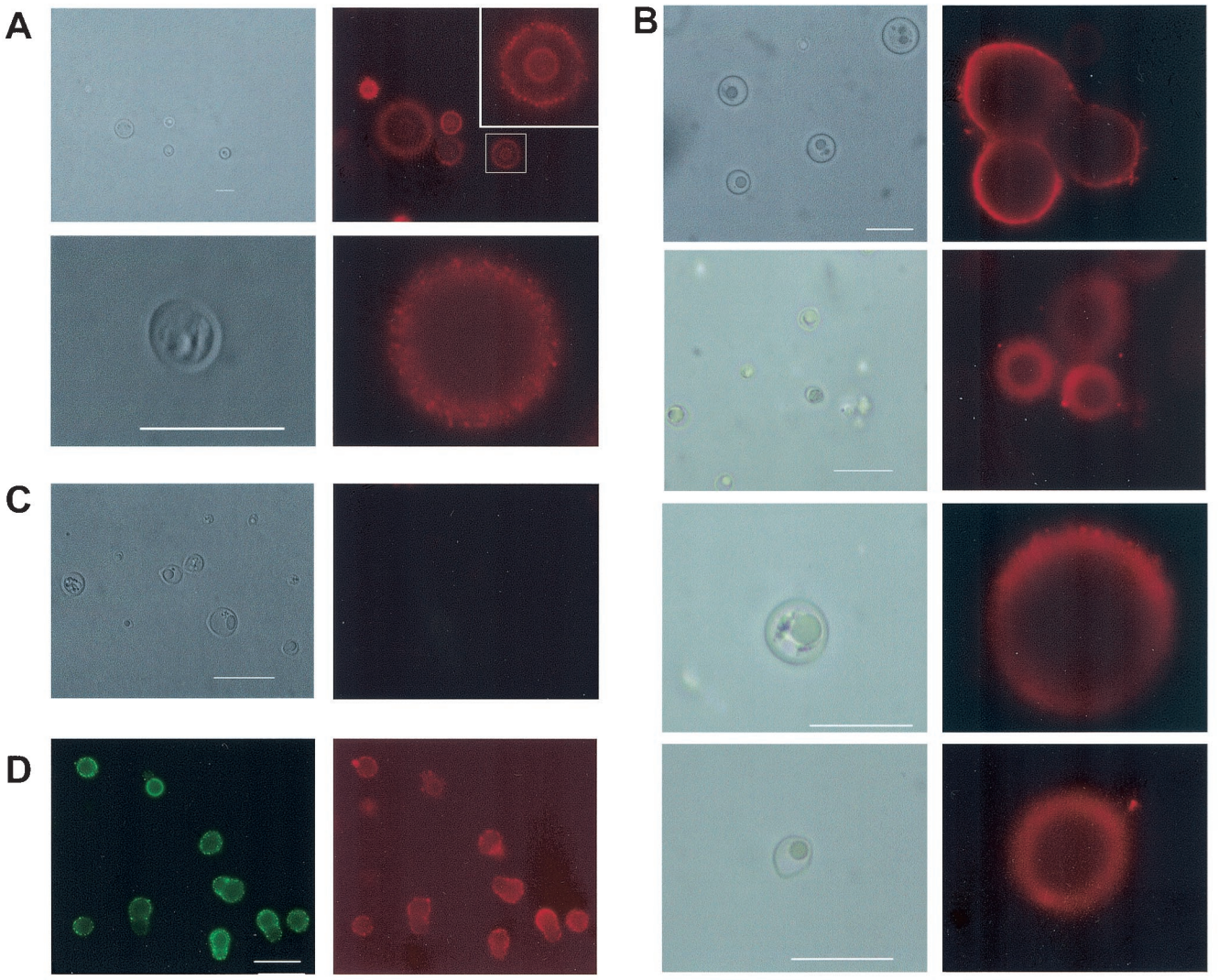

FIG. 1. Immunofluorescence patterns of Abs produced in response to *C. neoformans* in the lung. *C. neoformans* cells were isolated from mouse lungs after 7 days of infection as described in the text and stained for IgG (A) or IgM (C). (B) Yeast cells from mice given MAb 18B7 and stained for IgG bound to the cells. Four representative fields are shown. (A to C) Light microscopy (left panels) and rhodamine fluorescence patterns (right panels). In panel A, the two bottom panels are high magnifications of a representative cell. An enlargement of the boxed cell in the top right panel is shown in the inset. (D) Cells grown in Sabouraud medium, incubated in serum from infected mice (day 7 of infection), and then assayed for bound IgM (left panel) or IgG (right panel) by using secondary Abs conjugated to FITC or TRITC, respectively. Bars, 10 μm.

cells. Microscopy of the final product showed that most of the cells recovered were yeast cells. The cells were counted and incubated in blocking solution (0.5% horse serum–1% bovine serum albumin [BSA] in PBS) with goat Ab to mouse IgG or IgM conjugated to tetramethyl rhodamine isothiocynate (TRITC) or to fluorescein isothiocynate (FITC) (10 μg/ml; Southern Biotechnology Associates, Inc., Birminghan, Ala.) for 1 h at 37°C or overnight at 4°C. The cells were washed three times with blocking solution, suspended in mounting medium (50 mM *N*-propylgallate–50% glycerol in PBS), observed in an Olympus AX70 microscope, photographed with a QImaging Retiga 1300 digital camera by using the QCapture Suite V2.46 software (QImaging, Burnaby, British Columbia, Canada), and processed with Adobe Photoshop 7.0 for Windows (Adobe, San Jose, Calif.). Photographs of negative control to which no conjugated Ab was added were taken in parallel, at the same exposure time. In parallel, serum samples were taken from the infected mice, and the amount of Abs to GXM was measured by enzyme-linked immunosorbent assay (ELISA) (5). All the incubations were done for 1 h at 37°C or overnight at 4°C. Briefly, the plates were coated with purified GXM from H99 (10 μg/ml in PBS) and then blocked with 1% BSA in PBS (the rest of the incubations were done in this solution). Serum was added to the wells at a 1/20 dilution and then serially diluted in the plates. Goat anti-mouse IgG or IgM conjugated to phosphatase alkaline (2 μg/ml) was then added, and the wells were developed with 1 mg of *p*-nitrophenyl phosphate (Sigma, St. Louis, Mo.) per ml dissolved in substrate buffer (0.001 M $MgCl_2$–0.05 M Na_2CO_3 [pH9]). Parallel wells not coated with GXM but blocked with BSA served as controls for determination of the amount of Abs in serum that bound to BSA. The absorbance measured on BSA plates was subtracted from the

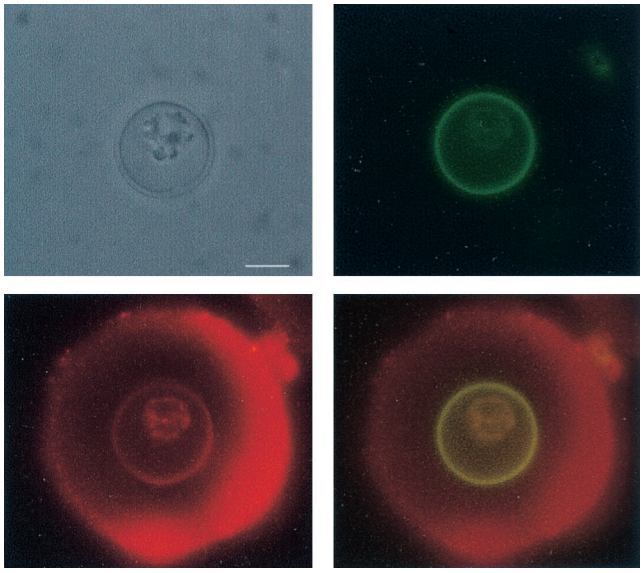

FIG. 2. Yeast cells isolated from mice at day 7 postinfection and incubated with FITC-conjugated Ab to C3. The capsule was visualized by staining with MAb 18B7 followed by goat anti-mouse IgG TRITC. (Top right) Light microscopy; (top left) complement localization (green, fluorescein). (Bottom left) Capsule (red, rhodamine); (bottom right) merge of C and MAb 18B7 localization. Bar, 10 μm.

absorbance measured on GXM plates for the final calculations. Finally, the serum samples were used in immunofluorescence studies of cells grown in Sabouraud medium to ascertain the fluorescence pattern of the Abs measured by ELISA. For immunofluorescence, yeast cells grown in Sabouraud were incubated for 1 h at 37°C in serum from mice infected for 7 days with *C. neoformans*. Abs bound to the capsule were detected by using the secondary conjugated Abs described above. Phagocytosis experiments were performed as described in reference 36.

IgG was found in the *C. neoformans* capsule on day 7 of infection (Fig. 1A) but not on day 3 (data not shown). The immunofluorescence pattern of IgG on the capsule was punctuate and strongly resembled that observed for nonprotective MAbs (22,29). Since a punctuate pattern is associated with lack of protection (22,29), we studied the fluorescence pattern of yeast cells isolated from mice infused with a protective Ab. We used MAb 18B7, which is an IgG1, which ordinarily produces annular immunofluorescence.

In contrast to the consistent annular pattern produced by MAb 18B7 binding to yeast cells grown in vitro, passive administration of MAb 18B7 produced a heterogeneous pattern of immunofluorescence for yeast cells in lung tissue, which was nevertheless different and distinct from that observed for the endogenously produced IgG. The majority of cells demonstrated a faint fluorescence pattern throughout the breath of the capsule that was different from the punctuate pattern observed for endogenously produced IgG Abs. We also found a lower proportion of cells with an annular pattern (around 5% of the cells) and of cells that did not show any fluorescence at all. This result confirms that the pattern of an exogenously administered protective Ab in vivo is different from that observed for IgG produced during infection (Fig. 1B). We suspect that this heterogeneity arises as a consequence of changes in the ratio of Ab to yeast cells with time of infection, since the amount of Ab and number of yeast cells change with time. In fact, it is likely that this ratio is a continuously changing variable since the amount of MAb decreases with time while at the same time yeast cells replicate and their capsules increase in size. When mice were given 1 (high dose), 0.1 (intermediate dose), or 0.01 (low dose) mg of MAb 18B7, we detected IgG1 only in the yeast cells of mice given the high dose. No difference in the fluorescence pattern produced by 18B7 was observed during the first 7 days of infection.

In contrast to IgG, we did not detect IgM on the capsule of yeast cells isolated from the lung (Fig. 1C), even after 14 days of infection. In mice treated with 1 mg of MAb 12A1 (IgM), we did not detect any IgM in the *C. neoformans* cells (data not shown). We surmised that the absence of IgM in yeast cells in the lung might be the result of poor penetration of IgM in the lung due to either its high molecular weight, a lack of IgM production locally or systemically, and/or lack of reactivity with the capsule. To discriminate between these possibilities, we analyzed the titer of serum GXM-binding Ab and demonstrated both specific IgM and IgG by ELISA (the inverse titers were approximately 1,500 for IgM and 1,000 for IgG). This result indicated that the absence of binding in the lung was not due to lack of IgM production. To insure that the IgM detected by ELISA bound to the capsule, we incubated H99 cells with serum from infected mice (after 7 days of infection) and detected both IgM and IgG on the capsule by using secondary Abs conjugated to rhodamine or fluorescein. As shown in Fig. 1D, these Abs produced punctuate immunofluorescence after binding to the capsule. Since a punctuate immunofluorescence pattern is associated with nonprotective antibodies (8, 23, 29; Cleare et al.) we explored whether these were opsonic in phagocytosis assays. To evaluate the opsonic efficacy of capsule-bound Abs, we isolated cells from the lungs of mice after 7 days of infection and used them in a phagocytosis assay in vitro with the macrophage-like cell line J774.16 (30). This cell line expresses both Fc and complement receptors (32). The cells isolated from the lung, which had capsule-bound IgG, were not phagocytosed in vitro (results not shown). We noted that a significant proportion of the yeast cells had a huge size (compare sizes in Fig. 1A, B, or C with D). These forms have been reported to emerge during in vivo infection (14), and it is likely that their large size impairs phagocytosis by the macrophage, even when opsonized in vitro with MAb 18B7 (results not shown). Finally, we evaluated whether Abs in serum were opsonic. For this purpose, we performed a phagocytosis experiment employing H99 cells grown in Sabouraud broth with J774.16 macrophage-like cells and 50% serum from mice infected for 7 days. As a negative control, we used serum from nonimmunized mice. No phagocytosis was detected, indicating that the Abs in serum were not opsonic during in vitro phagocytosis experiments (result not shown).

Given that IgM was not detected in the lung, we evaluated whether *C. neoformans* in lung tissue stained for the other major serum opsonin, complement component 3 (C3). C binding has been reported in *C. neoformans* cells recovered from a lung with systemic infection, but C binding is tissue dependent and does not occur in the brain (34). C3 staining with a FITC-conjugated goat Ab to mouse complement (5 μg/ml; Cappel) localized deep in the capsule of *C. neoformans* cells in the lung

(Fig. 2), a location which has been associated with an inability to interact with the receptor in the macrophage and inefficient opsonization (36).

Our findings provide insights about the role of Ab responses to cryptococcal polysaccharide during *C. neoformans* pulmonary infection. Both IgG and IgM are produced in response to pulmonary infection, but only IgG penetrates into the lung. IgM and IgG produced in vivo presented punctuate immunofluorescence patterns on *C. neoformans* capsules that were very similar to those observed for nonprotective MAbs. This result suggests that nonprotective Abs dominate the Ab response to pulmonary infection in these mice. The Abs produced in response to infection are not opsonic in a phagocytosis assay. In fact, murine pulmonary infection appears to be associated with a dearth of opsonins, since C3 in lung yeast cells was located deep inside the capsule and the IgG antibodies on cryptococcal cells did not promote phagocytosis. The problem of opsonization is further compounded by the emergence of large cryptococcal cells, which are more difficult to ingest. We propose that part of the difficulty in establishing an important role for humoral immunity to *C. neoformans* in pulmonary infection is due to both a lack of penetration into the alveolar space and the production of Abs that bind to the capsule in a manner that does not promote opsonization. The fact that some of the Abs isolated from infected mice as MAbs are protective (26) suggests that the pool of Abs produced in vivo is heterogeneous in function.

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