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Encapsulation of *Cryptococcus neoformans* Impairs Antigen-Specific T-Cell Responses

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The encapsulated yeast *Cryptococcus neoformans* is a significant cause of opportunistic infection in patients with impaired cell-mediated immunity. The major virulence determinant of the organism is an antiphagocytic polysaccharide capsule synthesized after entry into the host. Using both an encapsulated virulent strain and an acapsular avirulent mutant, we have demonstrated the reduced ability of the encapsulated strain to stimulate specific T-cell responses in vitro. This reduction was mediated by the antiphagocytic action of the capsule rather than by direct inhibition of antigen processing and presentation, since prior opsonization with complement enhanced the ingestion of encapsulated yeast cells by purified antigen-presenting cells and allowed significant T-cell activation. Once ingestion had occurred, cryptococci were efficiently processed by activated macrophages via a chloroquine-sensitive pathway. Cryptococcal antigens were available for T-cell recognition within 1 to 2 h of interaction with macrophages and presented in a major histocompatibility complex-restricted manner. Our results suggest that the antiphagocytic action of the polysaccharide capsule is an important determinant for the development of T-cell immunity to *C. neoformans*.

Cryptococcus neoformans is an encapsulated yeast that is an increasingly frequent cause of opportunistic infection as a consequence of human immunodeficiency virus infection (5, 7). The organism enters the host via inhalation of a small, acapsular form into the lung, where it can produce a localized subclinical infection or disseminate throughout other organ systems, frequently resulting in lethal meningitis. A primary virulence determinant of *C. neoformans* is the synthesis of an antiphagocytic polysaccharide capsule after inhalation by the host (9). Nonencapsulated mutants of *C. neoformans* are avirulent in experimental models of infection and are readily phagocytosed by host macrophages (8, 11, 26). In contrast, capsule synthesis by virulent *C. neoformans* impairs ingestion by phagocytic cells (2, 18, 21), although this can be partially overcome by opsonization with complement (6, 22) or specific anticapsule antibody (19).

Clinical and experimental evidence has implicated T lymphocytes in the resistance of the immunocompetent host to *C. neoformans*. Thus, although infection can occur in apparently immunocompetent individuals, the majority of cases are associated with impaired cell-mediated immunity states such as AIDS. Experimental evidence also supports a primary role for T cell-dependent resistance, since athymic nude mice show increased susceptibility to infection (4, 14) and resistance in normal animals is dependent on the presence of $CD4^+$ T cells in vivo (17, 27). However, previous studies of T-cell immunity to *C. neoformans* have focused on delayed-type hypersensitivity responses in vivo (10, 13, 16, 25) or the development of T cell-mediated suppressor cell networks (28). In the present study we have extended these observations by investigating the characteristics of T-cell activation by *C. neoformans* in vitro.

The mechanisms of antigen processing required for the development of T-cell immunity to fungal pathogens such as *C. neoformans* have not been defined. Since most processing events occur intracellularly (30, 31), *C. neoformans* poses unique problems to the host due to the antiphagocytic action of its polysaccharide capsule, which is also suggested to have immunosuppressive properties (3, 20, 28, 29). Cryptococcal polysaccharides are found at high concentrations in the serum and cerebrospinal fluid of patients (12) and have been correlated with suppression of delayed-type hypersensitivity in a murine model of infection (28). Furthermore, other defined polysaccharides such as Ficoll and dextran have been reported to inhibit the intracellular processing of protein antigen by macrophages (24).

The purpose of this study was to investigate the effect of capsule synthesis on the processing and presentation of *C. neoformans* to specific T cells. In comparison to an avirulent, acapsular mutant, encapsulated yeast cells were unable to stimulate the proliferation of antigen-specific T cells in vitro. This was due to the antiphagocytic effect of the capsule, preventing ingestion by antigen-presenting cells, rather than to an inhibitory effect of the polysaccharide capsule on intracellular antigen processing. Our results suggest that capsule synthesis is a potential obstacle to the uptake of *C. neoformans* by antigen-presenting cells and will therefore be an important determinant for the induction of T-cell immunity.

MATERIALS AND METHODS

C. neoformans. *C. neoformans* var. neoformans was used in all experiments. The encapsulated B3501 strain and an acapsular mutant, B4131, were stored at 4°C in water cultures and streaked onto Sabouraud dextrose agar when required. Both strains of the organism were kindly provided by J. Kwon-Chung, National Institutes of Health. Heat-killed organisms were prepared by scraping a 3- to 5-day-old culture into pyrogen-free saline and heating to 80°C for 1 h and then washing three times with pyrogen-free saline and storing at 4°C. Soluble cryptococcal antigen, obtained as the first wash supernatant of the heat killing process described above, was stored at −70°C. Purified capsular polysaccharide (CPS) serotype A (125 kDa) was kindly provided by

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Robert Cherniak, Georgia State University. Where applicable, complement opsonization of *C. neoformans* was achieved by incubating the organisms for 60 min at 37°C with normal mouse serum or medium alone and then washing three times. Confirmation of the surface properties of the strains was achieved by staining 10^6 organisms with a rabbit anti-*C. neoformans* capsule antibody (Alpha Laboratories, Hampshire, United Kingdom) or a rabbit anti-*C. neoformans* cell wall antibody kindly donated by R. Hay (Guys hospital, London) and then second-layer staining with a goat anti-rabbit immunoglobulin conjugated with fluorescein isothiocyanate (Nordic Immunologicals, Maidenhead, United Kingdom), and the cells were analyzed by using flow cytometry. The capsule size of the organism was increased by growing the organism on medium known to enhance capsule synthesis (8) and was confirmed by using flow cytometry.

Mice. Specific-pathogen-free CBA/CA and BALB/c female mice 8 to 10 weeks of age were obtained from the National Institute for Medical Research, Mill Hill, and housed at the London School of Hygiene and Tropical Medicine. For infection with *C. neoformans*, 5×10^7 organisms in pyrogen-free saline were injected intraperitoneally. For the induction of *C. neoformans*-specific T cells, 5×10^7 heat-killed acapsular organisms in pyrogen-free saline were emulsified in an equal volume of incomplete Freund adjuvant (Difco), and 50 μl was injected subcutaneously into each hind footpad.

Macrophage harvest and quantitation of *C. neoformans* ingestion by macrophages. Peritoneal exudate cells were harvested from mice by lavage with 10 ml of RPMI 1640 supplemented with 1% fetal calf serum, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (R1 medium; media and supplements from GIBCO) 5 days after intraperitoneal injection with 200 μg of concanavalin A (ConA, type IV; Sigma Chemical Co., St. Louis, Mo.). Cells were washed once and then plated at 3×10^5 per well in a 96-well flat-bottomed plate (Falcon) in R10 medium (like R1 but with 10% fetal calf serum). The cells were incubated at 37°C for 2 h and washed four times to remove nonadherent cells.

For analysis of macrophage ingestion of *C. neoformans*, heat-killed organisms were washed three times in R10 and added in 100-μl volumes to macrophage monolayers, the plates were spun at 1,100 rpm (300 × *g*) for 1 min and incubated at 37°C for 2 h, and the cells were washed to remove nonbound or ingested organisms. Absolute methanol (100 μl) was added to each well for 10 min at room temperature to permeabilize the cells, and the cell monolayer was washed with phosphate-buffered saline (PBS)–5% donor calf serum (PBS-DCS). All antibody staining was done in 35-μl volumes at 4°C for 30 min. Rabbit anti-*C. neoformans* capsule antibody (Alpha Laboratories) diluted 1:500 in PBS-DCS or rabbit anti-*C. neoformans* cell wall antibody at 100 μg/ml was added to each well. After the cells were washed with PBS-DCS, goat anti-rabbit immunoglobulin conjugated with tetramethylrhodamine isothiocyanate (Nordic Immunologicals) and diluted 1:40 in PBS-DCS was added; the cells were then washed and stored at 4°C in PBS. Fluorescence was visualized by using an inverted fluorescence microscope, and the number of organisms bound or ingested per 100 macrophages was calculated by using the following formula: (total number of organisms stained red/total number of macrophages per field) × 100.

***C. neoformans*-specific T-cell proliferation and antigen processing.** Popliteal lymph nodes were harvested from mice 7 days after priming as described above. The cells were washed three times in R10–10 mM nonessential amino acids (GIBCO)–1 mM sodium pyruvate (GIBCO) and diluted to 5×10^6/ml. Cells were added to flat-bottomed microtiter plates in 100-μl volumes, and 100 μl of stimulus or medium was added. After incubation at 37°C for 36 h, the cells were pulsed with 0.5 μCi of [*methyl*-^{3}H]thymidine (Amersham) per well and incubated for 32 h. Cells were harvested with a PHD cell harvester (Cambridge Technologies), and thymidine incorporation was measured by counting in a Beckman LS800 liquid scintillation counter.

To investigate antigen processing requirements, peritoneal exudate cells activated in vivo 5 days previously by intraperitoneal injection of 200 μg of ConA were harvested, plated at 3×10^5 per well in 96-well flat-bottomed plates in R10, allowed to adhere for 2 h at 37°C, and used throughout as antigen-presenting cells. All washing steps involved four changes of medium per well. After the macrophage monolayer was washed, *C. neoformans* preopsonized in normal mouse serum or medium alone was added, and the plates were spun at 1,100 rpm for 1 min. After incubation at 37°C for 2 h, monolayers were washed to remove nonbound organisms and incubated at 37°C to allow processing to occur. At appropriate times, processing was halted by the addition of 100 μl of 2% paraformaldehyde (Sigma) in PBS per well for 10 min at room temperature. Cells were washed, 100 μl of 0.1 M lysine (Sigma) diluted in R10 was added, the cells were incubated at 37°C for 15 min, and the washing was repeated. Further incubation in R10 at 37°C for at least 2 h removed the excess paraformaldehyde and prevented toxic effects from reducing the T-cell response. Primed popliteal lymph node cells were harvested and prepared at 5×10^6/ml in R10–10 mM nonessential amino acids–1 mM sodium pyruvate. The cells were added to antigen-pulsed presenting cell monolayers in 100-μl volumes, and 100 μl of medium was added per well. T-cell proliferation (thymidine incorporation) was measured after 3 days as described above. In inhibition experiments, chloroquine (Sigma) was added to the monolayers for 30 min before the yeasts were added and remained in culture for the entire processing time.

In experiments investigating the effect of purified CPS on the intracellular processing of an unrelated antigen, hen egg white lysozyme (HEL; Sigma), the procedure was followed as described previously (24). In summary, CPS was added to macrophage monolayers for 2 to 3 h before incubation with HEL for 90 min. The HEL-specific T-cell hybridoma 3A9 (kindly donated by P. Allen, Washington University, St. Louis, Mo.) was added, and interleukin-2 production was assayed after 24 h by a modification of the method previously described (1). Erythrocyte-depleted spleen cells from CBA mice were incubated at 10^6/ml in the presence of 1 μg of ConA per ml for 3 days. After three washes, the T-cell blasts were resuspended to 4×10^5/ml and plated in 50-μl volumes in round-bottomed Flow tissue culture plates. The supernatants to be assayed were added in 50-μl volumes, the cells were incubated at 37°C for 24 h (the last 4 h in the presence of [^{3}H]thymidine) and then harvested, and the proliferation was measured as described above.

RESULTS

Development of specific T-cell responses against *C. neoformans*. Initial experiments established optimal methods for enumeration and for determining the growth and characteristics of the organism. The two strains of *C. neoformans* used in this study differ in surface properties and virulence.

The virulent B3501 organism is encapsulated, whereas the avirulent B4131 strain is a stable, acapsular mutant derived from the encapsulated parent strain. Flow cytometric analysis indicated that 89% of B3501 cells stained positive with a rabbit anti-capsular antibody, compared with 3% of B4131 cells.

To determine the optimal conditions for the development of T cells that are reactive against *C. neoformans*, mice were primed with heat-killed acapsular organisms in vivo followed by in vitro stimulation of lymph node cells with intact *C. neoformans*. Initially, the acapsular form of the organism was selected to remove any potentially suppressive effects of the capsule. Injection of 5×10^7 heat-killed organisms in incomplete Freund adjuvant resulted in the formation of lymph node cells that were reactive against cryptococcal antigens when challenged in vitro with 1×10^6 heat-killed acapsular organisms. Control mice injected with saline and adjuvant showed no reactive cells, demonstrating the requirement for cell priming and the absence of any mitogenic activity of *C. neoformans* in vitro (*C. neoformans* plus adjuvant, 16.8 ± 0.6 kcpm; saline plus adjuvant, 2.6 ± 0.2 kcpm; medium alone, 1.4 ± 0.1 kcpm). Similar proliferative responses to soluble cryptococcal antigen were observed (data not shown). Spleen lymphocytes reactive against *C. neoformans* were also produced after infection with live acapsular organisms, although the magnitude of the proliferative response was less than that in adjuvant-primed animals (*C. neoformans*-infected mice, 10.1 ± 1.1 kcpm; saline-injected mice, 2.6 ± 0.2 kcpm). The production of the T cell-derived cytokines interleukin-2 and gamma interferon by the responding lymph node cells suggested that the response to cryptococcal antigens was T cell mediated (data not shown).

To assess the effect of the polysaccharide capsule on the stimulation of specific T cells, both forms of the organism were compared for their ability to stimulate primed lymph node cells in vitro. B4131-primed lymph node cells showed a proliferative response to the acapsular organism but no stimulation above background levels after the addition of intact encapsulated *C. neoformans* (Fig. 1). In other experiments, 30-fold more encapsulated organisms were required to induce cell proliferation (data not shown). This was not due to an obvious difference in antigenicity between the two organisms, because the proliferation in response to soluble antigen preparations derived from both strains were identical. These results suggested that the capsule of intact yeast cells inhibited the in vitro stimulation of *C. neoformans*-specific T cells.

Effect of the polysaccharide capsule on processing of *C. neoformans* antigens. Since intracellular processing of complex protein antigens is usually required before inducing T-cell activation, encapsulation of *C. neoformans* could inhibit ingestion or degradation by antigen-presenting cells. Therefore a system was established to characterize the requirements for processing of *C. neoformans* by macrophage antigen-presenting cells. Initial experiments with acapsular organisms showed that macrophages activated in vivo with ConA for 5 days processed intact yeast cells and presented cryptococcal antigens to primed lymph node cells in a dose-dependent manner (Fig. 2). Viable antigen-presenting cells were required for this response, since fixation with 2% paraformaldehyde before pulsing with antigen abolished lymphocyte proliferation, excluding the contribution of already degraded antigens in the preparation of the organisms used. Purification of the lymph node cell population by passage over nylon wool resulted in T-cell enrichment, as determined by flow cytometric analysis, and a subsequent enhancement of the proliferative response after interaction with the antigen-pulsed macrophage monolayer (data not shown), again demonstrating the T-cell nature of the response.

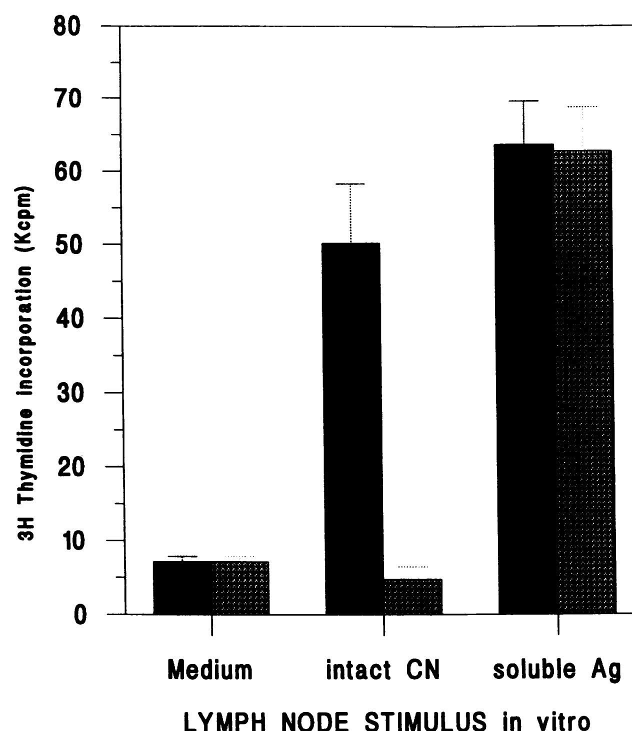

FIG. 1. Comparison of in vitro lymph node cell proliferation in response to encapsulated and acapsular *C. neoformans*. Primed lymph node cells were prepared as described in the text and incubated with medium alone, soluble antigen (1/30), or 3×10^5 encapsulated (■) or acapsular (■) heat-killed *C. neoformans*. Cell proliferation was measured after 3 days, and the results expressed as means ± standard deviations (SD) of triplicate cultures.

Since antigen processing first requires the antigen to be internalized by the presenting cell, a method to quantitate the ingestion of the two *C. neoformans* strains was established. Fluorescent antibody staining of intracellular organisms allowed ingestion of *C. neoformans* to be quantitated under conditions identical to those used to detect cryptococcal antigen processing. In this system, the antiphagocytic effects of the polysaccharide capsule were observed, reducing ingestion by 90%. Prior opsonization of encapsulated organisms in normal mouse serum increased ingestion to approximately that of the acapsular mutant (numbers ingested or bound per 100 macrophages: for B4131, 257 ± 50; for B3501, 25 ± 2; for preopsonized B3501, 253 ± 22).

The macrophage antigen-presenting cell system described above was then used to compare the efficiency of processing and presentation to primed lymph node cells of opsonized encapsulated versus acapsular *C. neoformans*. In terms of the actual number of organisms ingested per antigen-presenting cell, the capsule had no effect on the ability of macrophages to process cryptococcal antigens (Fig. 3); 10 organisms ingested per 100 macrophages were sufficient to induce T-cell proliferation. Furthermore, yeast cells cultured in conditions that increased capsule synthesis were also processed and presented with an efficiency equal to that for acapsular organisms once ingested by the macrophage pre-

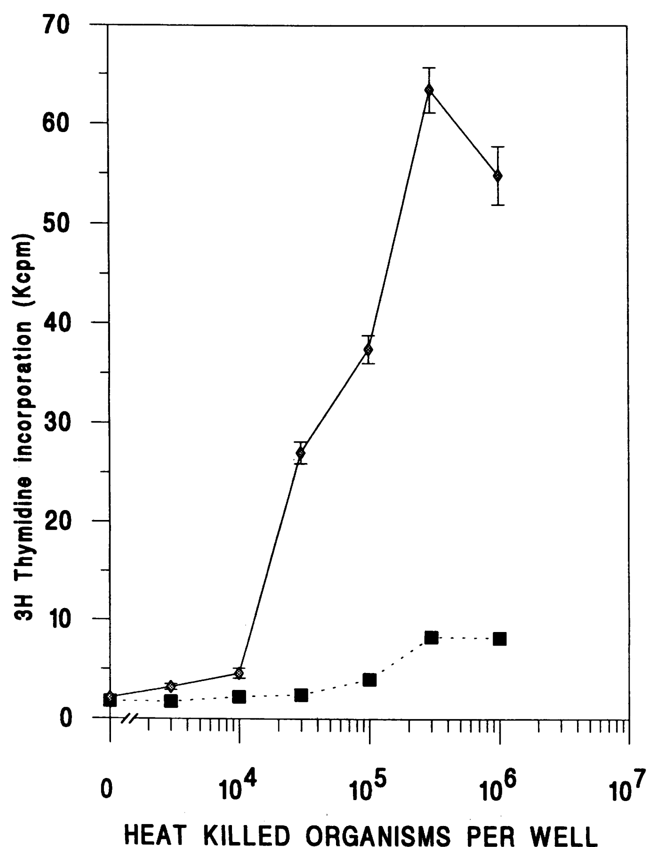

FIG. 2. Effect of fixation with paraformaldehyde on processing of *C. neoformans* by peritoneal macrophages. Macrophage monolayers were prepared and fixed with 2% paraformaldehyde before the addition of organisms (■), or the organisms added without fixation (◆). After 2 h, the monolayers were washed and the remaining macrophages were fixed. Primed lymph node cells were added, and cell proliferation was assayed after 3 days. The results are expressed as means ± SD of triplicate cultures.

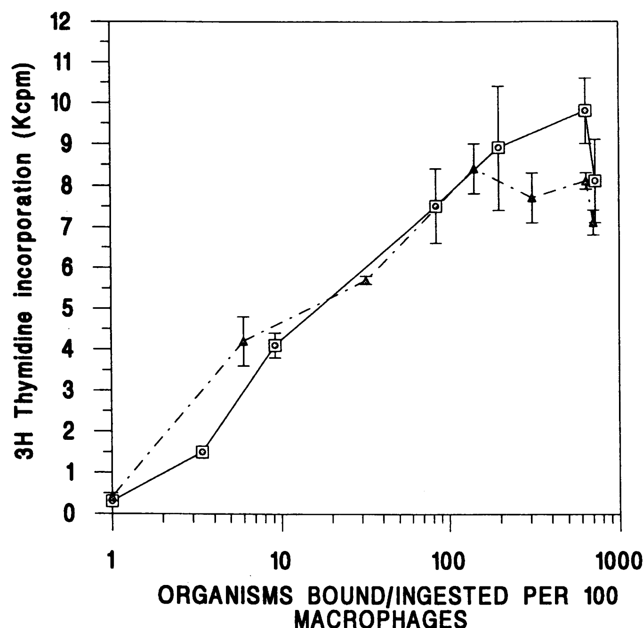

FIG. 3. Effect of the polysaccharide capsule on processing of *C. neoformans*. Encapsulated yeast cells preopsonized with normal mouse serum (⊡) or acapsular *C. neoformans* (▲) were added to macrophage monolayers and incubated for 2 h. The cells were washed and then fixed with 2% paraformaldehyde, and proliferation of primed lymph node cells was measured after 3 days. The results are expressed as means ± SD of triplicate cultures after adjustment for number of ingested yeasts per 100 macrophages.

senting cells (data not shown). In other experiments, equivalent ingestion of unopsonized and opsonized encapsulated *C. neoformans* was achieved by the addition of a 10-fold excess of unopsonized organisms. Under these conditions, no difference in lymph node proliferation was observed (8.1 ± 0.4 kcpm versus 7.8 ± 0.2 kcpm at 75 and 70 yeasts bound or ingested per 100 macrophages, respectively), demonstrating that opsonization per se had no intrinsic effect on the efficiency of antigen processing, other than to increase antigen uptake. Finally the addition of 1 mg of purified CPS from *C. neoformans* had no effect on the ability of ConA-activated macrophages to process and present an independent protein antigen, HEL (HEL plus medium, 12.1 ± 1.8 kcpm; HEL plus CPS, 11.6 ± 2.0 kcpm). Together, these experiments suggest that, provided ingestion of the organism by the presenting cell occurs, the capsule has no effect on the processing and presentation of cryptococcal antigens and subsequent T-cell activation.

Characterization of antigen-processing events for *C. neoformans*. The characteristics of antigen processing for T-cell responses to fungal pathogens have not been previously described. Here, processed cryptococcal antigen was available for T-cell recognition within 1 to 2 h after the interaction of intact yeast cells with the antigen-presenting cells (Fig. 4). These events were major histocompatibility complex (MHC) restricted, because proliferation of specific lymph node cells was reduced after interaction with *C. neoformans*-pulsed antigen-presenting cells prepared from mice with a different MHC haplotype (Fig. 5). It is established that class II MHC-restricted processing events are inhibited by the addition of lysosomotropic agents to the antigen-presenting cell. Antigen- presenting cells treated with chloroquine before the addition of live opsonized encapsulated yeast cells resulted in reduced proliferation of *C. neoformans*-specific lymph node cells (Fig. 6). Thus, presentation of *C. neoformans* antigens initially requires an intracellular processing event similar to that previously described for soluble protein antigens and bacteria (31, 32).

DISCUSSION

In the present study, T cells responsive to cryptococcal antigens were elicited after in vivo priming of mice with the acapsular form of the organism. Primed lymph node cells proliferated equally well in response to soluble antigens prepared from either acapsular or encapsulated *C. neoformans*. In contrast, proliferation induced by intact encapsulated yeast cells was greatly reduced in comparison with that induced by the acapsular mutant, suggesting that capsule synthesis impaired T-cell activation, possibly by interfering with either uptake or processing and presentation by antigen-presenting cells.

To address the mechanism of impaired T-cell responsiveness, we established a model to study the processing and presentation of cryptococcal antigens in vitro. In experiments with purified macrophages as antigen-presenting cells, encapsulation of *C. neoformans* was also associated with

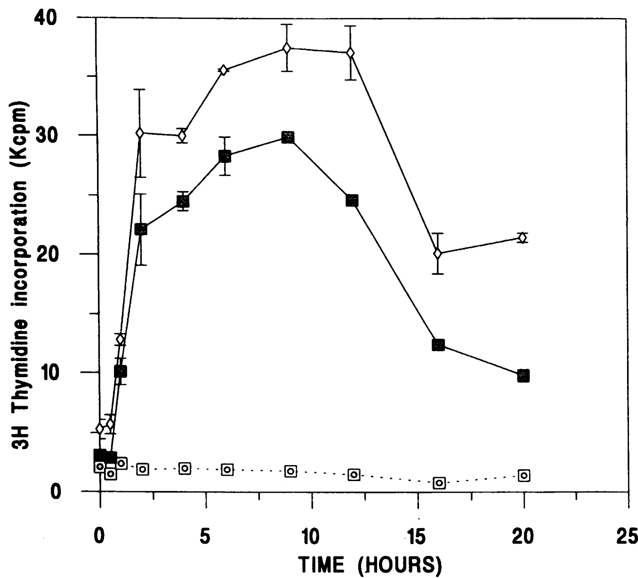

FIG. 4. Time course for processing of *C. neoformans*. Serum-opsonized encapsulated organisms at 3×10^4 (■) or 3×10^5 (◇) per well or medium alone (⊡) was added to macrophage monolayers, and antigen processing was stopped at various time points by paraformaldehyde fixation. At time zero cells were washed and fixed immediately after centrifugation, and at 2 h all plates were washed to remove nonbound organisms. Primed lymph node cells were added to all monolayers after fixation at the final time point, and proliferation was assayed after 3 days. The results are expressed as means ± SD of triplicate cultures.

reduced T-cell proliferation. However, prior opsonization with complement allowed encapsulated yeast cells to induce significant antigen-specific responses. Furthermore, when ingestion by antigen-presenting cells was quantitated in

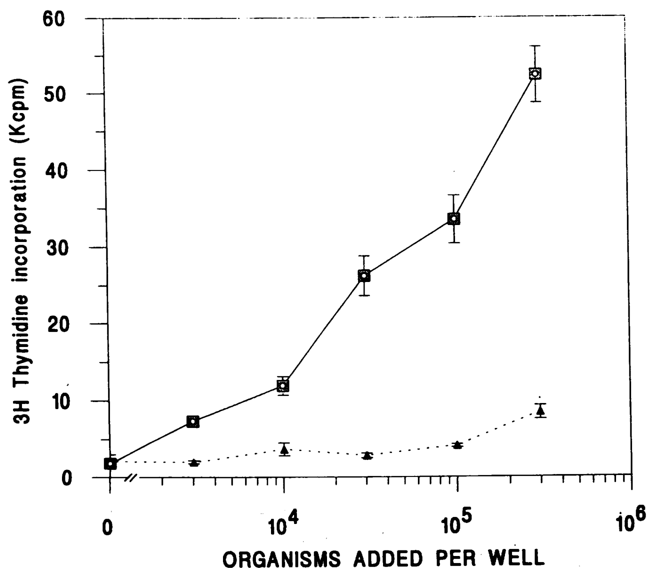

FIG. 5. MHC restriction of *C. neoformans* antigen processing and presentation. Macrophage monolayers were prepared from BALB/c mice (▲) and CBA/CA mice (⊡). Preopsonized encapsulated *C. neoformans* cells were added, and processing was measured after 16 h as described in the text. The results are expressed as means ± SD of triplicate cultures.

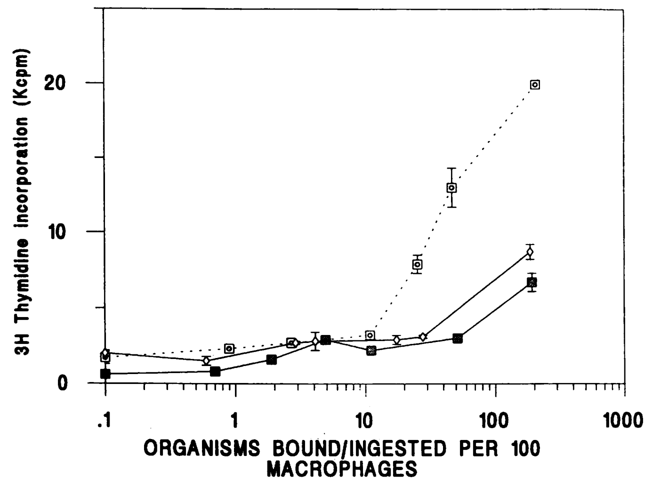

FIG. 6. Effect of chloroquine on macrophage processing of *C. neoformans*. Macrophage monolayers were prepared and incubated for 30 min in the presence of medium (⊡), 0.1 mM chloroquine (◇), or 0.3 mM chloroquine (■). Live opsonized encapsulated organisms were added in the presence of the appropriate concentration of chloroquine and incubated for 90 min. After fixation, primed lymph node cells were added and processing was measured as described in the text. The results are expressed as means ± SD of triplicate cultures.

parallel to T-cell activation, encapsulated and acapsular yeast cells induced identical T-cell responses. Thus, the inability of encapsulated yeast cells to stimulate T-cell proliferation is a consequence of the antiphagocytic action of the capsule on antigen-presenting macrophages. Finally, purified CPS did not inhibit macrophage processing of an unrelated protein antigen, HEL. Unlike the case with other polysaccharides (24), we have no evidence that the capsule of *C. neoformans* can interfere with processing of either cryptococci per se or heterologous protein antigens.

Despite the importance of T-cell immunity in cryptococcal infection (13, 14), the characteristics of antigen processing for *C. neoformans* or other fungal pathogens have not been described. After ingestion, the processing of *C. neoformans* by activated macrophages was efficient, with an average of only 10 organisms per 100 macrophages required to stimulate significant T-cell proliferation. Antigen presentation was MHC restricted and exhibited characteristics similar to those described for other organisms, including a requirement for viable antigen-presenting cells and inhibition by lysosomotropic agents (31, 32). Cryptococcal antigens were functionally available for T-cell recognition within 1 to 2 h of the interaction of intact yeast cells with antigen-presenting macrophages. This was longer than the 30-min processing time observed for *Listeria monocytogenes* under identical conditions (31; data not shown), possibly reflecting differences in the compositions of the cell walls of the organisms. This system will now permit further examination of the biology of antigen processing for this and other fungal pathogens. Future experiments will examine whether melanin synthesis, an additional determinant of *C. neoformans* virulence, influences the kinetics of antigen processing in this model. These results will also facilitate the production of *C. neoformans*-specific T-cell clones and the further identification of immunodominant antigens. Recent reports indicate that $CD4^+$ and $CD8^+$ T lymphocytes are important in controlling different aspects of resistance to *C. neoformans* in vivo (17). We are

characterizing the surface phenotype and cytokine profiles of the T cells responding here, and preliminary results suggest they secrete both gamma interferon and interleukin-2.

Our experiments have shown that acapsular *C. neoformans*, the form that initiates infection after inhalation into the lung, is efficiently ingested by macrophages and cryptococcal antigens processed and presented to stimulate specific T-cell proliferation. However, once in the pulmonary environment, *C. neoformans* rapidly synthesizes a polysaccharide capsule (9). Although alveolar macrophages can ingest encapsulated cryptococci (23), dissemination to extrapulmonary sites involves tissue macrophages in other organs. Our results suggest that opsonization will then be essential for efficient ingestion by antigen-presenting cells and the further development of specific T-cell immunity. Finally, it is important to note that complement-dependent phagocytosis is not a constitutive function of resting macrophages (15). We have recently demonstrated that activation of macrophages by tumor necrosis factor and granulocyte macrophage colony-stimulating factor or *C. neoformans*-specific T-cell supernatants is required for efficient ingestion of encapsulated yeast cells (5a). Thus, the activation of macrophages for ingestion, antigen presentation, and fungistasis of *C. neoformans* is essential for host resistance. In conclusion, capsule synthesis by *C. neoformans* inhibits T-cell activation in vitro by reducing the uptake of intact yeast cells by antigen-presenting cells, rather than by a direct effect on antigen presentation per se. Further studies on the pathways of macrophage activation and the characteristics of T-cell immunity should provide a greater understanding of resistance to this increasingly important organism.

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