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Differential Stimulation of Murine Resident Peritoneal Cells by Selectively Opsonized Encapsulated and Acapsular *Cryptococcus neoformans*

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Stimulation of murine resident peritoneal cells (RPCs) by encapsulated strain 52 and acapsular strain 602 of *Cryptococcus neoformans* was compared. Fresh serum was required for fungistasis of both strains. Encapsulated organisms were killed only if the RPCs were activated with gamma interferon (IFN-γ) or if the organisms were opsonized with anticapsular immunoglobulin G (IgG). In contrast, acapsular organisms were killed by unactivated RPCs, with enhanced killing seen if the cells were activated with IFN-γ. Except for unopsonized strain 52, all organisms of both strains were phagocytosed. The respiratory burst was stimulated in unactivated and IFN-γ-activated RPCs by encapsulated strain 52 only if organisms were opsonized with both IgG and serum. In contrast, the burst was stimulated by acapsular strain 602, with or without opsonization. Only unopsonized strain 602 stimulated significant lysosomal enzyme release. Nitrite synthesis by unactivated RPCs was seen only if strain 52 was opsonized with anticapsular IgG or if strain 602 was opsonized with serum. If RPCs were activated with IFN-γ, then serum-opsonized strain 52 was also able to stimulate nitrite synthesis. Thus, RPC killing, phagocytosis, respiratory burst, lysosomal enzyme release, and nitrite synthesis following challenge by both unopsonized and opsonized with serum or anticapsular IgG strains 52 and 602 varied according to the surface properties of the organisms, the state of activation of the RPCs, and the particular RPC event studied. However, stimulation of nitrite synthesis was the only RPC event which correlated with killing of both strains.

Both clinical and experimental data have linked cell-mediated immunity with containment of the yeast *Cryptococcus neoformans* (4–6, 33). However, the fine orchestration of the immune system that is able to control the fungus in the normal, but not compromised, host remains incompletely understood. *C. neoformans* is unique among fungi causing systemic human disease in its possession of a polysaccharide capsule (1), which has been shown to inhibit phagocytosis (17, 25, 28). Cryptococcal capsule or circulating capsular antigen may also have deleterious effects on the immune system by inducing suppressor T-cell networks (38, 39), activating complement (10, 34), and binding antibody. Circulating antibody against capsular polysaccharide has been associated with a favorable outcome in human cryptococcosis (9), and administration of monoclonal anticapsular antibody was protective in a murine model of disease (12).

While macrophages are thought to act as a major effector cell mediating the cell-mediated immune response against cryptococcosis, macrophages or macrophage cell lines from several species, including humans (8), mice (15, 22), and guinea pigs (3), have been reported to be incapable of killing *C. neoformans*. However, under certain conditions, such as a high macrophage-to-yeast ratio, the presence of fresh serum, and the addition of endotoxin, fungistasis has been obtained (15, 22). In vivo, the treatment of mice with silica to incapacitate the reticuloendothelial system has decreased resistance to experimental cryptococcosis (36), whereas nonspecific activation of macrophages has been protective (13). Nevertheless, fungicidal effects by macrophages have proven difficult to reproduce in vitro, perhaps because additional factors are required to mimic in tissue culture the optimal in vivo conditions for activation. Such factors could include interactions of macrophages with other cell types or with products of other cell types. The lymphokine gamma (immune) interferon (IFN-γ) has been shown to stimulate macrophages to inhibit or kill several species of parasites, bacteria, fungi, and tumor cells (2, 40, 42, 54). The mechanisms by which IFN-γ exerts its enhanced antimicrobial and antitumor activity in macrophages are incompletely worked out and may vary, depending on the particular target involved. In the present study, we define in vitro and in vivo conditions necessary for killing of *C. neoformans* by murine resident peritoneal cells (RPCs). Moreover, by comparison of an encapsulated strain with an acapsular mutant of *C. neoformans*, and by selective opsonization of these strains with either fresh serum as a source of complement or anticapsular immunoglobulin, we demonstrate that stimulation of phagocytosis, respiratory burst, lysosomal enzyme release, and nitrite generation is dependent upon the state of activation of the RPCs and the surface properties of the organism. However, only stimulation of nitrite synthesis correlated with RPC fungicidal activity.

MATERIALS AND METHODS

C. neoformans. Previously described, encapsulated serotype D *C. neoformans* 52 (ATCC 24067) (51) was a gift from Richard D. Diamond (Boston University Medical Center, Boston, Mass.) and acapsular strain 602 (24) was a gift from Eric Jacobson (Medical College of Virginia, Richmond, Va.). Yeast cells were maintained by serial passage at 30°C on asparagine minimal agar medium (30) and harvested from 2- to 3-day-old cultures by suspending a loopful from a single colony in phosphate-buffered saline (PBS). The organisms were washed three times with PBS, counted on a hemacy-

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tometer, and suspended at the desired concentration in PBS or other medium. Under these conditions, less than 2% of the organisms were in clumps of two or three, and the rest were single cells. Budding was not observed. Organisms were heat-killed by incubation in a boiling water bath for 30 min.

C. neoformans was opsonized with immunoglobulin G (IgG) by incubation with a subagglutinating concentration of a monoclonal IgG1 antibody highly reactive with serotype D capsule (a gift from Thomas Kozel, University of Nevada School of Medicine, Reno, Nev.) for 30 min at 37°C. Yeasts were opsonized with serum by incubating the organisms in fresh mouse serum, obtained by cardiac puncture, for 30 min at 37°C. Acapsular organisms were coated with capsule by incubation with 200 μg of purified serotype D capsular polysaccharide (kindly provided by Thomas Kozel) per ml for 2 h at 37°C (26). All organisms were washed three times in medium prior to use.

RPCs. Female, 5- to 8-week-old CD-1 mice (Charles River Breeding Laboratories, Inc., Kingston, N.Y.) were used in all experiments and were determined to be specific pathogen-free on the basis of negative serologies for 15 common murine pathogens. Mice were sacrificed by CO_2 inhalation, and the peritoneal cavity was lavaged with 8 ml of cold PBS. The RPCs obtained were centrifuged at 600 × *g* for 10 min, erythrocytes were removed by lysis with hypotonic saline, and the cells were counted with a hemacytometer, centrifuged, and suspended in medium at the desired concentration. Medium used for all experiments, unless stated otherwise, was RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.), supplemented with L-glutamine, penicillin, streptomycin, and 10% heat-inactivated, low endotoxin fetal bovine serum (Hyclone). A total of 4×10^6 to 7×10^6 RPCs were routinely obtained per mouse. In some experiments, cells had adhered to plastic petri dishes or to the bottom of cell wells for 2 h and nonadherent cells were removed by agitation, followed by three washes.

Cell differentials were performed by Giemsa and nonspecific esterase staining (21) on cytocentrifuge preparations of lavaged cells. RPCs averaged 94% macrophages, 4% lymphocytes, 1 to 2% mast cells, and less than 1% neutrophils and eosinophils. Cell differentials at 18 h following injection of IFN-γ or PBS were similar, with 90 to 95% of the cells macrophages, less than 1% neutrophils, and the rest lymphocytes, mast cells, and eosinophils. All media used were periodically measured by the *Limulus* amoebocyte lysate assay (Sigma Chemical Co., St. Louis, Mo.) and used only if 0.05 ng/ml or less of endotoxin was found.

Killing of *C. neoformans*. RPCs, isolated as outlined above, were allowed to settle in flat-bottom wells (0.32 cm^2) of 96-well plates (Costar, Cambridge, Mass.) at 10^6 cells per well in 150 μl of medium. A 15-μl sample of medium containing 16.5 U of recombinant IFN-γ (to give a final concentration of IFN-γ of 100 U/ml) or medium alone was added to each well, and the cells were incubated at 37°C in a 5% CO_2 atmosphere for 6 h. IFN-γ was a gift of Genentech, Inc. (South San Francisco, Calif.) and had a final concentration of less than 1 pg of endotoxin per ml. Effector cells were then challenged with 5×10^4 *C. neoformans* suspended in 15 μl of medium. In some experiments, 10% (final concentration) fresh serum or 100 ng of lipopolysaccharide (LPS; prepared from *Escherichia coli* O55:B5 by the phenol extraction method, [Difco Laboratories, Detroit, Mich.]) per ml was also added. Effectors and targets were incubated for an additional 2 to 72 h at 37°C in a 5% CO_2 atmosphere, when growth inhibition was determined qualitatively by careful examination of the cultures under an inverted microscope and, in selected experiments, quantitatively by hemacytometer counts of organisms per well at the start and finish of the experiment. Inhibition was defined as the absence of a significant difference between the number of organisms per well at the start compared with that at the finish of the experiment.

To measure killing of *C. neoformans*, the contents of the cell well and of two subsequent vigorous washes with 100 μl of H_2O to remove adherent cells were transferred to 1.5-ml polypropylene centrifuge tubes. Microscopic observation of cell wells confirmed that the entire contents of the cells were transferred. Tubes were placed in an ice water bath, and the contents were sonicated with the microtip attachment of a sonicator (VibraCell model; Sonics and Materials, Danbury, Conn.) for 16 0.5-s pulses at a setting of 2 in a biohazard hood. These conditions broke up the leukocytes into particles of less than 1 μm in diameter without affecting the viability of the *C. neoformans*. Sonication had the added benefit of breaking up the rare small clumps of yeast into single cells. The yeasts were washed once with distilled water and suspended at approximately 10^4/ml, and 30 μl of the suspension was added to triplicate wells of a 96-well flat-bottom plate. *C. neoformans* was allowed to settle to the bottom of the plate and overlaid with 120 μl of Sabouraud broth containing 1.5% molten agarose heated to 45°C. The plate was then incubated for 16 h at 23°C and read under an inverted microscope at a magnification of 600× by systematically scanning, looking for *C. neoformans*. Since only the live *C. neoformans* can grow into microcolonies, single cells (easily recognizable as a result of their size, refractile properties, and internal structures) were scored as dead, while microcolonies (generally approximately 30 to 50 cells) were scored as live. At this low concentration of *C. neoformans*, microcolonies rarely grew until they touched each other or dead cells. A total of 100 single yeast cells and microcolonies were counted, with the percent killing equal to the number of single yeast cells counted. Two sets of controls were used in these experiments. Firstly, *C. neoformans* was harvested, opsonized, sonicated, and plated as described above, but stored in PBS at 4°C in lieu of incubation with mammalian cells. These controls always had greater than 98% viability, even when sonicated for up to 5 min. The second set of controls was obtained by incubating *C. neoformans* in culture medium without mammalian cells at 37°C for 18 h. These controls also had greater than 98% viability. Wells were read with the observer blinded with respect to which experimental group the *C. neoformans* belonged. For the in vitro experiments, results were periodically correlated with an assay counting CFUs after dilutions and pour plates (31). Discrepancies between results obtained with the microcolony assay and the CFU assay averaged less than 10%.

For in vivo studies, mice received an intraperitoneal injection of 0.5 ml of PBS containing 10^6 U of IFN-γ followed 18 h later by an intraperitoneal injection of 10^6 *C. neoformans* in 1 ml of PBS. The mice were lavaged 18 h after fungal challenge. The lavaged cells were sonicated, and viability of the lavaged yeast was determined by the microcolony assay as described above.

Phagocytosis assays. A total of 8×10^5 RPCs were incubated for 2 h in 8-well glass tissue culture chambers (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.), washed free of nonadherent cells, and incubated an additional 16 h in RPMI 1640 with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. Following a 30-min challenge of

monolayers with 1.6×10^6 *C. neoformans* or zymosan, the number of stimuli associated per cell was determined by microscopic inspection of paraformaldehyde-fixed monolayers. To facilitate identification of stimuli, in some experiments yeasts and zymosan were labeled with fluorescein isothiocyanate by overnight incubation at 4°C with 1 mg of fluorescein isothiocyanate per ml in 20 mM borate buffer (pH 9.0), followed by washing the stimuli in PBS 10 times. The average number of stimuli associated per cell (phagocytic index) was determined by scoring 100 cells and represented both fully internalized and attached-only organisms.

To distinguish between fully internalized and attached-only organisms, a modification of the diaethanol assay, as previously described by us for zymosan and *Candida* particles (32), was used. Cell monolayers were incubated with *C. neoformans* for 30 min as above, exposed to 0.01% diaethanol (Uvitex 2B, a gift of Dietmar G. Braun, CIBA-Geigy Limited, Basel, Switzerland) for 1 min and washed free of the diaethanol by successively dipping the slide into five chambers containing PBS alone. With an epifluorescent microscope (Olympus model IMT2-RFL) equipped with UV objectives at a final magnification of 1,000×, microscope fields were systematically observed under bright-field illumination to determine the number of cell-associated particles and then under epifluorescence to determine which of those particles stained with diaethanol. Particles that were cell associated and fluorescent were considered attached but not internalized, whereas those that were not fluorescent were considered fully internalized (32). A total of 100 cell-associated particles were scored on each slide, with the percent internalized equal to the number of particles counted that were not fluorescent.

Respiratory burst, lysosomal enzyme release, and nitrite assays. Hydrogen peroxide production by RPCs was measured by the H_2O_2-dependent, horseradish peroxidase-mediated oxidation of homovanillic acid to the fluorescent dimer 2,2′-dihydroxy-3,3′-dimethoxydiphenyl-5,5′-diacetic acid (43). β-glucuronidase activity in RPC supernatants was assayed with phenolphthalein glucuronic acid as a substrate and by measuring formation of phenolphthalein with a spectrophotometer at 540 nm (49). Results were expressed as percent maximal release, with sonicated RPCs as the standard for maximal release. For both the H_2O_2 and β-glucuronidase assays, RPCs were washed and the medium was replaced with Hanks buffered saline solution prior to the addition of stimuli. Lactate dehydrogenase release was assayed colorimetrically by measuring the conversion of pyruvate to lactate (Sigma kit no. 500) by the instructions of the manufacturer. Nitrites were measured by the technique of Green et al. (16). The sample was allowed to react with Griess reagent (1% sulfanilamide–0.1% naphthylethylene diaminedihydrochloride–2.5% phosphoric acid) to form a chromophore absorbing at 546 nm. Nitrite in unknowns was determined by comparison with a standard curve constructed by using known concentrations of sodium nitrite.

Statistics. Means and standard errors of the means were compared by using the two-sample, two-tailed Student's *t* test for independent means.

RESULTS

Fungistasis. Initial experiments looked at conditions required for fungistasis of *C. neoformans* 52 and 602 by RPCs. Fungistasis was defined as complete inhibition of growth, as measured both by comparisons of hemacytometer counts of organisms per well at the start and finish of the experiment and by visual inspection of monolayers. Fungistasis of either strain was consistently achieved for 48 h when at least 5×10^5 RPCs and 10% fresh mouse serum were added to the system. Despite greater than 90% of the RPCs consisting of macrophages, fungistasis, even for just 18 h, was not achieved when up to 10^6 adherent cells were used, rather than a mixed-cell population, or if the RPCs were washed free of nonadherent cells 2 h after challenge with *C. neoformans*. Up to 10^6 nonadherent cells also were not fungistatic.

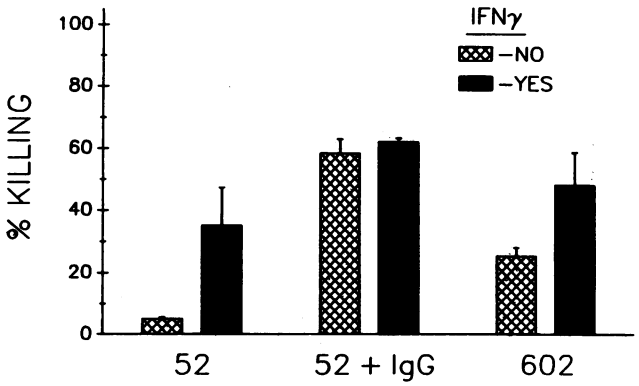

FIG. 1. In vitro killing of cryptococcal strains by RPCs in the presence of 10% serum. Each result is the mean ± the standard error of the mean for three to seven duplicate experiments. $P < 0.001$ comparing killing of strain 52 or 602 by unstimulated versus IFN-γ-stimulated RPCs and comparing IgG-opsonized 52 with unopsonized 52.

Fungicidal activity. Having established conditions sufficient for fungistasis, we next examined under what conditions killing occurred. For these experiments 10^6 RPCs were challenged with 5×10^4 *C. neoformans* in the presence of 10% fresh mouse serum. In preliminary experiments, we observed <10% killing of the yeasts following a 2-h incubation. Therefore, the incubation period was extended to 16 h. When organisms were opsonized with serum alone, 26% of acapsular strain 602 organisms were killed compared with only 6% of encapsulated strain 52 ($P < 0.001$ [Fig. 1]). When RPCs were stimulated for 6 h prior to fungal challenge with 100 U of IFN-γ per ml, a dose which had been shown to activate peritoneal macrophages to kill other fungi and parasites (2, 40), killing of strains 602 and 52 increased to 48 and 35%, respectively ($P < 0.001$ comparing strain 602 or 52 with and without IFN-γ). When strain 52 was opsonized with anticapsular IgG in addition to serum, 59 and 62% killing was seen with and without IFN-γ, respectively. IFN-γ or IgG failed to inhibit or kill either fungal strain in the absence of RPCs.

Although in vivo studies of RPC-mediated killing are potentially limited by difficulties determining the fate of the inoculum, including those organisms not lavaged back, they have the advantage of allowing in situ study of the RPC without the trauma of lavage and subsequent culture in an artificial environment. Therefore, we next examined in vivo killing. Mice received an intraperitoneal injection of 10^6 U of IFN-γ or placebo and were challenged 16 h later with 10^6 *C. neoformans*. At 24 h after fungal challenge, the peritoneal cavity was lavaged and viability of the lavaged organisms was determined. This dose of IFN-γ had previously been shown to stimulate RPC anti-protozoal activity (40). Prior to fungal challenge, RPCs averaged 94% macrophages, 4% eosinophils. At 24 h after challenge with either strain, the percentage of neutrophils among the peritoneal cells lavaged

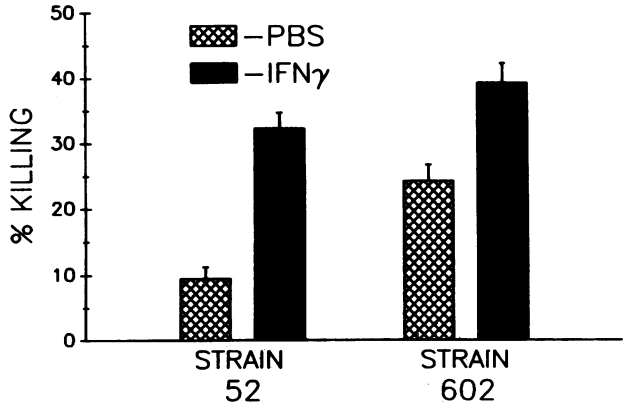

FIG. 2. In vivo killing of cryptococcal strains by RPCs. Each result is the mean ± standard error of the mean of three to four triplicate experiments. $P < 0.001$ comparing any two stimuli.

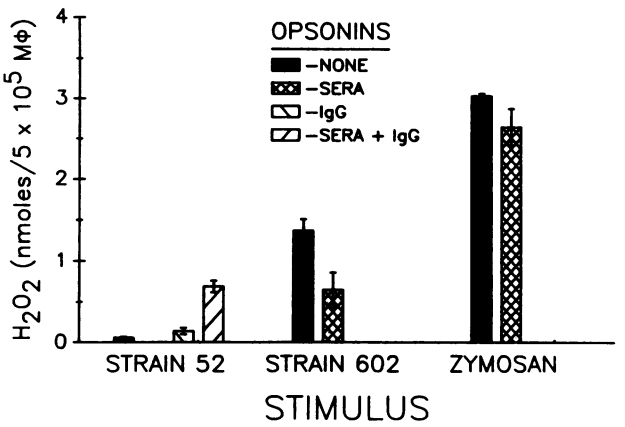

FIG. 3. H_2O_2 generation by RPCs stimulated with selectively opsonized particles. A total of 5×10^5 RPCs were incubated for 16 h, washed, and challenged with 5×10^6 particles for 2 h. RPC alone or strain 52 opsonized with serum failed to stimulate any detectable H_2O_2 release. Each result is the mean ± standard error of the mean of four triplicate experiments. $P < 0.001$ comparing (i) strain 52 opsonized with IgG plus serum with any other selectively opsonized 52, (ii) serum-opsonized 52 and 602, (iii) unopsonized strain 602 with any other stimulus, and (iv) opsonized or unopsonized zymosan with any other stimulus.

increased to 3 to 20%, while the percentage of lymphocytes remained the same. However, greater than 95% of the lavaged yeasts in all the groups had been phagocytosed by the macrophage population, as determined by nonspecific esterase staining of cytocentrifuge preparations, with the remainder of the yeasts not cell associated. Budding yeasts or clumps of yeasts were not observed. Similar patterns of killing to those seen in vitro were observed in vivo. Of the organisms lavaged back, 24% of strain 602 and 9% of strain 52 were killed in the placebo group versus 39 and 32%, respectively, in the group treated with IFN-γ ($P < 0.001$ comparing either strain with and without IFN-γ [Fig. 2]).

Phagocytosis. We next sought insights into the mechanisms of killing, or resistance to killing, of *C. neoformans* by RPCs. Therefore, several RPC functions that have been associated with cytocidal activity were examined following fungal challenge, including phagocytosis, generation of a respiratory burst, lysosomal enzyme release, and nitrite production. Some of the phagocytosis experiments were performed with fluorescein isothiocyanate-labeled fungal particles to facilitate identification of the organisms. Preliminary experiments established that fluorescent labeling of fungi did not influence phagocytosis. Moreover, nearly identical results were obtained with heat-killed versus live organisms. Phagocytosis by RPCs of unopsonized strain 52 was practically nil (Table 1), whereas the phagocytic index following challenge with unopsonized strain 602 was 3.11. However, if strain 602 was pretreated with cryptococcal polysaccharide, the phagocytic index decreased to 0.16. Opsonization of strain 52 with IgG or fresh sera as a source of complement markedly increased the phagocytic index. Similar phagocytic indices were obtained with or without incubation of the RPCs with IFN-γ.

TABLE 1. Phagocytosis of *C. neoformans* by RPCs

Stimulus	Opsonin	Phagocytic index	% Internalized[a]
Strain 52	None	0.01[b]	ND[c]
Strain 52	Serum	0.84	7
Strain 52	IgG	1.92	16
Strain 52	Serum + IgG	3.31	10
Strain 602	None	3.11	18
Strain 602	Serum	4.74	20
Strain 602 + CPS[d]	None	0.16	ND
Zymosan	None	2.38	ND
Zymosan	Serum	3.91	ND

[a] As measured by the diaethanol assay.
[b] Results are the means of two to four duplicate experiments.
[c] ND, Not determined.
[d] Strain 602 pretreated with cryptococcal capsular polysaccharide.

The above phagocytosis assays measured cell-associated organisms only but failed to distinguish between attached-only versus fully ingested organisms. Since this distinction is potentially important in triggering of cellular cytocidal mechanisms, we used the diaethanol assay to differentiate attached from fully internalized fungi (32). Regardless of whether strain 602 or 52 was tested or whether the yeast strains were opsonized with serum, IgG, or both, no more than 20% of the organisms were internalized (Table 1). Markedly different results were obtained when peritoneal cells were challenged in vivo. Mice were injected intraperitoneally with 5×10^6 *C. neoformans* and lavaged 2 h later, and the RPCs were adhered to glass and assayed by the diaethanol method. Over 70% of injected organisms were recovered in the lavage fluid, as measured by hemacytometer counts. We found that 41% of unopsonized strain 602, 28% of unopsonized strain 52, and 64% of IgG-coated strain 52 were fully internalized ($P < 0.001$ comparing IgG-opsonized strain 52 with unopsonized strain 52 or 602).

Respiratory burst. In preliminary experiments, we determined that the respiratory burst occurred within 2 h of stimulation and that similar amounts of H_2O_2 were generated by RPCs that were washed free of nonadherent cells compared with unwashed RPCs. Subsequent experiments measured H_2O_2 generation by RPCs that were cultured for 16 h with or without 100 U of IFN-γ, washed, and then challenged for 2 h with selectively opsonized strain 52, 602, or zymosan particles (Fig. 3). There was a poor correlation between fungicidal activity and stimulation of a respiratory burst. Not surprisingly, unopsonized encapsulated strain 52 yeast cells, which are not phagocytosed, did not stimulate a respiratory burst. However, strain 52 yeast cells opsonized with serum or IgG, which stimulated some phagocytosis, did not stimulate an appreciable burst. Only when the combina-

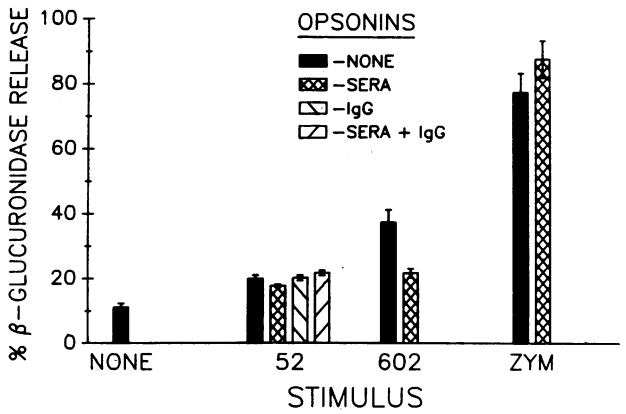

FIG. 4. β-glucuronidase release by RPCs stimulated with selectively opsonized particles. A total of 5×10^5 RPCs were incubated for 16 h, washed, and challenged with 5×10^6 particles for 2 h. Each result is the mean ± standard error of the mean of three duplicate experiments. $P < 0.001$ comparing unopsonized strain 602 with any other stimulus or comparing either unopsonized or serum-opsonized zymosan with any other stimulus. ZYM, Zymosan.

tion of serum and IgG opsonization was used did the organisms stimulate a modest burst. In contrast, unopsonized strain 602 stimulated a moderate burst which diminished by greater than 50% if the organisms were serum opsonized. Zymosan particles, with or without serum opsonization, stimulated the greatest amount of H_2O_2 release. IFN-γ had no significant effect on H_2O_2 generation by RPCs challenged with any of the stimuli. H_2O_2 release by the organisms themselves was not observed, and nearly identical results were obtained if heat-killed or live organisms were used as stimuli. H_2O_2 generation remained constant if the particle-to-RPC ratio was increased fivefold, suggesting that the cells were maximally stimulated. If strain 602 yeast cells were pretreated with cryptococcal polysaccharide prior to stimulating the RPCs, H_2O_2 generation decreased from 1.65 to 0.04 nmol ($P < 0.0001$). When cells were challenged at the same time with zymosan and either unopsonized or IgG-opsonized strain 52, H_2O_2 generation was 2.83 and 3.07 nmol, respectively, which was not significantly different from the 3.04 nmol generated by zymosan particles alone. Thus, strain 52 appeared to be unable to stimulate the burst but did not suppress it.

Lysosomal enzyme release. RPCs were challenged with selectively opsonized strain 52, strain 602, or zymosan particles, and β-glucuronidase release was determined as a measure of lysosomal enzyme release (Fig. 4). Of the cryptococcal stimuli, only the unopsonized, acapsular organisms significantly stimulated lysosomal enzyme release above that seen with unstimulated cells. Unopsonized and serum-opsonized zymosan particles both stimulated large amounts of β-glucuronidase release. Pretreatment of the RPC with 100 U of IFN-γ for 18 h prior to challenge with stimuli resulted in inhibition of β-glucuronidase release to base-line levels of unstimulated cells. For example, for unopsonized strain 602, percent release decreased from 39 ± 4 to 16 ± 1 without and with IFN-γ, respectively, ($P < 0.001$). Extending the incubation period beyond 2 h did not result in significantly greater release. Similar results were obtained with RPCs that were either washed free of nonadherent cells or unwashed. Thus, extracellular release of granule contents varied according to stimuli and culture conditions but did not correlate with observed fungicidal activity. Cell death, as

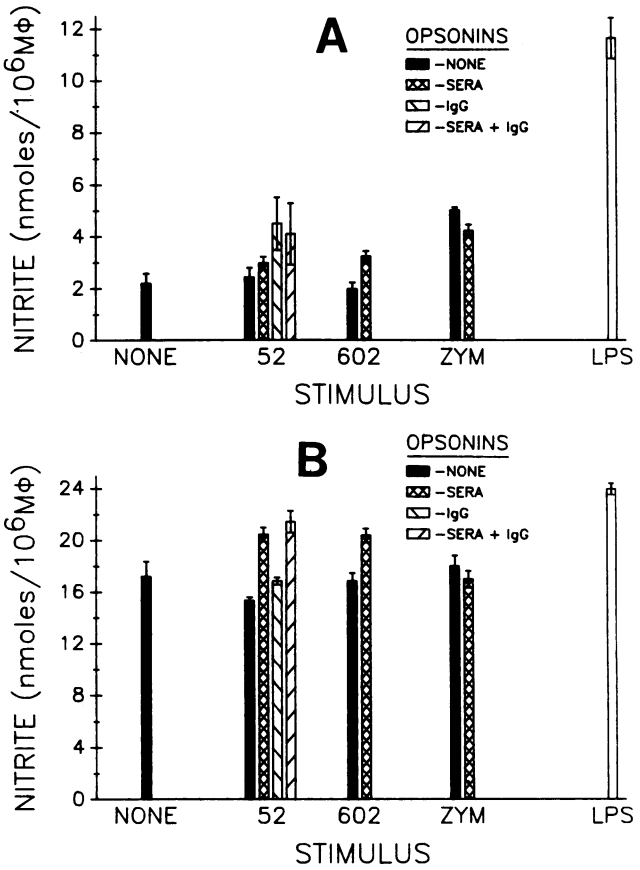

FIG. 5. Nitrite generation by RPCs stimulated with selectively opsonized particles at a 1:10 ratio for 18 h. Each result is the mean ± standard error of the mean of four duplicate experiments. LPS was used at 1 mg/ml. ZYM, Zymosan. (A) RPCs were incubated without IFN-γ. $P < 0.05$ comparing any stimulus except unopsonized strain 52, serum-opsonized strain 52, and unopsonized strain 602 with RPCs alone. (B) RPCs were incubated with 100 U of IFN-γ 6 h prior to stimulation. $P < 0.05$ comparing serum-opsonized strain 52, serum-plus-IgG-opsonized strain 52, serum-opsonized strain 602, or LPS with RPCs alone.

measured by release of the cytoplasmic enzyme lactate dehydrogenase, was less than 10%.

Nitrite. Finally, nitrite generation by RPCs stimulated for 6 h with or without IFN-γ, and then challenged for 18 h with heat-killed strain 52, strain 602, zymosan, or LPS was determined (Fig. 5). Nitrite forms an end product of a pathway that has been associated with macrophage killing activity (18, 45). When cells were incubated without IFN-γ, IgG- (or IgG-plus-serum-) opsonized strain 52 and serum-opsonized strain 602 were the only cryptococcal stimuli that triggered significant increases in nitrite synthesis over base-line levels. Incubation of RPCs with IFN-γ resulted in markedly increased base-line nitrite generation. However, only serum-(or serum-plus-IgG-) opsonized strains 52 and 602 stimulated significant increases over these base-line levels (Fig. 5B). Nearly identical results were obtained with or without incubation of cells and stimuli with 10 μg of polymyxin B per ml (data not shown), making it unlikely that these results were secondary to endotoxin contamination. Thus, while nitrite levels did not absolutely correlate with killing, fungicidal activity was only observed in situations in which increased nitrite synthesis over base-line unstimulated

levels was also seen. Reagent sodium nitrite, at concentrations up to 10 mM, was not fungicidal.

DISCUSSION

These data demonstrate, for the first time, that murine RPCs can kill an encapsulated strain of *C. neoformans*, if the cells have been activated with IFN-γ or if the yeast cells have been opsonized with anti-capsular IgG (Fig. 1 and 2). In contrast to encapsulated organisms, acapsular *C. neoformans* 602, which is weakly virulent in murine models of infection (24), could be killed by RPCs in the absence of IFN-γ or IgG (Fig. 1 and 2), with enhanced killing seen when the cells were activated with IFN-γ. Fungistasis required nonadherent cells in addition to macrophages, suggesting that interactions between various cell types formed an essential part of our system. The macrophage appeared to be the primary effector cell responsible for killing, as greater than 90% of the RPCs were macrophages and, upon nonspecific esterase staining, the yeasts were found to be phagocytosed by macrophages. However, it remains possible that nonadherent cells were responsible for the killing and the macrophages were simply acting as scavengers by phagocytosing the dead organisms. In addition to macrophages, several other effector cells may play important roles in killing or inhibiting *C. neoformans*. In vitro, human blood neutrophils and antibody-dependent killer cells can kill opsonized *C. neoformans* (7, 11, 48) and murine splenic natural killer and antibody-dependent killer cells, at high effector/target ratios, can inhibit cryptococcal growth (37, 41). In our system, the inability of nonadherent cells alone to kill *C. neoformans* and the low relative percentage of lymphoid or neutrophilic cells make it difficult to attribute killing to these cells. However, this does not preclude some direct contribution of fungicidal or fungistatic activity by cells other than macrophages. Current studies are, therefore, directed at defining specific cell requirements in the fungicidal process.

In order to gain insight into the mechanisms of stimulation and killing of *C. neoformans* by RPCs, four presumed components of the antimicrobial armamentarium of these cells (phagocytosis, respiratory burst, lysosomal enzyme release, and L-arginine-dependent nitrite generation) were assayed following challenge with selectively opsonized yeasts (Table 1 and Fig. 3 through 5). Stimulation was dependent upon both encapsulation and opsonization of the organisms. Moreover, the ability of a selectively opsonized strain to trigger one function, such as the respiratory burst, did not necessarily correlate with its ability to trigger the other three functions tested.

Consistent with previous observations (27, 29), phagocytosis of encapsulated strain 52 by RPCs was not seen unless the organisms were opsonized with either fresh serum as a source of complement or anticapsular IgG (Table 1). In contrast, acapsular strain 602 was phagocytosed in the absence of opsonization, with enhanced uptake seen if the organisms were opsonized with serum. Cryptococcal capsule may inhibit phagocytosis by presenting a barrier that prevents contact between ligands on the cell wall of the organism, such as mannans or β-glucans, and their receptors on the phagocyte. Moreover, under certain conditions, capsule may mask IgG and C3 deposited on the organisms and thus prevent recognition by receptors for these ligands (27). Regardless of which strain or opsonin were used, less than 20% of the organisms were completely internalized in vitro, even when IgG-opsonized strain 52 was used (Table 1). This result was surprising in light of data from laboratories, including our own, that macrophages completely internalize particles opsonized with IgG (32,35), and suggests that Fc receptors for IgG may need to act in synergy with receptors for other ligands in order for complete ingestion to occur. These other ligands could be masked from the surface of the yeast cell by capsule. Alternatively, physiochemical properties of the capsule surface, such as its hydrophilic nature or anionic charge, could have inhibited complete ingestion (23). In vivo, opsonization of strain 52 with IgG increased the percentage of internalized yeasts from 28 to 64%. The discrepancy between in vitro and in vivo results may be related to differences in extracellular environments between the two groups. Regulation of macrophage receptors, including those for Fc, can occur if cell culture conditions are varied (47, 52).

The respiratory burst, as measured by H_2O_2 generation, was not significantly stimulated by encapsulated strain 52 unless the yeast cells were opsonized with both serum and anticapsular IgG (Fig. 3). Moreover, the respiratory burst was inhibited by greater than 50% when RPCs were stimulated with serum-opsonized, acapsular strain 602 compared with unopsonized organisms, despite greater phagocytosis of the opsonized organisms. Previous investigators have shown that C3b- and iC3b-coated particles do not stimulate the burst (53). Thus, the diminished burst stimulated when strain 602 was opsonized with serum may be secondary to uptake of these organisms via macrophage receptors for C3b and iC3b. In contrast, immune complexes and IgG-coated particles generally do stimulate the respiratory burst (20, 50), although, under certain conditions, the Fc receptor-mediated burst can be down-regulated (20). Our data also suggest that Fc and complement receptors interact in a synergistic manner to stimulate the burst (Fig. 3). Consistent with previous observations with resident, but not elicited, peritoneal cells (44), IFN-γ had no significant effect on H_2O_2 release. These data (Fig. 3) argue against a primary role for the respiratory burst in RPC-induced killing of *C. neoformans*, as killing was not seen following a 2-h incubation, yet the respiratory burst had already taken place. Moreover, serum-opsonized strain 52 cells were killed by IFN-γ-treated RPCs but did not stimulate a detectable burst. However, oxidative mechanisms may still have played a role in some situations by damaging organisms and thus making them more susceptible to nonoxidative damage.

Of the different cryptococcal stimuli used, only unopsonized acapsular organisms stimulated lysosomal enzyme release, as measured by β-glucuronidase release (Fig. 4). Thus, binding of ligands to complement or Fc receptors on RPCs was not adequate to stimulate lysosomal enzyme release. Unopsonized and serum-opsonized zymosan particles both strongly induced lysosomal enzyme release. This is consistent with recent data suggesting that lysosomal enzyme release from human monocytes is mediated, at least in part, by β-glucan-inhibitable receptors (19). Interestingly, pretreatment of macrophages with IFN-γ strongly inhibited zymosan-stimulated lysosomal enzyme release. These data do not preclude a role for lysosomal enzymes in killing of *C. neoformans*, as phagosome-lysosome fusion may be taking place without extracellular enzyme release.

An L-arginine-dependent pathway synthesizing L-citrulline and nitrite has been associated with macrophage tumoricidal (18, 45) and, recently, fungistatic activity (14). Macrophages are the only mammalian cells known to generate nitrite. LPS and IFN-γ have both been shown to be potent stimulators of nitrite synthesis, with a 4- to 12-h lag time from stimulation of synthesis (46). As opposed to stimulation

of phagocytosis, the respiratory burst, or lysosomal enzyme release, nitrite generation correlated well with cryptococcal killing. Thus, nitrite generation and killing by untreated RPCs following cryptococcal stimulation were seen only when encapsulated strain 52 was opsonized with IgG or when acapsular strain 602 was opsonized with serum (Table 1 and Fig. 5). When the RPCs were treated with IFN-γ, base-line nitrite generation increased greater than 10-fold (Fig. 5B). However, further increases over these base-line levels occurred only when serum- or serum-plus-IgG-opsonized organisms were added. These same groups of selectively opsonized organisms were also killed by the IFN-γ-treated RPCs (Table 1). Experiments are currently under way to attempt to assess what role this L-arginine-dependent, nitrite-generating pathway plays in killing. If this pathway is indeed contributing to killing, it would appear that nitrite itself is not the direct mediator, as high concentrations of nitrite do not influence cryptococcal viability.

Thus, killing of *C. neoformans* by murine RPCs appears to depend on both the state of activation of the cells and the surface properties of the organisms. Moreover, stimulation of the components of the antimicrobial armamentarium of the RPC is not an all-or-none phenomenon. Rather, following receptor-ligand binding, specific activities including phagocytosis, generation of a respiratory burst, lysosomal enzyme release, and nitrite synthesis can be independently regulated. While only nitrite synthesis correlated well with killing, it still remains unclear how each of these separate events are related to fungicidal activity. It is possible that different effector mechanisms may mediate killing in different situations. The complex intracellular signaling events by which these receptor-ligand interactions are transduced, resulting in stimulation of the fungicidal apparati of the cell, also remain to be defined.

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