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Inhibition of *Cryptococcus neoformans* Replication by Nitrogen Oxides Supports the Role of These Molecules as Effectors of Macrophage-Mediated Cytostasis

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Activated macrophages are able to inhibit the replication of intracellular microbes and tumor cells. In the murine system, this cytostatic effect is associated with the oxidation of L-arginine to L-citrulline, nitrite, and nitrate and is thought to be mediated by an intermediate of this reaction, possibly nitric oxide (NO·). By exposing replicating *Cryptococcus neoformans* cells to conditions under which NO· is chemically generated, we have observed a cytostatic effect similar to that caused by activated murine macrophages. Nitric oxide is formed as a decomposition product of nitrite salts in acidic, aqueous solutions. Although *C. neoformans* replicates well in the presence of high nitrite concentrations at physiologic pH, its growth in acidic media can be inhibited by the addition of low concentrations of sodium nitrite. The degree of cytostasis is dependent on both the pH and the nitrite concentration of the NO· generating solution. The cytostatic effector molecule appears to be a gas since, in addition to inhibiting *C. neoformans* replication in solution, it is able to exert its inhibitory effect across a gas-permeable but ion-impermeable membrane. At high nitrite concentrations, a fungicidal effect occurs. We propose that the growth inhibition of *C. neoformans* upon exposure to chemically generated NO· or some related oxide of nitrogen represents a cell-free system simulating the cytostatic effect of activated murine macrophages.

Activated macrophages have been shown to produce a number of substances that are potentially harmful to foreign cells (20). Any one or a combination of these compounds might be responsible for the macrophage's ability to inhibit the growth of both microbial and tumor cells. However, the exact identity of the molecules used by the macrophage to inhibit the growth of cell targets is not fully known. Recent evidence indicates that nitric oxide (NO·) acts as one of the murine macrophage's cytotoxic effector molecules. In vitro, macrophage-induced cytostasis requires L-arginine and is associated with the oxidation of this amino acid to the nontoxic end products nitrite, nitrate, and L-citrulline (4, 10, 18, 19). Highly reactive oxides of nitrogen, including nitric oxide, are formed as intermediates in this reaction (14, 15, 17). Additionally, reagent NO· as well as chemically generated nitrogen oxides have recently been shown to simulate the cytotoxic effects of activated macrophages on neoplastic target cells (9, 20). The ability of these compounds to inhibit cell division may be related to their ability to induce cellular iron loss and to their affinities for the iron-sulfur centers in particular enzymes, including mitochondrial NADH dehydrogenase, succinate dehydrogenase, and aconitase, as well as cytosolic ribonucleotide reductase (2, 5, 8, 22). Once nitrosylated after reacting with NO·, these enzymes may lose iron and become inactive and thus render the cell incapable of replicating.

In cell culture, activated murine macrophages are able to inhibit the growth of the pathogenic fungus *Cryptococcus neoformans* (4, 7). This organism provides a good example for studying the cytostatic effector molecules of macrophages. These yeast cells can proliferate both intracellularly and extracellularly (7). They grow rapidly and equally well in culture media with pHs ranging from 3.5 to 7.5. This characteristic is especially useful since nitric oxide can be chemically produced by the addition of nitrite salts to acidic solutions (11). In addition, the cytostatic effects of activated macrophages on *C. neoformans* are well documented and characterized (7).

In this set of experiments, we have attempted to create a cell-free system that reproduces the antiproliferative effects of activated macrophages on this fungus. This was achieved by exposing the organism to various conditions in which NO· is generated. We present data suggesting that a gaseous nitrogen oxide(s) can effect fungistasis, that an NO· scavenger blocks this fungistatic activity, and that the generation of nitrogen oxide(s) induces a metabolic perturbation in static but living fungal target cells. Conditions of NO· generation leading to fungal cell death are also explored.

MATERIALS AND METHODS

Culture media. Unless otherwise stated, all experiments were conducted in minimal medium (MM) consisting only of those components necessary to sustain rapid proliferation of cryptococcal yeast cells. MM contains 109.4 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 25 mM D-glucose, 0.4 mM glycine, 4.0 mM glutamine, 5.0 mM asparagine, 248 nM $FeCl_3$, and 12 μM thiamine. Different buffering compounds were added depending on the medium pH desired: 25 mM succinic acid was used to prepare an acidic medium, and 25 mM sodium 3-(*N*-morpholino)propanesulfonic acid (MOPS) was used to prepare a neutral medium. All media were prepared in the laboratory from individual constituents, brought to the appropriate pH with 1 M HCl or 1 M NaOH, and filter sterilized (0.22 μm pore size; Costar, Cambridge, Mass.).

Microorganism. A single clone of the human strain H99 of

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serotype A *C. neoformans* was used in all experiments. This clone, designated C3D, was originally isolated because of its rapid proliferation in the presence or absence of CO_2 (6). Additionally, its growth inhibition by activated murine peritoneal macrophages has previously been studied and described (7). The organism was maintained in bicarbonate-free Dulbecco minimal essential medium in tissue culture flasks (Falcon, 25 cm^2) and incubated without CO_2 at 37°C. Log-phase yeast cells were isolated immediately prior to each experiment by centrifugation (4,000 × *g*) and three washes, followed by resuspension in MM.

Reagents. Unless otherwise stated, all reagents were obtained from Sigma Chemical Co., St. Louis, Mo. Human oxyhemoglobin was kindly supplied by Joe Bonaventura, Duke Marine Biology Station, Beaufort, N.C. Teflon membranes (0.001 in. [ca. 0.003 cm] thick) were purchased from Yellow Springs Instrument Co., Yellow Springs, Ohio.

Cell culture in NO· generating media. Washed, log-phase C3D cells were resuspended in MM of appropriate pH at 2.5 × 10^4 organisms per ml. The cell suspension was divided into experimental and control cultures. Sodium nitrite and any additional reagents were added to the experimental cultures at this time. One-milliliter aliquots of both the control and experimental cell suspensions were placed in 16-mm-diameter tissue culture wells and allowed to incubate at 37°C without CO_2 for 24 h. Cell replication was arrested by the addition of 0.9% (final concentration) sodium dodecyl sulfate (SDS). Cell counts were determined electronically with a Coulter ZB_1 counter (Coulter Electronics, Hialeah, Fla.) as previously described (7). The average number of replications experienced by the yeast cells in these cultures was determined by subtracting the $\log_2$ of the number of cells at the beginning of the experiment from the $\log_2$ of the number of cells at the end of the experiment.

Cytostasis mediated through a gas-permeable membrane. Log-phase cells were prepared as described above and resuspended at 2.5 × 10^4 organisms per ml in MM, pH 7.0. One milliliter of this suspension was placed in a glass test tube (10 by 75 mm) whose open end was then tightly covered with a Teflon membrane (0.001 in. [ca. 0.003 cm] thick) by stretching the membrane. The membrane was secured onto the tube with a rubber O-ring (9 mm). The tube was inverted and its membrane-covered end was submerged in either neutral (25 mM MOPS, pH 7) or acidic (25 mM succinic acid, pH 4) solutions containing various concentrations of sodium nitrite. The cells were incubated in this fashion at 37°C without CO_2. At 24 h, the tubes were shaken vigorously to resuspend the yeast cells, and 0.9% SDS was added to arrest cell replication. Cell counts were performed electronically as described above. Examination of the Teflon membrane showed that yeast cells were not adherent; thus, resuspension by this method gave the true cell density inside the inverted tube.

Assessment of cell viability. C3D cells were prepared as described above and resuspended in MM, pH 4.0. The cell suspension was divided into two equivalent portions, to one of which was added 750 μM (final concentration) sodium nitrite. Aliquots of both cultures were placed in tissue culture wells and allowed to incubate at 37°C without CO_2. At appropriate times, serial 10-fold dilutions of samples of both cell cultures were performed with phosphate-buffered saline, and 0.1 ml of the appropriately diluted cell suspension was spread onto Sabouraud agar. These culture plates were allowed to incubate for 3 days at 30°C. Colony counts were obtained from the average of triplicate plates with 20 to 200 colonies per plate.

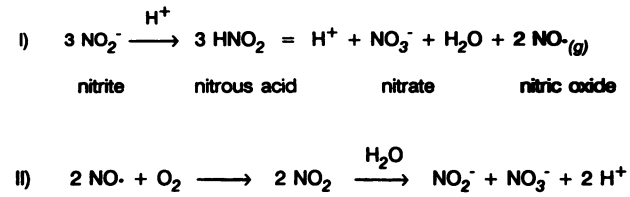

FIG. 1. Reactions of nitric oxide in aqueous solution. Reaction I shows the chemical generation of nitric oxide by the decomposition of nitrite salts in acidic solutions. Reaction II shows the reaction of nitric oxide with molecular oxygen, yielding the nontoxic end products nitrite and nitrate.

Quantitation of glucose metabolism. Glucose metabolism by the fungal cells was quantitated by measuring the evolution of $^{14}CO_2$ by C3D cells in culture media containing [^{14}C]glucose. Log-phase C3D cells were washed and resuspended at 2.5 × 10^6 cells per ml in MM with 5 mM D-glucose and 380 nM D-[U-^{14}C]glucose (specific activity, 348.2 mCi/mmol; New England Nuclear, Boston, Mass.). Aliquots (2.5 ml) of this suspension were placed in multiple reaction flasks (10 ml; Kontes Glassware, Vineland, N.J.), which were then sealed with rubber stoppers. A trapping solution of Protosol (0.1 ml) (New England Nuclear) was suspended in a plastic cup from each top stopper. The cell cultures were incubated with shaking at 37°C. At various times during the experiment, the $^{14}CO_2$ generation was determined by removing the Protosol-containing cups from the chamber and placing them in scintillation vials with 15 ml of Econofluor/methanol (9:1) (Econofluor from New England Nuclear). Liquid scintillation spectrometry was used to measure disintegrations per minute by the external ratio correction method. Each datum point represents the average of triplicate samples (see Fig. 5).

Quantitation of nitrite. The concentration of nitrite in the experimental solutions and cell cultures was determined colorimetrically after the addition of the Greiss reagents. To 0.5 ml of sample was added 1.0 ml of 1% sulfanilamide–2.5% phosphoric acid followed by 1.0 ml of 0.5% naphthylethylenediamine dihydrochloride–2.5% phosphoric acid. The absorbance of the mixture at 540 nm was measured, and the concentration was determined from a standard curve which was linear between 10 and 200 μM sodium nitrite. All samples were performed in duplicate.

RESULTS

Inhibition of *C. neoformans* growth with NO· generating media. Incubation of *C. neoformans* C3D in MM allows logarithmic replication of the yeast cells within the first 24 h. The ability of this microorganism to grow in acidic conditions simplifies the study of the effect of nitric oxide on its growth. Nitric oxide is spontaneously generated by the decomposition of nitrite salts in acidic, aqueous solutions (Fig. 1, reaction I). The addition of sodium nitrite to the acidic culture media of *C. neoformans* creates the condition in which NO· is actually produced by the environment surrounding the replicating fungal cells. The fact that nitrite itself has no growth-inhibitory effects is seen in experiments performed at neutral and slightly acidic pH conditions, in which the growth of C3D is undisturbed by the addition of high concentrations of sodium nitrite (Fig. 2a, closed symbols; Fig. 2b, open symbols). Growth inhibition at pH 7.0 occurs only at nitrite concentrations approaching 100 mM

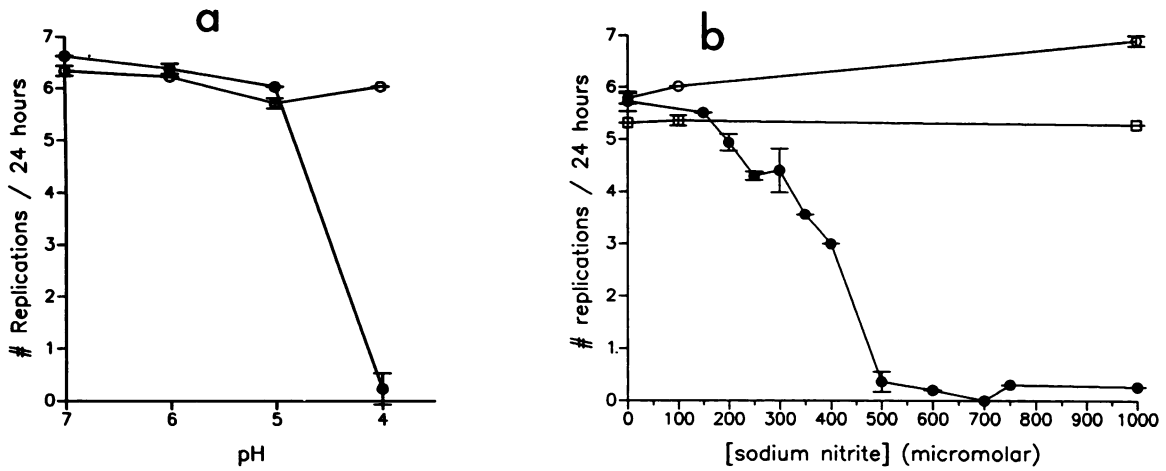

FIG. 2. (a) Cytostatic effect of sodium nitrite is dependent on pH. At time zero, 10^4 viable cryptococci were incubated with (closed circles) and without (open circles) 500 μM sodium nitrite in MM with different pH values. After 24 h, the number of yeast cells per milliliter was determined by electronic counting. The ordinate shows the number of times the yeast cells divided during the 24-h incubation. This was calculated by subtracting the $\log_2$ of the number of cells at time zero from the $\log_2$ of the number of cells at 24 h. Datum points are the arithmetic means of triplicate samples ± SEM. (b) Effect of NO· generating media on *C. neoformans* replication. Similarly, 10^4 viable, log-phase cryptococci were incubated in MM containing increasing concentrations of sodium nitrite at pH 6.5 (open squares), pH 5.5 (open circles), and pH 4.0 (closed circles). At the end of a 24-h incubation, cells were counted and replications were calculated as described above. Data represent means of duplicate samples ± SEM.

(data not shown). However, at more acidic pH levels, the addition of low concentrations of sodium nitrite causes marked cytostasis. At pH 4.0, the presence of 500 μM sodium nitrite causes complete inhibition of fungal growth (Fig. 2a, closed circles). At this acidic pH, there is a dose-response relationship between the amount of nitrite added to the medium and the degree of cytostasis observed (Fig. 2b, closed circles).

The only two variables that affected the degree of cytostasis also determined the amount of NO· produced, namely, pH and nitrite concentration. The cytostasis caused by nitrite salts in an acidic medium did not appear to be affected by other factors within the cell culture. Other nitrite salts produced an identical cytostatic effect in acidic medium, as did sodium nitrite. At similar concentrations, other sodium salts (nitrate, sulfate, hypochlorite, hypophosphite) had little or no effect on cell growth in either acidic or neutral conditions. No effect on the degree of observed cytostasis was seen either when different media with different buffers were used or when the fungal cell concentration was increased or decreased by 100-fold.

Effector molecule of cytostatic solution exists in the gas phase. To further characterize the mechanism by which acidic sodium nitrite solutions inhibit C3D growth, the cell cultures were physically separated from the nitrite-containing solutions by a Teflon membrane. This type of membrane allows the passage of dissolved gases of low molecular weights but is impermeable to ions. Various degrees of cytostasis were achieved by placing acidic solutions containing different concentrations of sodium nitrite on the opposite side of a Teflon membrane from a culture of C3D cells growing in MM, pH 7.0 (Fig. 3a). A graded effect of cytostasis was seen as the nitrite concentration of the "generating solution" was varied from 1 to 100 mM sodium nitrite (Fig. 3b, closed circles). This growth-inhibitory effect was associated with the appearance of nitrite and nitrate in the cell suspension. Presumably, NO· was formed in the nitrite-containing, acidic solutions. This gas was able to cross the Teflon membrane to the chamber containing the C3D cells. In this suspension, the nitric oxide gas either reacted with the cells, thereby causing inhibition of their growth, or it was oxidized to nitrogen dioxide, resulting in

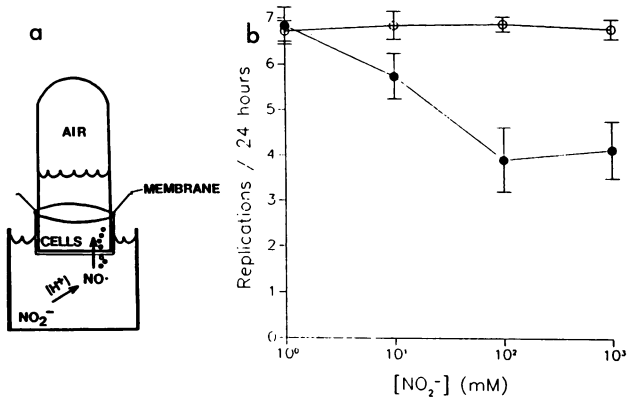

FIG. 3. (a) Diagrammatic representation of membrane apparatus. Each culture of C3D cells was placed in a glass test tube that was tightly sealed with a Teflon membrane (0.001 in. [ca. 0.003 cm] thick). Tubes were inverted and their membrane-covered ends were submerged in acidic and neutral solutions containing various concentrations of sodium nitrite. As shown here, gaseous NO· would be formed in acidic nitrite solutions, traverse the gas-permeable membrane, and interact with the cell culture, potentially leading to inhibition of replication. Since NO· would not be generated in neutral nitrite solutions, these conditions served as controls. (b) Effector molecule of cytostatic solution exists in the gas phase. Fungal replication in the cultures in the experimental membrane apparatus described above was assessed at 24 h with a Coulter counter. The number of replications of cells exposed across a membrane to acidic nitrite solutions (closed circles) was compared with the number of replications under control conditions of cells exposed to neutral nitrite solutions (open circles). Datum points represent means of five experimental samples ± SEM.

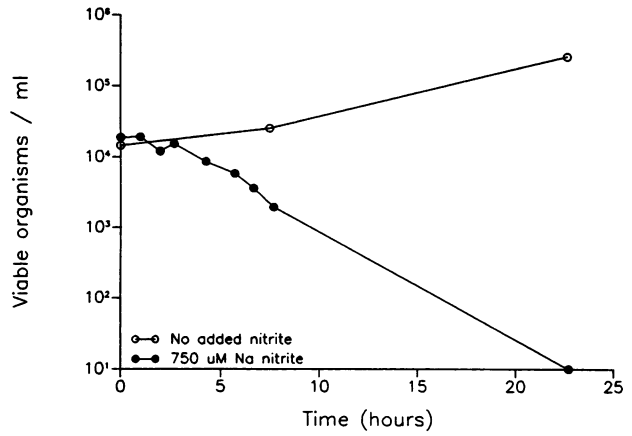

FIG. 4. Killing of *C. neoformans* by NO· generating media. To determine whether cell killing accompanies the cytostatic effect of acidic nitrite, C3D cells were cultured in media of pH 4.0 either without added nitrite (open circles) or with 750 μM sodium nitrite (closed circles). Cell viability over a 24-h period was quantified by serially removing samples from the cultures and plating them on Sabouraud agar. Results shown represent the means of triplicate plates.

the eventual formation of nitrite and nitrate (Fig. 1, reaction II).

This postulated mechanism was supported by control conditions. When a nitrite solution of neutral pH was placed in the chamber opposite the cell culture, no nitrite or nitrate was found in the cell suspension at the end of the experiment. Also, no cytostasis was seen when a neutral solution of up to 100 mM sodium nitrite was placed in the generating chamber (Fig. 3b, open circles). Thus, the ability to cause cytostasis in a physically separate cell culture required nitric oxide-generating conditions within the solution on the opposite side of the membrane from the growing cells.

Effect of NO· generating conditions on cell viability. Nitric oxide-generating media are able to inhibit the growth of C3D cells to various degrees, depending on the concentration of sodium nitrite added to the media and on the pH. To better assess the ultimate metabolic fate of these cells, viability testing was performed on C3D cell cultures growing in the presence and in the absence of NO· generating conditions. Cells grown in the absence of added nitrite replicated in accordance with the expected growth curve. Viability studies performed throughout the course of this experiment showed that nearly all of the cells in these cultures were viable (Fig. 4, open circles). However, those yeast cells that were exposed to nitric oxide-generating media did not replicate. Additionally, they retained their original viability for only 4 h, after which time a net decrease in cell viability was observed. Figure 4 shows this time-dependent killing reaction that occurred in the NO· generating media. At 24 h, complete sterilization of the cell cultures was accomplished within the NO· generating media.

Exposure of C3D cells to NO· generating conditions causes inhibition of glucose metabolism prior to cell killing. A culture of log-phase C3D cells was prepared in MM, pH 4.0, containing [^{14}C]glucose. This suspension was aliquoted into multiple reaction flasks which were subsequently sealed with rubber stoppers. A solution for trapping evolved CO_2 was also included in the apparatus. The flasks were then incubated with shaking at 37°C. After 30 min, one-half of the flasks were injected with 750 μM (final concentration) sodium nitrite. Samples were tested at time points after the injection, and the amount of trapped $^{14}CO_2$ was measured. Control cells continued to oxidize glucose at a relatively stable rate throughout the experiment. However, there was an immediate decrease in the amount of glucose oxidized among those cells exposed to NO· generating conditions (Fig. 5a). To ensure that this decrease in $^{14}CO_2$ production was not due to cell death in the NO· treated group, viability of the yeast cells was assessed at each time point. The total amount of trapped $^{14}CO_2$ in each of the cell populations was divided by the number of viable cells in that group. When corrected for the number of viable cells present in the cultures, there remains a marked reduction in glucose me-

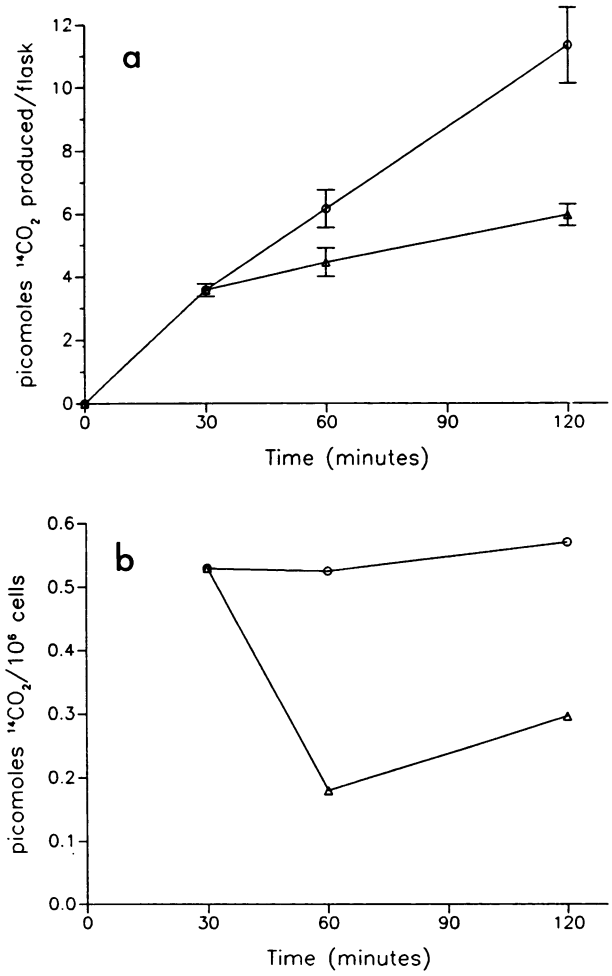

FIG. 5. Inhibition of cell glucose metabolism by NO· generating media. (a) Cells were grown in acidic medium containing [^{14}C]glucose. After 30 min of incubation, 750 μM (final concentration) sodium nitrite was injected through a side-arm rubber septum to one-half of the reaction flasks (open triangles), while the other half served as controls (open circles). The metabolic rate of these cells was assessed by measuring the elaboration of radiolabeled CO_2. Values represent means of triplicate flasks ± SEM. (b) To ensure that this effect was not secondary to cell death within the nitrite-containing cultures, the amount of $^{14}CO_2$ elaborated per 10^6 cells during each time period was calculated. The viability of the cells was determined by plating aliquots of the cell cultures onto Sabouraud agar plates at different time points during the experiment. The graph shows results from one representative experiment. Symbols are the same as in panel A.

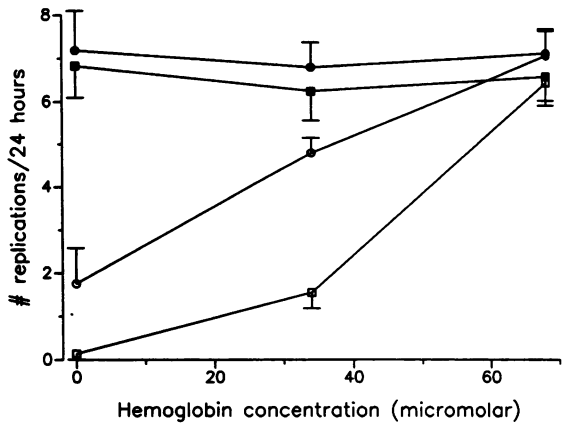

FIG. 6. The effect of hemoglobin on the proliferation of cryptococci in acidic media containing sodium nitrite. Log-phase cryptococcal cells were incubated with various concentrations of human oxyhemoglobin in acidic MM. To one-half of these cultures was added 500 μM sodium nitrite (open circles), while the rest was grown in nitrite-free media (closed circles). The growth of cells under these conditions was also assessed in the presence and absence of 1.0 mM ascorbic acid (open and closed squares).

tabolism by those cells exposed to NO· generating conditions (Fig. 5b).

Hemoglobin reverses the cytostatic effects of inhibitory media. Fe(II) oxyhemoglobin is known to act as a scavenger of NO· present in solution. It has also been shown to reverse the cytotoxic effects of activated macrophages on tumor cells in culture. Hemoglobin itself has no independent effect on cryptococcal growth. However, when added to the culture of C3D cells in NO· generating media (MM [pH 4.0] with 500 μM sodium nitrite), hemoglobin is able to reverse the cytostatic effects of this medium. The addition of 1.0 mM ascorbic acid [to maintain the hemoglobin iron in the Fe(II) form] augments the ability of hemoglobin to allow cell growth in otherwise inhibitory conditions (Fig. 6). Datum points represent the means of duplicate samples ± standard errors of the mean (SEM).

DISCUSSION

During the past few decades, much insight has been gained into the elaborate and intricate ways in which macrophages interact with foreign cells to protect the host from infection and neoplasm. Among the better described of the macrophage's effector functions are complement- and antibody-mediated phagocytosis and the oxygen-dependent respiratory burst. Recent experimentation has shown the ability in vitro of murine macrophages to inhibit the growth of neoplastic and tumor cells in the presence and in the absence of phagocytosis. This interaction does not require complement or immunoglobulin, nor does it appear to involve the respiratory burst. Useful experimental models designed to study this phenomenon have included the culture of macrophages with fungal cells. *C. neoformans* is unique among the pathogenic fungi in its elaboration of a thick polysaccharide capsule to provide protection against phagocytosis. Studying the interaction of activated murine macrophages with this fungus has therefore been useful in helping to define this potentially phagocytosis-independent arm of the macrophage's complex effector functioning.

An increasing body of evidence has implicated nitric oxide as the cytotoxic molecule used by the murine macrophage in this unique interaction with both microbial and tumor cells. However, in macrophages from nonrodent species, including humans, other mechanisms apparently independent of NO· lead to fungistasis (1). Early experiments with the murine system revealed that macrophage-mediated cytostasis required L-arginine and was associated with the oxidation of this amino acid to L-citrulline, nitrite, and nitrate (4, 10, 18). Since none of the products of this reaction could account for the inhibitory effects of the macrophage on the target cells, it was presumed that some intermediate in this process was the active agent. Intermediates thought to be likely candidates for this effector role included reactive nitrogen compounds. Marletta et al. (15) and Hibbs et al. (9) demonstrated by two separate methods that NO· was indeed elaborated by macrophages which had been activated by immunostimulatory agents. Subsequently, Stuehr and Nathan demonstrated the ability of reagent nitric oxide and chemically generated nitric oxide to inhibit the metabolism and growth of cultured tumor cells (20). Further support for this mechanism of cytotoxicity came from the isolation of nitrosylated iron complexes in activated macrophage cultures, indicating the reaction of nitric oxide with sources of cell iron (12).

When cultured together, murine cytotoxic activated macrophages effectively inhibit the growth of cryptococcal cells (4, 7). In this set of experiments, we have demonstrated the ability of a nitric oxide-generating system to inhibit the replication of *C. neoformans* in a manner attempting to simulate the effects of these macrophages. This cell-free system is useful in studying the cytostatic process for a number of reasons. The number of cryptococcal cells can be easily counted electronically, and the degree of replication can therefore be directly assessed. Additionally, the ability of this fungus to grow well in acidic conditions allowed us to create effective nitric oxide-generating conditions simply by the addition of nitrite salts to an acidic medium.

The compound responsible for this cytostatic effect appears to be nitric oxide or a related reactive nitrogen species. Micromolar concentrations of nitrite added to an acidic medium exert striking cytostatic effects. The only two variables that appear to influence the degree of growth inhibition of the cryptococcal cells in this system are the pH and the concentration of nitrite in the medium, both of which correlate with the amount of nitric oxide generated in the solution. Additionally, the cytostatic molecule in these experiments is able to exist as a gas, since it exerts its inhibitory effect in a dose-response manner on replicating *C. neoformans* cells on the opposite side of a gas-permeable but ion-impermeable membrane. Nitric oxide and other oxides of nitrogen exist as gases until they react with surrounding molecules (11).

Additional evidence supporting nitric oxide as the cytostatic effector molecule of this system was seen with the use of a nitric oxide scavenger, hemoglobin. Since hemoglobin effectively scavenges nitric oxide by the nitrosylation of ferrous heme (16, 20), it would be expected to inhibit a process mediated by this molecule. Previous experiments have shown that the cytostatic effect of activated macrophages on cultured tumor cells can be inhibited by the presence of hemoglobin (21). In our experiments, the addition of hemoglobin to the acidic, nitrite-containing media reversed the cytostatic effect normally observed.

The effect of the nitric oxide-generating solution on the cryptococcus involved metabolic derangements prior to cell killing. By measuring the rate of glucose oxidation, we were able to document an immediate, significant decrease in

cellular metabolism on the part of the organism after exposure to NO· generating conditions. Although cell death may eventually accompany the cytostatic effects of the acidic, nitrite-containing environment, the decrease in glucose metabolism was independent of killing. Inhibition of glucose oxidation could reflect mitochondrial injury in fungal cells. This finding requires further study to localize the metabolic defect(s) in these static cells.

Previous experimentation has attempted to determine the fate of cryptococcal cells when exposed in culture to activated macrophages. Under certain experimental conditions, activated macrophages can completely inhibit cryptococcal cell growth in a manner independent of phagocytosis and fungal cell killing (4, 7). Other investigators have been able to induce activated macrophages to cause actual cryptococcal cell death (3, 13). With this NO· generating system, we were able to demonstrate both fungal cell growth inhibition and killing. By varying the concentration of nitrite, we were also able to separate these two phenomena. Exposure of C3D cells to NO· generating conditions for 24 h caused variable degrees of cell killing. The addition of 750 μM sodium nitrite to acidic media resulted in immediate and complete inhibition of cellular replication. However, cell killing was not appreciable until after 4 h of exposure to NO· generating conditions, and sterilization of the cell culture was not accomplished for 24 h. At lower concentrations of added nitrite, a variable degree of cell death occurred. Among C3D cells exposed to nitric oxide-generating conditions just adequate to completely inhibit their growth (pH 4.0, 400 to 500 μM sodium nitrite), approximately one-half of these cells were viable and could actually be recultured after 24 h of exposure to the inhibitory medium. Potentially, this condition of replication inhibition in the absence of cell killing may be similar to that achieved by some organisms that are harbored within the macrophage in a nonreplicative state for extended periods of time. These microorganisms may then undergo reactivation of replication with the onset of a deficiency in cellular immunity.

In conclusion, we have shown the ability of NO· generating conditions to inhibit the metabolism and replicative ability of cryptococcal yeast cells. A similar cytostatic effect is observed when these cells are cocultured with cytotoxic activated macrophages. Nitric oxide appears to be the common mechanism of cytostasis in these systems. The development of this simple, cell-free system simulating macrophage activity on fungal cells may prove to be useful in further studying this unique aspect of macrophage function.

ACKNOWLEDGMENTS

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