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Fate of *Histoplasma capsulatum* in Guinea Pig Polymorphonuclear Leukocytes

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Guinea pig polymorphonuclear leukocytes (PMN) were parasitized in vitro with yeast cells of *Histoplasma capsulatum*. Preparations stained after 3 h at 37 C with May Greenwald-Giemsa revealed that 87% of the yeasts were tinctorially altered. Such alterations corresponded to those displayed by fungus cells intentionally killed by heat or other means and thus the altered yeast were presumed to be dead. A combination of 10^{-5} M H_2O_2, 10^{-5} M KI, and horseradish peroxidase killed *H. capsulatum*. Death was assessed by the eosin-y dye exclusion test. All of the listed components were required for death of the fungus. A granule lysate preparation derived from guinea pig PMN leukocytes could replace the horseradish peroxidase in the fungicidal system. The granule lysates behaved in keeping with the attributes of a myeloperoxidase. Thus, PMN leukocytes and certain extracellular peroxidase systems kill the yeast cell phase of the dimorphic fungus *H. capsulatum*.

Polymorphonuclear neutrophiles (PMN) maintained in cell culture kill ingested microbes (10). The range of microbicidal activity of these phagocytes includes bacteria, fungi, viruses, and *Mycoplasma* (17). An antimicrobial system comprised of myeloperoxidase (MPO), hydrogen peroxide, and a halide has been described as one of the ways in which PMN leukocytes deal with ingested microorganisms (10, 17). Fungi which are killed by neutrophiles or are susceptible to peroxidases under certain extracellular conditions are: *Candida albicans*, *C. krusei*, *C. parapsilosis*, *C. stellatoidea*, *C. pseudotropicalis*, *Saccharomyces cerevisiae*, *Geotrichum candidum*, *Rhodotorula* sp., *Aspergillus niger*, *A. fumigatus*, and *Cryptococcus neoformans* (1, 2, 11, 13, 19).

The facultative intracellular parasite *Histoplasma capsulatum*, survives and multiplies within mononuclear phagocytes maintained in vitro (7-9). However, the data in the present investigation will establish that PMN leukocytes kill the yeast cell phase of this dimorphic fungus. The contrast in behavior of *H. capsulatum* within MN and PMN phagocytes will be discussed.

MATERIALS AND METHODS

Fungi. The fungi used in this study were *C. albicans* strain no. 304 and *H. capsulatum* strain no. 505 (Mycology Stock Culture Collection of this Department). *C. albicans* was grown on slants of glucose peptone agar, stored, and subcultured as previously reported (9).

Preparation of yeast cell suspensions. *C. albicans* was grown 24 h at 37 C on glucose-peptone agar slants. *H. capsulatum* was grown 48 h at 37 C on glucose-cysteine blood agar slants. Growth from the slants was harvested in 0.15 M NaCl solution and standardized as described in earlier reports (6, 7). In work with horseradish peroxidase and in some of the work with guinea pig granule lysates the salt solution of low ionic strength (STM) previously reported (4) was used to prepare the suspensions. The STM contained 0.01 M KCl; 0.0024 M $CaCl_2$; 0.0025 M $MgCl_2$, and was sometimes used as a buffer by adding 0.05 M tris (hydroxymethyl)-aminomethane (Tris) base and hydrochloric acid. The low ionic strength of this diluent precludes its use with mammalian cells. Indication of which diluent was used to prepare yeast suspensions in the various experiments will be made in the appropriate portion of the text.

Harvest of guinea pig PMN. Guinea pigs of both sexes, which weighed 300 to 600 g, were used. In early experiments leukocytes were induced by the intraperitoneal injection of 15 ml of 0.1% sterile glycogen (Nutritional Biochemical Co., Cleveland, Ohio) in 0.15 M NaCl solution. After 16 to 18 h, the animal was killed by chloroform anesthesia and the exudate was aseptically harvested by lavage of the peritoneal cavity three times with 20 ml of Hanks' balanced salt solution (BSS). Suspensions of harvested cells were centrifuged in the cold, washed once in BSS, and counted in a haemocytometer. The number of cells was adjusted to 10^7 PMN/ml (80-90% of the harvested

cells were PMN). Later in the course of the studies leukocytes were induced by the intraperitoneal injection of 15 ml of 12% sodium casinate (Difco, Detroit, Mich.) in distilled water because casinate was found to be more effective than glycogen in producing PMN-rich exudates. The cells were harvested and counted in the same manner.

Assessment of intracellular killing. The phagocytic and fungicidal activities of guinea pig PMN were determined by modifications of the methods of Lehrer and Cline (13) and of Lehrer (12).

Fresh guinea pig serum (GPS) was mandatory to adequate phagocytosis of both *C. albicans* and *H. capsulatum*. Guinea pigs were anesthetized by the intraperitoneal injection of 0.05 ml/100 g (body weight) of stock sodium pentobarbital (Diabutal, Diamond Laboratories, Des Moines, Iowa). Ten to 15 ml of blood were obtained by cardiac puncture. The blood was set at room temperature for 1 h, rimmed, and centrifuged. Serum was withdrawn and used the same day.

Equal volumes (0.15 ml) of fresh GPS, leukocyte suspension (10^7 cells/ml), and BSS were placed aseptically into sterile plastic tubes (12 by 75 mm) (Falcon Plastics, Oxnard, Calif.). The tubes were incubated at 37 C for 10 min. A 0.25-ml volume of fungus cells at a concentration of 5×10^6 cells/ml was added and the tubes rotated (30 rpm) on a multi-purpose rotator (Model 105V, Scientific Industries, Inc., Springfield, Mass.) for 1 h at 37 C. After 60 min, 0.15 ml of 2.5% sodium deoxycholate (DOC) at pH 8.7 and 0.25 ml of deoxyribonuclease (DNase) (Schwarz/Mann, Orangeburg, N. Y.) were added to each tube. The DNase solution contained 1 mg of the enzyme per ml and 10^{-2} M MgCl. The released yeasts were centrifuged and their viability was determined by dye exclusion with 0.01% methylene blue (13) or 1% eosin-y in distilled water (22).

The DOC-DNase system worked well with *C. albicans* (see Results) but was toxic to *H. capsulatum*. Therefore, other concentrations of DOC (0.5-2.5%), and other detergents, i.e., sodium lauryl sulfate (SLS), Tween 40 (T40), and Triton X100 (X100), were studied singly (0.5-2.5%) and in various combinations, e.g., SDS/T40, SDS/X100, and T30/X100. None of these modifications provided a satisfactory way of releasing yeast cells whose eosin-y-excluding ability was not influenced by the detergents themselves. Those reagents or combination of reagents which efficiently lysed PMN also affected the dye uptake by *H. capsulatum*. Earlier unpublished experiments revealed that the plant glycoside saponin behaved in the same way. Distilled water lysis did not affect dye exclusion by *H. capsulatum* but also was not efficiently lytic to guinea pig PMN cells. Accordingly, the staining procedure described next was developed.

Tinctorial evidence for intracellular killing of H. capsulatum by guinea pig PMN cells. The viability of *H. capsulatum* in situ within guinea pig PMN was determined by their tinctorial appearance after staining samples by the May Greenwald-Giemsa (MG-G) method (9). The procedure for parasitization and incubation of leukocytes was the same as that reported in the preceding section, but no effort was made to recover yeasts from the PMN by lysis. Instead, after 15 min, 1, 2, and 3 h of incubation at 37 C on the rotator, 0.05-ml samples were removed and placed onto the plastic coated coverslips of a tissue culture chamber described in previous work (6, 7, 9). The samples within the chambers (16- by 125-mm screw-cap tubes) sat at room temperature for 30 min. The cover slips were washed once in BSS, fixed in methanol, and stained by the MG-G method (6). Cover slips were mounted in permount on glass slides. Controls consisted of similar preparation in which heat-killed (60 C for 30 min) yeasts were used in place of viable yeasts. Slides were scanned for tinctorially altered yeast cells (see reference numbers 6 and 12 for a full description of the appearance of such cells) and the percentage of altered cells among 300 intracellular yeasts determined.

Antifungal effects of HPO. Peroxidase (horseradish), RZ 3.11, was obtained from Schwarz/Mann. The lyophilized powder was dissolved in distilled water, diluted to contain 100 U/ml, and stored in a freezer.

Yeast cells were grown, washed, and standardized as described previously except that the final suspending fluid was the diluent being used in a particular experiment. Initially, 0.1 M citrate phosphate buffer (pH 5.0) was used in the studies on *C. albicans* in keeping with the procedure employed by Lehrer (11). This buffer was too toxic for *H. capsulatum* (see Results) and the toxicity was not due to the acidic pH. In preliminary observations on *C. albicans* it was noted that a pH of less than 6.5 was necessary for the optimum effect of horseradish peroxidase (HPO). Therefore, an alternative buffering system was sought. Since STM is known to maintain viability of *Histoplasma* yeasts very well, it was hoped that it could be used by incorporating 0.05 M Tris, but the buffer inhibited, for reasons unexplored, the activity of HPO. Thus in those experiments in which *H. capsulatum* was to be studied either unbuffered STM (pH 6.2) or 10^{-2} M phosphate buffer (pH 5.5) was employed. Viability was always better in STM than it was in phosphate buffer, but in some experiments it was desirable to remove chloride from the system and for such experiments the phosphate buffer worked reasonably well (see Results). The assay components, in a final volume of 0.5 ml, were placed in sterile, disposable plastic tubes (12 by 75 mm) and rotated for 3 h at 37 C on a rotator (30 rpm). Unless otherwise noted, components were added in the following concentrations: fungi, 5×10^6 cells/ml; hydrogen peroxide 10^{-5} M; HPO, 50 U/ml; KI, 10^{-4} M (with *C. albicans*) or 10^{-5} M (with *H. capsulatum*). A volume of appropriate diluent (citrate phosphate buffer, STM, or phosphate buffer in accordance with the particular system and fungus studied; see Results) was added to the reaction mixture to bring the total volume up to 2.5 ml. After 3 h of incubation, duplicate samples were withdrawn, mixed with eosin-y, and the percentage of eosin-y positive (dead) cells was recorded.

Preparation of granule lysates from guinea pig PMN. The procedure was adopted with some modifications from one published by McRipley and Sbarra (15). Leukocytes were induced, harvested, and washed as described in preceding paragraphs. Cells were counted and BSS suspensions were adjusted to contain 4×10^8 to 5×10^8 cells. The suspensions were centrifuged and the packed cells were suspended in 6

ml of 0.25 M sucrose solution containing 20 U of heparin per ml (Lipo-hepin, Riker Laboratories, Northridge, Calif.). The cells were mixed and broken by vigorous pipetting for 60 to 90 s, and by homogenization for 5 min in a motor-driven Teflon homogenizer (A. H. Thomas Co., Philadelphia, Pa.). The volume of the mixture was increased to 15 ml with 0.25 M sucrose. The disrupted cell suspension was centrifuged at 1,280 × *g* for 15 min, and the opalescent supernatant fluid was collected. The pellet was resuspended in 15 ml of 0.25 M cold sucrose and centrifuged again. The procedure was repeated three times or until the supernatant fluids were clear. The opalescent supernatant fluids were collected and centrifuged at 12,800 × *g* for 15 min. The supernatant fluid was discarded and the pellet which contained the granules was frozen and thawed six times in a dry ice-alcohol bath to release the activity sought (in our experiments the activity was most likely MPO; see Results). The lysate was kept in the freezer until used. The frozen material was thawed and suspended in 5 ml of the diluent appropriate to the system being studied.

Antifungal effects of lysed guinea pig granules. The activity of the lysates prepared as described in the preceding paragraph was estimated in an assay system very much like that employed with HPO. The assay components, in final volume of 0.5 ml, consisted of H_2O_2, yeast cells, and granule lysates. The halide requirement was met by the diluent or added as KI or KCl. The diluent varied in different experiments and will be recorded with the results. Samples were withdrawn, mixed with eosin-y, and observed as explained above.

RESULTS

Preliminary observation. Yeast cells of *C. albicans* were actively phagocytized by guinea pig PMN leukocytes in the presence of fresh guinea pig serum. Fresh serum was essential for prompt phagocytosis and serum stored in the freezer was unsatisfactory. One hour after phagocytosis, 60% of the ingested yeasts were dead as evidenced by methylene blue or eosin-y uptake by cells released after DOC lysis of the leukocytes. Appropriate controls established that the killing was an intracellular event.

Having achieved the expected results (13) with *C. albicans*, I turned next to study the fate of *H. capsulatum* within guinea pig PMN. Again fresh serum was mandatory for adequate phagocytosis. Difficulty was encountered in the detergent release phase of the experimental procedure. The DOC was obviously quite toxic to *H. capsulatum*. Extensive experiments were initiated to discover some modification of the procedure which would allow release of yeasts from PMN without influencing their ability to exclude eosin-y. None of the surface active substances tested nor any combination of the substances was discovered which achieved this goal. Distilled water lysis did not adversely affect viability of the fungus but the efficiency of release was so poor that quantitative assessments were frequently unreliable and always difficult. Nevertheless, some of the results obtained with the distilled water lysis modification suggested that *H. capsulatum* was killed by its encounter with guinea pig PMN and accordingly, a more reliable and readable method for establishing this fact was sought.

Tinctorial evidence for intracellular killing of H. capsulatum by guinea pig PMN. *Candida* cells killed by phagocytic leukocytes display alterations in Giemsa staining characteristics (12). Dead cells of *H. capsulatum* also are tinctorially different from living cells when stained by Giemsa or indeed any of the Romanovsky stains used with blood films (6). These facts led to the adoption of a technique for assessing viability of *Histoplasma* yeast cells in situ within guinea pig PMN by ascertaining changes in their staining characteristics after various periods of intracellular residence. The appearance of altered yeasts has been described in some detail (6). Briefly, yeasts lose nuclear differentiation and are coloured red or pink when observed shortly after death. The dead yeasts gradually lose all ability to retain the Romanovsky stains and become colourless ghosts (see also Lehrer's description of these events in *C. albicans*, reference 12).

The results from such an assessment of altered staining characteristics are shown in Table 1. *C. albicans* was included for the purpose of comparison. Clearly, the percentage of blastospores of each fungus which was tinctorially altered by intracellular residence within guinea pig PMN increased over the 3-h incubation period. The changes in staining characteristics are identical in appearance to those seen in yeasts killed by a variety of other means such

TABLE 1. *Tinctorially altered yeast cells within guinea pig PMN leukocytes*

Yeast	Yeast cell with aberrant staining (%) properties after: (min)[a]			
	15	60	120	180
C. albicans	8	4	19	56
H. capsulatum	8	21	52	87

[a] Cells stained by the MG-G procedure. Percentages based on three separate counts of the contents of 100 PMN. For description of the appearance of aberrantly stained cells see references 6 and 12 and the text. The yeasts were harvested and washed in saline before final dilution in BSS containing fresh guinea pig serum.

as heat or chemicals (6). Accordingly, these data were interpreted, in keeping with the results of others (12), to mean that the yeasts were killed by the leukocytes. An interesting unintentional control in these experiments was the intracellular behavior of *H. capsulatum* within MN phagocytes. It is known that *Histoplasma* yeast cells survive and multiply within such cells and that they do not display aberrations in Giemsa staining characteristics therein (7). Although the phagocytes harvested in the manner indicated (see Materials and Methods) contained mostly PMN, a few MN were also present. No alteration of the staining characteristics of *H. capsulatum* was seen within MN which were in the same microscope fields as PMN containing altered yeasts.

Effect of horseradish peroxidase on H. capsulatum. The data presented in the preceding section of this paper suggested that the antifungal effects of PMN clearly demonstrated with *Candida* (11) and with *C. neoformans* (2, 19) were also operative against yeast cells of *H. capsulatum*. Several functional bases for the antimicrobial effects of PMN leukocytes have been suggested, e.g., acids, H_2O_2, lysosomal enzymes, cationic proteins, MPO, and lactoferrin (10). Among these putative microbicidal agents, a system involving the mutual activities of H_2O_2, halide, and MPO is the one most thoroughly explored with the fungi (2, 11, 12, 14). Thus, experiments were undertaken to establish the sensitivity of *H. capsulatum* to peroxidase systems. Studies were conducted employing horseradish peroxidase functioning in a medium containing H_2O_2 and KI in order to establish certain variables before embarking on efforts to isolate granules supplying MPO activity from guinea pig leukocytes.

The data in Table 2 establish that H_2O_2-killed *Histoplasma* blastospores at a concentration of 10^{-2} M but not at lower concentrations. KI, acting alone, was not toxic at any concentration examined. The data in Table 3 show

TABLE 2. *Fungicidal effect of H_2O_2 and KI on H. capsulatum*

Reactant	Eosin-y-positive (dead) yeast cells at each molar concentration of reactant (%)[a]					
	0	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
H_2O_2	10	100	23	14	5	6
KI	10	9	10	10	10	10

[a] 10^{-2} M Phosphate buffer (pH 5.5) was used as diluent in the experiments and to prepare cell suspensions used in them. Five-15% eosin-y-positive yeast cells after 3 h at 37 C in this diluent.

TABLE 3. *Augmentation by KI of the fungicidal effect of H_2O_2 on H. capsulatum*

KI (M)	Eosin-y-positive (dead) yeast cells at indicated molar concentrations of reactants (%)[a]			
	H_2O_2			
	10^{-3}	10^{-4}	10^{-5}	10^{-6}
10^{-2}	100	100	100	100
10^{-3}	100	100	100	100
10^{-4}	100	73	15	16
10^{-5}	80	12	11	3
10^{-6}	17	12	8	9

[a] 10^{-2} M Phosphate buffer (pH 5.5) was used as diluent in the experiments and to prepare cell suspensions. Five-15% eosin-y-positive yeast cells after 3 h at 37 C in this diluent.

that the fungicidal effect of H_2O_2 was markedly augmented by KI when the two reactants were mixed. A concentration of 10^{-5} M of each reactant was not toxic and accordingly was chosen for studies on the combined effect of H_2O_2, iodide, and HPO. The results recorded in Tables 2 and 3 were obtained from experiments in which 10^{-2} M phosphate buffer was employed as the diluent. In essentially identical experiments in which STM was used as the diluent in place of the phosphate buffer the same results were obtained. Thus, chloride ion did not augment the fungicidal effect of H_2O_2 beyond that seen with KI (see also Table 4).

The antifungal effects of peroxidase systems have been explored most thoroughly with *C. albicans* (11). Therefore, this fungus was chosen for a pilot study of HPO and was used as a positive control throughout the remainder of the experiments. The effect of HPO, H_2O_2, and iodide on *C. albicans* is shown by the data in Table 4. HPO in the presence of H_2O_2 and iodide kills *C. albicans*. The levels of H_2O_2 and KI were chosen on the basis of experiments similar to those reported in Table 3. The data are not shown here for the sake of brevity. All three of the ingredients were required for the fungicidal effect under the conditions employed.

A few difficulties were encountered in studies with *H. capsulatum* which had to be resolved. The citrate-phosphate buffer employed by Lehrer (11) and used in the experiments with *C. albicans* reported in Table 4 was toxic to *Histoplasma* blastospores. Phosphate buffered saline did not support as good *Histoplasma* survival as did STM over the 3-h incubation period. The STM diluent described in the Materials and Methods section was therefore

TABLE 4. *Effect of HPO on C. albicans and H. capsulatum*

Mixture[a]	Eosin-y-positive yeast cells (%)	
	C. albicans	*H. capsulatum*
Diluent	0	8[b]
HPO	1	9
H_2O_2	1	10
KI	0	7
HPO + H_2O_2	3	10
H_2O_2 + KI	2	18
HPO + H_2O_2 + KI	100	100

[a] Reaction mixture contained 5×10^6 yeast cells, in citrate phosphate (pH 5.5) buffer with *C. albicans* or STM (pH 6.2) with *H. capsulatum*, to a final volume of 2 ml and the following supplements as indicated in the table: HPO, 50 U (Schwartz/Mann); H_2O_2, 10^{-5} M; KI, 10^{-4} M (*C. albicans*), or 10^{-5} M (*H. capsulatum*). Incubation at 37 C for 3 h on a rotator.

[b] Freshly prepared yeast cells of *H. capsulatum* always contained some (5–15%) eosin-y-positive (dead) cells (6, 7). Cells were prepared in STM and diluted in the indicated diluent.

substituted for the citrate-phosphate buffer. The results recorded in Table 4 establish that a combination HPO, iodide, and H_2O_2 kills *H. capsulatum*. All of the ingredients are required under the conditions outlined. The chloride ion of the STM at the concentration present (0.015 M) did not substitute for iodide as the halide (see Table 4, row 5). Lehrer also reported that chloride at 0.05 M (a level approximately 4 times greater than that in STM) would not substitute for iodide as the halide in the candidacidal activity of HPO (11).

Use of PMN granule preparations in an antihistoplasma peroxidase system. The fact that the yeast cell phase of *H. capsulatum* was sensitive to HPO led to efforts to substitute granule lysates prepared from PMN cells as a source of peroxidase in the H_2O_2-halide system. Once again, *C. albicans* was adopted for preliminary experiments and the efficacy of granule preparations was determined by their ability to kill this fungus in the presence of H_2O_2 and KI. No effort was made to purify such preparations for MPO activity and the amount of lysate used in the various experiments was recorded as the number of cells used in its preparation (4×10^8 to 5×10^8 PMN in most experiments). The number of PMN to be used in preparing lysates was established by preliminary experiments (data not shown) in some of which the preparations were toxic to *Candida* without the presence of the H_2O_2-KI supplements. Numbers of PMN were adjusted until granule lysates were obtained which were not by themselves highly toxic for *C. albicans* or for *H. capsulatum* (Table 5). STM was used as the diluent of choice with *H. capsulatum*. The chloride of the STM met the halide requirements of H_2O_2 to kill *C. albicans* and *H. capsulatum*.

By changing the diluent to 10^{-2} M phosphate buffer (pH 5.5) it was possible to study the relative efficiency of chloride and iodide ions in the system even though the survival of *H. capsulatum* over the 3-h incubation period was not quite as good in phosphate as it was in the STM diluent over several experiments (the data in Tables 2, 3, 5, 6 were from experiments in which survival of blastospores in diluent alone was good). KI was more efficient as the halide in the antifungal activity of H_2O_2 and guinea pig granule lysates. A concentration of 10^{-5} M KI was sufficient for killing both *H. capsulatum* and *C. albicans* in a peroxidase system comprised of granule lysate, H_2O_2, and halide but 10^{-2} M KCl was required to meet the halide requirement under the same experimental conditions (Table 6). This concentration of chloride ion is somewhat less than that present in the STM diluent (0.015 M) but the disparity is not large.

The granule lysate preparations were used in relatively crude form. No effort was made to purify the enzyme activity or to characterize it precisely as MPO. Nevertheless, the granule activity observed was very like that of the purified MPO used by Lehrer (11) and that of the carefully characterized material of McRipley and Sbarra (15) in that it worked in an H_2O_2 system, was heat sensitive and was inhibited by 10^{-3} M NaN_3 (Table 6).

TABLE 5. *Antifungal effect of granule lysates from guinea pig PMN on C. albicans and H. capsulatum*

Mixture[a]	Eosin-y-positive yeast cells (%)	
	C. albicans	*H. capsulatum*
Diluent	0	15
Granule lysate	12	15
H_2O_2	0	16
Granule lysate + H_2O_2	100	100

[a] Reaction mixture contained 5×10^6 yeast cells in STM (pH 6.2) to a final volume of 2 ml and the following supplements as indicated in the table: granule lysate prepared from 4×10^8 to 5×10^8 PMN in a final volume of 5 ml, 0.5 ml in each experimental situation; H_2O_2 10^{-5} M. Incubation at 37 C for 3 h on a rotator. Suspensions of blastospores prepared in STM.

TABLE 6. *Effect of heat and NaN_3 on the antifungal activity of guinea pig granule lysate*

Mixture[a]	Eosin-y-positive yeast cells of *H. capsulatum* (%)
Diluent	14
Granule lysate	9
H_2O_2 + KCl	16
Heated granule lysate	11
Granule lysate + H_2O_2 + KCl	75
Heated granule lysate + H_2O_2 + KCl	11
Granule lysate + H_2O_2 + KCl + NaN_3	23

[a] Reaction mixture contained 5×10^6 yeast cells in 10^{-2} phosphate buffer (pH 5.5) to a final volume of 2.5 ml and the following supplements as indicated in the table: granule lysate prepared from 2.5×10^8 PMN/ml in a final volume of 5 ml, 0.5 ml in each experimental situation; heated granules, the same as just outlined but heated in a boiling water bath for 30 min before use; H_2O_2, 2.5×10^{-6} M; KCl, 10^{-2} M; NaN_3, 10^{-3} M. Incubation at 37 C for 3 h on a rotator. Suspensions of blastospores prepared in 10^{-2} M phosphate buffer.

DISCUSSION

It has long been recognized that the yeast cell phase of *H. capsulatum* is fragile. Blastospores in saline (0.15 M NaCl solution) suspensions rapidly die (22) and efforts have been made to improve the keeping qualities of this widely used diluent by adding other substances to it (16). However, it is likely that the sodium ion is toxic (3), and thus solutions in which this ion does not occur are preferably used in studies where the quantitative viability of the yeast cells is crucial (4, 22). The starvation medium (STM) of Williamson and Scopes (21) has proven of great value as a diluent for blastospores of *H. capsulatum* (4, 22).

A not unexpected extension of this sort of ion toxicity was that noted with various phosphate buffers because phosphate is known to inhibit growth of the fungus (18). Nevertheless, the STM diluent was easily substituted into most of the experiments and phosphate could be employed at low molarity, when necessary, with a somewhat reduced survival of yeast cells in the diluent controls. This fact was obvious on a day to day experimental basis even though not as clear from the data shown in Tables 1, 2, 5, and 6 which were constructed from experiments in which blastospores survival in diluent controls was the best observed.

A corollary of the diluent problem was the problem of the sensitivity of *H. capsulatum* to detergents. It was a great disappointment not to be able to adopt the detergent release method that Lehrer used with *C. albicans* (11) and that Diamond et al. employed with *C. neoformans* (2). Nevertheless it was clear, after a very long series of experiments, that no single detergent or combination of them could be discovered which, at an efficient concentration, would release *H. capsulatum* from the PMN without also affecting the dye exclusion ability or the yeast cells. Distilled water which is not without long-term effect on the viability of *Histoplasma* blastospores (22) was a relatively inefficient lytic agent and attempts to do plate counts were thus not as reliable as tinctorial changes observed in situ in assessing viability of the yeasts (Howard, D. H. and Gupta, R. K., 1972. Bacteriol. Proc., p. 133).

Even though the lethal effect of PMN phagocytes has been displayed for a broad range of fungi (2, 11, 13, 14, 19) the outcome of our observations on *H. capsulatum* was not all assured because some fungal spores, i.e., *A. fumigatus* conidiospores, are not killed by their encounter with human neutrophiles (14). The MPO-H_2O_2 system is antimicrobial with either chloride or iodide functioning as the halide (15, 17). However, chloride ion cannot substitute for iodine in systems mediated by other peroxidases such as lactoperoxidase or salivary peroxidase (10) and it does not serve the halide requirement of HPO (11). Chloride ion is also a relatively ineffective substitute for iodide in an extracellular MPO-H_2O_2 system which kills *A. fumigatus* spores and this fact was suggested as an operational basis for the inability of neutrophiles to kill those spores (14). Indeed, chloride ion is much less efficient, i.e., required in much higher concentrations, than iodide ion in the MPO-H_2O_2 system operative against *C. albicans* and *H. capsulatum*. However, the chloride ion level in vivo are more than adequate to support intracellular MPO activity (10). Recognition of this fact has led to speculation that the sometimes successful by unexplained action of iodide therapy in mycoses was predicated on the efficiency of that halide in the MPO-H_2O_2 system within leukocytes (11).

The antifungal effect of H_2O_2 observed by Diamond et al. (2) on *C. neoformans* was manifest at lower concentrations of peroxide than that seen with blastospores of *H. capsulatum*. However, these investigators performed their assays in phosphate buffered saline (0.15 M chloride ion) and the synergistic effect of H_2O_2 and chloride was not measured. In the present study KI increased the antifungal effect of H_2O_2 orders of magnitude (Tables 2

and 3), and chloride at a level of 0.015 M did not substitute for this potentiating effect.

The results obtained in this study establish that the fate of *H. capsulatum* in PMN leukocytes is different from that within MN cells. Guinea pig PMN phagocytes readily engulf yeast cells of the fungus and promptly kill them. In contrast, blastospores of *H. capsulatum* not only survive within normal MN phagocytes but thrive therein, growing at a rate comparable to that on lifeless media (7) and manufacturing macromolecules unimpeded (8). *H. capsulatum* is sensitive to a combination of H_2O_2, halide, and a peroxidase-like activity derived from PMN granule lysates. But mouse MN cells maintained in cell culture are devoid of peroxidase activity (unpublished observation and reference 20). Thus, one of the reasons that *H. capsulatum* can survive with a MN phagocyte in cell culture is that such cells lack MPO. However, it is simplistic to suggest that this deficiency is the sole basis for the growth of *Histoplasma* blastospores within mouse MN cells. It is clear that the MPO-H_2O_2-halide system is only one of several antimicrobial systems found in mammalian PMN phagocytes (10, 17). The type of microbicidal activity studied depends on the conditions chosen and the methods employed to prepare cell lysates. The fact that preparation of guinea pig granules used in concentrations higher than that found optimum for the H_2O_2-halide system (Table 5) killed *H. capsulatum* in the absence of exogenously supplied peroxide or halide reveals toxicities other than those specifically referable to the MPO-H_2O_2-halide system. Indeed, such a result would have been presumed on the basis of (i) the observation of an anticryptococcal effect by NaN_3 treated leukocytes (2), (ii) the observation of antifungal effects by MPO deficient cells (1, 2, 12), (iii) the description of antifungal cationic proteins from PMN cells (23), and (iv) the early descriptions of the antimicrobial phagocytin extracted from PMN granules (5). Several of these reports related experiences from granule suspensions prepared from PMN cells in much the same manner as those used in this study. Thus just as MPO is clearly not the only possible antihistoplasma factor in PMN so also the absence of MPO from cultured MN cells is probably not the sole basis of the survival and growth by *H. capsulatum* within such cells.

MN phagocytes freshly harvested from immunized animals restrict the intracellular growth of *H. capsulatum* and the lymphocyte is the most likely mediator of this form of cellular immunity (9). However, lymphocyte activated macrophages do not become MPO positive (unpublished observation) and the fungus is seemingly inhibited but not killed by its encounter with such MN cells (Howard, D. H. and R. K. Gupta, 1972, Bacteriol. Proc., p. 133). Thus, the intracellular fate of *H. capsulatum* within MN phagocytes from an immunized animal is different from that within PMN phagocytes as reported in this investigation.

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