Further Studies on the Inhibition of *Histoplasma capsulatum* Within Macrophages from Immunized Animals

DEXTER H. HOWARD

Department of Microbiology and Immunology School of Medicine, University of California, Los Angeles, California 90024

Mononuclear phagocytes freshly harvested from immunized animals restrict the intracellular growth of *Histoplasma capsulatum*. The fate of the inhibited fungus is the subject of this report. Macrophages harvested from mice immunized by sublethal infection were parasitized in vitro with yeast cells of *H. capsulatum*. The subsequent fate of the fungus was assessed by staining characteristics, viability on subculture, and radioisotopic techniques. No tinctorial distinction could be made between yeasts within normal or immune phagocytes stained by the May Greenwald-Giemsa or by the Gridley techniques. Yeast cells recovered after 24 h of residence within the cytoplasm of immune monocytes excluded the dye eosin-Y, germinated on Casamino Acid agar incubated at 30 C, and initiated growth at a normal rate within phagocytes from normal animals. Thus even though the growth of *H. capsulatum* was inhibited within macrophages from immunized animals, the fungus was not killed by the encounter. However, autoradiographic studies established that protein synthesis by the fungus in situ was impaired.

Mononuclear phagocytes freshly harvested from the peritoneal cavity of mice immune to histoplasmosis restrict the intracellular growth of *Histoplasma capsulatum*, whereas the same cells maintained in culture for 48 h do not. Parasitized macrophages do not restrict the multiplication of the fungus for more than 24 h after harvest, and cultures maintained for longer than that period of time are soon overwhelmed by the resumed growth of the fungus (unpublished data). Thus *H. capsulatum* is seemingly inhibited but not killed by its encounter with an immune macrophage in vitro. The data presented in this report will document this assumption.

MATERIALS AND METHODS

Fungus. *H. capsulatum*, strain no. 505 (stock culture collection of this Department), which has been employed in experiments previously reported was used in these studies (10). The yeast cell phase was maintained and subcultured as noted in other reports (7, 10).

Macrophage cultures. Suspensions of mononuclear cells (MN) were obtained from the peritoneal cavities of unstimulated Webster-Swiss mice. Methods for the harvest and maintenance of such

cultures have been published (7, 10, 11). The tissue culture medium (TCM) was 40% normal human serum (NHS) (Flow Laboratories, Inc., Inglewood, Calif.) in Hanks balanced salt solution (BSS).

Preparation of inocula. Cultures of *H. capsulatum* were grown on glucose-cysteine-blood-agar slants incubated at 37 C for 48 h. Saline (0.15 M NaCl solution) suspensions of the growth from the cultures were prepared and standardized as described in an earlier report (7).

Parasitization of macrophages. Macrophage cultures were exposed to dispersed small inocula (2×10^5 yeasts/ml) of *H. capsulatum* in TCM and incubated at 37 C for 3 h. Approximately 60% of the yeast cells in the inoculum were phagocytized during this incubation period. Fifteen to 20% of the macrophages were parasitized by the fungus, and the average number of yeasts per infected cell was 2.5. The cultures were washed three times in 5 ml of BSS which removed approximately 85 to 90% of the extracellular yeasts remaining after phagocytosis. In this manner, the extracellular population was reduced below a level at which it could influence significantly the subsequent observation (7, 8). TCM (1.5 ml) was placed over the washed, parasitized macrophages, and the cultures incubated at 37 C for an additional 18 to 21 h. At the conclusion of the incubation period the intracellular fate of the fungus was assessed as described below.

Immunization of mice. In a previous report (11) it

was established that partially purified lymphocytes, from the peritoneal cavity of mice immunized by sublethal infection, mediated the suppression of intracellular growth of the fungus in normal mouse macrophages in vitro. However, it is much simpler to study the fate of inhibited yeast cells within mixed populations of cells (MN and lymphocytes) from immunized animals. Thus the immune macrophages studied were the exudate cells freshly harvested from the peritoneal cavities of mice immunized by sublethal infection. The techniques have been reported (11).

Tinctorial studies on the intracellular fate of H. capsulatum. After the indicated periods of incubation, cover slips were fixed in methanol (Giemsa) or 10% Formalin (Gridley) and stained by the May Greenwald-Giemsa (7) or Gridley (16) procedure.

Culture studies on the intracellular fate of H. capsulatum. Blastospores of this dimorphic fungus initiate a mycelial form of growth by germinating. They generally germinate when the temperature of incubation is reduced from 37 to 25 C, provided that certain nutrients are provided (22). A convenient medium which allows a high percentage of blastospore germination is 1% casein hydrolysate (acid hydrolyzed) (Nutritional Biochemicals Corp., Cleveland, Ohio), 2% glucose, and agar (CHA).

After appropriate incubation periods, parasitized macrophages were washed in BSS, scraped from the surfaces of the cover slips into distilled water, agitated for 2 to 3 min, and plated in dilution series on CHA. Duplicate plates of each dilution were incubated at 25 C, and the mycelial colonies which arose were counted after 7 days of incubation. The relatively inefficient distilled water lysis procedure was adopted because *H. capsulatum* was sensitive to all other methods of release attempted.

Autoradiographic study of protein synthesis by H. capsulatum. A technique for displaying protein synthesis by yeast cells of *H. capsulatum* contained within MN phagocytes has been reported (10). The methods used in the present study were those developed in this earlier report. Briefly, macrophages from normal and immunized mice were parasitized with *H. capsulatum*. After a 3-h period allowed for phagocytosis, and in another set of parasitized cultures after 21 h of incubation at 37 C, the medium over the cells was replaced with one containing 5 to 10 μCi of ^{3}H-leucine/ml. The cultures were pulse labeled for 15 to 30 min, washed three times in BSS to remove isotope not taken up, and then were fixed in methanol for 5 min. The cover slips were removed, air dried, and prepared for autoradiography. Controls consisted of macrophage cultures parasitized with heat-killed yeasts, pulsed, and prepared for autoradiography as with the viable yeasts. Puromycin, which inhibits protein synthesis by many types of cells, does not affect *H. capsulatum* at certain concentrations, probably because it does get into the fungus cells. Thus, in some studies 20 μg of puromycin per ml was incorporated into the pulse medium to impede protein synthesis by the mouse macrophages while permitting uninterrupted synthesis by the yeasts (10).

Cover slips prepared as above were affixed, cells

facing upward, to gelatin sub-bed (19) glass slides with a drop of Permount (Fisher Scientific Co., Fair Lawn, N.J.). Stripping film, Kodak AR-10 (Kodak Ltd., London, England), was applied to the cover slips in the usual fashion (19). The film-covered slides were exposed, developed, and eventually stained through the film in a manner previously described in detail (10). Grain distributions were assessed as described before (10).

L-Leucine-*4,5-^{3}H* (specific activity 50 Ci/mmol) was obtained from Schwarz/Mann, Orangeburg, N.Y.

RESULTS

The tinctorial response of phagocytized yeasts to the Giemsa and Gridley staining procedures. Observations made previously have established that viable cells of *H. capsulatum* within macrophages from normal animals give a typical metachromatic effect when stained with any of the common Romanovsky stains used with blood films (7). The yeasts have a magenta-colored nucleus and a blue to blue-grey cytoplasm, surrounded by a broad halo which represents the unstained cell wall. In contrast, death of the fungus, either induced by heat or chemicals or occurring naturally upon aging, causes a loss of tinctorial nuclear differentiation and eventual inability to retain Romanovsky stains at all (7). This change in stain ability has been documented by Lehrer (14) with *Candida albicans* and developed by him into a semiquantitative measure of intracellular fate of yeasts within leukocytes.

Throughout all of the observations in this work and those reported in a preceding publication (11), the tinctorial appearance of Giemsa-stained yeast cells phagocytized by and maintained for 21 h within macrophages from immunized animals was normal. The fungus cells stained as if they were viable at the time of fixation. The cell walls of Gridley-stained yeasts contained within immune macrophages for 21 h also appeared the same as yeasts within normal macrophages. Thus, the tinctorial behavior of *H. capsulatum* in response to a Giemsa or Gridley stain is not altered by a 21-h residence within macrophages from immunized animals. However, the reproduction of the fungus within such cells was severely restricted over this period of time.

Germination of yeasts recovered from immune macrophages. The ability of yeasts recovered from immune macrophages to germinate and thereby to initiate a mycelial form of growth was measured by semiquantitative plate counts. Macrophages were parasitized with yeast cells of *H. capsulatum*. At 2 and at 21 h, parasitized macrophages were washed, scraped

from the glass surface of the tissue culture chamber into distilled water, and vigorously agitated for 2 to 5 min. Unfortunately, the relatively inefficient water lysis was the only method of release discovered which did not harm the fungus (D. H. Howard, in press). Accordingly, colony counts probably most often represented clones from an entire macrophage rather than individual yeasts. Nevertheless, the lysates were sampled and plated on CHA at 25 C. Data from four such experiments are shown in Table 1. Visual counts on stained material established that growth of the fungus was restricted by 90% over controls in these cultures. The same or slightly increased numbers of yeast capable of germination were recovered from the immune macrophages. Because the mean number of yeasts per infected macrophages was approximately two, both at 2 and 21 h, it is unlikely that significant killing was overlooked because of failure of the techniques to assess viability of individual yeasts among the contents of a single macrophage. Thus, death of yeasts, if it occurred at all, would have to have transpired during the 2-h period set aside for phagocytosis to occur. However, even this is unlikely because 85 to 90% of the recovered yeasts (at 2 and at 21 h) were viable as assessed by their ability to exclude the dye eosin-Y (22).

Yeast cells recovered after 21 h from immune MN phagocytes by distilled water lysis were capable of initiating yeast phase growth within normal macrophages, and the growth rate within such normal phagocytes was not retarded (Table 2).

Autoradiographic observation on protein synthesis by phagocytized yeast cells. The data in Table 3 were derived from experiments in which the uptake of leucine by phagocytized yeast cells was studied. It is clear that the

TABLE 2. *Intracellular growth of Histoplasma capsulatum yeasts recovered from immune phagocytes*

Type of yeast cell	Mean no. of yeasts per infected MN		Generation time (h)
	0 time	18 h	
Grown in vitro[a]	2.4	8.8	10.2
Recovered from normal MN[b]	2.9	10.1	9.9
Recovered from immune MN[b]	2.4	9.8	9.4

[a] (See Materials and Methods) 48 h, 37 C, blood-glucose-cysteine-agar.

[b] Macrophages (MN) from normal or immunized mice parasitized with *H. capsulatum*. After 24 h at 37 C, the cultures were washed in BSS, the MN cells were scraped from the glass surface of the tissue culture chambers into distilled H_2O, and vigorously agitated for 2 to 5 min. Samples were withdrawn and used to parasitize macrophages from normal animals; 0 time = after the 3 h allowed for phagocytosis. Generation time was calculated after 18 h of incubation at 37 C.

amount of amino acid utilized by fungi housed within immune macrophages was substantially reduced. Multiplication was reduced by 90% in these MN cells. Even after 3 h within immune macrophages, the amount of amino acid incorporation was 30 to 40% below the amount incorporated by yeast cells within normal macrophages. By 21 h the amount of reduction was 50 to 65%. The initial experiments were conducted in the presence of puromycin in order to observe amino acid incorporation by *Histoplasma* in the relative absence of macrophage protein synthesis (10). A similar experiment was performed without puromycin to ensure that the inhibitory effect was not due to increased permeability of yeast cells to puromycin engendered by the encounter with an immune cell. The same results were obtained (Table 3).

These experiments lead to the conclusion that protein synthesis by *H. capsulatum* was impaired within the immune macrophage. Experiments are in progress to define this impairment more precisely.

DISCUSSION

The fate of microorganisms within phagocytic cells has been the subject of considerable experimentation. Polymorphonuclear neutrophiles express a potent microbicidal function operative against both gram-positive and negative bacteria, fungi, viruses, and *Mycoplasma* (13, 20). Eosinophiles are apparently less well

TABLE 1. *Recovery of yeast cells from immune macrophages 2 and 21 h after phagocytosis*

Time within macrophage (h)	Experiment no. (colonies/ml, $\times 10^2$)[a]			
	1	2	3	4
2[b]	36	87	246	440
21[c]	62	83	282	560

[a] Mycelial colonies on CHA after 7 days at 25 C. Variations in 2-h results were due to different initial inocula in the four experiments.

[b] Period of phagocytosis

[c] Intracellular growth restriction of *H. capsulatum* was determined by visual counts on stained material approximately 90% over normal controls in these experiments.

TABLE 3. *Apparent reduction of protein synthesis by yeast cells of Histoplasma capsulatum within immune macrophages*

Type of macro-phage	Time of yeasts within MN (h)	Mean no. of grains per phago-cytized yeast cell		
		With puromycin[a]		Without puromycin[b]
		1	2	
Normal	3	7.0	6.9	4.8
	21	7.8	6.2	5.2
Immune	3	4.0 (42)[b]	4.9 (29)	2.1 (56)
	21	3.6 (46)	2.1 (66)	1.8 (65)

[a] Cultures were pulsed with 10 μCi of L-leucine-4,5-^{3}H (specific activity 50 Ci/mmol) and 20 μg of puromycin per ml. Results from two experiments are shown.

[b] Cultures were pulsed with 5 μCi of L-leucine-4,5-^{3}H (specific activity 50 Ci/mmol). Mean number of grains were corrected for background MN cell protein synthesis by subtracting the number of grains over heat-killed yeast cells in control cultures from the number of grains over viable cells in experimental cultures (10). Figures in parentheses represent the percentage reduction of mean grain counts above yeasts within immune MN compared with that above yeasts within normal MN.

endowed (2). The interaction between pathogenic bacteria and MN phagocytes is variable, but the following facts appear to be established: (i) only certain species of bacteria are capable of survival and multiplication within macrophages, (ii) the species of animal from which the macrophages are obtained is fundamental to conclusions about intracellular survival and multiplication, (iii) some bacteria survive, but do not multiply within phagocytes, (iv) the virulence of an organism is correlated with its ability to survive and multiply intracellularly, and (v) macrophages from immunized animals restrict the intracellular multiplication of virulent microorganisms (17).

The fate of fungi within macrophages appears to correspond to the fate of bacteria with regard to each of the aforementioned points, even though the literature is not so abundant in support of the conclusions regarding fungi. Accordingly, in reference to the assertions numbered i to v in the preceding paragraph: (i) *H. capsulatum* and *Cryptococcus neoformans* survive and replicate within MN phagocytes (3, 7, 15), whereas the growth of *Torulopsis glabrata* is markedly restricted therein (9); (ii) *H. capsulatum* survives, but appears to multiply more slowly within frog macrophages than it does within mouse macrophages (8), and some differ-

ences in intracellular survival of *C. neoformans* within rat peritoneal exudate cells and human monocytes have been recorded (3, 15); (iii) *T. glabrata*, though impeded in growth, is not apparently killed (9); (iv) various strains of *C. neoformans* differ in their resistance to intracellular killing (15); and (v) macrophages from immunized animals restrict the growth of *H. capsulatum* (11) and *C. neoformans* (5).

Some of these points, particularly the last, deserve further consideration. First, *T. glabrata* has a limited ability to grow within macrophages (9). About 55% of an inoculum of this yeast is completely inhibited within mouse macrophages, whereas 30% grow to a limited extent and 15% grow quite well (unpublished data). The disparity seems to be a function of macrophages and not the inoculum. Conclusions from these observations are complicated by the fact that serum has an inhibitory effect on the fungus (9, 21). However, antifungal effects of serum do not appear to operate against intracellular growth of fungi, because the anticryptococcal of human serum (6, 21) does not preclude growth of *C. neoformans* in macrophages (3). Whatever the role of serum in the intracellular growth of *T. glabrata* (a problem currently under investigation in this laboratory), it is clear that the intracellular behavior of the fungus is remarkably similar to that recorded for the protozoan *Toxoplasma gondii* in which two populations of microorganisms were observed, one thriving intracellularly and the other destroyed in a few hours (12).

The data in the present study establish that although the intracellular growth of *H. capsulatum* is markedly inhibited within macrophages from immunized animals (the fact that this is actually a lymphocyte-mediated effect has been reported), the fungus is not killed. This fact is in keeping with the observations of others (23). Evidence continues to accumulate, therefore, that acquired immunity need not be accompanied by death of intracellular organisms (18). The recovery and continued well-being of a host with respect to a particular pathogen would depend on the continuous inhibition of the growth of the microorganism (18, 23). Nonproliferating, but viable, organisms are known to persist in vivo for long periods (18). Perhaps inhibited microbes would eventually succumb to accumulated nonspecific factors which obtain in the granulomatous lesions, i.e., acids, anaerobiasis, caseation, fibrosis, and calcification (4). It is easy to imagine the continuous suppression of microbial growth within hosts subjected to repeated lymphocyte stimulating

events (1), and especially in view of the presumed nonspecific nature of macrophage activation (17).

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