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INTRACELLULAR BEHAVIOR OF *HISTOPLASMA CAPSULATUM*

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ABSTRACT

HOWARD, DEXTER H. (University of California, Los Angeles). Intracellular behavior of *Histoplasma capsulatum*. J. Bacteriol. **87**:33-38. 1964. —The tinctorial characteristics and intracellular generation time of *Histoplasma capsulatum* within mouse histiocytes maintained in tissue culture were studied. The appearance of viable and nonviable cells of *H. capsulatum* was observed after staining with the May Grunwald-Giemsa, Methenamine Silver, and Periodic acid-Schiff (PAS) procedures. Death of the fungus, either induced by heat or occurring naturally in saline suspension, caused a loss of nuclear differentiation. This loss was not accompanied by changes in the PAS or Silver staining capacities of the cell walls of the fungus. There was no tinctorial evidence for intracellular death of *H. capsulatum* in normal mouse histiocytes. The intracellular generation time of the fungus was estimated to be 10.3 ± 1.5 hr, and was not affected by the age of the histiocytes. The generation time was approximately the same in histiocytes from Webster-Swiss and A/J mice.

Histoplasma capsulatum is a facultative intracellular parasite. The intracytoplasmic behavior of the fungus in cells maintained in vitro has been studied by a number of investigators, notably, Larsh and Shepard (1958), Howard (1959, 1960), Hill and Marcus (1960), and Miya and Marcus (1961). The tinctorial characteristics and the generation time of *H. capsulatum* within mouse histiocytes are the subjects of this report.

MATERIALS AND METHODS

Animals. Webster-Swiss albino mice, from a random-bred but long-closed colony, were employed as the source of peritoneal exudate cells. In a small series of comparative experiments, A/J mice were also used.

Fungus. A single strain of *H. capsulatum* (no. 505) maintained in the yeast cell phase on potato-egg medium (Kurung and Yegian, 1954) was used throughout the studies. Bimonthly transfers were made of stock cultures stored in a refrigerator.

Histiocyte cultures. Suspensions of mononuclear cells were obtained from glycogen-induced peritoneal exudates by methods previously described (Howard, 1959). A 0.5-ml volume of washed cells was pipetted into screw-cap tubes (16 by 125 mm) containing cover slips (5 by 43 mm) which had been coated and fixed to the side of the tubes with a few drops of Formvar (0.5% polyvinyl formol in ethylene dichloride). After the cells had settled onto the cover slips, the suspending fluid [Hanks' balanced salt solution (BSS)] was replaced with 1.5 ml of a medium consisting of 40% normal human serum in Hanks' BSS. The caps of the tubes were replaced with white rubber stoppers, and the cultures were incubated in a slanted position at 37 C. By 24 to 48 hr, the cultures consisted of monolayers of histiocytes free from cells of other morphological types (Howard, 1959). At this stage of development, they were ready for use.

Parasitization of histiocytes. Saline suspensions of yeast cells were prepared from the growth on potato-egg slants incubated at 37 C for 48 hr. The suspensions were adjusted photometrically to contain the desired number of cells in a manner previously described (Howard, 1959). The medium over the cultures was replaced with one containing an appropriate number of fungi. The procedure at this point varied, depending on whether the experiment was designed to study the tinctorial properties or the intracellular generation time of the engulfed yeasts.

In experiments on generation times, the cultures were incubated at 37 C for 3 hr. Cultures were then washed three times with Hanks' BSS to remove uningested organisms. A series of cultures were immersed in warm saline, fixed in methanol, and the cover slips were removed and stained with May Grunwald-Giemsa. A volume of 1.5 ml of a medium composed of 20% normal human serum in Hanks' BSS was added to the remaining cultures, and incubation was con-

tinued at 37 C for an additional 21 hr, after which the cover slips were fixed and stained as before.

Cultures devoted to studies on the tinctorial behavior of *H. capsulatum* were not washed to remove uningested fungi. Cover slips were immersed in saline, fixed, and stained after 3, 24, 48, 72, 96, and 120 hr of incubation at 37 C. The medium was the same.

Fixation and staining procedures. The staining characteristics of *H. capsulatum* within histiocytes were observed after application of the May Grunwald-Giemsa, Methenamine Silver, and Periodic acid-Schiff (PAS) stains. Cultures to be stained were immersed in warm saline for 20 min and fixed for 5 min in methanol (May Grunwald-Giemsa), in 95% alcohol (Methenamine Silver), or in 10% formalin (PAS). After fixation, the cover slips were removed and stained by a standard procedure: Giemsa (Howard, 1959); Silver and PAS (Mowry and Frenkel, 1960).

Enumeration of fungi within histiocytes. Intracellular populations were surveyed by observation of 100 infected cells (1,000 to 2,000 histiocytes in most experiments). Counts were made on transits through areas of the culture most densely populated with histiocytes. An oil-immersion objective and a magnification of 970 × were employed. The number of yeast cells per infected histiocyte was recorded.

Enumeration of fungi in extracellular fluid. Numbers of viable fungi in the supernatant culture medium were estimated by plating 0.1-ml samples of serial tenfold dilutions of the fluid in duplicate on the surface of glucose (1%)-peptone (2% Difco proteose peptone no. 3)-agar plates. Mycelial colonies were counted after 14 days of incubation at 28 C.

Heat-killed suspensions. Saline suspensions prepared from the growth on potato-egg slants incubated at 37 C for 48 hr were heated at 60 C for 1 hr in a water bath. Tests for viability of the heated cells were made by plating 0.5 ml of the suspension on glucose-peptone-agar incubated for 14 days at 28 C.

RESULTS

Tinctorial behavior of H. capsulatum. Tissue cultures of histiocytes were prepared from the peritoneal exudates of Swiss mice, and were exposed to suspensions of yeast cells of *H. capsulatum*. The intracellular morphology of the fungus in Giemsa-stained preparation from such cultures has been reported (Howard, 1959). Briefly, yeast cells measured 2 to 4 μ in greatest diameter, and were oval, round, or pyriform. The carmine-colored nucleus was most often eccentric and stained unevenly. The cytoplasm was dark blue and surrounded by a halo of unstained material. Cell walls were not stained by the Giemsa procedure. The granularity of the cytoplasm of the histiocyte was more pronounced in the areas bordering on the engulfed yeasts [For other details of the staining characteristics of *H. capsulatum*, see Mowry and Frenkel (1960).]

It was noted previously (Howard, 1959) that yeast cells, heat-killed before phagocytosis, were markedly altered in their staining reaction with the Giemsa procedure. This phenomenon was examined further. Yeast cells of *H. capsulatum* killed by heating at 60 C for 1 hr were introduced into 48-hr cell cultures. Cultures were also inoculated with viable cells for comparative purposes. At intervals thereafter, cover slips were removed and stained by the May Grunwald-Giemsa, Methenamine Silver and PAS procedures.

Heat-killed cells were phagocytized as readily as were viable cells by the histiocytes (Table 1). However, the tinctorial behavior of nonviable cells in the Giemsa preparations was quite different from that of viable cells. (The terms *viable* and *nonviable* refer to the condition of the fungus before fixation and staining.) The size and shape of the two forms was the same, but there was no differentiation of the nucleus of the dead cells. The yeasts were brick-red or pale pink with red granules. Colorless vacuoles, which were the same size and shape as that of yeasts, were occasionally noted in the cytoplasm of some of the histiocytes. The relative number of yeasts displaying each of these staining aberrations depended on the length of time after phagocytosis. Thus, 4 hr after engulfment most of the fungus cells were brick-red. By 24 to 48 hr, a large proportion of the yeasts was pale pink with and without red granules. Unstained vacuoles were more prominent in histiocytes that had been parasitized for 96 to 120 hr.

The PAS and Silver stains did not reveal any differences between heat-killed and untreated yeast cells. Thus, the loss of nuclear differentiation by heat-killed cells was not accompanied by changes in the PAS or Silver staining capacities of the cell walls of the fungus.

Rowley and Huber (1955) found that the via-

bility of yeast cells of *H. capsulatum* decreased rapidly in saline suspensions at 25 C. In the present study, it was determined that only 1 to 5% of the cells in saline suspensions incubated at 37 C were viable after 18 to 24 hr. Such essentially nonviable suspensions were introduced into histiocyte cultures, and the same altered staining behavior of the fungi was noted. Thus, death of the yeast cells of *H. capsulatum*, either induced by heat or occurring naturally in saline suspensions, causes a loss of nuclear differentiation of the cells.

The remarkable tinctorial aberrations displayed by nonviable cells of *H. capsulatum* provided a basis for study of the intracytoplasmic fate of the fungus. It was ascertained that approximately 10 to 15% of the cells comprising saline suspensions of the growth from 48-hr cultures are dead. Since viable and nonviable cells are phagocytized equally well (see Table 1), it was anticipated and subsequently demonstrated that the percentage of intracellular forms abnormally stained by Giemsa would be approximately the same, after a 3-hr phagocytosis period, as the percentage of dead cells in the original suspension. It was also reasonable to presume that, if the intracellular sojourn of *H. capsulatum* resulted in death of the fungus, this fact would be reflected in the altered staining of affected

TABLE 1. *Phagocytosis of viable and nonviable cells of Histoplasma capsulatum**

Inoculum	Time after addition	Percentage of histiocytes infected	Percentage of infected histiocytes with indicated number of yeast cells per histiocyte†		
			5-5	6-10	>10
	hr				
Viable cells (10^6/ml)	1	31	70	20	10
	2	54	55	27	18
	3	61	52	19	29
Nonviable cells (10^6/ml)	1	28	73	24	3
	2	52	58	23	19
	3	54	54	17	29

* Results from a typical experiment. Percentage of histiocytes infected estimated from observations on approximately 500 cells. Medium over histiocytes composed of 20% inactivated normal human serum in Hanks' BSS.

† Distributions estimated from observations of approximately 100 infected histiocytes.

TABLE 2. *Efficiency of phagocytosis of Histoplasma capsulatum by 48-hr histiocytes*

Inoculum (cells/ml)*	Tissue culture medium 3 hr after exposure to monolayers of histiocytes (cells/ml)*	Efficiency†
		%
6×10^3	1.33×10^3	78
2.75×10^3	7.75×10^2	72

* Determined by plate count.

† Efficiency of phagocytosis = $\frac{\text{no. of cells in inoculum} - \text{no. of cells in medium after 3 hr}}{\text{no. of cells in inoculum}} \times 100$

cells. However, over an observation period of 120 hr, there was no increase in the percentage of intracellular yeasts showing a loss of nuclear differentiation. Actually, there was a slight decrease in the percentage of abnormal forms. Thus, there was no tinctorial evidence for intracellular death of *H. capsulatum* within normal mouse histiocytes. On the contrary, the fungus gradually destroyed the phagocytes, and the rate of destruction was dependent upon the number of yeasts introduced (*see* Howard, 1959).

Intracellular multiplication of H. capsulatum. When monolayers of mouse histiocytes were exposed to dispersed small inocula of *H. capsulatum*, the efficiency of phagocytosis after 3 hr was approximately 75% (Table 2). Washing the cultures three times in BSS, after the 3 hr period of phagocytosis, removed 90 to 95% of the remaining extracellular forms. Enumeration of the intracellular populations showed that 5 to 10% of the histiocytes had engulfed yeast cells, and that of these infected phagocytes 90% or more contained 1, 2, or 3 cells of the fungus (Table 3). This distribution of intracellular forms is such that intracytoplasmic reproduction during the next 24 hr would not result in bursting of the histiocytes with release of yeasts (Howard, 1959).

Under these conditions, intracellular multiplication was ascertained by counting the number of yeasts per infected histiocyte at zero time (3 hr after addition of inoculum) and again at 24 hr (21 hr after washing of cultures to remove uningested forms). A comparison of the mean number of yeasts per histiocyte at zero time with that at 24 hr was used to compute the generation time.

Intracellular generation time of H. capsulatum.

Monolayers of histiocytes were exposed to approximately 2×10^4 yeast cells of *H. capsulatum* per ml of medium. After 3 hr of incubation at 37 C, cover slips were removed and stained. The distribution of yeast cells within infected histiocytes (5 to 10% of the total histiocyte population) based on four separate experiments is shown in Table 3. Approximately 90% of the infected cells contained 1 to 3 yeasts per phagocyte; the mean ranged from 1.57 to 1.69 (based on those phagocytes containing 1 to 3 yeasts). The percentage of infected histiocytes did not increase during the next 21 hr, but the distribution of yeast cells clearly demonstrated intracellular proliferation (Table 4); the mean ranged from 6.23 to 7.36 (based on those phagocytes containing 1 to 12 yeasts). The generation time was calculated by using the following formula: $G = T/n$, where n is the number of generations in time T. From the data presented in Tables 3 and 4, the intracellular generation time was determined to be 10.3 ± 1.5 hr. It was considered that the observed variations in a nonsynchronously dividing population were not extreme.

TABLE 3. *Distribution of Histoplasma capsulatum within histiocytes*

Expt no.	Distribution of yeasts among 100 infected histiocytes at 0 time*					Mean no. of yeasts per infected histiocyte†
	1	2	3	4	5 or >5	
1	49	35	9	4	5	1.57
2	48	33	10	5	4	1.58
3	40	34	13	6	7	1.69
4	42	38	18	5	6	1.62

* Results are expressed as percentage of histiocytes showing indicated number (1, 2, 3, etc.) of yeasts.

† Based on those histiocytes with one to three yeast cells (approximately 90% of the infected population).

TABLE 4. *Distribution of Histoplasma capsulatum within histiocytes after 24 hr of incubation*

Expt no.*	Distribution of yeasts among 100 infected histiocytes at 24 hr†						Mean no. of yeasts per infected histiocyte‡
	1–2	3–5	6–8	9–11	12	>12	
1	3	24	37	17	8	10	7.36
2	4	28	38	11	9	10	6.78
3	2	34	37	12	6	7	6.25
4	2	43	37	10	6	6	6.23

* Number of experiment corresponds to those in Table 3.

† Results are expressed as percentage of histiocytes showing indicated number (1–2, 3–5, etc.) of yeasts.

‡ Based on those histiocytes with 1 to 12 yeast cells (approximately 90% of the infected population).

TABLE 5. *Effect of age of histiocytes on intracellular generation time of Histoplasma capsulatum*

Time of infection of culture	Mean no. of yeasts per histiocyte*		Generation time
	0 time	24 hr	
hr			*hr*
1	1.65	5.76	12.0
24	1.78	6.65	11.3
48	1.65	6.24	11.1

* Data represent averages from several observations at each time increment.

TABLE 6. *Intracellular generation time of Histoplasma capsulatum in histiocytes from Webster-Swiss and from A/J line mice*

Strain of mice	Mean no. of yeasts per histiocyte*		Generation time
	0 time	24 hr	
			hr
Webster-Swiss	1.65	6.24	11.1
A/J	1.62	6.80	10.3

* Data represent averages from several observations.

The data presented in Table 5 summarize the observations on generation times in populations of histiocytes of different ages. Phagocytes were inoculated with approximately 2×10^4 yeasts per ml at 1, 24, and 48 hr after preparing the cell cultures. The intracellular generation times ranged from 11.1 to 12.0 hr, a range of variation which was expected from the previous experiments (Table 4). The age of the histiocyte did not influence markedly the intracellular generation time.

The results of experiments performed on histiocyte cultures from A/J line mice are shown in Table 6. The cultures were prepared and inoculated as in the preceding experiments. The observations were made on 48-hr cultures. No sig-

nificant difference in the generation time of *H. capsulatum* within phagocytes of Webster-Swiss or A/J line was noted.

DISCUSSION

The loss of nuclear differentiation by cells of *H. capsulatum* in hematoxylin- and eosin-stained paraffin sections from cases of histoplasmosis is well documented (*see* Mowry and Frenkel, 1960). That such a loss is associated with death of the cell was presumed by early investigators (quoted by Mowry and Frenkel, 1960), and is clearly substantiated by the results in tissue culture reported here. The reasons for the staining aberrations noted are not known. Indeed the basis of metachromasia of the nuclei of certain intracellular parasites is yet to be completely explained. [For a discussion of the various theories regarding the metachromasia of nuclei of intracellular protozoan parasites, see Baker (1958).] Nevertheless, it is clear that such tinctorial characteristics hold some promise in the study of the intracytoplasmic behavior of *H. capsulatum*, in that any effect which influences the viability of the cells is reflected in their Giemsa-staining properties. Thus, it has been shown that antifungal drugs (nystatin and amphotericin B) added to the culture medium over histiocytes infected with *H. capsulatum* will penetrate the phagocytes and inhibit the yeast cells therein, and that this event is accompanied by a loss of nuclear differentiation of the yeasts (Howard, 1960). [Nystatin and amphotericin B are generally considered to be fungistatic rather than fungicidal (Emmons, 1960). However, the staining behavior of drug-inhibited cells within histiocytes was the same as that of heat-killed cells (Howard, 1960).]

The loss of nuclear differentiation by heat-killed cells was not associated with changes in the PAS or Silver staining capacities of the cell walls of the fungus. The persistence of the cell wall of *H. capsulatum* in the tissues of a host has been repeatedly observed (Schwarz, 1960). Thus, in essential aspects, the tinctorial behavior of *H. capsulatum* within mouse histiocytes in vitro mimics its recorded tinctorial behavior in mammalian hosts.

The intracellular generation time of *H. capsulatum* was 10.3 ± 1.5 hr. Larsh and Shepard (1958) estimated the intracellular generation time in HeLa cells to be "about one day." On blood agar, the generation time of the fungus ranges from 6 to 8 hr (Rowley and Huber, 1955; Pine, 1955) and in liquid shake cultures, under optimal conditions, from 9 to 11 hr (Pine and Peacock, 1958). Therefore, *H. capsulatum* multiplies within mouse histiocytes nearly as well as it does in artificial culture media, and significantly better than it does in HeLa cells. There is one interesting difference in growth behavior in tissue culture: as few as 2 to 30 yeast cells will initiate intracellular yeast-phase growth in tissue cultures, whereas a considerably larger inoculum is generally required in other culture media or tissue culture media without histiocytes (Larsh and Shepard, 1958; Howard, 1959). Thus, the toxic factors, which preclude yeast-phase growth from small inocula in vitro and which various culture techniques tend to remove (Salvin, 1947; McVickar, 1951), are effectively excluded from the internal milieu of cultured cells.

The in vivo generation time of *H. capsulatum* was estimated by Rowley and Huber (1956) to be 15 to 19 hr. However, the technique employed in these studies was such that the time is probably too long (Rowley and Huber, 1956; Pine, 1960). Therefore, the in vivo generation time in the early stages of experimental infection may not be greatly different from the generation time in histiocytes maintained in culture.

The tinctorial characteristics and intracellular generation time are useful in assessing the behavior of *H. capsulatum* within histiocytes. Further observation in which these attributes of the fungus are studied may help in a more thorough understanding of the pathogenesis and mechanism of acquired resistance in histoplasmosis.

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