EFFECT OF MYCOSTATIN AND FUNGIZONE ON THE GROWTH OF HISTOPLASMA CAPSULATUM IN TISSUE CULTURE[1,2]

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Rous and Jones (1916) demonstrated that typhoid bacilli phagocytized by leucocytes were protected from the bactericidal activity of normal rabbit serum and of 0.01N potassium cyanide. These workers were among the first to emphasize that "living phagocytes are able to protect ingested organisms from the action of destructive substances in the surrounding fluid. . . . " Many years later, Mackaness (1952) reported that cells of *Mycobacterium tuberculosis* within macrophages were protected from bactericidal concentrations of several antibiotics. More recently it has been shown that penicillin, chloramphenicol, erythromycin, and novobiocin fail to kill phagocytized gonococci in tissue culture (Thayer *et al.*, 1956/1957). Suter (1952) has shown, however, that intracellular tubercle bacilli are as susceptible to isonicotinic acid hydrazide as are those suspended in Tween-albumin medium. This latter observation suggests that at least some drugs will be found that are equally effective against intracellular and extracellular organisms. Murat *et al.* (1959) have proposed a technique involving tissue cultures to be used for screening antibiotics and other drugs for their activity against intracellular bacteria.

The observation that intracellular organisms remain essentially unaffected by concentrations of many drugs which are bactericidal or bacteriostatic to extracellular forms has led to the development of an experimental technique whereby host-parasite relationships may be studied on a cellular level. The technique has many modifications, but consists essentially of allowing phagocytic cells (macrophages) in tissue culture to ingest microorganisms, stopping extracellular multiplication by the addition of an appropriate antibiotic, and studying the sequence of intracellular events either microscopically or by suitable culture techniques. This experimental approach has been applied to studies on the host-parasite relation in tuberculosis (Mackaness, 1952, 1954; Suter, 1952, 1953; Berthrong and Hamilton, 1958, 1959), in brucellosis (Holland and Pickett, 1956, 1958; Braun *et al.*, 1958; Pomales-Lebròn and Stinebring, 1957; Freeman and Vana, 1958), in salmonellosis (Furness, 1958; Furness and Ferriera, 1959), in gonorrhea (Thayer *et al.*, 1956/1957), and in staphylococcal infections (Kapral and Shayegani, 1959).

In a previous publication (Howard, 1959) it was suggested that the value of tissue culture techniques in a study of host-fungus relationships might depend upon a study of the use of fungistatic or fungicidal agents for controlling extracellular multiplication of fungi in such cultures. Mycostatin and Fungizone are antifungal drugs which might be employed for this purpose. Mycostatin (Squibb nystatin), an antifungal antibiotic derived from *Streptomyces noursei*, has had extensive experimental trial as an antifungal agent in various laboratory techniques (Wigmore and Henderson, 1955; Squibb Institute, 1956) and has demonstrated effective therapeutic action against certain clinical manifestations of Candida infections (Squibb Institute, 1957). Fungizone (Squibb amphotericin B) is a relatively new antifungal antibiotic derived from an unidentified species of *Streptomyces* and is currently under investigation as a therapeutic agent for systemic mycoses (Newcomer *et al.*, 1959).

The present work is concerned with observations on the effect of Mycostatin and Fungizone on the growth of *Histoplasma capsulatum* in tissue cultures of mouse peritoneal exudates.

MATERIALS AND METHODS

Most of the materials and methods used in these experiments were described in detail in a

previous report (Howard, 1959), and are therefore considered only briefly in this paper.

Tissue culture methods. Suspensions of mononuclear cells were obtained by washing the peritoneal cavity of mice with chilled Hanks balanced salt solution (BSS)[3] 5 days after intraperitoneal injection of 1.0 ml of a 10 mg per cent solution of glycogen. Cells were washed twice in balanced salt solution and pipetted into 16 by 125 mm screw cap tubes containing 5 by 43 mm cover slips which had been coated and fixed to the side of the tubes with a few drops of Formvar (0.5 per cent polyvinyl formol in ethylene dichloride). After the cells had settled onto the plastic coated cover slips, the balanced salt solution was replaced with 1.5 ml of a nutrient growth medium which consisted of 40 per cent filtered normal human serum, 5 per cent chick embryo extract, and 55 per cent balanced salt solution.[4]

Inoculation of cell cultures. The same strain of *H. capsulatum* employed in the previous report (Howard, 1959) was used in this study. The organism was grown in the nutrient growth medium incubated at 37 C. The density of suspensions of yeast cells was determined spectrophotometrically and appropriate dilutions containing the desired numbers of fungi were inoculated into the cultures of mononuclear cells.

Preparation and addition of drugs. Mycostatin was obtained as a sterile, amorphous powder in vials which were stored before use in the refrigerator. Although Mycostatin is practically insoluble in water, the small particle size of the powder permits the preparation of a very fine suspension with excellent antifungal properties. Mycostatin suspended in balanced salt solution (100,000 units/ml) was stored in the frozen state at −20 C in 1-ml lots. Frozen suspensions not used within a period of 1 month were discarded and new lots prepared. Dilutions of the stock suspension were made in balanced salt solution and added to the tissue cultures at intervals after inoculation. In a few experiments, the dry powder

[3] Five units of Heparin Sodium (Darwin Lipo-Hepin) were added per ml of balanced salt solution. This represents a modification of the previously reported procedure (Howard, 1959).

[4] The balanced salt solution used in preparing the nutrient growth medium was the supernatant balanced salt solution from the first washing of the exudate cells ("conditioned BSS" of Barski *et al.*, 1955).

Mycostatin was dissolved in N,N-dimethylformamide and dilutions were prepared in balanced salt solution as before.

Fungizone was obtained as a sterile, lyophilized powder in vials containing 50 mg amphotericin B and approximately 41 mg sodium deoxycholate with sodium phosphate as a buffer. This mixture was dissolved in balanced salt solution and stored in the same manner and for the same period of time as the Mycostatin suspensions. Dilutions of the stock suspension of drug were made in balanced salt solution and added to the cell cultures at intervals after inoculation.

Examination of cultures. Cover slips were removed at periodic intervals after preparation of cell cultures. The cover slips were immersed in warm saline for 20 min, fixed in absolute methanol for 5 min, and stained by the May Greenwald-Giemsa technique (Hanks, 1955).

Cell numbers were determined by averaging the number of cells in 20 consecutive fields. The proportion of infected cells after inoculation was determined by observing a total of 100 cells.

Plate counts of the viable cells of *H. capsulatum* per cell culture tube were made at various intervals after addition of the drug-containing medium. This was effected by plating 0.1 ml of serial 10-fold dilutions of the tissue culture medium on Sabouraud's dextrose agar. Colony counts were made after 1 week of incubation at room temperature.

RESULTS

Toxicity of Mycostatin and Fungizone for mouse mononuclear cells. Initial experiments were designed to determine the cytotoxicity of Mycostatin and Fungizone for the cells of mouse

TABLE 1

Effect of Mycostatin on mouse mononuclear cells in tissue culture

Conc of Mycostatin	Avg No. of Macrophages/Oil Immersion Field (Hr after Incubation)			
	24 hr	48 hr[a]	72 hr	96 hr
units/ml				
0	25.3	22.7	23.9	23.3
50	22.9	23.5	23.0	23.7
100	23.5	22.9	24.6	21.7
250	20.5	21.8	13.2	9.9

[a] Medium over cells changed and drug replaced after the 48 hr observations.

peritoneal exudates maintained in tissue culture. Both drugs were suspended in balanced salt solution, diluted to the desired concentrations in balanced salt solution, and added to the growth medium at the time of preparation of the cell cultures. Observations on stained cover slips removed from the cultures at 24-hr intervals re-

TABLE 2

Effect of Fungizone on mouse mononuclear cells in tissue culture

Conc of Fungizone	Avg No. of Macrophages/Oil Immersion Field (72 Hr Incubation)
µg/ml	
0	19.2
0.05	18.3
0.5	16.6
2.5	15.2
5.0	14.5

TABLE 3

Suppression of the growth of Histoplasma capsulatum by various concentrations of Mycostatin in tissue culture

Conc of Mycostatin	Viable Cells of *H. capsulatum* per Ml of Culture Medium after Addition of Drug	
	24 hr	48 hr
units/ml		
0	5.0×10^6	$>1.0 \times 10^8$
10	1.0×10^5	1.5×10^8
15	4.0×10^3	4.0×10^5
20	1.0×10^3	4.0×10^3
25	1.0×10^3	3.0×10

vealed that Mycostatin at a concentration of 250 units/ml was markedly toxic to the macrophages. Levels of drug of 100 units/ml or less had no observable deleterious effects on the cultures. Data from which these conclusions were drawn are shown in table 1.

Concentrations of Fungizone greater than 5.0 µg/ml were highly toxic to mouse macrophages. Levels of drug ranging from 0.5 to 5.0 µg/ml were slightly toxic to the cell cultures, whereas 0.05 µg/ml was not toxic to the cells. The data from which these conclusions were drawn are shown in table 2. Only data relative to the critical levels of drug are shown in table 2, since the observations extended over a period of time and involved a number of separate experiments. Sodium deoxycholate was not toxic to cell cultures at concentrations comparable to those that were present in the diluted Fungizone preparation (4.0 to 0.04 µg/ml).

Effect of Mycostatin on the growth of H. capsulatum in tissue culture. The initial observations were based on experiments designed as follows. Cell cultures were prepared and inoculated with approximately 2×10^4 yeast cells of *H. capsulatum*. After 4 hr of incubation at 37 C, Mycostatin was added at various nontoxic levels. Under these experimental conditions, it was found that 100, 50, and 25 units of Mycostatin/ml completely suppressed the extracellular multiplication of *H. capsulatum*. Observations of the stained cover slips revealed that these concentrations of the drug had also apparently completely suppressed the intracellular multiplication of the parasite. Yeast cells could not be found within the

TABLE 4

Influence of various levels of Mycostatin on the destructive effects of Histoplasma capsulatum on mouse mononuclear cells

Treatment of Cells	Mycostatin	Avg No. of Macrophages/Oil Immersion Field (Hr after Addition of Drug)		
		24 hr	48 hr	72 hr
	units/ml			
Uninfected controls	—	24.5	18.6	18.7
2×10^5 cells of *H. capsulatum*	—	10.0 (45)[a]	2.8 (73)	CD[b]
2×10^5 cells of *H. capsulatum*	10	13.2 (14)	11.6 (18)	6.4 (50)
2×10^5 cells of *H. capsulatum*	15	18.9 (8)	17.4 (13)	8.6 (30)
2×10^5 cells of *H. capsulatum*	20	21.4 (8)	20.0 (5)	15.1 (<1)
2×10^5 cells of *H. capsulatum*	25	21.0 (2)	19.3 (<1)	18.2 (<1)

[a] Figure in parentheses indicates per cent of cells showing intracellular *H. capsulatum*.
[b] CD = complete destruction of cell cultures.

macrophages of those cultures to which these levels of drug had been added, although control cultures without drug showed that proliferation of the parasite had taken place.

Further experiments were undertaken in which the concentration of Mycostatin was reduced and the number of yeast cells in the original inoculum was increased to approximately 2×10^5 cells/ml. In addition the period of parasitization of the macrophages was increased to 24 hr. The results of these studies are shown in tables 3 and 4. The addition of 25 units of Mycostatin/ml produced nearly complete suppression of the extracellular growth of *H. capsulatum*, whereas levels of drug ranging from 10 to 20 units/ml produced an initial suppression of growth which was followed by an increase in the number of viable cells (table 3). Thus Mycostatin at concentrations of 10 to 20 units/ml failed adequately to control the extracellular multiplication of *H. capsulatum* under these experimental conditions. Examination of stained cover slips (table 4) revealed that 25 units of drug/ml again completely suppressed the intracellular multiplication of the yeast cells. Ten and fifteen units of drug/ml produced a reduction in the number of intracellular yeast cells followed by an increase in numbers which occurred at a time when cultures from the supernatant culture medium indicated that the drug was no longer effective in controlling extracellular multiplication. Twenty units of drug/ml produced a marked reduction in the number of intracellular forms and this reduction was cor-

related with a noticeable but not complete suppression of the extracellular multiplication of the parasite.

Two possible explanations may be advanced to account for the data thus far presented. Either Mycostatin is capable of entering the macrophages and inhibiting the cells of *H. capsulatum* contained therein or the parasitized macrophages undergo lysis and release the intracellular yeast cells into the extracellular environment during the first 24 hr of incubation after addition of the drug. Experiments were devised to attempt to distinguish between these two possibilities. Cell cultures were inoculated with 2×10^4 yeast cells of *H. capsulatum*. After 24 and 72 hr of incubation, 25 units of Mycostatin/ml were added to the cultures. Control cultures were observed so that the extent of parasitization and destruction of the cultures could be ascertained at the time of the addition of the drug. To examine a larger area of each cover slip and thus obtain a more critical evaluation of the changes in the cell population, the method of analysis was altered. Numbers of macrophages were determined on the basis of observations made with the high power objective of the microscope ($450 \times$) and the per cent of macrophages showing intracellular *H. capsulatum* was determined on the basis of observation of 1000 instead of the usual 100 cells. The results of a typical experiment are shown in table 5. After 24 hr of incubation, approximately 13 per cent of the macrophages contained intracellular yeast cells and there was a loss of

TABLE 5

Growth of Histoplasma capsulatum in tissue cultures to which 25 units Mycostatin per ml of medium were added 24 and 72 hr after infection of the cultures

Treatment of Macrophages	Addition of 25 Units Mycostatin per Ml of Medium (Hr after Infection of Cultures)	Avg No. Macrophages/Microscope Field[a] (Hr after Infection)				
		24 hr	48 hr[b]	72 hr	96 hr[b]	120 hr
Uninfected controls	—	80.6	84.2	73.2	68.0	59.7
2×10^4 cells *H. capsulatum*	—	66.7 (13)[c]	59.4 (19)	6.2 (50)	CD[d]	CD
	24	—	68.6 (3)	74.4 (<1)	55.7 (<1)	54.8 (<1)
	72	—	—	—	5.2 (17)	CD

[a] High power objective of the microscope ($450 \times$).

[b] Medium over cells changed and drug replaced after 48 and 96 hr. observations.

[c] Figure in parentheses indicates the per cent of the macrophages showing intracellular *H. capsulatum*. Percentage calculated from observation of 1000 macrophages. Observations made with $970 \times$ magnification.

[d] CD = complete destruction of the cell cultures.

approximately 17 per cent of the macrophages. Addition of the drug at this time protected the cultures from any further destructive effects of *H. capsulatum* and markedly reduced the percentage of parasitized macrophages. If this reduction of parasitized macrophages were the result of lysis of the cells with the release of the yeast cells, a reduction in the number of macrophages should have resulted. Such a reduction of numbers of macrophages was not observed.

After 72 hr incubation, there was a marked destruction of the cultures with 50 per cent of the remaining macrophages showing parasitization. The addition of drug at this time likewise prevented the complete destruction of the cultures noted with the controls during the next 24 hr and greatly reduced the percentage of the remaining macrophages showing parasitization (table 5).

Examination of cultures to which drug had been added revealed some rather striking morphologic forms. These forms resembled yeast cells of *H. capsulatum* but were markedly altered in their staining properties. The structures were distorted and were pale blue in color. Similar

forms had been observed when heat-killed cells of *H. capsulatum* were added to macrophage cultures (Howard, 1959). Figure 1 shows a field in which several of these forms are present in the cytoplasm of the macrophages. It is possible that these structures represent drug-inhibited organisms undergoing destruction or at least tinctorial alteration within the macrophages. Since it was often impossible to distinguish these forms from other artifacts in the cell cultures, only readily recognizable cells of *H. capsulatum* were counted in estimating the per cent of infected macrophages (tables 4 to 6).

The data in table 5 and the observations recorded in figure 1 strongly suggest that Mycostatin is capable of entering macrophages in cell culture and of inhibiting the cells of *H. capsulatum* contained therein.

Since Mycostatin was in suspension rather than solution, it is possible that the drug's apparent ability to enter macrophages is related to its particulate nature. Attempts were made to dissolve the Mycostatin before addition of the drug to the cell cultures. Mycostatin is quite soluble
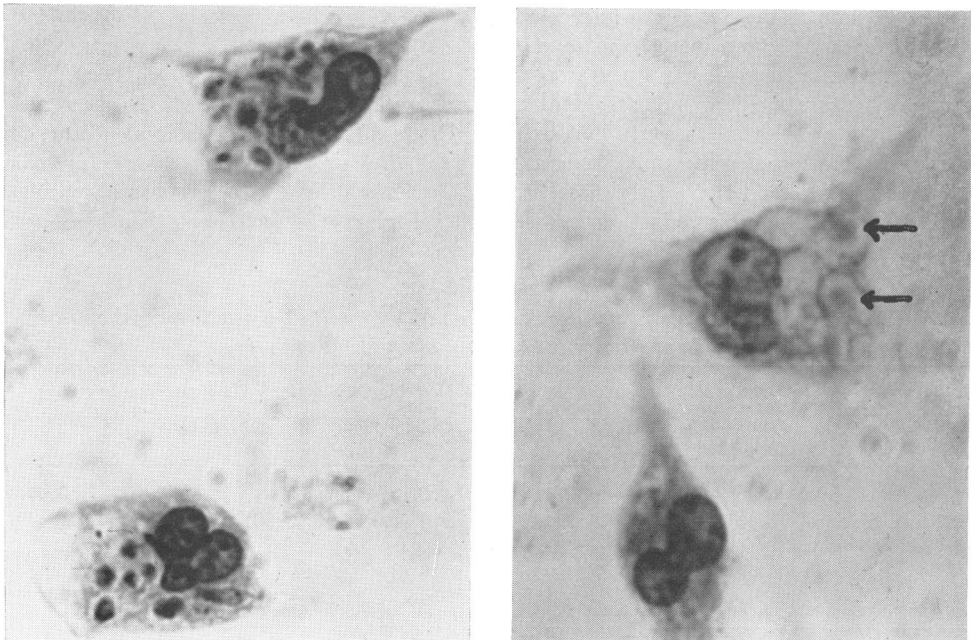

Figure 1. Photomicrographs of mouse peritoneal exudate cells parasitized by *Histoplasma capsulatum*. *Left*, macrophages from a control tissue culture 24 hr after parasitization of the culture with cells of *H. capsulatum*. *Right*, macrophage from a culture to which 25 units of Mycostatin had been added 24 hr after parasitization of the cultures with cells of *H. capsulatum*. Note the faded appearance of the yeast cells, the lack of internal detail, and the distortion of the cell walls. 970X.

TABLE 6

Influence of various levels of Fungizone on the destructive effects of Histoplasma capsulatum on mouse mononuclear cells

Treatment of Cells	Fungizone	Avg No. of Macrophages/Oil Immersion Field (Hr after Addition of Drug)		
		24 hr	48 hr[a]	72 hr
	$\mu g/ml$			
Uninfected controls	—	14.6	14.3	11.0
2×10^4 cells *H. capsulatum*	—	14.0 (15)[b]	5.8 (24)	2.4 (60)
	0.05	14.6 (12)	8.8 (21)	6.5 (31)
	0.10	14.0 (6)	15.7 (2)	13.0 (2)

[a] Medium not replaced after 48 hr readings as in previous experiments.

[b] Figure in parentheses indicates per cent of cells showing intracellular *H. capsulatum*. Observations made with 970X magnification.

in *N,N* - dimethylformamide, *N,N* - dimethyl-acetamide, and propylene glycol. The first of these solvents was tested in tissue culture and found toxic at levels of 1 to 100 or more. Nevertheless, it was postulated that dissolving the dry powder Mycostatin in *N,N*-dimethylformamide and then making subsequent dilutions in balanced salt solution might effect a different colloidal configuration which might not enter the macrophages. However, Mycostatin prepared in this manner was found to produce the same effects in tissue culture as did Mycostatin prepared in the usual manner. Other solvents were not tried, since they would probably have been toxic in undiluted form to the macrophages and it did not seem promising to pursue this approach further.

Effect of Fungizone on the growth of H. capsulatum in tissue culture. Although Mycostatin and Fungizone are similar in biologic activity, it was postulated that some differences in their behavior in tissue culture might appear, since Fungizone is a partially soluble product because of its content of sodium deoxycholate. Cell cultures were inoculated with 2×10^4 cells of *H. capsulatum*. After 24 hr of incubation, 0.1 and 0.05 μg of Fungizone/ml were added to the cultures. Subsequent observations were made as described previously. Cultures of the supernatant culture medium revealed that 0.1 $\mu g/ml$ markedly reduced the extracellular multiplication of the parasite and that 0.05 μg of drug/ml only partially suppressed the growth of *H. capsulatum*. The results from the stained cover slips are shown in table 6. The results are quite comparable to those obtained with Mycostatin. Levels of drug which only partially suppressed extracellular

multiplication had but little effect on the intracellular parasites. Levels of drug which markedly suppressed the extracellular growth of *H. capsulatum* also suppressed the intracellular development of the parasite.

DISCUSSION

Mycostatin has been used extensively to control fungal growth in a variety of laboratory procedures involving tissue culture techniques. Hemphill *et al.* (1957/1958) studied the comparative antifungal activity of nystatin (Mycostatin) and amphotericin B in tissue cultures of monkey kidney epithelium (M.B.A.), KB (Earle), HeLa (Gey), human intestine (Henle), bovine kidney (AFIP), and feline kidney (AFIP). They reported that 100 units of nystatin/ml was not toxic for any of the cell lines studied whereas 200 units/ml was slightly toxic for the HeLa cells but not for the other cell lines. Three-hundred units/ml was toxic for the four cell lines studied (monkey kidney, KB, HeLa, and human intestine). These data are in general agreement with those of other investigators who have also shown that Mycostatin at a concentration of 100 units/ml is not toxic for a variety of types of cells in culture, and that the toxicity levels of the drug are related to the type of cell cultured and the experimental techniques employed (Squibb Institute, 1956). The results of the present work show that nonproliferating mouse macrophages in tissue culture have approximately the same degree of sensitivity to the toxic action of Mycostatin as do rapidly proliferating cell lines.

Hemphill *et al.* (1957/1958) in their comparative study also showed that 40 $\mu g/ml$

amphotericin B was not toxic to the same cell lines as those studied with nystatin. Eighty $\mu g/$ml was toxic, however, to those cell cultures studied. It is known that 1 mg Mycostatin is roughly equivalent to 2500 units (Squibb Institute, 1956). On the basis of these figures, the data of Hemphill et al. (1957/1958) show that Mycostatin and amphotericin B have very similar toxicity levels for various tissue cell lines. The author is not aware of any data relating to the toxicity of Fungizone (a mixture of amphotericin B and sodium deoxycholate) for cells in culture. However, the data from the present work suggest that Fungizone is more toxic for mouse macrophages than is Mycostatin. It was not possible to relate the cellular toxicity of Fungizone to the content of sodium deoxycholate. Since amphotericin B as such was not used in the present study, the results can not be compared with those of Hemphill et al. (1957/1958).

It has been repeatedly shown that living phagocytes protect ingested organisms from the destructive action of antibodies, simple chemicals, and some antibiotics (discussed previously). In a few instances it has been demonstrated that at least some drugs may enter intact macrophages (Suter, 1952; Murat et al., 1959). The data presented in the present report suggest that both Mycostatin and Fungizone are capable of entering macrophages maintained in tissue culture and of inhibiting the cells of H. capsulatum contained therein. Whether or not these conclusions are substantiated by further investigation of these two drugs, the fact remains that the drugs are not suitable for the original purpose for which they were studied. It is clear that there was no level of Mycostatin or Fungizone which would completely inhibit the extracellular multiplication of H. capsulatum in tissue cultures of mouse macrophages without at the same time affecting to some degree the intracellular parasites.

Murat et al. (1959) have pointed out that tissue culture techniques may be useful in screening newly discovered drugs for their activity against intracellular bacteria. The data presented in this and the previous report (Howard, 1959) indicate that the tissue culture method may be equally useful in the study of antifungal drugs.

SUMMARY

The effect of Mycostatin and Fungizone on the growth of Histoplasma capsulatum in tissue cultures of mouse peritoneal exudates has been studied. Preliminary observations reveal that Mycostatin was toxic to the macrophages at concentrations of 250 units/ml or greater but was relatively nontoxic to these cells at concentrations of 100 units/ml or less. Fungizone was found to be somewhat more toxic than Mycostatin on a weight for weight basis. The Fungizone preparation was markedly toxic at 10 $\mu g/ml$, partially toxic at 5 μg to 0.5 $\mu g/ml$ and relatively nontoxic at 0.1 μg to 0.05 $\mu g/ml$.

The destructive effects of H. capsulatum on cell cultures could be completely suppressed by the addition of 25 units of Mycostatin or 0.1 μg of Fungizone/ml 24 hr after initial infection. The per cent of macrophages showing parasitization was markedly reduced in cultures to which the drugs had been added, whereas control cultures inoculated at the same time showed that extensive proliferation of the organism had taken place. This reduction in the number of parasitized macrophages was not correlated with a reduction in the total cell population. Intracellular structures resembling yeast cells were observed in the cytoplasm of macrophages from cultures to which drug had been added. These forms were distorted and failed to stain in a manner characteristic of viable cells of H. capsulatum. The structures were interpreted as representing drug-inhibited organisms undergoing intracellular degeneration.

These data suggest that both Mycostatin and Fungizone were capable of entering macrophages and inhibiting the cells of H. capsulatum therein. There was no level of these drugs which would completely inhibit extracellular multiplication of H. capsulatum in tissue cultures of mouse macrophages without at the same time affecting to some degree the intracellular parasites.

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