

Lymphocyte-Mediated Cellular Immunity in Histoplasmosis¹

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Mononuclear phagocytes freshly harvested from immunized animals restrict the intracellular growth of *Histoplasma capsulatum*, whereas the same cells maintained in culture for 48 hr do not. Experiments established that within the mixed cell population of the mouse peritoneal cavity the lymphocyte was the most likely mediator of the intracellular growth restriction observed. 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 cell culture.

Mononuclear phagocytes from mice immunized against *Histoplasma capsulatum* restrict the intracellular growth of the fungus (7, 13, 20). However, some inconsistencies in the in vitro expression of this type of inhibition have been reported (8, 9, 20). The results presented in this report explain these inconsistencies and show that lymphocytes mediate macrophage suppression of intracellular growth by *H. capsulatum* in vitro.

MATERIALS AND METHODS

Fungus. *H. capsulatum*, strain 505, employed in previous experiments (8-10; D. H. Howard and V. Otto, *Bacteriol. Proc.*, p. 121, 1970), was used in these studies. The yeast cell phase of the fungus was maintained at 37 C on glucose-cysteine-blood-agar (4).

Macrophage cultures. Suspensions of mononuclear cells were obtained from the peritoneal cavities of unstimulated Webster-Swiss mice. A 0.5-ml volume of cells washed in Hanks balanced salt solution (BSS) 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). The cells were allowed to settle onto the cover slips for 1 hr at room temperature. In those cultures to be used 48 hr after preparation, the suspending fluid was replaced with 1.5 ml of a medium consisting of 40% normal human serum (Flow Laboratories, Inc., Inglewood, Calif.) in BSS. The caps of the tubes were replaced with rubber stoppers to prevent loss of CO₂, and the cultures were incubated in a slanted position at 37 C. Those cultures to be used immediately after harvest were parasitized with *H.*

capsulatum (see below), washed three times in BSS, overlaid with 1.5 ml of medium, and incubated as described above.

Preparation of inocula. Cultures of *H. capsulatum* were grown on glucose-cysteine-blood-agar slants incubated at 37 C for 48 hr. Saline suspensions of the growth from the cultures were prepared and standardized as described in earlier reports (8, 9).

Parasitization of macrophages. Macrophage cultures were exposed to dispersed small inocula (2×10^6 yeasts/ml) of *H. capsulatum* in tissue culture medium (40% normal human serum in BSS) and incubated at 37 C for 3 hr. Approximately 60% of the yeast cells in the inoculum were phagocytized during this incubation period. Fifteen to twenty per cent 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 observations (8, 9).

Immunization of mice. Mice were immunized by intravenous injection of a sublethal number of washed yeast cells of *H. capsulatum*. The median lethal dose (LD₅₀) of the strain used in these studies was 2×10^6 cells per mouse. The sublethal dose chosen was 2.5×10^6 yeasts per mouse. Resistance of the mice was assessed by intravenous injection of 1.4×10^7 cells per mouse (7 times LD₅₀). The general procedures used were those widely adopted for such work (6). Our results with such immunization techniques have been reported previously (D. H. Howard and V. Otto, *Bacteriol. Proc.*, p. 121, 1970).

Preparation of lymphocytes. Lymphoid cells were prepared from peritoneal populations by a modification of a procedure used by Mackaness (12). Cells harvested from the peritoneal cavity of mice were

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washed once in BSS and suspended in BSS containing 1% fetal calf serum. The suspensions were passed through a 4-cm column of acid-cleaned cotton wool. After a second passage through cotton wool, the cells were counted and brought to the desired concentration by dilution in tissue culture medium. The final population of cells was heterogeneous but consisted mainly of lymphoid cells of varying sizes. There were no cells which could properly be called macrophages.

Intracellular growth. Methods for fixation and staining of cover slips and for enumeration of fungi within macrophages have been reported in detail previously (8-10). The intracellular growth of the fungus is expressed either as a generation time or as per cent inhibition of growth compared to controls.

RESULTS

Inhibition of intracellular growth. The key observation leading to the studies to be reported was that documented by the data, from a typical experiment, shown in Table 1. The intracellular generation time of *H. capsulatum* was prolonged threefold within immune macrophages parasitized 1 hr after harvest from the mice. However, immune macrophages maintained in cell culture for 48 hr before being infected did not inhibit the intracellular growth of the fungus (Table 1). The percentage of macrophages parasitized was approximately the same in tissue cultures from normal and immunized mice. Although some cells left the cover slip surface during incubation, the percentage of infected macrophages did not change appreciably during the 21-hr observation period. Thus there was not a significant loss of infected macrophages. The conclusion reached after numerous repetitions of the experimental results in Table 1 was that macrophages freshly harvested from mice inhibited intracellular growth but that macrophages from the same animals maintained in culture for 48 hr did not.

Persistence of immune macrophages in peritoneal cavities of immunized mice. The data in Table 2 establish that mice injected intravenously with

TABLE 2. Resistance of mice sublethally infected with *Histoplasma capsulatum*^a

Day challenged ^b (after sublethal infection)	Infected mice	Control mice
4	3/4 ^c	4/4
7	0/4	4/4
14	0/4	4/4
21	0/4	4/4
28	0/4	4/4
60	0/4	4/4
90	3/4	4/4

^a Injected intravenously with 2.5×10^5 yeasts per mouse ($LD_{50} = 2 \times 10^6$ per mouse).

^b Challenge dose was 1.4×10^7 yeast cells per mouse ($7 LD_{50}$) injected intravenously.

^c Results recorded as number dead/number injected.

2.5×10^5 cells per animal first clearly displayed resistance at 7 days. The resistance to challenge was still obvious at 60 days but had largely disappeared 90 days after infection. Macrophages harvested from mice 4, 7, 14, 21, and 30 days after sublethal infection and studied immediately after harvest inhibited the intracellular growth of *H. capsulatum*; cells obtained 60 days after sublethal infection did not. The inhibitory effects were manifested most strongly in cells harvested 7 to 21 days after sublethal infection. Accordingly, "immune" mononuclear cells were obtained from infected mice during this period of time in all subsequent experiments.

Effect of washing cell cultures on expression of intracellular growth inhibition. The observation that the inhibitory effect of immune macrophages was displayed only by cells freshly harvested from mice suggested that some type of cell or labile factor did not persist during incubation of the tissue cultures. This suggestion was the basis for the experiment recorded in Table 3. Macrophages harvested from immunized mice and allowed to settle for 1 hr onto cover slips in culture chambers lost most of their inhibitory capacity if they were washed three times with BSS before parasitization with *H. capsulatum*. The wash procedure did not always completely remove the inhibitory capacity of immune macrophages. The per cent reduction of intracellular growth in washed immune macrophages ranged from <1 to 25% in a series of repeated experiments. However, washed immune macrophages were always at least 50% less inhibitory than unwashed cells. Intracellular growth suppression was restored in part to the washed cultures by the addition of partially purified lymphocytes from immunized animals (Table 3).

The washing procedure was effective only when

TABLE 1. Intracellular generation time of *Histoplasma capsulatum* within macrophages maintained in cell cultures for 1 hr and 48 hr

Type of macrophage	Time in culture (hr)	Intracellular generation time ^a (hr)
Normal	1	9.9
Immune ^b	1	33.0
Normal	48	9.7
Immune	48	10.5

^a Results of a single experiment recorded.

^b Cells harvested 14 days after mice were injected intravenously with a sublethal dose of yeast cells of *H. capsulatum*.

TABLE 3. Effect of washing on capacity of macrophages to inhibit intracellular growth of *Histoplasma capsulatum*

Type of macrophage	Wash ^a in cell culture	Lymphocytes ^b added after washing	Per cent reduction of intracellular growth ^c
Normal	—	—	0
Immune	—	—	77
Immune	+	—	<1
Immune	+	+	44

^a Wash was with 3X Hanks balanced salt solution.

^b Lymphocytes were peritoneal cells from immunized animals passed through cotton wool columns and added with inoculum of *H. capsulatum*. Number of lymphocytes added was 10⁷/ml.

^c Compared to controls after 18 hr of incubation. Results are from a typical experiment. See text for description of variations observed.

performed on cell cultures, i.e., the effect was not removed by washing cells in suspension before they had attached to glass. The wash fluids did not restore activity to the cultures. The BSS used to harvest the cells from the peritoneal cavity did not inhibit the intracellular growth of *H. capsulatum*. The tentative conclusion was that the key cell in growth suppression of *H. capsulatum* was a lymphocyte.

Lymphocyte-macrophage interaction. The washing experiments reported in the preceding paragraphs were extended to gain additional information on lymphocyte-macrophage interactions. The results reported in this paragraph are not documented in detail and were derived from observations on variations of experiments like the one shown in Table 3. Cell cultures washed 1 hr after preparation lost most of their antifungal capacity (Table 3), but cell cultures washed 3 hr after preparation did not. The inhibitory capacity was more efficiently washed from cultures of cells harvested from mice injected 14 days previously than from those of mice injected 21 days previously. Immune lymphocytes did not affect the viability of yeast cells of *H. capsulatum* suspended in buffer. The intracellular growth of the fungus was still inhibited in cultures washed after parasitization rather than before parasitization. The general conclusions were that lymphocytes exerted their effect on the macrophage and not directly on the fungus, that lymphocyte macrophage interaction took place within 1 hr in culture, and that in vivo interaction was manifested by cell population harvested from animals which had been infected for longer periods of time. These tentative conclusions led to the experiments reported next.

Normal macrophages or immune macrophages that had been in culture for 48 hr [at which time they were no longer inhibitory (Table 1)] restricted the intracellular growth of the fungus after exposure to lymphocytes from immunized animals (Table 4). Thus exposure to lymphocytes from immunized animals activated macrophages to suppress the intracellular growth of the fungus regardless of the source of those macrophages or the period of time they were in cell culture. Heat inactivation (60 C, 30 min) destroyed the activity of immune lymphocytes, and activity was not recovered from cells treated sonically.

Macrophages were activated to inhibit *H. capsulatum* by addition of lymphocytes to cell cultures already parasitized by the fungus (Table 5). This observation further documents the fact that the lymphocyte effect is on the macrophage and not directly on the fungus. The data

TABLE 4. Effect of immune lymphocytes on growth of *Histoplasma capsulatum* within normal and immune macrophages

Type of macrophage ^a	Lymphocytes added ^b	Mean no. of yeasts per infected macrophage ^c	Per cent reduction of intracellular growth ^d
Normal	—	10.1	0
Normal	+	3.9	62
Immune	—	10.8	0
Immune	+	3.3	70

^a Macrophages in cell culture 48 hr before parasitization.

^b Lymphocytes were peritoneal cells from immunized animals passed through cotton wool columns and added with inoculum of *H. capsulatum*. Number of lymphocytes added was 10⁷/ml.

^c After 18 hr of incubation.

^d Compared to controls.

TABLE 5. Effect of immune lymphocytes on intracellular growth of *Histoplasma capsulatum* within normal macrophages^a

Type of lymphocyte added ^b	Mean no. of yeasts per infected macrophage ^c	Per cent reduction of intracellular growth ^d
None.....	12.7	0
Normal.....	10.6	17
Immune.....	3.3	74

^a Macrophages harvested from normal animals and maintained in cell culture for 48 hr.

^b Peritoneal cells from immunized or normal animals passed through cotton wool columns and added after parasitization of cultures with *H. capsulatum*. Number of lymphocytes was 10⁷/ml.

^c After 21 hr of incubation.

^d Compared to controls.

TABLE 6. *Effect of numbers of immune lymphocytes on intracellular growth of Histoplasma capsulatum within normal macrophages^a*

No. of lymphocytes ^b added per ml	Mean no. of yeasts per infected macrophage ^c	Per cent reduction of intracellular growth ^d
None	9.9	0
10 ⁵	10.5	0
3 × 10 ⁵	8.6	13
10 ⁶	8.7	12
3 × 10 ⁶	5.6	45
10 ⁷	2.8	72

^a Macrophages harvested from normal animals and maintained in cell culture for 48 hr.

^b Peritoneal cells from immunized animals passed through cotton wool columns and added after parasitization of cultures with *H. capsulatum*.

^c After 16 hr of incubation.

^d Compared to controls.

in Table 5 also suggest that lymphocytes from normal animals had a slight capacity to activate macrophages. The effect was small and did not exceed variation in the estimates of intracellular growth recorded previously (8, 9). Glycogen stimulation of peritoneal exudates, which produces macrophages with enhanced antimicrobial activity (17), did not elicit cells capable of restricting intracellular growth by *H. capsulatum*.

The dose-response relationships between numbers of lymphocytes added and intracellular growth suppression in normal macrophages are shown in Table 6. At concentrations of 3 × 10⁶/ml and 10⁷/ml, the added lymphocytes clearly inhibited intracellular growth of the fungus. Addition of fewer than 3 × 10⁶ lymphocytes per ml produced either no obvious effects or effects which did not exceed normal variations seen in other types of experiments (8, 9). The conclusion was that partially purified lymphocytes from immunized animals activated macrophages to suppress the intracellular growth of *H. capsulatum*.

DISCUSSION

Acquired immunity to infection by the facultative intracellular parasite *H. capsulatum* is probably mediated by cells. This tentative conclusion is based upon two sorts of in vitro information: (i) the lack of any compelling evidence for the role of humoral antibody (8, 9, 19) and (ii) the observation that macrophages from immunized animals "digested" *H. capsulatum* in the absence of specific antibody (7, 13, 20). The in vitro expression of cellular immunity is variable. Thus Wu and Marcus (20) found little difference in phagocytic and "digestive" capacity of im-

mune and normal phagocytes when these cells were maintained in cell culture for 24 hr. Furthermore, Howard (9) reported that the rate of growth of *H. capsulatum* within macrophages maintained in cell culture for 48 hr was identical in cells from immune and normal mice or guinea pigs. Wu and Marcus (20) suggested that these reported inconsistencies in the in vitro behavior of the macrophages were related to the length of time the cells were maintained in culture. The data from the present report (Table 1) establish this suggestion as correct. Thus cells freshly harvested from immunized animals restrict the intracellular growth of *H. capsulatum*, whereas the same cells maintained in culture for 48 hr do not.

The techniques employed in this paper are not well suited to a quantitative appraisal of efficiency of phagocytosis. Nevertheless, the number of yeasts phagocytized by cells from normal and immune animals was very similar, a result in keeping with the observations of Miya and Marcus (13).

The percentage of macrophages parasitized by the fungus remained constant over the period of observation. Experimental conditions have been developed that reduce extracellular multiplication to a minimum (8-10); accordingly, there should have been no increase in the number of parasitized cells. Furthermore the constancy of the percentage of parasitized cells ruled out a significant loss of cells into the medium (3).

The results from this study suggest that the cell mediator of in vitro intracellular growth restriction was a lymphocyte. This suggestion was originally held because the effect was observed in freshly harvested mixed cell populations but was not manifested by cell cultures prepared in a manner that would deplete the number of lymphocytes. Subsequent experiments established that it was important not that lymphocytes failed to persist in culture, but rather that macrophages, activated by interaction with lymphocytes, did not retain their acquired antifungal property for very long in cell culture. The reason for this fact has not been found. Nevertheless, our assumption that the lymphocyte was the key cell was reinforced by the observations that: (i) in keeping with the well known fact that lymphoid cells do not attach to glass, the inhibitory effects could be washed away from cells attached to cover slips but not from suspended cells prior to attachment to glass and (ii) the effect could be added back to washed immune macrophage cultures or induced in normal macrophage cultures by means of cell populations consisting predominantly of lymphoid cells but not by the wash fluids from such cells.

Accordingly, in keeping with current immunologic concepts (2, 11, 12, 15, 18) the lymphocyte in the mixed cell population of the peritoneal cavity was the most likely mediator of the intracellular growth restriction observed.

The argument for lymphocyte mediation is augmented by analogy with other infectious diseases and by derivation from other experimental approaches to acquired resistance in histoplasmosis. Thus, acquired cellular immunity to infection with other facultative intracellular parasites is clearly lymphocyte-mediated (5, 12, 15, 17, 18). Furthermore, antilymphocyte serum is known to influence adversely the response of animals to infection with *H. capsulatum* (1). Finally, factors which inhibit the growth of *H. capsulatum* have been reported from splenic homogenates of immunized animals (16).

In the present studies, lymphocytes had no detectable direct effect on the viability of *H. capsulatum*, and intracellular growth was inhibited by adding immune lymphoid cells to normal macrophages after the phagocytic event. Thus, the primary event was macrophage activation by lymphoid cells from immunized animals. The concept of "macrophage activation" has received considerable review in the literature (2, 11, 12, 14, 15, 18). It is clear that one of the best ways to produce macrophages which will inhibit intracellular parasites is to immunize animals in such a way as to induce a solid state of delayed hypersensitivity. Under these circumstances the cell mediating activation is clearly a lymphocyte (12, 14, 17). *H. capsulatum* can now be added to the growing list of facultative intracellular parasites whose intracellular growth can be shown to be restricted by lymphocyte-activated macrophages (5, 12, 15, 17, 18).

Since lymphocytes activate macrophages and since the peritoneal cavity consists of a mixed population of cell types, it was somewhat puzzling that removal of lymphocytes from in vitro cell cultures substantially removed macrophage inhibitory capacity (Table 3). It is not clear why macrophages were not already activated within the peritoneal cavity before they were harvested. Activation obviously took place rapidly in cell culture. Although this enigma was not completely resolved by the present data, a partial answer is suggested by the observation that the inhibitory effects were more efficiently washed from cell cultures of mice infected for 14 days than from cultures of mice infected for 21 days. Thus, in vivo interaction was manifested by cell populations harvested from animals that were infected for longer periods of time. One may tentatively conclude that macrophage activation

by contact with lymphocytes does take place in vivo but that the tissue culture chamber provides an opportunity for maximum early cell interactions.

Of equal significance is the fact noted by Wu and Marcus (20) and confirmed in this report that the fungistatic activity of immune macrophages is obvious only during the first few hours in vitro. Cells maintained in culture for 24 hr or longer (Table 1) are not able to restrict the intracellular growth of *H. capsulatum*. In contrast, the antibacterial effects of activated macrophages are manifested over longer periods of cell cultivation (17, 18). Moreover, Gentry and Remington (5) have shown that macrophages activated by *Listeria monocytogenes* and *Besnoitia jellisoni* infections inhibited the intracellular growth of *Cryptococcus neoformans* in cells cultivated for 24 hr in vitro. Thus, it would seem worthwhile to study fungistatic or fungicidal properties of more examples of macrophages activated by bacterial infections. Equally worthwhile would be the study of bacteristatic or bactericidal properties of macrophages activated by fungus infections.

Macrophages can be activated to inhibit the intracellular growth of *Mycobacterium tuberculosis* by soluble substances elaborated by lymphoid cells in contact with antigen to which they are specifically sensitized (17). Efforts to demonstrate a similar soluble substance in our system have failed consistently to date. Nevertheless the ever increasing number of substances released by lymphocytes interacting with specific antigens (2, 11, 12, 14) and the results of Patterson and Youmans (17) in the *M. tuberculosis* system clearly encourage continued efforts to reveal an active soluble substance in our cultures. Moreover, Mackaness (12) reported data in support of the view that acquired resistance in *Listeria* infections "depends upon the activation of host macrophages through a product resulting from the specific interaction between sensitized lymphoid cells and the organism or its antigenic products." Mackaness also discussed the possibility that macrophage activation could depend on the interaction of cells sensitized by cytophilic antibody (12). The data from observations on fungus-macrophage interactions are too few to permit more than speculative recognition of such a modality for activation of the fungus inhibitory property.

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