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Immunological Studies on *Histoplasma capsulatum*

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Alveolar macrophages freshly harvested from normal and immunized rabbits were parasitized with yeast cells and protoplasts of *Histoplasma capsulatum*. Macrophages obtained from either normal or sensitized rabbits failed to phagocytize protoplasts, whereas the yeast cells were actively ingested. There was no detectable intracellular killing by macrophages. A serological similarity was found between the whole yeast cell, the purified isolated cell wall, and the protoplasts of the fungus. A precipitin test of the protoplasts of the fungus gave a positive band, whereas immunodiffusion in agar was negative. Addition of immune sera activated phagocytosis, the immune sera against cell walls being the most active.

Antigens from the dimorphic fungus *Histoplasma capsulatum* have been studied extensively by different investigators (3, 5, 9, 16, 18); however, in the majority of reports the authors used the mycelial and the yeast phases of the fungus. Consequently, there is very little information concerning the fate of the protoplasts of the fungus within the phagocyte and its immunogenic properties (17). Evidence exists concerning the necessity of promoting factors (c' or other heat-labile substances) for phagocytosis of the yeast phase of *H. capsulatum* by normal and immune mouse peritoneal macrophages (15, 19).

In previous reports the antibody did not seem to play a significant role in enhancing phagocytic or digestive rates of peritoneal phagocytes from either normal or immune mice. However, the results obtained in this report are different. The present work was carried out to demonstrate: (i) where in the yeast cell of *H. capsulatum* each antigen is located, since the whole cell has proven useful and active as an antigen; (ii) the role of the immune sera in phagocytosis; and (iii) the fate of the protoplasts of *H. capsulatum* as well as the yeast cell of the fungus within the alveolar macrophages of rabbits. Special attention was paid to the mode of phagocytosis and immunogenic properties of the protoplasts of the fungus, since electron microscopy of this preparation did not reveal the presence of protoplasts as a whole or traces of residual cytoplasm inside the macrophage.

MATERIALS AND METHODS

Fungus. *H. capsulatum* strain SWB was grown in the following liquid culture media: (i) brain-heart infusion (BBL, Cockeysville, Md.) (37 g/1,000 ml of distilled water); or (ii) modified Sabouraud broth (BBL, Cockeysville, Md.) (30 g/1,000 ml of distilled water) on a reciprocal shaker for 18 h.

Preparation of protoplasts. Protoplasts were obtained by the method of Berliner and Reca (1, 2) and were separated from the yeast by centrifugation at 3,000 rpm for 30 min. The supernatant was recentrifuged at 3,000 rpm for 1 h.

Animals. Rabbits of either sex weighing from 2 to 3 kg were used.

Sensitization. The rabbits were sensitized intravenously and via footpads with a single injection of 100 μg of heat-killed, lyophilized whole yeast cell, isolated cell wall, and protoplasts, and finally with alkaline-soluble and -insoluble fractions of isolated cell wall of *H. capsulatum* strain SWB. All these fractions were suspended in 0.1 ml of Freund incomplete adjuvant. The same number of animals was maintained under the same conditions as control animals (6).

Macrophage culture. Animals were sacrificed 21 days after sensitization. The alveolar cells were procured by flushing the lungs of rabbits by the technique of Myrvik et al. (14). Studies of the viability of the cell population were done by the trypan blue exclusion technique (13). The macrophages were washed twice in tissue culture medium (TCM) 199 without sera and recovered by centrifugation at 400 × *g* for 10 min. The cells were resuspended in TCM 199 and counted in a Levy hemocytometer, and the concentration was adjusted to 2×10^6 cells/ml. All media used in the experiments contained 100 μg of streptomycin and 100 U of penicillin per ml. When the macrophages were parasitized by protoplasts, 3% peptone (Difco) was added to the medium as an osmotic stabilizer. These phagocytic cells were used in two ways. In one procedure, the cell cultures from normal and sensitized animals were exposed to inocula (9×10^6 yeast or protoplasts/ml) in TCM and incubated at 37 C in plastic culture flasks (Falcon Plastics, Oxnard, Calif.) for 3 h. The culture flasks were washed three times in 5 ml of TCM to remove

the extracellular organisms remaining after phagocytosis. The adhered macrophages were released with trypsin every 15 min and fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 0.8 M mannitol. The latter was used as a stabilizer for the protoplasts. The pellet was set for electron microscopic observation by post-fixation in 2% osmium tetroxide for 2 h followed by dehydration in a series of alcohols and was embedded in Maraglas. All experiments were performed at least in triplicate. Results were recorded at 0.25, 0.5, 0.75, and 2 h after incubation. Continuous microscopic observations were performed under a Nikon inverted microscope, model M, at 37 C. The second cell maintenance technique was only developed in order to determine the rate of phagocytosis. Suspensions of pulmonary macrophages (2×10^6/ml) obtained from normal and sensitized rabbits were washed in medium 199 and pipetted into Leighton culture tubes (Pyrex Laboratory Glassware) containing cover glasses (5 by 43 mm) fixed to the side of the tubes. After the phagocytic cells were allowed to attach to the cover glass, the cultures were incubated with yeast or protoplasts (9×10^6/ml) of *H. capsulatum*. The caps of the tubes were replaced with rubber stoppers to prevent loss of CO_2 and were incubated in a flat position at 37 C (18). The fluid was poured off and the cover glasses were removed every 15 min and fixed in glutaraldehyde. The phagocytosis of the fungus was quantitated by periodic acid-Schiff reaction and Grocott staining procedure. Digested yeasts, both from normal and immunized macrophages, were recovered after 24 h of incubation, scraped from tissue culture flasks into distilled water, and agitated for 6 h. More than 80% of the recovered cells were viable, since they could initiate growth in the culture media.

Antisera. Antisera against the different fractions of the fungus were produced in rabbits. The animals were injected as previously described, but using 0.2 ml of the antigen (1 mg of the lyophilized material per ml of Freund complete adjuvant) followed by two injections in Freund incomplete adjuvant spaced 1 week apart. Seven days later they were exsanguinated by cardiac puncture, and the serum was recovered.

Preparation of antigens. The yeast cells were grown for 3 days under constant rotation at 37 C in a liquid medium (brain-heart infusion or modified Sabouraud broth), washed by centrifugation several times, killed by heating at 65 C for 45 min, and lyophilized. The dry weight of a portion was determined. This was used to immunize animals as well as in the serology tests.

Protoplasts obtained as described before were lysed in water and treated in an Omnimixer, heat killed, lyophilized, and used as another type of antigen in the course of experiments.

Purified isolated cell wall as antigen. Formalin-killed yeast cells were treated three times in a Ribi cell fractionator, model RF-1 Sorvall, under 30,000 lb/in^2 of pressure, suspended in water, and treated in an Omnimixer for 5 min. The crude cell walls were collected by centrifugation at $1,500 \times g$ for 10 min. The above treatment was repeated several times until the supernatant solution was clear. Cell walls were obtained as a sediment and ribonucleic acid (11) was determined. The cell walls were washed, dialyzed against several changes of water, and lyophilized. Alkaline-soluble and -insoluble fractions of cell wall were obtained according to a published schedule (10).

Adsorption of sera. Immune sera against the different fractions of the fungus were adsorbed by 100 μg of the corresponding lyophilized materials. They were mixed with the sera and incubated at 37 C overnight. The mixture was centrifuged and the supernatant, which was the adsorbed sera, was used in the experiments.

Immunodiffusion test. The test was carried out in 2-mm layers of 0.7% agarose made up in merthiolate-Barbitol buffer. One central well and eight outer wells were cut. Diluted antigen was used in the outer wells and undiluted serum was used in the inner well, or vice versa. The antigens used from 5 to 25 mg/ml in 0.01 M phosphate buffer, pH 7.6, with 37% ClNa, and the first dilutions to be tested were 1:2, 1:4, 1:8, etc., incubated at room temperature for 24 h and placed in the refrigerator at 4 C for 48 h.

Precipitin test. A ring test was performed where the antigen overlayed the immune sera against protoplasts, the whole yeast cell, and the isolated cell wall. Diluted histoplasmin (1:100), supplied by the Center for Disease Control, Atlanta, Ga., and other fractions of *H. capsulatum* were used as antigens.

RESULTS

In an attempt to observe the phagocytosis of *H. capsulatum* by alveolar macrophages, a microscopic observation of these materials was carried out. Macrophages obtained from either normal or rabbits sensitized against killed protoplasts or yeast cells of *H. capsulatum* strain SWB failed to phagocytose protoplasts. However, the same phagocytes ingested yeast cells of the fungus even in the absence of immune sera in the first 15 min of contact (Fig. 1). The protoplasts did not persist in the culture medium once they came in contact with the macrophages; almost 95% were destroyed within the first 30 min. However, in the control medium there was no destruction observed during the incubation period (3 h). In the experiments in which macrophages sensitized against the isolated cell wall were used, the highest rate of phagocytosis was achieved. Approximately 65% of the yeast cells in the inoculum were ingested during the first 15 min of incubation, with an average of 6.5 yeast cells per macrophage. This percentage was reduced to 58.6% in the phagocytes sensitized against the whole yeast cells, with an average of five yeast cells per macrophage, to 45.7% in the cells sensitized against protoplasts, with an average of 1.5 yeast cells per macrophage (Tables 1 and

2). The sensitized macrophages against two fractions of the cell wall showed almost the same rate of phagocytosis (58%) as the whole yeast cell. The phagocytic rate remained constant from 30 min to 3 h. Addition of the immune sera against any of the following fractions: (i) protoplasts, (ii) yeast, and (iii) isolated cell wall, to either normal (Fig. 2) or immunized phagocytes (Fig. 3) increased the rate of phagocytosis by macrophages. The immune sera against protoplast and alkaline-insoluble fractions of the cell wall activated the macrophages equally well. The percentage of phagocytosis in the phagocytes sensitized against the yeast cells was increased up to 59.6% by using the immune serum against these two fractions of *H. capsulatum*. However, when the immune serum against the purified isolated cell wall was used, the phagocytic activity increased notably (69.8%) (Fig. 3). The specific antibody was detected in the immune serum against the whole cell as well as the protoplasts, which was demonstrated as a ring-form precipitate in the classical precipitin test, whereas the immune sera against protoplasts failed to show any precipitation band in immunodiffusion in agar (Table 3).

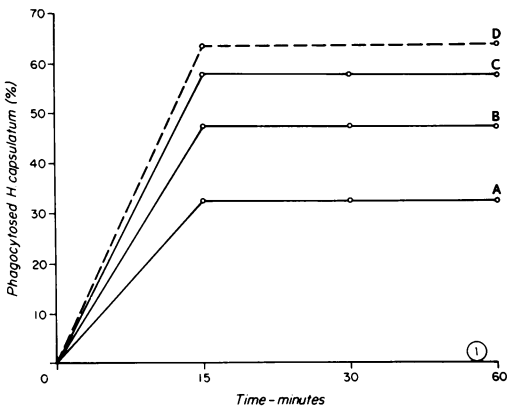

FIG. 1. *Mean percentage of phagocytosed yeast cells per cover glass by (A) normal cells; (B) cells immunized against protoplasts; (C) cells immunized against the whole yeast; and (D) cells immunized against pure isolated cell wall.*

The adsorbed sera (obtained from immune sera against any fractions of the fungus) behaved as normal sera and showed almost the same rate of phagocytosis in both normal and immune cells (Fig. 2 and 3).

DISCUSSION

In the majority of the reported experiments in vitro concerning phagocytosis of *H. capsulatum* by any kind of phagocyte, the authors used the yeast phase of the fungus. Consequently, there is no or very little information on the protoplasts and their immunogenic properties. The purpose of the present report was to study the

TABLE 1. *Percentage of phagocytosis of Histoplasma capsulatum by immune and normal rabbit macrophages*

Fraction	Normal macrophage (%)	Macrophages immunized against protoplasts (%)	Macrophages immunized against yeast (%)	Macrophages immunized against the cell wall (%)
Phagocytized yeast cell	32.4	45.7	58.6	65
Parasitized macrophages	48.7	63	65.8	65.5
Avg no. of yeast/macrophage	1.5	3.5	5	6.5

TABLE 2. *Average percentage of phagocytosis of Histoplasma capsulatum by normal and immune rabbit macrophages*[a]

Fraction	Avg percentage of phagocytosis								Mean ± standard deviation
	1[b]	2	3	4	5	6	7	8	
Normal macrophages	32.2	32	31.9	32.1	31.6	32	31.8	32.1	32.4 ± 1.7
Macrophages immunized against protoplasts	47.5	46.2	45.5	44.6	46.8	45.8	44.4	45.1	45.7 ± 1.64
Macrophages immunized against yeast cells	58.6	58	59.5	58.8	58.3	57.8	59.2	57.6	58.6 ± 0.57
Macrophages immunized against the cell wall	66.6	67.3	63.2	61.9	68.2	67	63.3	62.6	65 ± 2.52

[a] Data are from eight experiments.
[b] Number of experiments.

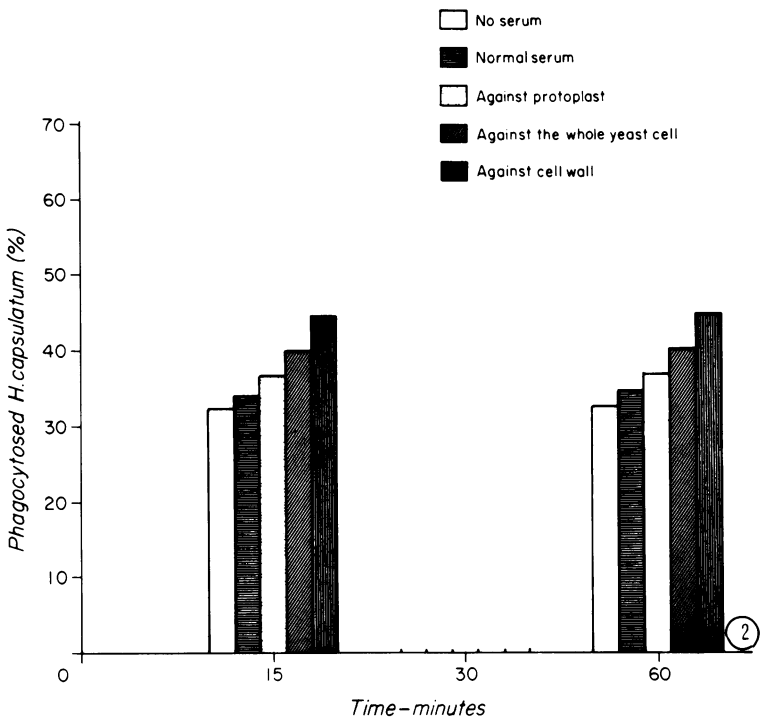

FIG. 2. *Mean percentage of phagocytosed yeast cells per cover glass by normal macrophages incubated with different types of immune sera.*

immunology of the protoplasts of *H. capsulatum*. The correlation study between the cell count by examination of the stained cover glass preparations and electron microscopy examination indicated that phagocytosis and digestion of the protoplasts do not occur at all.

A working hypothesis to explain this phenomenon was that the macrophages apparently release a substance into the medium that is toxic to the protoplasts. Evidence was gained from the fact that the protoplasts could survive for at least 2 h in the control medium. By changing once more the macrophage culture medium and incubating the protoplasts with macrophage, the destruction of protoplasts occurred in the first 15 min of contact. We once observed two destroyed protoplasts within the macrophages from the sample that was taken immediately after incubation for 15 min, but we are not completely sure that the cell wall was indeed absent (Fig. 4). The yeast cells were phagocytosed by cells from normal and immune animals (Fig. 5–8), but the rate of phagocytosis was almost double by immune cells. The present report shows results different from that of Miya and Marcus (15) concerning the similarity found in the phagocytic rates between immune and normal macrophage. The conflicting results are probably due to differences in the techniques used.

Immune sera against protoplasts increased the rate of phagocytosis compared with the normal serum and activated the phagocytes as well as did alkaline-insoluble fractions of the cell wall.

The working hypothesis that phagocytes from immunized animals and in the presence of different sera are endowed with an increased capacity to phagocytose in the presence of antibody was studied.

In our experiments, the constants were: (i) alveolar macrophage from normal and immunized animals; (ii) culture medium 199; and (iii) incubation temperature. The variants were: (i) normal serum; (ii) immune serum against protoplasts; (iii) immune serum against the whole yeast cell; (iv) immune sera against isolated cell wall; and (v) adsorbed sera used above.

From the results obtained in the experiments and in the precipitin and immunodiffusion tests, antibodies appeared to play a great role in phagocytosis in either normal or immune macrophages. However, in the case of protoplasts this is very doubtful, since we think that the increased rate of ingestion and the ring-type

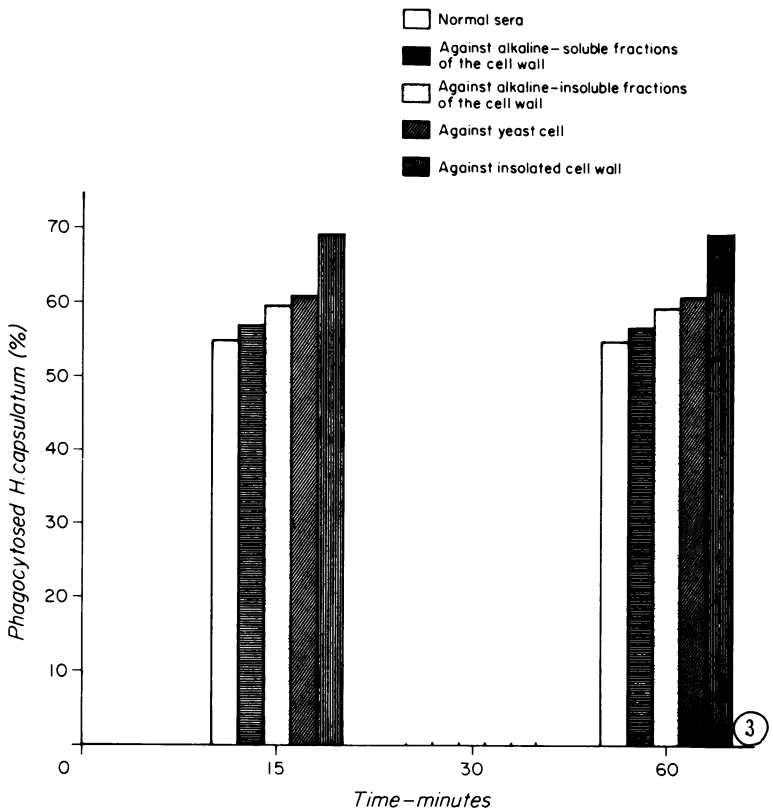

FIG. 3. *Mean percentage of phagocytosed yeast cells per cover glass by immunized macrophages against yeast cells incubated with different types of immune sera.*

TABLE 3. *Results obtained in serological tests with Histoplasma capsulatum using three different fractions of the fungus and histoplasmin as antigens in the presence of three different antisera from experimentally infected rabbits*

Antigen	Immune sera against:	Immuno-diffusion	Precipitin
Complete yeast cell	Complete yeast cell	+	+
Protoplasts	Protoplasts	–	+
Isolated cell wall	Isolated cell wall	+	+
Histoplasmin	Protoplast	–	+

response in the precipitin test using immune sera against protoplasts could be due to impurity of the antigens used to sensitize animals. This may be caused by difficulties in separating protoplasts from the yeast. We could not often obtain 100% pure protoplasts. The main conclusion to be drawn is that in spite of the differences in the phagocytosis, the serological behaviors of cell wall and protoplasts are rather similar. The increased rate of ingestion by the immunized rabbit macrophage against protoplast, the effect of antiprotoplast sera on normal as well as immune phagocytes, and a positive definite precipitation ring, using histoplasmin as well as lyophilized protoplasts, may indicate that the protoplast possesses substances serologically related to those of the cell wall, or that an antigen(s) active in initiating a response in sensitized rabbits is present in both cell wall and protoplast. Nevertheless, isolated cell wall proved to be a much stronger antigen than the whole yeast cell and protoplasts. It is interesting to note that, in the present report, the viability of the yeast cells within the macrophage was also studied by assessing the ability of yeasts recovered from immune macrophages to germinate in a suitable medium and to initiate intracellular growth in normal macrophage. Seventy per cent of the yeast cells recovered after 24 h from immune phagocytes were viable and able to grow within normal macrophage.

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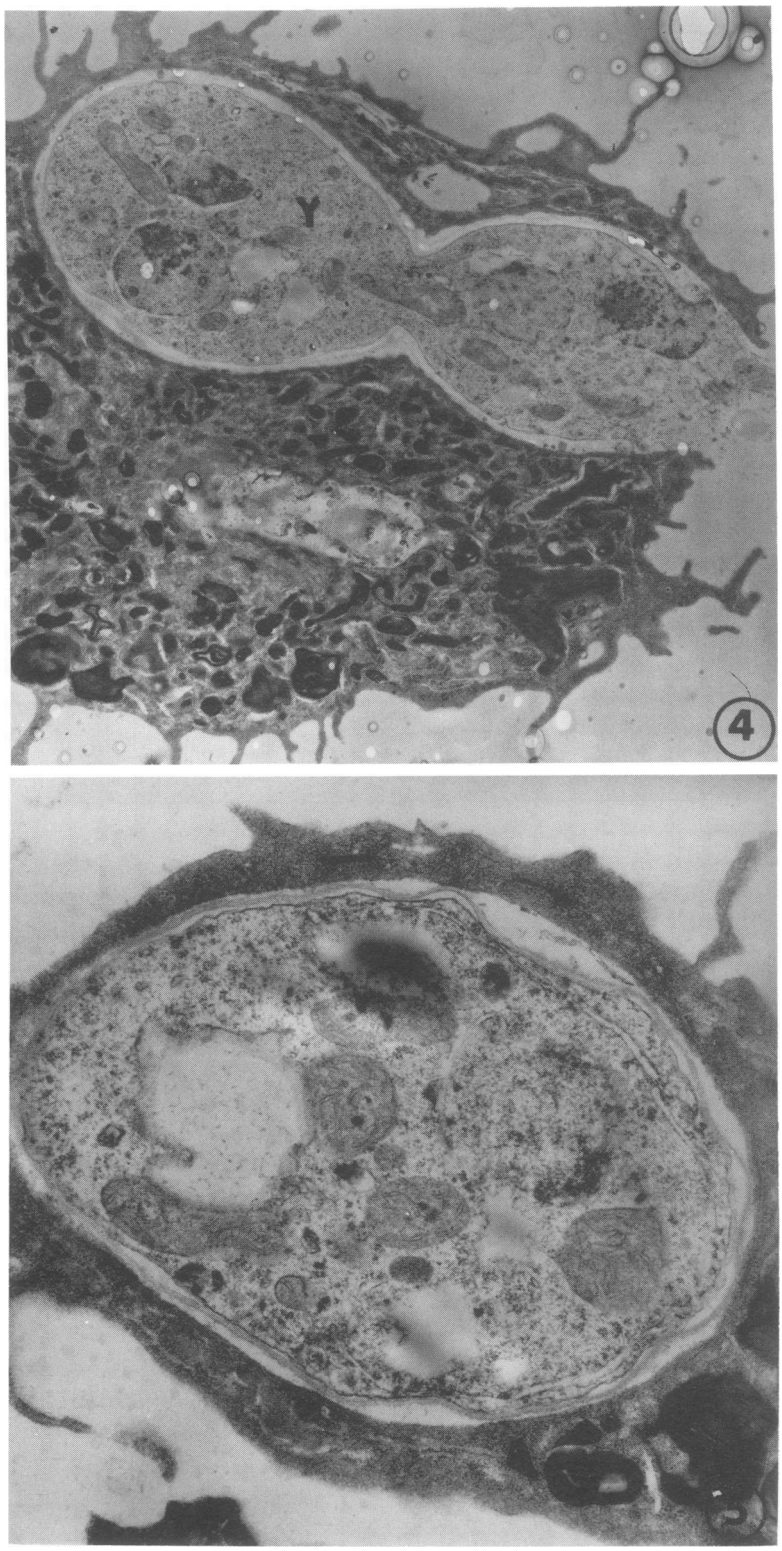

FIG. 4 AND 5. *Yeast cells (Y) phagocytosed by alveolar cells from a normal rabbit. Macrophages photographed 15 min after incubation with yeast cells of Histoplasma capsulatum.*

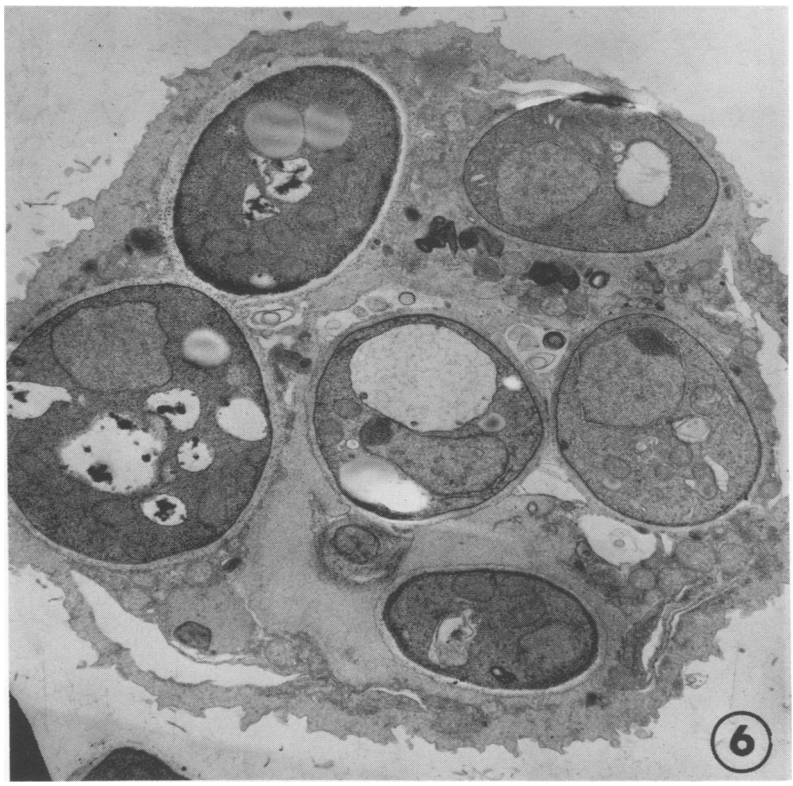

FIG. 6. *Phagocytosis of yeast cells of Histoplasma capsulatum by the alveolar macrophages of a rabbit sensitized against the whole yeast cells of the fungus.*

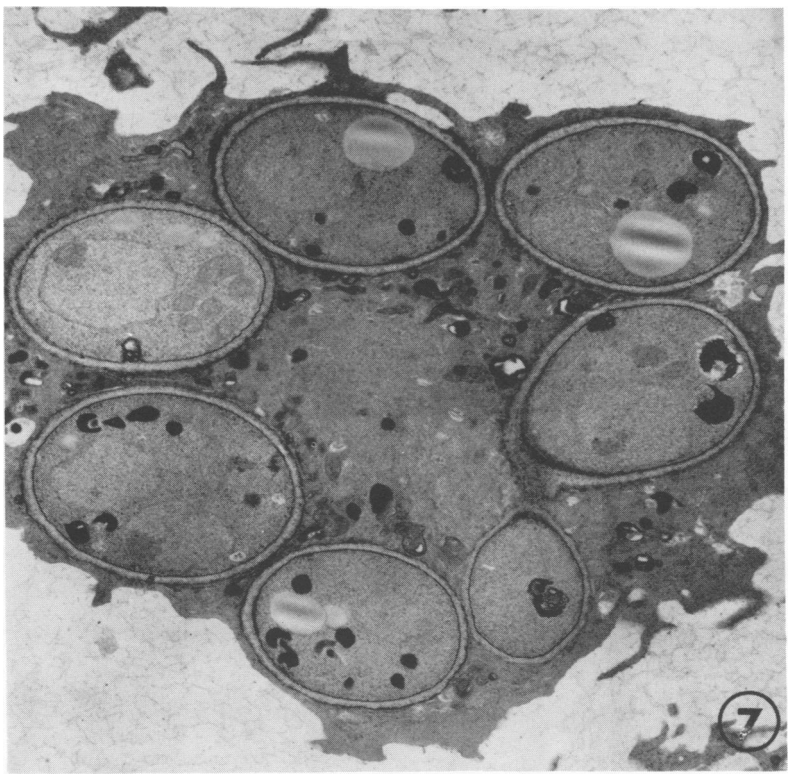

FIG. 7. *Yeast cells phagocytosed by macrophages sensitized against the isolated cell wall of the yeast.*

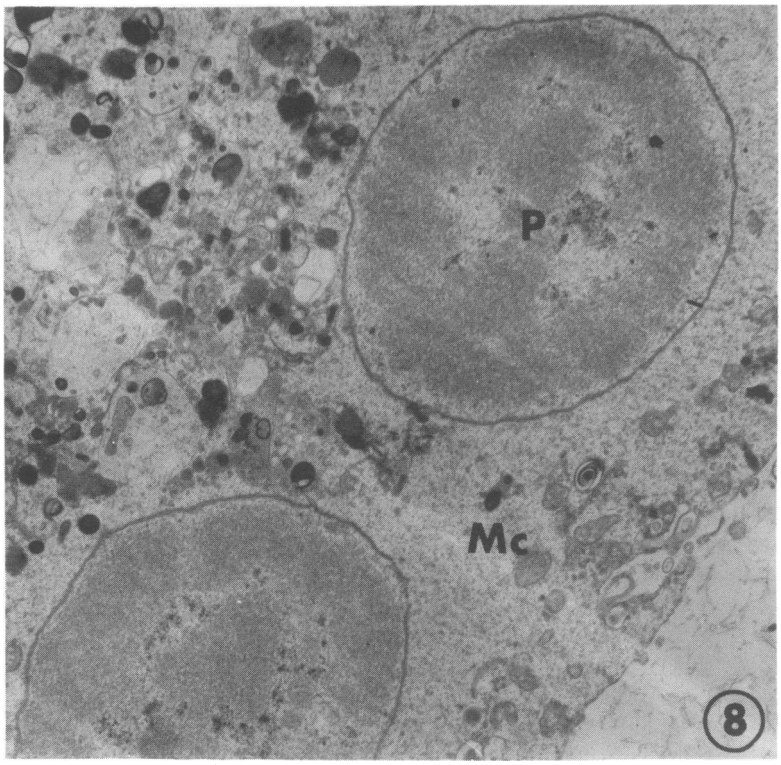

FIG. 8. *Two unhealthy protoplasts (P) phagocytosed by the normal macrophages (Mc). The protoplasts were photographed immediately after the first contact with the alveolar cells.*

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