INFECTION AND IMMUNITY, June 1980, p. 963-971
0019-9567/80/06-0963/09$02.00/0

Vol. 28, No. 3

In Vitro Phagocytosis of *Candida albicans* by Peritoneal Mouse Macrophages

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The ability of sensitized mouse peritoneal macrophages to phagocytose and inhibit *Candida albicans* was studied in an in vitro system. Mice were sensitized to *C. albicans* by intraperitoneal infection with viable organisms or by intracutaneous injection of heat-inactivated cells in Freund complete adjuvant. Development of delayed hypersensitivity to *C. albicans* was evaluated by footpad tests with cytoplasmic and cell wall antigens as well as by macrophage migration inhibition by these antigens and by whole heat-inactivated cells. Inhibition of macrophage migration by heat-inactivated cells was significantly greater when the mice were sensitized by viable organisms. The macrophages from these mice were also larger and showed a greater ability to inhibit germ tube production by phagocytosed yeasts. This suggests that macrophages may play a protective role in infection by *C. albicans*.

Phagocytosis of *Candida albicans* by human leukocytes has been studied in both healthy and immunodeficient individuals (3-6, 14, 16-22, 40). It appears that the role of the neutrophil in resistance to *C. albicans* is related to its general bactericidal capacity (32) and depends partially on an active myeloperoxidase-halide-H_2O_2 system (17-19). Other candidacidal systems also seem to be present in human neutrophils and monocytes (15, 16). Investigation of phagocytosis of *C. albicans* by neutrophils in animals has confirmed the ability of these cells to ingest and kill yeast organisms (1, 8, 20, 29, 39). However, observations on in vitro phagocytosis of *C. albicans* by peritoneal macrophages (30, 33, 36, 43) suggest that ingestion of the invading yeast by these phagocytes may help to spread rather than contain the infection (36).

Studies with various microorganisms other than *C. albicans* have shown that phagocytosis by macrophages and increased host resistance result from a state of sensitization, as reflected by delayed hypersensitivity (24-27). It was thus considered of interest to study the effect of sensitizing mice to *C. albicans* by infection and by immunization on the ability of their macrophages to phagocytize the organisms in vitro. The delayed hypersensitivity reactions elicited were correlated with two macrophage functions: inhibition of migration and phagocytic activity.

MATERIALS AND METHODS

C. albicans strain B311 serotype A (obtained from H. F. Hasenclever, National Institutes of Health, Bethesda, Md.) was maintained by monthly transfers on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.). Female albino Swiss mice (TO strain) weighing 20 to 25 g were used.

Cultures of *C. albicans* grown for 48 h on Sabouraud dextrose agar in large petri dishes were harvested and washed by centrifugation at 1,500 × *g* for 10 min. Four antigens were prepared from these cells.

To prepare whole heat-inactivated cells (antigen 1), cells suspended in saline were inactivated by heating to 65°C three to four times for 6 to 7 h, freeze-dried, and made up to 50 mg/ml (2×10^8 cells/ml). Viability was tested by staining with 0.1% methylene blue.

To prepare cytoplasmic antigen (CPA; antigen 2), packed cells were suspended in normal saline containing rust-resisting steel balls and vibrated in a Mickle apparatus for 20 min at maximal amplitude. After this time 90% of the cells had disintegrated, and the cell debris was separated by centrifugation at 2,000 × *g* for 30 min. The supernatant fluid was dialyzed, freeze-dried, and made up to 50 mg/ml. This antigen contained 52.4% protein (determined by the method of Lowry et al. [23]) and 32% carbohydrate (determined by the anthrone method).

To prepare cell wall antigens (CWA; antigen 3), packed cells were extracted with 33% (wt/vol) chloral hydrate at 80°C for 80 min and the extract was precipitated in cold acetone (−20°C) overnight. The precipitate was separated by filtration, washed with acetone and ether, and made up to 50 mg/ml. This antigen contained 1.4% protein and 93.6% carbohydrate.

To prepare viable cells (antigen 4), cells were suspended in saline to give 8×10^6 viable cells per ml (equal to 2 mg/ml, dry weight). Viability was tested with methylene blue.

These antigens were used as follows: antigens 1 and 4, for sensitizing the mice; antigens 2 and 3, for skin (footpad) testing; antigens 1, 2, and 3, for the macrophage migration inhibition (MMI) test; antigens 2 and 3, to estimate precipitating antibodies; and antigen 1, to estimate the agglutination titer.

The mice were divided into three groups and treated as follows: group M_1 (40 mice) was sensitized with one intraperitoneal injection of 0.5 ml of antigen 4; group M_2 (60 mice) was sensitized by inoculating the front footpads and shoulders with 1.5 to 20 mg of antigen 1 in Freund complete adjuvant; and group M_3, the control group (60 mice), was treated the same way as group M_2, but with normal saline instead of antigen.

Skin tests (footpad). Three weeks after sensitization, samples of 11 to 20 mice from each group were tested by injecting one rear footpad with 0.01 ml of saline containing 50 mg of antigen 2 and the other rear footpad with a similar amount of antigen 3. Footpads were measured with a Schnelltaster Caliper before injection, and the net thickening was recorded at 4, 24, 48, and 72 h.

MMI (7, 10). Macrophages were obtained from peritoneal exudates of 20 mice in each group 3 days after intraperitoneal injection of 1 ml of sterile light mineral oil (Bayol F). The mice were killed and exsanguinated, and the opened abdomens were washed out twice with 5-ml portions of cold Hanks balanced salt solution. The wash fluids in each group were pooled, and the aqueous and oily phases were separated. The cells were separated by centrifugation, washed twice with Hanks solution, suspended in medium 199 containing antibiotics and 15% heat-inactivated fetal calf serum, and made up to a 10% suspension, estimating that 0.1 ml of packed cells equals 7×10^7 cells when centrifuged for 5 min at 1,000 rpm ($95 \times g$) at 4°C. Capillary tubes (1.3 to 1.5 by 75 mm) were filled with the cell suspension (2×10^7/ml), centrifuged at $95 \times g$ for 5 min, and cut at the cell-fluid interface. The portions containing the cells were placed on the bottom cover slip of Mackaness-type chambers of 1-ml capacity, two tubes in each chamber. Chambers were closed with cover slips, sealed, and filled through side holes with medium 199 containing 15% fetal calf serum and various antigen concentrations; control chambers contained the medium and fetal calf serum only. Duplicate chambers were set up with each concentration of each antigen. The following antigens were used: antigen 1 at concentrations of 2×10^6, 1×10^7, and 4×10^7/ml; and antigens 2 and 3 at concentrations of 125, 250, and 500 μg/ml and also at 1 and 2 mg/ml. Chambers were incubated at 37°C and examined after 24 and 48 h. Areas of migration were measured by planimetry after projecting microscopic images of fans of migrating cells from capillary tubes onto graph paper. The absolute area of migration was calculated by reference to a projected standardized square millimeter. The extent of migration of cells was expressed according to the following formula:

$$\left(\frac{\text{average area of migration with antigen}}{\text{average area of migration without antigen}}\right) \times 100 = \text{percentage migration with antigen.}$$

The average area was calculated from four capillary tubes.

Phagocytosis by peritoneal macrophages. Macrophages were obtained from oil-induced peritoneal exudates as described for MMI, but using 3-ml portions of medium 199 instead of Hanks solution. Fluids from two mice were pooled, the cells were counted, and suspensions containing 10^6 macrophages were placed in duplicate Leighton tissue culture tubes containing specially washed cover slip pieces. The tubes were incubated for 1 h at 37°C, and the fluid was poured, leaving behind those cells that adhered to the cover slip. One-milliliter portions of fresh medium 199 containing 2×10^6 viable cells of a 24-h-old *C. albicans* culture and 0.1-ml portions of immune or normal serum were introduced into the tubes. Tubes were then gassed with a mixture of 5% CO_2 in air, tightly plugged, and incubated for periods of 1 to 4 h at 37°C. After incubation the cover slips were fixed in methanol for 1 min, rinsed with distilled water, stained by the Jenner-Giemsa method, and examined under oil immersion. For estimating germ tube (pseudomycelium) production, 50 macrophages containing phagocytosed *C. albicans* were counted and germ tube-producing cells were expressed as a percentage of the total number of yeast cells.

Antibody detection. Precipitating antibodies were detected in pooled sera of 10 mice (collected at the same time that peritoneal exudates were obtained), using double-diffusion tests in agar gel with antigen 2 (10 mg/ml) and antigen 3 (1 mg/ml). Agglutinating antibodies were detected by titrating doubling dilutions of serum (0.2-ml volumes) with antigen 1 (0.04-ml volumes of 40×10^6 cells/ml).

RESULTS

Skin (footpad) reactions. Skin reactions to both antigens measured by footpad swelling were maximal at 24 to 48 h and were generally stronger with antigen 2 (CPA) than with antigen 3 (CWA). The average swelling to CPA at 48 h in treated groups was 0.43 mm (0.02 mm in controls) compared with 0.29 mm with CWA (nil in controls).

The most pronounced reactions to both these antigens were seen in group M_2 (immunized mice), averaging 0.68 mm (standard deviation [SD] ± 0.18) with CPA and 0.46 mm (SD ± 0.11) with CWA. Group M_1 (infected mice) had an average footpad swelling of 0.18 mm (SD ± 0.05) with CPA and 0.12 mm (SD ± 0.04) with CWA (Fig. 1). There was a mild reaction in the M_3 control group, especially with CWA at 24 h. All reactions declined sharply after 72 h, except that to CPA in group M_1.

Circulating antibodies. Agglutination titers in the pooled sera of both groups M_1 and M_2 were >256. Precipitin reactions with CPA were strong in group M_2 and weak in group M_1 sera; CWA did not elicit a reaction in these sera. Group M_3 (control) sera gave no reaction to any of the antigens.

MMI. The inhibition of migration of peritoneal macrophages in the presence of three of the antigens is shown in Fig. 2. Inhibition was most pronounced in group M_1 in the presence of heat-inactivated cells (70%). There was far less inhibition with CWA (38%), and none with CPA.

Macrophages of group M_2 showed strongest inhibition in the presence of CPA (46%), less inhibition with CWA (39%), and none with heat-inactivated cells. (The above figures represent the average MMI obtained from various concentrations of each antigen.) Macrophages of control group M_3 were also inhibited by the three antigens. Only MMI of group M_1 in the presence of heat-inactivated cells and of group M_2 in the presence of CPA was statistically significant as compared with that of control group M_3 ($0.01 < P < 0.05$ and $P < 0.01$, respectively).

Phagocytosis of *C. albicans* yeast cells by peritoneal macrophages and germ tube production. One hour after the addition of *C. albicans* yeast cells to peritoneal macrophages of groups M_2 and M_3, many yeast cells were seen inside the macrophages (Fig. 3A). Most contained between one and three yeast cells, though up to seven cells were seen. No significant differences in phagocytic activity were noticed between the two groups, and in both a number of the phagocytosed cells had started to germinate. After an additional hour, the number of germ tubes had increased (Fig. 3B), and the maximal countable number of germ tubes was reached after 3 h (Fig. 4A). By the end of 4 h long mycelial filaments were produced, forming a network that completely engulfed the macrophages (Fig. 4B). A significant difference was observed in the timing of events in group M_1 macrophages compared with those of groups M_2 and M_3: 1 h after the addition of *C. albicans* yeast cells no germination had taken place, and after 2 h most intracellular yeasts were still dormant with only an occasional germ tube (Fig. 5A). By 3 h the number of germinating cells had increased (Fig. 5B), and at 4 h the picture was similar to that shown in Fig. 4A for groups M_1 and M_2. There was thus a lag period of about 2 h in the germination of phagocytosed yeast cells in group M_1 macrophages. Another difference between group M_1 and groups M_2 and M_3 was in the morphology of the macrophages: those in group M_1 were larger and possessed expanded nuclei. The percentage of germ tube production by phagocytosed *C. albicans* yeast cells in the different groups is shown in Fig. 6. The differences observed between normal and immune mice (M_2 and M_3) and infected mice (M_1) were statistically significant ($0.01 < P < 0.05$).

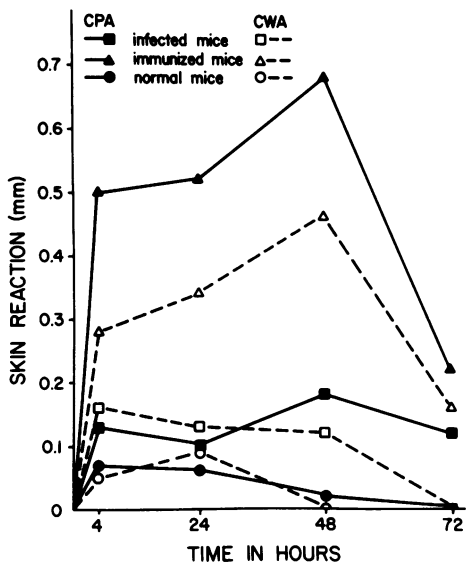

FIG. 1. *Skin (footpad) reactions to CPA and CWA of C. albicans in normal mice (M_3) and in mice immunized (M_2) and infected (M_1) with C. albicans.*

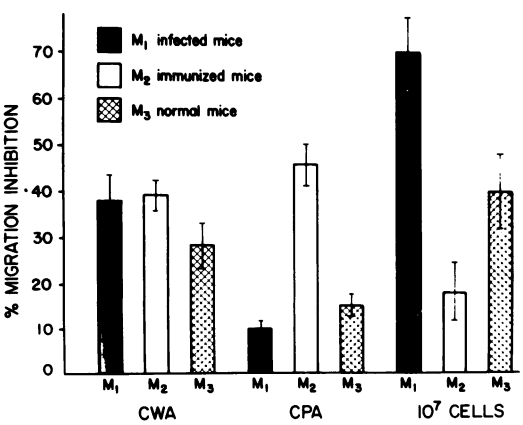

FIG. 2. *Percentage MMI of normal mice (M_3) and of mice immunized (M_2) and infected (M_1) with C. albicans by CPA and CWA and heat-inactivated cells of C. albicans.*

DISCUSSION

Many microorganisms die within minutes of being phagocytosed by macrophages; others are facultative intracellular parasites that are able to multiply and survive (i.e., species of *Mycobacterium*, *Brucella*, and *Salmonella*), and these latter invariably cause delayed hypersensitivity toward the corresponding microbial antigens (24). A definite relationship exists between acquired cellular resistance to infection and delayed hypersensitivity, but the peak of the former does not always correspond with that of the latter. In some infections, resistance is at its peak at the onset of the delayed reaction; in others, it occurs when this reaction is already fading (25, 28, 33). In all cases, macrophages of actively infected animals show greatly enhanced nonspecific microbicidal properties (11, 24, 27, 28).

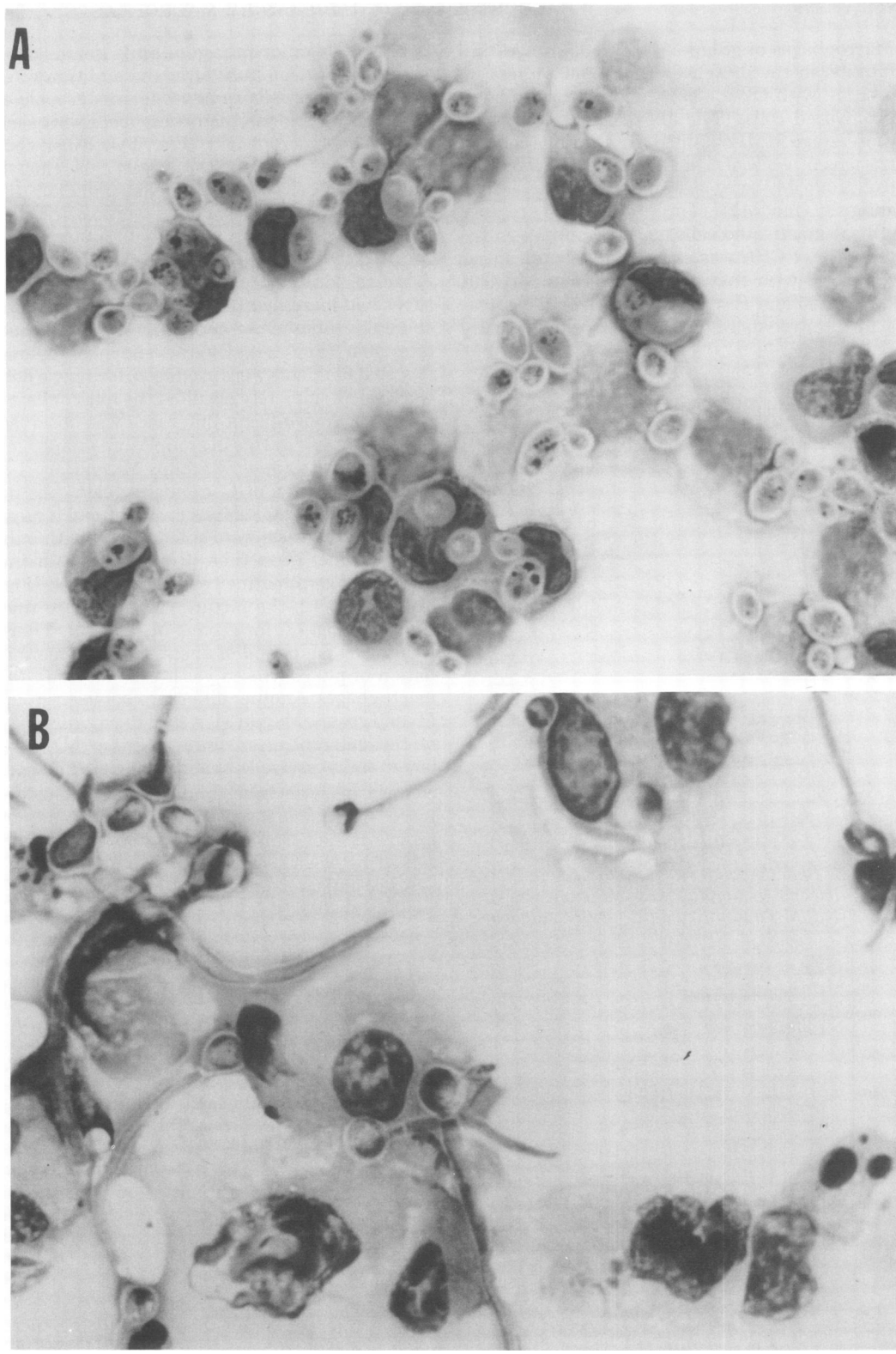

FIG. 3. *Phagocytosis of C. albicans 1 h (A) and 2 h (B) after addition of peritoneal macrophages of mice immunized with C. albicans (M_2).*

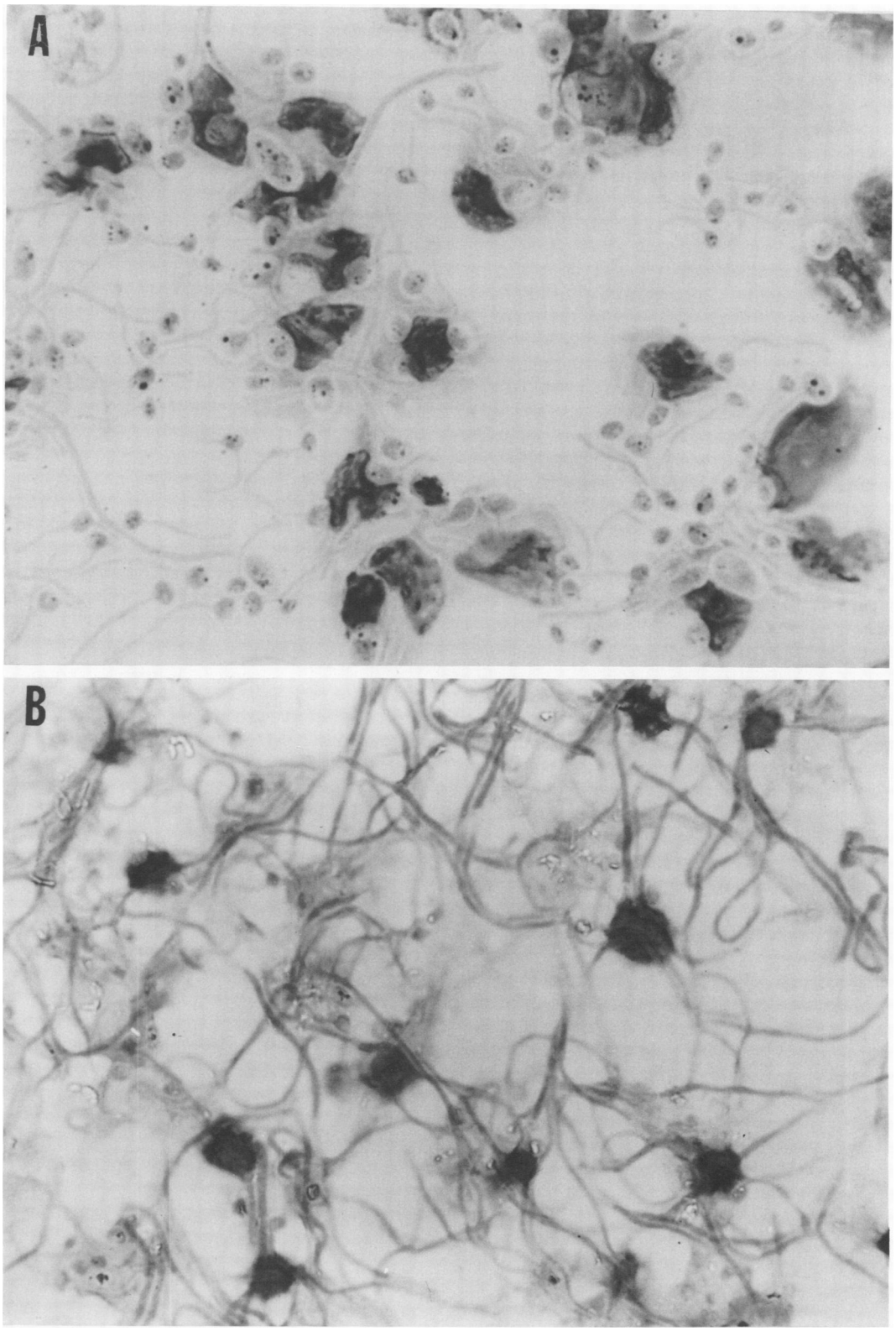

FIG. 4. *Phagocytosis of C. albicans 3 h (A) and 4 h (B) after the addition of macrophages of mice immunized with C. albicans (M_2).*

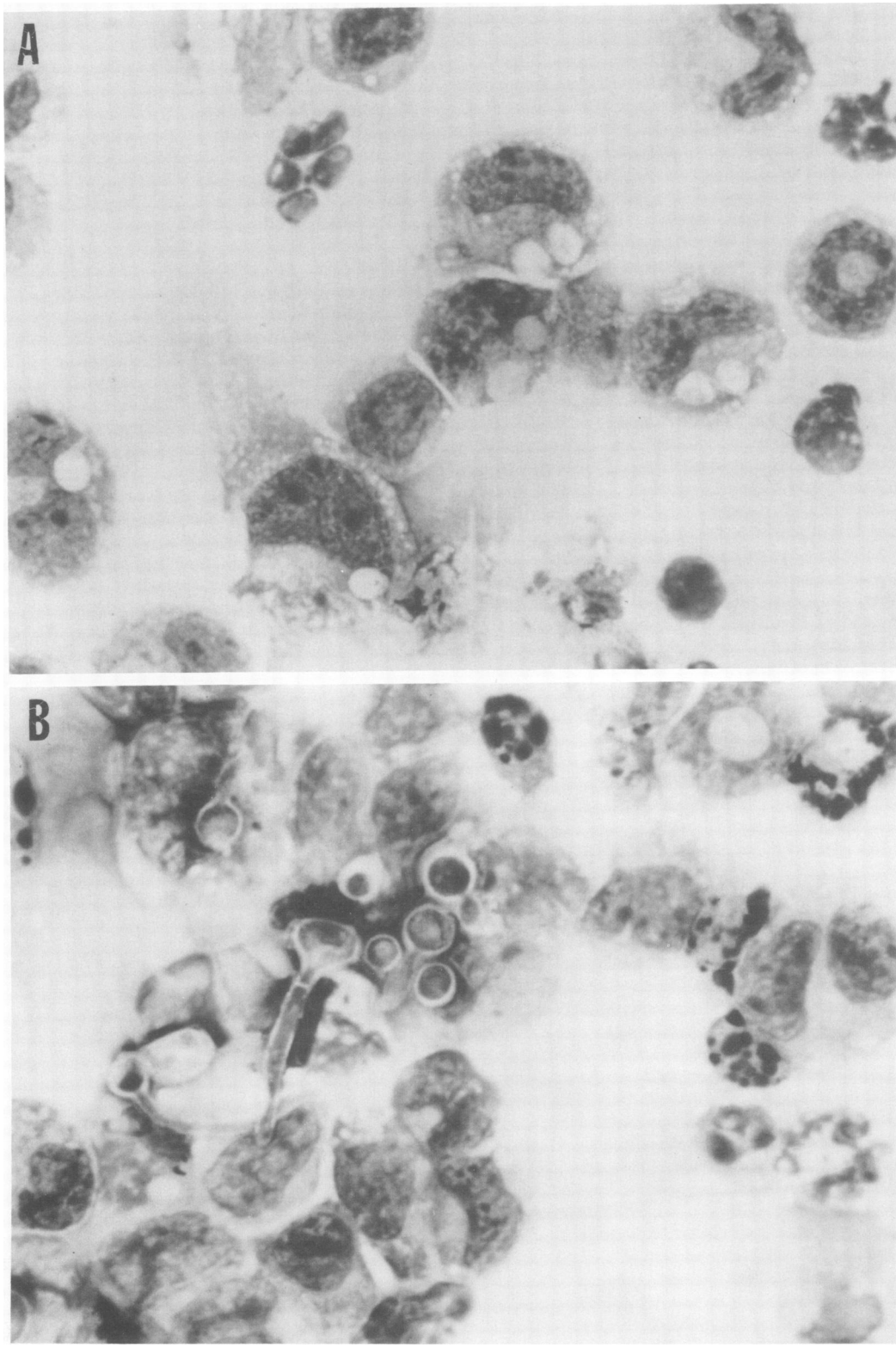

FIG. 5. *Phagocytosis of C. albicans 1 h (A) and 3 h (B) after the addition of macrophages of mice infected with C. albicans (M_1).*

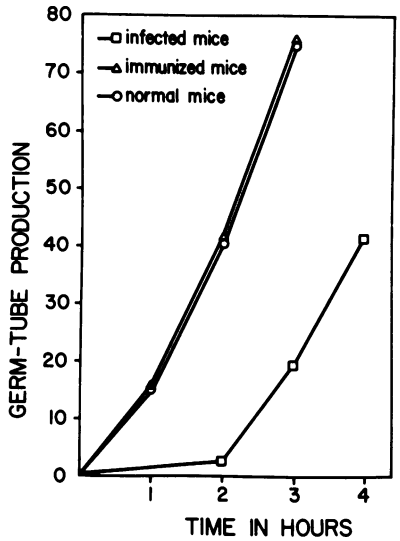

FIG. 6. *Germ tube production by C. albicans yeast phagocytosed by macrophages of normal mice (M_3) and by macrophages of mice immunized (M_2) and infected (M_3) with C. albicans.*

C. albicans, a dimorphic organism existing in both yeast and mycelial forms, exhibits a unique behavioral pattern with regard to macrophages. After being engulfed as a yeast, it survives and causes delayed hypersensitivity in the infected host. Although behaving in this respect like other intracellular parasites, it does not multiply in its yeast form inside the macrophages. After a short dormant stage, the ingested yeasts convert into a mycelial (or pseudomycelial) phase, producing germ tubes that grow out from the phagocytes and eventually completely engulf and destroy them (36, 43). The speed with which this chain of events occurs was found to correspond to the degree of virulence of the *Candida* strain (36). Thus, a basic question concerning macrophage function in regard to *C. albicans* is whether phagocytosis is an enhancing factor in infection which assists in the process of dissemination, or whether phagocytosis helps to destroy the invading yeast.

In the experiments described here, skin (footpad) reactions in all three groups were maximal at 24 to 48 h. They were therefore classified as a delayed hypersensitivity response, although they probably contained a strong Arthus component as suggested by the presence of circulating antibodies.

Skin reactions were considerably weaker in mice infected with viable cells than in those immunized with heat-inactivated cells. It is possible that delayed hypersensitivity develops more slowly in the former and that it had not yet reached its peak after 3 weeks. Alternatively, the different methods of sensitization used might have been responsible. There seemed to be a stronger Arthus component in the mice sensitized with heat-inactivated cells, and this was accompanied by a more prominent precipitin reaction. In infected mice the skin reaction to cytoplasmic antigen did not decline sharply after 72 h, and it is possible that these mice showed a true delayed reaction.

Inhibition of macrophage migration by heat-inactivated cells was significantly higher in infected than in immunized and control mice. Macrophages from infected mice were abnormally large with swollen nuclei and showed enhanced phagocytic activity and enhanced inhibition of germ tube formation of ingested yeast cells compared with macrophages from mice immunized with heat-inactivated cells and control mice.

Failure of immunization to increase candidacidal activity has been demonstrated in alveolar macrophages from rabbits (1). Mackaness (25) has shown infection to be the best way of sensitizing macrophages and of potentiating a cell-mediated response to intracellular facultative parasites. Wagner (41) has demonstrated that only macrophages from an infected peritoneal cavity exhibit a high degree of in vitro inhibition toward certain microorganisms. His interpretation was that only local inflammation caused by intraperitoneal infection leads to the influx of T-cell mediators, converting the peritoneal cavity to one rich in T cells and poor in B cells and producing macrophages with inhibitory capacity. This interpretation would fit the present data. Nevertheless, such macrophages tested in an isolated system are not a representative sample of those encountered by microorganisms in the intact host. The inhibition displayed by macrophages from infected mice in vitro might be weaker than that which occurs in intact animals, where "cooperating" cells are available (38). This agrees with studies showing that preinfection of mice with *C. albicans* resulted in increased host resistance to infection (30, 31) as well as with histological evidence of lesions containing phagocytosed yeasts within macrophages (37).

The in vitro inability of the macrophages of infected mice to kill ingested *C. albicans*, as compared with the killing of this yeast by normal neutrophils, could be the result of the absence of a myeloperoxidase-halide-H_2O_2 system in the former (1, 12, 13). In humans whose neutrophils lack this system, destruction of phagocytosed yeast cells is minimal and probably carried out by other, less effective, hydrolytic mechanisms

(chymotrypsin-like cationic proteins, lysosomal cationic proteins [9], and C3 [42]).

The presence of a weak skin response in unsensitized mice to *C. albicans* antigens, particularly to CWA, could be due to the fact that mice normally harbor yeasts in their gastrointestinal tracts. These yeasts probably have common antigens with *C. albicans* (28, 34). This might also explain the weak inhibition of macrophages of control mice by *C. albicans* antigens.

ACKNOWLEDGMENT

This study was supported by a grant from the Chargin Endowment.

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