INFECTION AND IMMUNITY, Feb. 1973, p. 231-236

Vol. 7, No. 2
Printed in U.S.A.

Growth of *Cryptococcus neoformans* Within Human Macrophages *In Vitro*

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Received for publication 4 August 1972

Macrophages cultured from human peripheral blood monocytes were infected with *Cryptococcus neoformans* in vitro. Although *C. neoformans* were actively ingested, there was no detectable intracellular killing by macrophages. Over 2 days or more, intracellular fungi grew more rapidly than a corresponding inoculum of extracellular cryptococci growing in tissue culture medium containing human serum. Macrophages were induced to develop into cells which appeared to be activated by morphological and phagocytic criteria. However, these activated cells did not acquire an ability to kill or inhibit intracellular growth of *C. neoformans*. There were no detectable differences between macrophages from normal subjects and those from cryptococcosis patients.

As reviewed elsewhere, cellular host defense mechanisms against *Cryptococcus neoformans* are probably more important than humoral mechanisms (7). Previous studies have shown that neutrophils and monocytes from cryptococcosis patients had no defect in intracellular killing of *C. neoformans* when compared with leukocytes from normal volunteers (7). However, possible defects in other cellular host defense mechanisms might still explain an increased susceptibility to *C. neoformans* infection in certain patients. The central role of macrophages in resistance to intracellular infections has been emphasized (10). In vitro studies on resistance to *C. neoformans* have suggested the importance of macrophages interacting with lymphocytes in this infection (8). However, viability of *C. neoformans* within human macrophages has never been studied. Therefore, in the present study, macrophages were derived in culture in vitro from peripheral blood monocytes (3, 5), and infected with *C. neoformans*.

MATERIALS AND METHODS

Leukocyte cultures. As previously outlined, human monocytes were separated from heparinized peripheral blood (7) from 12 normal subjects and 14 cryptococcosis patients. Cells were then suspended in Eagle minimal essential medium (MEM) enriched as outlined elsewhere (15), containing 100 units of penicillin per ml and 100 μg of streptomycin per ml. Cell cultures were performed in 35-mm plastic tissue culture dishes (Falcon Plastics, Los Angeles, Calif.). Each dish contained 2×10^6 monocytes in 2 ml of media (enriched MEM with 15-20% human serum from a normal blood type AB donor). Macrophage cultures were incubated with and without nonadherent cells (mostly lymphocytes) up to 7 days at 37 C in 5% CO_2 before use in phagocytic studies. In selected experiments, leukocytes were cultured with and without 1:100 or 1:200 dilutions of a soluble cryptococcal skin test antigen (1), or with 100/25 units of streptokinase-streptodornase (SK-SD, Lederle Laboratories, Pearl River, N.Y.). Before use, SK-SD was dialyzed twice against phosphate-buffered saline. In some experiments using SK-SD, the nonadherent lymphoid cells were decanted just prior to infection of the monolayer and used for lymphocyte transformation studies. Tritiated thymidine was added overnight to antigen-treated and control cells, and thymidine uptake measured by the technique of Newberry et al. (13).

Inoculum. A poorly encapsulated isolate of *C. neoformans* previously described (7) was grown overnight on Sabouraud agar at 30 C. Phagocytosis of the fungus was quantitated by examination of Wright-stained smears and, in certain experiments, by examination of radiolabeled cryptococci. For the latter, inocula were grown for 7 days on glucose-ammonia agar slants with 200 μCi of ^{14}C-glucose (4.6 mCi/mmole) as the sole glucose source. In other studies, to allow use of a lower inoculum, *C. neoformans* was cultured on Sabouraud agar slants containing 200 μCi of $H_3{}^{32}PO_4$ layered on the surface. Viability of *C. neoformans* was not affected by these levels of radioactivity.

Infection of leukocyte cultures. Leukocyte cultures were infected with *C. neoformans* inocula

ranging from 5×10^3 to 5×10^7. After allowing 20 to 90 min for phagocytosis, culture dishes were washed six times with warm Hanks balanced salt solution to remove organisms. Fungi remaining on the plate were termed "cell associated." Visual counts done on randomly selected Wright-stained plates in each experiment confirmed that 90% or more of these cell-associated fungi appeared to be intracellular. Macrophages usually contained only one organism, though rare macrophages contained three cryptococci with the lowest inoculum and never more than six cryptococci with the highest inoculum. Macrophage monolayers on randomly selected culture dishes were then lysed at timed intervals by exposure to distilled water for 15 min and scraping with a rubber policeman. This did not alter cryptococcal viability. The number of live, cell-associated organisms was then determined by tube dilutions and plate counts. In addition, all washes were saved for dilution and plating to quantitate live organisms in the supernatant fluid. For each subject, duplicate or triplicate culture dishes were harvested at each time interval. When radiolabeled organisms were used, fractions of distilled water lysates from each dish were digested overnight in 0.2 N sodium hydroxide and dissolved in Aquasol (New England Nuclear, Boston, Mass.) for liquid scintillation counting. Such digests were also used for protein determinations by the Lowry technique (9), modifying the reagents to compensate for the sodium hydroxide already present.

RESULTS

Survival of macrophage monolayer. Macrophage monolayers were more dense and more resistant to destruction by cryptococci when nonadherent lymphocytes were not removed during the preparation of the culture. Density of the macrophage population was determined after washing off nonadherent cells, by using grid counts and protein determinations. Loss of macrophages from the monolayer during infection with *C. neoformans* was further decreased when cultures contained SK-SD. In five experiments, cultures containing SK-SD had 1.5 to 8 (mean 6.4) times more adherent macrophages after 5 days in culture than identical cultures without SK-SD. Such macrophage monolayers ingested proportionally larger numbers of *C. neoformans* and resisted destruction by fungi better than control macrophages. Morphologically, macrophages cultured with SK-SD appeared to have more projections and granules than macrophages of the same age from the same subject cultured without SK-SD (Fig. 1). The presence of lymphocytes in cultures was required for expression of this effect of SK-SD. Studies of thymidine uptake by the nonadherent cells in macrophage cultures indicated stimulation of SK-SD-treated cells under these culture conditions. The mean ratio of the thymidine uptake by SK-SD-stimulated compared with unstimulated control cells was 15.8. Stimulated and unstimulated lymphocytes had comparable viability as determined by trypan blue exclusion.

Viability of fungi in leukocyte cultures. By two separate techniques, there was no detectable killing of *C. neoformans* by 3- to 7-day-old macrophages. First, killing was de-

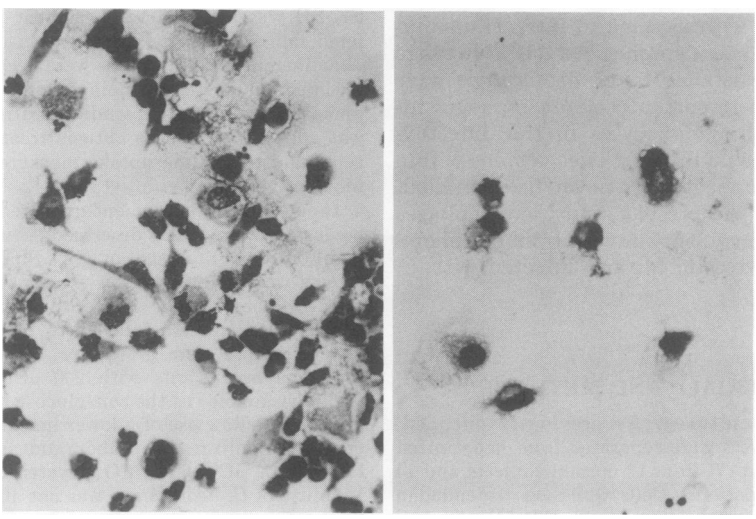

FIG. 1. *Macrophages cultured 5 days with SK-SD (left) and without SK-SD (right). Note the larger size and increased cytoplasmic projections of individual cells and greater cell density of the SK-SD treated monolayer (×500).*

termined by uptake of radiolabeled fungi by macrophages, as outlined by Cline (3). With this technique, the amount of radiolabel associated with viable fungal cells is determined both before and after incubation with macrophages. Excess radiolabel compared to plate counts of cell-associated fungi was considered evidence of killing. In 45 experiments using ^{14}C and 8 experiments using ^{32}P, the disparity between expected and observed radiolabel never amounted to more than 16% of the expected value. As a second means for measuring viability of cryptococci, plates were washed to remove free fungal cells after the preliminary period of phagocytosis, and viable fungi were quantitated at timed intervals by plate counts. In 144 experiments using macrophages from 26 different subjects, the number of total viable fungal cells in the petri dish (both cell monolayer and supernatant fluid) at 1 to 4 hr never decreased more than 13% below the number of viable fungi after the first washing procedure. Variables tested included inoculum size, age of macrophage cultures, presence or absence of lymphocytes, presence or absence of antigen (SK-SD or cryptococcin) during differentiation of macrophages, use of different sera, presence or absence of anticryptococcal antibody, as well as time course of the experiment. Control studies were performed to determine whether absence of killing of *C. neoformans* could have been due to functional impairment of macrophages. Killing of *Listeria monocytogenes* was studied by using the system of Simon and Sheagren, modified to quantitate the number of live intracellular *Listeria* at 20 and 120 min after infection (15). At 20 min, there was a mean of 8.2×10^5 live *Listeria* per petri dish. This fell to a mean of 3.5×10^5 *Listeria* at 120 min, a 57% decrease (range in six experiments 48–72%). Grid counts, protein determinations, and examination of gram-stained smears indicated that loss of adherent macrophages from the culture dishes could not account for this apparent killing. These percentages are similar to values previously reported by Cline (3) using a slightly different system. In addition to the *Listeria* studies, viability of macrophage cultures was determined on randomly selected petri dishes in each experiment prior to infection with cryptococci. All cultures were discarded if viability was 90% or less by neutral red ingestion and trypan blue exclusion. Not only did macrophages fail to kill *C. neoformans*, but they appeared to provide a favorable environment for intracellular growth of the fungi. After the first 3 to 4 hr, the number of organisms in cultures increased steadily. This is best illustrated by representative experiments using low inocula of *C. neoformans*. In 12 subjects, macrophages were cultured for 5 days with lymphocytes but without added antigens (Fig. 2). In these studies, the mean inoculum in both macrophage cultures and media controls was 1.27×10^6 *C. neoformans* per culture. After

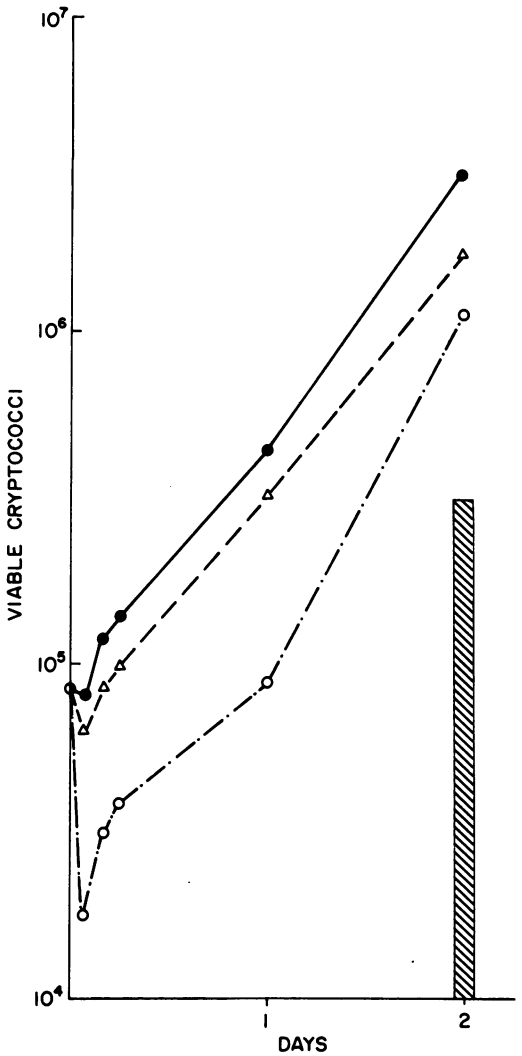

FIG. 2. *Growth of cryptococci within 5 day old macrophages (log scale). The original inoculum in the media control was 1.27×10^6. Although the same inoculum had been added to the macrophage cultures, a portion of the inoculum was removed when extracellular organisms were washed off. The total number of live fungi (●) represents the sum of cell-associated (Δ) plus supernatant (○) fungi. This is contrasted with the number of live fungi in simultaneous controls without macrophages at 2 days (bar).*

initial phagocytosis and washing, the inoculum retained by macrophages averaged 7.01×10^4 cryptococci per culture. After 48 hr, the number of live fungi in macrophage cultures was 10-fold higher than the number of fungi in simultaneous cultures containing only MEM with serum but no leukocytes. This was true even though the media control was not subjected to washing and removal of a portion of the original inoculum. Thus, the macrophages appeared to be more favorable media for growth of cryptococci than tissue culture media containing serum (Fig. 3). It is probable that the large number of cryptococci cultured from the supernatant fluids of these macrophage cultures predominantly represented organisms which grew within macrophages and were later released, rather than extracellular fungi adherent to macrophages which grew extracellularly. Though they exhibited the more advanced morphological changes noted above, macrophages cultured with cryptococcin or SK-SD were no less favorable media for growth of *C. neoformans*.

Attempts were then made to stimulate differentiation of specifically activated macrophages from monocytes by in vitro infection with a low inoculum of cryptococci. Normal subjects with positive cryptococcin skin tests appeared the most likely source of sensitized or "immune" leukocytes on the basis of probable previous exposure to *C. neoformans*. Freshly separated mononuclear cells from eight such subjects were mixed with 7×10^3 *C. neoformans*, placed in cultured dishes, and followed for 6 days (Fig. 4). When cultured with cryptococci in this manner, monocyte monolayers were more dense, and individual cells were larger with more cytoplasmic projections than in control cultures of monocytes without cryptococci. After 3 days in culture, the rate of increase in live cryptococci was almost identical to that seen in the macrophage studies noted above (Fig. 2). The number of live fungi in macrophage cultures at 6 days was almost 100 times greater than the number of fungi in simultaneous control cultures containing MEM and serum but no leukocytes. The apparent fungicidal activity within the first 24 hr of the study is consistent with values previously noted for human monocytes in a different in vitro system (7).

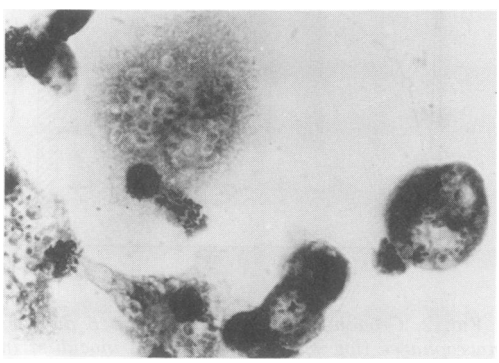

FIG. 3. *Macrophages cultured 5 days and then infected with cryptococci for an additional 48 hr, showing large numbers of intracellular fungi (×1,000). A substantial increase in the number of fungi per macrophage had occurred over the prior 48 hr. At 2 hr after infection, only rare macrophages had more than one intracellular organism, and none more than three.*

Studies with cryptococcosis patients. In parallel studies of phagocytosis, killing, and growth inhibition of cryptococci, as well as macrophage monolayer survival, macrophages from 14 cryptococcosis patients (3 with active disease prior to antifungal therapy, 11 cured) were not significantly different from macrophages from 12 normal control subjects.

DISCUSSION

Studies by Cline have indicated that the human monocyte-derived macrophage was a less effective killer of *Listeria* than the blood neutrophil (3). In the present studies, differentiation in culture from monocyte to macrophage was accompanied by an apparent loss of ability to kill *C. neoformans*. Differentiation from monocyte to macrophage in other systems has been shown to be accompanied by loss of detectable myeloperoxidase (4). Generation of hydrogen peroxide by macrophages has never been demonstrated (3). In view of the central role of the myeloperoxidase-peroxide-halide system in killing of *C. neoformans* by human neutrophils (7), it is tempting to speculate that the inability of macrophages to kill *C. neoformans* might be related to the absence of these mechanisms.

Acquired immunity need not be accompanied by killing of intracellular organisms. For example, in animals with acquired immunity to tuberculosis, intracellular mycobacteria may be viable but nonmultiplying (14). Cellular immunity may then be primarily dependent upon intracellular bacteriostasis (17). However, in the present studies, cultured human macrophages provided a favorable medium for intracellular growth of *C. neoformans*. It was not possible to produce activated macrophages capable of inhibiting intracellular growth of cryptococci. It may be argued that the method of macrophage activation used here

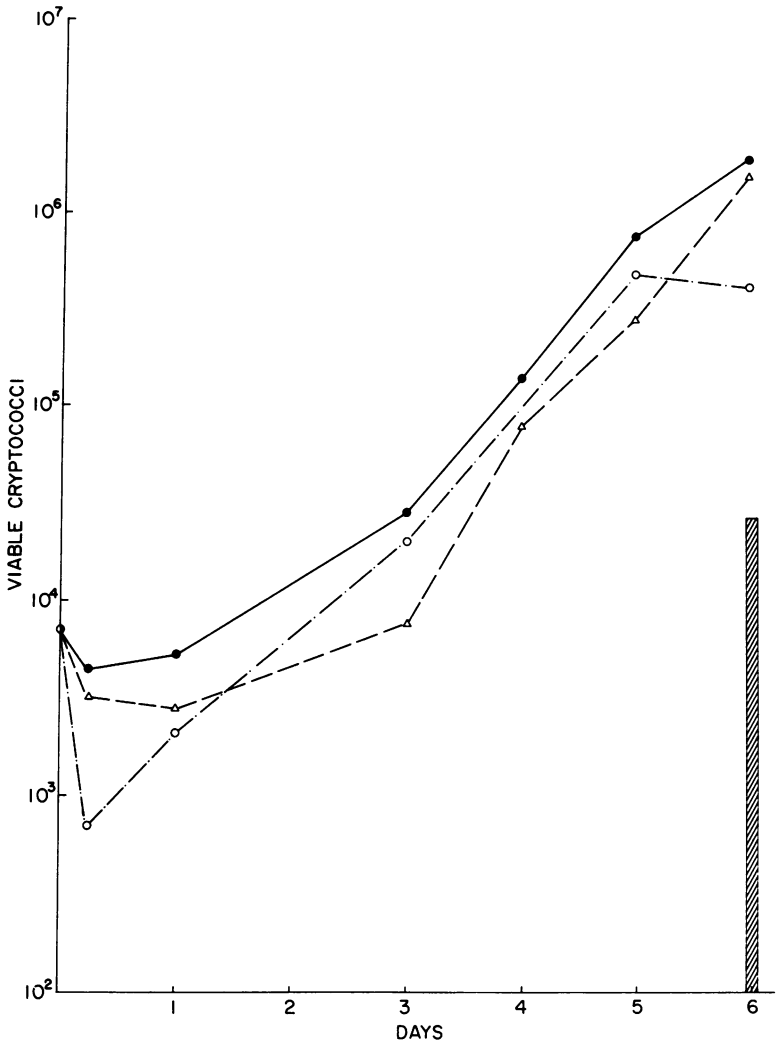

FIG. 4. *Growth of cryptococci within differentiating monocytes (log scale). The mean inoculum in both monocyte cultures and media controls was 7 × 10^3. The total number of live fungi (●) represents the sum of cell-associated (△) plus supernatant (○) fungi. This is contrasted with the number of live fungi in simultaneous controls without macrophages at 6 days (bar).*

was ill-chosen. Several observations mitigate this possibility. In a similar in vitro system, soluble antigens have been shown to induce activated macrophages from sensitized guinea pigs (15). Such macrophages had an increased capacity to kill *Listeria*. In the present study, human macrophages similarly cultured with SK-SD appeared activated by morphological and phagocytic criteria used by others to identify activated macrophages (6, 12, 15). Furthermore, thymidine uptake by nonadherent cells suggested lymphocyte stimulation by SK-SD in this culture system. Finally, in other experiments, macrophages which also appeared to be activated by morphological criteria were produced when fresh monocytes were cultured with low inocula of live cryptococci.

Gentry and Remington have noted that macrophage monolayers in vitro resisted destruction by *C. neoformans* if the macrophages were obtained from animals with a nonspecifically increased resistance to intracellular infections. Killing of *C. neoformans* by these cells was not studied (8). In the present study, stimulated macrophage monolayers were similarly resistant to destruction by cryptococcal infection. However, survival of macrophages was accompanied by increased survival and growth of

cryptococci in the culture because of the apparently favorable intracellular environment for growth. Using different methodology from the present study, Mitchell and Friedman have noted killing of *C. neoformans* by rat peritoneal macrophages (11). However, the amount of killing they observed was extremely low. Most macrophages were unable to kill their phagocytized yeasts, and average killing was only 3 to 49%, depending on the fungal isolate used, despite the fact that macrophages containing multiple organisms were excluded from counts. Thus, these extremely low killing percentages are little different from values in the present study and appear to be easily explainable by differences in isolates, species differences in leukocytes, and experimental methodology. Mitchell and Friedman did not study intracellular growth inhibition of fungi beyond 48 hr.

As reviewed elsewhere, the majority of tissue macrophages appear to be derived from circulating blood monocytes (2). Despite this, there are obvious limitations in making conclusions based upon an in vitro system using cultured peripheral blood cells. However, within these limitations, the present study does not support a central role for the monocyte-derived macrophage in resistance to cryptococcosis in man. Stanley and Hurley noted that *Candida* grew readily within mouse peritoneal macrophages and suggested that such cells might contribute to extension and dissemination of infections (16). Results in the present study raise this as a possibility in cryptococcosis.

ACKNOWLEDGMENT

We would like to thank Charlotte Davis for expert technical assistance and Harvey Simon for helpful advice during the studies.

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