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Nature of the Effector Cells Responsible for Antibody-Dependent Cell-Mediated Killing of *Cryptococcus neoformans*

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Studies were performed to identify the types of human peripheral blood leukocytes capable of killing *Cryptococcus neoformans* in the presence of anticryptococcal antibody in vitro. A total of 24.1 ± 2.7% (mean ± standard error of the mean of four experiments) of the original cryptococcal inoculum survived in a mixed mononuclear cell preparation (approximately 30% monocytes) after 4 h of incubation at 37°C with rabbit anticryptococcal antibody. When phagocytic cells were removed, there was 36.4 ± 4.6% survival in six experiments, compared with 52.8% survival in the presence of purified granulocytes (mean of two experiments) and 96.9 ± 1% survival in the presence of purified T cells. There was never any significant killing in control mixtures that contained leukocytes with normal rabbit serum nor in those that contained anticryptococcal antibody without effector leukocytes. Significant antibody-dependent fungicidal activity was seen with ratios of effector to target cells as low as 6.25:1. These observations indicate that multiple types of peripheral blood leukocytes, excluding T cells, are capable of antibody-dependent fungicidal activity.

Previous studies established that mononuclear cells separated from human peripheral blood could kill the yeastlike fungus *Cryptococcus neoformans* in the presence of anticryptococcal antibody (7). Neither leukocytes in the absence of antibody nor anticryptococcal antibody (with or without complement) killed the fungus. Killing appeared to occur by a nonphagocytic mechanism. The limited capacity of human peripheral blood neutrophils, monocytes (10, 26), and "activated" macrophages derived from monocytes (8) to ingest and kill cryptococci had been noted in previous studies. From these studies, as well as the known histopathology of cryptococcal lesions, it appeared that many organisms, particularly large capsule forms, might not be subject to the intracellular killing mechanisms of phagocytes. In clinical studies, it was also found that the presence of anticryptococcal antibody in the serum of patients with cryptococcosis could be correlated with eventual cure (3, 9). In previous studies of antibody-dependent cell-mediated killing of cryptococci, a mixed population of mononuclear leukocytes was used as effector cells (7). Therefore, further studies were done to determine which types of human peripheral blood leukocytes were capable of fungicidal activity in this system.

MATERIALS AND METHODS

Organisms. Two isolated of *C. neoformans* were used: a small capsule isolate previously described (10), and a large capsule isolate (21710, Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine) with a mean diameter of 18 μm. Organisms were subcultured for 48 h on Sabouraud agar, washed three times in phosphate-buffered saline, and counted in a hemocytometer chamber. Inocula were then diluted in Eagle minimal essential medium (MEM) supplemented as outlined elsewhere (25). The viability of the inocula was then quantitated by tube dilutions and plate counts.

Antiserum. Anticryptococcal antibody (a gift from J. E. Bennett) was raised in rabbits by multiple courses of injection of a small capsule cryptococcal isolate in Freund complete adjuvant. The titer of the antiserum used was 1:512, as determined by the procedure for slide agglutination outlined by Wilson et al. (28). Agglutination was further tested by microscopic examination of specimens handled under the conditions used for incubation with leukocytes (vide infra). The final dilution of antiserum used in incubations (1:4,000) was twice that which gave no discernible microscopic agglutination. Control serum was obtained from the same rabbit before development of antibody. Sera were heated at 56°C for 30 min before use.

Leukocytes. Human peripheral blood was obtained from normal donors and defibrinated with glass beads. From this, mixed populations of mononuclear cells (approximately 30% monocytes plus

70% lymphocytes) were separated by centrifugation on a mixture of Triosil (sodium metrizoate; Glaxo, London, England) and Ficoll (Pharmacia, Uppsala, Sweden) as outlined by Böyum (4). Granulocytes were then separated from remaining erythrocytes by dextran sedimentation, as previously described (4, 10). In some cases phagocytic cells were eliminated by modifications of the technique of Lundgren et al. (20). Defibrinated blood in a siliconized flask was mixed with 1 mg of carbonyl iron (GAF, Manchester, England) per ml that had been sonically treated just before use. Flasks were incubated at 37°C for 60 min on a rocking platform and were swirled by hand every 5 min to ensure mixing. After multiple passages through a magnetic field, the remaining mononuclear cells were separated, using a Triosil-Ficoll mixture as noted above. Such preparations contained 1.5% or less phagocytic cells (usually <1.0%), as judged by Giemsa-stained smears after exposure to 1-μm latex particles or by neutral red ingestion or vital staining with Euchrysine 3R (Ed. Gurr, London, England) (1). The viability of remaining leukocytes was 98.0% or more by trypan blue exclusion or vital staining with fluorescein dibutyrate (6). T cell-enriched populations were prepared by modifications of the technique of Julius et al. (17). Mononuclear cells were separated by using Triosil-Ficoll, diluted in MEM containing 5% fetal calf serum, and incubated at 37°C for 45 min in columns containing washed, sterile nylon wool (LP-1 Leuko-Pak leukocyte filters, Fenwall Laboratories, Morton Grove, Ill.). Effluent cells were washed three times in serum-free MEM before use. By trypan blue exclusion and fluorescein dibutyrate staining, 98.5% of the remaining leukocytes were viable. A total of 89.5% were T cells, as judged by the technique of Jondal et al. for formation of spontaneous rosettes with sheep erythrocytes (16). Only 1.0% had detectable surface immunoglobulins when stained with a rabbit anti-human immunoglobulin preparation according to the method of Grey et al. (14), and no phagocytic cells were detectable by latex particle or neutral red ingestion.

Assay of fungicidal activity. Two-milliliter, flat-bottomed plastic vials each contained approximately 1×10^4 cryptococci plus either anticryptococcal antibody or control normal rabbit serum. To this were added varying numbers of effector leukocytes or equivalent volumes of MEM as controls. All materials were diluted with MEM with no added serum, except for the 1:4,000 dilution of rabbit serum. Each tube contained only 80 μl in total volume, which was the minimum volume required to completely cover the bottom surface of the tubes. Tubes were incubated at 37°C with 5% CO_2 on a rocking platform and were removed at timed intervals. Leukocytes were lysed using a 1:100 final dilution of Triton X-100 as previously described (7), and mixed vigorously for 15 min, and the contents were quantitatively removed by repeated washing with distilled water. Remaining live cryptococci were determined by plate counts, which were compared with the number of viable organisms in the original inoculum to determine how much killing had occurred.

RESULTS

Under the conditions used in these studies, there was no significant growth of cryptococci during the first 4 h of incubation. Thereafter, organisms grew in different tubes at variable rates, making quantitative comparisons extremely difficult. Therefore, results of 4-h incubations are summarized here.

Separated subpopulations of leukocytes were tested for their relative ability to kill cryptococci in the presence of anticryptococcal antibody (Fig. 1). As determined by tube dilutions and plate counts, significant killing of cryptococci occurred only in the presence of anticryptococcal antibody, as in previous studies (7). In addition, there was never any decrease in the original cryptococcal inoculum in incubation tubes that contained antibody but no effector cells, and microscopic examination confirmed

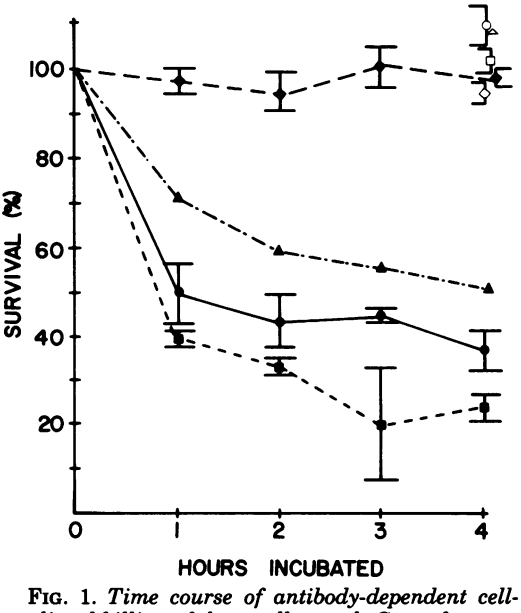

FIG. 1. *Time course of antibody-dependent cell-mediated killing of the small capsule C. neoformans isolate by mixed mononuclear cells (■), lymphoid cells depleted of phagocytic cells (●), granulocytes (▲), and T cells (◆). Corresponding values are shown at 4 h for mixed mononuclear cells (□), phagocyte-depleted lymphoid cells (○), granulocytes (△), and T cells (◇) incubated without antibody. For granulocytes, each point represents the mean of two separate experiments. All other points represent the means and bars represent the standard errors of the means of three or more separate experiments. There were approximately 50 leukocytes to one cryptococcus in each experiment. Although not shown, there was never killing of cryptococci in incubations that contained anticryptococcal antibody or normal rabbit serum but no leukocytes.*

that clumping of organisms could not explain the apparent killing which was observed when effector cells were present. Because incubation tubes contained only 80 μl, it was possible to examine the entire contents of tubes for clumps. After 4 h, leukocyte preparations which had been depleted of phagocytes killed fewer cryptococci than those which contained monocytes ($P < 0.05$ by one-tailed, 2-sample t test), although the time course of killing was similar. Preparations that contained 99.0- to 99.5% granulocytes also killed cryptococci at a slower rate than did mononuclear cells. No killing of organisms occurred in incubations that consisted primarily of T cells. These data were obtained by using the small capsule cryptococcal isolate. Comparable killing of the large capsule isolate occurred. A total of 28.5% of the large capsule isolate survived after 4 h of incubation when a 50:1 ratio of mixed mononuclear cells to cryptococci was used versus 41.3% when phagocytic cells were eliminated from effector cell populations.

Although most experiments were performed with a 50:1 ratio of leukocytes to cryptococci, significant killing was noted at much lower ratios (Table 1). At all but the lowest ratio studied, cell preparations which contained monocytes consistently killed more cryptococci than those which did not, even though the total number of leukocytes in each incubation was the same. Therefore, although nonphagocytic lymphoid cells appeared to kill cryptococci in the presence of anticryptococcal antibody, it seemed that monocytes also had the capacity to kill in this system.

As in earlier studies (7), minimal phagocytosis of cryptococci was usually noted, as expected in a system nearly free from serum. However, electron microscope studies indicated that purified lymphocyte preparations sometimes contained more monocytes than was indicated by uptake of latex particles or neutral red and that some phagocytosis of cryptococci occurred during the experiments. Therefore, additional experiments were performed to verify that most cryptococci were killed by a nonphagocytic mechanism in this system. Careful quantitative counts of intracellular organisms were performed at timed intervals, using Giemsa-stained smears and phase-contrast microscopy. Usually 0 to 5% (and never more than 20%) of the organisms were intracellular, even in preparations containing many phagocytic cells. Few intracellular organisms were noted after 1 h of incubation. Most of the phagocytosis was observed at later time intervals, after maximum killing (determined by tube dilutions and plate counts of viable fungi) had already occurred (Fig. 1). Interaction of cryptococci with leukocytes in the presence of anticryptococcal antibody was observed continuously, using phase-contrast microscopy with a stage incubator (Lab Line Instruments, Inc., Melrose Park, Ill.) to maintain cells at 37°C. Cryptococci were observed in close association with monocytes, neutrophils, and cells that appeared to be small lymphocytes. Over 1 or more h, many of these leukocyte-associated fungi exhibited striking changes in internal morphology, suggesting cell death. Such association of leukocytes and yeasts occurred only in the presence of anticryptococcal antibody.

TABLE 1. *Effect of the ratio of effector leukocytes to cryptococci on antibody-dependent fungicidal activity*

Type of effector leukocyte	No./cryptococcus[a]	Survival of cryptococcal inoculum (%)[b]
Mixed mononuclear (monocytes plus lymphocytes)	100	20.4
	50	19.3
	25	27.3
	12.5	35.6
	6.25	73.1
	1.0	95.8
Lymphocytes(phagocytic cells removed by iron treatment)	100	43.5
	50	37.7
	25	41.3
	12.5	46.9
	6.25	71.6
	1.0	101.2
Granulocytes	100	49.3
	50	52.8
	25	48.1
	12.5	53.3
	6.25	55.8
	2	69.1
	1	82.7

[a] A total of 1×10^4 of the small capsule *C. neoformans* isolate were incubated for 4 h at 37°C with varying ratios of effector leukocytes and a 1:4,000 dilution of anticryptococcal antibody.

[b] Mean of two separate experiments (each performed in triplicate). Survival in control tubes containing effector leukocytes plus normal rabbit serum or anticryptococcal antibody without effector cells was 94.7 to 129.2%.

DISCUSSION

This report confirms an earlier study that human peripheral blood leukocytes in the presence of specific antibody can kill a microorganism, *C. neoformans*, by a nonphagocytic mechanism (7). The current study suggests that nonphagocytic lymphoid cells have this

fungicidal capacity. However, monocytes appeared to be active in this system as well. This was suggested by the increment in killing of cryptococci that occurred when monocytes were present in preparations of mononuclear leukocytes. Because high ratios of leukocytes to cryptococci are necessary for killing and leukocyte separation procedures are imperfect, it remains possible that nonphagocytic monocytes account for most of the observed killing. Nevertheless, direct observations under phase-contrast microscopy suggest that some of the fungicidal cells are lymphocytes. In addition to monocytes and nonphagocytic lymphoid cells, granulocytes were active in this system. However, no killing was observed when using a population enriched in T cells. In these respects, antibody-dependent killing of cryptococci resembles antibody-dependent cell-mediated lysis of erythrocytes or mammalian target cells. In that system, when phagocytic cells are eliminated, a non-T cell that has a surface receptor for the Fc portion of immunoglobulin G appears to be responsible for cytotoxicity (19, 21, 22, 29). These effector cells share some properties with B cells (11, 21), although others have implicated non-immunoglobulin-bearing cells of the monocyte series (13) or some other type of lymphoid cell still to be identified. In addition, monocytes (15) and granulocytes (12, 24) appear to be capable of antibody-dependent lysis of target cells as well, which is consistent with the data in the present study.

Since fungicidal activity in this study appeared to occur in the absence of complete ingestion, possible mechanisms of killing are of interest. Studies by Kalina et al. (18) further extended by Aronson and Kletter (2) established that phagocytic cells formed rosettes around cryptococci that had not been ingested. Fungi surrounded by monocyte rings appeared to be destroyed, whereas those surrounded by granuloyces remained viable. They suggested that fungicidal activity might be due to secretion of lysosomal enzymes and perhaps other substances by monocytes. Because cryptococci are surrounded by thick polysaccharide capsules in addition to cell walls, some digestion might be necessary before killing can occur.

In the present study, however, it appeared likely that at least some fungicidal activity occurred by other mechanisms. First, cryptococci were killed in some experiments by lymphoid cells which contained few lysosomes and in other experiments by granulocytes, which failed to kill fungi in the studies of Aronson and Kletter. In addition, in preliminary studies, preparations rich in lysosomal enzymes released from monocytes and macrophages (5) failed to kill *C. neoformans* and did not enhance killing by other mechanisms (R. D. Diamond, C. J. Cardella, P. Davies, and A. C. Allison, manuscript in preparation). These studies also suggested that detectable lysosomal enzyme release could be induced by cryptococci, but required the presence of heat-labile opsonins. In contrast, heat-labile opsonins were not required for antibody-dependent killing of cryptococci in the present study. In any case, there may be several host defense mechanisms against infectious agents that are not readily ingested by phagocytic cells (7, 18, 23). Further studies will be necessary to establish their relative importance in host immunity to cryptococcosis and other infections.

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