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Activation of Human Peripheral Blood Mononuclear Cells by Interleukin-2 and Granulocyte-Macrophage Colony-Stimulating Factor To Inhibit *Cryptococcus neoformans*

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The abilities of selected cytokines to activate human peripheral blood mononuclear cells (PBMC) to inhibit and kill the opportunistic fungus *Cryptococcus neoformans* were studied. PBMC were cultured for 7 days in cell wells containing no cytokines, tumor necrosis factor (TNF), gamma interferon (IFN-γ), 1,25-dihydroxycholecalciferol (vitamin D_3), granulocyte-macrophage colony-stimulating factor (GM-CSF), or interleukin-2 (IL-2) and were then challenged for 24 h with a fixed number of CFU of *C. neoformans*. The number of CFU increased in wells containing no cytokines, TNF, IFN-γ, or vitamin D_3 and remained about the same in wells containing GM-CSF. In contrast, the number of CFU in wells containing IL-2-stimulated PBMC decreased, suggesting fungicidal activity. Optimal conditions for IL-2 stimulation included a minimum of 5 days of incubation of PBMC with IL-2, a concentration of 100 U of IL-2 per ml, and a high ratio of effectors to fungi. Separation of IL-2-stimulated PBMC based upon their adherence to plastic revealed that antifungal activity resided in the nonadherent fraction. These data demonstrate that IL-2 and GM-CSF are capable of stimulating PBMC-mediated antifungal activity and suggest that these cytokines may play physiological or pharmacological roles in host defenses against cryptococcosis.

Impaired cell-mediated immunity predisposes patients to infections with the encapsulated fungus *Cryptococcus neoformans* (3, 22). Clinical and experimental evidence suggests that lymphocytes and macrophages are necessary for optimal antifungal activity (3, 5, 8, 11, 14, 16, 20). However, the specific phenotypes of immune cells that are critical and the mechanisms by which these immune cells are activated remain to be determined. The availability of highly purified recombinant and synthetic cytokines has made possible studies examining whether physiological or pharmacological amounts of cytokines can activate specific effector cell populations to inhibit or kill *C. neoformans*. Such studies provide insights into how the normal immune system controls *C. neoformans* infection, and they may provide a rationale for immunotherapy of this difficult-to-treat fungal infection.

While gamma interferon (IFN-γ) has been shown to activate mouse macrophage populations to kill *C. neoformans* (5, 11), previous studies have failed to demonstrate the capacity of cytokines to activate human effector cells for enhanced activity against *C. neoformans*. IFN-γ, tumor necrosis factor (TNF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and vitamin D_3 each failed to stimulate human monocyte-derived macrophages for enhanced antifungal activity (13). Similarly, the combination of interleukin-2 (IL-2) and IFN-α had no effect on the ability of purified human natural killer (NK) cells to inhibit *C. neoformans* (19). These negative results do not preclude a role for cytokines in the stimulation of human anticryptococcal effector cells, since the optimal dosage of cytokines and duration of cytokine therapy may not have been achieved. Moreover, many cytokine-effector cell combinations have yet to be tested. Therefore, in the present study, the abilities of selected cytokines to stimulate human peripheral blood mononuclear cells (PBMC) to inhibit and kill *C. neoformans* were examined.

MATERIALS AND METHODS

Materials. All reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.), except where otherwise noted. The cell culture medium was RPMI 1640 (GIBCO, Grand Island, N.Y.) supplemented with L-glutamine, penicillin, streptomycin, and 10% human heat-inactivated AB male serum. Flat-bottom, 96-well, polystyrene tissue culture plates (no. 25860; Corning Glass Works, Corning, N.Y.) were used for assays involving cell wells. Pooled human serum was obtained by combining serum from at least 10 healthy volunteers under conditions preserving complement activity. Anticapsular antibody (a gift from John Bennett, National Institutes of Health, Bethesda, Md.) was prepared by immunizing rabbits with serotype D capsular polysaccharide and was used at a subagglutinating concentration (final dilution, 1:4,000). Recombinant human IL-2 was purchased from Genzyme (Boston, Mass.). Recombinant human IFN-γ and TNF were gifts of Genentech, Inc. (South San Francisco, Calif.). Recombinant human GM-CSF was a gift of Genetics Institute (Cambridge, Mass.). Vitamin D_3 was a gift of Milan R. Uskokovic (Hoffmann-La Roche, Nutley, N.J.).

C. neoformans. Encapsulated, serotype D strain MP415 (also known as strain B3501) and acapsular, isogenic mutant strain CAP67 (ATCC 52817) were obtained from Eric Jacobson (Medical College of Virginia, Richmond, Va.) (6, 10). Fungi were harvested from 4-day-old cultures that had been grown on asparagine minimal agar medium, as in previous studies (11–15). Under these conditions, >95% of the *C. neoformans* isolates were present as single cells, viability of the cells as measured by a microcolony assay averaged >98%, and clumping of organisms was not observed (14). The average capsule thickness on the encapsulated strain was 1.5 μm (13).

Isolation of PBMC. Human peripheral blood was obtained by venipuncture from healthy volunteers. For each set of experiments, the same blood donor was not used more than

once. Blood was anticoagulated with heparin, and the PBMC were purified by centrifugation on a Ficoll-Hypaque density gradient (17). PBMC were washed twice in phosphate-buffered saline containing 0.1% bovine serum albumin, counted, and suspended in medium at the desired concentration.

Culture of PBMC. In cell wells containing 200 μl of medium, 2×10^6 (or fewer, where indicated) PBMC were cultured for 7 days in a humidified 37°C, 5% CO_2 environment. During this incubation period, PBMC were either stimulated with cytokines or left unstimulated. In some experiments, where indicated, PBMC (10^6/ml in medium) were cultured with or without IL-2 (100 U/ml) in tissue culture flasks (no. 25106; Corning Glass Works) for 7 days. PBMC were then harvested, washed, counted, and resuspended at 10^7/ml in fresh medium with or without IL-2, and 200 μl of the cell suspension was added per cell well.

Separation of PBMC into adherent and nonadherent populations. Cultured PBMC were separated on the basis of their adherence to plastic, as in prior studies (14). Briefly, PBMC (2×10^6) were cultured for 7 days in cell wells containing IL-2. Wells were then agitated, and the medium containing the suspended nonadherent cells was transferred to a new well. The transfer procedure was repeated two more times. Fresh medium containing IL-2 was added to the wells, and the cells were then challenged with live *C. neoformans*. Antifungal activity was determined as outlined below.

Antifungal activity of cultured PBMC. Antifungal activity was determined as described previously (13, 14). Pooled human serum (final concentration, 10%), 5×10^3 CFU of *C. neoformans*, and, where indicated, anticapsular antibody were added to each well. Wells were incubated an additional 24 h in a 37°C, 5% CO_2 atmosphere. CFU of *C. neoformans* were then determined by sonication, dilution, and spread plating. For each experiment, two sets of cell wells containing *C. neoformans*, medium, and pooled human serum but no PBMC were included. The first set was sonicated, diluted, and plated immediately. The CFU counted were used to calculate the inoculum of live organisms added per well. The second set was incubated at 37°C for 24 h before being processed and plated. The CFU counted were used to calculate fungal replication in medium not containing PBMC. The growth of controls was not affected by anticapsular antibody.

Results are expressed as percent change in the experimental group from the inoculum according to the following formula: [(CFU in experimental sample/CFU in inoculum) − 1] × 100. Therefore, a negative value indicates that there were fewer live fungi in the experimental group than in the inoculum and that killing had taken place. However, because some of the yeast cells may have replicated while others were killed, the exact amount of killing cannot be calculated. Thus, a result of −20% means that a minimum of 20% of fungi in the inoculum were killed. A positive value indicates that there were more live fungi in the experimental group than in the inoculum and therefore that fungal replication had ensued. A value of zero indicates that the numbers of CFU at the start and the conclusion of the experiment were the same (14).

Statistics. The means and standard error (SE) of sample groups were compared by the two-sample, two-tailed Student *t* test.

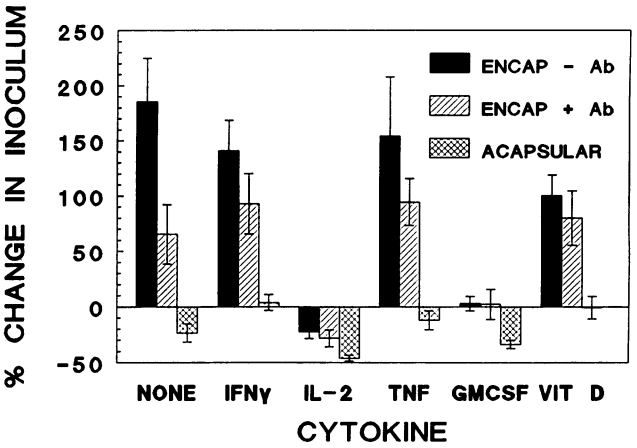

FIG. 1. Effect of cytokines on the antifungal activity of PBMC. PBMC (2×10^6) were stimulated with cytokines for 7 days and then challenged with *C. neoformans* (5×10^3). The fungal inoculum was an acapsular strain (ACAPSULAR) or an encapsulated strain in the presence (ENCAP + Ab) or absence (ENCAP − Ab) of anticapsular antibody. After 24 h, the number of live yeast cells per well was determined by counting CFU following dilutions and spread plates. Results represent means ± SE of four separate experiments, each of which was performed in triplicate, and are expressed as percent change in inoculum, with a negative number indicating that killing has taken place. In cell wells incubated under identical conditions except for the absence of PBMC, the number of CFU of the encapsulated and acapsular strains increased by 799% and 542% over the inoculum size, respectively. NONE, no cytokines; IFNγ, 500 U of IFN-γ per ml; IL-2, 100 U of IL-2 per ml; TNF, 500 U of TNF per ml; GMCSF, 250 pM GM-CSF; VIT D, 5 μM vitamin D_3.

RESULTS

In initial experiments, I examined the anticryptococcal activity of PBMC stimulated in culture by various cytokines (Fig. 1). Both encapsulated and acapsular strains of *C. neoformans* were studied. Overall, fungal replication occurred when the encapsulated strain was incubated with unstimulated PBMC in both the presence and the absence of anticryptococcal antibody. However, the degree of fungal replication, as measured by the percent change in the inoculum, was significantly less in wells containing PBMC than in wells containing medium alone (186% versus 799%, respectively, $P < 0.001$). The degree of fungal replication detected in wells containing PBMC stimulated with 500 U of IFN-γ per ml, of 500 U of TNF per ml, or 5 μM vitamin D_3 was similar to that seen in wells containing unstimulated PBMC. In contrast, the number of CFU of *C. neoformans* in wells containing PBMC stimulated with 250 pM GM-CSF remained approximately constant during the 24-h incubation ($P < 10^{-4}$, comparing PBMC stimulated without cytokines versus PBMC stimulated with GM-CSF). Moreover, there was a decrement in the number of CFU following incubation of encapsulated *C. neoformans* with PBMC stimulated with 100 U of IL-2 per ml, suggesting that overall fungal killing had taken place ($P < 10^{-4}$, comparing PBMC stimulated without cytokines versus PBMC stimulated with IL-2). Consistent with results of previous studies (14), the acapsular strain of *C. neoformans* was killed by unstimulated PBMC. Killing of the acapsular strain was significantly augmented in the presence of IL-2 ($P = 0.015$).

Since IL-2 stimulates proliferation of PBMC populations, experiments were next performed to determine whether the

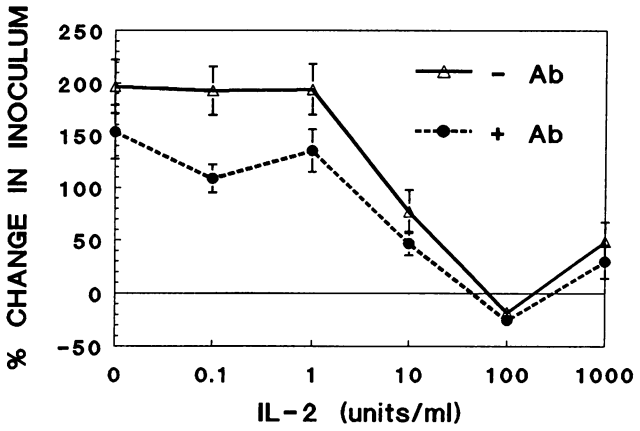

FIG. 2. Influence of IL-2 concentration on PBMC antifungal activity. PBMC (2×10^6) were stimulated with various concentrations of IL-2 for 7 days and then challenged with encapsulated *C. neoformans* (5×10^3) in the presence (+ Ab) or absence (− Ab) of anticapsular antibody. After 24 h, the number of live yeast cells per well was determined by counting CFU following dilutions and spread plates. Results represent means ± SE of two separate experiments, each of which was performed in triplicate, and are expressed as percent change in inoculum, with a negative number indicating that killing has taken place. In cell wells incubated under identical conditions except for the absence of PBMC, the number of CFU increased by 745% over the inoculum size. $P \leq 0.001$, comparing 10, 100, or 1,000 U of IL-2 per ml with no IL-2 both with and without antibody.

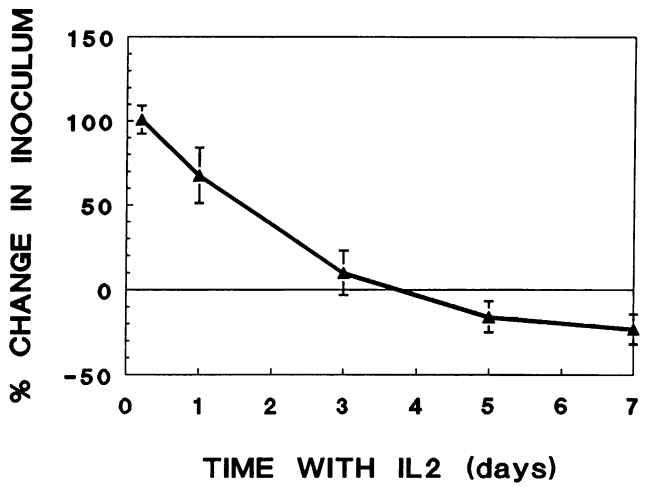

FIG. 3. Effect of duration of IL-2 treatment on antifungal activity of cultured PBMC. PBMC (2×10^6) were cultured in cell wells for 7 days. IL-2 (100 U/ml) was added to wells during the last 2 hours, 1 day, 3 days, 5 days, or 7 days of the culture period. Cells were then challenged with encapsulated *C. neoformans* (5×10^3) for 24 h. The number of live yeast cells per well was determined by counting CFU following dilutions and spread plates. Results represent means ± SE of three separate experiments, each of which was performed in triplicate, and are expressed as percent change in inoculum, with a negative number indicating that killing has taken place. In cell wells containing PBMC cultured without IL-2, the number of CFU increased by 168% over the inoculum size ($P \leq 0.002$, comparing PBMC cultured without IL-2 with any other group). In cell wells incubated under identical conditions except for the absence of PBMC, the inoculum increased by 522%.

beneficial effects seen with IL-2 were simply secondary to an increased number of cells per well. PBMC were cultured for 7 days in flasks with or without IL-2, harvested, and washed, and 2×10^6 cells were added per well prior to challenge with encapsulated *C. neoformans*. Similar results were obtained by comparing the antifungal activity of IL-2-stimulated and unstimulated PBMC cultured in flasks with that of PBMC cultured in wells (data not shown).

The next sets of experiments sought to determine the conditions under which IL-2 could stimulate PBMC-mediated inhibition and killing of the encapsulated strain of *C. neoformans*. Figure 2 depicts the influence of IL-2 concentration on PBMC antifungal activity. IL-2 concentrations ranging from 0.1 to 1,000 U/ml were tested. In both the presence and the absence of anticryptococcal antibody, significant augmentation of PBMC antifungal activity was seen with IL-2 concentrations of 10, 100, and 1,000 U/ml. However, optimal results were obtained with 100 U/ml. This concentration was used for subsequent experiments.

The effect of the duration of IL-2 treatment on antifungal activity of cultured PBMC was examined next (Fig. 3). The length of time of exposure of PBMC to IL-2 was varied from 2 h to 7 days. Even stimulation of PBMC with IL-2 for only 2 h prior to fungal challenge resulted in some beneficial effect, as the percent increase in the inoculum was diminished compared with that seen with unstimulated PBMC ($P = 0.002$). To demonstrate an overall reduction in CFU, a minimum of 5 days of incubation of PBMC with IL-2 was required.

The effect of varying the ratio of IL-2-stimulated PBMC to *C. neoformans* was studied next (Fig. 4). For these experiments, the fungal inoculum was kept constant while the number of PBMC per well was varied. The ability of IL-2-stimulated PBMC to inhibit *C. neoformans* increased in direct proportion to the ratio of PBMC to fungus. However, even at 6:1, some inhibitory effects could be seen, as the increase in CFU was about half that seen in wells containing no effector cells. Overall killing, as assessed by a decrease in CFU compared with the inoculum, was seen at only 400:1.

Finally, preliminary characterization of the nature of the IL-2-induced effector cells was attempted. IL-2-stimulated PBMC were separated on the basis of their adherence to plastic. Preliminary studies established that >90% of the adherent cells were of monocyte-macrophage lineage on the basis of their morphology and their capacity to phagocytose antibody-coated particles. Approximately the same degree of antifungal activity was seen in the nonadherent fraction as was seen in the unfractionated cells (Fig. 5). In contrast, the adherent fraction had significantly diminished antifungal capacity. These effects were seen even when the numbers of adherent and nonadherent cells per well were equalized (data not shown).

DISCUSSION

The lymphocytotropic cytokine IL-2 is critical for the generation of an effective immune response (26). IL-2 is required for clonal T-cell proliferation after antigen stimulation and for B-cell differentiation into antibody-secreting cells (26, 27). In response to pharmacological concentrations of IL-2, specific lymphocyte populations proliferate and differentiate into lymphokine-activated killer cells capable of mediating non-major histocompatibility complex-restricted cytotoxicity against a broad array of tumor targets (4, 23, 29). The data presented here establish that IL-2 is competent to activate PBMC to inhibit and probably kill *C. neofor-*

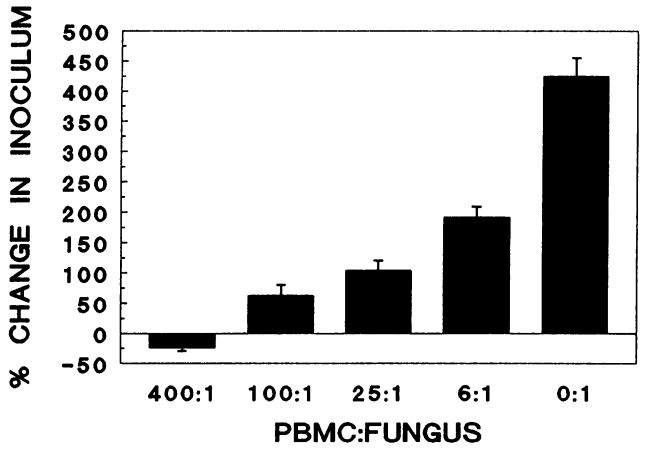

FIG. 4. Effect of varying the ratio of PBMC to *C. neoformans* on IL-2-stimulated antifungal activity. Various numbers of PBMC (ranging from 2×10^6 to 0) were cultured in cell wells with 100 U of IL-2 per ml for 7 days and then challenged with a fixed inoculum of encapsulated *C. neoformans* (5×10^3) for 24 h. The number of live yeast cells per well was determined by counting CFU following dilutions and spread plates. The ratio of 0:1 represents wells containing fungi but no PBMC. Results represent means ± SE of three separate experiments, each of which was performed in triplicate, and are expressed as percent change in inoculum, with a negative number indicating that killing has taken place. $P < 0.001$, comparing any two groups except for 100:1 and 25:1.

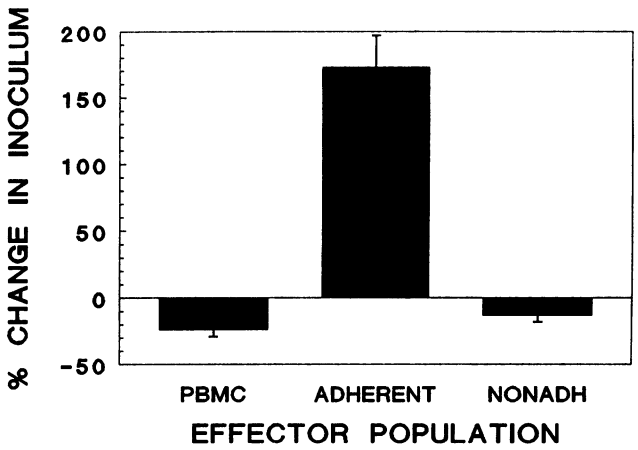

FIG. 5. Cell populations responsible for the antifungal activity of IL-2-stimulated PBMC. PBMC (2×10^6) were cultured in cell wells for 7 days with 100 U of IL-2 per ml and then separated into two populations on the basis of their adherence to plastic. Cells were then challenged with encapsulated *C. neoformans* (5×10^3). After 24 h of incubation, the number of live yeast cells per well was determined by counting CFU following dilutions and spread plates. Results represent means ± SE of three separate experiments, each of which was performed in triplicate, and are expressed as percent change in inoculum, with a negative number indicating that killing has taken place. In cell wells incubated under identical conditions except for the absence of PBMC, the number of CFU increased by 347% over the inoculum size. PBMC, unfractionated cells; ADHERENT, adherent fraction; NONADH, nonadherent fraction. $P < 0.001$, comparing adherent fraction with unfractionated cells or with nonadherent fraction.

mans. Optimal conditions for antifungal activity included a minimum of 5 days of incubation of PBMC with IL-2, a concentration of 100 U of IL-2 per ml, and a high ratio of PBMC to fungi.

Lymphokine-activated killer cells are a phenotypically heterogeneous group of cells, including those derived from NK-cell precursors ($CD3^-$ $CD56^+$ $CD16^+$) and from T-cell precursors ($CD3^+$ $CD56^+$ and $CD3^+$ $CD56^-$) (4, 7, 23, 28, 29). There are significant differences between the requirements for activating PBMC with IL-2 to kill tumor targets and the requirements for activation to kill *C. neoformans*. For example, whereas IL-2 activates PBMC for tumoricidal activity within 3 days (28, 29), activation for optimal antifungal activity took at least 5 days (Fig. 3). Moreover, whereas 1,000 U of IL-2 per ml is an optimal or near optimal concentration for activating PBMC to kill tumor targets (29), 100 U/ml proved to be ideal for fungal targets (Fig. 2). Both length of culture periods and concentration of IL-2 influence the phenotypes of lymphocytes cultured with IL-2. Thus, longer culture periods result in a higher percentage of lymphocytes expressing CD3 (24, 30). In addition, incubation with 100 U of IL-2 per ml favors expansion of $CD4^+$ cells, while a concentration of 1,000 U/ml preferentially expands $CD8^+$ cells (30). The disparate conditions required for IL-2-mediated activation of PBMC for antitumor activity as opposed to antifungal activity suggest that the phenotypes of the effector cells active against tumor and fungal targets may be different. Ongoing studies in my laboratory are directed at determining the phenotype(s) of lymphokine-activated fungicidal cells.

To my knowledge, the data presented here are the first to demonstrate the capacity of IL-2 to directly augment human effector-cell activity against a fungal target. Recently, Miller and colleagues reported the inability of human NK cells activated with IL-2 and IFN-α to inhibit growth of *C. neoformans* in the absence of specific antibody (19). Their studies differed from those reported herein in several important respects, including their use of a purified NK-cell population nearly devoid of $CD3^+$ lymphocytes. In contrast, as discussed above, conditions in the investigations presented herein favored expansion of $CD3^+$ cells. Beno and Mathews demonstrated in vitro growth inhibition of *Candida albicans* by murine lymph node cells stimulated with IL-2 (1). However, with in vivo murine models, the beneficial effects of IL-2 were not seen in experimental histoplasmosis or coccidioidomycosis (2, 9).

IL-2 production has been demonstrated in vitro following antigenic stimulation of lymphocytes from cryptococcosis patients (18). In addition, an increase in the percentage of lymphocytes expressing IL-2 receptors is seen following stimulation of PBMC with *C. neoformans* (14, 18). However, it is not known whether the concentrations of IL-2 reached in tissue sites infected by *C. neoformans* in vivo are sufficient for activation of fungicidal effector cells. Moreover, the specific cells recruited to aid infected tissue in response to cryptococcal antigens may be incapable of IL-2-stimulated fungicidal activity. Patients at increased risk for cryptococcosis, especially those with AIDS, often have severe defects in their ability to produce IL-2 (21, 25). The studies presented here, although preliminary, support the possibility that IL-2, either as immunoprophylaxis or as immunotherapy, could be beneficial in such patients.

In addition to IL-2, GM-CSF had a beneficial effect, as the number of CFU of *C. neoformans* did not significantly increase in wells containing PBMC stimulated by GM-CSF (Fig. 1). Future studies are planned to determine the specific population(s) of PBMC possessing GM-CSF-stimulated an-

tifungal activity and the optimal conditions for their activation. In previous studies, GM-CSF failed to activate monocyte-derived macrophages to inhibit cryptococcal growth (13). The other cytokines tested did not augment the antifungal activity of PBMC.

Thus, the data presented here demonstrate that IL-2, under defined conditions, is capable of activating PBMC to inhibit and probably kill *C. neoformans*. Antifungal activity was mediated by nonadherent cells, although the specific phenotypes of the responsible effector cells remain to be determined. In addition, these data suggest that IL-2 and GM-CSF may play a physiological and/or pharmacological role in host defenses against cryptococcosis.

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