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Conditioned Medium from Stimulated Mononuclear Leukocytes Augments Human Neutrophil-Mediated Killing of a Virulent *Acanthamoeba* sp.

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Human neutrophils in the presence of serum containing anti-amoeba antibody either lacked amoebicidal activity or were poorly amoebicidal for *Acanthamoeba culbertsoni*. In contrast, neutrophils preexposed for 1 h to supernatants from human peripheral blood mononuclear leukocytes (MNLs) stimulated with phytohemagglutinin demonstrated significant amoeba killing in the presence of serum containing anti-acanthamoeba antibodies. Supernatant from MNL cultured in the absence of phytohemagglutinin were not effective in stimulating significant activity in the neutrophils. Serum containing antibody promoted the adherence of many neutrophils to one amoeba. There was no significant difference between the ability of neutrophils treated with supernatants from stimulated MNLs (stimulated conditioned medium [sCM]) and supernatants from nonstimulated MNLs (nonstimulated conditioned medium [nsCM]) in their binding to acanthamoeba. The effects of sCM on neutrophils was a general phenomenon. For example, the sCM but not the nsCM enhanced the antibody-dependent neutrophil-mediated cytotoxicity against three tumor targets (K562 erythroid myeloid leukemia cell line, B16 melanoma, and P815 (DBA/2 mastocytoma). Furthermore, the sCM but not the nsCM increased the bactericidal (against *Staphylococcus aureus* and *Streptococcus pneumoniae*) and fungicidal (against *Torulopsis glabrata*) activity of the neutrophil. The sCM but not the nsCM contained activities which inhibited neutrophil migration and stimulated a respiratory burst in these leukocytes. These results suggest that the neutrophil antimicrobial power can be increased by exposing the leukocytes to MNL mediators.

The neutrophil has often been considered important in host resistance to bacterial and fungal infections, and recent evidence suggests its importance in resistance to helminthic, protozoal, and malignant diseases. However, it was only most recently that serious consideration was given to the possible modulation of neutrophil antimicrobial functions by mediators, released by lymphocytes and monocyte-macrophages, which are distinct from antibodies and their fragments and which can be tentatively referred to as lymphokines and monokines (3, 11, 25, 27, 28, 33, 43). Accordingly, these factors are likely to influence the outcome of diseases in which neutrophils play an important role, but unfortunately this area of neutrophil biology is ill defined and controversial.

In this laboratory we have been interested in defense systems against free-living amoebae which cause disease in humans. These are considered to belong to two genera, *Naegleria* and *Acanthamoeba*. Only one species in the former, *Naegleria fowleri*, has been shown to infect humans, producing a well-defined, acutely fatal meningoencephalitis (1, 4, 24, 40) resembling a fulminating bacterial meningitis. Species of *Acanthamoeba* cause either a chronic meningoencephalitis or chronic eye infections (29, 30). To date there is no effective treatment for these diseases (15, 24; Y. H. Thong and A. Ferrante, Experimental Pharmacology, *in* E. G. Rondanelli (ed.), *Amphizoic Amoebae: Human Pathology*, in press). For this reason we have pursued studies on host interaction with these amoebae and have recently presented evidence that for human neutrophils to kill *N. fowleri* cells the leukocytes must be pretreated with conditioned medium (CM) from phytohemagglutinin (PHA)-stimulated mononuclear leukocytes (MNLs) (11). This finding supports the concept that neutrophil antimicrobial properties can be augmented by lymphokines or monokines or both. We now extend these findings by showing that human neutrophils have a similar requirement in their ability to kill a virulent *Acanthamoeba* sp., *A. culbertsoni*. In addition, the study shows that the active principle(s) in the conditioned medium augments the neutrophil-mediated killing of tumor cells, bacteria, and fungi.

MATERIALS AND METHODS

Amoebae. *Acanthamoeba culbertsoni* A-1 was originally purchased from the American Type Culture Collection, Rockville, Md. The amoebae were maintained axenically in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) in tissue culture flasks (LUX Scientific Corp.) as described previously (36). Amoebae for use in experiments were obtained by vigorous agitation of flasks and low-speed centrifugation of the amoebae suspension.

Tumor cells. Three tumor cell lines were used: a K562 human erythroid myeloid cell line (10), a mouse B16/C57BL melanoma cell line (18), and a P815 (DBA/2 mastocytoma) cell line (kindly provided by Paul R. Wood, Department of Microbiology, University of Melbourne, Melbourne, Victoria, Australia). These cells were stored frozen at −170°C in aliquots, and cultures were established from these 2 to 3 days before use in experiments. The B16 melanoma cells are adherent and were detached by treatment with 1× Trypsin-EDTA (Flow Laboratories, Australia Pty. Ltd., Sydney, Australia). All cells were maintained in culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS). The cultures were maintained at 37°C in the

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presence of 5% CO_2–air and under conditions of high humidity.

Bacteria and fungi. The bacteria and fungi used in this study were those used previously in this laboratory. These were *Staphylococcus aureus* (41), *Streptococcus pneumoniae* (32), and *Torulopsis glabrata* (42). Viable bacteria and fungi were determined by their ability to form colonies on blood agar and Sabouraud dextrose agar, respectively.

Preparation of leukocytes. Blood from healthy volunteers was drawn into tubes containing lithium heparin. MNLs and neutrophils were prepared by a rapid single-step technique (16, 19, 20). The blood was layered onto a Ficoll-Hypaque solution of density 1.114 g/ml and centrifuged at 400 × *g* for 20 min. After centrifugation, the leukocytes resolved into two distinct bands. The MNLs settled and formed a band at the interface. The neutrophils collected in a second band approximately 1 cm below the first. During the centrifugation the erythrocytes sedimented to the bottom. The methodology allows the preparation of neutrophils in high yields and at a purity of greater than 96%. The viability of both leukocyte populations was greater than 99%, as judged by the ability of cells to exclude trypan blue under previously described conditions (14).

Preparation of antiserum. Rabbits were immunized with sonicated *A. culbertsoni* cells under conditions previously described for mice (35). Rabbits received three to four injections, each of 10^6 amoebae, given subcutaneously 2 weeks apart. In mice this antigen has been shown to induce a high level of resistance to acanthamoeba infection (35). Antiserum against K562 and B16 melanoma was also raised in rabbits. Rabbits were given a primary injection (subcutaneously) of 5×10^6 tumor cells in incomplete Freund adjuvant. The animals were given three more injections of tumors, each 2 weeks apart and in the absence of adjuvant. Blood was taken from these rabbits 1 week after the final immunization, and antiserum was prepared.

Amoeba agglutination assay. Twofold dilutions were made of the serum or serum fractions in medium 199 (volume, 0.1 ml) in wells (flat bottomed) of Linbro microtiter plates (Flow Laboratories, McLean, Va.). To each was added 0.1 ml of a solution containing 5×10^5 amoeba per ml. The plates were incubated at 37°C and observed microscopically for amoeba agglutination after 2 h of incubation. The titer was arbitrarily chosen as the reciprocal of the highest serum dilution at which agglutination could be observed.

Adsorption of serum. The adsorption procedure was carried out at 2 to 4°C. A total of 10^8 amoebae were pelleted by centrifugation, and the pellet was suspended in 1 ml of heat-inactivated antiserum and incubated for 1 h. After incubation, the amoebae were removed and the serum was subjected to two additional adsorption steps. The serum was finally filtered through a membrane filter (pore size, 0.22 μm; Millipore Corp.). After adsorption the serum contained an agglutination titer of <2 (the original titer was 32).

Preparation of MNL-conditioned medium. The MNLs, at a concentration of 2×10^6 cells per ml, were incubated in RPMI 1640 medium containing 10% heat-inactivated FCS in either the presence or absence of PHA. The mitogen was used at optimal concentration of 1 μg/ml (19). The cells were incubated at 37°C for 24 h in an atmosphere of 5% CO_2–air and under conditions of high humidity. The MNLs were washed three times with medium, suspended in fresh medium containing 10% FCS, and reincubated for 48 h. The cell-free supernatants were prepared by centrifugation and filtration through a (0.22-μm-pore-size Millipore filter). The supernatants from PHA-stimulated MNLs will be referred to as stimulated conditioned medium (sCM), and those from MNL cultured in the absence of mitogen will be referred to as nonstimulated conditioned medium (nsCM).

Preparation of trinitrophenyl (TNP)-labeled P815 tumor cells. P815 cells were labeled with TNP essentially as described by Vadas et al. (44). The P815 cells were reacted with 10 mM trinitrobenzenesulfonic acid in phosphate-buffered saline containing 1% FCS at room temperature for 20 min at pH 7.3. After incubation the cells were washed and were ready for isotope labeling.

Labeling of tumor cells with $Na_2{}^{51}CrO_4$. The cells were suspended in RPMI 1640 medium (with 10% FCS) to a concentration of 5×10^6/ml, and to these was added 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham, Australia Pty. Ltd.) After an incubation period of 1 h, the cells were washed and adjusted to 10^5/ml for use in cytotoxicity assays.

Treatment of neutrophils with CM. To 0.25 ml of a solution containing 4×10^7 neutrophils per ml was added 0.75 ml of sCM or nsCM, and these solutions were incubated at 37°C for 1 h. After incubation, the leukocytes were washed three times in medium 199 and used immediately in assays.

Amoebicidal assay. The amoebicidal assay was conducted in the same way as that with *N. fowleri* (11). In the assay, 2.5×10^6 neutrophils were added to 2.5×10^4 amoebae. The contents were made up in a total volume of 0.5 ml by using medium 199 in tissue culture tubes (16 by 125 mm; LUX Scientific). The sera, FCS, normal rabbit serum, rabbit anti-amoeba antisera, or normal human serum was added to a final concentration of 4%. All sera used were heated at 56°C for 30 min. The interaction was allowed to proceed at 37°C for 4 h with regular mixing. After 4 h of incubation, 10 ml of sterile distilled water was added to the tubes. The amoebae were washed in water, counted, and finally suspended in amoeba culture medium. Neutrophils are killed in this medium. The amoebae were then added to tissue culture petri dishes to test for growth (6). After 24 h of incubation at 37°C, the amoebae were counted with the aid of an inverted microscope. Approximately 20 fields were randomly counted (magnification, ×200). The percentage of amoebae killed was estimated as follows: percent killed = $[(C - T)/C] \times 100$, where C is the number of amoebae in control cultures and T is the number of amoebae in test cultures. Although we have used the terminology "killed," it is possible that the method measures cytostasis.

Examination of neutrophil-amoeba interaction. Microscope slide chambers were prepared by sticking a cover slip to the slide with double-sided sticky tape on two of the four sides. The amoeba-neutrophil suspension was applied to the chamber through one of the open sides, and both of the open sides were sealed with paraffin wax. The neutrophils were mixed with the amoebae at a ratio of 50:1 in the presence or absence of antiserum. A count of the number of amoebae containing adhered neutrophils and the number of neutrophils adhered per amoeba was made after 30 min of incubation. Observations were made with a phase-contrast microscope (Leitz, Orthoplan). Approximately 200 amoebae were screened.

Transmission electron microscopy. The cells were pelleted by centrifugation and fixed in 3% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer with 0.002 mM $CaCl_2$ (pH 7.2) for 2 h. This was followed by suspension in 1% OsO_4 in 0.1 M sodium cacodylate buffer and dehydration of the sample by changes at 10-min intervals in graded ethanol (100%) series. After several changes in ethanol, the material was infiltrated with low-viscosity epoxy resin (39) and then polymerized overnight at 65°C. Ultrathin sections were cut

with a diamond knife on a Reichert Ultracut Ultramicrotome. Sections were collected on copper grids, stained with uranyl acetate and lead citrate, and examined in a JEOL JEM 100C transmission electron microscope.

Cytotoxicity assays. The cytotoxicity reaction was carried out in U-bottom microtiter plates (Linbro, Flow Laboratories, Inc., Sydney, Australia) in a manner described previously (10). To 10^4 ^{51}Cr-labeled target cells (volume, 0.1 ml) were added 10^6 neutrophils (0.05 ml) and 0.01% rabbit antiserum against either B16 or K562 cells, or of the anti-dinitrophenyl bovine serum albumin (anti-DNP-BSA) antiserum (raised in rabbits; Miles-Yeda Ltd., Rehovot, Israel) for the TNP-labeled P815 cells. The latter antiserum cross-reacts with TNP. All assays were carried out in triplicate. The plates were incubated at 37°C in an atmosphere of 5% CO_2–air and under conditions of high humidity. The reaction times were 4 h for K562 and B16 cells and 2.5 h for TNP-P815 tumor cells. The supernatants in the wells were collected with a Titertek Supernatant Harvester (Flow Laboratories, Sydney) and assayed for radioactivity in an auto-γ-counter (Nuclear-Chicago Corp., Des Plaines, Ill.). The degree of cytotoxicity was calculated as follows: % ^{51}Cr release = [(cpm supernatant)/(cpm total)] × 100; and % lysis (cytotoxicity) = [(test release − spontaneous release)/(maximum release − spontaneous release)] × 100.

Bactericidal and fungicidal assays. Bactericidal and fungicidal assays were performed essentially as described previously (32, 41, 42). Neutrophils (10^6) were mixed with a concentration of *S. aureus* in the presence of 5% human AB serum in a 0.5-ml final volume with Hanks balanced salt solution. The tubes were gassed with 5% CO_2–air and incubated at 37°C with end-to-end mixing. At the times indicated in Results, 0.1-ml samples were taken and diluted in sterile distilled water, and serial dilutions were plated on blood agar to determine the number of viable bacteria. Assays for measuring neutrophil-mediated killing of *Streptococcus pneumoniae* cells were conducted as for *S. aureus*, with the exception that 10% human serum from a volunteer who had received a pneumococcal vaccine was used. The opportunistic human fungus *T. glabrata* was used in the fungicidal assay. This assay was conducted in a manner similar to the measurement of *S. aureus* killing (5% human AB serum was used). The fungi were plated onto Sabouraud dextrose agar plates to determine viability.

Hexose monophosphate (HMP) shunt assay. The neutrophil-HMP shunt activity was measured as described previously (12). A 20-ml and 5-ml volume glass scintillation vial was used to make a container with an inner and an outer vessel. The outer vessel received 2 × 10^6 neutrophils, 0.024 μCi of L-[1-^{14}C]glucose (3.96 mCi/mmol), and 4% autologous serum. All solutions were made up in glucose-free Earle balanced salt solution, and the final volume in each outer vessel was 1.0 ml. To the inner vessel was added 0.1 ml of 5 N NaOH to absorb the $^{14}CO_2$ evolved. The containers were placed in a shaking water bath at 37°C for various periods. After incubation the radioactivity in the NaOH was counted by liquid scintillation. Assays were conducted in triplicate.

Chemiluminescence. Neutrophils were suspended in Hanks balanced salt solution (with no phenol red). The leukocyte concentration was adjusted to 5 × 10^6/ml, dispensed in 0.1-ml aliquots (containing 5% human AB serum), and incubated at 37°C in the dark. After 15 min of incubation, 0.9 ml of 10^{-6} M luminol was added, the tubes were placed in a lightproof chamber of the luminometer (LKB Wallac), and the resulting light output in millivolts was recorded by digital printout set to 1- or 10-s recording intervals. Assays were conducted in duplicate.

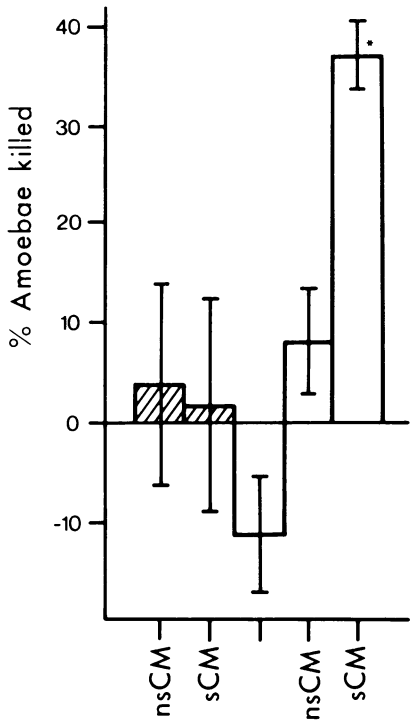

FIG. 1. Killing of *A. culbertsoni* cells by neutrophils treated with sCM. Results are the mean ± standard error of 7 to 14 experiments. Assays were conducted in the presence of either FCS (▨) or antiserum (□) and in the presence or absence of neutrophils treated with either sCM or nsCM. Asterisk, $P < 0.001$.

Measurement of neutrophil migration. Neutrophil migration was measured as described by Nelson et al. (31) with some modification (9). Wells (diameter, 2.5 mm) were cut with a template into 1% agarose gel in medium 199 (supplemented with 10% heat-inactivated FCS) contained in tissue culture petri dishes (60 by 15 mm). A 5-μl portion of a neutrophil suspension (2 × 10^7/ml) was added to each well, and the dishes were incubated at 37°C for 90 min in an atmosphere of 5% CO_2–air and under conditions of high humidity. After incubation the migration distance was measured under an inverted microscope (with phase) by using an eyepiece grid. Each testing involved setting up each sample in two separate dishes and in four wells per dish.

Statistical analysis. In all cases the data were analyzed by the unpaired *t* test. In the amoebicidal assays, all reactions were compared with the control (amoebae incubated in the presence of FCS only). In other assays the comparisons made are indicated in the figure legends.

RESULTS

Effect of sCM-treated neutrophils on amoebae. Neutrophils preincubated with sCM or nsCM were examined for amoebicidal activity against *A. culbertsoni*. The results showed that nsCM-treated neutrophils failed to cause significant killing of amoebae in the presence of either FCS or antiserum (Fig. 1). Similarly, neutrophils treated with sCM failed to cause killing of acanthamoebae in the presence of FCS. However, the sCM-treated neutrophils caused approximately 40% killing in the presence of rabbit anti-amoeba antiserum ($P < 0.001$).

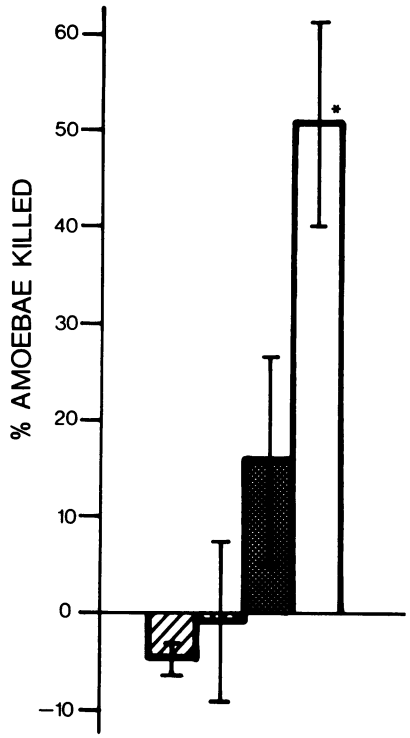

FIG. 2. Effect of MNLs treated with nsCM on acanthamoebae. The bars represent the mean ± standard error of three experiments. The amoebae were incubated with either antiserum only (▨), antiserum plus MNLs treated with nsCM (▩), antiserum plus MNLs treated with sCM (▩), or antiserum plus neutrophils treated with sCM (□). The effector-to-target-cell ratio used was 50:1. Asterisk, $0.01 < P < 0.02$.

Effect of sCM-treated MNLs on amoebae. In an endeavor to exclude the possibility that the killing demonstrated under the above conditions was due to an effect of sCM on contaminating MNLs, the ability of sCM to induce amoebicidal activity in MNLs was examined. The MNLs were treated with the same sCM as used above and under the same conditions. The ratio used was 50 MNLs to one amoeba. In addition a control group was added, representing the highly purified neutrophil population. The effect of this cell type was examined also at a ratio of 50 neutrophils to one amoeba. The results showed that the highly purified neutrophil population caused significant (approximately 50%) killing of amoebae (Fig. 2). Although the MNLs showed some effect (approximately 15% killed), this was not significant (Fig. 2).

Adsorption of the serum promoting activity. It was evident that serum factors in the antiserum were important in promoting the killing of amoebae by sCM-treated neutrophils. To see whether these factors were specific, the effect of prior adsorption of the antiserum with acanthamoebae was examined. Adsorption of the serum with acanthamoebae reduced the amoeba agglutination titer from 1/32 to 1/2. The killing of amoebae by sCM-treated neutrophils was completely abolished if the adsorbed antiserum was used in lieu of the nonadsorbed serum (Fig. 3).

Effects of direct PHA treatment of neutrophils. Since PHA contamination of the sCM is conceivable, the likelihood that PHA was the active agent in the sCM had to be excluded. The ability of various concentrations of PHA (0.002 to 2 μg/ml) to enhance the amoebicidal properties of neutrophils was examined. The results showed that neutrophils treated with PHA in a similar manner to their treatment with sCM caused no significant killing of amoebae in the presence of antiserum (data not presented).

Effect of sCM on acanthamoebae. Supernatants from stimulated MNLs are likely to contain cytotoxic substances, e.g., lymphocytotoxin. The killing of amoebae by neutrophils may be merely a result of adsorption of the lymphokine by the neutrophils, which then present it directly to the amoebae.

To exclude this possibility, the sCM was examined for the presence of amoebicidal activity. In these experiments, the same setup as used for the experiments involving neutrophils was adopted. The amoebae were incubated with 0.1, 1.0, or 10.0% sCM. The results showed that these concentrations of sCM had no amoebicidal effect. In fact, at the higher concentration the sCM increased the growth of acanthamoebae in culture by approximately 20% ($0.01 > P > 0.001$).

Effect of changing the neutrophil-to-target-cell ratio. Assays were conducted at effector-to-target-cell ratios of 80:1, 40:1, 10:1, and 1:1. The results showed that a related decrease in the degree of killing occurred, with a decrease in effector cells (Fig. 4). Significant killing was still observed at a ratio of 1:1.

Adherence of neutrophils to amoebae. Examination of neutrophil-amoeba interaction showed that comparable adherence of nsCM- and sCM-treated neutrophils to amoebae occurred (Fig. 5). Adherence was markedly increased in the presence of antiserum. A number of neutrophils were seen to adhere to a single amoeba (Fig. 6).

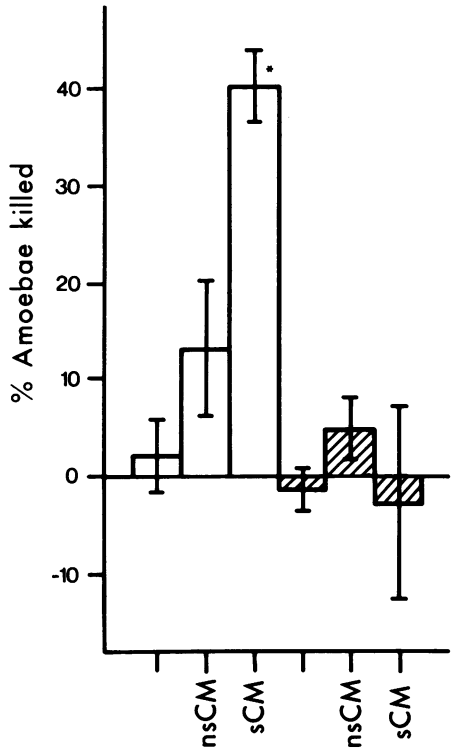

FIG. 3. Ability of amoeba-adsorbed antiserum to aid sCM-treated neutrophils in the killing of acanthamoebae. Results are expressed as mean ± standard error of five to seven experiments. Assays were conducted in the presence of either antiserum (□) or adsorbed antiserum (▨) and either in the presence of neutrophils treated with sCM or nsCM or in the absence of neutrophils. Asterisk, $P < 0.001$.

Effects of normal serum. Since it is known that normal serum may contain natural antibodies to *A. culbertsoni* (5, 13), the effect of substituting normal rabbit serum for the antiserum was examined. The results showed that sCM-treated neutrophils were capable of killing acanthamoebae in the presence of normal rabbit serum (percent killing, 30; $0.01 > P > 0.001$). Normal rabbit serum adsorbed with the amoebae failed to aid the sCM-treated neutrophils in killing (percent killing, 4.5). Similarly, 5% human serum (heated at 56°C for 30 min) was capable of promoting amoeba killing by sCM-treated neutrophils (percent killing, 39.2; $P < 0.001$).

Modification of neutrophil-mediated cytotoxicity for tumor cells. The neutrophil-mediated antibody-dependent cytotoxicity against three tumor cell types was examined for neutrophils treated with either nsCM or sCM. Studies with the B16 melanoma tumor cell line showed that in the presence of rabbit antiserum to the tumor neither nonconditioned medium (NCM)- nor nsCM-treated neutrophils showed any significant cytotoxicity against the target (Fig. 7). However, when neutrophils were pretreated with sCM, a significant level of cytotoxicity was observed in the presence of antiserum but not in its absence (Fig. 7). Similar experiments with the K562 erythroid myeloid cell line showed that there was a low but nonsignificant level of neutrophil-mediated antibody-dependent cytotoxicity by neutrophils treated with either NCM or nsCM. Treatment of neutrophils with sCM, however, resulted in an increase in the cytotoxicity (Fig. 8).

In a third set of experiments, the antibody-dependent cell-mediated cytotoxicity was examined by a system previously adopted to demonstrate enhancement of neutrophil antibody-dependent cell-mediated cytotoxicity by colony-stimulating factor (44). This was the TNP-tagged P815 tumor target. The results of our experiments demonstrated that neutrophils were cytotoxic for the P815 target in the presence of antibody (Fig. 9). In addition, the neutrophil-mediated cytotoxicity against the target was enhanced by prior treatment of leukocytes with sCM (Fig. 9).

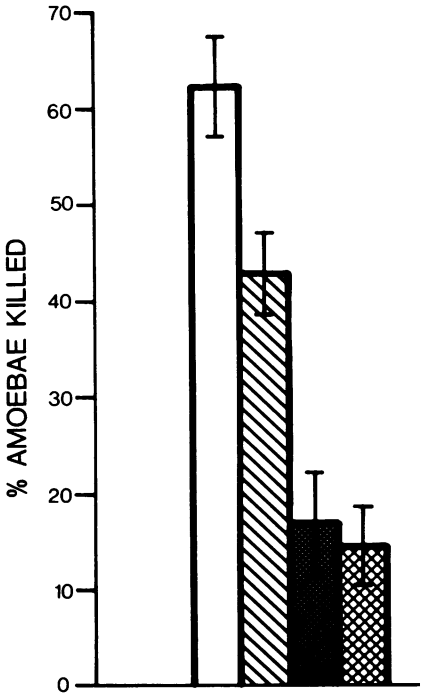

FIG. 4. Effect of changing the neutrophil-to-amoeba ratio. Neutrophils treated with sCM were added to amoebae in the presence of antisera to give effector-to-target-cell ratios of 80:1 (□), 40:1 (▨), 10:1 (▩), or 1:1 (▨). Assays were conducted in the presence of antiserum. Results are the mean ± standard error of four experiments. Statistics: 80:1, $P < 0.001$; 40:1, $P < 0.001$; 10:1, $0.02 < P < 0.05$; 1:1, $0.02 < P < 0.05$.

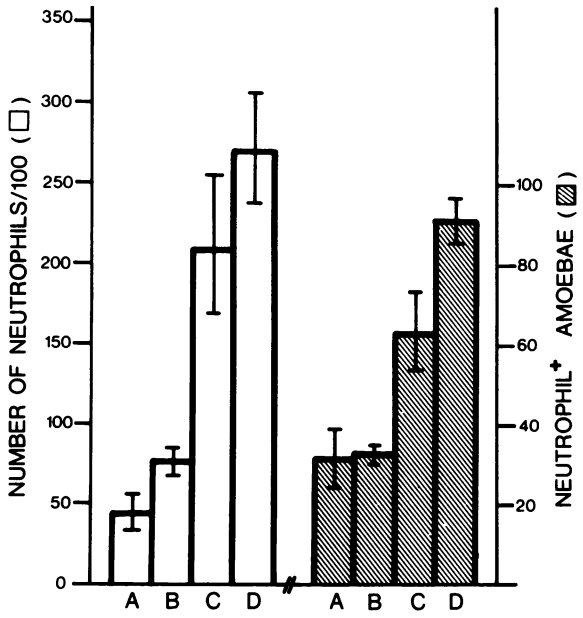

FIG. 5. Adherence of neutrophils to acanthamoebae in the presence or absence of immune rabbit serum (IRS). The results are presented as the number of neutrophils bound per 100 amoebae and as the percentage of amoebae containing at least one attached neutrophil. Amoebae were mixed with FCS and either (A) nsCM- or (B) sCM-treated neutrophils, or IRS and either (C) nsCM- or (D) sCM-treated neutrophils. The results are presented as mean ± standard error of three experiments. Statistical significance for both panels was obtained between D and A, D and B, C and A; $0.01 > P > 0.001$.

With respect to all three antibody-dependent cell-mediated cytotoxicity systems, it was shown that sCM had no direct tumoricidal activity (data not presented). In addition it was found that MNLs used at their highest possible contamination (5%), i.e., effector-to-target-cell ratio of 5:1, showed no significant cytotoxicity when these leukocytes were treated with sCM (data not presented).

Alteration of neutrophils in the killing of phagocytosable microorganisms by sCM. Since the amoeba is relatively large compared with the neutrophil, an extracellular killing mechanism is involved, and it was therefore of importance to see the status of the neutrophil treated with sCM in relation to killing of microorganisms which are killed after their confinement within phagocytic vacuoles. In this study, the bacteria *S. aureus* and *Streptococcus pneumoniae* and the opportunistic fungal pathogen *T. glabrata* were used.

The data presented in Fig. 10 showed that at a ratio of 20 *S. aureus* cells to one neutrophil, there was a significant difference between neutrophils treated with nsCM or sCM in the killing of *S. aureus* cells. Neutrophils treated with nsCM reduced the bacterial inoculum by 15% after 60 min, whereas leukocytes treated with sCM reduced it by 60% ($0.05 > P > 0.02$). In a separate set of experiments we found no significant difference in the killing of *S. aureus* cells by neutrophils treated with nsCM and NCM (data not presented). In a third set of experiments the ratio of bacteria to neutrophils was

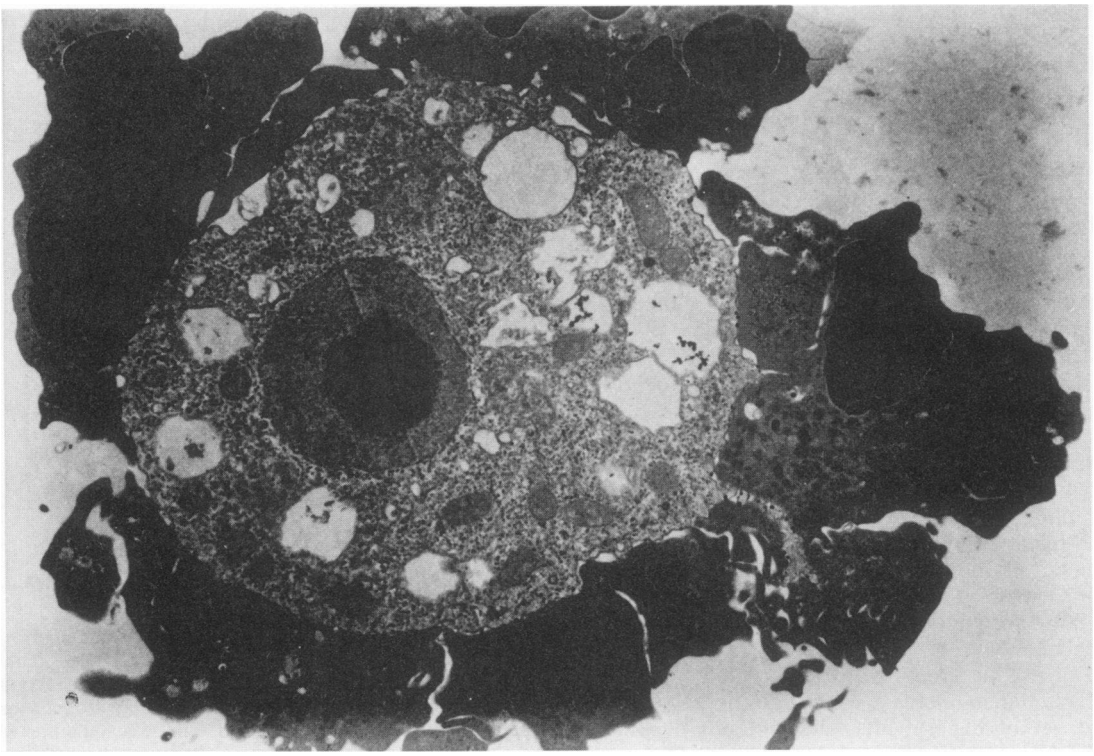

FIG. 6. Electron micrograph showing binding of many neutrophils to an acanthamoeba. sCM-treated neutrophils were reacted with amoebae in the presence of IRS.

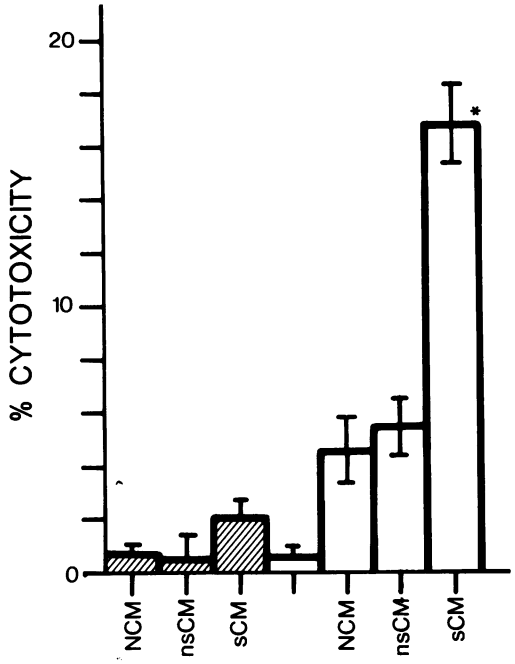

FIG. 7. Enhancement of antibody-dependent neutrophil-mediated cytotoxicity against B16 melanoma cells by sCM. Assays were conducted in either the presence of FCS (▨) or IRS (□). The targets were reacted with neutrophils treated with NCM, nsCM, or sCM. the ratio of effector to target used was 50:1. The results are presented as mean ± standard error of three experiments. Asterisk, $P < 0.001$.

changed; ratios of 20:1, 10:1, and 2:1 were used. We found that a difference between neutrophils treated with nsCM and sCM, although apparent at a ratio of 10:1, was clearly evident at a ratio of 20:1 (Table 1). At a ratio of 2:1, at which the neutrophils killed a high proportion of bacteria, a difference was not obtained.

Similar studies conducted with *Streptococcus pneumoniae* showed that the killing of this organism by neutrophils could be enhanced by treating the leukocytes with sCM (Fig. 11). Again, it was shown that the ability to show a difference between the killing by sCM- and nsCM-treated neutrophils was dependent on the ratio of bacteria to neutrophils used (data not presented).

Neutrophils treated with sCM showed significant enhancement of killing of *T. glabrata* compared with nsCM-treated leukocytes (Fig. 12). After 90 min of incubation the percentage killed with nsCM and sCM was 5 and 55%, respectively. This effect by sCM, as in the studies with bacteria, was observed only at the higher ratio of organism to neutrophil.

Effect of sCM on neutrophil migration and metabolic activity. A well-characterized lymphokine with activity on neutrophils is leukocyte inhibitory factor (LIF). The principal effect of LIF on neutrophils is to inhibit their migration rate. The sCM was examined for the presence of neutrophil migration inhibitory activity. The results showed that neutrophils treated with sCM but not nsCM were inhibited in their locomotion (Fig. 13).

Activation of the NADPH oxidase activity in neutrophils is an important step in the production of oxygen metabolites which may directly or in combination with other components (such as myeloperoxidase) of the neutrophil kill microorganisms and tumor cells. The HMP shunt activity and light emission (chemiluminescence) are closely linked to the

NADPH oxidase activity. The effects on these two parameters of treating neutrophils with sCM or nsCM were examined. The results showed that the sCM induced both a burst of chemiluminescence and HMP shunt activity in neutrophils (Fig. 14 and 15).

DISCUSSION

The virulent *Acanthamoeba* sp. *A. culbertsoni* was poorly killed or not killed by NCM- or nsCM-treated neutrophils in the presence of antibody. However, the same neutrophils exposed for 1 h to sCM demonstrated significant killing of acanthamoebae in the presence of antiserum. Previously a similar result was obtained with another pathogenic amoeba, *N. fowleri* (11). Normal rabbit serum also contained a principle(s) which aided sCM-treated neutrophils in killing acanthamoebae. This, as well as the activity in the antiserum, could be adsorbed with the amoebae, suggesting that it is antibody.

Consistent with our previous findings with naegleriae (11), the addition of antiserum to acanthamoebae in the absence of neutrophils enhanced the growth of amoebae. It may therefore be argued that comparisons should be made with this result, in which case neutrophils treated with NCM or nsCM would give higher killing values. However, we are against this interpretation because, as we argued for naegleriae, amoebae have the ability to redistribute, cap, and internalize surface-bound antibody (11, 17). This phenomenon is unlikely to occur if neutrophils bind to the amoebae via the Fc receptor.

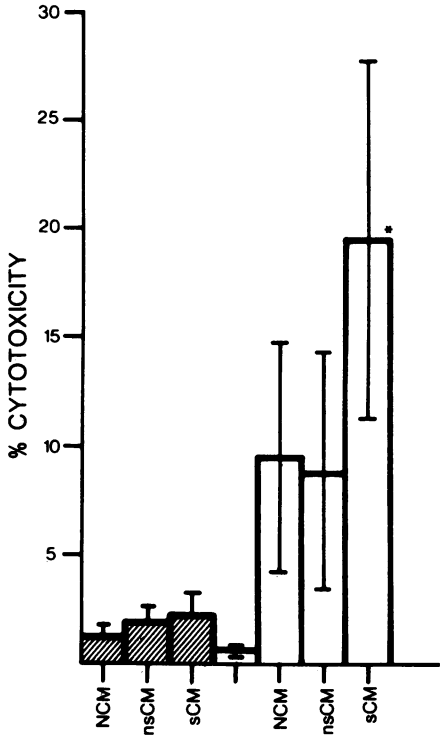

FIG. 8. Enhancement of antibody-dependent neutrophil-mediated cytotoxicity against K562 target cells by sCM. Assays were conducted in the presence of either FCS (▨) or IRS (□). The targets were reacted with neutrophils treated with NCM, nsCM, or sCM. The effector-to-target-cell ratio was 50:1. The results are presented as mean ± standard error of four experiments. Asterisk, $P < 0.001$.

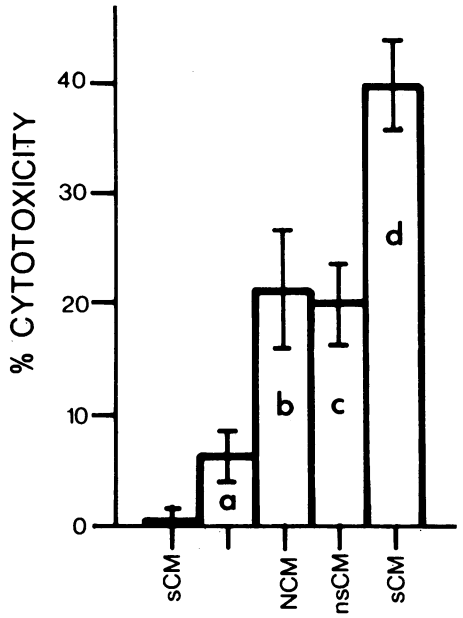

FIG. 9. Enhancement of antibody-dependent neutrophil-mediated cytotoxicity against TNP-P815 target cells by sCM. Assays were conducted in the presence of either FCS (■) or IRS (□). The target cells were reacted with neutrophils treated with NCM, nsCM, or sCM. The effector-to-target-cell ratio was 50:1. The results are presented as mean ± standard error of three experiments, each conducted in quadruplicate. Statistics: b and c are significantly different from a: $0.02 < P < 0.05$ and $0.01 < P < 0.02$, respectively; d is significantly different from b and c: $0.01 < P < 0.02$.

Alteration of neutrophils by sCM is unlikely to be due to possible PHA contamination. First, the MNLs were washed after PHA stimulation and supernatants were collected after a subsequent reculture in the absence of this mitogen. Second, it was shown that neutrophils exposed to a range of concentrations of PHA did not display enhaced killing of amoebae.

The neutrophil preparation used in these studies was of high purity (>96%) (16), and it is unlikely that any MNL contamination was responsible. Evidence against the involvement of MNLs is that the neutrophil preparation was still effective at an effector-to-target-cell ratio of 1:1. Second, exposure of MNLs to sCM did not endow these leukocytes with amoebicidal activity. This finding also suggests that MNLs do not display natural killing against this protazoon. Previously it was demonstrated that *Giardia lamblia* is killed by normal human peripheral blood monocytes in the absence of antibody or complement (38).

Owing to the relatively large size of the amoeba, it can bind many neutrophils to its surface. Although binding was promoted by the antiserum, there was no significant difference between the ability of sCM- and nsCM-treated neutrophils to bind to amoebae. This demonstrates another example of antibody-dependent, neutrophil-mediated killing involving an extracellular mechanism and may be similar to that demonstrated with other targets, including tumor cells (2, 23), virus-infected cells (21, 37), and helminths (8). Since neutrophils treated with sCM did not display the stimulated amoebicidal properties in the absence of antibody, the results suggest that close contact is required for the killing.

The sCM was neither amoebicidal nor tumoricidal. It is therefore unlikely that the activity conferred on neutrophils was a result of passive transfer of amoebicidal activity to the

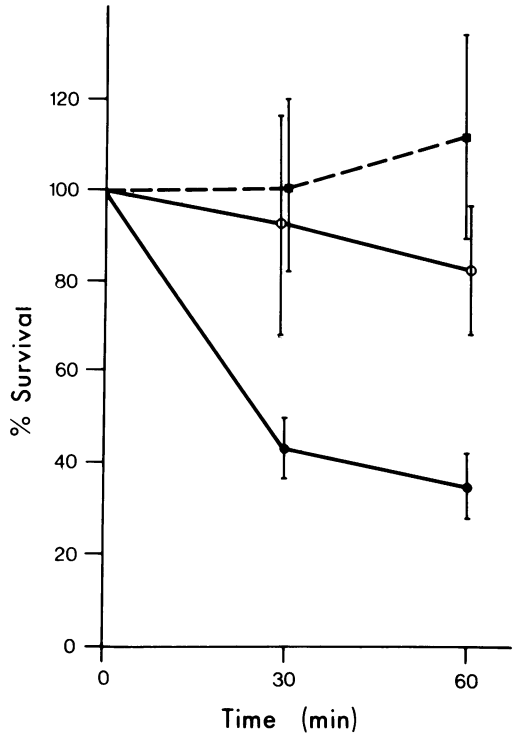

FIG. 10. Enhanced killing of *S. aureus* cells by sCM-treated neutrophils at a ratio of 20 bacteria to one neutrophil in the presence of human serum. The curves represent bacteria incubated with medium and human serum (■), human serum plus nsCM-treated neutrophils (○), or human serum plus sCM-treated neutrophils (●). The results are expressed as mean ± standard error of four experiments. Statistics: sCM is significantly different from nsCM after 30 and 60 min ($0.02 < P < 0.05$).

leukocyte. This is further supported by the finding that MNLs treated in the same manner as neutrophils were not amoebicidal. It is most probable that the stimulated antimicrobial property of the neutrophil is a result of a modulated function of this leukocyte.

Among the factors released by MNLs which modify neutrophil function are those which inhibit the migration of these leukocytes and includes LIF (25, 34) and neutrophil migration inhibition factor T, which appears to be distinct from LIF (45, 46). The relationship between these activities and neutrophil-mediated antimicrobial activities has received very little attention. Lomnitzer et al. (28), using supernatants from PHA-stimulated MNLs, demonstrated that neutrophils treated with these supernatants displayed decreased migration, increased phagocytosis, and a stimulated respiratory burst, but not increased killing of *Candida albicans*. More recently, Klempner and Rocklin (26) showed that highly purified LIF inhibited the phagocytosis of *S. aureus* cells and did not stimulate superoxide generation (26). This suggests that LIF cannot account for the enhanced respiratory burst that phagocytosis induced by supernatants from MNLs cultured in the presence of PHA. The sCM used in this study contained neutrophil migration inhibition activity and principles which stimulated a respiratory burst. However, whether these changes on the neutrophils are responsible for, or merely associated with, neutrophil-augmented killing, remains to be established.

Killing of acanthamoebae and naegleriae probably requires exocytosis of granular material from neutrophils. It is

TABLE 1. Stimulation of neutrophil killing of *S. aureus* by sCM

Conditions[a]	% killing at bacterium/neutrophil ratio[b]		
	20:1	10:1	2:1
No neutrophils	−5.62 ± 7.5	2.62 ± 9.07	−0.70 ± 4.86
NCM-neutrophils	26.40 ± 5.27	78.73 ± 7.08	84.9 ± 4.15
nsCM-neutrophils	23.75 ± 12.19	79.87 ± 7.90	96.66 ± 0.59
sCM-neutrophils	73.17 ± 1.15	84.44 ± 5.06	97.87 ± 0.67

[a] Neutrophils were treated with NCM, nsCM, or sCM just before use in bactericidal assays.

[b] The figures represent the percentage bacteria killed after 60 min of incubation and are expressed as mean ± standard error of four experiments.

therefore possible that LIF, by stimulating degranulation (26), is responsible for the augmentation of the neutrophil-mediated killing properties. However, it is most likely that, if this notion is true, it is not the only factor that stimulates neutrophil-mediated cytotoxic mechanisms. It has been shown by Vadas et al. (44) that colony-stimulating factors increase the neutrophil-mediated killing of tumor cells in the presence of antibody. We have also found that sCM but not nsCM increases the neutrophil cytotoxicity against tumor cells in the presence of antibody. Colony-stimulating activity was also detectable in the sCM (unpublished data). However, our preliminary studies have shown that colony-stimulating factor does not stimulate the neutrophil-mediated killing of *N. fowleri* (A. Ferrante, T. J. Abell, M. A.

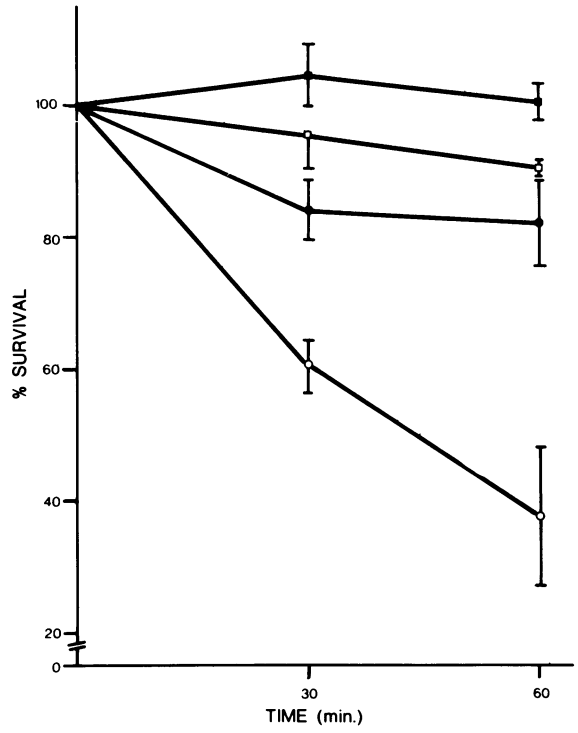

FIG. 11. Enhanced killing of *Streptococcus pneumoniae* cells by sCM treated neutrophils at a ratio of 15 bacteria to one neutrophil. The curves represent bacteria incubated in the presence of serum and either no neutrophils (■), NCM-treated neutrophils (□), nsCM-treated neutrophils (●), or sCM-treated neutrophils (○). The results are expressed as mean ± standard error of three experiments. Statistics: sCM was significantly different from NCM and nsCM (30 min, $0.01 < P < 0.02$; 60 min, $0.02 < P < 0.05$).

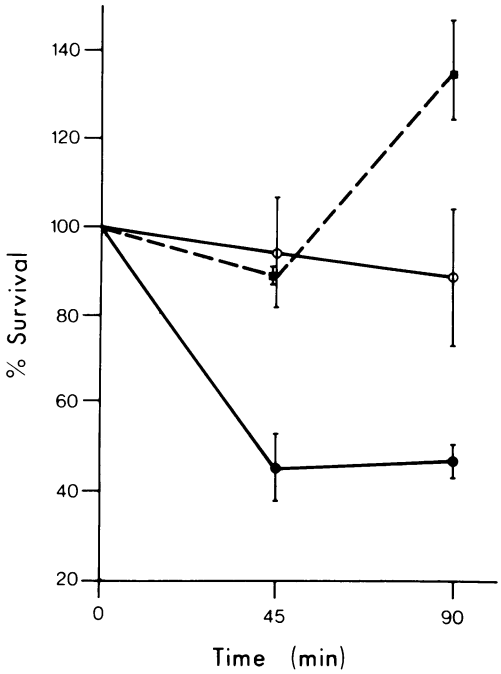

FIG. 12. Enhanced killing of *T. glabrata* cells by sCM-treated neutrophils at a ratio of 15 fungi to one neutrophil. The curves represent fungi incubated with medium and human serum (■), human serum plus nsCM-treated neutrophils (○), or human serum plus sCM-treated neutrophils (●). The results are expressed as mean ± standard error of four experiments. Statistics: sCM is significantly different from nsCM at 45 min ($P < 0.05$) and 90 min ($0.01 > P > 0.001$).

Vadas, and N. A. Nicola, unpublished data). Beta interferon could also be responsible. Hockland and Berg (23) have shown that interferon enhanced the antibody-dependent neutrophil cytotoxicity against erythrocytes and tumor targets, and we have found that human interferon stimulates the HMP shunt activity of human neutrophils (12).

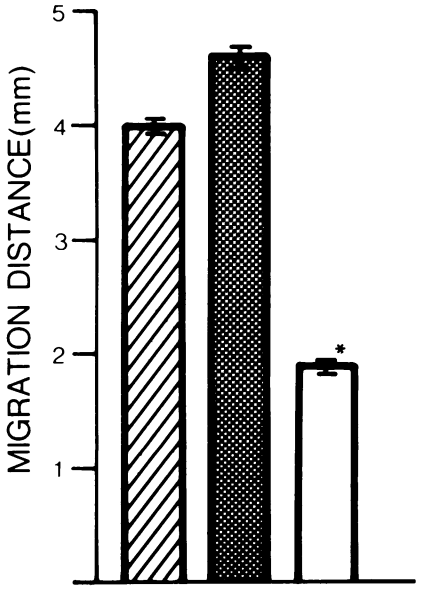

FIG. 13. Inhibition of neutrophil migration by sCM. Neutrophils were treated with either medium plus FCS (▨), nsCM (▩), or sCM (□). The results are presented as mean ± standard error of four experiments. Asterisk, $0.001 < P < 0.01$.

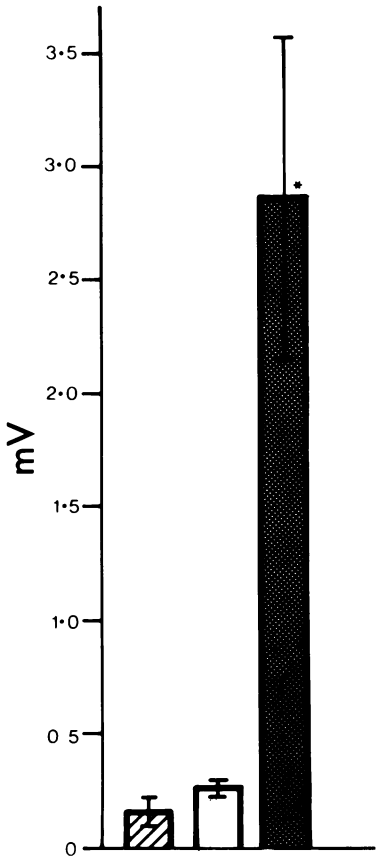

FIG. 14. Stimulation of chemiluminescence response in neutrophils by sCM. Neutrophils were treated with either medium plus FCS (▨), nsCM (□), or sCM (▩). The results are presented as mean ± standard error of six experiments. Asterisk, $0.01 < P < 0.01$.

This study raises the possibility that neutrophils could participate in host resistance to acanthamoebae. Normal human serum has been shown to contain antibodies to *A. culbertsoni* (5, 13), and these may function to aid neutrophils in the killing of amoebae. Since the amoebae are able to activate complement by the alternative pathway (13), complement may also act as a recognition factor in serum in the absence of antibody, as occurs with naegleriae (11). Hematogenous spread by acanthamoebae most likely occurs in individuals who are chronically ill or who are undergoing immunosuppressive therapy (29, 30); it is probably a result of the immunocompromised status of the individuals. Healthy individuals, besides having adequate levels of antibodies and complement, have the potential to produce lymphokines rapidly because of preexisting sensitization (6). These factors may be responsible for the pathogenic behavior of acanthamoebae.

Our findings show that neutrophils can be influenced by MNL mediators in their expression of antimicrobial killing properties. Although results of different investigators do not concur regarding the ability of supernatants such as those studied here to increase neutrophil killing of smaller microorganisms such as bacteria, it is evident from our study that sCM increases the killing of bacteria and fungi. It is, however, important to note that the conditions under which enhancement of neutrophil-mediated killing of the smaller

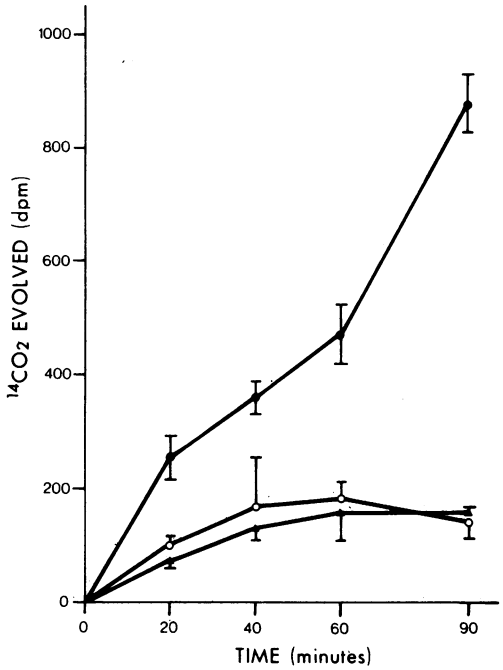

FIG. 15. Stimulation of the neutrophil HMP shunt by sCM. Neutrophils were treated with either medium plus FCS (○), nsCM (▲), or sCM (●). The results are expressed as mean ± standard error of three experiments.

organisms was demonstrated required the neutrophils to be subjected to high ratios of microorganism to leukocyte.

Potentiation of neutrophil activity by lymphokines or monokines or both may be of major significance in those circumstances in which microorganisms evade antimicrobial activities of neutrophils (7, 22).

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