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Apoptosis as a Mechanism of Cytolysis of Tumor Cells by a Pathogenic Free-Living Amoeba

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Previous studies have shown that trophozoites of the pathogenic free-living amoeba *Acanthamoeba castellanii* rapidly lysed a variety of tumor cells in vitro. Tumor cells undergoing parasite-mediated lysis displayed characteristic cell membrane blebbing reminiscent of apoptosis. The present investigation examined the role of apoptosis (programmed cell death) in *Acanthamoeba*-mediated tumor cell lysis. The results showed that more than 70% of tumor cell DNA was fragmented following exposure to *Acanthamoeba* cell extracts. By contrast, only 7% of untreated control cells underwent DNA fragmentation. DNA fragmentation increased significantly in a dose-dependent fashion following concentration of the parasite extract. Apoptosis was also confirmed by DNA ladder formation. Characteristic DNA ladders, consisting of multimers of approximately 180 to 200 bp, were produced by tumor cells exposed to *Acanthamoeba* cell extracts. The morphology of tumor cell lysis was examined by light and scanning electron microscopy. Tumor cells exposed to parasite extract displayed morphological features characteristic of apoptosis including cell shrinkage, cell membrane blebbing, formation of apoptotic bodies, and nuclear condensation. By contrast, similar effects were not found in tumor cells exposed to extract similarly prepared from normal mammalian cells (i.e., human keratocytes). The results suggest that at least one species of pathogenic free-living amoeba is able to lyse tumor cells by a process that culminates in apoptosis.

Acanthamoeba species are ubiquitous pathogenic free-living protozoal organisms that have been isolated from an unbelievably diverse range of habitats (17). Pathogenic free-living amoebae can cause life-threatening meningoencephalitis or sight-threatening keratitis (1, 9, 14). The corneal epithelium is affected early in the pathogenesis of *Acanthamoeba* keratitis. Epithelial thinning and necrosis are evident in both humans and experimental animals, suggesting that the parasite produces direct cytopathic effects (CPE) during the initiation of infection (1). This conclusion is further supported by in vitro studies which have shown that *Acanthamoeba* trophozoites produce extensive CPE on a wide variety of tumor cells (21) as well as corneal epithelial cells (13, 25, 26).

Although the underlying mechanisms for *Acanthamoeba*-mediated CPE are poorly understood, several studies have examined the cytopathic mechanisms for other pathogenic free-living amoebae. Brown (3) described the piecemeal engulfment of target cells by *Naegleria fowleri* as "troglocytosis," from the Greek meaning "to nibble." Subsequent studies confirmed the role of troglocytosis in producing CPE on a variety of target cells (10, 16). In addition to troglocytosis, pathogenic free-living amoebae injure nerve cells by contact-dependent lysis (16). Recently, Young et al. (27) described a cytolytic pore-forming protein produced by *N. fowleri*, which may have a direct lytic function in target cell killing. Thus, *N. fowleri* can damage host cells by either troglocytosis, contact-dependent lysis, or a combination of the two.

The rationale for the present study was based on recent observations that *Acanthamoeba castellanii* trophozoites produce extensive CPE on tumor cell and corneal epithelial cell monolayers (13, 21, 25, 26). Since *Acanthamoeba* trophozoites do not form amebostomes and are presumably unable to mediate troglocytosis (9), a likely mechanism to explain target cell damage by *Acanthamoeba* spp. is through the elaboration of pore-forming proteins similar to those described for *Naegleria* spp. (27) and *Entamoeba histolytica* (5). However, we have recently reported that melanoma cells and corneal epithelial cells exposed to either *Acanthamoeba* trophozoites or aqueous extracts of trophozoites undergo lysis by a process involving extensive cell membrane blebbing reminiscent of apoptosis (21). The goal of the present study was to determine whether the CPE produced by *Acanthamoeba* trophozoites and soluble products elaborated by trophozoites was due, at least in part, to apoptosis. The results reported here involve studies using tumor target cells because tumor cells were easier to propagate than corneal epithelial cells and CPE developed more swiftly in tumor cell cultures compared with normal cell monolayers. However, similar results were found with pig corneal epithelial target cells.

MATERIALS AND METHODS

Parasite cultivation. A pathogenic strain of *A. castellanii* (ATCC 30868), originally isolated from a diseased human cornea, was obtained from the American Type Culture Collection (Rockville, Md.). Parasites were grown axenically in peptone-yeast-glucose medium at 35°C as previously described (7).

Cell culture. Murine neuroblastoma cells (NB41A3; ATCC 147) were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum. Two sublines of B16F10 murine melanoma (Queens melanoma and D5.1G4 melanoma) were propagated in minimal essential medium (MEM) as described previously (12). A murine UV light-induced murine fibrosarcoma, UV 5497, was generously provided by Margaret Kripke, M.D. Anderson Cancer Center, Houston, Tex., and was cultured in minimal essential medium (12). Two human uveal melanoma cell lines, designated OCM1 and OCM3, were

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generously provided by June Kan Mitchell (University of Los Angeles Medical School, Los Angeles, Calif.) and were cultured in Ham's F12 medium (11). Murine mastocytoma, P91, was a generous gift from Thierry Boone (Ludwig Cancer Institute, Brussels, Belgium) and was cultured in MEM (11). A human keratocyte (HK) cell line was originally isolated from human corneal stroma and was kindly provided by Steven Wilson (University of Texas Southwestern Medical Center, Dallas, Tex.). Keratocyte cultures were propagated in minimal essential medium. Rat pre-T-cell lymphoma cell lines (NB2) were generously provided by P. W. Gout (British Columbia Cancer Agency, Vancouver, B.C., Canada) and were treated with glucocorticoid as described elsewhere (4).

Preparation of *Acanthamoeba* lysate. Log-phase cultures of *A. castellanii* trophozoites were collected in siliconized centrifuge tubes and washed with Hanks' balanced salt solution to remove growth medium (peptone-yeast-glucose). Sterile siliconized 0.5-μm glass beads were added, and the resulting mixture was vortexed at high speed for 1 min and then placed in ice for 1 min. After five cycles of vortexing and chilling were completed, the glass beads were allowed to settle, and supernatant was removed and stored in siliconized tubes at −70°C until used. The protein concentration of the lysate was determined by the Bradford assay (24). Control lysate was prepared from HK cells in a manner identical to that for the parasite lysate. In DNA extraction and electrophoresis experiments, the lysates were filtered through a 0.45-μm-pore-size low-protein-binding filter (Gelman Science, Ann Arbor, Mich.).

Propidium iodide (PI) staining and flow cytometry. The percentage of apoptotic nuclei was quantified by flow cytometry as previously described (19). Target cells at a concentration of 10^5 cells per well in 96-well microtiter plates were treated with 100 μl of 1.0-mg/ml parasite lysate (100 μg of protein per well) for 16 h at 37°C (21). The cells were centrifuged at 200 × *g*, and the cell pellet was resuspended in 1.0 ml of hypotonic fluorochrome PI solution (Sigma Chemical Co., St. Louis, Mo.). The tubes were placed at 4°C in the dark overnight before the flow cytometric analysis. The PI fluorescence of the cell preparation was measured in a fluorescence-activated cell sorter (FACS) Scan (Coulter Electronics, Inc., Hialeah, Fla.). Apoptotic nuclei appeared as a broad hypodiploid DNA peak, which was easily distinguishable from the narrow peak of target cells with normal (diploid) DNA content in the red fluorescence channels. The results are expressed as percentage of apoptosis (percentage of cells with hypodiploid DNA content). Controls consisted of target cells incubated in medium only or in a similar protein concentration of bovine serum albumin (BSA). HK lysate served as a control for comparison with parasite lysate.

DNA extraction and electrophoresis. The various tumor cells were treated with filtered lysate for 16 h, washed with Ca^{2+}-Mg^{2+}-free Dulbecco's phosphate-buffered saline, and then lysed in 10 mM Tris-HCl (pH 7.5) containing 1% Triton X-100 and 5 mM $MgCl_2$ for 30 min. The resulting mixture was centrifuged at 400 × *g* for 10 min at 4°C. The DNA content in the supernatant and pellet was resuspended in guanidine isothiocyanate solution containing 4 M guanidine isothiocyanate, 25 mM sodium acetate, and 84% 2-mercaptoethanol. DNA was precipitated with isopropanol, dried, and resuspended in electrophoresis buffer. DNA (1.0 μg per well) was separated by electrophoresis on a 1% agarose gel containing 10 mg of ethidium bromide per ml at 100 V for 2 h (23). Lambda DNA *Hin*dIII digest containing 23,130, 9,416, 6,557, 1,361, 2,322, 2,027, 564, and 125 bp (Sigma Chemical Co.) was used as a molecular weight standard.

Light and electron microscopy. Target cells treated with or without live trophozoites or *Acanthamoeba* lysate for 16 h were stained with Giemsa stain for light microscopy. Cells were also processed for scanning electron microscopy as previously described (20).

Isolation of soluble factors secreted by *A. castellanii*. Log-phase cultures of *A. castellanii* trophozoites were collected in centrifuge tubes and washed with Hanks' balanced salt solution as described above. Trophozoites at a concentration of 2 × 10^6 were cultured in Ham's F12 medium for 24 h at 35°C. The supernatants were removed and centrifuged at 500 × *g* for 10 min. The cell-free supernatants from HK cultures were prepared in a similar fashion. Target cells were treated with 200 μl of the cell-free supernatants.

RESULTS

Apoptosis of tumor cells by *Acanthamoeba* lysate. We have recently reported that *Acanthamoeba* trophozoites lyse a wide variety of murine and human tumor cells by a process reminiscent of apoptosis (21). The present study considers the hypothesis that *Acanthamoeba* trophozoites produced a lytic factor that mediates tumor cell lysis by apoptosis. Preliminary studies indicated that *Acanthamoeba* trophozoites elaborated lytic factors that were constitutively secreted and could be isolated from the culture medium. Lytic factors were also present in cell-free lysates produced by rupturing *Acanthamoeba* trophozoites. Lysates were used for most of the studies described below because they contained significantly greater lytic activity than culture supernatants and were much easier and more economical to prepare. Accordingly, murine neuroblastoma cells were exposed to *Acanthamoeba* lysate (1.0 mg/ml) for 16 h at 37°C. FACS analysis of PI staining demonstrated that more than 70% of tumor cells exposed to parasite lysate underwent apoptosis on the basis of fragmentation of tumor cell DNA (Fig. 1). By contrast, DNA fragmentation occurred in only 7% of tumor cells exposed to medium alone. *Acanthamoeba*-induced apoptosis of tumor cells was confirmed by demonstrating DNA ladders by gel electrophoresis. DNA was extracted from murine neuroblastoma cells following 16 h of incubation (37°C) with either *Acanthamoeba* lysate or medium alone, and the development of DNA ladders was assayed. Positive apoptosis controls consisted of rat thymocytes exposed to corticosteroids. Parasite lysate served as a background negative control. Characteristic DNA ladders consisting of multiple 180- to 200-bp nucleosome fragments were produced by neuroblastoma cells exposed to parasite lysate (Fig. 2). Similar DNA ladders were formed by rat thymocytes treated with corticosteroid. By contrast, DNA fragmentation did not occur in neuroblastoma cells similarly incubated in medium alone. Likewise, parasite lysate did not contain DNA fragments (Fig. 2).

Ultrastructural changes during parasite-induced apoptosis. The notion that tumor cells exposed to *Acanthamoeba* trophozoites undergo apoptosis was also confirmed by light and scanning electron microscopy. Following incubation with live trophozoites or parasite lysate, murine neuroblastoma cells underwent morphological changes characteristic of classical apoptosis. By light microscopy, tumor cells treated with either live trophozoites or parasite lysate developed extensive cell membrane blebs, nuclear condensation, and overall cell shrinkage (Fig. 3). Identical morphological alterations were demonstrated by scanning electron microscopy. Apoptotic bodies (protuberances that appear to pinch off from the plasma membrane of the apoptotic cell), which are diagnostic features of apoptosis, were detected by scanning electron microscopy

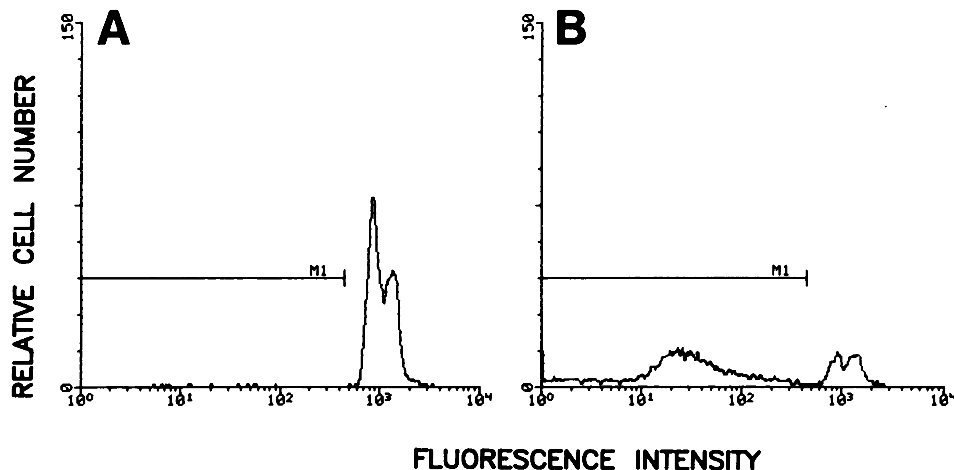

FIG. 1. Histogram of FACS analysis of neuroblastoma cells stained with PI. (A) FACS profile of cells incubated in medium only. (B) FACS profile of cells 16 h after incubation with *Acanthamoeba* lysate. Bracket M1 gate encompasses cell number with hypodiploid DNA.

(Fig. 3). However, there was no evidence of amebostome formation or troglocytosis in any of the experimental samples.

Dose-dependent induction of apoptosis by *Acanthamoeba* lysate. In order to determine an optimal dose of parasite lysate that induces apoptosis, human uveal melanoma cells (OCM1) were incubated with 100 μl of various concentrations of lysate, ranging from 0.0625 to 1.0 mg/ml (6.25 to 100 μg of protein per well). Sixteen hours after incubation, the melanoma cells were assayed for apoptosis by PI staining and flow cytometry. A dose-dependent effect of parasite lysate was clearly evident. Maximum apoptosis (73%) was detected in melanoma cells incubated with the highest concentration of parasite lysate

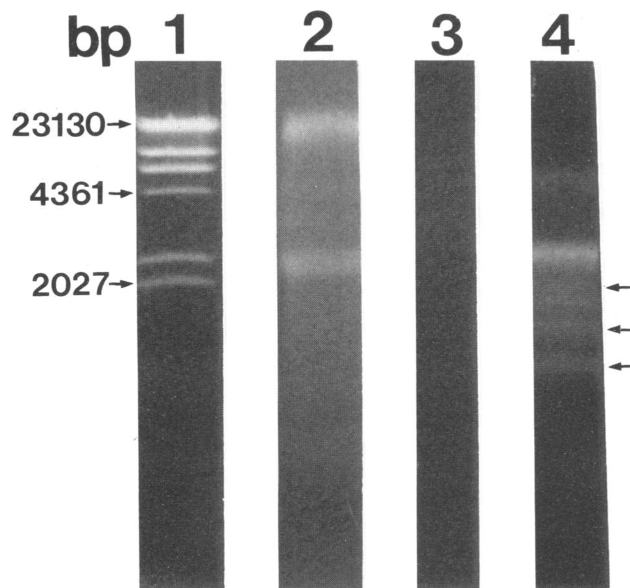

FIG. 2. Gel electrophoresis demonstrating DNA fragmentation in neuroblastoma cells after 16 h in culture. Lane 1, standard; lane 2, cells exposed to medium only; lane 3, parasite lysate; lane 4, cells exposed to *Acanthamoeba* lysate. Arrows indicate characteristic DNA ladder formation, consisting of nucleosomal fragments that occurred in cells exposed to *Acanthamoeba* lysate.

(Fig. 4). By contrast, incubation with a similar concentration of irrelevant protein (i.e., BSA) did not result in significant DNA fragmentation. Since 1.0 mg (100 μg per well) of parasite lysate per ml produced the greatest amount of apoptosis, all subsequent experiments utilized this concentration of lysate.

Constitutive secretion of apoptosis-inducing factors by *A. castellanii*. Preliminary cinematography studies suggested that extensive tumor cell lysis occurred without direct contact between the target cells and *Acanthamoeba* trophozoites (unpublished findings). The possibility that *Acanthamoeba* trophozoites secrete soluble factors that mediate tumor cell apoptosis was examined. Cell-free supernatants from 24-h *Acanthamoeba* cultures (2×10^6 cells per ml) were collected, filtered, and assessed for apoptotic activity by the previously described FACS assay for apoptosis. For comparison, the cell-free supernatant from HK cultures was similarly prepared and tested. Murine neuroblastoma cells incubated with the cell-free culture supernatants from *Acanthamoeba* trophozoites underwent extensive apoptosis (Fig. 5). By contrast, only 5.5% of the neuroblastoma cells exposed to HK culture medium underwent apoptosis. Thus, *Acanthamoeba* trophozoites constitutively elaborate soluble factors that produce extensive apoptosis of tumor cells in vitro.

Susceptibility of a variety of cell types to parasite-mediated apoptosis. Experiments were designed to determine susceptibility of several neoplastic cells to parasite-induced apoptosis. The apoptosis assay described above was used to assess apoptosis of the following target cells: (i) two murine melanomas (Queens and D5.1G4), (ii) two human uveal melanomas (OCM1 and OCM3), (iii) a murine fibrosarcoma (UV 5497), (iv) a murine mastocytoma (P91), and (v) a murine neuroblastoma.

Tumor cells were exposed to *Acanthamoeba* lysate, and apoptosis was evaluated by PI staining and flow cytometry. The results indicate that all tumor cells were susceptible to apoptosis induced by *Acanthamoeba* lysate (Table 1). To determine whether the observed apoptosis was specific for *A. castellanii*, neuroblastoma cells were exposed to an aqueous extract of normal mammalian HK cells. The results indicate that extract of HK control cells did not produce detectable apoptosis of murine neuroblastoma cells.

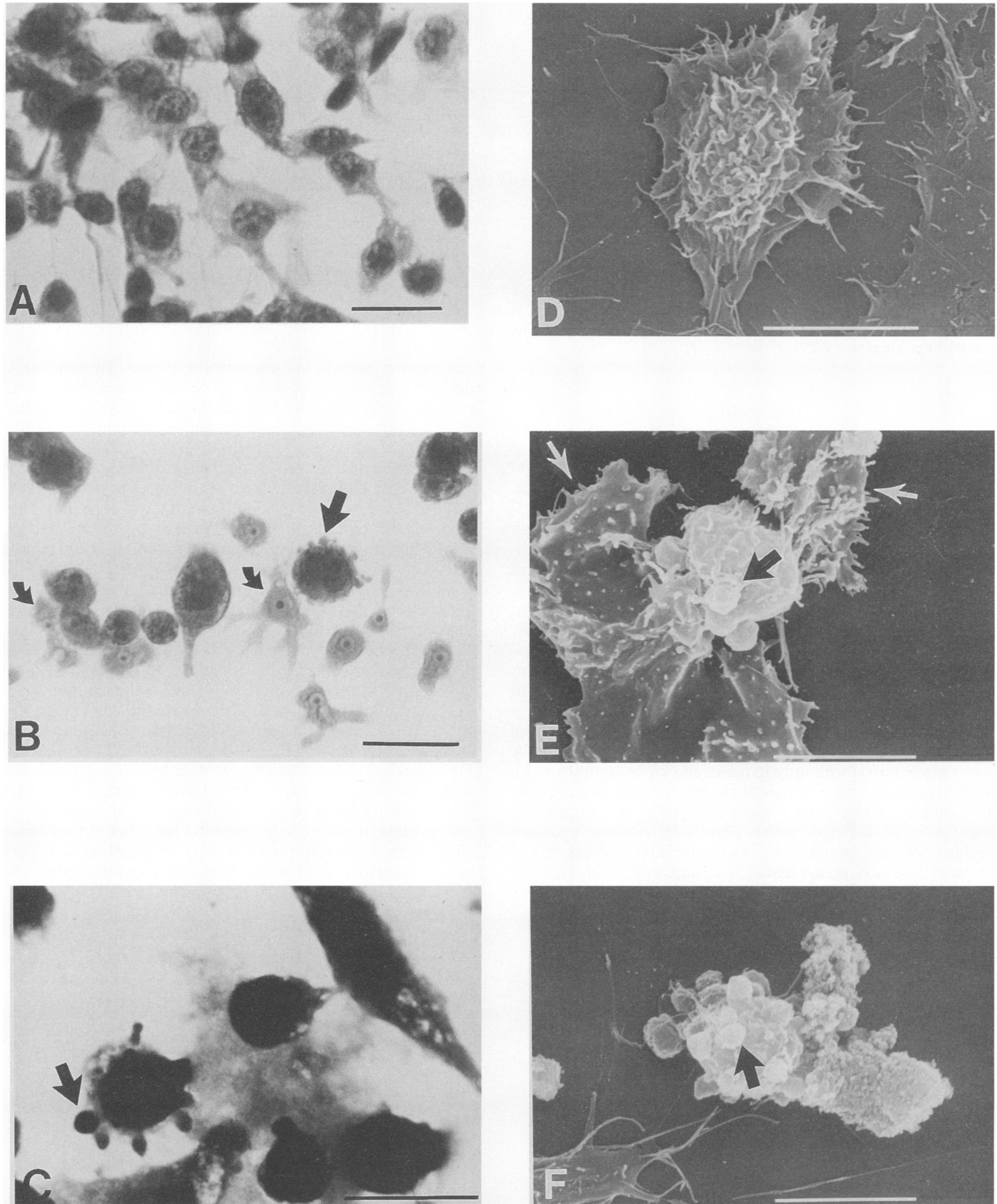

FIG. 3. Photomicrograph of neuroblastoma cells exposed to either *Acanthamoeba* trophozoites or *Acanthamoeba* lysate. (A, B, and C) Light microscopy. (A) Control neuroblastoma cells. (B and C) Cells exposed to *Acanthamoeba* trophozoites (curved arrows) or lysate. Bar = 50 μm. (D, E, and F) Scanning electron microscopy of normal and apoptotic neuroblastoma cells. (D) Control neuroblastoma cells (exposed to medium only). (E and F) Cells were induced to undergo apoptosis by *Acanthamoeba* trophozoites (white arrows) or lysate. Bar = 10 μm. Target cells exhibit blebbing (shown with arrows) after 16 h of exposure to lysate.

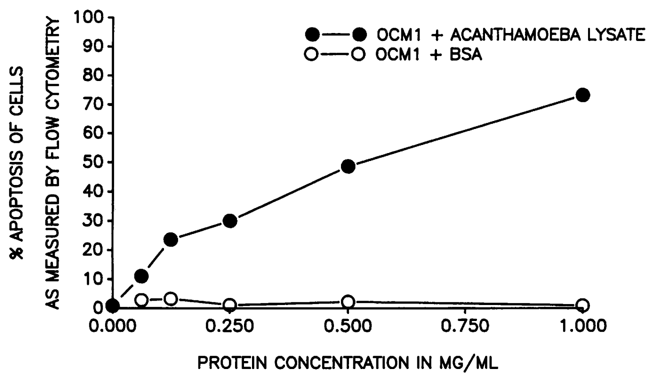

FIG. 4. The percentage of apoptotic OCM1 cells, assessed by staining with PI and FACS analysis. Cell cultures were exposed for 16 h to different concentrations of *Acanthamoeba* lysate or BSA. The standard deviation was <2%.

TABLE 1. Apoptosis induced by *Acanthamoeba* lysates[a]

Target cell	% Apoptosis (control)	% Apoptosis (lysate)
NB41A3 (murine neuroblastoma)	7.2	78
OCM1 (human ocular melanoma)	8.5	80
OCM3 (human ocular melanoma)	20.3	89
D5.1G4 (murine melanoma)	18.7	85
Queens (murine melanoma)	16.5	66
UV 5497 (murine fibrosarcoma)	17.5	45
P91 (murine mastocytoma)	16.8	93
HK (human keratocyte)	7.5	96

[a] Target cells, at a concentration of 10^5 cells per well, were treated with 100 μl of parasite lysate (100 μg of protein per ml) for 16 h at 37°C. The percentage of apoptosis was quantified by PI staining and flow cytometry. The standard deviation for each group was <2%.

DISCUSSION

It is well recognized that pathogenic free-living amoebae inflict remarkable CPE on mammalian cells in vitro and in vivo. *Naegleria* trophozoites can produce CPE by troglocytosis, contact-dependent lysis, or a combination of the two (8, 10, 15, 16). Although trophozoites of *Acanthamoeba* spp. lyse both neoplastic and nonneoplastic target cells, the underlying mechanisms have not been previously characterized. The present results indicate that, like *Naegleria* spp., *Acanthamoeba* trophozoites are capable of producing extensive CPE on a variety of tumor cells. However, unlike *Naegleria* spp., *Acanthamoeba* trophozoites do not form amebostomes or utilize troglocytosis as a mechanism for target cell destruction. *Acanthamoeba*-mediated CPE is, therefore, mediated by direct cytolysis of target cells.

In general, cytolysis can occur by one of two fundamental mechanisms, either by disruption of cell membrane integrity by necrosis via pore-forming lytic molecules or by apoptosis (6). Necrosis refers to morphological changes found when cells die from sudden injury. The plasma membrane is typically the major site of damage, which results in osmotic changes, swelling, rupture of the cell membrane, and spilling of cell contents into juxtaposed tissues. Necrotic cell death typically results in significant inflammatory responses. By contrast, cell death by apoptosis is more subtle and does not normally provoke inflammation. Cell membranes undergo extensive blebbing not seen in necrosis. Instead of swelling, the apoptotic cell shrinks significantly, resulting in a vast reduction in the cytoplasm-to-nucleus ratio. The nucleus undergoes remarkable changes culminating in nuclear shrinkage, chromatin condensation, and extensive DNA fragmentation. It is not known whether the parasite-mediated cytolysis of normal and neoplastic target cells is produced by both apoptosis and necrosis. However, the results of the present study indicate that *Acanthamoeba* trophozoites elaborate soluble factors that elicit apoptosis of a variety of human and murine tumors in a dose-dependent manner. Although significant tumor cell lysis may also occur by necrosis, the data do not rule out the possible additive effects of necrosis and apoptosis in cell death.

The present findings offer compelling evidence that apoptosis contributes to *Acanthamoeba*-mediated CPE. The most widely used and readily identifiable marker of apoptosis is the cleavage of DNA into oligonucleosomally sized fragments (i.e., DNA ladder). Characteristic DNA ladders were observed in neuroblastoma cells exposed to *Acanthamoeba* lysate. Moreover, ultrastructural changes characteristic of apoptosis were also evident in tumor cells exposed to either *Acanthamoeba* lysate or factors constitutively secreted by trophozoites. Tumor cells displayed extensive shrinkage, chromatin condensation,

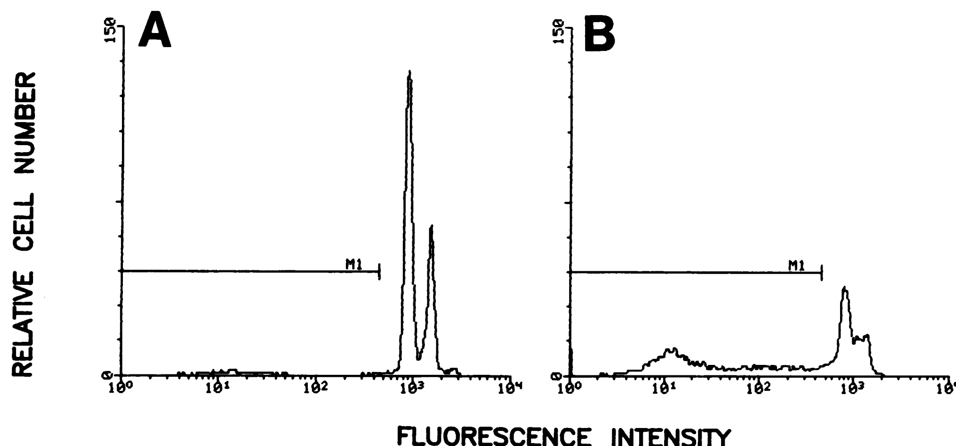

FIG. 5. Histogram of FACS analysis of neuroblastoma cells stained with PI. (A) FACS profile of cells incubated in medium. (B) FACS profile of cells 16 h after incubation with soluble factors secreted by *A. castellanii*. Bracket M1 gate encompasses cell number with hypodiploid DNA.

cell membrane blebbing, and the formation of apoptotic bodies.

It is tempting to assume that cytolysis mediated by *A. castellanii* occurs by the same process employed by cytolytic T lymphocytes (CTL). Although CTL-mediated lysis results in the fragmentation of the target cell DNA (22), direct binding of the CTL to the target cell is a critical prerequisite for cytolysis (18). It is not known whether binding of soluble factors produced by *Acanthamoeba* trophozoites is necessary for *Acanthamoeba*-mediated cytolysis. The morphological hallmarks of apoptosis—extensive cell membrane blebbing, cell shrinkages, and the formation of apoptotic bodies—are clearly evident in tumor cells exposed to either *Acanthamoeba* trophozoites or parasite-derived soluble factors. Similar morphological changes do not typically accompany CTL-mediated lysis, which occurs by rapid cell membrane disruption and rupture of the target cell (2).

The pathogenic free-living amoeba *Naegleria fowleri* inflicts extensive CPE on neuroblastoma cells by two mechanisms, troglocytosis and contact-dependent lysis, that are not mutually exclusive (16). Troglocytosis results in CPE by piecemeal consumption of neuroblastoma target cells by a sucker apparatus present on the surface of *Naegleria* trophozoites (8, 10, 15). The exact mechanisms involved in contact-dependent lysis of target cells by *N. fowleri* remain unclear, but it is possible that *N. fowleri*, like *A. castellanii*, produces significant tumor cell lysis by apoptosis.

In summary, the present results indicate that *A. castellanii* trophozoites constitutively elaborate soluble factors that elicit apoptosis in a variety of human and murine tumors. To our knowledge, this represents the first published report of apoptosis mediated by a protozoal parasite. The identity and characterization of the apoptosis-inducing molecule are under investigation and will hopefully yield further insights into the pathogenesis of pathogenic free-living amoeba infections.

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