**Acanthamoeba culbertsoni** Elicits Soluble Factors That Exert Anti-Microglial Cell Activity

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*Acanthamoeba culbertsoni* is an opportunistic pathogen that causes granulomatous amoebic encephalitis (GAE), a chronic and often fatal disease of the central nervous system (CNS). A hallmark of GAE is the formation of granulomas around the amoebae. These cellular aggregates consist of microglia, macrophages, lymphocytes, and neutrophils, which produce a myriad of proinflammatory soluble factors. In the present study, it is demonstrated that *A. culbertsoni* secretes serine peptidases that degrade chemokines and cytokines produced by a mouse microglial cell line (BV-2 cells). Furthermore, soluble factors present in cocultures of *A. culbertsoni* and BV-2 cells, as well as in cocultures of *A. culbertsoni* and primary neonatal rat cerebral cortex microglia, induced apoptosis of these macrophage-like cells. Collectively, the results indicate that *A. culbertsoni* can apply a multiplicity of cell contact-independent modes to target macrophage-like cells that exert anti-amoeba activities in the CNS.

*Acanthamoeba culbertsoni* belongs to a group of free-living amoebae, such as *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia pedata*, that can cause disease in humans (46, 56). *Acanthamoeba* spp. are found worldwide and have been isolated from a variety of environmental sources, including air, soil, dust, tap water, freshwater, seawater, swimming pools, air conditioning units, and contaminated contact lenses (30). Trophozoites feed on bacteria and algae and represent the infective form (47, 56). However, under unfavorable environmental conditions, such as extreme changes in temperature or pH, trophozoites transform into a double-walled, round cyst (22, 45).

*Acanthamoeba* spp. cause an infection of the eye known as amoebic keratitis (AK), an infection of the skin referred to as cutaneous acanthamoebiasis, and a chronic and slowly progressing disease of the central nervous system (CNS) known as granulomatous amoebic encephalitis (GAE) (22, 23, 30, 56). GAE is most prevalent in humans who are immunocompromised (30, 33, 40) and has been reported to occur among individuals infected with the human immunodeficiency virus (HIV) (28). It has been proposed that *Acanthamoeba* trophozoites access the CNS by passage through the olfactory neuroepithelium (32) or by hematogenous spread from a primary nonneuronal site of infection (23, 24, 33, 53).

In immune-competent individuals, GAE is characterized by the formation of granulomas. These cellular aggregates consist of microglia, macrophages, polymorphonuclear cells, T lymphocytes, and B lymphocytes (24, 30). The concerted action of these immune cells results in sequestration of amoebae and is instrumental in slowing the progression of GAE. This outcome is consistent with the observation that granulomas are rarely observed in immunocompromised individuals (34) and in mice with experimentally induced immune suppression following treatment with the cannabinoid delta-9-tetrahydrocannabinol (Δ^9-THC) (8).

Microglia are a resident population of macrophages in the CNS. These cells, along with CNS-invading peripheral macrophages, appear to play a critical early effector role in the control of *Acanthamoeba* spread during GAE (4, 5, 29, 31). *In vitro*, microglia have been shown to produce an array of chemokines and cytokines in response to *Acanthamoeba* (31, 51). However, these factors appear not to have a deleterious effect on these amoebae (29).

*Acanthamoeba* spp. produce serine peptidases, cysteine peptidases, and metallopeptidases (1, 2, 9, 10, 26, 37, 38, 41, 42, 52). In the present study, it is demonstrated that serine peptidases secreted by *A. culbertsoni* degrade chemokines and cytokines that are produced by immortalized mouse BV-2 microglia-like cells. In addition, soluble factors present in cocultures of *A. culbertsoni* and BV-2 cells induced apoptosis of the BV-2 cells. Collectively, these results suggest a mode through which *A. culbertsoni* can evade immune responsiveness in the CNS.

**MATERIALS AND METHODS**

*Amoebae. A. culbertsoni* (ATCC 30171), *Acanthamoeba astronyxis* (ATCC 30137), and *B. mandrillaris* (ATCC 50209) were acquired from the American Type Culture Collection (ATCC; Manassas, VA). *A. astronyxis* is a free-living amoeba that does not cause GAE (45). *B. mandrillaris* was isolated originally from the brain of a mandrill that died of amoebic meningoencephalitis (55). Axenic *acanthamoebae* (i.e., grown in culture free of contaminating microorganisms) were maintained in Oxoid medium (0.55% [wt/vol] Oxoid neutralized liver digest, 0.3% [wt/vol] dextrose, 0.5% [wt/vol] proteose peptone, 0.25% [wt/vol] yeast extract, 1% fetal bovine serum [FBS], and 0.1% hemin) at 37°C (*A. culbertsoni*) and 25°C (*A. astronyxis*). *B. mandrillaris* was maintained at 35°C in BM-3 medium (49).

*Microglia-like cells. The immortalized mouse BV-2 microglial cell line was a gift from Michael McKinney of the Mayo Clinic (Jacksonville, FL). BV-2 cells were maintained in complete Dulbecco’s modified Eagle medium (i.e., DMEM supplemented with 10% heat-inactivated FBS [HI-FBS], 100 U/ml penicillin G, 100 μg/ml streptomycin, and 25 mM HEPES) at 37°C and 5% CO_2._

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High performance liquid chromatography (HPLC) was used to analyze for the percent adjusted volume of total enzyme activity using Quantity-

... 100 ng/ml). In a second set of experiments, experiments, cell supernatants from BV-2 cells (10^6) maintained (8 h or 18 h) in Neurobasal-A medium in the presence of LPS (100 ng/ml). Additionally, cell-free culture supernatant from BV-2...
**RESULTS**

*A. culbertsoni* secretes serine peptidases. *Acanthamoeba* cells (10^6) were incubated in 5 ml of Neurobasal-A medium to yield *Acanthamoeba*-conditioned medium. In order to identify peptidases secreted by *A. culbertsoni* (Fig. 1A and B) and *A. astronyxis* (Fig. 1C) into this medium, gelatin zymography was performed. Each aliquot of medium was analyzed at a protein concentration of 0.70 mg/ml and was subjected to gel electrophoresis at a constant volume. For *A. culbertsoni*, bands indicative of peptidase activity were identified at positions corresponding to approximately 150, 100, 90, 70, 50, and 40 kDa (Fig. 1A). Percent adjusted volume analysis indicated that these corresponded, respectively, to 39%, 11%, 8%, 10%, 20%, and 7% of the total activity within the lane. In order to identify the class of peptidases associated with the respective bands observed on zymograms, peptidase class-specific inhibitors were employed. Incubation of samples prior to electrophoresis with 1 mM PMSF resulted in abrogation of enzymatic activity. On the other hand, incubation of gels with 5 mM 1,10-phenanthroline resulted in abrogation of enzymatic activity of the 210-, 80-, 55-, and 50-kDa bands (Fig. 1C). Complete abrogation of activity associated with the 80-, 45-, and 30-kDa bands was obtained upon treatment with 1 mM PMSF; lane 3, treated with 5 mM 1,10-phenanthroline; lane 4, treated with 5 μM E-64. The lower case letters and arrows indicate protein species of approximately 150, 100, 90, 70, 50, and 40 kDa. Molecular mass was calculated from the mean center of the respective band on gelatin zymograms. (B) Supernatant from coculture of BV-2 cells and *A. culbertsoni* subjected to no treatment (lane 1) or incubated in the presence of the inhibitor PMSF (lane 2). The lower case letters and arrows indicate protein species of approximately 150, 70, and 50 kDa. (C) Gel zymograms of *A. astronyxis*-conditioned medium. Lane 1, untreated; lane 2, treated with 1 mM PMSF; lane 3, treated with 5 mM 1,10-phenanthroline; lane 4, treated with 5 μM E-64. The lower case letters and arrows indicate protein species of approximately 210, 80, 55, 45, and 30 kDa. (D) Densitometric comparison of lane A1 for *A. culbertsoni* and lane C1 for *A. astronyxis*. The major bands of activity are indicated by the corresponding letter designations.

Data analysis. For chemokine/cytokine protein arrays, the background was subtracted and the density of duplicate chemokine and cytokine immunoreactive spots (i.e., the sum of pixels per array spot), which corresponded to the relative amount of chemokine or cytokine in each culture supernatant, was normalized to the average density of the internal standard control spots consisting of the biotin-conjugated IgG included in each membrane. The average density of the internal standard control spots of each membrane was normalized to that of other membranes to allow for assessment of individual chemokine/cytokine spots between membranes. Density values were graphed as the mean intensity value, and a fold difference of ≥2.1 compared to the control was considered significant. For graphic depiction of chemokine/cytokine species, the normalized density of each spot was determined using SigmaGel gel analysis software (SPSS Science, Chicago, IL) and plotted using GraphPad Prism V software (GraphPad Software, San Diego, CA). For ELISA, the optical density of aliquots from coculture supernatants at time of harvest (i.e., t = 0) was considered the 100% value. Aliquots of the same cocultures were assessed at temporal intervals thereafter, and their optical densities at t = x were represented as percentages of the optical densities obtained at the time of harvest (t = 0). Samples from each time point were assessed in triplicate, and the average optical density was obtained to calculate the percent maximum response. For MTT assays, data were graphed based on 4 separate experiments with ±standard deviations (SD). For TUNEL analysis, the graphs of 2 separate experiments performed in triplicate showing ±SD for 1,000 counted cells/well were constructed. Western immunoblotting results were representative of 3 separate experiments.
A. culbertsoni elicits the expression of chemokine and cytokine protein by BV-2 cells. In order to determine whether A. culbertsoni induced expression of a novel pattern of chemokines and cytokines from BV-2 cells at the protein level, the RayBio mouse cytokine antibody array III was employed (Fig. 3). BV-2 cells (10^6) were cultured for 9 h, and the cell-free supernatant was harvested for analysis. In addition to several species that were produced at relatively low levels, BV-2 cells maintained in the presence or absence of amoebae constitutively expressed or inducibly expressed chemokine and cytokine mRNAs by BV-2 cells.

**Factors present in A. culbertsoni-conditioned medium degrade cytokines and chemokines produced by BV-2 cells.** Supernatants from BV-2 cells cultured for 8 h or 18 h were assessed for the presence of chemokine and cytokine protein. The 18-h time point was shown to yield a robust level of constitutively expressed protein, including MCP-1, MIP-1α, MIP-1γ, MIP-2, platelet factor 4 (PF-4), P-selectin, soluble tumor necrosis factor receptor I (sTNFRI), and sTNFRII (Fig. 3A). These soluble factors, with the exception of MIP-2, which was identified at an augmented level, were also identified at approximately comparable levels in supernatants of BV-2 cells cocultured with A. culbertsoni for 9 h. In addition, the novel expression of MIP-3α, IL-1α, and TNF-α was observed. The pattern of chemokines and cytokines elicited by BV-2 cells in response to 8 h of exposure to LPS also was examined. A differential profile was observed and compared with that recorded for BV-2 cells maintained in the presence or absence of A. culbertsoni. In particular, robust or augmented levels of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-6 were produced, while diminished levels of PF-4, P-selectin, and M-CSF were observed. These observations indicate that BV-2 cells respond to A. culbertsoni by eliciting a distinctive, possibly signature pattern of cytokines and chemokines at the protein level, at least during the time frame of exposure employed in this study.

**MCP-1, gamma interferon-inducible 10-kDa protein (IP-10), macrophage inflammatory protein 2 (MIP-2), MIP-1α, and MIP-1β.** Of these, the highest levels observed were for MIP-1α. Similarly, using a riboprobe template set to assess expression of a select set of cytokine mRNAs, an augmented level of interleukin 1 receptor antagonist (IL-1Ra) mRNA was observed for BV-2 cells (10^6) cultured with A. culbertsoni (10^6 cells) compared to that for BV-2 cells cultured alone. The mRNA expression pattern of BV-2 cells maintained (6 h) in the presence of B. mandrillaris (10^6 cells) or LPS (100 ng/ml) also was examined. Distinctive mRNA profiles were obtained for BV-2 cells cocultured with B. mandrillaris compared with A. culbertsoni and for BV-2 cells treated with LPS compared with cells cultured with A. culbertsoni. B. mandrillaris elicited a minimal level of de novo chemokine mRNA expression compared to A. culbertsoni. On the other hand, LPS induced a robust cytokine mRNA response compared to A. culbertsoni. Relatively high levels of IL-1α, IL-1β, and IL-6 were obtained, at least at the 6-h time point of culture that was selected for assessment. Thus, although a large-scale screening of chemokine and cytokine mRNA species was not performed, these observations are consistent with the conclusions that (i) A. culbertsoni elicits a chemokine/cytokine mRNA profile distinctive from that elicited by B. mandrillaris and LPS and (ii) the presence of A. culbertsoni does not result in suppression of constitutively expressed or inducibly expressed chemokine and cytokine mRNAs by BV-2 cells.

**Chemokine and cytokine protein** was observed in culture supernatants of BV-2 cells treated with A. culbertsoni-conditioned medium. For supernatants of BV-2 cells incubated (8 h) with A. culbertsoni-conditioned medium, the only proteins that could be identified were MCP-1, MIP-1α, MIP-1γ, and sTNFRII (Fig. 4C). Compared to the results with the control, 8-h supernatant from BV-2 culture maintained in Neurobasal-A medium, the levels of these proteins were reduced respectively by approximately 32%, 91%, 75%, and 89%. Examination of supernatants from the 18-h BV-2 cell cultures maintained in A. culbertsoni-conditioned medium indicated that BV-2 cells constitutively expressed or inducibly expressed chemokine and cytokine mRNAs by BV-2 cells.

**Supernatants from BV-2 cells maintained in the presence or absence of A. culbertsoni** also were assessed for the presence of chemokine and cytokine protein. The 18-h time point was shown to yield a robust level of constitutively expressed protein, including MCP-1, MIP-1α, MIP-1γ, MIP-2, platelet factor 4 (PF-4), P-selectin, soluble tumor necrosis factor receptor I (sTNFRI), and sTNFRII (Fig. 3A). These soluble factors, with the exception of MIP-2, which was identified at an augmented level, were also identified at approximately comparable levels in supernatants of BV-2 cells cocultured with A. culbertsoni for 9 h. In addition, the novel expression of MIP-3α, IL-1α, and TNF-α was observed. The pattern of chemokines and cytokines elicited by BV-2 cells in response to 8 h of exposure to LPS also was examined. A differential profile was observed and compared with that recorded for BV-2 cells maintained in the presence or absence of A. culbertsoni. In particular, robust or augmented levels of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-6 were produced, while diminished levels of PF-4, P-selectin, and M-CSF were observed. These observations indicate that BV-2 cells respond to A. culbertsoni by eliciting a distinctive, possibly signature pattern of cytokines and chemokines at the protein level, at least during the time frame of exposure employed in this study.

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conditioned medium revealed a virtually total degradation of chemokine and cytokine protein species (Fig. 4D). In contrast, incubation of BV-2 cells in the presence of Neurobasal-A medium supplemented with 0.70 mg/ml conditioned medium from nonpathogenic *A. astronyxis* had a minimal effect on constitutively expressed chemokines and cytokines (Fig. 4E). The soluble factors produced by *A. culbertsoni* that degrade BV-2 cell chemokines and cytokines are serine peptidases. In order to link the soluble factors in *A. culbertsoni*-conditioned medium that degraded chemokines and cytokines to serine peptidases, cell-free supernatant from BV-2 cells (10^6) cultured for 18 h was incubated with 0.70 mg/ml *A. culbertsoni*-conditioned medium in the presence or absence of 1 mM PMSF for 8 h and assessed using the RayBio mouse cytokine antibody array (Fig. 5). Treatment with PMSF prevented the degradation of chemokines and cytokines in the BV-2 supernatant (Fig. 5C). To confirm these data, BV-2 cells (10^6) were cocultured with *A. culbertsoni* (10^6 cells) for 9 h, and the cell-

FIG. 3. *A. culbertsoni* induces a distinctive pattern of chemokine and cytokine protein from BV-2 cells. Cell-free culture supernatants were assessed for chemokine and cytokine protein using the RayBio mouse cytokine antibody array III. (A and B) Membrane array depicting chemokines and cytokines in culture supernatant of BV-2 cells (10^6) maintained (9 h) in the absence (A) or presence (B) of *A. culbertsoni*. (C) Membrane array depicting chemokines and cytokines in culture supernatant of BV-2 cells (10^6) maintained (8 h) in the presence of LPS (100 ng/ml). The relative amount of each chemokine or cytokine was normalized to the average density of standards (Std). A graphic representation of select protein species is paired with each membrane array. Chemokines and cytokines are designated as follows: 1, cutaneous cell-attracting chemokine (CTACK); 2, granulocyte colony-stimulating factor (G-CSF); 3, granulocyte macrophage colony-stimulating factor (GM-CSF); 4, insulin-like growth factor binding protein 3 (IGFBP3); 5, IL-1α; 6, IL-6; 7, IL-12p40/70; 8, MCP-1; 9, macrophage colony-stimulating factor (M-CSF); 10, MIP-1α; 11, MIP-1γ; 12, MIP-2; 13, MIP-3α; 14, PF-4; 15, P-selectin; 16, regulated upon activation, normal T cell expressed and secreted (RANTES; CCL5); 17, TNF-α; 18, sTNFRI; and 19, sTNFRI.
free supernatants were incubated an additional 12 to 48 h in the presence or absence of PMSF and examined by ELISA. The 9-h time period for assessment of coculture supernatant was selected since a longer coculture period could have led to degradation of protein, thereby precluding identification of chemokines or cytokines targeted by the peptidases. For these experiments, MIP-1α, MIP-2, and TNF-α were selected as representative species, and their levels were measured at the 9-h time point of coculture harvest \((t = 0)\) and designated the 100% base for comparison of levels of the respective species at defined incubation time points postharvest. In the absence of PMSF, all three protein species in cell-free culture supernatants underwent a rapid time-related decrease in level (Fig. 6). At the 12-h time point postharvest, approximately 10% of MIP-1α levels and 50% of MIP-2 levels remained. By 48 h postharvest, minimal levels of MIP-1α or MIP-2 were detected. A similar outcome was obtained for TNF-α. The level of TNF-α at 12 h postharvest was approximately 20% of that recorded at the time of harvest of supernatant (Fig. 6C). By 48 h postharvest of supernatant, minimal levels of TNF-α were detected.

In contrast, for culture supernatants treated with PMSF, a relatively high level of MIP-1α or MIP-2 protein was maintained postharvest. For MIP-1α, 80% and 48% of levels obtained at the time of harvest were recorded at 12 h and 48 h postharvest, respectively. For MIP-2, 100% of the level recorded at the time of harvest was maintained for the 12- to 48-h postharvest incubation period. Similarly, for supernatants treated with PMSF, 80% of the level of TNF-α recorded at the time of culture supernatant harvest was retained at the 12-h time point. At the 48-h time point, approximately 40% of the level of TNF-α recorded at the time of culture harvest was obtained. Collectively, these results indicate that the principle factors present in \(A.\ culbertsoni\)-conditioned medium that account for the degradation of microglia-secreted chemokines and cytokines are serine peptidases.

**Soluble factors in \(A.\ culbertsoni\)-conditioned medium induce microglial apoptosis.** Light microscopy revealed that BV-2 cells maintained for 18 h in Neurobasal-A medium displayed a spindle morphology (Fig. 7A). However, these cells, when maintained in Neurobasal-A medium supplemented with \(A.\ culbertsoni\)-conditioned medium for 8 h, displayed a round morphology (Fig. 7B). Maintenance of BV-2 cells in the presence of \(A.\ culbertsoni\)-conditioned medium for 18 h resulted in...
membrane blebbing in greater than 50% of the BV-2 cells, rupture in cell membranes, and extrusion of cytosol (Fig. 7C). Consistent with the light microscopy observations, TUNEL assay demonstrated that 47.8% and 66.4% of the BV-2 cells maintained, respectively, for 8 h and 18 h in Neurobasal-A medium supplemented with *A. culbertsoni*-conditioned medium were apoptotic (Fig. 7F, G, and I). At the latter time point, electron microscopy revealed the presence of membrane blebbing and apoptotic bodies, indicating that soluble factors in *A. culbertsoni*-conditioned medium induced apoptosis of BV-2 cells (Fig. 7J). To confirm these results using a primary cell type, neonatal rat cerebral cortex microglia (pMG cells) were incubated with *A. culbertsoni*-conditioned medium for 8 h and assessed using the TUNEL assay. Comparable with results obtained with BV-2 cells, approximately 50% of the pMG cells were apoptotic in the presence of *A. culbertsoni* (10°) cells. Cultures were maintained for 9 h, and the cell-free medium was harvested and incubated in the presence or absence of 1 mM PMSF for defined periods thereafter. At the time of harvest, 3,983 pg/ml of MIP-1α, 5,247 pg/ml of MIP-2, and 1,695 pg/ml of TNF-α were obtained and were considered the respective 100% values. (Top) MIP-1α; (middle) MIP-2; (bottom) TNF-α. Experiments were performed in triplicate, and the average optical density of each sample was used to calculate the percent maximum response.

In order to gain insight as to whether the intrinsic versus the extrinsic apoptotic pathway was induced, Western immunoblot assessment of Fas ligand (FasL) and endonuclease G (Endo G) proteins was performed. FasL (CD95L) is a type II transmembrane protein of the TNF superfamily that is able to activate the extrinsic pathway of apoptosis, while Endo G is a mitochondrial protein released from the intermembrane space together with other proapoptotic pro-
teins and is a constituent element of the intrinsic pathway of apoptosis. BV-2 cells were maintained in Neurobasal-A medium for 18 h as a negative control or in Neurobasal-A medium supplemented with 0.70 mg/ml \textit{A. culbertsoni}-conditioned medium for 8 h (D). The arrows designate blebs extruding from the cell surface. Magnification, ×40. (E to H) Light microscopy assessment of terminal dUTP nick end labeling (TUNEL assay) for BV-2 cells cultured (18 h) in medium (E) or in medium supplemented with \textit{A. culbertsoni}-conditioned medium for 8 h (F) or for 18 h (G) or with \textit{A. astronyxis}-conditioned medium for 18 h (H). The percentage of apoptosis for each culture is designated at the bottom left corner of each panel. Magnification, ×20. (I) Graphical representation of the TUNEL assays shown in panels E through H. BV-2 cells maintained only in medium served as a negative control (CT) for apoptosis, while cells treated with DNase I (5 U/ml) served as a positive control for apoptosis. BV-2 cells were cultured in \textit{A. culbertsoni}-conditioned medium for 8 h (Cultb 8 h) or for 18 h (Cultb 18 h) or in \textit{A. astronyxis}-conditioned medium for 18 h (Astro 18 h). One thousand cells/well were counted, and bars represent the standard deviations of results of three separate experiments performed in triplicate; ***, \( P < 0.001 \). (J) Electron micrograph of a BV-2 cell maintained in medium supplemented with \textit{A. culbertsoni}-conditioned medium that has undergone apoptosis. Arrows indicate the electron-dense apoptotic bodies. Bar, 1 \( \mu \)m. (K) Graphical representation of TUNEL assays for pMG cultured (8 h) in medium supplemented with \textit{A. culbertsoni}-conditioned medium or \textit{A. astronyxis}-conditioned medium. One thousand cells/well were counted, and bars represent the standard deviations of results of two experiments performed in duplicate; ***, \( P < 0.001 \).
that are secreted by A. culbertsoni cells were cultured, was also observed in medium in which only pattern of peptidase activity resident in corresponded to molecular masses of 150, 70, and 50 kDa. The disparity in molecular masses may be due to the of activity corresponded to molecular masses of approximately 150, 100, 90, 70, and 50 kDa. However, as anticipated, the relative levels of enzyme activity in medium from these cocultures were lower since fewer amoebae (i.e., 10^6) were used. Nevertheless, these latter results suggest that the presence of BV-2 cells, at least at the 1:1 ratio that was used for cocultures, does not result in a differential induction of peptidases.

In contrast, a distinctive pattern of secreted proteases was obtained for A. astronyxis, a nonpathogenic amoeba within the same genus. Upon zymography, A. astronyxis maintained bands of activity with molecular masses corresponding to 210, 80, 55, 50, 45, and 30 kDa. Through the use of class-specific inhibitors, it was demonstrated that both serine and metallopeptidases were released by these amoebae. The serine peptidase activity, however, was associated only with bands of approximate molecular masses of 55 kDa and 50 kDa, compared with A. culbertsoni, for which the full spectrum of enzyme activity was associated with that of serine peptidases. Thus, A. culbertsoni also exhibited a distinctive pattern of serine peptidase activity compared with A. astronyxis. These results suggest that a specified subclass of serine peptidases emitted from A. culbertsoni, or a higher level of total serine peptidase produced by these amoebae, accounts for chemokine/cytokine degradation. This supposition is consistent with the observation that A. astronyxis-conditioned medium did not degrade chemokines/cytokines constitutively expressed by BV-2 cells, at least during the 8-h time frame employed for assessment. The collective zymography data are generally consistent with those derived from previous studies that indicated that pathogenic Acanthamoeba spp. produce serine peptidases that upon zymography exhibit three major bands of activity (9, 50). However, in these studies, the bands of activity corresponded to molecular masses of 188, 97, and 55 kDa. The disparity in molecular masses may be due to the amoeba variants used, to the medium conditions, or to the zymographic analysis applied and attendant extrapolation of molecular masses.

Microglia, as resident macrophages in the CNS, act as early responders to amoebic invasion (30, 31). Yet, although these cells elicit a plethora of proinflammatory mediators (31), it is apparent that they do not affect the amoebae (29). In this study, by using BV-2 cells that retain morphological, phenotypical, and functional properties of freshly isolated microglia (6), it is demonstrated that the serine peptidases released from A. culbertsoni degrade chemokines and cytokines, articulating one mode by which amoebae may evade host immunity. BV-2 cells exposed to A. culbertsoni were shown to produce chemokines and cytokines at the mRNA level, as identified by an RNase protection assay. This pattern of chemokine/cytokine mRNAs was distinctive from that of BV-2 cells maintained in the absence of A. culbertsoni, from that elicited in response to the amoeba B. mandrillaris, which also causes GAE, and from that produced in response to the potent bacterial immune modulator LPS. This pattern of differential chemokine/cytokine gene expression was replicated at the protein level, as shown by protein microarrays revealing a distinctive pattern of protein species for BV-2 cells compared with BV-2 cells cocultured with A. culbertsoni and, in turn, compared with BV-2 cells treated with LPS.

Although chemokine and cytokine protein was produced
and released by BV-2 cells in response to A. culbertsoni, it was found to undergo a time-related degradation in culture supernatants. This temporal degradation process was linked to the action of serine peptidases secreted from the amoebae, since treatment of cell-free culture supernatants with PMSF reversed the degradative process. These results are in agreement with those that demonstrated that Acanthamoeba-secreted serine peptidases can degrade exogenously introduced immunoglobulins and recombinant cytokines (26, 41, 42). The present studies, however, are novel in that they demonstrate that Acanthamoeba-secreted serine peptidases degrade chemokines and cytokines that are natively secreted by microglia-like cells.

The ability to drive degradation of soluble “immune” factors through the mediation of peptidases does not appear to be a feature unique to Acanthamoeba. For example, group A streptococcal serine proteases have been shown to degrade the chemokine MIP-2 (17). Also, the neutrophil-derived serine peptidases cathepsin G, elastase, and proteinase 3 have been reported to degrade human chemokine MIP-1α (48). In addition, Echinococcus multilocularis metacestode metabolites have been shown to contain a cysteine protease that digests eotaxin (35), a CC (or B-) proinflammatory chemokine. Thus, it is apparent that the ability to degrade chemokines and cytokines through the use of secreted proteases is an “immune evasion” property that is shared by a variety of microbial agents.

Soluble factors present in A. culbertsoni culture supernatants also were shown to induce apoptosis of the BV-2 cells and primary neonatal rat cerebral cortex microglia; however, no apoptosis was observed following treatment of the cells with conditioned medium from A. astronyxis. These results indicate, at least in terms of apoptosis, that results obtained with the BV-2 cells were replicated using a primary microglial cell type. Furthermore, the capacity to induce apoptosis of microglia-like cells may articulate a mode by which A. culbertsoni, and not A. astronyxis, elicits neurocytopathogenesis. This induction of apoptosis on the part of A. culbertsoni-conditioned medium could be due to several factors. BV-2 cells express protease-activated receptors (PARs) (3). These G protein-coupled receptors also are expressed on platelets, endothelial cells, epithelial cells, monocytes, T lymphocytes, natural killer (NK) cells, astrocytes, neurons, and microglia (54). Once activated, PARs lead to the production of cytokines and chemokines (11, 20, 27, 43, 44, 54, 57) and can regulate cell death (13, 54). In this context, it has been suggested that peptidases released by bacteria and protozoa can activate PARs, although the mechanisms that are operative remain obscure (13). Alternatively, exposure of BV-2 cells to Acanthamoeba spp. may elicit a chemokine/ cytokine “storm” early in the exposure process. The protein species thus elicited, while susceptible to eventual serine peptidase degradation, could nevertheless simultaneously act in an autocrine or paracrine fashion to trigger the extrinsic pathway to apoptosis in BV-2 cells. In this context, it could be anticipated in vivo that a relatively high level of chemokine and cytokine protein would be found locally within focal granulomas containing amoebae. That this outcome is a possibility is based on the identification of increased levels of FasL protein observed for BV-2 cells maintained in the presence of A. culbertsoni-conditioned medium. This protein is a homotrimERIC type II membrane protein which has a C-terminal region outside the cell and an extracellular region that has homology to the corresponding region of members of the TNF family. FasL, as a trimer binds three Fas receptor molecules on the surface of target cells, which results in activation of downstream caspases committing the cell to apoptosis. In this context, it is noted that supernatants of BV-2 cells cultured with A. culbertsoni contained TNF-α.

In summary, it is likely that A. culbertsoni uses both cell contact-dependent and contact-independent modalities to evade host immunity. In the present study, a BV-2 cell in vitro model was used to gain insight into the microglial cell-Acanthamoeba interaction. The results indicate that serine peptidases emitted from A. culbertsoni may play a cell contact-independent role in immune evasion in the CNS in their capacity to degrade chemokines and cytokines produced by microglia. Such degradation could be anticipated at focal sites of infection, where it would be anticipated that relatively high levels of secreted peptidases would be found. Soluble factors produced during the Acanthamoeba–BV-2 cell interaction also led to apoptosis of the microglia-like cells. Whether serine peptidases are linked to this phenomenon remains to be defined. Most probably, the concerted action of A. culbertsoni-specified serine peptidases and yet-to-be-defined soluble factors leads to the demise of microglia-like cells. The collective results indicate that A. culbertsoni, in addition to being able to target immune cells in a contact-dependent manner, can apply a multiplicity of cell contact-independent modes to evade the immune response in the CNS.

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REFERENCES


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