Clinical and Diagnostic Laboratory Immunology, July 2001, p. 837–840 Vol. 8, No. 4
1071-412X/01/$04.00+0 DOI: 10.1128/CDLI.8.4.837–840.2001
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Cytopathic Changes in Rat Microglial Cells Induced by Pathogenic *Acanthamoeba culbertsoni*: Morphology and Cytokine Release

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Received 22 December 2000/Returned for modification 13 April 2001/Accepted 2 May 2001

To determine whether pathogenic *Acanthamoeba culbertsoni* trophozoites and lysate can induce cytopathic changes in primary-culture microglial cells, morphological changes were observed by transmission electron microscopy (TEM). In addition, the secretion of two kinds of cytokines, tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β), from microglial cells was observed. Trophozoites of pathogenic *A. culbertsoni* made contact with microglial cells and produced digipodia. TEM revealed that microglial cells cocultured with amoebic trophozoites underwent a necrotic process, accompanied by lysis of the cell membrane. TEM of microglial cells cocultured with amoebic lysate showed that the membranes of the small cytoplasmic vacuoles as well as the cell membrane were lysed. The amounts of TNF-α secreted from microglial cells cocultured with *A. culbertsoni* trophozoites or lysate increased at 6 h of incubation. The amounts of IL-1β secreted from microglial cells cocultured with *A. culbertsoni* trophozoites at 6 h of incubation was similar to those secreted from the control group, but the amounts decreased during cultivation with *A. culbertsoni* lysate. These results suggest that pathogenic *A. culbertsoni* induces the cytopathic effects in primary-culture rat microglial cells, with the effects characterized by necrosis of microglial cells and changes in levels of secretion of TNF-α and IL-1β from microglial cells.

Acanthamoeba spp. are small, free-living limax amoebae which can cause chronic granulomatous amoebic encephalitis (GAE) and acanthamoebic keratitis in humans and experimental animals (7, 18, 21). Generally, a virulent amoeba that causes GAE in mice is cytotoxic to target cells (12, 13). Moore et al. (11) suggested that the cytopathic effects (CPEs) induced by *Acanthamoeba castellanii* against human corneal epithelial cells was the result of cytolytic enzymes released from trophozoites and subsequent phagocytosis by amoebae. The CPE of *A. castellanii* trophozoites against target cells involves calcium channels, cytoskeletal elements necessary for phagocytosis, and amoeba motility (17). Alizadeh et al. (1) and Dove Pettit et al. (4) reported that apoptosis was also a mechanism of cytolysis of corneal epithelial cells and murine neuroblastoma cells by pathogenic *Acanthamoeba*.

Microglial cells, a type of brain macrophage, have been cultured from rats and mice to understand the pathogenesis responsible for infection with various microorganisms (6, 19). Microglial cells occur in three morphological forms following cell differentiation, i.e., an amoeboid form during embryogenesis, a ramified shape in the mature normal brain, and a rod-shaped morphology around inflammatory lesions in the central nervous system (CNS) (6, 15). Moreover, they function as phagocytotic cells and produce cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha (TNF-α) (3, 16). Thus, it has been suggested that microglial cells play an important role as inflammatory cells or immunoregulatory cells in the protective immune system of the CNS (16). The purpose of present study was to determine whether primary-culture rat microglial cells show cytopathic changes in response to pathogenic *Acanthamoeba* trophozoites and lysate. Morphological changes of microglial cells were observed by transmission electron microscopy (TEM). In addition, the levels of secretion of TNF-α and IL-1β from microglial cells were determined.

A pathogenic strain of *Acanthamoeba culbertsoni* (donated by J. B. Jardin in 1977) was axenically cultured in peptone-yeast-glucose medium (20) at 37°C. The pathogenicity of this strain had previously been confirmed, in which 60% mortality occurred when mice were infected with 10^4 trophozoites (8). To prepare the amoeba lysate, trophozoites (10^8) of amoeba were harvested and washed with phosphate-buffered saline (PBS; pH 7.4). Trophozoites suspended in 1 ml of PBS were frozen (−70°C) and thawed (37°C) three times and centrifuged at 10,000 × *g* for 2 h. The supernatant was filtered with a 0.25-μm-pore-size disk filter, and the protein concentration (adjusted to 10 mg/ml) was determined by the assay described by Bradford (2).

Microglial cells were prepared by the method of Giulian and Baker (6), with some modifications (14). Briefly, the cortex of the brain was obtained from a newborn Sprague-Dawley rat and homogenized with a 21-gauge syringe. The mixture was centrifuged at 300 × *g* for 10 min and resuspended in Eagle's minimal essential medium (EMEM) with 10% fetal bovine serum (FBS). The suspension was put onto 75-cm^2 tissue culture flasks pretreated with polylysine (Sigma Chemical Co., St.

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Louis, Mo.), in order to increase the level of adherence of the cells, and the flasks were incubated at 37°C in a 5% CO_2 atmosphere for 1 week. Microglial cells were harvested by vigorously shaking each culture flask and were then filtered with nylon wool to remove any remaining astrocytes and centrifuged at 300 $\times$ *g* for 10 min. The pellet was resuspended in EMEM with 10% FBS, and the mixture was incubated at 37°C for 2 h. The attached microglial cells were harvested and adjusted to a concentration of 10^5 cells per well in a 24-well culture plate for the treatment of *Acanthamoeba* trophozoites (1:1) or lysate.

Following coincubation of the microglial cells with trophozoites or amoeba lysate, the culture was fixed in modified Karnovsky's fixative solution in cacodylate buffer (pH 7.4) and postfixed in 1% osmium tetroxide–1.5% potassium ferrocyanide. The cells were stained en bloc in 0.5% uranyl acetate, dehydrated through a graded ethanol series, and embedded in resin (Polyscience, Warrington, Pa.). Then, the blocks were sectioned with a Reichert-Jung Ultracut S ultramicrotome and stained with Ultrostain 1H and 2 (Leica, Vienna, Austria). The specimens were observed and photographed with a Zeiss EM 902 A electron microscope (Leo, Oberkohen, Germany).

Observations by TEM revealed that trophozoites of pathogenic *A. culbertsoni* characterized by a large karyosome in the nucleus made contact with microglial cells and produced a digipodium (Fig. 1B). TEM showed that microglial cells underwent necrotic processes which were accompanied by clumping of chromatin materials in the nucleus and lysis of the cell membrane (Fig. 1C).

When microglial cells were cultured with amoeba lysate at concentrations from 1 to 0.25 mg/ml, lysis of the cell membrane was detected (Fig. 2A). In addition, many food vacuoles containing pathogenic *A. culbertsoni* lysate were detected in the cytoplasm, and the membranes of these food vacuoles were lysed (Fig. 2B); in contrast, food vacuoles were not detected in untreated microglial cells (Fig. 1A). In some microglial cells, small vacuoles condensed into larger vesicles (Fig. 2C).

To determine whether microglial cells showed any change in cytokine release as a result of a CPE induced by pathogenic *Acanthamoeba* trophozoites and lysate, assays for cytokines such as TNF-α and IL-1β were performed with culture supernatants with enzyme-linked immunosorbent assay kits (Endogen, Woburn, Mass.). The amounts of TNF-α secreted from microglial cells cultured in EMEM and PBS, used as volume controls, were 32.5 and 40.5 pg, respectively, at 6 h of incubation (Table 1). In microglial cells cocultured with *A. culbertsoni* trophozoites for 6 h, the amount of TNF-α secreted significantly increased to 114.3 pg in comparison with the amount secreted by the control group (by Student's *t* test, $P < 0.01$). In comparison with the amount secreted by the control group, the amount of TNF-α secreted from microglial cells cocultured with a lysate (1 mg/ml) of *A. culbertsoni* increased to 95.6 pg at 6 h ($P < 0.01$). In addition, the degree of increase revealed the same patterns by treatment with 0.5 mg of amoeba lysate per ml (Table 1). In microglial cells cocultured with trophozoites of *A. culbertsoni* for 6 h, the amount of IL-1β secreted was 129.6 pg, which was similar to the amounts secreted by the control groups (Table 1). By contrast, the amounts of IL-1β secreted from microglial cells cocultured with 1 and 0.5 mg of *A. culbertsoni* lysate per ml for 6 h decreased to 18.2 and 20.6 pg, respectively ($P < 0.01$).

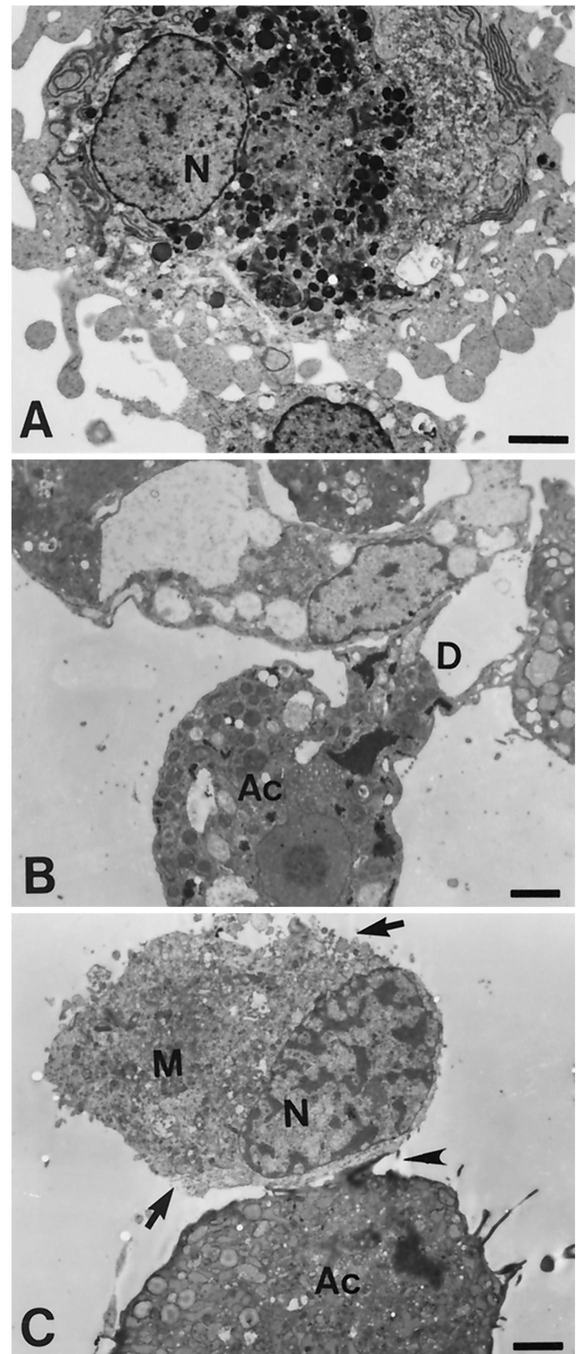

FIG. 1. Findings for microglial cells and *Acanthamoeba* spp. obtained by TEM (A) A primary-culture microglial cell shows numerous pseudopodia and a large nucleus (N) containing marginally scattered chromatin materials. (B and C) Coculture of microglial cells (M) with *A. culbertsoni* trophozoites (Ac) for 6 h. An amoeba is attached on the surface of a microglial cell (arrowhead). A trophozoite of *A. culbertsoni* produced a digipodium (D) on a microglial cell. Lysis of the cell membrane of a microglial cell was also apparent (arrow). Bars, 2.5 μm.

Pathogenic *Acanthamoeba* produces a CPE on various cells both in vitro and in vivo. Dove Pettit et al. (4) demonstrated

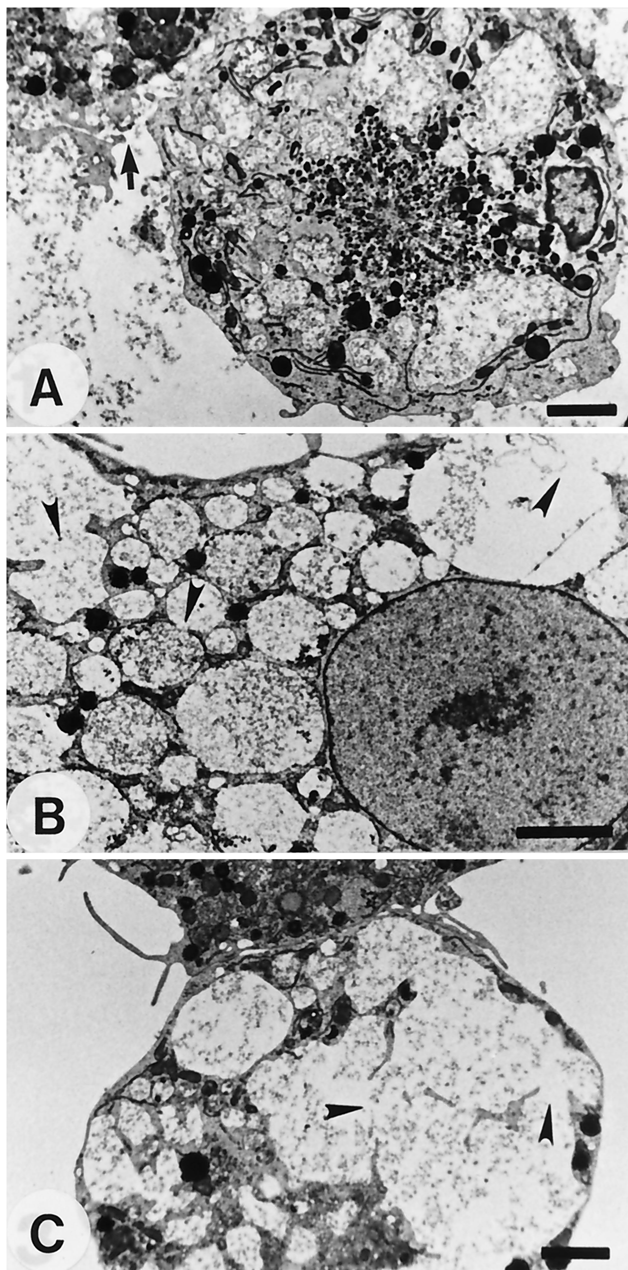

FIG. 2. TEM studies of microglial cells cocultured with 1 mg of a lysate of *A. culbertsoni* per ml for 6 h. (A) A microglial cell showed numerous food vacuoles containing amoeba lysate and a lysed cell membrane (arrow). (B) The membranes of cytoplasmic vacuoles were lysed (arrowheads). (C) The lysed membranes (arrowheads) were integrated into a large one. Bars, 2.5 μm.

that a digipodium of pathogenic *Acanthamoeba* made contact with rat neuroblastoma cells, similar to the CPE of *Naegleria fowleri*, a kind of contact-dependent cell lysis referred to as troglocytosis (10). In the present study, microglial cells cultured from newborn rat brain were used as target cells because these cells could play a protective role during *Acanthamoeba* infection in the CNS. When amoebae were cocultured with microglial cells, digipodia were shown on pathogenic *A. culbertsoni* trophozoites. Thus, this result could be considered apparent evidence of the CPE induced by pathogenic *Acanthamoeba*.

TABLE 1. Amounts of TNF-α and IL-1β secreted from microglial cells cocultured with *A. culbertsoni* trophozoites or lysate for 6 h

Culture conditions	Amt (pg/ml) secreted	
	TNF-α	IL-1β
Microglial cells:		
In EMEM medium	32.5 ± 2.97[a]	131.4 ± 43.91
With PBS	40.5 ± 4.38	150.4 ± 13.44
Microglial cells with:		
A. culbertsoni trophozoites (1:1)[b]	114.3 ± 7.99	129.6 ± 19.30
1 mg of lysate per ml	95.6 ± 0.71	18.2 ± 6.05
0.5 mg of lysate per ml	119.9 ± 1.41	20.6 ± 1.69

[a] Values are means ± standard deviations.
[b] The ratio is the number of microglial cells to the number of trophozoites.

The two fundamental mechanisms in the cytolysis of target cells by pathogenic free-living amoebae are the disruption of cell membrane integrity by necrosis via pore-forming lytic molecules or the disruption of cell membrane integrity by apoptosis, or both (1, 4). In the present study, microglial cells cocultured with amoeba trophozoites died mostly as a result of the necrotic process that accompanied the clumping of chromatin materials in the nucleus and lysis of the cell membranes. In addition, in microglial cells cocultured with pathogenic *A. culbertsoni* lysate, the membranes of small food vacuoles were lysed and integrated. This membrane lysis seems to be affected by lytic molecules released from *Acanthamoeba*, but further study of that mechanism is needed. In a previous study, it was reported that *Naegleria* lysate contained pore-forming lytic molecules which affected the lysis of target cells (9).

Apoptosis was characterized by various morphological features, such as cell shrinkage, cell membrane blebbing, the formation of apoptotic bodies, nuclear chromatin condensation, and DNA fragmentation, as determined by electrophoresis and flow cytometry (1, 4). In the present study, cell membrane blebbing and nuclear condensation were observed for less than 10% of microglial cells cocultured with pathogenic *A. culbertsoni* (14).

Lipopolysaccharide (LPS) stimulated the release of TNF-α and IL-1 from primary murine microglial cell cultures, whereas inhibitors such as pentoxifylline and dexamethasone blocked their release (3). In microglial cells infected with *Toxoplasma gondii*, the secretion of IL-1 was triggered by bradyzoites and tachyzoites in a time- and a dose-dependent manner and depended on an LPS stimulus (5). In the present study, the level of secretion of TNF-α from microglial cells cocultured with pathogenic *A. culbertsoni* trophozoites or lysates was increased at 6 h of incubation. The level of secretion of IL-1β from microglial cells cocultured with lysate for 6 h decreased. More extensive studies on the cytokine responses of microglial cells due to pathogenic *Acanthamoeba* are necessary.

In conclusion, our results demonstrate that pathogenic *A. culbertsoni* trophozoites make contact with microglial cells and produce digipodia. The primary-culture rat microglial cells cocultured with amoeba lysate undergo a necrotic process following the lysis of the cell membrane and the membranes of the inner vacuoles. The level of secretion of TNF-α from mi-

croglial cells increased at 6 h postincubation, whereas the level of secretion of IL-1β decreased. These findings are regarded as the CPE induced by pathogenic *Acanthamoeba* against microglial cells and may be partly important for understanding of the interaction of pathogenic *Acanthamoeba* with microglial cells in the development of GAE.

This study was supported by a research grant of the Korea Science and Engineering Foundation (grant 981-0701-001-1) and in part by a research grant from Shinheung College (in 1999).

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