Acanthamoeba castellanii Promotion of In Vitro Survival and Transmission of Coxsackie B3 Viruses

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This work was undertaken to determine whether Acanthamoeba could play a role in the survival and transmission of coxsackieviruses and focused on in vitro interactions between Acanthamoeba castellanii and coxsackie B3 viruses (CVB-3). Residual virus titer evaluations and immunofluorescence experiments revealed a remarkable CVB-3 adsorption on amoeba surfaces and accumulation inside cells. The survival of viruses was independent of the dynamics of amoeba replication and encystment. In addition, our results indicated that virus-infected amoebas can release infectious viruses during interaction with human macrophages. On the basis of these data, Acanthamoeba appears to be a potential promoter of the survival of coxsackieviruses and their transmission to human hosts.

Free-living amoebas of the genus Acanthamoeba are ubiquitous in nature (34); they have been isolated from air (19, 32, 33), soil (2, 3, 6, 29), and water environments, including chlorinated swimming pools (10), drinking water (17, 28), cooling towers (5), natural thermal water (31), hospital water networks (35), and marine water (4). Acanthamoeba is characterized by a feeding and replicating trophozoitic stage which under adverse conditions can develop to a dormant cyst stage (34). Cysts are highly resistant forms capable of withstanding disinfection, desiccation, and extremes of temperature. When favorable conditions occur, the cysts hatch and the trophozoites emerge to feed and replicate. Acanthamoeba species are not parasites, as they do not require the infection of a host organism to complete their life cycles (11, 37). However, these amoebae can infect a variety of mammals, including humans, thereby producing severe and often fatal diseases. They act as opportunistic as well as nonopportunistic pathogens, are the causative agents of granulomatous amoebic encephalitis and amoebic keratitis, and have been associated with cutaneous lesions and sinusitis (18, 20, 37–39). Both trophozoites and cysts have been shown to be resistant to chlorination used for disinfecting water systems (30, 35, 36).

In addition, Acanthamoeba amoebas play a role as reservoirs for Legionella pneumophila and other amoeba-resistant microorganisms that include bacteria, fungi, and viruses (15).

Enteroviruses (polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses) are naked small RNA viruses of the Picornaviridae family. They are important human pathogens often causing mild febrile illness, but clinical manifestations of enterovirus infections also include meningitis, encephalitis, paralysis, and myocarditis (16, 27). Gastrointestinal involvement may also result. Transmission from person to person proceeds through the fecal-oral route. These viruses are widespread in marine water and also may be acquired by eating contaminated aquatic organisms (14). Although labile, they may persist in free-flowing estuarine or marine waters for several months and in some cases during the winter months (21). Although their life span in water may be prolonged by the influence of estuarine sediments (7, 12), it has been hypothesized that free-living amoebas may host these viruses. However, up to now the role of amoebas as vectors for enteroviruses has not been confirmed, since viruses were found only on amoeba surfaces (9).

In the order to study whether Acanthamoeba could play a role in the survival and transmission of coxsackieviruses, we evaluated under reproducible cultural conditions interactions between Acanthamoeba castellanii and coxsackie B3 viruses (CVB-3).

In this paper, we report that CVB-3 are able to adhere to amoeba surfaces and that most virions adsorbed on the cell surface can penetrate the trophozoites without changing their infective activity. Residual infectious CVB-3 are detectable in Acanthamoeba trophozoites even after a complete 6-month cycle of encystment and excystment. Furthermore, our data show that virus-infected amoebas coincubated with human macrophages can release infectious CVB-3.

Therefore, our findings suggest that Acanthamoeba may well play an active role as a vector in the survival of coxsackieviruses.

MATERIALS AND METHODS

Amoeba cultivation. Our study was performed using trophozoites of A. castellani isolated from the corneal ulcer of a soft contact lens wearer (in Ancona, Italy) that were axenically grown at 25°C in peptone-yeast extract-glucose (PYG) medium (13). The species identification of this isolate was based on cyst morphology and indirect immunofluorescence microscopy. Amoebas used as inocula were taken from logarithmic-phase cultures.

Viruses. The prototype strains of CVB-3 (Nancy) and echovirus type 31 (ECHO-31) were obtained from the American Type Culture Collection (Rockville, MD). Virus stocks, in both cases, were prepared in serum-free RPMI 1640 medium (Gibco-BRL/Life Technologies) as described previously (25) using human KB cells infected at a multiplicity of infection of 0.5. Virus titers were determined in KB cells and expressed as 50% tissue culture infective doses per ml (25).

KB cell cultivation. The KB cell line, derived from a human epidermoid carcinoma, was maintained in continuous culture in RPMI medium containing
10% heat-inactivated fetal calf serum (FCS) (Gibco-BRL/Life Technologies), 100 U penicillin G per ml, and 100 μg of streptomycin per ml and was grown in 25-cm² sterile plastic flasks at 37°C in a humid atmosphere containing 5% CO₂.

**Amoeba virus infection.** Experiments were performed in sterile polystyrene test tubes (Falcon) containing 3 × 10⁶ trophozoites washed twice in sterile phosphate-buffered saline (PBS) buffer (pH 7.2) and suspended in 0.45 ml of RPMI medium. Fifty microliters of the viral stock suspension was added to each test tube (multiplicity of infection of 0.33); controls received the same volume of RPMI medium. After 1 h of incubation at 37°C or 25°C, amoebas were centrifuged (at 100,000 × g for 10 min) in order to recover both cell-free supernatants and pellets. All samples were stored at −70°C and then used for virus titration on KB cells. Before titration, pellets were suspended in 1 ml of PBS, the trophozoites were disrupted by six freeze-thaw cycles, and cell debris was removed by centrifugation. The experiments were performed in sterile 96-well plates (Corning). In one set of experiments, before being frozen at −70°C, the amoebic suspension was centrifuged (at 400 × g for 10 min) in order to recover both cell-free supernatants and pellets. All samples were stored at −70°C and then used for virus titration on KB cells. Before titration, pellets were suspended in 1 ml of PBS, the trophozoites were disrupted by six freeze-thaw cycles, and cell debris was removed by centrifugation. The experiments were performed in sterile 96-well plates (Corning). To quantify CVB-3, serial dilutions were prepared in quadruplicate in 100 μl of RPMI medium; the wells for control KB cells received the same volume of RPMI medium. After 1 h of incubation at 37°C or 25°C, amoebas were centrifuged (at 100,000 × g for 5 min) and pellets were washed three times in 8 ml sterile PBS in order to remove unadsorbed virus, suspended in 1 ml of RPMI medium supplemented with 5% heat-inactivated FCS and antibiotics, and maintained at 25°C or 37°C for various lengths of time (times postinfection; see below).

**Virus titer determination.** Immediately (time zero) and after 6, 24, 48, 72, 96, 120, and 144 h of incubation, these amoebic suspensions were centrifuged (at 400 × g for 10 min) in order to recover both cell-free supernatants and pellets. All samples were stored at −70°C and then used for virus titration on KB cells.

**Evaluation of growth curves.** In these experiments, trophozoites at 6 days postinfection were washed, suspended in 10 ml of PYG medium (1.5 × 10⁹ cell/ml), and incubated in 25-cm² sterile plastic flasks at 25°C. At selected time intervals (2, 4, 6, 8, 10, and 12 days), amoebas were counted with a hemacytometer. The obtained trophozoites were recovered and used for virus titration as described above.

**Evaluation of virus localization in the trophozoites.** (i) Virus titration after mild digestion of amoebas with trypsin. In one set of experiments, before being frozen at −70°C, the amoebic suspension was centrifuged (at 400 × g for 10 min) with 0.25% trypsin-EDTA (Sigma-Aldrich, Milan, Italy) at 37°C in order to inactivate viruses present on the amoeba surface. The enzyme was then removed by centrifugation, and the amoebas, washed twice in sterile PBS, were processed as described above. The same experimental conditions were employed to evaluate virus titers for echo 31 viruses.

(ii) **Confocal immunofluorescence studies.** Suspensions of infected trophozoites at 1 h and 24 h postinfection were centrifuged, and trophozoites were then resuspended in sterile PBS, seeded into chamber slides (Lab-Tek), and incubated for 1 h at 25°C. Amoebas were then treated for 1 h with mouse immunoglobulin G (IgG) anti-cocssackie (light diagnostic cossackievirus B blend reagent; CHEMICON). After 1 h of incubation at 25°C, the samples, suitably washed with PBS, were fixed for 45 min with freezing methanol and incubated with anti-mouse IgG rhodamine-conjugated antibody (tetramethyl rhodamine isocyanate antibody; Sigma-Aldrich, Milan, Italy). Afterwards, the washed amoebas were again exposed to the mouse IgG anti-cocssackie and incubated with anti-mouse IgG fluorescein-conjugated antibody (fluorescein isothiocyanate antibody; Sigma-Aldrich, Milan, Italy). After washings with PBS, samples were mounted in Gelvatol (Monsanto Corp.) and examined with a Nikon Eclipse-600 microscope equipped with a Bio-Rad Microradiance confocal system.

**Evaluation of infectious CVB-3 in Acanthamoeba trophozoites after a complete cycle of encystment and excystment.** In these experiments, trophozoites at 6 days postinfection were washed, suspended in 3 ml of sterile PBS, and incubated at 25°C in order to induce their encystment. After 6 months, the cysts were recovered, twice washed in PBS, and cultured in PYG medium at 25°C for 8 days to induce their differentiation in the trophozoitic form. The obtained trophozoites were then lysed and used for virus titration as described above.

**Study of the release of infectious CVB-3 from trophozoites incubated with human macrophages.** Tests were performed in 24-well plates (Corning). Amoebas not exposed to CVB-3 and virus-infected trophozoites at 6 days postinfection were washed twice with sterile PBS and suspended in RPMI 1640 medium containing 10% heat-inactivated FCS at a density of 1 × 10⁶/ml. Each amoebic suspension was seeded in aliquots of 1 ml/well into wells of sterile plates that contained human macrophages (amoeba-to-cell ratio, 1:2). The plates were then incubated at 37°C in a 5% CO₂ atmosphere. Immediately (time zero), after 6 h, and at time intervals of 24 h for 5 days in succession, plates were centrifuged (at 100,000 × g for 3 min) in order to recover cell-free supernatants. All samples were stored at −70°C and used for virus titration on KB cells. At the selected time intervals, wells were observed by use of an Olympus TX-71 inverted microscope equipped with a differential interference contrast mechanism.

**Human macrophages.** For human macrophages, we used the human myelomonocytic cell line THP-1, which represents a nonadherent population of early monoblasts (40, 42). These cells were maintained in continuous culture in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U penicillin G per ml, and 100 μg of streptomycin per ml and were grown in 25-cm² sterile plastic flasks at 37°C in a humid atmosphere containing 5% CO₂. Before experiments, cells were exposed for 48 h to 10 nM phorbol 12-myristate 13-acetate (Sigma-Aldrich, Milan, Italy) to induce their differentiation in macrophages. In each experiment, 2 × 10⁶ activated cells suspended in 1 ml of RPMI 1640 medium containing 10% FCS were seeded into each well of a sterile 24-well plate. After 24 h of incubation at 37°C in a 5% CO₂ atmosphere, the culture medium was aspirated and replaced with 1 ml/well of amoebic suspension as described above.

**Statistics.** Statistical differences between groups were determined using a two-tailed Student’s t test. The difference was considered significant when P was <0.05.

**RESULTS**

**Quantitative evaluation of residual virus.** To study the survival of CVB-3 in A. castellanii, after 1 h of infection and suitable washings, amoebas were suspended in the culture medium, and then at selected postinfection time intervals the amounts of infectious virus present in both trophozoites and cell-free supernatants were evaluated.

Of primary importance is the fact that the virus titration performed on the KB cells indicated that about 53% (n = 24) of the input virus was recovered in the time zero trophozoites, independent of the temperature (25°C or 37°C) used during the adsorption phase.

**The kinetic study of the survival of cossackie B3 viruses in amoeba cultures during the period after infection.** After 1 h of infection at 25°C or 37°C, washed trophozoites were suspended in RPMI medium (3 × 10⁶/ml) and incubated at the same temperatures. At selected time intervals, the amounts of infectious virus present in both trophozoites and cell-free supernatants were evaluated. Virus titration was performed in KB cells. Values are means ± standard errors from at least nine experiments at 25°C and three experiments at 37°C. The input virus value is log₁₀ 6 ± 0.2 (n = 12). TCID₅₀: 50% tissue culture infective dose.

**FIG. 1.** Survival of cossackie B3 viruses in A. castellanii cultures during the period after infection. After 1 h of infection at 25°C or 37°C, washed trophozoites were suspended in RPMI medium (3 × 10⁶/ml) and incubated at the same temperatures. At selected time intervals, the amounts of infectious virus present in both trophozoites and cell-free supernatants were evaluated. Virus titration was performed in KB cells. Values are means ± standard errors from at least nine experiments at 25°C and three experiments at 37°C. The input virus value is log₁₀ 6 ± 0.2 (n = 12). TCID₅₀: 50% tissue culture infective dose.
dition, comparing the results obtained at 25°C and 37°C, we observed no significant difference.

**Growth evaluation.** To study possible modifications induced by CVB-3 on *A. castellanii*, the growth rates of noninfected and virus-infected trophozoites were evaluated. After 12 days of incubation at 25°C (optimum temperature for *Acanthamoeba* growth), control cultures produced about 1.9 × 10⁷ amoebas/flask, with doubling times of about 24 h. Under the same experimental conditions, cultures of infected trophozoites did not show any significant difference in growth rate (Fig. 2A). In addition, phase-contrast microscopy indicated that trophozoites exhibited the same morphological features and cell viability values of >95% in both cases. The virus titration performed during the growth evaluation confirmed the survival of infectious CVB-3 in amoebas (Fig. 2B).

**Virus localization in trophozoites.** To establish the localization of viruses after contact with *A. castellanii* trophozoites, we used two different experimental methods.

(i) **Virus titration after a mild trypsin digestion of amoebas.** Trypsin, a proteolytic enzyme, is usually used to selectively inactivate viruses adsorbed on the cell surface with respect to those inside the cell. Comparing viral titers obtained from infected amoebas that were exposed to trypsin and from those not so exposed, we found significant differences in trophozoites only at 0 h and 6 h postinfection (Fig. 3A). On the contrary, in similar experiments carried out using amoebas infected with ECHO-31 viruses under the same experimental conditions, significant differences were observed for all kinetic studies (Fig. 3B).

(ii) **Confocal immunofluorescence studies.** To further investigate the location of CVB-3 in *A. castellanii* trophozoites, we performed a double indirect immunofluorescence using mouse IgG anti-coxsackie and anti-mouse IgG-tetramethyl rhodamine isothiocyanate-conjugated antibody and IgG-fluorescein isothiocyanate-conjugated antibody that distinguishes viruses adsorbed on cell surface (red) from those that entered the cell (green) (45). Compared to control cells (Fig. 4A), trophozoites at 1 h postinfection showed an evident red fluorescence localized along the cell surface in particular (Fig. 4B), while at 23 h postinfection they appeared as an intense green fluorescence throughout the cell (Fig. 4C).

**Evaluation of infectious CVB-3 in *Acanthamoeba* trophozoites after a complete cycle of encystment and excystment.** To further investigate CVB-3 survival in *A. castellanii* after a longer time postinfection and to analyze the effect exerted on this virus by a complete cycle of amoebic encystment and excystment, we compared viral titers obtained from trophozoites at 6 days postinfection with those evaluated after their encystment-excystment cycle. The results indicated that at 6 months after the infection, a significant amount of residual infective CVB-3 was still detectable in trophozoites (Fig. 5).

**Study of the release of infectious CVB-3 from trophozoites incubated with human macrophages.** To elucidate whether *Acanthamoeba* may play an active role as a vector in the survival and transmission of coxsackieviruses, we investigated the
in vitro ability of virus-infected amoebas to release infectious CVB-3 following coincubation with human macrophages, since these are important effector cells in Acanthamoeba infections (23, 41). Our experiments showed that in cell-free supernatants of these cocultures, the amount of residual infectious CVB-3 increased significantly even after just 24 h of incubation, with a peak increase at 48 h maintained for up to 120 h of coincubation (Fig. 6). As shown by differential interference contrast microscopy, the detection of infectious CVB-3 was time correlated with morphological alterations of and cell damage to both Acanthamoeba trophozoites and human macrophages (Fig. 7).

FIG. 4. Immunofluorescence pictures of uninfected A. castellanii trophozoites (A) and of CVB-3-infected A. castellanii trophozoites at 1 h (B) and 23 h (C) postinfection, showing the different localizations of CVB-3 at the times observed. Magnification, ×000.

FIG. 5. Survival of CVB-3 in A. castellanii trophozoites after 6 months spent in the cystic form at 25°C. The encystment of $3 \times 10^9$ virus-infected trophozoites at day 6 postinfection was obtained by incubation in PBS buffer; excystment was induced by incubation of cysts in PYG medium. Values are means ± standard errors from at least three experiments. TCID$_{50}$, 50% tissue culture infective dose.

DISCUSSION

Acanthamoebae are some of the most ubiquitous protozoa and are especially widespread in water and highly resistant to
otics at 37°C and in 5% CO2 atmosphere. Cell-free supernatants of these cocultures were used as samples for virus titration. Supernatant A, samples obtained by incubation of virus-infected amoebas; supernatant B, samples obtained by incubation of uninfected amoebas. Values are means ± standard errors from at least six experiments. TCID50, 50% tissue culture infective dose.

FIG. 6. Time course of the infectious CVB-3 release from A. castellanii trophozoites coincubated with human macrophages (amoeba-to-cell ratio, 1:2) in RPMI medium containing 10% FCS and antibiotics at 37°C and in 5% CO2 atmosphere. Cell-free supernatants of these cocultures were used as samples for virus titration. Supernatant A, samples obtained by incubation of virus-infected amoebas; supernatant B, samples obtained by incubation of uninfected amoebas. Values are means ± standard errors from at least six experiments. TCID50, 50% tissue culture infective dose.

decommission. Contact with humans is frequent; in fact, it has been shown that >80% of the normal human population exhibits antibodies against Acanthamoeba (8).

It has also been shown that enteroviruses present in polluted waters can be accumulated by naturally resistant aquatic organisms (14), increasing their capacity to survive in this environment.

To verify whether these free-living amoebas can play a role as carriers or vectors for coxsackieviruses, we studied the in vitro interactions between Acanthamoeba castellanii and CVB-3 in order to determine the extent of virus adsorption to protozoan cells and/or virus accumulation in this microorganism and the role of virus-amoeba contact on survival and transmission of virus.

To quantify the residual virus present in amoebas, we used virus titration in permissive cells, which offers the advantage of distinguishing the infectious virus.

Our results on viral adsorption indicate that the binding of CVB-3 to the amoeba cell surface occurs at temperatures as low as 25°C, a temperature similar to those found for polluted natural water environments at the end of summer, when cox- sackie B viruses are isolated with greater frequency. Our experiments also demonstrate that the virus binding occurs at 37°C: this is an interesting result, since both CVB-3 and enteroviruses might differ according to the specific viral genus.

Kinetic findings on the virus survival in amoeba cultures studied for an overall period of 18 (i.e., 6 plus 12) consecutive days suggest that under both experimental conditions, A. castellanii, while nonpermissive for virus replication, is unable to inactivate cox sackie B3 viruses. The infectivity of residual CVB-3 is not influenced by the dynamics of amoeba replication and encystment. On the other hand, the presence of CVB-3 does not modify the cell morphology, the viability, or the growth of trophozoites.

Both methods employed to discriminate virions adsorbed on amoeba surfaces from those accumulated inside cells clearly indicate that CVB-3, within 24 h of adsorption, were localized mainly inside trophozoites. In fact, by comparing viral titers obtained from trophozoites that were digested with trypsin and those not digested with trypsin, we found that this proteolytic enzyme caused a significant decrease of virus titers only at time zero and at 6 h postinfection. Furthermore, confocal immunofluorescence pictures clearly showed that the majority of CVB-3 was internalized in amoebas at postinfection times ranging from 1 h to 23 h.

On the contrary, in similar experiments carried out using trophozoites infected with the ECHO-31 virus, we found that trypsin digestion of amoebas caused a significant decrease in viral titers throughout the whole kinetic study. These results seem to indicate that ECHO-31 enteroviruses also can bind to the cell surface of A. castellanii while maintaining their infective activity; nevertheless, they are not internalized into trophozoites.

This finding is in agreement with that obtained by Danes and Cerva about 25 years ago (9). Studying the in vitro survival of polioviruses (type 1 and type 3) and echoviruses (type 4 and type 30) on A. castellanii, those authors found that tested enteric viruses persisted in trophozoites from 10 days to 75 days and were bound most probably to amoeba surfaces only, since all were sensible to neutralization with specific antisera.

All these data consequently suggest that relationships between A. castellanii and enteroviruses might differ according to the specific viral genus.

In addition, our study demonstrates that virus-infected trophozoites, after 6 months spent in the cystic form, still contain a significant amount of infectious CVB-3.

It has been shown that encysted amoebas are resistant to a variety of physical (heat, freezing, UV radiation) and chemical (disinfectants and antimicrobials) agents, desiccation, and starvation (1), and cysts have been known to survive for ≥20 years (26). Therefore, we suggest that the encystment of Acanthamoeba may promote the survival of coxsackieviruses in the aquatic environment as well.

Many studies have indicated that macrophages play an important role in the control of Acanthamoeba infections (22, 23, 24). Reports on amoeba-macrophage interactions have established that activated macrophages are capable of injuring Acanthamoeba species and that trophozoites can destroy phagocytic cells by contact-dependent cytolysis and by the use of cytolytic factors (22, 24). Therefore, to elucidate whether Acanthamoeba may play an active role as a vector in the transmission of coxsackieviruses, we conducted an in vitro investigation into CVB-3 release following amoeba coincubation with human macrophages. Our findings indicate that the interaction of Acanthamoeba with human macrophages can induce the release of residual infectious CVB-3 from trophozoites.

We can exclude the possibility that virus titer increases in these experiments might depend on CVB-3 replication for two main reasons. (i) Our results show that A. castellanii is nonpermissive for virus replication. (ii) Previous reports indicate that monocytic cell lines are nonpermissive for CVB-3 infection (43, 44). On the other hand, our results would confirm that these increases may be due to lysis occurring in trophozoites, as the release curve is time dependent, exhibits a plateau phase, and, as shown by differential interference-contrast microscopy, correlates with the cell damage observed.

In conclusion, this study demonstrates that free-living amoebas of the genus Acanthamoeba are able to interact with cox sackie B viruses; that after adhesion to the amoeba surface,
FIG. 7. Morphological alterations and cell damage concerning both virus-infected trophozoites and human macrophages at different times of coincubation in RPMI medium containing 10% FCS and antibiotics at 37°C and in 5% CO₂ atmosphere. (A) Control human macrophages; (B) control trophozoites. Also shown are human macrophages plus trophozoites at time zero (C), 24 h (D), 48 h (E), and 72 h (F). Magnification, ×400.
virions are internalized by trophozoites in which they survive, maintaining their infectivity independently of the dynamics of amoeba replication and encystment; and that virus-infected amoebas can release infectious viruses during interaction with human macrophages.

Further studies are necessary to identify the nature of amoebic surface molecules involved in CVB-3 binding and to characterize the mechanism(s) involved in virus internalization. Nevertheless, on the basis of all data reported here, Acanthamoeba appears to have the potential capacity to promote the survival of coccacieviruses and to play a role as a vector in their transmission to human hosts.

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