In Vitro Activity of *Acanthamoeba castellanii* on Human Platelets and Erythrocytes[∇]

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The effect of *Acanthamoeba* on human platelets and erythrocytes has not been fully elucidated. This paper reports that cell-free supernatants prepared from *A. castellanii* can activate human platelets, causing both a significant increase in the cytosolic free-calcium concentration and platelet aggregation. In addition, we demonstrated that platelet activation depends on the activity of ADP constitutively secreted into the medium by trophozoites. This study also showed that *A. castellanii* can affect human red blood cells, causing hemolysis, and provided evidence that hemolysis occurs in both contact-dependent and contact-independent ways; there are differences in kinetics, hemolytic activity, and calcium dependency between the contact-dependent and contact-independent mechanisms. Partial characterization of contact-independent hemolysis indicated that ADP does not affect the plasma membrane permeability of erythrocytes and that heat treatment of amoebic cell-free supernatant abolishes its hemolytic activity. These findings suggest that some heat-labile molecules released by *A. castellanii* trophozoites are involved in this phenomenon. Finally, our data suggest that human platelets and erythrocytes may be potential cell targets during *Acanthamoeba* infection.

Over the last two decades, *Acanthamoeba* species have been increasingly recognized as human pathogens causing serious, even life-threatening, infections. They are the causative agents of granulomatous amoebic encephalitis, a fatal disease of the central nervous system, and amoebic keratitis, a painful sight-threatening disease of eyes (18, 22, 34). *Acanthamoeba* spp. have also been associated with cutaneous lesions and sinusitis in AIDS patients and other immunocompromised individuals (31, 38, 40, 41).

The pathogenesis of *Acanthamoeba* infection is highly complex and involves several mechanisms. In general, the ability of the amoebas to bind to host cells is the first crucial step in the pathogenesis of *Acanthamoeba* infections (5, 17). This leads to secondary events, such as interference with host intracellular signaling pathways, toxin secretion, and the ability to phagocytose host cells, ultimately leading to target cell death, which can occur by necrosis or apoptosis (15, 26–28, 35). Furthermore, in order to produce damage to the host cell and/or tissue migration, pathogenic *Acanthamoeba* species rely upon their ability to produce hydrolytic enzymes (proteases and phospholipases) (1, 7). Studies performed by using different human cell lines demonstrated that there are both contact-dependent and contact-independent mechanisms of cell injury (2, 21, 30, 37).

Several studies have shown the potential role of erythrocytes and platelets in the tissue damage caused by these protozoa. Microcirculation disorders in patients with granulomatous amoebic encephalitis that lead to disseminated intravascular coagulation have often been described (3, 8, 20, 23). In addition, a case report suggested that thrombosis of small vessels in the periventricular regions and necrosis and hemorrhage of the

periventricular tissue, cerebellum, and brain stem can develop during cerebral amebiasis (10). However, to our knowledge, the action of *Acanthamoeba* on erythrocytes and platelets has not been elucidated.

The present investigation was undertaken to study whether *Acanthamoeba* and/or its soluble metabolites released in conditioned medium can affect human blood platelets and erythrocytes.

The aims of the study were (i) to examine the effect of amoebic metabolites on the platelet cytosolic free-calcium concentration ($[Ca^{2+}]_i$) and aggregation and (ii) to analyze the hemolytic activity of trophozoites and soluble protozoan molecules.

Our results strongly suggest that amoebas lead to platelet activation by ADP release. In addition, *Acanthamoeba castellanii* is able to cause both contact-dependent and contact-independent hemolysis. The latter seems to depend on heat-labile molecules released by trophozoites during growth.

MATERIALS AND METHODS

Reagents and materials. HEPES buffer, Fura 2-AM, ADP, and suramin were obtained from Sigma-Aldrich Srl, Milan, Italy. Amicon Centriprep-YM10 microconcentrators were obtained from Millipore SpA, Milan, Italy.

Amoebas. Our study was performed using trophozoites of *A. castellanii* that were isolated from a patient with amoebic keratitis (in Ancona, Italy) and were axenically grown at 25°C in PYG medium (12). Species identification of this isolate was based on cyst morphology and PCR analysis using primers JDP1 and JDP2 (specific for 18S rRNA gene stretch ASA.S1) (33). Our previous observations showed that at 37°C this *A. castellanii* isolate is able to kill human-derived epithelial (Wish) and myelomonocytic (THP-1) cell lines by contact-dependent and contact-independent mechanisms (26–28).

Preparation and fractionation of amoebic cell-free supernatant. Amoebic cell-free supernatant was prepared as previously described (27, 28). Briefly, amoebas were washed twice in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} at pH 7.4, resuspended (6×10^6 cells/ml) in the same buffer containing 20 mM HEPES, and finally incubated for 2 h at 25°C. Cell-free supernatant was obtained by centrifugation at $500 \times g$ for 15 min and was designated entire conditioned PBS (ePBS). For some experiments ePBS was centrifuged ($3,000 \times$

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g for 20 min) by using Centricon-10 microconcentrators (10-kDa cutoff) to differentiate fractions containing amoebic metabolites having molecular masses of >10 kDa (rPBS) and fractions containing amoebic metabolites having molecular masses of <10 kDa (fPBS). The first fraction was obtained by resuspending the pellet from the bottom chamber of microconcentrators in the starting volume of PBS, while the second fraction was obtained by removing supernatant from the top chamber of microconcentrators. To establish the heat resistance of secreted molecules in the conditioned medium, ePBS, prepared as described above, was treated at 95°C for 10 min to obtain a heat-treated fraction (htPBS). All samples were used immediately after processing.

Isolation of hematic elements. Platelets and erythrocytes were obtained by centrifugation of venous whole blood obtained from healthy human donors at the SS Annunziata Hospital Transfusion Centre (Sassari, Italy), where all of the relevant ethical reviews and approvals were granted for collection and manipulation at the time. Patients had taken no medication for at least 2 weeks before they donated blood.

Blood was gently mixed in 3.8% trisodium citrate (ratio, 9:1 [vol/vol]), and platelet-rich plasma (PRP) and erythrocytes were obtained by centrifugation at $100 \times g$ for 10 min at room temperature. The residual plasma was then centrifuged at $3,000 \times g$ for 10 min to obtain platelet-poor plasma. Washed platelets were isolated from PRP by centrifugation at room temperature for 5 min at $450 \times g$. The pellets were resuspended in Tyrode-HEPES buffer (145 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4 , 5.6 mM glucose, 3 mM EGTA, 10 mM HEPES; pH 7.4). Removed erythrocytes were washed three times in PBS and stored at 4°C in the same buffer for no more than 24 h. All experiments were performed with fresh blood.

$[\text{Ca}^{2+}]_i$ measurement. Intracellular calcium in platelets was monitored by using the fluorescent calcium probe Fura 2-AM, as described by Ivanova et al. (16). The evaluation was performed using washed platelets suspended in Tyrode-HEPES buffer and a Hitachi F-2000 spectrophotometer. Dye loading was standardized by incubating platelets with 3 μM Fura 2-AM for 30 min at room temperature. Fura 2-AM-loaded platelets were then washed twice with the same buffer, and the assay was performed with a stirrer aliquot (1.5 ml) containing 100,000 platelets μl^{-1} at 37°C. The excitation and emission wavelengths were 340 to 380 nm and 510 nm, respectively; these wavelengths were detected every 500 ms, and the results were stored in separate memories of the F-2000 spectrophotometer. A data manager was used to monitor the fluorescence signal of Fura 2-AM-loaded platelets. Platelets were preincubated at 37°C for 1 min in the presence of 1 mM CaCl_2 . Basal and stimulated cytosolic calcium levels were quantified by the method described by Grynkiewicz et al. (14) using the ratio technique and a dissociation constant of 224 nM for Fura 2-AM; the maximal and minimal fluorescence values were determined after addition of 30 μl of 0.3% Triton X-100 and 10 mM EGTA, respectively. Hitachi F-2000 software was used for calculation.

In order to evaluate the effect of secreted protozoan products on the calcium concentration, platelets were stimulated with 80 μl of fPBS, rPBS, htPBS, and ADP. The intracellular Ca level was evaluated as described above. In some experiments, platelets were preincubated for 5 min with 25 or 50 μM suramin before stimulation with secreted protozoan products or ADP.

Platelet aggregation evaluation. Platelet aggregation was estimated using the turbidimetric method described by Born and Cross (4) and an Aggrecopter PA-3210 aggregometer. The evaluation was performed at 37°C using a stirrer aliquot (0.5 ml) of PRP containing approximately 250,000 platelets μl^{-1} . To express the aggregation of platelets, for each assay the absorption of PRP was defined as 0%, while the absorption of the corresponding platelet-poor plasma was defined as 100%. Curves for platelet aggregation were obtained by evaluating the increase in light transmission after PRP stimulation with 80 μl of PBS alone, ADP, or conditioned cell-free medium (fPBS, rPBS, or htPBS). The results, expressed as percentages of the maximum aggregation, were provided directly by the instrument, along with the times necessary to evoke the peak increase.

Evaluation of hemolytic activity of trophozoites. For the experiments in which the hemolytic activity of trophozoites was evaluated, isolated erythrocytes were suspended (6×10^6 cells/ml) either in PBS buffer alone or in PBS buffer containing 15 mM glucose and 0.33 mM CaCl_2 (to promote amoebic metabolism and to study the role of calcium ions). Amoebas from exponentially growing cultures (viability, >98%) that had been washed twice in PBS buffer were mixed with erythrocytes using an effector/target ratio of 1:2. Cell suspensions were incubated at 37°C and harvested at fixed times (0, 30, 60, 90, and 120 min). After centrifugation ($300 \times g$ for 10 min) hemoglobin release was quantified with a spectrophotometer (UV/VIS lambda-3; Perkin-Elmer) by determining the absorbance at 546 nm of the supernatant. Erythrocytes suspended (6×10^6 cells/ml) either in PBS alone or in PBS containing 15 mM glucose and 0.33 mM CaCl_2 were used

FIG. 1. Increase in $[\text{Ca}^{2+}]_i$ in human platelets loaded with 3 μM Fura 2-AM and suspended in Tyrode-HEPES buffer at a density of 100,000 platelets μl^{-1} at 37°C after stimulation with 80 μl of PBS buffer (control), fPBS, htPBS, rPBS, and the same volume of a 20 μM ADP solution. The bars indicate the means and the error bars indicate the standard errors for nine experiments. *, $P < 0.01$ versus the control; #, $P < 0.01$ versus the ADP solution; ^, not significant versus the ADP solution.

as controls. To evaluate total hemolysis, erythrocytes (6×10^6 cells/ml) were suspended in sterile distilled water for 15 min at 37°C; the cell suspensions were centrifuged, and the supernatants were used for quantification of hemoglobin (100% hemolysis).

Evaluation of hemolytic activity of amoebic cell-free supernatants. To evaluate the hemolytic effect of amoebic cell-free supernatants, erythrocytes were suspended (1.2×10^7 cells/ml) either in PBS buffer alone or in PBS buffer containing 30 mM glucose and 0.66 mM CaCl_2 ; 0.5 ml of both suspensions was mixed with 0.5 ml of ePBS, htPBS, or 20 μM ADP and incubated at 37°C. At 30-min intervals, samples were centrifuged, the hemoglobin released into the supernatants was quantified as described above, and the results were compared with the results for control red blood cells incubated with PBS or with PBS containing Ca and glucose.

Statistics. All experiments were performed in duplicate and were repeated at least six times. Statistical differences between groups were determined by using a two-tailed Student t test (GraphPad Prism, version 4.02; GraphPad Software Incorporated). Differences were considered significant at a P value of <0.05; the "n value" included the mean from each experiment.

RESULTS

Activity of amoebic cell-free supernatants in platelets. Our previous sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis performed with ePBS obtained using the same *Acanthamoeba* isolate and under the same experimental conditions revealed 10 bands at molecular masses ranging from 97 to 16.4 kDa (26). In additional experiments, capillary electrophoresis revealed the presence of significant micromolar concentrations of ADP in ePBS (27). To characterize the role of ADP and molecules released from *A. castellanii* during incubation, we examined the effects of rPBS, fPBS, and htPBS on (i) the platelet $[\text{Ca}^{2+}]_i$ and (ii) platelet aggregation.

(i) Measurement of $[\text{Ca}^{2+}]_i$. In our experiments the basal value of platelet $[\text{Ca}^{2+}]_i$ was 96.3 ± 4.6 nM ($n = 14$), and addition of PBS alone resulted in no variation ($[\text{Ca}^{2+}]_i$, 97.2 ± 4.6 ; $n = 12$). After stimulation of platelets with 80- μl portions of samples obtained from the amoebic cell-free supernatant, only conditioned fPBS and conditioned htPBS changed the basal value significantly; the increase in $[\text{Ca}^{2+}]_i$ was not significantly different from that obtained by stimulation with 20 μM ADP and represented the same concentration released into the medium by trophozoites (Fig. 1). In all cases stimulation led to a rapid biphasic increase in $[\text{Ca}^{2+}]_i$, which consisted of an initial transient increase, which was maintained for several

FIG. 2. Time courses of the increase in $[Ca^{2+}]_i$ in human platelets loaded with 3 μ M Fura 2-AM and suspended in Tyrode-HEPES buffer at a density of 100,000 platelets μ L $^{-1}$ at 37°C after stimulation with 80 μ L of fPBS (A), htPBS (B), or a 20 μ M ADP solution (C). The times when the stimulus (S), 0.3% Triton X-100, and 10 mM EGTA were added are indicated by arrows.

seconds, followed by additional increases (Fig. 2). On the other hand, no increase in the calcium concentration was detected in platelets exposed to conditioned rPBS (containing high-molecular-weight molecules [Fig. 1]). To investigate the role of trophozoite-secreted ADP on the increase in $[Ca^{2+}]_i$, we evaluated the effects of suramin (a P2-purinoreceptor antagonist) on platelets stimulated with both ADP and amoebic cell-free supernatants. Suramin (50 μ M) nearly abolished the effect of an ADP solution, fPBS, and htPBS on $[Ca^{2+}]_i$ (Table 1). This clearly indicated that conditioned fPBS and conditioned htPBS, as well as purified ADP, reacted with purinergic receptors expressed in platelet membranes, inducing calcium overload.

These findings strongly suggested that the effect on the cytosolic free calcium of heat-resistant low-molecular-weight components in amoebic cell-free supernatant was very probably caused by ADP secretion.

(ii) Platelet aggregation. For evaluation of platelet aggregation, each sample of PRP, after calibration, was stimulated with 80 μ L of PBS alone (negative control), with conditioned

TABLE 1. Effect of the purinergic antagonist suramin on the increase in the peak $[Ca^{2+}]_i$ induced in platelets stimulated with htPBS conditioned for 2 h by addition of *A. castellanii* trophozoites (6×10^6 cells/ml), fPBS, or PBS containing 20 μ M ADP^a

Sample	Increase in $[Ca^{2+}]_i$ (nM) (mean $\pm$ SE)		
	No treatment	Treatment with:	
		25 μ M Suramin	50 μ M Suramin
htPBS	29.70 $\pm$ 3.08	15.20 $\pm$ 0.96 ^b	0 $\pm$ 0 ^b
fPBS	27.20 $\pm$ 2.68	14.64 $\pm$ 0.48 ^b	0 $\pm$ 0 ^b
ADP (20 μ M)	36.30 $\pm$ 3.50	17.32 $\pm$ 1.66 ^b	0 $\pm$ 0 ^b

^a Cell stimulation was determined by using 80 μ L of each of the samples indicated. Evaluation was performed at 37°C. The data are from six experiments. The basal value for $[Ca^{2+}]_i$ in platelets was 96.30 $\pm$ 4.58 nM ($n = 14$).

^b $P < 0.01$ versus the corresponding control.

rPBS, fPBS, or htPBS, or with equal volumes of a 20 μ M ADP solution (positive control). Under our experimental conditions the maximum level of aggregation obtained after addition of PBS alone was 6.5% $\pm$ 1.3% ($n = 8$) after 36 s. In about 8 s, stimulation with the 20 μ M ADP solution induced a significant increase in the maximum level of aggregation (20.5% $\pm$ 2.6%; $n = 8$) (Fig. 3). In about 8 s, stimulation with fPBS and conditioned htPBS caused a significant increase in platelet aggregation compared to PBS alone ($P < 0.01$; $n = 8$) (Fig. 3). In addition, the maximum level of aggregation obtained for both these samples did not differ significantly from the level observed with the same volumes of the 20 μ M ADP solution; furthermore, there was no significant difference between fPBS and htPBS ($P = 0.3$; $n = 8$) (Fig. 3). In contrast, the conditioned rPBS was unable to induce significant platelet aggregation ($P = 0.4$ versus PBS alone; $n = 8$) (Fig. 3). These findings suggested that *A. castellanii* can also cause platelet aggregation by way of ADP release.

Activity of *A. castellanii* trophozoites and amoebic cell-free supernatants in erythrocytes. In order to establish whether the amoebas can affect human erythrocytes, we tested the hemolytic activities of both (i) trophozoites and (ii) amoebic cell-free supernatant. The data reported below are data for "group O" erythrocytes, but similar results were obtained for preliminary experiments performed with "group AB" erythrocytes (data not shown).

FIG. 3. Levels of maximum aggregation in 0.5 ml of human PRP containing about 250,000 platelets μ L $^{-1}$ evaluated at 37°C after stimulation with 80 μ L of PBS buffer, 20 μ M ADP, rPBS, fPBS, and htPBS. The bars indicate the means and the error bars indicate the standard errors for eight experiments. *, $P < 0.01$ versus PBS buffer; #, $P < 0.01$ versus the ADP solution; ^, not significant versus the ADP solution.

FIG. 4. Hemolytic activity of *A. castellanii* trophozoites (Trophoz.) (3×10^6 cells/ml) with human erythrocytes (Erythroc.) (6×10^6 cells/ml) during incubation in 1 ml of PBS buffer or in the same volume of PBS buffer containing 15 mM glucose and 0.33 mM CaCl_2 (§) at 37°C. As a control, erythrocytes suspended in PBS buffer or PBS (§) at a density of 6×10^6 cells/ml were used. The data are the means $\pm$ standard errors of nine experiments. *, $P < 0.01$ versus the corresponding control; #, $P < 0.01$ versus the corresponding hemolysis value obtained with trophozoites in the absence of glucose and CaCl_2 . The absorbance at 546 nm after complete hemolysis was 0.095 ± 0.002 ($n = 20$).

(i) **Hemolytic activity of trophozoites.** The absorbance of hemoglobin released from erythrocytes incubated in sterile distilled water was 0.095 ± 0.002 ($n = 20$), representing 100% hemolysis.

The results showed that incubation in PBS buffer in the absence or presence of 15 mM glucose and 0.33 mM CaCl_2 did not modify the hemolytic effect (Fig. 4). *A. castellanii* trophozoites in PBS alone and *A. castellanii* trophozoites in PBS supplemented with 15 mM glucose and 0.33 mM CaCl_2 caused time-dependent hemolysis, and the maximum value was reached 60 min after the beginning of incubation with red blood cells (the absorbance values were 0.040 ± 0.003 [$n = 9$] and 0.088 ± 0.005 [$n = 9$], respectively). Longer exposure times did not increase hemolysis (Fig. 4). The presence of glucose and CaCl_2 in the medium significantly increased the hemolytic activity of trophozoites (Fig. 4). Light microscopy examination during hemolysis experiments showed that there was agglutination between trophozoites and erythrocytes within 10 to 15 min.

(ii) **Hemolytic activity of amoebic cell-free supernatant.** Our experiments performed to evaluate the hemolytic effect of ePBS showed that *A. castellanii* was able to induce contact-independent hemolysis in human erythrocytes, but only in the presence of 15 mM glucose and 0.33 mM CaCl_2 . This phenomenon was time dependent, but the highest hemolytic activity was observed after 30 min of exposure (absorbance, 0.034 ± 0.002 ; $n = 9$), and a longer time incubation did not increase hemolysis (Fig. 5).

To characterize the amoebic molecule(s) that is able to induce hemolysis, we evaluated the hemolytic activities of htPBS and 20 μM ADP. The results showed that heating at 95°C for 10 min totally abolished the hemolytic activity of cell-free supernatant conditioned by *A. castellanii* and that ADP did not lyse erythrocytes (Fig. 5). These results suggest that the amoebic effector(s) able to induce hemolysis is thermolabile and is not ADP.

Comparison of hemolysis in the presence of glucose and

FIG. 5. Hemolytic activity with human erythrocytes (Erythroc.) (6×10^6 cells/ml) of cell-free PBS conditioned for 2 h with 3×10^6 *A. castellanii* trophozoites at 25°C, PBS not treated (ePBS) or treated at 95°C for 10 min (htPBS), and PBS containing 20 μM ADP. As a control, erythrocytes suspended in PBS buffer at a density of 6×10^6 cells/ml were used. Evaluation was performed in the presence of 15 mM glucose and 0.33 mM CaCl_2 at 37°C. The data are the means $\pm$ standard errors for nine experiments. *, $P < 0.01$ versus the control. The absorbance at 546 nm after complete hemolysis was 0.095 ± 0.002 ($n = 20$).

CaCl_2 highlighted the finding that the hemolytic activity of amoebic cell-free supernatant (conditioned with 3×10^6 amoebas) was less than the contact hemolytic activity (with the same number of trophozoites).

DISCUSSION

Numerous workers have described the role of platelets in inflammation and tissue injury. Activated platelets have previously been demonstrated to upregulate endothelial cell adhesion molecule expression and inflammatory cytokine secretion and therefore may play an important role in promoting vascular inflammation and coagulation (6, 19, 39). In addition, blood platelets actively participate in organism defense reactions, particularly in antiparasitic immunity (24, 25). During infections, platelet stimulation can be induced by contact with parasites or parasite metabolites and by the presence of immunoglobulins, complement, or cytokines.

Previous studies by our group showed that *A. castellanii* is able to release ADP into the culture medium, demonstrating that release of ADP plays an important role in its contact-independent cytotoxicity (27, 28).

ADP is a well-known activator of platelet aggregation (9). Stimulation with ADP causes the release of Ca^{2+} from intracellular stores, Ca^{2+} influx, phospholipase C activation, inhibition of stimulated adenylate cyclase, and aggregation (11). Therefore, we investigated the putative role of parasite-derived ADP obtained from amoebic cell-free supernatant in platelet activation.

Our findings demonstrate, albeit indirectly, that *A. castellanii* trophozoites can activate human platelets by ADP release, inducing both overload of cytosolic free calcium and aggregation. In fact, purified ADP and *A. castellanii*-conditioned PBS in vitro cause similar patterns of calcium flux and aggregation on human platelets. Furthermore, treatment with the specific P2-purinoceptor antagonist suramin completely abolished the effect of protozoan-secreted metabolites on Ca release. It was not possible to further evaluate ADP-induced platelet aggregation using suramin as a specific inhibitor because of exten-

sive binding of suramin to plasma proteins that interfered with aggregation complexes.

To our knowledge, this is the first report that demonstrates that platelets could be a cellular target for pathogenic *Acanthamoeba* isolates.

The potential capacity to modify platelet functionality could also play an important role in vivo, contributing to tissue damage in *Acanthamoeba* infections. In fact, platelet aggregation triggered by ADP released by trophozoites might contribute to cerebral microcirculation disorders during *Acanthamoeba* meningoencephalitis.

The ability to activate platelets has been exploited by some pathogenic bacteria to promote colonization and avoidance of the host immune response.

Despite the prolonged clinical course of the disease, several features suggest that there is an unusual host response during *Acanthamoeba* keratitis, including a lack of corneal vascularization and a lack of a lymphocyte response in human and experimental disease (13, 42). The activation of platelets might explain both the lack of vascularization and the scarce lymphocytic infiltration in corneal stroma during *Acanthamoeba* keratitis. Recent evidence indicates that platelets significantly contribute to the leukocyte recruitment in acute inflammation, and data reported by Zhijie and coworkers (44) suggested a role for platelets in the inflammatory and epithelial responses to corneal injury.

Further studies are necessary to conclusively demonstrate whether ADP released from *A. castellanii* trophozoites comes from exocytotic granules and/or vesicles, from the cytosolic ADP pool via intrinsic plasma membrane channels or pores, or from the extracellular hydrolysis of released ATP. A previous study (36) revealed the presence of divalent-cation-dependent ecto-ATPases in *Acanthamoeba* isolates belonging to genotypes T1, T2, T3, T4, and T7 that play an important role in the pathogenesis of *Acanthamoeba*. Ecto-ATPases are glycoproteins that are present in plasma membranes whose active site faces the external medium rather than the cytoplasm; therefore, it is likely that *Acanthamoeba* uses these enzymes to produce extracellular ADP from ATP.

Data presented in this work concerning the effects of amoebic cell-free supernatant on human platelets were obtained by stimulation of platelets with a single contact with protozoan-secreted metabolites; consequently, our data could not show putative effects of chronic exposure to amoebic soluble metabolites. Further in vitro studies are necessary to elucidate the interaction between human platelets and *Acanthamoeba* trophozoites.

Red blood cells are known to be a very susceptible target for cytolytic effectors, and the lytic effect can be easily quantified by estimating the amount of hemoglobin released. Hemolytic factors from several protozoa, including *Entamoeba histolytica* (32) and *Naegleria fowleri* (43), have been identified. Hemolytic activity, however, has not been well characterized in *Acanthamoeba*.

The present findings demonstrate that *A. castellanii* can lyse human red blood cells. In this study we obtained evidence that hemolysis can occur in both contact-dependent and contact-independent ways, indicating that amoeba adhesion to target cells is not a prerequisite for hemolytic activity. Differences in the kinetics and in the values for the two types of hemolysis

were observed. In addition, trophozoites had hemolytic activity both in the absence and in the presence of calcium ions, even though under the latter conditions, their cytolytic effect on human erythrocytes was significantly increased. In contrast, the action of a hemolytic factor(s) released into the medium was strictly calcium dependent. Therefore, the mechanism by which amoebic soluble metabolites released into the medium trigger hemolysis appears to differ from the mechanism used by trophozoites during direct interaction with erythrocytes. These data suggest that *A. castellanii* trophozoites can trigger hemolysis by using at least two different strategies, only one of which requires calcium ions.

These findings corroborate previous findings (29) showing that clinical *Acanthamoeba* isolates exhibit significantly higher erythrocyte adhesion and hemolytic activity than environmental *Acanthamoeba* isolates.

We still do not know the chemical nature of the amoebic hemolytic molecules; however, this study demonstrates that the effectors involved in contact-independent hemolysis can be inactivated by heat treatment. In addition, although it has been shown that human erythrocytes express purinergic receptors (9), our results indicate that ADP at the same concentrations in amoebic secretions does not affect the plasma membrane permeability of erythrocytes. These findings, therefore, led us to suppose that some proteins released by *A. castellanii* trophozoites might be involved in this phenomenon. Further studies are necessary to identify these molecules and to characterize the mechanism of hemolysis. Nevertheless, human platelets and erythrocytes seem to play a role as potential cell targets during *Acanthamoeba* infections.

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