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Cap Formation Induced by Concanavalin A in Pathogenic Free-living Amoebae

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Summary. Morphological differences in cap formation were found when trophozoites of different free-living amoebae were treated with the lectin Concanavalin A, which resulted in a rapid redistribution of certain surface components to form small clusters and membrane-folded structures of diverse sizes. *Acanthamoeba castellanii*, *Acanthamoba polyphaga* and *Naegleria lovaniensis* exhibited characteristic caps, however, in *A. castellanii* this structure was larger and included several folds of the plasma membrane; furthermore, some of these caps had vacuoles containing a fibro granular content. In contrast, the caps formed by *A. polyphaga* and *N. lovaniensis* lacked vacuoles. Regarding *Naegleria fowleri*, the trophozoites did not produce a defined cap, and only small patches of lectin-bound surface receptor complexes were observed at one pole of the cell body. In the free-living amoebae studied, it was not possible to correlate the shape and size of cap with pathogenicity .

Key words: Free-living amoebae, Con A, capping, polar redistribution.

INTRODUCTION

Free-living amoebae are protozoa found worldwide in a broad variety of wet habitats; in fact, encysted forms have even been isolated from the atmosphere (Visvesvara and Stehr-Green 1990, Rivera *et al*. 1991, John D. T. 1993, Martinez and Visvesvara 1997, Schuster and Visvesvara 2004). *Naegleria fowleri* is a free-living amoeba that produces an infection known as primary amebic meningoencephalitis (PAM) (Lawande *et al*. 1980; Martinez 1993, 1997; Cursons *et al*. 2003; Cogo *et al*. 2004), a rapid fatal infection of the central nervous system, while *Acanthamoeba castellanii* and *Acanthamoeba polyphaga* are the causative agents of granulomatous amebic encephalitis (GAE) (Martínez 1980, 1991; Khan 2009). In addition, *Acanthamoeba castellanii* may cause an eye infection known as *Acanthamoeba* keratitis (AK), a painful vision-threatening infection predominantly found in contact lens users (Ma *et al*. 1990, John 1993, Schaumberg 1998, Clark and Niederkorn 2006).

Apparently, the first description of capping phenomenon was reported by Ray (1951) in the soil amoebae

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Hartmanella sp. Afterwards, a similar observation was done by Taylor *et al*. (1971); they found that antibodies reacting with lymphocyte surface immunoglobulin molecules caused these to gather over one pole of the cell.

In *Entamoeba histolytica*, the causative agent of the human intestinal amebiasis, the capping phenomenon is characterized by a great mobilization of cell surface components that interact with certain external ligands such as lectins, polyspecific antibodies and cationic ferritin, which under certain conditions of temperature and ligand concentrations are polarized toward the uroid region, where they accumulate and are released spontaneously without damage to the trophozoite (Pinto da Silva *et al*. 1975; Trissl *et al*. 1977; Calderón *et al*. 1980, 1986).

Capping formation has also been reported in other protozoan parasites such as *Trypanosoma brucei* (Barry 1979), *Trypanosoma cruzi* (Schmuñis *et al*. 1980), *Leishmania enriettii* (Doyle *et al*. 1974), *Leishmania donovani* (Dwyer 1976), *Toxoplasma gondii* (Dzbenski *et al*. 1976) and in the free-living amoebae *Naegleria fowleri* (Ferrante and Thong 1979), *Naegleria gruberi* (King and Preston 1977), *Hartmanella* sp. (Ray 1951) and *Acanthamoeba castellanii* (Preston and King 1984, Gun *et al*. 1988). It has been suggested that the rapid disappearance of antigen-antibody complexes from the parasite cell surface may be an effective means of evading the host immune response (Dzbenski *et al*. 1976, Aus-Kettis and Sundqvist 1978, Ferrante and Thong 1979, Martínez-Palomo 1982).

In order to establish if the pathogenic free-living amoebae in culture *Acanthamoeba castellanii*, *Acanthamoeba polyphaga* and *Naegleria fowleri* used in this study were able to form caps after incubation using Concanavalin A as an external ligand, and to find possible morphological differences in cap formation that could be related to their pathogenicity, experiments were carried out simultaneously with this lectin and observed by means of fluorescence, transmission and scanning electron microscopy.

MATERIALS AND METHODS

Cell cultures

Trophozoites of the axenically grown strain *Acanthamoeba castellanii* (isolated from the contact lens of a patient), *Acanthamoeba polyphaga* (associated with an *Acanthamoeba* keratitis case), *Naegleria fowleri* (ATCC 30808) and *Naegleria lovaniensis*, a non pathogenic amoeba (ATCC 30569) were used.

All the *Acanthamoeba* strains as well as *Naegleria lovaniensis* were cultured at 30°C in 2% Bactocasitone (Becton Dickinson Detroit Michigan, USA) supplemented with 10% fetal bovine serum (Equitech-Bio, INC. Texas, USA), while *Naegleria fowleri* trophozoites were grown at 36.6ºC in the same culture medium. *Entamoeba histolytica* trophozoites (HM-1: IMSS) were used as a positive control, and were grown at 36.6°C in TYI -S33 Diamond medium supplemented with 10% fetal bovine serum.

Cap induction and fluorescence assays

Trophozoites obtained during the logarithmic phase of growth were allowed to adhere to chamber slides (Lab-Tek, Nalge Nunc Rochester, N Y) for 1 h. Afterwards, the parasites were washed three times with phosphate-buffered saline (PBS) and incubated with 10 µg/ml fluoresceinated-Concanavalin A (Vector Burlington, CA, USA) in the same buffer for 5 min. at culture temperature. After washing with PBS, cells were fixed with a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde for 30 min. After washing the samples multiple times, they were mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) and observed in a fluorescence equipped Zeiss Axiophot photomicroscope (Carl Zeiss Oberkochen, Germany).

Scanning electron microscopy

Amoebas induced to form caps as above were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, dehydrated with increasing concentrations of ethanol and placed on 13 mm diameter Thermanox plastic coverslips (Nunc Rochester, NY, USA). Samples were critically-point dried using a Samdri-780 apparatus (Tousimis Research Corporation, Rockville, MD) and lightly coated with gold (approximately 30 nm thick) using a JEOL JFC-110 ion sputtering device. All samples were observed in a Philips XL-30 ESEM scanning electron microscope.

Transmission electron microscopy

Cap formation was induced by treating trophozoites with non-fluoresceinated Con A (100 µg/ml) for 15 min. except for *A. polyphaga* which was incubated with 10 µg/ml Con A for 5 min. Cells were washed once with PBS followed by an incubation with 50 µg/ml horseradish peroxidase (Polysciences Inc. Warrington, PA, USA) for 15 min. at culture temperature. Samples were then fixed with 2.5% glutaraldehyde in PBS, washed three times and incubated for 15 min. with 0.5 mg/ml 3,3'-diaminobenzidine (Gibco BRL, Carlsbad, CA, USA) in 0.1 M Tris-buffer pH 7.4 containing 0.2% H_2O_2. Glutaraldehyde-fixed samples were post-fixed with 1% OsO_4 in sodium cacodylate buffer. Dehydration was performed with increasing concentrations of ethanol and finally with propylene oxide. Samples were then embedded in epoxy resins. Thin sections were placed on copper grids, and contrasted with 5% uranyl acetate in 50% ethanol and Reynold's lead citrate. Observations were carried out in a JEOL JEM-1011 transmission electron microscope.

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RESULTS

Epifluorescence microscopy

After treatment with fluoresceinated Concanavalin A, the different amoebae strains showed a rapid redistribution of certain surface components to form arrangements that ranged from small clusters to rounded membrane folded structures or caps. In *A. castellanii*, Con A receptors rearranged at one pole of the cell (Fig. 1A) while in *A. polyphaga*, a redistribution in certain areas of the cell surface was observed (Fig. 1B). Similar results were observed on the surface *N. fowleri* (Fig. 2A). In contrast, *N. lovaniensis* showed an evident redistribution of lectin-bound surface receptors, which concentrated as a cap at one pole of the cell (Fig. 2B).

Transmission electron microscopy

In *A. castellanii* the cap was formed by numerous membranous structures observed cross and longitudinally sectioned, with some membrane complexes joined to the cell body, while others were separated; a curious finding was that certain folded membranes presented vacuoles with membrane remnants and fibrogranular inclusions inside (Fig. 1C). On the other hand, in *A. polyphaga* the redistribution of lectin-bound receptors was observed as inconspicuous electron-dense deposits around the cell surface, ending in a small cap devoid of large membrane complexes (Fig. 1D). In *N. fowleri* a typical cap structure was not observed, instead, the redistribution of lectin-bound receptors was very similar to that previously described for *A. polyphaga* (Fig. 2C). In contrast, in *N. lovaniensis* the cap was clearly defined but was smaller in size compared with *A. castellanii* (Fig. 2D).

Scanning electron microscopy

By scanning electron microscopy, the caps from *A. castellanii* (Fig. 1E), *A. polyphaga* (Fig. 1F) and *N. lovaniensis* (Fig. 2F) were clearly identified in their typical fashion, forming an irregular structure produced by several folds of the plasma membrane, resembling those found in *Entamoeba histolytica*. In contrast, *N. fowleri* exhibited some small globular membrane patches on its surface, which accumulated at a pole of the cell in a discrete way resembling a cap. These globular patches are likely to correspond to lectin-bound membrane surface receptors since they presented a random distribution and a few of them were practically detached from the cell surface (Fig. 2E). This image is very similar to that observed in the fluorescence assays (Fig. 2A).

In *Entamoeba histolytica* trophozoites used as control, the cap was clearly visible both by epifluorescence and electron microscopy (Fig. 3).

DISCUSSION

The surface antibody-Con A complexes usually form small clusters or patches that are collected into a large aggregate at one pole of the cell, forming a cap, which may be constituted by numerous folded membranes. From this pole the caps are usually shed into the extracellular surroundings. It has been suggested that the formation of the ligand-receptor complex involves a cross-linking process that depends on the valence of the ligand. To form the cap a tetrameric form of Con A is a requisite; however, strong evidences also implicate the cytoskeleton of the cell, particularly the actin microfilaments, in the formation of the cap (reviewed in Bourguignon and Bourguignon 1984, Espinosa-Cantellano and Martínez-Palomo 1994, Tavares *et al*. 2000). Moreover, Kwiatkowska and Sobota (1997) reported the presence of α-spectrin and filamentous actin comigrating with Con A receptors that were accumulating in the region of cap formation in *Acanthamoeba* spp.

In various protozoa, the polar redistribution of Con A receptors and membrane antigens is followed either by the rapid shedding and sometimes internalization of the antigen-antibody complexes. This event is considered an effective mechanism to evade the host immune response (Doyle *et al*. 1974, Dzbenski *et al*. 1976, Trissl *et al*. 1977, Aust-Kettis and Sundqvist 1978, Ferrante and Thong 1979, Schmuñis *et al*. 1980, Martínez-Palomo 1982, Calderón and Avila 1986, Espinosa-Cantellano and Martínez-Palomo 1994, Tavares *et al*. 2000).

Capping events has also been implicated as a feeding method as demonstrated by the works of Ray (1951) and Preston and King (1984) in which amoebae bind large numbers of bacteria. The accumulated bacteria were then shifted to a polar localized area forming a clump and were either endocytosed or released spontaneously.

Results of the present study using various free-living amoebae have demonstrated the Con A-induced aggregation and movement of surface receptors, which temporarily modify the membrane structure of trophozoites without damaging them. A remarkable morphological finding was that the rearrangement of membrane

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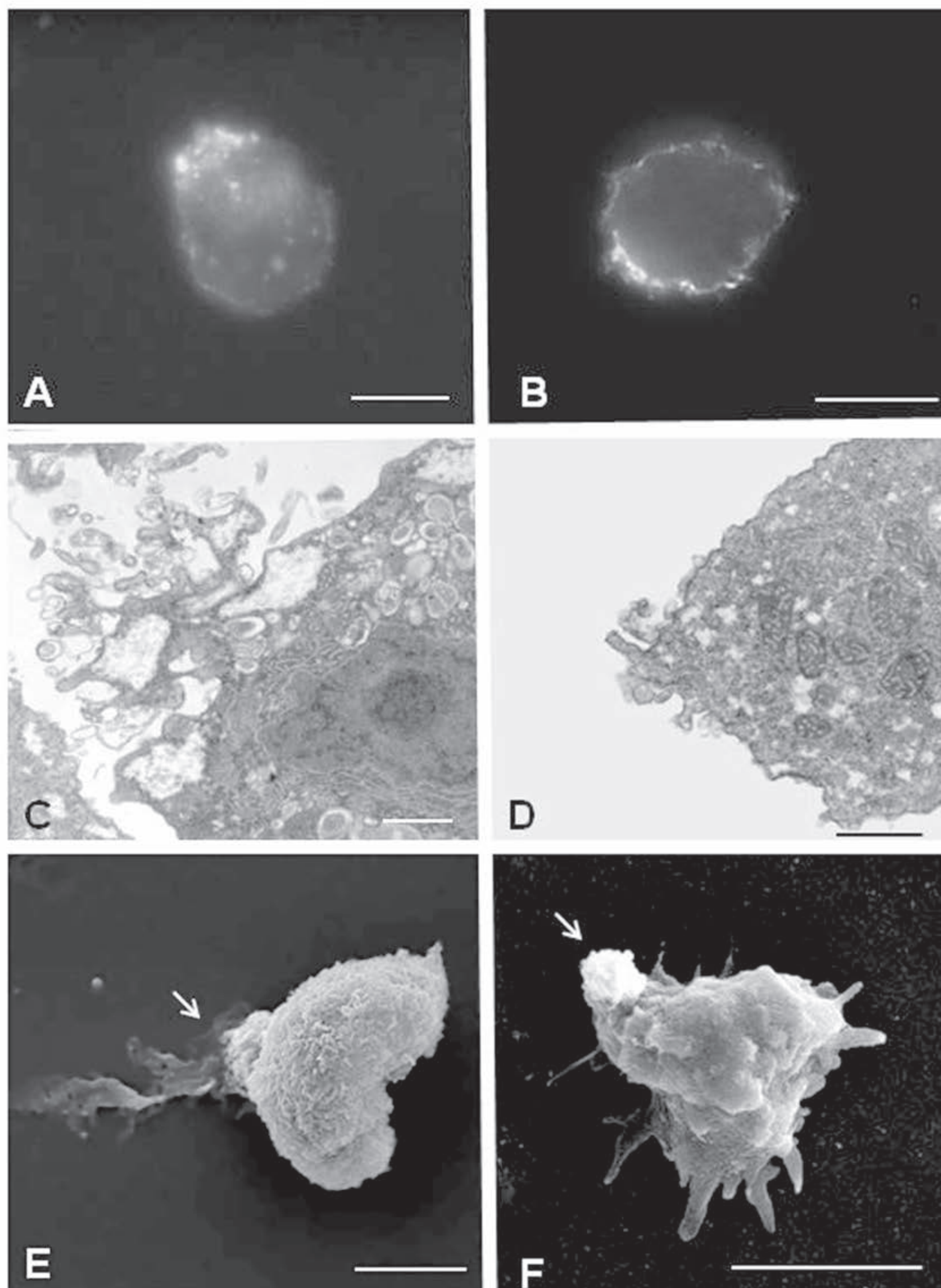

Fig. 1. Fluoresceinated Concanavalin A surface receptors complexes. **(A)** *Acanthamoeba castellanii* exhibits a large fluorescent area at one pole of the cell. Bar: 10 µm, while in *Acanthamoeba polyphaga* **(B)** there is a minor polar concentration of fluorescent reaction that is also present all over the trophozoite. Bar: 5 µm. Transmission electron microscopy of Con A receptors complexes. **(C)** Portion of an *A. castellanii* trophozoite showing a well-defined cap where folded portions of plasma membrane are visible. Some of the caps have irregular vacuoles filled with fibrogranular and membrane debris. Bar: 10 µm. **(D)** In *A. polyphaga* a smaller cap is present at one pole with minor folds of the membrane; the electrondense Con A-peroxidase-benzidine reaction on the surface of the cell is observed close to it. Bar: 10 µm. Scanning electron microscopy images of *A. castellanii* **(E)** and *A. polyphaga* trophozoites **(F)** showing the polar accumulation of Con A-surface receptors (arrows). E. Bar: 1 µm; F. Bar: 5 µm.

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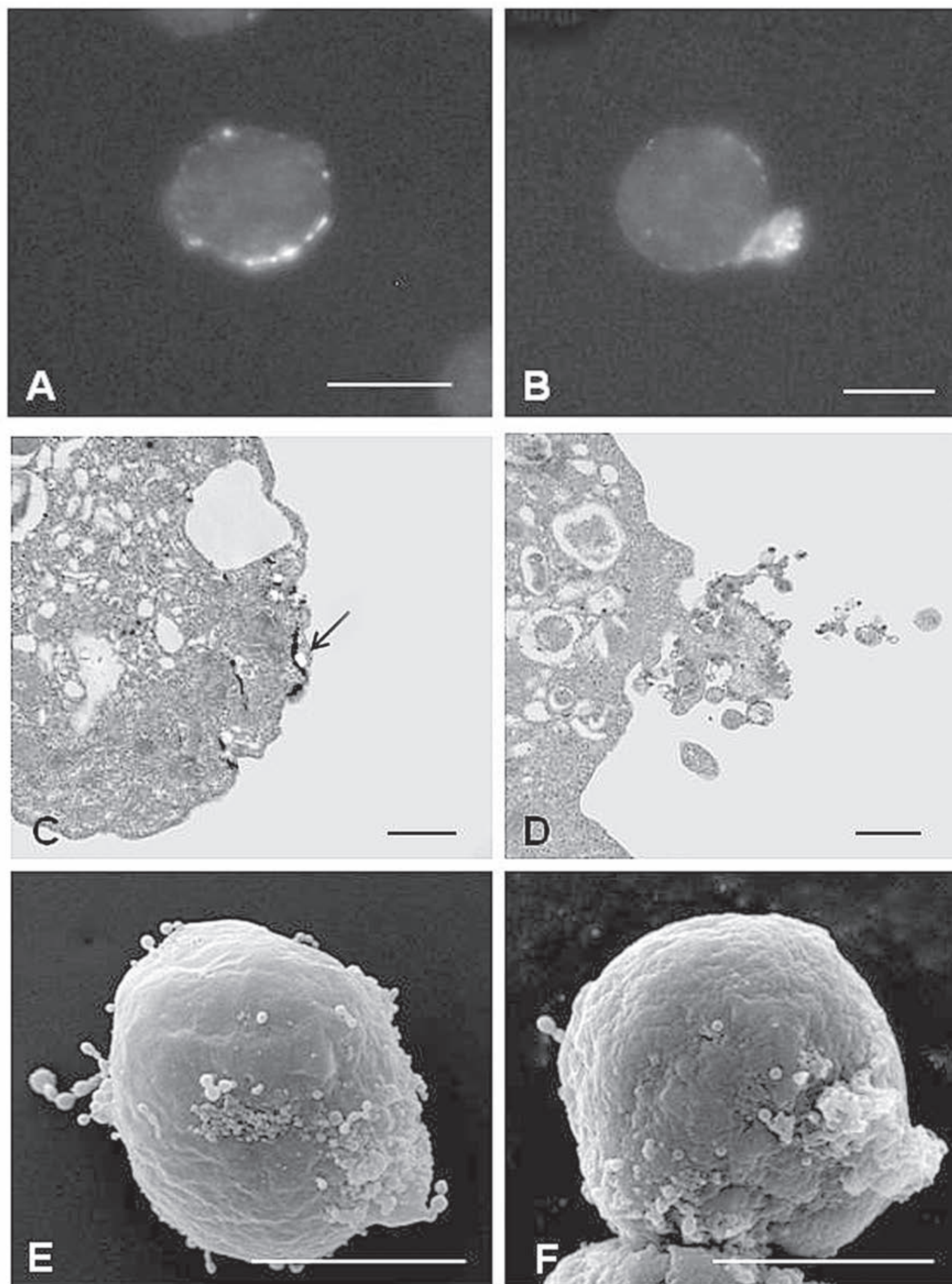

Fig. 2. Redistribution of antibody-fluoresceinated Con A compounds. In *N. fowleri* **(A)** capping is present mainly at a pole of the cell but is not condensed like a classic cap, as observed in *N. lovaniensis* **(B)**. A. Bar: 10 µm; B. Bar: 1 µm. **(C)** Thin section of a trophozoite exhibiting only a slight electrondense Con A-peroxidase-benzidine deposit (arrow), contrasting with *N. lovaniensis* **(D)**, where a small cap with folds on its plasma membrane is observed. Some portions detaching from this structure are also observed. C. Bar: 5 µm; D. Bar: 10 µm. As observed by transmission electron microscopy, at one pole of a *N. fowleri* trophozoite **(E)**, an inconspicuous cap structure is observed. The cap is constituted by small globular units, some of which are apparently being released from the cell surface. *N. lovaniensis* **(F)** presents a similar appearance but with a noticeable cap. E. Bar: 2 µm; F. Bar: 5 µm.

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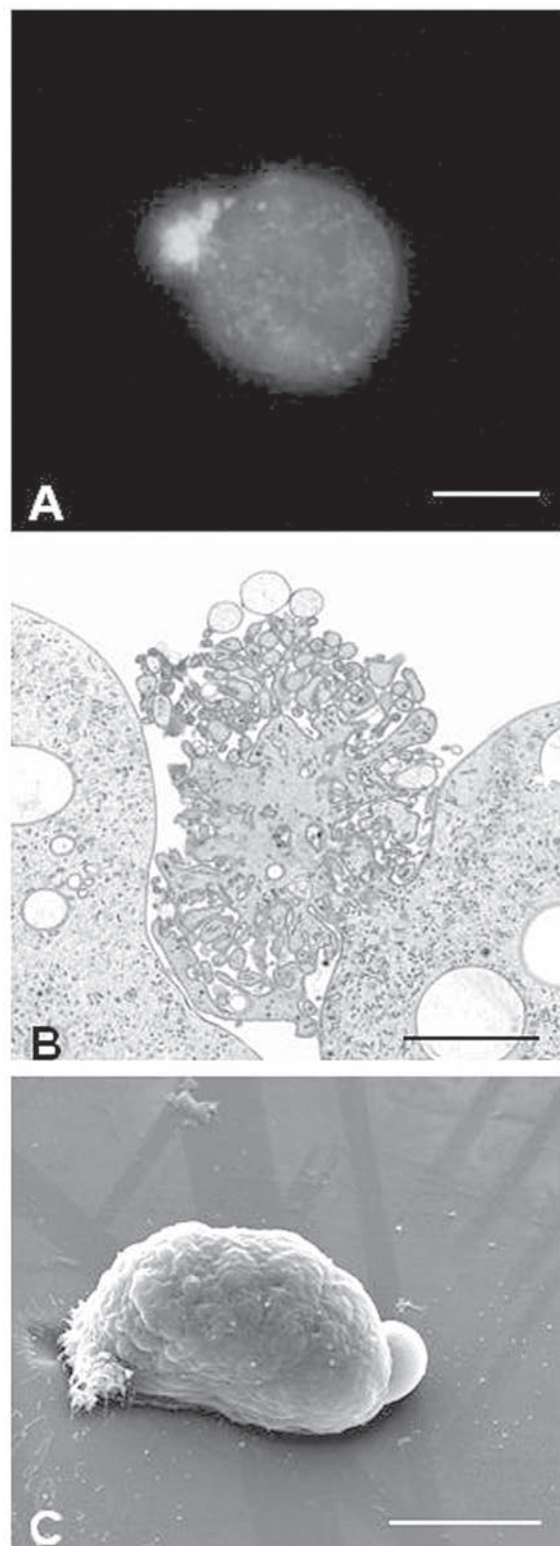

Fig. 3. *E. histolytica* trophozoites observed by fluorescence microscopy **(A)**, transmission microscopy **(B)** and scanning electron microscopy **(C)**. In all of them a well-defined cap structure is observed. A. Bar: 10 µm; B. Bar: 1 µm; C. Bar: 10 µm.

components ranged from small patches to well defined membrane folded caps. As observed by transmission electron microscopy, *Acanthamoeba castellanii*, *Acanthamoba polyphaga* and *Naegleria lovaniensis* exhibited morphologically typical caps consisting of membrane folded structures bulging from the surface of the cell. Remarkably, in *A. castellanii* these structures had a much larger size and resembled those of *Entamoeba histolytica*, which include several folds of plasma membrane, and occasionally, vacuoles with fibrogranular content and membrane remnants were observed inside the cap. In contrast, the caps produced by *A. polyphaga* and *N. lovaniensis* always lacked vacuoles.

Concerning *Naegleria fowleri*, trophozoites did not produce a defined cap; instead, only small patches of lectin-bound surface receptor complexes were identified at one pole of the cell body. It must be noted that internalization of cap fragments was not observed in the different amoeba studied. In previous assays (González-Robles *et al*. 2007), when the pathogenic *N. fowleri* and the non-pathogenic *N. lovaniensis* amoebae were treated with surface markers, they exhibited differences in agglutination capacity, capping, detection of anionic sites, carbohydrate-containing components as well as differences in the nature and distribution of plasma membrane proteins. The dissimilarities found in the cell surface coat of both species suggested the presence of diverse carbohydrate residues, as well as differences in the nature and distribution of proteins. Thus, the chemical nature of the cell surface components of the strains studied in this assay may be responsible for the different capping patterns found.

Although the morphological results of this study did not allow us to correlate the presence and size of caps with the pathogenicity of the different amoebae, our results demonstrate that the free-living species studied are also able to form caps in response to the presence of Con A. Exploration into the chemical nature of the cell surface components responsible for these differences may shed some light in our understanding of the factors involved in the different capping processes.

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