SURFACE REDISTRIBUTION AND RELEASE OF ANTIBODY-INDUCED CAPS IN ENTAMOEBAE*

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Tissue damage in human amebiasis is produced by the motile form of Entamoeba histolytica, the trophozoite (1). The structural components of this protozoan are immunogenic during infection (2, 3), but little is known about the role of immunity in invasive amebiasis. If there is an immune rejection of E. histolytica during infection, such as antibody-dependent cell cytotoxicity, or a lymphocyte-mediated response, it is reasonable to assume that the specific interaction must involve the surface antigens of the trophozoite. Accordingly, the study of the dynamic properties of the plasma membrane components, and the identification of externally located antigens should provide information on the mechanisms involved in the interaction of the trophozoite with the host immune system, as well as on the means through which the parasites survive in the human host.

The fine structure of the plasma membrane (4) and various surface properties that appear to be related to the virulence of the amebas, such as undetectable repulsive charges and higher agglutinability with concanavalin A of pathogenic strains in comparison to nonpathogenic trophozoites of E. histolytica, isolated from human asymptomatic carriers have already been reported (5). These results suggested that dynamic surface properties of Entamoeba could be related to the invasive capacity of the parasite.

The purpose of this work is to describe the behaviour of surface membrane components of E. histolytica and E. invadens when antibodies bind to the cell surface, and the events that follow this interaction.

Materials and Methods

Entamoeba Strains and Cultures. E. invadens originally obtained from Dr. L. S. Diamond (National Institutes of Health, Bethesda, Md.) was axenically cultured in TPS-1 and TYI-S-33 media (6, 7), in glass culture tubes by inoculating $10^6$ exponentially growing amebas in 10 ml of culture media. E. histolytica strain HM1:IMSS, was obtained from Dr. A. Martínez-Palom from this department. For all experiments we used trophozoites harvested at the end of the log phase and washed twice in cold phosphate-buffered saline for amebas (PBS-A), 1 (one part of 0.15 M phosphate buffer, pH 7.2, and nine parts 0.205 M NaCl).

Antibodies. Rabbits were immunized subcutaneously every 2 wk with $10^6$ trophozoites for 3 mo. IgG from immune rabbits was prepared by ammonium sulfate precipitation, dialyzed, and fractionated by DEAE-cellulose chromatography as described elsewhere (8). Goat serum anti-rabbit IgG was prepared by the standard procedures of immunization.

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1 Abbreviations used in this paper: FITC, fluorescein isothiocyanate; PBS-A, phosphate-buffered saline for amebas.
Fluorescent Antibodies. Rabbit IgG anti *E. invadens* or anti *E. histolytica* HM1 were conjugated with fluorescein isothiocyanate (FITC) as indicated by Johnson et al. (9).

Iodination. IgG anti-*E. histolytica* HM1 was radiolabeled by the chloramine T method (10) with Na $^{125}$I (New England Nuclear Co., Boston, Mass.). Fractionation from free iodine was done by gel filtration. TCA precipitation studies consisted of three consecutive precipitations with 10% TCA.

Cell Surface Redistribution. $2 \times 10^8$ trophozoites were resuspended in 0.2 ml cold TYI-S-33 medium (for *E. histolytica*) or PBS-A (for *E. invadens*) that contained 250 µg of FITC-IgG, normal and immune at fluorescein:protein ratio 2.5, or non-FITC-Ig when indicated, incubated for 15 min at 4°C, washed once, and centrifuged at 450 g for 2 min at 4°C. The pellet was suspended in 4 ml of PBS-A or TP-S-1 medium in the presence or absence of 5% vol/vol goat serum anti-rabbit IgG and incubated under constant shaking to avoid cell sedimentation, for different times indicated in Results. After incubation, cells were fixed in 2.5% glutaraldehyde for 15 min at room temperature, washed, and examined with an epifluorescence IV/F microscope (Carl Zeiss, Ober Kochen, Wurtemberg, West Germany). In some experiments, indirect staining was done after fixation.

Successive Surface Redistribution. Cycles of capping (see below) of *E. invadens* were induced by repeating several times the following steps: (a) Antibody binding to cell surface. $5 \times 10^8$ trophozoites were exposed to 500 µg/ml of rabbit IgG anti-*E. invadens* for 10 min at 4°C, and unbound Ig was washed out twice by centrifugation and suspension of the cells. (b) Incubation for assay of cell surface redistribution. Amoebas with antibody bound to its plasma membrane were incubated 30 min at 22°C in the presence of 5% vol/vol decomplemented goat serum anti-rabbit IgG. This step rendered a uniform cell surface vesiculation. After one cycle of redistribution, trophozoites were successively exposed to antibodies by repeating the above-mentioned steps. To ascertain whether in the last cycle of antibody binding redistribution of membrane components still took place, each of the assayed groups was tested with fluoresceinated antibodies. Also, to determine if segregation of membrane-bound antibodies still occurred at the various steps of the experimental procedure, samples were fixed after steps a and b.

**Results**

Cap Formation Induced by Antibodies in *E. histolytica* and *E. invadens*. Fluoresceinated Ig anti-*E. histolytica* HM1 bound to the surface membrane of *E. histolytica* HM1 and HK9 induced a polar redistribution of surface components (Fig. 1). As capping progressed, the polar accumulation of antibodies was concentrated on the uroid region of the cells. The caps formed a vesiculated membranous structure during the first 15 min; the latter also became visible under light microscopy, as shown in Fig. 2, with *E.
invadens. The morphology of the caps was heterogeneous. It varied from surface crescents to well-defined vesicles in a proportion of 25 and 54%, respectively, when observed at the end of a 15-min incubation period. These varied forms of surface redistribution represent a progression of the capping phenomenon, which ended with the gradual appearance of a constriction ring, until the fluorescent cap was released into the culture medium after 60 min in E. histolytica HM1 and 90 min in E. invadens, without loss of cell viability.

Kinetics of Capping and Fate of Segregated Caps. The kinetics of the cell surface redistribution induced by antibodies on the amebas was explored using single and double layers of antibodies, previously bound in cold for 10 min, and, afterwards, the excess Ig was washed. Other cells were also assayed in continuous exposure to antibodies.

Under these conditions, the kinetics of cap formation and the rate of cap release from the cell surface showed a similar behavior in the three above-mentioned experimental conditions in E. histolytica and E. invadens (Fig. 3). Uniform caps were observed in all cells treated with a double layer of antibodies. In experiments where the cells were continuously exposed to antibodies, the trophozoites formed the polarized cap, but the whole cell surface was also fluorescent, which indicates the appearance of newly exposed antigens during the capping process.

3 h after the occurrence of capping, most of E. histolytica HM1 population had released their caps into the culture medium, and 60% in E. invadens had lost their
caps. These appeared as fluorescent vesicles 2-5 μm in diameter (Fig. 4). Capping in *E. histolytica* HM1 and HK9 was tested with different concentrations of Ig anti-HM1, and cells were stained indirectly after fixation. Optimal redistribution was induced with 3-6 mg of Ig/ml (Fig. 5).

**Successive Capping Induced with Antibodies.** Cycles of capping using optimal times (30 min) were used to know to which extent the newly uncovered surface membrane antigens that become available to antibodies could be redistributed.

Repeated exposure to anti-*E. invadens* antibodies induced the accumulation of successive caps on the cell surface. The last cycle of redistribution, tested with the probe FITC-antibody, revealed primarily a fluorescent staining on the whole cell surface, which included the cap previously formed on the ameba at the time before the last surface segregation took place.

FITC-antibodies tested at any cycle were always localized in a cap restricted to the uroid region. The remaining surface membrane only showed a slight fluorescent background (Fig. 6). This polarized cap accumulated a significant amount of folded
Caps liberated from *E. invadens*. After complete cell surface redistribution induced by antibodies, caps were spontaneously liberated into the medium. They appeared as free vesicles 2–5 μm in diameter. ×2,000.

Fig. 5. Surface redistribution in *E. histolytica* with different concentrations of antibodies. Conditions were as described in Materials and Methods. (Δ) *E. histolytica* HM1, (○) *E. histolytica* HK9.

membrane, which almost reached the size of the remaining surface of the ameba (Fig. 6F).

Cell viability was not affected during the first three successive cycles of capping,
but 15, 30, and 40% of cell lysis was observed in cycles 4, 5, and 6, respectively. The damage was also found in cells that were not exposed to antibodies, which suggests that it was not related to repeated capping. Moreover, cumulative capping was only detected in motile cells.

**Release of \(^{125}\text{I}-labeled Antibodies Bound to the Surface of E. histolytica.** Trophozoites with \(^{125}\text{I}-labeled antibodies bound to their surface were distributed in tubes with 2 ml of
culture medium at a cell density of $1.25 \times 10^5$/ml. Triplicate samples were incubated for 0, 0.5, 2, and 5 h at 37°C, and the cells were collected by centrifugation. Fig. 7 shows the amount of radioactive material released from the cells into the medium as a function of the time of incubation. In the control sample 17% of the radioactive protein was released into the medium, but this percentage increased to 68% after 2 h of incubation. These results were very similar for both strains of Entamoeba.

Discussion

Membrane redistribution (capping) was induced in *E. histolytica* and *E. invadens* with a single layer of polyspecific antibodies directly bound to surface antigens. This phenomenon was not the result of a spontaneous segregation because antibodies reacted homogeneously on the whole surface of trophozoites that had been previously fixed.

The differences between the process of surface segregation in *E. histolytica* and *E. invadens*, as compared with capping in lymphocytes (11–13) are the following: (a) A marked ability to accumulate large amounts of folded plasma membrane on the uroid region in amebas, especially when the trophozoites were repeatedly induced to cap with brief antibody exposures (Fig. 6 F). Most, if not all surface antigens that bound polyspecific antibodies, coalesced towards the extruded cap. (b) A spontaneous release from the trophozoite of the evaginated cap took place after 60 min without cell damage; small tight masses of fluorescent material were left on the cell surface where the cap detached. Apparently, these vesicles, 2-5 μm in diameter, contained integral plasma membrane as microvesiculated structures, as observed by electron microscopy (data not shown). In this respect, Pinto da Silva et al. (14) described vesicular and tubular structures at caps of trophozoites, induced by concanavalin A peroxidase treatment.

Several possibilities arise regarding the source of the released $^{125}$I-labeled protein into the medium. This material could be derived from cells partially or totally autolyzed. Because there were not significant losses of cell density and viability during 2 h of incubation, and by this time most cells have lost their caps, we think that most of the radioactivity found in supernate was a result of the released caps.

Aust-Kettis and Sundqvist (15) reported that a double layer of antibodies was required to induce capping in *E. histolytica*. We have observed that in experiments in which the amount of Ig was lower than 1 mg/ml, no capping was induced, unless a second antibody was added, confirming the observations of these authors. However, as shown in Fig. 5, when the concentration of immune Ig is ~3 mg/ml, capping was induced with a single layer of antibody.

It is known that a single layer of antibodies produce surface redistribution of Ig receptors on mouse spleen lymphocytes (16). The capping process has been mainly studied in lymphocytes with antibodies specific for defined receptors (12, 13, 17) to examine the possible mechanism by which surface segregation takes place. It has been considered (18) that a continuous and oriented flow of lipid molecules or the integral membrane components (19) across the cell surface collects the patches of receptor-ligand complexes as a cap on the cell.

Because of the distinctive lateral mobilities of different membrane antigens in regard to the kinetics of capping, of the requirements of one or two layers of antibodies for cap induction, and of their relationship with cell movement, it has been postulated
(20) that there are two distinct mechanisms for redistribution of lymphocyte surface antigens. One, that involves a direct link of surface molecules, such as Ig, Fc receptor, and thymus leukemia antigen with the cytoplasmic contractile apparatus, based on the finding that myosin becomes concentrated in the cytoplasm underlying the spontaneous cap formed (21, 22). This kind of redistribution stimulates cell movement. The second form of capping simply results from the aggregation of cross-linked molecules in the plane of membrane, as evidence for H2 and Thy-1 surface antigens on mouse lymphocytes (20, 23) that do not cap spontaneously. In the latter kind of segregation there is no redistribution of cytoplasmic myosin, nor stimulation of cell movement, and surface redistribution does not require a direct association between surface molecules and cytoplasmic contractile molecules.

In Entamoeba, we have found that most of the surface antigens that bound polyclonal antibodies were polarized in <5 min. Pseudopods were always present on the opposite side of the vesiculated cap, which indicates that motile amebas were almost always associated with the capping process. On the other hand, a defined constriction ring, under the protruded cap, was noticed during its detachment from the cell. Accordingly, it is possible that an active contractile process is taking place during these events of surface redistribution and release. With these characteristics, it seems that only one mechanism of capping is taking place in the redistribution induced with antibodies in the Entamoeba. The vesicular conformation and constriction of the cap suggest that contractile forces originated from the cap side are responsible for this phenomenon. It remains to be demonstrated if the evaginated and detached cap is rich in actin and myosin.

The surface properties here shown by these Entamoebae make these protozoa an excellent model to study the turnover and biosynthesis of membrane components, especially under conditions where repeated capping produced an enormous accumulation of folded surface membrane. In addition, the properties of surface redistribution, liberation of caps, and rapid surface membrane turnover demonstrated here may contribute to the survival of the parasite in the host during infection.

Summary

Polyspecific antibodies bound to Entamoeba induced surface redistribution of membrane components toward the uroid region. Capping of surface antigens was obtained with a single layer of antibodies in E. histolytica and E. invadens. This surface segregation progressed to a large accumulation of folded plasma membrane that extruded as a defined vesicular cap. A spontaneous release of the cap at the end of the capping process took place. These released caps contained most of the antibodies that originally bound to the whole cell surface. Two-thirds of radiolabeled antibodies bound to the surface of E. histolytica were released into the medium in 2 h.

Successive capping induced by repeated exposure of E. invadens to antibodies produced conglomerates of folded surface membrane, visualized as stacked caps, in proportion to the number of antibody exposures. These results indicate the remarkable ability of Entamoeba to rapidly regenerate substantial amounts of plasma membrane. The properties of surface redistribution, liberation of caps, and plasma membrane regeneration, may contribute to the survival of the parasite in the host during infection.
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