SURFACE PROPERTIES RELATED TO
CONCANAVALIN A-INDUCED AGGLUTINATION
A Comparative Study of Several Entamoeba Strains*

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Invasive amebiasis is one of the common diseases caused by protozoan parasites in humans. Its importance derives from the high incidence of clinical manifestations, such as dysentery and liver abscesses (1-3). The trophozoites, or motile forms of the causative agent of amebiasis, *Entamoeba histolytica*, express their pathogenicity by invasion and lysis of epithelial tissues. In many instances, however, the trophozoites dwell in the intestinal lumen without invading the colonic mucosa, thus clinical symptoms are not apparent. The reasons for this variable behaviour of *E. histolytica* are not known.

A better understanding of the properties of the parasite has been facilitated recently with the development of axenic cultures of *E. histolytica* (4). Several strains of *E. histolytica* isolated from human dysentery cases and grown in monoxenic or axenic culture have been found to induce liver abscesses in hamsters (5-9), while invasion of the intestinal epithelium seems to be restricted to organisms maintained in monoxenic cultures (10, 11).

In contrast to the above mentioned pathogenic strains, two types of *Entamoeba* cultures are in general unable to induce liver abscesses in rodents, and therefore, are considered as nonpathogenic for mammals. These two groups include trophozoites isolated from asymptomatic human carriers (12), and several types of *Entamoeba* that grow at room temperature, such as *E. histolytica* Laredo type, *Entamoeba moshkovskii*, and *Entamoeba invadens* (13-15). The latter strain is invasive only in reptiles (14).

Pathogenic and nonpathogenic strains of *Entamoeba* are morphologically indistinguishable, even when studied by electron microscopy (16, 17). We have analyzed various surface properties of pathogenic and nonpathogenic trophozoites. Initial evidence was obtained indicating a greater sensitivity of pathogenic strains to agglutination with concanavalin A (Con A) in comparison with strains isolated from asymptomatic carriers (18). In this report we extend the agglutination studies by using *Entamoeba* strains which grow at room temperature, and compare various surface properties which might be involved in the agglutination process.

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1 Abbreviations used in this paper: Con A, concanavalin A; FITC-Con A, fluorescein-labeled Con A; PBS, phosphate-buffered saline.
Materials and Methods

Cell Cultures

Trophozoites of Entamoeba from the following strains were studied: (a) E. histolytica, HK9:NIH, cultured in axenic medium; (b) E. histolytica, HM2:IMSS, cultured in monoxenic conditions with Bacteroides symbiosus; (c) E. histolytica, HM15:IMSS, cultured with Bacteroides symbiosus; (d) E. histolytica, HM2:IMSS, cultured in axenic medium; (e) E. invadens, PZ strain, clone IV; (f) E. moshkovskii, FIC strain; (g) E. histolytica-like, Laredo strain. The last three strains were kindly provided by Dr. L. D. Diamond and were developed in axenic medium. All cultures were maintained in the same medium (4) in 25 cm² plastic bottles. Strains a to d were cultured at 37°C and studied at 48-72 h of culture. Strains e to g were cultured at room temperature and studied from 6 to 12 days of culture. To detach trophozoites from the culture flask, bottles were incubated for 5 min in an ice-water bath. Cells were pelleted by centrifugation and washed twice with phosphate-buffered saline (PBS), pH 7.3, 296 mosmol, either at 37°C or at room temperature, according to the type of culture studied. During all experiments and was always higher than 80%. When present, dead cells could be easily identified both with light or electron microscopy and were excluded from the results.

Agglutination Reaction

The agglutination was carried out in siliconized glass plates, 3 cm in diameter and 4 mm in depth. Cells (5 × 10⁵/ml, 0.4 ml) were incubated at room temperature with Con A (Miles Yeda, Israel) for 30 min, with intermittent shaking. Because of their smaller size, E. moshkovskii and E. histolytica Laredo strain were used at a concentration of 10⁶ cells/ml. At the end of the incubation period, photomicrographs were obtained by the use of a 1 × planachromatic objective with a Zeiss II photomicroscope (Carl Zeiss, West Germany) with a final magnification of 4 ×. The size of the cell clumps was measured directly on the negative film with the aid of a calibrated magnifying lens. For each experiment the diameter of at least 10 cell clumps was measured. A mean diameter was calculated from two to four experiments. The results shown in Fig. 1 are expressed as the ratio between the mean diameter of trophozoite clumps induced by Con A, and the mean diameter of trophozoite clumps suspended in PBS, since the Laredo strain shows a small degree of spontaneous agglutination. Con A was used at final concentrations of 5 or 50 µg/ml. The specificity of the agglutination reaction was tested in all cases by addition of the reaction by previous incubation of Con A with 0.1 M α-methylmannoside (Sigma Chemical Co., St. Louis, Mo.). All Con A solutions were prepared freshly before each experiment. Agglutination of fixed cells was studied with glutaraldehyde-treated trophozoites (2.5% glutaraldehyde in PBS 15 min at 37°C, twice washed in PBS).

Capping of Con A Receptors

To find out the optimal experimental conditions to obtain a polar redistribution of surface receptors for Con A in trophozoites of Entamoeba histolytica, and as a step before the comparison of the relative mobilities of receptors in pathogenic and nonpathogenic strains, several parameters were studied using the strain HK9:NIH. These included: influence of cell density, time of culture, Con A concentration, peroxidase concentration (19), fixation after or before peroxidase, time of incubation with Con A, and time of incubation with peroxidase. The results indicated that the highest percentage of trophozoites with clearly defined caps was observed at a cell density of 1-2 × 10⁶ cells/ml, total vol 10 ml, using 3-day cultures. The optimal concentration of Con A found was 10 µg/ml and that of peroxidase 50 µg/ml. Best results were obtained by incubation in Con A and peroxidase 15 min each. Cap formation of Con A receptors occurred whether fixation was carried out after incubation with Con A or after incubation with Con A and peroxidase. However, the degree of redistribution, as judged both by light microscopical examination and by ultrastructural observation of thin sections, indicated that caps are best defined in trophozoites treated both with Con A and peroxidase before fixation.

The final procedure used to estimate the degree of redistribution in all strains was as follows. Washed trophozoites were incubated for 15 min with 10 µg/ml Con A in PBS. After washing with PBS cells were incubated with 50 µg/ml horseradish peroxidase (Sigma Chemical Co.) in PBS for 15 min, washed again with PBS, and fixed subsequently with 2.5% glutaraldehyde in PBS during 15 min. Fixed cells were reacted for 15 min with 3,3′-diaminobenzidine (0.5 mg/ml) in 0.1 M Tris buffer, pH 7.4, containing 0.2% H₂O₂ (19). The percentage of capped cells was determined in at least 100 trophozoites in the light microscope. It was also determined in thick (5-10 µm) uncontrasted Epon sections studied with light microscopy and in thin sections studied with a Zeiss EM 10 electron microscope without counterstaining.
The normal distribution of Con A receptors was determined in cells fixed in 2.5% glutaraldehyde for 15 min before treatment with the Con A-peroxidase-benzidine sequence. In addition, the normal distribution and the induced redistribution of Con A receptors was estimated by fluorescence microscopy in cells fixed in 2.5% glutaraldehyde and in living cells, respectively, reacted with fluorescein-labeled Con A (FITC-Con A, Miles Yeda) at a concentration of 50 μg/ml, using a photomicroscope II (Carl Zeiss), with dark contrast illumination. All experiments were repeated at least three times.

Cell Microelectrophoresis. The surface charge of trophozoites in PBS was studied by measuring the electrophoretic mobilities in the cylindrical cell electrophoresis apparatus (Rank Brothers, Cambridge, England) (20) using the 4-ml vol chamber fitted with platinum electrodes and immersed in water at 25 ± 1°C. Migration rates were measured by timing the passage of trophozoites through a calibrated graticule when a current of 2.5 mA and a gradient of 4 V/cm were applied to the electrophoresis chamber. Trophozoites were timed in alternate directions to minimize electrode polarization. Calibration was controlled before each experiment by measuring the electrophoretic mobility of human red blood cells.

Surface Labeling with Cationized Ferritin. Binding of cationized ferritin was studied at 0°C. Cells were washed twice in PBS, incubated with cationized ferritin (21) (Miles Yeda) at a concentration of either 1.0 or 1.5 mg/ml for 10 min, washed three times with PBS, fixed with 2.5% glutaraldehyde for 15 min, and washed again with PBS. After treatment with OsO₄, cells were dehydrated and embedded in Epon. The surface reaction was studied with an electron microscope in thin sections contrasted with lead nitrate. Labeling with cationized ferritin of cells fixed in glutaraldehyde gave nonspecific binding in all strains even in the presence of ammonium chloride which is supposed to inhibit unspecific binding of proteins to free aldehyde groups (22). If living cells were labeled at room temperature before fixation, a redistribution of ferritin particles occurred in nonpathogenic strains. For these reasons, the normal distribution of the negative charges could only be examined in living cells at 0°C.

Results

Con A-Induced Agglutination. The quantitative estimation of Con A-induced agglutination demonstrates clearly that the pathogenic strains of Entamoeba (HM15:IMSS monoxenic, HM2:IMSS monoxenic, and HK9:NIH) form much larger agglutinates than the other strains in the presence of 50 μg/ml of lectin (Fig. 1). The intensity of the agglutination reaction of pathogenic amebas is particularly striking, since there is a 40-60-fold increase in the size of cell clumps when Con A is added to the cells, thus each large clump may be formed from more than one hundred thousand cells. The measurement of the mean diameter of cell clumps in photomicrographs, however, provides a faster and more reliable method to quantitate differences in agglutination than to determine the total number of cells per clump.

As shown in Table I, prefixation with glutaraldehyde reduced, but did not eliminate, the agglutination of susceptible strains in the presence of Con A.

Normal Distribution of Con A Receptors. In pathogenic strains of E. histolytica a strong and uniform binding of Con A was detected over the cell surface, both with light and electron microscopy of prefixed cells reacted with Con A-peroxidase-benzidine sequence, or with the use of fluorescein-tagged Con A (Figs. 2-4). E. invadens showed a much weaker, but still uniform, surface reaction on all cells (Fig. 5), whereas in E. histolytica HM2 axenic, only about 10% of the cells were strongly stained, the rest showing faint or no surface reaction (Fig. 6). The nonpathogenic amebas showed a weak and irregular reaction, most resembling the control obtained by addition of 0.5 M α-methylmannoside to fluorescein-tagged Con A before incubation with pathogenic cells (Fig. 7).
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Fig 1. Quantification of Con A-induced agglutination. Cells were incubated in PBS or Con A for 30 min at room temperature. The mean clump size was determined in microphotographs taken from three experiments with each strain.

| Strain | Living | Fixed |
|--------|--------|-------|
|        | PBS    | Con A | PBS    | Con A |
| E. histolytica HK9 axenic | 0.04   | 1.30   | 0.04   | 0.17   |
| E. histolytica HM2:NIH axenic | 0.04   | 0.36   | 0.04   | 0.13   |
| E. histolytica HM2:NIH monoxenic | 0.04   | 1.15   | 0.04   | 0.29   |

Cells were incubated in PBS or Con A (50 μg/ml) for 30 min at room temperature before or after fixation with 2.5% glutaraldehyde for 15 min. Results refer to the mean diameter from two experiments measuring 10 cell clumps each.

Cap Formation of Con A Receptors. Treatment of living trophozoites of *Entamoeba* with Con A resulted in the polar redistribution of surface receptors (cap formation, [23]) whether fixation was done before or after peroxidase, as shown in Table II. Treatment of living trophozoites with fluorescent Con A with or without subsequent treatment with peroxidase also resulted in cap formation (Fig. 8).

A comparison between the three different methods used to determine the percentage of capped cells revealed that the most convenient one was the light
Different Entamoeba strains reacted with FITC-Con A after fixation visualized with dark field fluorescence microscopy. Fig. 2, HK9 strain; Fig. 3, HM2 monoxenic; Fig. 4, HM15, monoxenic; Fig. 5, E. invadens; Fig. 6, HM2 axenic; Fig. 7, HK9 strain. FITC-Con A was previously incubated with α-methyl-mannoside. All micrographs × 300.

This allows the rapid study of many cells and gives the real percentage of capped cells in contrast to that obtained in sections studied with light (Fig. 10) or electron microscopy (Fig. 11) of cell sections. However, the last two methods were necessary to assess the actual degree of redistribution of Con A surface receptors.

The results of the comparative evaluation of the percentage of capped cells in the different strains of Entamoeba are shown in Table III. It is evident that all strains of Entamoeba tested were able to form caps, and no correlation exists between the number of caps and the sensitivity to agglutinate with Con A.

Cell Microelectrophoresis. As shown in Table IV the pathogenic strains HK9:NIH and HM2:IMSS monoxenic have a surface charge so low that it cannot be measured with the technique of cell electrophoresis under the conditions of pH and ionic strength used. On the other hand, the remaining strains E. moshkovskii, E. invadens, E. histolytica Laredo, and HM2 axenic showed a negative surface charge. The results obtained for the strain HM2:IMSS cultured under axenic or monoxenic conditions are particularly interesting since monox-
Table II

Percentage of Caps Detected with Different Methods

| Strain                  | Light microscopy | Electron microscopy |
|-------------------------|------------------|---------------------|
|                         | Whole cells      | Thick sections      | Thin sections      |
|                         | CPG*             | CPG$               | CPG*              | CPG$             |
| * E histolytica HK9 axenic | 62               | 20                 | 16                | 12               |
| $ E histolytica HM2 IMSS monoxenic | 53              | 20                 | 9                 | 15               |
| * E histolytica HM2 IMSS axenic | 21              | 12                 | 7                 | 15               |

* Con A ---> peroxidase ----> glutaraldehyde  
$ Con A ---> glutaraldehyde ---> peroxidase

Caps were induced by incubation with Con A (10 μg/ml) during 15 min at 37°C and peroxidase (50 μg/ml) 15 min at 37°C. Fixation was carried out with 2.5% glutaraldehyde for 15 min at 37°C. The numbers refer to the mean of two experiments. For light microscopy at least 100 cells were counted for each experiment. For electron microscopy a mean of 30 cells was studied for each experiment.

![Fig. 8](image)

HK9 trophozoite reacted with FITC-Con A for 15 min before fixation; a polar cap is clearly identified. x 300.

Enric cultures of this strain have no detectable surface charge, while in axenically grown trophozoites the largest value for electrophoretic mobility of Entamoeba was obtained (1.56 μm⁻¹·V⁻¹·cm⁻¹). The relative virulence of this strain under axenic conditions is presently being evaluated.

Normal Distribution of Cationized Ferritin Receptors. Figs. 12–18 show the results of a typical experiment of the distribution of cationized ferritin binding sites on Entamoeba trophozoites treated in the cold with 1.0 mg/ml of cationized ferritin. The binding corresponded well with the results of cell electrophoresis in that cells without measurable electrophoretic mobility at pH 7.3 (HK9 axenic and HM2 monoxenic) did not bind the polycation (Figs. 12 and 13). The strain HM15 showed a patchy distribution of the ferritin on most cells, with large areas of surface membrane devoid of grains (Fig. 14). In contrast, E. moshkovskii, HM2 axenic, and E. histolytica Laredo strains showed a strong uniform reaction all over the surface (Figs. 15–17). In E. invadens, ferritin bound to the cell surface appeared in few clusters (Fig. 18). When the concentration of cationized ferritin was increased to 1.5 mg/ml, the results were similar with the exception...
TABLE III

| Strain                        | Percentage of caps | Number of experiments |
|------------------------------|--------------------|-----------------------|
|                              | Mean   | Range |                      |
| *E. histolytica* HK9:NIH axenic | 51     | 36-71 | 17                    |
| *E. histolytica* HM2:IMSS monoxenic | 49     | 41-65 | 4                     |
| *E. histolytica* HM2:IMSS axenic | 22     | 16-26 | 4                     |
| *E. invadens*                 | 69     | 53-90 | 6                     |
| *E. moshkovskii*              | 16     | 7-29  | 4                     |
| *E. histolytica* Laredo        | 40     | 28-54 | 4                     |

Percentage of cells with caps was estimated by light microscopy of whole cells, treated with Con A-peroxidase-benzidine. Cells were fixed after Con A and peroxidase incubation.

TABLE IV

| Strain                        | Mobility   | Number of observations |
|------------------------------|------------|------------------------|
|                              | Mean       | SD         |                      |
|                              | μm·s⁻¹·V⁻¹·cm |           |                      |
| *E. histolytica* HK9:NIH axenic | NM*       | --        | 30                    |
| *E. histolytica* HM2:IMSS monoxenic | NM*       | --        | 30                    |
| *E. histolytica* HM2:IMSS axenic | -1.56     | 0.28      | 46                    |
| *E. invadens*                 | -0.87      | 0.13      | 50                    |
| *E. moshkovskii*              | -0.85      | 0.10      | 87                    |
| *E. histolytica* Laredo        | -0.84      | 0.08      | 100                   |

Electrophoretic mobilities were measured in PBS, pH 7.3, 296 mosmol at 25°C.

* Not measurable.

of strain HK9 which showed a clustered labeling in some cells while the remaining cells were negative. At the higher concentration of ferritin, *E. invadens* showed a more uniform distribution of the label.

Discussion

The major finding to emerge from the present study is the fact that pathogenic strains of *E. histolytica* (HK9, HM2 monoxenic, and HM15 monoxenic) show several cell surface properties different from those of nonpathogenic strains of *Entamoeba*. Strong agglutination induced by Con A, high Con A surface binding, and low surface charge are only present in strains that have been shown by others (6, 8) to induce liver abscesses in rodents. The possible link between virulence of pathogenic protozoa and the parasite surface properties was suggested by our earlier finding of a striking lectin-induced agglutination of *E. histolytica* pathogenic strains isolated from human dysentery cases. In contrast,
nonpathogenic strains obtained from human asymptomatic carriers (18), and several *Entamoeba* strains (*E. invadens*, *E. moshkovskii*, *E. histolytica* Laredo) which are not pathogenic for mammals (13, 14), lack this characteristic, as shown here. It remains to be demonstrated if these special surface properties of pathogenic *Entamoeba* are merely coincidental or if they have any bearing on the virulence of the amebas.

A variety of protozoa have now been found to agglutinate with Con A. The promastigote form of *Leishmania donovani* forms clumps at a concentration of Con A higher than that needed for agglutinating *E. histolytica* (24), and the infective stage of *Leishmania braziliensis* also agglutinates with Con A (25). A pathogenic strain of *Acanthamoeba*, a free living amoeba that causes meningoencephalitis in mammals (26), is also sensitive to Con A, but requires double the concentration of lectin necessary for maximal agglutination of an avirulent strain (27). A different nonpathogenic strain of *Acanthamoeba* does not agglutinate with Con A (28). In contrast, the pathogenic blood forms of *Trypanosoma cruzi* are not agglutinated by Con A, whereas the nonpathogenic culture form agglutinates readily (29). Therefore, although virulence of pathogenic protozoa is not necessarily associated with agglutination by Con A, this lectin nevertheless reveals consistent differences in surface properties between certain pathogenic and nonpathogenic protozoa.

Lectin-induced agglutination has been studied extensively in recent years mainly in tumor cells. In neoplastic cells, agglutination appears to depend, at least in part, on the redistribution of surface Con A receptors to form a clustered pattern (30, 31), although some doubts have been raised on this interpretation (32). The susceptibility of pathogenic *E. histolytica* strains to Con A-induced agglutination is strikingly higher than that of tumor cells in terms of the total number of clumped cells. Several alternatives may explain the high sensitivity of *E. histolytica* pathogenic strains to clumping with Con A. An increased mobility of surface receptors could be responsible, as has been suggested for tumor cells (33). The present study, however, demonstrates that the degree of receptor redistribution, estimated as the percentage of capped cells, is not related to the susceptibility of a given strain to agglutinate with Con A. Therefore, long range membrane movements of surface molecules, such as those present in cap formation, are equally found in pathogenic and nonpathogenic strains. Short range movements, in turn, could be involved, since we have found that fixed cells are less sensitive to agglutination. However, fixation does not only alter the mobility of membrane components, but also other parameters that may be involved in agglutination, such as cell rigidity, cell shape, and surface charge.

The phenomena of rapid aggregation and capping of surface components found in all strains of *E. histolytica* studied may be of significance for the understanding of host-parasite immunological interactions. Disappearance of parasite surface antigen after interaction with antibody has been reported...
recently in both amastigote and promastigote forms of Leishmania (34). The polar redistribution of Con A receptors (35), and of E. histolytica membrane antigens (36, Trissl and Calderón, in preparation), is followed either by the rapid internalization or shedding of the surface complexes from the parasite. Therefore, the rapid disappearance of the antigen-antibody complex from the parasite surface may render the ameba less susceptible to the immune response.

Another explanation for the higher sensitivity to agglutination of pathogenic Entamoeba strains may be that surface receptors differ in quantity, affinity, or accessibility for Con A. Either of these factors seems to be responsible, since we could show by labeling with FITC-Con A, that pathogenic amebas stain much stronger and more uniformly than nonpathogenic strains.

A third parameter which might be involved in cell agglutination is the surface charge. The lack of detectable surface charge in pathogenic strains of E. histolytica demonstrated by cell electrophoresis and by absence of binding of cationized ferritin is of interest, since the absence of repulsive forces represented by like negative charges could well facilitate the bridging of cells by Con A molecules (37). Thus, in addition to variations in the amount of Con A bound, the differences in surface charge may render the pathogenic strains more sensitive to agglutination. The nature of the ionogenic surface groups responsible for the negative surface charge in nonpathogenic Entamoeba is not known, since sialic acid, which is the main component related to the surface charge of mammalian cells, has not been found in Entamoeba fractions (38).

In summary, the present observations suggest that the high sensitivity of pathogenic Entamoeba to agglutinate with Con A is caused by a high capacity to bind Con A and a low surface charge. Although it would be premature to attempt to link these differences with the virulence of E. histolytica, the possible role of variations in surface charge and Con A binding in relation to pathogenicity can now be approached experimentally. In this respect, it is of interest that the strain HM2:IMSS varied in susceptibility to agglutinate with Con A and in surface charge according to the absence or presence of associated bacteria in the culture, since bacteria are known to be instrumental in determining the virulence of E. histolytica (10, 11, 39, 40). The present observations suggest that bacteria may interact with the amebas by changing certain surface properties of the parasite.

**Summary**

Pathogenic strains of Entamoeba histolytica are more easily agglutinated with concanavalin A (Con A) than strains isolated from human asymptomatic carriers. All three pathogenic strains studied here were found to agglutinate with low concentrations of Con A in contrast to various nonpathogenic axenic strains of amebas, characterized by their ability to grow at room temperature. Our present observations suggest that the extreme susceptibility of pathogenic strains of E. histolytica to agglutinate with Con A is related to their higher capacity for lectin binding and to their lack of detectable repulsive charges at the cell surface. The amount of fluorescein-tagged Con A bound to the surface was much higher in pathogenic strains. Only nonpathogenic strains showed a detectable negative surface charge as studied both by means of cell microelectro-
phoresis and by labeling cells with cationized ferritin at 0°C. The mobility of
surface Con A receptors estimated as the percentage of caps was comparable in
all strains. Results of one strain cultured in axenic and monoxenic conditions
suggested that bacteria can modify the behaviour of E. histolytica trophozoites
by altering surface properties of the amebas.

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