SURFACE PROPERTIES OF *ENTAMOEBA*: INCREASED RATES OF HUMAN ERYTHROCYTE PHAGOCYTOSIS IN PATHOGENIC STRAINS*

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Amebiasis is one of the most frequent protozoan infections of man, because an estimated 10% of the world population is affected (1). The disease is caused by *Entamoeba histolytica,* the only *Entamoeba* known to be pathogenic for humans. The motile form or trophozoite can live in the intestinal lumen as a harmless commensal, but occasionally, and for unknown reasons, it can invade the colonic mucosa giving rise to amebic dysentery and subsequently to liver abscesses (2). In addition to *E. histolytica,* nonpathogenic *Entamoeba* species like *Entamoeba coli,* and *Entamoeba hartmanni* may inhabit the human intestine. The reasons for the different behavior of pathogenic and nonpathogenic *Entamoeba* are not known.

Basic structural or biochemical differences which could account for the erratic invasive behavior of *Entamoeba* have not been found (3, 4). However, pathogenic strains of *E. histolytica* differ from strains isolated from asymptomatic carriers and other nonpathogenic *Entamoeba* in certain surface properties, such as the susceptibility to agglutinate with the plant lectin concanavalin A, and the lack of surface charge (5, 6).

Another surface property characteristic of *Entamoeba* is the ability to phagocytize a variety of particulate material including starch grains, bacteria, various protozoa, inert polyestyrene beads, and erythrocytes (7–16). More than 100 yr ago Lesh (Lösch) (17) demonstrated that amebas in stool samples from human dysentery contained erythrocytes. Since then, it has been claimed repeatedly that invasive strains of *E. histolytica* are the only intestinal amebas of humans able to ingest erythrocytes. Erythrophagocytosis has been traditionally considered as one of the most important criteria in identifying pathogenic *E. histolytica* trophozoites, as a perusal of texts on human protozoology will reveal (1, 18–25) with few exceptions (2).

In view of the importance given to the presence of ingested erythrocytes for the identification of invasive *E. histolytica,* the lack of systematic comparative studies is striking. On one hand, *Entamoeba* other than *E. histolytica* have occasionally been shown to engulf erythrocytes both in vivo and in vitro (16, 26, 27). On the other hand, studies on *E. moshkovskii* (the only free-living *Entamoeba* known) are contradictory, because it has been reported to be able (28) or unable (29) to phagocytize erythrocytes. Shaffer and Ansfield (30) studied the erythrophagocytosis in a number of *E. histolytica* strains without specifying their virulence.

In continuation of our studies on the surface properties of *Entamoebae* (5, 6) we have

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compared the in vitro ingestion of human erythrocytes (HRBC)[1] in various strains of *Entamoeba,* to determine whether or not phagocytosis indeed correlates with pathogenicity.

Materials and Methods

Strains and Conditions of Cultivation. Cultures of the following *Entamoeba histolytica* were used: (*a*) HK9, axenic, (*b*) HM2:IMSS, axenic and monoxenic, (*c*) HM15:IMSS, monoxenic, (*d*) HM22:IMSS, monoxenic, isolated on July 1976, from a patient with amebic rectocolitis, (*e*) HM27:IMSS, monoxenic, isolated on March 1977, from an asymptomatic patient, and (*f*) *E. histolytica*-like ameba, Laredo strain, axenic. In addition, cultures of *E. invadens* isolated from a snake, strain PZ, clone IV, and *E. moshkovskii* isolated from sewage effluent, FIC strain, cultured under axenic conditions, were used. All cultures were grown in TP-S-1 medium (31). *Fusobacterium symbiosum* was used for monoxenic cultures. *E. histolytica* Laredo, *E. invadens,* and *E. moshkovskii* were cultured at 25°C and tested at room temperature (21–24°C) at 8–11 days of culture unless stated otherwise. All other strains were grown and tested at 37°C and used after 2–3 days of cultivation. Cells were detached from the culture flask by chilling in an ice-water bath for 5 min. After centrifugation, the number of cells was adjusted to 10^6/ml in the medium in which the cells had grown. Usually, the pH of the medium was from 6.3 to 6.5. Since the rate of phagocytosis did not vary considerably within this range, the pH was adjusted only if it fell outside of this range. Cultures were used only when viability was higher than 95% as shown by trypan blue exclusion.

Pathogenic strains originated from cases of human dysentery (HM2:IMSS, HM15:IMSS, HM22:IMSS). Their degree of virulence, judged in the laboratory by the size of the inoculum required to induce liver abscesses in hamsters, varies from strain to strain depending on various factors such as time in culture and association with bacteria. HK9 was considered an attenuated strain (32). HM27:IMSS was isolated from a human asymptomatic carrier. All other strains were considered as nonpathogenic for mammals (11).

Human erythrocytes (group A, Rh negative) were used throughout the experiments. Similar results were obtained with human erythrocyte groups A, Rh positive; B, Rh positive; and O, Rh positive. Blood was stored under sterile conditions in Alsever's solution (33) with heparin, at 4°C for not more than 1 wk. Before use, the blood was washed three times in the medium from which the amebas had been harvested. The number of HRBC was adjusted to 10^8/ml.

Phagocytosis. For these experiments, 0.2 ml of amebas (10^6/ml) were incubated with 0.2 ml of HRBC (10^8/ml) for the desired times at 37°C, or at room temperature, according to the ameba type. 10 ml of distilled water were added rapidly to stop the reaction by lysing free and attached HRBC (12). It was assured by trypan blue exclusion that the amebas resisted the osmotic shock. After centrifugation (1 min, 600 *g*) the pellet was resuspended and fixed for 15 min in glutaraldehyde (2.5% in phosphate-buffered saline [PBS], pH 7.0, 296 mosmol). Cells were washed in PBS.

Staining. The method of Novikoff et al. (34) to visualize peroxisomes was used to stain the engulfed erythrocytes. Amebas were incubated for 30 min at 37°C in 2 ml of 3,3-diaminobenzidine (Sigma Chemical Co., St. Louis, Mo.) 2 mg/ml, plus 0.2% H_2O_2 in 2-amino-2-methyl-propanediol-HCl buffer (Merck-Schuchardt, Darmstadt, W. Germany) 0.05 M, adjusted to pH 917. After washing in PBS, the cell suspension was placed on a microscopic slide and 100 amebas and the engulfed HRBC were counted at random at a final magnification of 400. Various strains of *Entamoeba* have been shown to be devoid of peroxisomes by this staining method (35). Therefore, the presence of benzidine positive components in the cytoplasm of the amebas was due exclusively to ingested HRBC.

Results

Microscopic Evaluation of Phagocytosis. Fig. 1 shows the different stages of HRBC phagocytosis by *E. histolytica trophozoites.* HRBC attached first to the ameba surface, frequently forming large clumps. During the engulfment step, the HRBC were

[1] *Abbreviations used in this paper*: HRBC, human erythrocytes; PBS, phosphate-buffered saline.

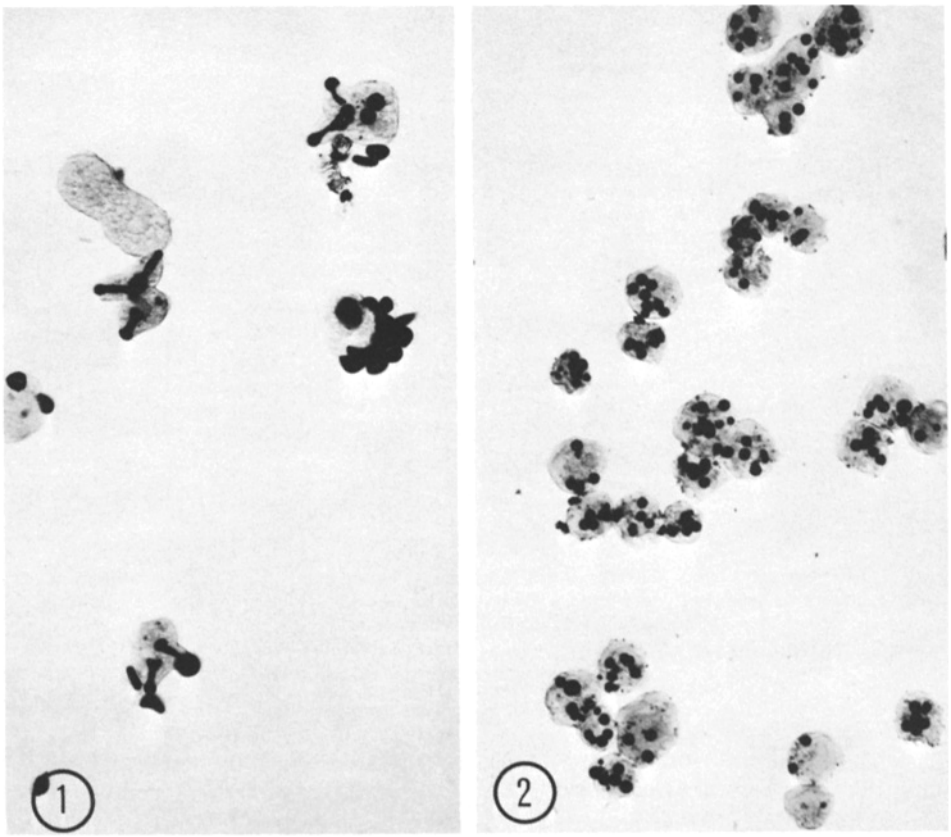

FIG. 1. Trophozoites of *E. histolytica* HM2:IMSS monoxenic (2×10^6/10 ml) were incubated in a Petri dish at 37°C for 30 min until they attached to the bottom of the dish. 7.5×10^8/ml HRBC were added. After 2 min phagocytosis was stopped by 2.5% glutaraldehyde. Excess of HRBC was decanted and the amebas detached with a rubber policeman and stained with benzidine. × 350.
FIG. 2. *E. histolytica* HM2:IMSS monoxenic. For quantification, 0.5×10^6/ml amebas were incubated with 0.5×10^8/ml HRBC in suspension for 10 min. The reaction was stopped by osmotic lysis of free and attached HRBC and by subsequent fixation in 2.5% glutaraldehyde. Benzidine stain. × 120.

extremely deformed and entered the ameba through long phagocytic channels. Several HRBC could be taken up simultaneously at different sides of the ameba. Once inside the ameba, the HRBC assumed a spherical shape. A detailed account on the ultrastructure of erythrophagocytosis will be presented elsewhere.

Quantification of phagocytosis was facilitated by stopping the reaction with osmotic shock before fixation. Amebas remained intact, whereas free and attached erythrocytes lysed rapidly. Fig. 2 shows amebas treated with distilled water after a 2-min incubation with HRBC. Since the ingested HRBC were clearly detected with the benzidine reaction, quantification became easier and more reliable than counting of unstained ingested HRBC.

Time Dependence. Individual amebas from a given culture showed highly different rates of phagocytosis, whereas within a given strain the average rate was a more or less constant property up to 30 min. At longer times, the average number of HRBC per ameba varied considerably from experiment to experiment. As shown in Fig. 3,

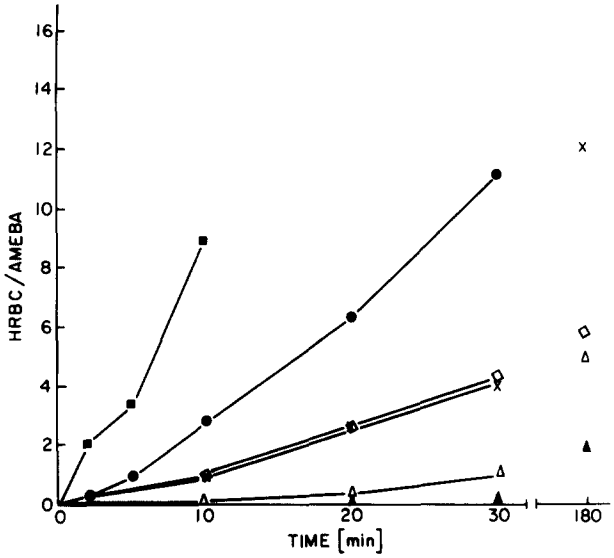

FIG. 3. Time dependence of phagocytosis. Amebas were incubated with HRBC, fixed, and stained as described in Materials and Methods. In each experiment the average number of HRBC per ameba was counted in 100 amebas at random. Strains HM2:IMSS, monoxenic, HM15:IMSS, monoxenic, and HM2:IMSS axenic gave values comparable to those shown for strain HM22:IMSS. Mean values of three to five experiments were plotted ■, HM22 mon; ●, HK9; ◇, HM27 mon; ×, *E. Inv* ax; △, Laredo ax; ▲, *E. mosh* ax.

all *Entamoeba* studied were able to phagocytize HRBC. After 30 min, only *E. moshkovskii* in particular, occasionally contained HRBC. After longer incubation times, all strains studied, including *E. moshkovskii,* had ingested large amounts of HRBC. However, the number of ingested HRBC varied considerably (Fig. 4), being highest for strains HM22:IMSS, HM15:IMSS, and HM2:IMSS, the latter strain under both axenic and monoxenic conditions. HK9 gave an intermediate rate. *E. invadens, E. histolytica,* Laredo, *E. moshkovskii,* and the carrier strain *E. histolytica* HM27:IMSS showed a less pronounced phagocytic activity.

Since the culture and test conditions were not the same for all amebas with regard to temperature and age of culture, additional experiments were performed to find out whether these differences could be responsible for the slower phagocytic rates found in nonpathogenic amebas.

Temperature Dependence Laredo strain can be cultured in a wide temperature range, from 20 to 37°C (11). Thus phagocytosis could be compared at 37°C and at room temperature (21–23°C). There was no difference in the phagocytic rate at these temperatures.[2]

Age of Culture. To test the influence of the time of culture, phagocytic rates of *E. invadens* at days 2–16 were measured. Cultures seeded on different days were compared

[2] Occasionally cultures of the Laredo strain were found not to phagocytize HRBC during 3 h of incubation, even in experiments where other strains showed the usual engulfment rates. This effect was independent of the age of culture or growth conditions, age of HRBC, pH, or the incubation in fresh or used medium. Attachment of HRBC to the ameba cell surface seemed to occur in the usual manner. All stock culture tubes proved to be positive for phagocytosis. No explanation for this erratic behavior can be given. These experiments were excluded from the graphs.

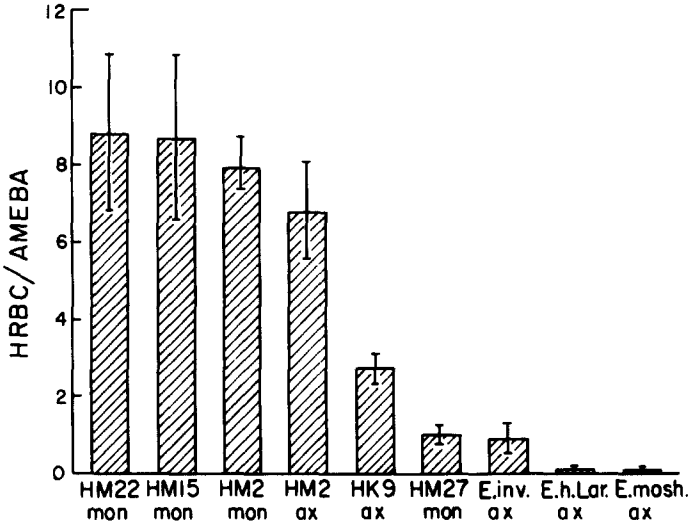

FIG. 4. Differences of phagocytic rates between pathogenic and nonpathogenic *Entamoeba*, after 10 min of incubation. Data taken from Fig. 3. Bars indicate standard deviation.

in the same experiment. Fig. 5 shows the results. Whereas the percentage of amebas that contain HRBC varied only slightly on different days of cultures (Fig. 5, right), the average number of HRBC per ameba increased considerably from day 2 to day 16 of culture (Fig. 5, left). Thus, under culture conditions comparable to those of *E. histolytica* (1–4 days), the rate of phagocytosis in *E. invadens* was even lower than that observed in older cultures. Clear dependence of phagocytosis in time of culture could not be found in either *E. moshkovskii* or in the Laredo strain.

Influence of Medium. Phagocytosis was normally tested in the medium from which the amebas had been harvested. Depletion of substances necessary for phagocytosis or accumulation of substances released from the amebas could influence the phagocytic rate. Therefore, a comparison was made of phagocytic rates in fresh and used medium. In contrast to all other strains studied, *E. invadens* showed a two to threefold increase of phagocytosis in fresh medium compared to that present in 8–15 day old culture media (Fig. 6). Therefore, this ameba under suitable conditions (25 days of culture and fresh medium) may well reach the phagocytic rates of pathogenic *E. histolytica.*

Discussion

The present results demonstrate that *E. histoytica* isolated from cases of human dysentery show a significantly higher phagocytic rate of HRBC ingestion in vitro, than *E. histolytica* isolated from a healthy carrier and other *Entamoeba* not pathogenic for mammals. However, all *Entamoeba* tested are able to ingest HRBC.

We have quantitated the erythrophagocytosis by means of direct microscopic counting. Averaging biochemical techniques have not been employed because abundant HRBC become adhered to the surface of the amebas. In addition, the uptake of fragmented HRBC, and the presence of partially digested HRBC inside the amebas may give rise to misleading results. To facilitate microscopic quantification of HRBC internalization we used: (*a*) a 100-fold excess of HRBC to maximize the possibilities of interaction at short times, (*b*) the lysis of attached and free HRBC by means of a

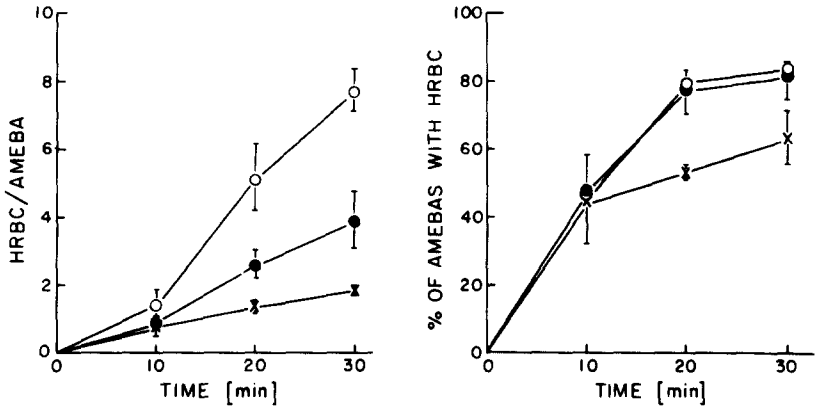

FIG. 5. Dependence of phagocytosis on age of culture *E. invadens* from 1 to 15-day old cultures were compared. Mean values of five experiments were plotted. Bars indicate standard deviation. ×, 1–4 days; ●, 8–11 days; ○ 14–15 days.

short hypotonic shock (12), which does not damage the trophozoites, and (*c*) the staining of the engulfed HRBC by means of benzidine, a procedure that facilitates visualization of the strongly stained HRBC against the pale background of the ameba cytoplasm. This technique is useful for up to 30 min of interaction. After longer times, the larger number of attached ghosts and the cytoplasmic degradation of HRBC render an exact quantification difficult.

Under the experimental conditions used, the quantitative difference found in the erythrophagocytic rate between pathogenic and nonpathogenic amebas was particularly evident comparing two recently isolated strains; cultured, and tested under identical conditions. The highest phagocytic rate was shown by strain HM22:IMSS, isolated from a case of dysentery, in comparison to the low rate exhibited by HM27:IMSS, isolated from an asymptomatic carrier. With respect to other nonpathogenic *Entamoeba*, the low phagocytic rate is not due to variations in the incubation temperature, since in *E. histolytica* Laredo, the phagocytic rate was similar either at room temperature or at 37°C. The age of the culture and the age of the medium used was found to affect only *E. invadens*. Thus, the basic difference found between pathogenic and nonpathogenic *Entamoebae* is the rate of HRBC phagocytosis.

Differences in the attachment phase could play a role in the observed variations of the phagocytic rates between pathogenic and harmless amebas. The lack of repulsive negative surface charge (6) at neutral pH in the former strains could conceivably facilitate the adhesion to erythrocytes. However, we have found that the rate of attachment of HRBC to HM2 monoxenic and HM2 axenic trophozoites is the same, even though these two strains possess clear-cut differences in their surface charge (6). The adhesive properties of pathogenic and nonpathogenic strains of *E. histolytica* to epithelial surfaces, and the possible existence of receptors at the surface of HRBC are being explored at present.

It remains to be demonstrated if the capacity of all types of *Entamoeba* to phagocytize HRBC, irrespective of their degree of virulence, as demonstrated here in vitro, holds also in the in vivo situation. In this respect, the trophozoites of *E. coli*, a harmless inhabitant of the human intestinal lumen have been occasionally reported to ingest HRBC (26, 27), when blood was present in the intestinal lumen. If so, the presence of

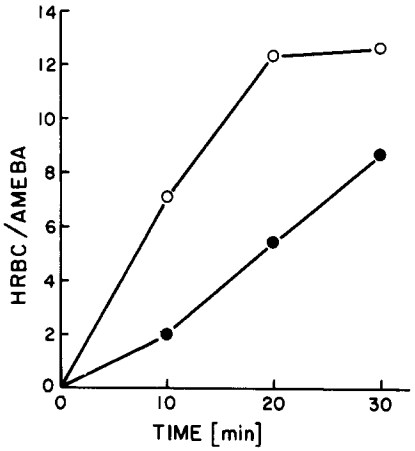

FIG. 6. Dependence of phagocytosis on culture medium. Phagocytosis of *E. invadens* from a 15-day old culture was compared in the medium from which the amebas had been harvested, and in fresh culture medium. ●, used medium; ○, fresh medium.

HRBC in the cytoplasm of *Entamoeba* trophozoites isolated from human stools may not always be diagnostic of invasive *E. histolytica* trophozoites.

The fact that pathogenic trophozoites of *E. histolytica* incorporate large numbers of HRBC in a shorter time may indicate that the phagocytic process in general is faster and more efficient compared to nonpathogenic *Entamoeba.* This difference could have some bearing on the pathogenicity towards mammalian hosts.

Other surface properties of *E. histolytica,* in addition to phagocytosis, which may be related to pathogenicity are the selective adhesiveness towards host intestinal and liver epithelial cells, the much searched but little understood cytopathic contact effect (3), the lack of repulsive negative surface charge (6), and the capacity to evade the immune response through antigenic modulation indicated by redistribution of surface components after interaction with concanavalin A or specific antibodies (6). These and other possibilities should be explored to obtain an understanding of the operative factors in the establishment of invasive amebiasis.

Summary

The assertion that ingestion of human erythrocytes is restricted to invasive strains of *Entamoeba histolytica* has not been evaluated previously by comparative studies. In this report we describe the in vitro ingestion of human erythrocytes by pathogenic and nonpathogenic *Entamoeba.* Microscopic evaluation of erythrophagocytosis by eight different *Entamoeba* grown in culture revealed that strains of *E. histolytica* isolated from cases of human dysentery show a much higher rate of erythrocyte ingestion than nonpathogenic strains. However, all strains are able to phagocytize erythrocytes. The extremely high rate of phagocytic activity shown by pathogenic *E. histolytica* could be one of the properties related to the pathogenicity of this parasitic protozoan.

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