ENTAMOEBA HISTOLYTICA

Phagocytosis as a Virulence Factor*

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One of the fundamental questions of the biology of *Entamoeba histolytica* directly related to the understanding of human amebiasis concerns the nature of the factors that determine the virulence of the parasite. The initiation of invasive amebiasis may result from the rupture of a host-parasite equilibrium that is maintained while *E. histolytica* is restricted to a commensal phase. No specific host factor has been shown to play a decisive role in the establishment of intestinal or liver lesions in those countries in which invasive amebiasis represents a common and important health problem. For these reasons, the emphasis of recent investigators has concentrated on the study of parasite virulence factors (1).

The degree of virulence of cultured *E. histolytica* varies according to the strain (2, 3) and culture condition (4). The factors responsible for these variations remain obscure. Despite a large amount of information on the subject, ultrastructural (5) and biochemical (6) studies have not been able to demonstrate differences that could explain the variable degree of virulence. Certain cell surface properties appear to characterize pathogenic strains: adhesion to epithelial cells (7), susceptibility to agglutinate with concanavalin A (8), ability to produce lytic effect on cultured cells (9–11), and phagocytosis of erythrocytes (3, 12). Recently, a correlation between collagenase production and virulence has been found (13).

Traditionally, erythrophagocytosis has been the main laboratory criterion to identify pathogenic amebas (14, 15). Furthermore, a correlation between the rate of erythrocyte (RBC)[1] phagocytosis and virulence of various amebic strains has been found (3, 12). The results have been obtained with strains isolated directly from amebic patients and cultured for a long time, which therefore could differ in more than one property. Our aim was to isolate, from a virulent and phagocytic strain, a nonphagocytic clone and then ask how the virulence has changed. The reduction of phagocytosis was matched by a dramatic loss in virulence. Furthermore, virulent revertants isolated by serial passage through

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[1] *Abbreviations used in this paper:* BUdR, 5-bromo,2′-deoxyuridine; CFE, colony-forming efficiency; MM, maintenance medium; PBS, phosphate-buffered saline; RBC, erythrocytes; TdR, thymidine.

hamster liver simultaneously recovered the high phagocytic rate characteristic of the wild strain. Since the phagocytic capacity of cultured amebas was closely related with their virulence, the results strongly suggest that phagocytosis is an important factor involved in the aggressive mechanism of pathogenic amebas.

Materials and Methods

Cells. Trophozoites of *E. histolytica* strains HM1:IMSS, HM3:IMSS, and HK9, as well as clones A and L-6, were axenically cultured in BI-S-33 medium (16). Trophozoites were harvested during the logarithmic growth phase by chilling the culture tubes for 5–10 min in an ice-water bath and then collecting by centrifugation at 360 *g* for 10 min. The cell number was adjusted in culture medium to 1×10^6 cells/ml.

Cloning of E. histolytica. Monodispersed diluted suspensions of trophozoites (1 cell/μl) were plated in plastic tissue-culture flasks (Falcon Labware, Oxnard, CA) filled with agar (Difco Laboratories Inc., Detroit, MI) (0.48 wt/wt) in BI-S-33 medium. The cultures were incubated at 37°C for 7 d. The colony-forming efficiency (CFE) was measured as the number of colonies grown divided by the number of trophozoites inoculated, multiplied by 100 (17). In other cases, microdrops of diluted suspension of trophozoites were placed on pieces of sterile coverglasses and examined with an inverted microscope. Coverglasses containing a single ameba were placed into culture tubes containing medium. Cultures were incubated at 37°C for 6–10 d. The clonal growth efficiency in liquid medium was calculated by dividing the number of tubes showing growth by the number of tubes inoculated, multiplied by 100.

Selection of Phagocytosis-deficient Amebas. The strain CR34-Thy$^-$ derived from *Escherichia coli* was grown by shaking at 37°C in saline medium 56/2 plus glucose (0.5%) (Sigma Chemical Co., St.Louis, MO), vitamin-free casaminoacid (Difco Laboratories Inc.) (0.5%), thiamine (1 μg/ml), and thymidine (TdR) (50 μg/ml) or 5-bromo,2′-deoxyuridine (BUdR) (Sigma Chemical Co.) (50 μg/ml). After 5 h of incubation at 37°C, bacteria were collected, washed twice with 56/2 saline medium, and resuspended in the maintenance medium (MM) described for amebas (18).

Interaction of Amebas and Bacteria-BUdR. Bacteria-BUdR were resuspended in MM at a density of 1×10^9 cells/ml. Trophozoites were washed twice and resuspended in MM at a density of 1×10^6 cells/ml. 1 ml of trophozoite suspension was added to culture tubes with 1 ml of bacteria-BUdR. Incubation was carried out at 37°C for 3 h. Free bacteria were discarded by centrifugation and amebas were placed into culture tubes with medium supplemented with streptomycin (300 μg/ml). Trophozoites were incubated for 24 h at 37°C to allow for amebic replication and for BUdR incorporation into amebic DNA. The amebas were collected by chilling and centrifugation at 360 *g*, washed three times, and resuspended in MM at 0.5×10^6 cells/ml.

Irradiation of BUdR-containing E. histolytica. Aliquots of amebas treated with bacteria-BUdR or bacteria-TdR (0.5×10^6/ml) were placed into 35 × 10-mm plastic petri dishes. As a filter to protect amebas from irradiation of <310 nm, the lids of the petri dishes were inverted, filled up with a 1 mg/ml solution of TdR (19, 20), and placed on top of the dishes containing the amebas. Dishes were placed on a shaker at 4°C, 4 cm below a Westinghouse fluorescent sun lamp (No. F520) that emits its peak intensity at 310 nm. The trophozoites were irradiated for 120 min and then inoculated in BI-S-33 medium plus penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were counted at different times after irradiation. The selection protocol was repeated after surviving trophozoites reached the logarithmic growth phase. The population surviving three treatments was axenically subcultured in BI-S-33 medium and cloned.

Adhesion and Phagocytosis Assays. The assays for adhesion and phagocytosis of RBC were carried out as previously described (21, 12). Briefly, 0.4 ml of trophozoites suspension (1×10^6/ml) were mixed with 0.4 ml of previously washed human RBC (1×10^8/ml of BY1-S-33). Cell suspensions were incubated at 0°C for adhesion assays, and at 37°C for phagocytosis assays. Cells were fixed at 2, 5, and 10 min with glutaraldehyde (2.5% in phosphate-buffered saline [PBS]) for 30 min at room temperature. For erythrophagocy-

tosis assays, 10 ml of distilled water were added to each tube with amebas and RBC to lyse free and attached RBC, before glutaraldehyde fixation. Cells were washed three times with PBS, and RBC were contrasted with benzidine for 30 min at 37°C (22). The adherent or engulfed RBC were counted in 100 randomly selected amebas at a final magnification of 160×.

In assays of phagocytosis of latex beads, colored latex (7 μm diam; Sigma Chemical Co.) were washed five times with PBS and resuspended in BI-S-33 at 1×10^8 beads/ml. A monodispersed latex bead suspension was obtained by sonication. Afterwards, 0.4 ml of the trophozoite suspension (1×10^6/ml) were mixed with 0.4 ml of latex bead suspension. Incubation was carried out at 37°C. At the desired times the trophozoites were washed five times with cold PBS (4°C) and fixed with glutaraldehyde (2.5% in PBS) for 30 min at 37°C. Trophozoites were washed again with PBS and the number of ingested latex beads was counted with an optic microscope in 100 randomly selected amebas.

Virulence of E. histolytica. Virulence was estimated by intrahepatic inoculation of amebas to newborn golden hamsters. Inocula of 1×10^4 to 2×10^5 trophozoites from strain HM1:IMSS wild type, and from clones A and L-6 were used. All newborns of a given litter were inoculated with the same number of trophozoites of the same strain. The inoculation volume was 0.03 ml. All procedures were carried out under sterile conditions. Hamsters were sacrificed 8 or 16 d postinoculation and the number and size of liver abscesses recorded. The amebic origin of the absecesses was demonstrated by histopathological examination. The number of trophozoites able to produce abscesses in 50% of the inoculated animals was defined as AD_{50}.

Isolation of L-6 Vir$^+$ Subpopulation from L-6 Clone. 10 young hamsters (100 g) were intrahepatically inoculated with 3×10^6 trophozoites of the L-6 clone. 3 d after inoculation, animals were anesthetized and livers were extracted and cut into small fragments with a sterile knife. The liver fragments were placed in culture tubes with BI-S-33 medium plus penicillin (300 U/ml) and streptomycin (300 μg/ml), and incubated at 37°C for 72 h. The medium was replaced and culture tubes were incubated at 37°C until logarithmic growth phase was achieved. 1.8×10^4 trophozoites selected from the L-6 population that had been four times passed by hamster liver (L-6-Liv_4) were inoculated in newborn hamsters. Each of the L-6-Liv_4 subpopulations independently obtained was assayed in newborn hamsters. Animals were killed at 8 d postinoculation and the livers examined.

Results

The selection method used for the isolation of subpopulations deficient in phagocytosis is based on the scheme devised by Clarke (19) to obtain motility mutants of *Dictyostelium discoideum*, except that in our system mutagenesis was not required. The approach is based on the development of sensitivity to near visible light irradiation by amebas that had incorporated bacterial BUdR into their DNA. We have thus isolated from a pathogenic and highly phagocytic wild strain of *E. histolytica* (HM1:IMSS) a poorly phagocytic subpopulation (L) by selective killing through irradiation of amebas that had ingested bacteria-BUdR. A clone from subpopulation L was obtained (clone L-6) and tested in comparison with a nonselected pathogenic and phagocytic clone (clone A) for erythrophagocytosis, adhesion, and virulence.

Isolation of a Phagocytosis-deficient Subpopulation. Amebas of the wild strain HM1:IMSS, fed with bacteria-BUdR or bacteria-TdR, were allowed to duplicate for 24 h in culture. After irradiation with 310 nm light, surviving amebas were grown until they reached the logarithmic phase. They were subsequently submitted to a second and a third selection treatment that included the same scheme as the first treatment: feeding with loaded bacteria, culture, irradiation, and subculture of surviving amebas. Fig. 1 shows that the first treatment with bacteria-

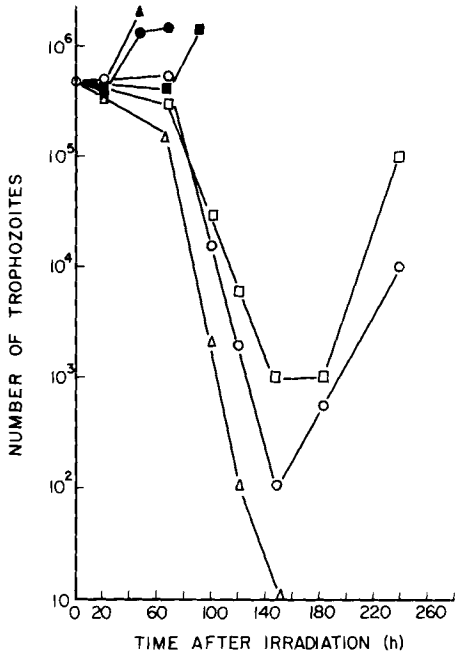

FIGURE 1. Effect of 310 nm irradiation on the growth of trophozoites fed with *E. coli* CR34 Thy⁻. Trophozoites from strain HM1:IMSS and clone A were fed with bacteria-TdR or bacteria-BUdR, incubated for 24 h, and irradiated with 310 nm light for 2 h. The number of viable trophozoites was counted every 24 h. (●) HM1:IMSS fed with bacteria-TdR and irradiated; (○) HM1:IMSS fed once with bacteria-BUdR and irradiated; (□) HM1:IMSS fed twice with bacteria-BUdR and irradiated; (▲) clone A fed with bacteria-TdR and irradiated; (△) clone A fed once with bacteria-BUdR and irradiated; (■) HM1:IMSS fed three times with bacteria-BUdR and irradiated.

BUdR resulted in the reduction of the number of viable trophozoites by 3.5 log units; the second treatment resulted in a reduction of 2.5 log units. At the end of the third treatment, trophozoites showed a growth rate similar to that of amebas that underwent one selection treatment using bacteria-TdR instead of bacteria-BUdR. Trophozoites that survived three selection treatments were subcultured and named subpopulation L. The viability of control cells fed with bacteria-BUdR without irradiation, or those fed with bacteria-TdR and irradiated, was reduced 20% or less in all experiments.

The above-mentioned experiments were also carried out with clone A isolated from HM1:IMSS wild strain. Clone A trophozoites fed with bacteria-BUdR failed to grow after irradiation with 310 nm light, while trophozoites fed with bacteria-TdR were not sensitive to irradiation (Fig. 1). These results suggest that the HM1:IMSS wild strain is composed of a heterogeneous cell population while clone A is homogeneous. The treatment does not seem to produce changes in the amebic phenotype, but rather to select the less phagocytic trophozoites present in the heterogeneous HM1:IMSS wild strain.

Cloning of L Subpopulation. Cloning was first attempted by culture in semisolid agar (17). Even though several pathogenic strains of *E. histolytica* multiplied in semisolid agar with a CFE of ~75%, the subpopulation L is defective for cloning

in this medium (Table I). For this reason, cloning of this subpopulation was carried out in liquid medium. One resulting clone, (L-6), selected at random, was successfully subcultured. It showed a growth rate similar to that of clone A (data not shown) obtained from the wild strain HM1:IMSS, thus indicating that trophozoites were not damaged by the selection treatment.

Phagocytic Efficiency of Selected Subpopulations. The erythrophagocytosis of the selected subpopulations was measured after each treatment with bacteria-BUdR and irradiation. After one selection treatment, the efficiency of erythrophagocytosis of the surviving population clearly diminished in comparison to an

TABLE I

Cloning Efficiency of E. histolytica Trophozoites in Semisolid Agar

Strain	Cloning efficiency
HM1:IMSS*	80 ± 6
HM1:IMSS‡	78 ± 7
HM3:IMSS	67 ± 10
HK9	68 ± 12
L	3 ± 1

* HM1:IMSS wild-type strain.

‡ HM1:IMSS fed twice with bacteria-TdR and irradiated at 310 nm.

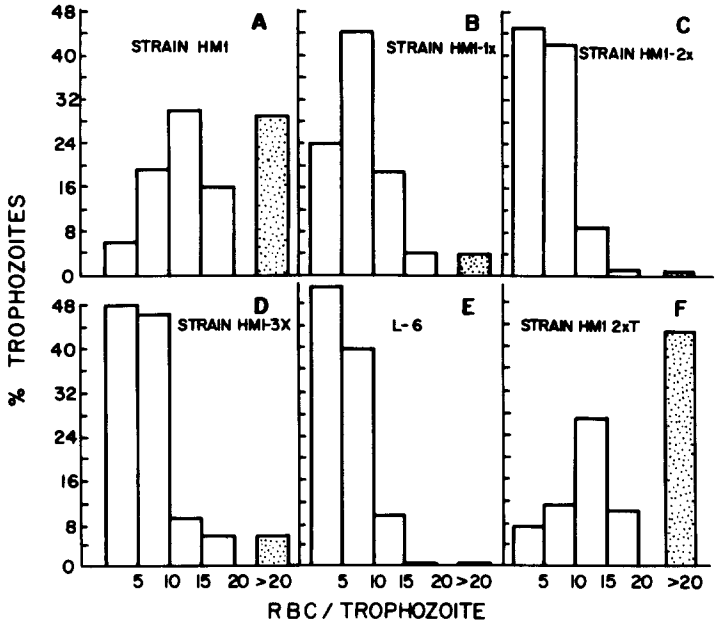

FIGURE 2. Efficiency of erythrophagocytosis of *E. histolytica* fed once, twice, and three times with bacteria-BUdR and irradiated with 310 nm light. Trophozoites from the subpopulations treated different times were mixed with RBC for 10 min at 37°C. Before fixation with glutaraldehyde, 10 ml of water was added to lyse the noningested RBC. The cell mixture was contrasted with benzidine, and RBC were counted in 100 randomly selected amebas. Trophozoites were grouped according to the number of RBC ingested. (*A*) HM1:IMSS; (*B*) HM1:IMSS treated once (bacteria-BUdR + irradiation); (*C*) HM1:IMSS treated twice; (*D*) HM1:IMSS treated three times; (*E*) L-6 clone; (*F*) HM1:IMSS treated twice but fed with bacteria-TdR instead of bacteria-BUdR.

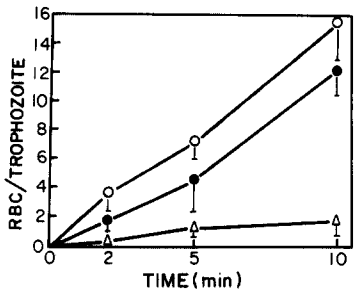

FIGURE 3. Erythrophagocytosis of strain HM1:IMSS and clones A and L-6. Trophozoites from strain HM1:IMSS (○), clone A (●), and clone L-6 (△) were mixed with RBC in a ratio of one trophozoite to 100 RBC. Incubation was carried out at 37°C for 2, 5, and 10 min. The erythrophagocytosis indices were obtained by multiplying the mean number of RBC per ameba by the number of trophozoites that showed at least one ingested RBC.

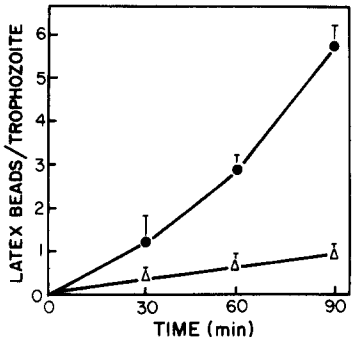

FIGURE 4. Phagocytosis of latex beads by clones A and L-6. Trophozoites from clone A (●) and clone L-6 (△) were mixed with colored latex beads (7 μm diam) at a ratio of one trophozoite to100 latex beads. Incubation was carried out at 37°C. At the desired times, trophozoites were washed with cold PBS (4°C) and fixed with glutaraldehyde. The ingested latex beads were counted in 100 randomly selected amebas.

untreated culture (Fig. 2*A* and *B*). A more pronounced reduction in the average number of phagocytosed RBC per ameba was found after two or three rounds of treatment (Fig. 2*C* and *D*). The phagocytic activity of clone L-6, obtained from the amebas that survived three treatments, was reduced even more drastically (Fig. 2*E*). In contrast, amebas fed with bacteria-TdR continued to engulf RBC with an efficiency similar to that of the wild strain.

When the rate of phagocytosis of clones A and L-6 was compared, the lower erythrophagocytosis index (23) of L-6 clone became evident (Fig. 3). The reduced capacity of the L-6 clone to phagocytose was not restricted to RBC, but applied also to the rate of engulfment of latex particles (Fig. 4). To determine whether or not the deficiency in erythrophagocytosis of the L-6 clone was due to impairment of trophozoite adhesion to RBC, the adhesion efficiency of clones L-6 and A was compared. Fig. 5 shows that trophozoites of the L-6 clone adhered RBC with an efficiency similar to that shown by clone A. Therefore, the impairment in the rate of erythrophagocysis of clone L-6 is not related to the adhesion step of RBC to the surface of amebas.

Virulence of Clones A and L-6. To define if there was a relationship between

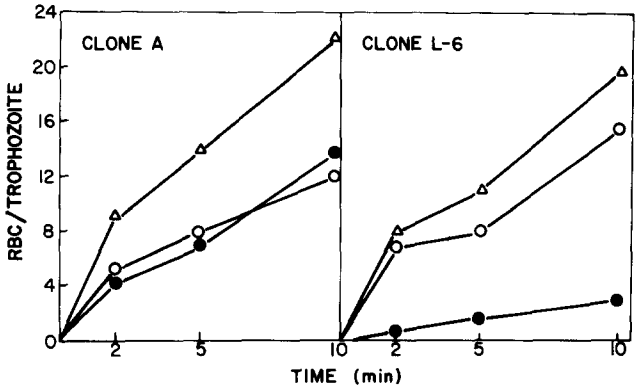

FIGURE 5. Adhesion and phagocytosis of RBC by clones A and L-6. Trophozoites were mixed with RBC (1:100). For adhesion assays (○), incubation was carried out at 0°C. For phagocytosis assays (●), incubation was carried out at 37°C, and adherent and noningested RBC were lysed, adding 10 ml of distilled water. The sum of adherent and ingested RBC (△) was measured at 37°C but without hypotonic shock. Cells were fixed with glutaraldehyde, washed five times with PBS, and contrasted with benzidine. The adherent and engulfed RBC were counted in 100 randomly selected amebas.

TABLE II

Virulence of E. histolytica (HM1:IMSS and Derivative Clones)

Strain	Number of trophozoites	Number of animals*	Percent of hamsters with abscesses
HM1:IMSS	2×10^4	20	95
HM1:IMSS‡	1.5×10^4	20	50
Clone A	2×10^4	20	90
Clone A‡	1.8×10^4	20	50
Clone L-6	2×10^5	30	0
Clone L-6	5×10^5	20	0

* Newborn hamsters were intrahepatically inoculated. At 8 or 16 d postinoculation, animals were sacrified.

‡ AD_{50} was defined as the number of trophozoites required to produce abscesses in 50% of the animals.

the diminished rate of phagocytosis of the L-6 clone and its virulence, newborn hamsters were inoculated intrahepatically with living trophozoites. Table II shows that with inocula of 2×10^4 trophozoites of strain HM1:IMSS or of clone A, hepatic abscesses were detected in ~90% of the inoculated hamsters. The AD_{50} for HM1:IMSS in newborn hamsters was 1.5×10^4 trophozoites, and for clone A, 1.8×10^4. However, 2×10^5 trophozoites of clone L-6 failed to produce hepatic abscesses. These results show a direct correlation between the rate of phagocytosis and the degree of virulence in *E. histolytica*.

Virulence and Erythrophagocytosis of Subpopulations Passed Through Hamster Liver. One of the time-honored procedures to increase the virulence of a given strain of *E. histolytica* is the serial passage through hamster liver (24). We wanted to determine with this procedure if the possible augmentation of virulence of the clone L-6 would correlate with an increased rate of phagocytosis. Seven

TABLE III
Virulence of L-6 Subpopulations After Serial Liver Passage

Subpopulations	Hamsters with abscesses/hamsters inoculated*	E/A‡
L-6-LIV$_1$	6/10	14
L-6-LIV$_2$	5/10	12
L-6-LIV$_3$	6/10	13
L-6-LIV$_4$	0/10	5
L-6-LIV$_5$	4/10	12
L-6-LIV$_6$	1/10	6
L-6-LIV$_7$	0/10	4

* Newborn hamsters were intrahepatically inoculated with 1.8×10^4 trophozoites.
‡ Erythrocytes/ameba; T = 37°C, t = 10 min.

subpopulations independently obtained from L-6 trophozoites that were passed four times through hamster liver (L-6-Liv$_4$) were assayed for virulence in newborn hamsters. 1.8×10^4 trophozoites of each one of the L-6-Liv$_4$ subpopulations were intrahepatically inoculated. Table II shows that four of seven subpopulations indeed recovered the ability to produce abscesses in hamster liver. The rate of erythrophagocytosis of the various subpopulations was also measured; recovery of virulence correlated with a recuperation of the erythrophagocytosis rate, similar to HM1:IMSS. The subpopulations that failed to produce abscesses in the liver of newborn hamsters remained deficient in erythrophagocytosis (Table III).

Discussion

Phagocytosis appears to be one of the major cellular functions that determine the virulence of the protozoan parasite *E. histolytica* (3, 12). In free-living pathogenic amebas, phagocytosis has been also reported as one of the important means of tissue aggression of the parasite (25). It has been reported that association with bacteria is required for the expression of pathogenicity in *E. histolytica*. Thus, an increase in virulence occurs when trophozoites are grown in association with nonpathogenic bacteria (26, 27), and axenization has been reported to reduce the virulence of various strains (4). Furthermore, serial passage of trophozoites in the liver of rodents, followed by subculturing trophozoites from the amebic lesions has been reported to increase the virulence of *E. histolytica* (24). These results may be interpreted if we take into consideration that the association with bacteria and serial liver passage could act through selection of the more phagocytic amebas from the relatively heterogeneous population found in noncloned cultures.

We have analyzed the role of phagocytosis on the virulence of *E. histolytica* through the selection, from a pathogenic wild strain, of a clone with a decreased rate of phagocytosis. The isolated clone showed a dramatic reduction in virulence. These results, and the analysis of virulence in revertant subpopulations, showed a direct correlation between virulence and rate of phagocytosis of *E. histolytica*.

To isolate phagocytosis-deficient subpopulations of *E. histolytica*, the highly phagocytic trophozoites of strain HM1:IMSS were eliminated by near-visible light irradiation after BUdR incorporation into their DNA through phagocytosis of labeled bacteria (19). Among the survivors, the phagocytosis-deficient clone L-6 was isolated readily without using mutagenic agents. Clone A, a phagocytic and virulent clone isolated from the parental strain HM1:IMSS, did not yield phagocytosis-deficient survivors after the same selection treatment. We interpret these results to mean that a phagocytosis-deficient subpopulation was already present in the original HM1:IMSS strain and that the selection method did not induce the observed change in the phagocytic ability of the isolated clone.

Phagocytosis is a complex phenomenon involving, among other events, adhesion of trophozoites to the target cell. Therefore, we determined whether or not the deficiency in phagocytosis showed by L-6 clone was due to impairment of RBC adhesion to trophozoites. L-6 trophozoites showed an adhesion efficiency to RBC similar to the wild-type strain HM1:IMSS and to clone A. This suggests that the low phagocytic rate showed by L-6 clone is not due to impairment of adhesion, but occurs in a subsequent step during the uptake of particles into the cytoplasm. This conclusion is also supported by the lower rate of latex bead phagocytosis showed by clone L-6, compared with strain HM1:IMSS and clone A. L-6 probably is not a simple mutant; in addition to its defect in phagocytosis, L-6 is unable to form clones in semisolid agar medium (data not shown), unlike the virulent clone A. These two traits of clone L-6 may not be phenotypes of the same mutation because revertants of L-6 able to phagocytose do not recover the ability to clone in semisolid medium. These results indicate that clone L-6 may be a variant that has accumulated two or more mutations.

The low rate of phagocytosis of L-6 clone correlated with a marked decrease in its virulence. Likewise, the recovery of virulence of the subpopulations obtained from L-6 after serial passage of the trophozoites through hamster liver correlated with an increase in the rate of phagocytosis. These results confirm the notion that phagocytosis is an important factor in the virulence of *E. histolytica*.

In conclusion, the close relationship between phagocytic rate and virulence of the *E. histolytica* suggests that phagocytosis is involved in the aggresive mechanism of the invasive trophozoite. We also show evidence for the heterogeneity of the cell population of *E. histolytica* in the HM1:IMSS strain. On the other hand, the methodology here described to study the relationship between a given surface property of *E. histolytica* trophozoites and virulence introduces a novel approach for the understanding of the cellular and genetic factors related to the virulence of *E. histolytica*.

Summary

In this paper, we attempted to define the role of phagocytosis in the virulence of *Entamoeba histolytica*. We have isolated, from a highly phagocytic and virulent strain, a clone deficient in phagocytosis. Trophozoites of wild-type strain HM1:IMSS were fed with *Escherichia coli* strain CR34-Thy$^-$ grown on 5-bromo,2′-deoxyuridine. The trophozoites that had incorporated the base analog through phagocytosis of the bacteria were killed by irradiation with 310 nm light. The survivors, presumably trophozoites defective in phagocytosis, were

grown until log phase and submitted two more times to the selection procedure. Clone L-6, isolated from a subpopulation resulting from this selection procedure, showed 75–85% less erythrophagocytic activity than the wild-type strain. The virulence of clone L-6 and strain HM1:IMSS was measured. The inoculum required to induce liver abscesses in 50% of the newborn hamsters inoculated (AD_{50}) of HM1:IMSS was 1.5×10^4 trophozoites. Clone L-6 trophozoites failed to induce liver abscesses in newborn hamsters even with inocula of 5×10^5 trophozoites. Virulence revertants were obtained by successive passage of L-6 trophozoites through the liver of young hamsters. The trophozoites that recovered the ability to produce liver abscesses simultaneously recuperate high erythrophagocytic rates. These results show that phagocytosis is involved in the aggressive mechanisms of *E. histolytica*.

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