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Adhesion of *Entamoeba histolytica* Trophozoites to Human Erythrocytes

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To understand the mechanism of *Entamoeba histolytica* adhesion, we characterized the binding of trophozoites to human erythrocytes (RBC) in suspension by measuring the kinetics of amoeba-RBC complex formation. Adhesion was very efficient, since most of the amoebae were complexed with RBC after only 5 min at 37°C in mixtures containing 10^4 amoebae and 10^6 RBC per ml; the adhesion rate depended on amoeba and RBC concentrations, but not on the A, B, and O human blood groups, and was maximal at 37°C and pH 7.3 in the presence of 4 mM Ca^{2+} and 1 mM Mg^{2+}. Adhesion was prevented if trophozoites were fixed with glutaraldehyde, but only decreased slightly if RBC were previously fixed; it decreased in the absence of glucose and was inhibited as a function of the concentration of cytochalasin B and of the metabolic inhibitors bathophenanthroline and 8-hydroxyquinoline. From these results we conclude that *E. histolytica* adhesion is an active process that depends on the amoebal cytoskeleton and metabolic energy and on the mobility of both amoebal and RBC surface ligands.

Adhesion of *Entamoeba histolytica* trophozoites to animal cells is the initial event of their most prominent cytopathogenic activities, contact-mediated cytolysis (7, 14, 16, 22) and phagocytosis (24, 27), and might be also involved in intestinal colonization and invasion by amoebae. Kobiler and Mirelman (17) recently found in *E. histolytica* trophozoites a lectin activity that is inhibited by *N*-acetylglucosamine oligomers and proposed that it may mediate the adhesion of amoebae to other cells.

We have determined optimal conditions for the binding of *E. histolytica* trophozoites to human erythrocytes (RBC) and the effect that some chemicals have on this phenomenon. Our results indicate that *E. histolytica* adhesion is an active process that depends on the amoebal cytoskeleton and metabolic energy.

MATERIALS AND METHODS

Chemicals. Bathophenanthroline, cytochalasin B (CB), colchicine, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), and dimethyl sulfoxide were obtained from Sigma Chemical Co. (St. Louis, Mo.); glutaraldehyde was obtained from J. T. Baker Chemical Co. (Phillipsburg, N.J.), 8-hydroxyquinoline came from Merck, and reagent-grade inorganic salts were obtained from J. T. Baker Chemical Co.

***E. histolytica* trophozoites.** We used trophozoites of strain HK-9, axenically cultured in TP-S-1 medium (6).

Human RBC. Unless otherwise stated, human RBC of blood group O (Rh^+) were used. Human blood was obtained by venipuncture and immediately mixed with 1 volume of glucose-citrate buffer (1); the RBC were sedimented by 10 min of centrifugation at 2,000 × *g*, washed three times with 0.15 M NaCl, and, unless indicated otherwise, suspended in HEPES-saline (20 mM HEPES, 5.5 mM glucose, 120 mM NaCl, 4 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.3) to a concentration of 2 × 10^6/ml. To fix the washed RBC, they were diluted with 0.15 M NaCl to a 50% (vol/vol) suspension, mixed with 50 volumes of fixing solution (9 mM Na_2HPO_4, 85 mM NaCl, 1% glutaraldehyde) at 4°C, and gently agitated for 30 min. The fixed RBC were centrifuged for 20 min at 2,000 × *g*, washed five times with 0.15 M NaCl and five times with distilled water, and finally suspended in 0.15 M NaCl to a concentration of 4 × 10^8/ml.

Adhesion assays. Log-phase trophozoites were harvested by chilling the cultures at 4°C and centrifuging the tubes for 10 min at 600 × *g*; packed amoebae were washed immediately twice with phosphate-buffered saline (111 mM NaCl, 16 mM K_2HPO_4, 3 mM KH_2PO_4; pH 7.4) and then washed and suspended in HEPES-saline to a concentration of 2 × 10^4/ml.

Unless noted otherwise, adhesion was started by mixing equal volumes of cell suspensions to obtain 10^4 amoebae and 10^6 RBC per ml and incubating the mixtures at 4, 25, or 37°C under gentle agitation (30 cycles per min in a Lab-Tek Aliquot Mixer). In samples taken at 0.2, 2, 5, 10, 15, 20, 30, and 40 min, adhesion was stopped by 2.5% glutaraldehyde fixation. Adhesion was calculated as the percentage of amoeba-RBC complexes (i.e., trophozoites firmly attached to at least one erythrocyte) found after counting 200 amoebae in each fixed sample under a phase-contrast microscope (see Fig. 1). The initial rate of adhesion was calculated with the formula: $(\text{adhesion}_{2\ \text{min}} - \text{adhesion}_{0.2\ \text{min}})/1.8$ min.

In experiments done at variable pH, 20 mM maleic

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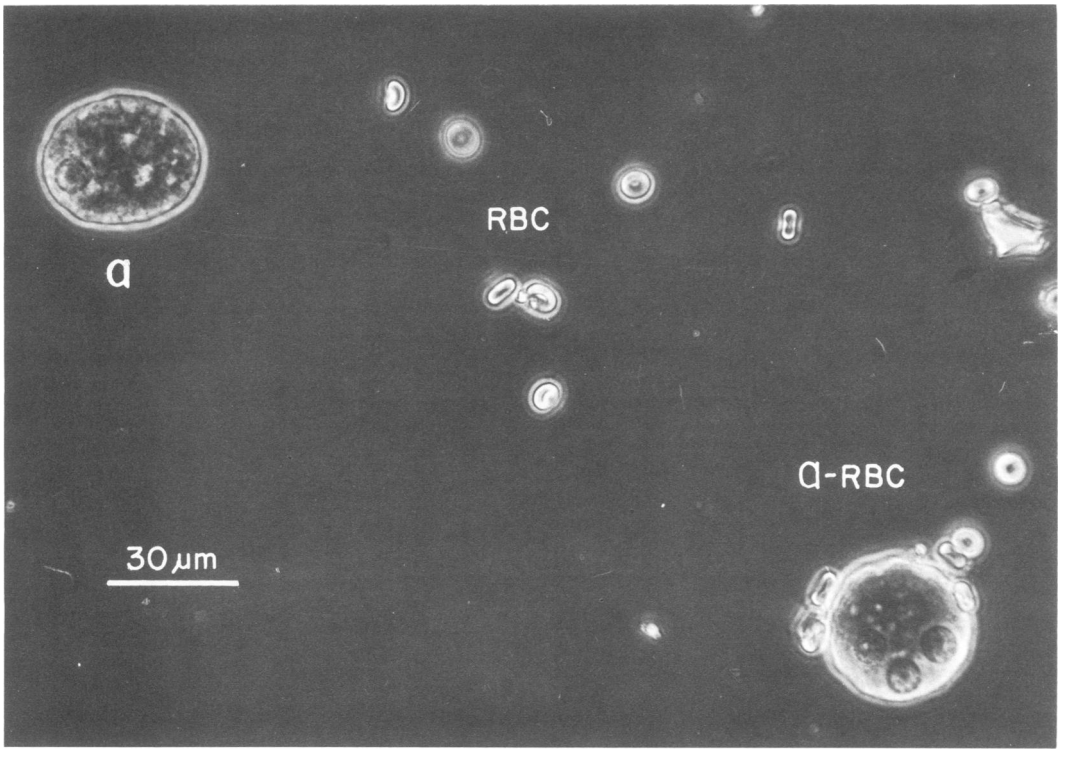

FIG. 1. Microscopic appearance of amoeba-RBC complexes. Free amoeba (a), free RBC (RBC), and an amoeba-RBC complex (a-RBC) are clearly distinguished.

acid was also added to HEPES-saline and the pH was adjusted at fixed intervals from 5 to 8.5; osmolarity was always adjusted with NaCl to 300 mosmol/kg.

To determine the effect of chemicals on adhesion, we prepared suspensions in HEPES-saline alone or containing (i) 5% dimethyl sulfoxide or CB in 5% dimethyl sulfoxide, (ii) colchicine, or (iii) 2% ethanol, bathophenanthroline in 2% ethanol, or 8-hydroxyquinoline in 2% ethanol. We mixed equal volumes of RBC and amoeba suspensions to obtain 1-ml mixtures and maintained them at 25°C; after 3 min or the indicated periods, we stopped adhesion by glutaraldehyde addition. Statistical significance was determined by means of one-tailed Student's t test.

RESULTS

Effect of trophozoite and RBC concentration. In preliminary experiments we found that adhesion of amoebae to RBC depended on the concentration of both cell types and that we could best quantitate it in mixtures containing at most 10^4 amoebae and 10^6 RBC per ml because higher cell concentrations resulted in 100% initial adhesion.

The kinetics obtained by mixing 1 volume of packed RBC with 340 volumes of diluted amoebae consisted of a high initial rate of adhesion as well as an elevated initial proportion of amoeba-RBC complexes that increased asymptotically with time to reach a maximum after 20 min of incubation (Fig. 2). However, by decreasing the original RBC concentration and starting the reactions with equal volumes of amoeba and RBC suspensions, the initial proportion of complexes fell from 30% to 8%, and we could measure the initial rates more precisely because they also decreased (Fig. 2).

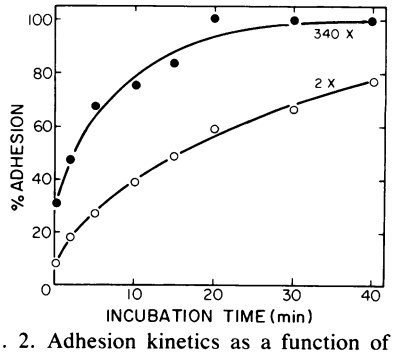

FIG. 2. Adhesion kinetics as a function of initial RBC concentration. Addition of packed RBC concentrated 340-fold (6.45×10^8/ml) before mixing (●). Addition of diluted RBC concentrated only twofold (2×10^6/ml) before mixing (○). Experiments were done at 25°C in mixtures with 10^4 trophozoites, 10^6 RBC per ml, 2.25 mM Ca^{2+}, and 1.05 mM Mg^{2+}.

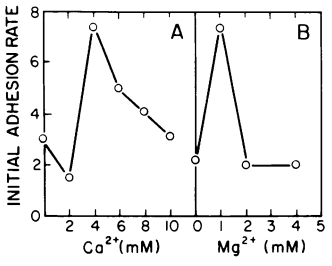

FIG. 3. Effect of Ca^{2+} and Mg^{2+} concentration on the initial rate of adhesion. Experiments were done at 4°C in mixtures with 10^4 trophozoites and 10^6 RBC per ml. (A) 1 mM Mg^{2+}, variable Ca^{2+} concentration. (B) 4 mM Ca^{2+}, variable Mg^{2+} concentration.

Effect of calcium and magnesium concentration. Similar results were obtained with variable cation concentrations at any temperature, but they were less disperse at 4°C. Maximal initial rates of adhesion occurred with 4 mM Ca^{2+} (Fig. 3A) and 1 mM Mg^{2+} (Fig. 3B), and the rate was significant even if cations were not added to the medium. With 4 mM or higher Mg^{2+} concentrations, trophozoites were vacuolated or lysed.

Effect of pH. The maximum initial rate of adhesion was obtained at pH 7.3. At pH lower than 6.5 or higher than 8, trophozoites and RBC were damaged or lysed.

Effect of temperature. Adhesion was clearly dependent on temperature, its initial rate and the proportion of amoeba-RBC complexes being maximal at 37°C (Fig. 4).

Influence of blood group. There were no differences in the rate of adhesion of trophozoites to RBC of the human blood groups A, B, and O.

Effect of glutaraldehyde fixation. Fixation of amoeba-RBC mixtures completely prevented further adhesion, whereas previous fixation of RBC with glutaraldehyde decreased the initial rate only slightly in mixtures incubated at 25°C but not at 4°C.

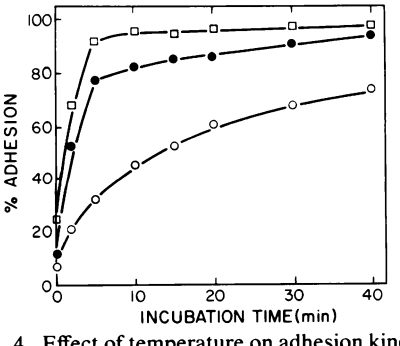

FIG. 4. Effect of temperature on adhesion kinetics. Mixtures with 10^4 trophozoites and 10^6 RBC per ml. Incubation at 4°C (○), 25°C (●), and 37°C (□). Averages of three (25°C and 37°C) or six (4°C) independent experiments.

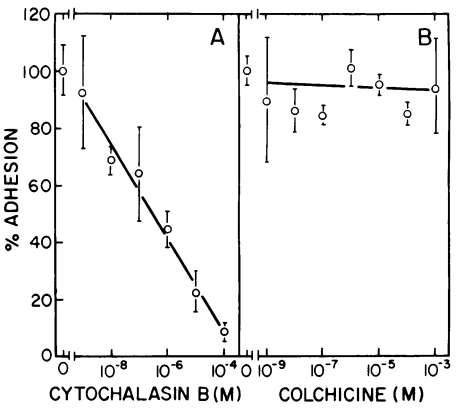

FIG. 5. Effect of CB and colchicine on adhesion. Mixtures with 10^4 trophozoites and 10^6 glutaraldehyde-fixed RBC per ml; adhesion was stopped with glutaraldehyde after 3 min. The symbols represent normalized averages and standard deviation from three experiments.

Since glutaraldehyde fixation of RBC did not prevent adhesion, we used previously fixed RBC in the experiments done with inhibitors, to insure that the chemicals affected only amoebae and not the RBC.

Effect of CB and colchicine. In 3-min assays, the adhesion of trophozoites to fixed RBC was inhibited as a linear function of the logarithm of CB concentration (Fig. 5A), whereas colchicine did not have any significant inhibitory effect (Fig. 5B). The 50% inhibitory dose of CB was 3×10^{-7} M.

When we analyzed the kinetics of mixtures containing 0.2 mM CB, we observed that adhesion was only 9% after 40 min, whereas it was 68% in mixtures containing dimethyl sulfoxide only (used as a vehicle for CB) and 88% in untreated controls.

Effect of glucose and metabolic inhibitors. Adhesion of amoebae to fixed RBC was lower in the absence of glucose and decreased as the concentration of bathophenanthroline and 8-hydroxyquinoline increased (Table 1).

DISCUSSION

It has been extensively shown that adhesion of pathogenic bacteria to animal cells results mainly from the interaction of specific surface proteins, called "adhesins," with molecules containing specific carbohydrate residues that are responsible for the binding (25) and that may be found on the surface of cells from infected organs (4, 11, 20) as well as of human RBC (3, 5, 8–10, 15).

Adhesion is essential for the most prominent

TABLE 1. Effect of metabolic inhibitors on amoebal adhesion

Inhibitor and concn	Relative adhesion	
	With glucose[a]	Without glucose
Bathophenanthroline		
0	1.00[b]	0.72
2×10^{-8} M	0.91	0.67
2×10^{-6} M	0.61	0.63
2×10^{-4} M	0.19	0.17
8-Hydroxyquinoline		
0	1.00[c]	0.59
4.6×10^{-7} M	0.91	0.41
4.6×10^{-5} M	0.33	0.31
4.6×10^{-3} M	0.19	0.09

[a] At 5.5 mM.
[b] 23% amoeba-RBC complexes in 3 min.
[c] 32% amoeba-RBC complexes in 3 min.

in vitro cytopathogenic activities of *E. histolytica* trophozoites, i.e., contact-dependent cytolysis (7, 14, 16, 22) and phagocytosis (24, 27), and might also be involved in intestinal invasion since amoebae appear to require contact with epithelial cells to penetrate the colonic mucosa either through the glands (26) or the interglandular epithelium (19).

To analyze amoebal adhesion we used human RBC as target cells because they are easy to obtain and have been successfully used to characterize several adhesins (5, 9, 15). We selected HEPES and maleate buffers because they do not chelate cations (12, 13), and we added glucose to the incubation medium because it is the major energy source of *E. histolytica* trophozoites (28).

With concentrations above 10^4 amoebae and 10^6 RBC per ml, 100% initial adhesion was found; trophozoites had caps of polarized RBC on their surface and formed aggregates among them (data not shown). The capping suggests that amoebal surface components bound to RBC move actively in the plane of the membrane, just like those that bind concanavalin A (21) and antibodies (2).

The addition of packed RBC to amoeba suspensions resulted in high initial adhesion rates and high initial proportions of amoeba-RBC complexes. By mixing equal volumes of diluted cellular suspensions we could also better quantitate the initial rate (Fig. 2).

Adhesion was cation dependent, and its rate was maximal in mixtures containing 4 mM Ca^{2+} and 1 mM Mg^{2+} (Fig. 3).

Twenty minutes after mixtures originally containing 10^7 tumor cells and 10^6 natural killer cells per ml are sedimented, 30% of the natural killer cells may be bound to the tumor cells (23). In contrast, after only 5 min, amoebal adhesion approached 100% in mixtures of much lower initial cell concentration that were not packed but maintained in suspension through constant agitation (Fig. 4). Since we did not find significant differences in the rate of adhesion to RBC of the human blood groups A, B, and O, it is possible that their determinants are not involved in the binding to amoebae.

With fixed RBC the adhesion rate was lower at 25°C but not at 4°C, suggesting that mobility of RBC ligands increases the efficiency of adhesion. Since at 4°C the rate of adhesion was identical with fixed and unfixed RBC, the affinity of RBC ligands appeared not to be affected by glutaraldehyde. The abolishment of further adhesion by the addition of glutaraldehyde to the assay mixtures suggested that the binding was an active process.

The binding to glass (18) and the contact-dependent cytolethal effect (22) of *E. histolytica* trophozoites are both inhibited by CB, but not by colchicine. Since we also found that CB, but not colchicine, blocked the adhesion to RBC (Fig. 5), it appears that active amoebal cytoskeleton microfilaments are required for efficient binding of amoebae to glass surfaces and to other cells, and therefore, that inhibition of contact-dependent amoebal cytopathogenicity by CB may be due to the blocking of adhesion.

The metal chelators bathophenanthroline and 8-hydroxyquinoline inhibit metabolic energy production by *E. histolytica* (28). Inhibition of adhesion in the absence of glucose and by addition of the metabolic inhibitors (Table 1) again indicated that adhesion requires metabolic energy.

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