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Adherence and Ingestion of *Escherichia coli* Serotype 055 by Trophozoites of *Entamoeba histolytica*

RIVKA BRACHA AND DAVID MIRELMAN*

Department of Biophysics and Unit for Molecular Biology of Parasitic Diseases, Weizmann Institute of Science, 76100 Rehovot, Israel

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Carbohydrate-binding activity present on the *Entamoeba histolytica* cell surfaces was found to mediate the adherence of two types of bacteria, *Escherichia coli* serotype 055 and *Salmonella greenside* 050. Adherence was inhibited by low-molecular-weight carbohydrates (10 mg/ml) such as galactose, lactose, and *N*-acetylgalactosamine, as well as by asialofetuin and the lipopolysaccharide extracted from *E. coli* 055. Mild periodate oxidation of the bacteria inhibited their adherence, whereas heat inactivation, glutaraldehyde fixation, or γ-irradiation had no effect. On the other hand, pretreatment of trophozoites with glutaraldehyde, cytochalasin B, or cold (5°C) abolished adherence. None of these treatments, however, affected the attachment of bacteria that contain on their cell surface type I pili with mannose-binding capacity. These findings lend further support to our earlier observations on how amoebae interact with bacteria.

It is generally recognized that the pathogenicity of *Entamoeba histolytica* is related in part to association of the amoebae with suitable bacterial species. Early observations by Westphal (14), Phillips and Gorstein (8), Wittner and Rosenbaum (16), and others, demonstrated that various bacterial species, when allowed to associate with amoebae, augment virulence, as evidenced by their ability to produce hepatic abscesses in hamsters.

In a previous study, we found that *E. histolytica* trophozoites were very selective in their interactions with bacteria (1). Two principal mechanisms were shown to be responsible for these interactions. Certain bacteria, such as a number of *Escherichia coli* strains which are known to possess mannose-binding components on their cell surface (4), bound to mannose receptors on the amoeba membrane. Other bacterial species, such as *Shigella flexneri* and *Staphylococcus aureus*, which do not possess mannose-binding capacity, attached to the amoeba, but only after precoating the bacteria with concanavalin A or after opsonization of the bacteria with immune serum. The latter attachment, in contrast to the former, was markedly inhibited by galactose, lactose, and *N*-acetylgalactosamine (1). In the present study, we found that *E. histolytica* trophozoites may also directly bind certain bacteria that do not possess mannose-binding capacity and are not opsonized.

MATERIALS AND METHODS

Trophozoites of *E. histolytica* strains NIH:200, HK-9, and HM1:IMSS were axenically grown in TYI-S-33 medium by the methods of Diamond et al. (2). Trophozoites were grown for 44 to 72 h (exponentially growing culture) and harvested by chilling in an ice water bath for 10 min to release those attached to the culture tubes. The trophozoites were washed twice in saline by low-speed centrifugation (600 × *g* for 5 min) and resuspended in saline to a final concentration of 10^6 amoebae per ml. Counting of the amoeba was done under a microscope with a hemacytometer.

Growth of bacterial cells. *E. coli* serotype 055, *Salmonella greenside* serotype 050, and *E. coli* 7343 were clinical specimens isolated and characterized by G. Altmann, Department of Microbiology, Sheba Medical Center, Tel Hashomer, Israel. Bacteria were grown overnight at 37°C in a medium containing yeast extract (0.5%), Bacto-Peptone (Difco Laboratories, Detroit, Mich.) (1.0%), NaCl (0.5%), and [^{14}C]glucose (1 μCi/ml; specific activity, 329 mCi/mmol; Radiochemical Centre, Amersham, England). Bacteria were harvested by centrifugation at 9,000 × *g* for 10 min, washed three times with saline, and resuspended in saline to a concentration of 5×10^9 to 1×10^{10} bacteria per ml based on turbidity measurements at 660 nm. Fixation of bacteria was done as described previously (1). The specific radioactivity of the bacteria obtained varied between 3 and 10 cpm/10^4 bacteria. *E. coli* 055 and *S. greenside* 050 were nicely agglutinated upon addition of soybean agglutinin (Miles-Yeda, Rehovot, Israel), at a concentration of 1 mg/ml with 10^9 bacteria.

Preparation of immune sera. Antisera were prepared against *E. coli* 055 in rabbits inoculated in the footpads

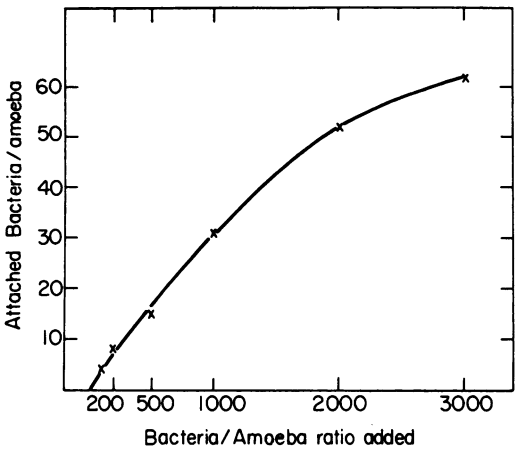

FIG. 1. Axenically grown trophozoites of *E. histolytica* strain HM-1 were incubated with ^{14}C-labeled *E. coli* 055 at different multiplicities for 30 min at 37°C in 0.5 ml of saline. The number of trophozoites was 5 × 10^5 per assay. Radiolabeled bacteria found in the Percoll gradient band in which the amoebae accumulate (30 to 50% band) were counted in a scintillation counter with a Triton X-100-based scintillation fluid. For experimental details, see the text.

with 10^{10} glutaraldehyde-fixed bacteria. After 2 weeks, the rabbits were bled, and the sera were separated and checked for agglutination titer. A positive specific agglutination at a 1:100 dilution was considered satisfactory. Coating of bacteria with immune or nonimmune sera was done as described previously (1).

Attachment of bacteria to amoebae. Bacteria (5 × 10^8 to 1 × 10^9) were incubated with trophozoites (10^6) in a total volume of 1 ml of saline solution for the desired time and temperature in glass tubes as described previously (1).

Separation of the bacteria that attached to the amoebae and those that did not was performed by discontinuous density gradient centrifugation with Percoll (Pharmacia, Uppsala, Sweden). Bacteria layered between 80 and 60% Percoll, whereas amoebae layered between 50 and 30%. Only bacteria that were attached to amoebae were observed in the amoeba layer.

Inhibitors. *D*-Galactose, lactose, α-methylmannoside, and thiodigalactoside were from Sigma Chemical Co., St. Louis, Mo. Crabshell chitin (Sigma) was purified by refluxing in 1 M HCl for 2 h to extract protein contaminants. Partial acid hydrolysis of chitin and preparation of the *N*-acetylglucosamine oligosaccharides was done by the method of Rupley (11). Extraction of the lipopolysaccharide from *E. coli* 055 and *E. coli* 7343 was done by the method of Westphal and Jann (15). *N*-Acetyl-*D*-galactosamine was from Pfanstiehl Laboratories, Waukegan, Ill. Cytochalasin B was obtained from Sigma.

RESULTS

A large number of *E. coli* as well as other gram-negative or gram-positive bacteria that do not have the ability to bind to mannose residues did not adhere to trophozoites of *E. histolytica* (1). As shown in our previous study, adherence of such microorganisms, however, could take place upon coating with immune sera. The attachment of such opsonized bacteria was not mediated by Fc receptor activity on the amoeba but by a membrane-associated lectin which apparently bound to specific *N*-acetyl amino sugars on the immunoglobulin chain.

Cells of *E. coli* serotype 055, which do not have mannose-binding capacity, readily attached to trophozoites of *E. histolytica* strains HK-9, HM1:IMSS, or NIH:200 without precoating with immune sera. The attachment was found to be dependent on the amount of bacteria added, as well as on time (1 h optimal time) (Fig. 1 and 2). In contrast to our previous observations with bacteria that had mannose-binding activity, very little attachment of *E. coli* 055 occurred at low temperature (5°C) or with trophozoites in which the formation of filopodia was blocked by cytochalasin B (9). Moreover, glutaraldehyde fixation of amoebae completely abolished their ability to attach *E. coli* 055. On the other hand, inactivation of the bacteria by glutaraldehyde, heat denaturation, or γ-irradiation had no noticeable effect on their attachment to the amoebae (Table 1). A number of carbohydrates were found that would inhibit the attach-

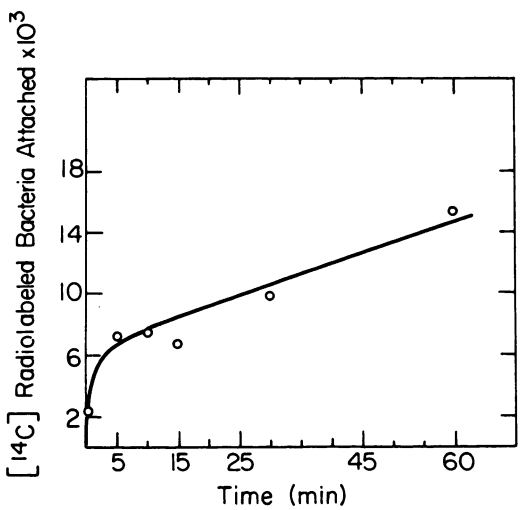

FIG. 2. Time-dependent attachment of *E. coli* 055 to trophozoites of *E. histolytica*. Amoebae (strain HM-1) (5 × 10^5 cells) were incubated with ^{14}C-labeled bacteria (specific activity, 6.4 cpm/10^4 bacteria), 5 × 10^8 bacteria per assay, in 0.5 ml of saline at 37°C for different periods of time. The reaction was stopped by dilution of tubes with saline (5 ml), and further separation of bacteria attached to amoebae was done as described in the text.

TABLE 1. Effects of pretreatment of bacteria and amoebae on attachment[a]

Pretreatment of amoebae	Pretreatment of bacteria	Incubation temp (°C)	Amt of bacteria attached (% of control)
None	None	37	100
None	Glutaraldehyde fixation	37	100
None	Heat (121°C; 15 min)	37	120
None	γ-irradiation (500 krad)	37	79
None	Periodate oxidation	37	57
Glutaraldehyde fixation	None	37	3.7
Cytochalasin B μg/ml	None	37	36
Cold (5°C)	None	5	57

[a] Amoebae (strain HM-1; 5×10^5 cells) were incubated with ^{14}C-labeled *E. coli* 055 (5×10^8) in saline (0.5 ml) for 30 min. For details on pretreatments and other experimental procedures, see the text.

ment of the bacteria (Table 2). The most prominent inhibitors were galactose, lactose, thiodigalactoside, and *N*-acetylgalactosamine, as well as asialofetuin, in which galactose is exposed at the terminal position. Fetuin itself had low inhibitory activity. Another efficient inhibitor of bacterial attachment was the lipopolysaccharide extracted from *E. coli* 055. No inhibition was observed with the lipopolysaccharide from *E. coli* 7343, a mannose-binding strain, which was used as control (Table 2). This was not surprising, as *E. coli* 7343 cells were not agglutinated by soybean agglutinin, indicating that they are most likely devoid of galactose or *N*-acetylgalactosamine residues on their lipopolysaccharide.

Supporting evidence that a carbohydrate component of the *E. coli* 055 lipopolysaccharide is the receptor for the amoeba lectin was obtained from the inhibition of attachment observed after mild periodate oxidation of the bacteria (Table 1).

E. coli 055 and *S. greenside* 050 are known to have common antigens (7). It was therefore interesting to see whether this *Salmonella* strain could also attach to the trophozoite. As shown in Table 3, the data obtained on attachment are very similar to those with *E. coli* 055.

In contrast to *E. histolytica* trophozoites, bacteria are usually not disrupted or solubilized to a significant extent by detergents such as Triton X-100 (0.2%). In the case of the bacteria that were metabolically labeled by growing them overnight with [^{14}C]glucose, the detergent treatment solubilized less than 5% of the radioactive components. Moreover, preincubation of the radiolabeled bacteria with cell-free extracts of amoebae did not render them more sensitive to solubilization by the detergent. On the other hand, and as previously observed (1, 13), a large proportion of the radiolabeled *E. coli* 055 that attached to the intact amoebae became very sensitive to detergent treatment. Approximately 50% of their radioactive content solubilized and upon centrifugation was found in the non-sedimentable fraction. This finding indicated that *E. coli* 055 became sensitive to the detergent due to ingestion and initial degradation by the amoebae.

The attachment of *E. coli* 055 to the trophozoites was not significantly affected by the simultaneous adherence of other types of bacteria, such as *E. coli* 7343, which bind to mannose residues on the amoeba surface (1) and vice versa (Table 4). The attachment of *E. coli* 055 was somewhat affected, however, by the addition of opsonized bacteria such as *Shigella* species, which most likely compete for the same carbohydrate-binding component on the surface of the amoeba (1). It was hard to demonstrate, however, a direct competition between opsonized and nonopsonized *E. coli* 055, since the antibody-coated 055 tend to bind and aggregate some of the nonopsonized 055 bacteria added. This may explain the enhancement in total adherence when nonlabeled opsonized *E. coli* 055 was added to labeled *E. coli* 055 (Table 4).

TABLE 2. Inhibitors of attachment of *E. coli* 055 to trophozoites of *E. histolytica*[a]

Substance added	Concn (mg/ml)	Amt of bacteria attached (% of control)
None		100
Galactose	10	20
N-Acetylgalactosamine	10	56
Lactose	10	55
α-Methylmannoside	10	100
(*N*-Acetylglucosamine)$_{3-4}$	1	90
Fetuin	1	92
Asialofetuin	1	70
Lipopolysaccharide		
E. coli 055	4	49
E. coli 7343	4	130

[a] Amoebae (strain HM-1; 5×10^5 cells) were incubated with ^{14}C-labeled *E. coli* 055 in saline (0.5 ml) for 30 min at 37°C. Compounds were added to incubation tubes to final concentrations as indicated.

TABLE 3. Properties of adherence of *S. greenside* serotype 050 to trophozoites of *E. histolytica*[a]

Substance added	Concn (mg/ml)	Amt of bacteria attached (% of control)
None		100
α-Methyl-mannoside	10	105
Galactose	10	38
Lactose	10	32
N-Acetylgalactosamine	10	28
Asialofetuin	1	55

[a] Amoebae (strain HM-1; 5×10^5 cells) were incubated with ^{14}C-labeled *S. greenside* 050 (5×10^8; 1 cpm = 2,770 bacteria) in saline at 37°C for 30 min. Under control conditions, the average number of *S. greenside* 050 cells that attached per each trophozoite was 30.3.

DISCUSSION

In a previous study, we found that *E. histolytica* trophozoites are very selective in their interaction with bacteria and attach them by two mechanisms. Since trophozoites of *E. histolytica* had been shown to contain receptors for concanavalin A (12) and many gram-negative bacteria are known to have mannose-binding proteins on their surface (4), it was not surprising to find that adherence of such bacteria to the amoebae proceeded via such a mutual recognition. A wide variety of bacterial strains, however, such as *S. flexneri* and *S. aureus*, which do not possess the mannose-specific cell surface lectin, did not interact with the amoebae. Precoating of such bacteria with specific antisera enabled their attachment and ingestion by the amoebae. This interaction, however, was not mediated by an Fc binding system but by an amoeba surface lectin which recognized carbohydrate moieties, such as galactose and *N*-acetylgalactosamine (1, 9, 10), that are present on the coating immunoglobulin molecules (5).

The finding that amoebae have a surface carbohydrate-binding protein was the basis for the present work. We searched, therefore, for bacteria which would have any of the sugar moieties recognized by the amoeba as a component of its outer membrane. Two such bacteria were *E. coli* serotype 055 and the serologically similar *S. greenside* 050. These bacteria displayed no mannose-binding activity toward *Saccharomyces cerevisiae* yeasts (6) and very poorly adhered to guinea pig intestinal cells (M. Izhar, Y. Nuchamowitz, and D. Mirelman, unpublished observations). On the other hand, they were agglutinated by soybean agglutinin, a lectin which has a specificity for galactose and *N*-acetylgalactosamine residues (3) and were very readily bound and ingested by the amoebae. The attachment of *E. coli* 055 as well as that of *S. greenside* 050 was significantly inhibited by galactose, lactose, and *N*-acetylgalactosamine. The lipopolysaccharide extracted from *E. coli* 055, which is known to contain *N*-acetylgalactosamine and galactose (7), was also an excellent inhibitor of the attachment of the bacteria. A control lipopolysaccharide preparation from another bacterium (*E. coli* 7343) that does not have such carbohydrates and which was not agglutinated by soybean agglutinin was ineffective as an inhibitor. Further evidence that the carbohydrate on the bacterial cell surface is the receptor for the amoebic lectin was obtained after mild periodate oxidation of *E. coli* 055 which markedly affected its attachment to the amoebae. Both mannose-binding bacteria (i.e., *E. coli* 7343) and *E. coli* 055 were attached and ingested simultaneously and without competition, and their sensitization to detergents was comparable (1, 13). Opsonized *S. flexneri* did, however, compete with the attachment of *E. coli*

TABLE 4. Effect of various types of bacteria on the attachment of labeled *E. coli* 055 to trophozoites of *E. histolytica*[a]

Labeled bacteria added (5×10^8)	Unlabeled bacteria added (10^9)	Amt of radioactive bacteria attached per amoeba	% Adherence of radiolabeled *E. coli* 055 (% attached)
^{14}C-labeled *E. coli* 055		50	100
	E. coli 055	33.8	68
	E. coli 7343	48	96
	Opsonized *E. coli* 055	68	136
	S. flexneri	45	89
	Opsonized *S. flexneri*	38	76
Opsonized ^{14}C-labeled *E. coli* 055		125	100
	Opsonized *E. coli* 055	85	68
	E. coli 055	114	91
	E. coli 7343	154	123
	S. flexneri	125	100
	Opsonized *S. flexneri*	98	79

[a] Amoebae (strain HM-1; 5×10^5 cells) were incubated with ^{14}C-labeled *E. coli* 055, either opsonized or nonopsonized (5×10^8 bacteria), in saline (0.5 ml) for 30 min at 37°C in the presence or absence of nonlabeled bacteria to test for competition. Opsonized cells were obtained by incubation of bacteria with their specific antisera followed by washing with saline in an Eppendorff centrifuge. Microscopic observation of opsonized bacteria revealed that they were in agglutinates.

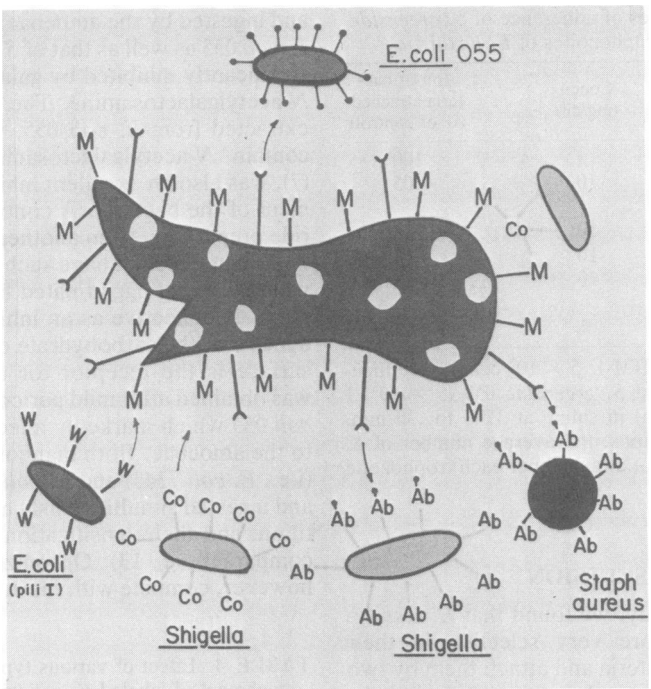

FIG. 3. Summary of the various possibilities of attachment of different bacteria to amoebic trophozoites. M, Mannose-containing receptors on the amoeba surface; —⊂, carbohydrate-binding (lectin) (3) activity; W, *E. coli* surface pili which have mannnose-binding properties; Co, concanavalin A bound to receptors on the bacteria; Ab, antibodies; and —•, *N*-acetylgalactosamine or galactose on bacterial surfaces or on opsonins.

055, and this lends further support to our contention that the amoebic lectin recognized the same carbohydrate moieties on the immunoglobulin molecule that coats the bacterial surface, as well as on the surface of *E. coli* 055 (5). The amount of opsonized versus nonopsonized *E. coli* 055 that attached to the amoebae was much higher, probably due to the aggregates formed upon interaction with immune sera.

Further evidence that the amoebae possess the carbohydrate-binding protein for attachment of both *E. coli* 055 and opsonized bacteria was obtained from the fact that bacterial adherence was abolished when trophozoites were glutaraldehyde fixed, treated with cytochalasin B, or kept at 5°C. These conditions did not affect the attachment of mannose-binding bacteria such as *E. coli* 7343, since the mannose residues on the surface of the amoebae, which serve as the inert receptors for the bacterial lectin, were not modified by the treatments (6).

In summary, our studies indicate that *E. histolytica*, which inhabits the colon region of the human intestine, has a variety of options by which to bind and ingest bacteria (Fig. 3). The ability of amoebae to interact with bacteria is apparently an important fact in pathogenesis, since preliminary experiments indicate that both *E. coli* 055 and *E. coli* 7343 cause a significant augmentation of virulence, especially in a nonvirulent strain (HK-9) of axenically grown *E. histolytica* (D. Mirelman, C. Feingold, A. Wexler, and R. Bracha, Ciba Found. Symp. Cytopathol. Parasitic Dis., in press). Investigations on the molecular contribution of the ingested bacteria to the amoebae and the mechanism of virulence augmentation are in progress.

ACKNOWLEDGMENTS

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