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Chlorine-Induced Damage to Surface Adhesins During Sublethal Injury of Enterotoxigenic *Escherichia coli*†

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A comparison of the adhesive ability of noninjured and chlorine-injured enterotoxigenic *Escherichia coli* was made by in vitro attachment to human peripheral leukocytes. Chlorination selected for noninjured cells with greater capabilities for colonizing the small intestine. Injured populations exhibited reduced association with leukocytes. Maximum reduction was seen in populations with greater than 80% injury. These cells demonstrated less adhesive ability than nonpiliated populations. Electron micrographs suggested that reduced adhesive ability was due to the loss of surface structures as a consequence of sublethal chlorination. The data imply a reduced ability among chlorine-injured pathogens to colonize the small intestine and initiate disease.

Proper assessment of the sanitary quality of water depends on the accurate detection and enumeration of fecal indicator organisms. Sanitary evaluations, however, may be complicated by the presence of sublethally injured indicator organisms. Injured organisms are characterized by the inability to form colonies on selective media, with concomitant capabilities for colony formation on nonselective media. Consequently, a portion of the viable population may escape detection by most methods prescribed for the bacteriological examination of water. The health risk associated with these undetected indicator organisms in aquatic environments is presently speculative, owing to the lack of information concerning the status of the pathogen population. Pathogenicity of injured foodborne pathogens has been investigated (8, 18, 33, 35). Application of these findings to aquatic systems, however, may not be valid owing to the comparative differences in nutrient availability provided by the two environments.

Enteric pathogens, which cause disease by enterotoxigenesis, must colonize and proliferate in the small intestine for disease to occur (23, 25). Colonization is accomplished through surface adhesins which allow bacteria to attach to various human and animal tissues (9, 27). Among gram-negative bacteria, this function is carried out by proteinaceous thread-like structures called pili or fimbriae which project outward from the surface of the cell in close proximity to the environment (5, 17).

Adherence as a prerequisite to pathogenesis is well-documented for a number of infections (13, 20, 28, 29), including gastroenteritis due to enterotoxigenic *Escherichia coli* (ETEC) (23). Reportedly, ETEC accounts for a significant portion of diarrhea cases previously undifferentiated with respect to infectious agent and for at least 30% of all cases of diarrhea in travelers to tropical and subtropical countries (12, 19). Additionally, this organism has been associated with at least two major outbreaks of waterborne disease in North America since 1975, the most notable of which occurred at Crater Lake National Park in Oregon (30).

The relationship between adhesion and disease due to ETEC is supported by in vivo studies. ETEC lacking adhesins failed to colonize the small intestine and did not cause disease in human volunteers (15). In this paper, we describe the effect of chlorine injury on the ability of ETEC to initiate disease by assessing colonizing ability with an assay system of in vitro attachment to human peripheral leukocytes.

MATERIALS AND METHODS

Test organism. ETEC H10407 (O78:H11), a Bangladesh isolate, which produces heat-stable and heat-labile enterotoxins and type 1 pili, was donated by C. C. Brinton, Jr., University of Pittsburgh, Pittsburgh, Pa. Stock cultures were grown under conditions which stimulate (P^+) or depress (P^-) type 1 pili (10, 11). P^+ cultures were obtained by repeated subculture at 48-h intervals in Trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, Md.) at 37°C without agitation. The P^+ characteristic was verified by mannose-sensitive hemagglutination of 2.5% guinea pig erythrocytes and by colony morphology (4). P^- cultures were obtained by subculture on

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Trypticase soy agar supplemented with 0.5% glucose and 0.3% yeast extract (TGY) at 30°C until no hemagglutination was noted. The final subculture of each was stored at 5°C for use as stock cultures.

Preparation of cells for exposure. Before each assay, 0.1 ml of P^+ or four isolated colonies of P^- culture were inoculated into 100 ml of TSB and incubated at 37°C and 120 rpm for 18 h. For addition to a test system, 36-ml portions were removed, centrifuged at 3,020 × *g* for 10 min, washed once in 0.1% peptone (1), and suspended in 20 ml KH_2PO_4 buffer (1) at pH 6.7.

Chlorination procedure. Prepared inoculum was added to each of three flasks containing 480 ml of KH_2PO_4 buffer to provide approximately 1.5×10^8 cells per ml and stirred at a constant slow speed. A 200-ml chlorine stock solution, freshly prepared by volumetric addition of 1.0 or 1.5 ml of 5.25% sodium hypochlorite (commercial Clorox) to buffer, was added to one flask to provide a final chlorine concentration of 0.5 or 0.75 mg/liter. Exposure time was varied from 15 s to 6 min, and treatment was then terminated by the addition of 10 ml of 20% sodium thiosulfate (1). Cells in the second flask received no chlorine treatment but were exposed to buffer only for comparable times.

Chlorine-killed cells were prepared in the third flask by 2 h of exposure to 1 ml of 5.25% sodium hypochlorite followed by neutralization. Nonviability was verified by the absence of turbidity after incubation of 0.5 ml of treated cells in 10 ml of TSB for 24 h at 35°C. The optical density of the killed cell suspension was compared spectrophotometrically at 625 nm with dilute buffer-exposed cells. Buffer-exposed suspensions with the appropriate absorbance were enumerated on TGY as an estimate of the killed population.

Measurement of chlorine. Free and total chlorine were measured by the *N,N*,-diethyl-*p*-phenyl-enediamine colorimetric method (Hach Chemical Co., Ames, Ia). Measurements were made for both chlorine stock solutions and at maximum exposure time in the injury flask for each chlorine concentration to ensure the presence of free chlorine under the conditions of the system.

Assessment of injury. Survivors of the buffer- and chlorine-exposed systems were enumerated by the spread plate technique. Injury was assessed by comparison of growth on TGY and m-Fecal Coliform (m-FC) agar (BBL Microbiology Systems, Cockeysville, Md.) after 18 to 24 h of incubation at 35°C and expressed as a percentage of the total surviving population, using the following equation: % injury = [(CFU on TGY − CFU on m-FC agar)/CFU on TGY] × 100.

Preparation of bacterial cells for attachment assay. Injured, chlorine-killed, and buffer-exposed cells were removed, centrifuged at 3,020 × *g*, and suspended in Hanks balanced salt solution (HBSS) (GIBCO; Grand Island, N.Y.) with or without 50 mM α-methyl-D-mannoside (aMM) at twice the original volume. Cell suspensions containing 100, 90, 75, 50, 14, 4, and 0% viable cells were prepared by diluting P^+ buffer-exposed cells with various volumes of P^+ chlorine-killed suspensions.

Preparation of leukocytes. Blood was collected from a single donor in 10-ml heparinized (143 U/10-ml tube) Vacutainer tubes (Becton, Dickinson & Co., Rutherford, N.J.). Leukocytes were separated by dextran sedimentation (3). Equal volumes of blood and 4.5% dextran in 0.9% saline were mixed and then allowed to stand at room temperature for 30 min. The upper third of the resulting leukocyte-rich plasma layer was removed, centrifuged at 250 × *g* for 10 min, and washed in HBSS with 0.4% bovine serum albumin at 4°C. Residual erythrocytes were removed by hypotonic lysis (7) with 0.2% NaCl for 30 s. Isotonicity was restored by addition of an equal volume of 1.6% NaCl. The cells were rewashed and resuspended in 0.4% bovine serum albumin–HBSS. A sample of leukocyte suspension was diluted in 1% glacial acetic acid (36) and counted in a hemacytometer. The suspension was adjusted to 10^6/ml with 0.4% bovine serum albumin–HBSS and held at 4°C until used (usually within 60 min). Suspensions typically contained 70 to 75% neutrophils.

ETEC-leukocyte association. Leukocytes were attached to cover slips by the method described by Swanson et al. (34). One drop (0.05 ml) of leukocyte suspension was pipetted onto alcohol-cleaned cover slips (22 by 22 mm) and incubated in a moist chamber at 37°C for 30 min. Nonadherent leukocytes were removed by dipping the cover slips in cold HBSS. Triplicate cover slips were placed in plastic petri dishes and overlaid with 0.5 ml of the bacterial suspension to be tested. The dishes were incubated at 37°C and 60 rpm for 20 min. Nonadhering bacteria were removed by dipping the cover slips in cold HBSS. The cover slips were rinsed with water, air dried, and mounted on a glass slide with a drop of Permount (Fisher Scientific Co., Pittsburgh, Pa.). Cells were examined under oil immersion (× 1,000) and scored positive if two or more bacteria were seen within the cytoplasm or attached to the cytoplasmic periphery. A minimum of 200 leukocytes were counted for each cover slip, and the results were expressed as a percentage of total leukocytes with associated bacteria.

Electron microscopy. Buffer- and chlorine-exposed cells were fixed in situ with 1% gluteraldehyde for 20 min at 4°C. Cells were centrifuged onto cover slips at 2,000 × *g*, treated with OsO_4, alcohol dehydrated, and subjected to critical point drying. Replicas were prepared by shadowing with platinum-carbon at 30°, followed by 90° with spectral grade carbon. Replicas were scored, floated off the cover slips, and recovered on uncoated grids. The replicas were examined by transmission electron microscopy at 100 kV with an RCA EMU 3G electron microscope.

RESULTS

Chlorine-induced injury of P^+ cells. P^+ cultures showed two distinct colony morphologies when grown on TGY. Both colony types demonstrated mannose-sensitive hemagglutinating activity in screening procedures. Colony type A, a small white colony, consistent with descriptions of P^+ colonies reported by Brinton (4), produced a thick pellicle in TSB within 24 h. Colony type B, a larger grey colony similar to those seen in P^- cultures, produced a pellicle only after two subcultures in TSB. The difference in colony morphology was attributed to different degrees of piliation within the P^+ population. The P^+ test culture typically contained a greater percentage of the less heavily piliated type B cells.

TABLE 1. Survival of colony type A after chlorination

% Viable[a]	% Injury[b]	% Type A survival[c]
100[d]	6.2	30.9
79.0	54.0	34.5
67.4	21.5	43.4
57.9	18.0	43.0
41.1	29.0	42.2
2.8	84.7[e]	40.5

[a] Calculated from CFU on TGY before and after exposure to chlorine.

[b] Percent of remaining population sublethally injured as demonstrated by failure to form colonies on m-FC agar.

[c] Percent of total population (TGY culture) exhibiting colony morphology consistent with piliated cells and determined representative of the more heavily piliated portion of the population by pellicle-producing capabilities.

[d] Exposed to buffer only.

[e] Colonies growing on m-FC agar tested for pellicle producing capabilities in TSB. 65.0% noninjured cells consistent with type A colonies.

Exposure to 0.5 or 0.75 mg of chlorine per liter caused sublethal injury to a portion of the exposed population, as demonstrated by the failure of injured organisms to form colonies on m-FC agar after incubation at 35°C. Observations of colony morphology before and after exposure to chlorine (Table 1) showed significantly better survival of colony type A in comparison with buffer-exposed cells ($P < 0.01$ as determined by the linear regression F-test). The percentage of colony type A cells increased from 30.9 to 43% of the total population as killing approached 50%, then decreased slightly as viability continued to decrease. Under conditions of severe injury (84.7%), colonies growing on m-FC agar were examined for pellicle-producing capabilities by inoculating into TSB. Within 24 h, 65% of the noninjured population produced the thick pellicle, consistent with type A pellicle production, compared to 40.5% of the total population. These results suggest greater resistance to injury among the more heavily piliated cells.

ETEC-leukocyte association. In preliminary experiments, we determined that chlorine-killed cells possessed some adhesive ability independent of pili. A similar phenomenon was seen among P^- populations. For this reason, we constructed a curve which took into consideration percent viability and association of unlysed dead cells. This curve, hereafter referred to as the control, was prepared by diluting volumes from the buffer-exposed system with volumes from the chlorine-killed system (see above).

As expected, the percent attachment of control cells decreased with decreasing viability (Table 2). The addition of 50 mM aMM reduced attachment of the control population but in no instance was completely inhibitory. Chlorine-killed cells demonstrated no hemagglutinating activity but exhibited a 31.4% leukocyte association which was irreversible by aMM. Similar type 1 pili-independent associations were observed among P^- cells and 100% viability controls. These findings are consistent with mannose-resistant adhesion in chlorine-injured populations with less than 2.5% viable cells. Attachment increased as viability approached 0% and became less sensitive to reversal by mannose (Table 3). These data support the concept that there is an outer membrane component capable of interacting in this assay and justify the inclusion of chlorine-killed cells in the control population.

Chlorine-injured populations associated with leukocytes to a lesser extent than did control cells (Table 4). Maximum reduction was seen in populations with greater than 80% injury. These cells demonstrated a lower percentage of leukocyte association than did P^- cells and may indicate damage at the cell surface sufficient to additionally reduce outer membrane-associated adherence. Leukocyte associations of control and chlorine-injured populations are compared graphically in Fig. 1. The adherence characteristics of both populations differ significantly ($P < 0.03$ as determined by the linear regression F-

TABLE 2. ETEC-leukocyte interactions of control populations

% Viable cells[a]	% ETEC-leukocyte association[b]		
	Without aMM	With aMM	% aMM resistant[c]
P^+			
100	91.1	35.3	38.7
90	87.5	27.9	31.9
75	79.4	30.8	38.8
50	70.4	39.8	56.5
14	60.5	6.8	11.2
4	40.4	6.5	16.1
0	31.4[d]	36.6	116.6
P^-			
(100)	31.5		

[a] Calculated from volume of buffer-exposed and killed cell suspensions used in the mixture (represents the percentage of viable cells in the total cell population).

[b] Percentage of leukocytes with two or more associated bacteria.

[c] Calculated as percent difference in association in the presence and absence of aMM.

[d] Hemagglutination-negative cells (association not due to pili).

TABLE 3. Mannose inhibition of ETEC-leukocyte interactions of chlorine-injured populations

% Viable cells[a]	% Injury[b]	% ETEC-leukocyte association[c]		
		Without aMM	With aMM	% aMM resistant[d]
2.5	87.0	14.3	0.1	0.2
1.6	88.0	15.8	3.3	20.8
0.7	81.0	22.7	10.9	48.0
0.2	80.6	20.9	16.1	77.0

[a] Calculated from CFU on TGY before and after exposure to chlorine. Results of four of six experiments used in the calculation of 1.1% viable population of Table 4.

[b] Percentage of remaining viable cells sublethally injured as demonstrated by failure to grow on m-FC agar.

[c] Percentage of leukocytes with two or more associated bacteria.

[d] Calculated as percent difference in association in the presence and absence of aMM.

test); however, evidence does not support the hypothesis that injury alone is responsible for the reduction. Electron micrographs of P^+ populations suggest that the reduced attachment of chlorine-exposed cells is due to the loss of surface structures as a consequence of sublethal chlorination (Fig. 2).

DISCUSSION

The cellular sites of chlorine injury, for the most part, have been investigated by using indicator populations (6, 22, 32). In assessing the public health significance of undetected indicator organisms in the environment, however, consideration must be given to the possibility that a portion of the pathogen population will also be injured and that virulence may be affected as a consequence of injury. The experiments reported in this paper describe the effect of chlorine injury on the attachment of ETEC by type 1 pili. Although there is little evidence to implicate type 1 pili as a definitive virulence factor for this organism, the appearance of these structures both alone (26) and in conjunction with other adhesins (2, 14) on strains isolated from cases of acute diarrhea suggests that they have some role in colonization of the small intestine. Additionally, on the basis of the common proteinaceous nature, stability (21, 24, 31), and physical location of these structures, we believe that our findings can be extrapolated to include other surface structures which may have a more important role in the pathogenesis of ETEC (16).

Our results show that survival and injury of ETEC was influenced by the degree of piliation, suggesting that chlorination selects for noninjured pathogens with the capability for colonizing the small intestine. This may increase the health risk under conditions in which the sanitary evaluation is in error. The mechanism for selection is not known, but heavily piliated cells may derive advantage by limiting access of HOCl to the cell surface.

The inability to completely inhibit leukocyte attachment of the 100% viability control with aMM suggests the presence of a second adhesin. We believe this adhesin to be an outer membrane component capable of interacting in the absence of pili. Since bacterial populations are subject to phase variation, which determines whether pili are synthesized by a particular bacterium (5, 11), not every organism in a P^+ culture will necessarily be piliated. A small percentage of P^- cells in the P^+ population may account for the observed mannose-resistant adherence. Support for an outer membrane-associated adhesin comes from our results with P^- and chlorine-killed populations. Both cell types lacked hemagglutinating activity and therefore

TABLE 4. ETEC-leukocyte interactions of chlorine-injured populations

% Viable cells[a]	% Injury[b]	% ETEC-leukocyte association[c]	% Control[d]
57.1	16.9	67.6	91.4
45.4	34.2	65.0	92.9
24.7	56.0	44.5	69.5
1.1	80.6	14.3	40.9

[a] Calculated from CFU on TGY before and after exposure to chlorine.

[b] Percentage of remaining viable cells sublethally injured as demonstrated by failure to grow on m-FC agar.

[c] Percentage of leukocytes with two or more associated bacteria.

[d] Percent association of injured cells compared with the appropriate control.

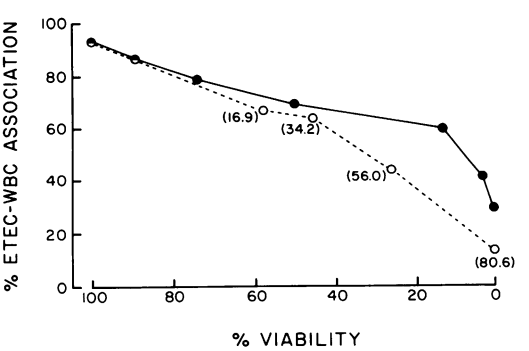

FIG. 1. Comparison of ETEC-leukocyte interactions of control (●) and chlorine-exposed (○) populations. Parenthetical values indicate percent injury.

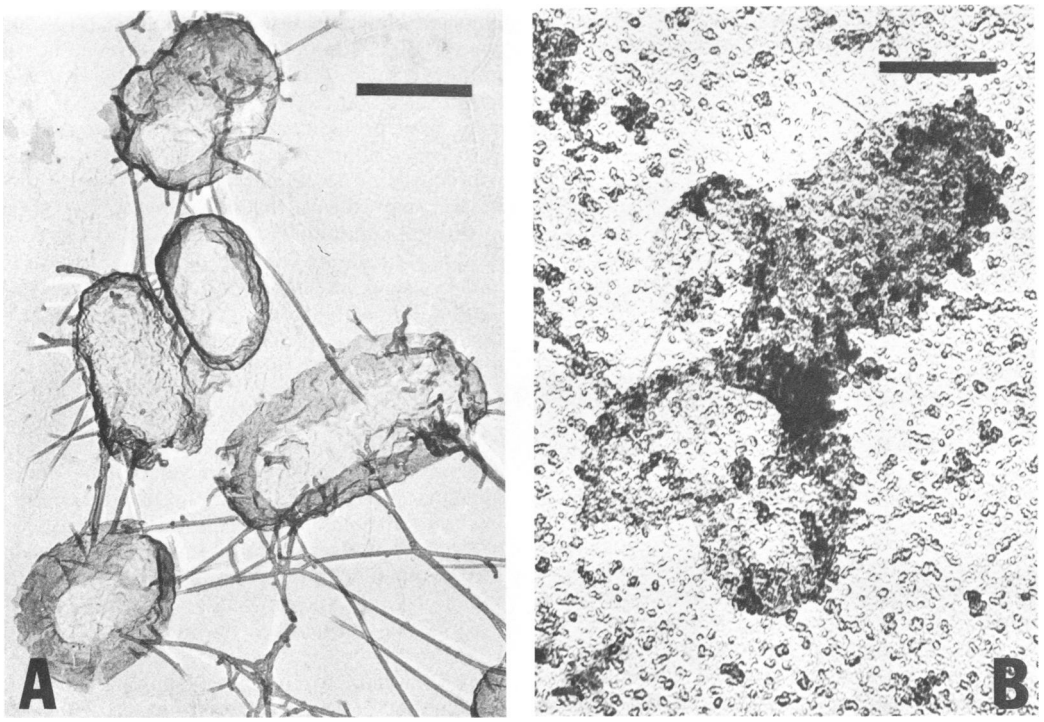

FIG. 2. Electron micrograph replicas of platinum-shadowed buffer-exposed ETEC (A) showing pili and flagella, compared with chlorine-exposed cells (B) lacking surface structures. Bar, 0.5 μm.

pili but exhibited similar adhesive ability. The increasing adherence of injured populations with less than 2.5% viable cells, in conjunction with decreasing mannose sensitivity, suggests that adhesive sites are made available as pili are removed and lends further support to the hypothesis of an outer membrane adhesin. Since organisms lacking surface structures do not cause disease (15), it is unlikely that this adhesin has a significant role in pathogenesis, but it may be an important consideration in this assay system. Chlorine-exposed populations consistently demonstrated reduced association with leukocytes. Greater reduction was obtained as the amount of injury increased. Exact correlation between injury and reduction was not expected, owing to the scoring requirements which dictated two or more associated bacteria per leukocyte for a positive score. This, together with the adherence characteristics associated with the outer membrane, may account for the inability to statistically relate percent injury to the observed reduction. It is clear, however, that ETEC undergoes a functional loss in its adhesive ability as a consequence of sublethal chlorination. Our results with 80% injured populations showed a lower leukocyte association than that found for P^- and killed cells, suggesting that surface damage is of such a magnitude to affect the outer membrane-associated adhesin. The comparatively higher adherence of the killed cells may be related to the selective nature of chlorination. The unlysed killed cells in this population are likely derived from the more heavily piliated cells, whereas at lower chlorine exposure, heavily piliated cells make up the majority of the noninjured population.

Damage to the outer envelope of indicator organisms during chlorine injury has previously been reported (6). Our electron micrographs of ETEC show that adhesins are among the sites of surface damage. The results presented in this paper imply a reduced ability among chlorine-injured ETEC to colonize the small intestine and initiate disease. Chlorine-injured pathogens which rely on surface structures to establish themselves in a host may therefore be less virulent than their noninjured counterparts.

In light of the increasing attention being given to enrichment and resuscitation methods to improve detection of environmentally stressed microbes, studies of the type reported in this paper are essential. Enrichment techniques usually require more time and expense. If chlorine-injured pathogens retain their virulence, these efforts would undoubtedly be justified. Our results suggest that virulence factors may be among the targets of sublethal injury; however,

selection of heavily piliated cells during chlorination may also be important under conditions in which the sanitary evaluation is in error. The effect of aquatic stress on the pathogen population should therefore be considered in assessing the public health significance of injured indicator organisms in the environment. Studies of other virulence factors, however, will be necessary to evaluate the significance of our findings with respect to the use of improved recovery methods for indicator organisms in chlorinated waters.

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