Assessment of In Vivo Revival, Growth, and Pathogenicity of *Escherichia coli* Strains after Copper- and Chlorine-Induced Injury

AJAIB SINGH, R. YEAGER, AND G. A. MCFETERS*

Department of Microbiology, Montana State University, Bozeman, Montana 59717

Received 24 April 1986/Accepted 27 June 1986

Cells of one enteroinvasive and three enterotoxigenic strains of *Escherichia coli* were exposed to sublethal concentrations of copper and chlorine to produce 85 to 94% injury. Injured cells were intraluminally inoculated into ligated ileal loops of anesthetized mice, and injury was assessed at timed intervals. Substantial recovery (72–84%) of copper- and chlorine-injured cells was observed in the inoculated loops at 4 and 3 h, respectively. No appreciable increase in total numbers was observed during these time intervals. In vitro revival of copper-injured cells in phosphate-buffered saline alone after incubation at 35°C for 4 h was not observed. However, a 60 to 70% revival occurred when 200 μg of protein per ml of mouse intestinal mucosal homogenate was incorporated into saline cell suspensions. The enterotoxigenic activity of copper-injured cells in rabbit ileal loops was somewhat reduced compared with that of chlorine-injured or uninjured cells. These results show that injured pathogenic *E. coli* cells can revive in the small intestine and appear to retain their enterotoxigenic activity.

*Escherichia coli* is considered a normal inhabitant of vertebrate intestinal tracts and indicates potential fecal contamination when present in foods and water. However, several strains have been implicated in diarrheal disease in humans and domestic animals (28, 33). Such organisms harbor plasmids that are responsible for encoding enterotoxigenic (8, 32), enteroinvasive (9), enterohemorrhagic (37), and enteropathogenic (14) traits in the respective strains. Enterotoxigenic *E. coli* (ETEC) strains are the most commonly reported organisms in diarrheal outbreaks and are the leading cause of infant death in underdeveloped countries, as well as a significant cause of morbidity in adults (28). Foods (25, 29) and water (11, 15, 27) have been implicated as the major vehicles of transmission of these organisms. Disinfectants applied to drinking water cause a portion of the microbial population to become injured owing to sublethal lesions (2). As a result, they fail to grow in the presence of selective agents commonly incorporated in isolation media (6, 12, 19, 20). Injury may also result from environmental stress factors (1, 38) or from the presence of trace amounts of metals (5, 16) in water. The injury process can lead to a significant underestimation of indicator organisms, since more than 90% of the viable population may become injured in treated drinking water (21). Such an occurrence can result in serious potential health risks when waterborne pathogens are also present. However, pathogens are likewise injured when exposed to stress factors such as sublethal doses of chlorine and copper (13, 30), heat (3), and the process of freeze-drying (7). Sublethally injured *Staphylococcus aureus* (7) and *E. coli* strains (31), as well as "viable but not recoverable" ETEC (23) cells, have been shown to regain their pathogenic traits when transferred to suitable in vitro environments. However, there appeared to be no information on the in vivo repair process of pathogenic *E. coli* cells after copper- and chlorine-induced injury. Thus, the objective of this investigation was to examine the recovery, growth, and pathogenicity of copper- and chlorine-injured *E. coli* after inoculations to animal models.

MATERIALS AND METHODS

*Strains of E. coli.* Strain H10407 (O78:H11), a human isolate that produced both heat-stable (ST) and heat-labile enterotoxins, and strain E7 (O111), an invasive strain that did not produce either toxin, were obtained from R. A. Wilson, *E. coli* Reference Center, Pennsylvania State University, University Park, Pa. An ST-producing strain, TX432 (O78:H12), associated with an acute diarrheal outbreak in children, was provided by D. J. Evans, University of Texas Medical School, Houston, Tex. Strain E6 (O9:K35:K99), which produced only ST, was isolated from a calf with diarrhea and was obtained from L. L. Myers, Veterinary Science Department, Montana State University, Bozeman, Mont. Stock cultures were stored at −70°C in 1% peptone water containing 40% glycerol.

**Injury procedures.** In our study, the test organisms were injured under conditions similar to those described for coliforms injured by copper and chlorine in treated distribution water (5, 12, 20). However, to obtain comparable and reproducible levels of injury in the cell suspensions, we used more uniform and controlled conditions (temperature, pH, cell density, etc.). Injury by copper was induced in different strains by the method described previously (31). Washed cells (ca. 5 × 10⁶ cells per ml) were suspended in 1.68 × 10⁻³ M inorganic carbon buffer (5), pH 7.0, and were exposed to different concentrations of copper at 4°C. The concentration that caused maximal injury with minimal cell death in the exposed population after 3 days was chosen to induce injury in subsequent experiments.

To induce injury by chlorine, we grew cultures in tryptic soy broth without glucose (Difco Laboratories, Detroit, Mich.) but supplemented with 1% lactose and 0.3% yeast extract (TLY broth) and was incubated at 35°C for 24 h. The cells were harvested by centrifugation at 6,000 × g for 10 min at 4°C, washed once with sterile reagent-grade water (Milli Q Water System; Millipore Corp., Bedford, Mass.), and suspended in sterile water to a cell density of approximately 5 × 10⁶ cells per ml. One milliliter of chlorine solution (10 times the final concentration desired) was added slowly to 9 ml of cell suspension which was constantly stirred to achieve the desired concentration, while being held...
in an ice bath. The exposed cell suspension was held for 10 min at 4°C, and the chloride was then neutralized by the addition of sodium thiosulfate (final concentration, 0.01%) before determination of the extent of injury.

Assessment of injury and lethality. Injury in copper- and chloride-exposed cells was assessed by determining the difference between the number of CFU per milliliter on nonselective agar, TLY broth containing 1.5% agar (TLY), and selective TLY agar supplemented with 0.1% sodium deoxycholate (TLYD) and was expressed as a percentage; for strain TX432, 0.075% sodium deoxycholate was used, since this organism was sensitive to higher concentrations. Lethality was determined from the viable counts obtained with TLY agar before and after exposure of the cells to copper and chloride and was expressed as the percentage of cells that were killed.

Revival of injured cells after intraluminal inoculation. Sixto eight-week-old female CD-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were used. Food was withheld for 24 h prior to inoculation, and animals were anesthetized by intraperitoneal administration of sodium pentobarbital (0.06 mg/g of body weight). The abdomen was opened by midline incision, and the small intestine was externalized. One milliliter of sterile phosphate buffer (PBS), pH 7.2, was injected into the lumen of the small intestine and was gently flushed into the posterior end, and a clamp was applied to prevent backward flow of the contents. The proximal portion of the small intestine was ligated to make three test and one control (5 to 7 cm) loops. Ligated loops were then suspended in 0.2 ml of PBS and were incubated for 0, 2, and 4 h. Sterile saline (0.2 ml) was used for inoculating the control segment. The abdominal opening was clamped immediately after each inoculation, and additional doses of anesthesia were administered whenever necessary. A minimum of three animals were used for each experiment, and the anatomical positions of the control and test segments used for inoculation were varied in each animal.

Immediately after the last inoculation, the animal was killed and the intestinal loops were carefully removed and placed in 100 ml of sterile PBS at 4°C. The intestinal tissue was then homogenized for 1 min in a laboratory homogenizer (VirTis Hi-Speed model 45; The VirTis Co., Inc., Gardiner, N.Y.). The homogenate was surface plated on TLY and TLYD agar after appropriate dilutions, and the percentage of injured cells was determined. Recovery of the inoculated cells varied from 60 to 80%. Colonies of strain H10407 from TLYD agar plates were confirmed by the slide agglutination test with a homologous antiserum (1:10). An increase in CFU on TLYD agar without a substantial increase in the log CFU of <1 on TLY agar represented the revival of injured cells. This increase was considered to be due to the repair of the lesion which in turn led to increased tolerance of sodium deoxycholate.

Survival and sodium deoxycholate tolerance of copper-injured cells in vitro systems. The copper-injured cells (8 × 10⁶ to 9 × 10⁶ cells per ml) were suspended in PBS alone or in PBS containing 200 µg of intestinal mucosal homogenate protein and incubated at 35°C. Samples were withdrawn at timed intervals and surface plated to assess the cell growth (increase in TLY CFU count) and percent sodium deoxycholate tolerance [(TLYD CFU count/TLY CFU count × 100)].

The mucosal homogenate was prepared from the small intestines of 6- to 8-week-old CD-1 mice. Each animal was sacrificed by cervical dislocation, and the small intestine was excised and placed in a sterile petri dish containing PBS at 4°C. The lumen was flushed with 2 ml of PBS and incised longitudinally. Any feces and partially digested food were removed by using a sterile cotton swab, and the split sections were transferred to another petri dish. The sections were then gently scraped with a glass slide to remove the layer of mucosal gel covering the intestinal lumen. Mucosal contents from four to five mice were pooled and homogenized for 3 min in a laboratory homogenizer (VirTis Hi-Speed model 45) and then centrifuged for 15 min at 500 × g at 4°C. The supernatant was incubated with gentamicin (5 mg/ml) for 3 h at 35°C and centrifuged at 10,000 × g for 20 min. The pellet was washed once and dissolved in 5 ml of PBS. Protein content was determined by the method of Markwell et al. (17), with crystalline bovine serum as a standard. The sterility of the preparation was checked by transferring 0.1 ml of the preparation to 10 ml of brain heart infusion, which was incubated at 35°C for 4 h and then surface plated on TLY agar.

Ileal loop inoculation test. New Zealand White rabbits, each weighing 1.0 to 1.5 kg, were fasted for 24 h before the test and anesthetized by intramuscular administration of ketamine and xylazine (40 and 10 mg/kg of body weight, respectively). The test was performed by the method of De and Chatterje (4) with minor modifications. After externalizing the small intestine, 10 to 15 ml of PBS was placed intraluminally at the proximal end and was flushed back into the large intestine by gentle manipulation. The small intestine was then ligated into six test segments (ca. 8 cm each) separated by approximately 4-cm segments. Various dilutions of the test cell suspension in PBS (1.0 ml) were inoculated into the test segments. Sterile PBS (1.0 ml) was inoculated into the control segment. The abdomen was closed, and the animal was allowed to recover from anesthesia. The rabbits were sacrificed after 18 h by being given overdoses of sodium pentobarbital. The volume of fluid accumulated (milliliters) per centimeter of each loop was determined.

Production of antiserum. Two New Zealand White rabbits, each weighing 2.5 to 3.0 kg, were used for immunization. Strain H10407 cells suspended in PBS (10⁶ cells per ml) were placed in a water bath at 80°C for 1 h and used as a source of antigen. An initial dose of 0.1 ml was administered intravenously and repeated two to three times a week. The dose
was increased to 1.5 ml over a period of 5 weeks. The animals were bled on day 6 after the final injection, and the serum was separated and stored at -20°C after determination of the titer by the slide agglutination method.

RESULTS

Injury induced by copper and chlorine. The effects of copper and chlorine on E. coli H10407, E6, TX432, and E7 are shown in Fig. 1 and 2. We observed that all the strains tested were not injured to the same extent by a single concentration of either copper or chlorine. Relatively low concentrations of copper (0.6 mg/liter) and chlorine (0.4 mg/liter) were required to induce injury in strain TX432. However, higher copper and chlorine concentrations, of 1.0 and 1.6 mg/liter, respectively, were required to cause appreciable injury (84.8 and 94.3%, respectively) in E7 cells. Lethality caused by these concentrations of copper and chlorine varied from 63.4 to 82.9% and 48.2 to 85.0%, respectively.

Growth of uninjured and copper-injured H10407 cells in the mouse intestinal lumen. Growth was monitored by determining colony formation on TLY and TLYD after appropriate in vivo incubation of the inoculated cells (Fig. 3). After intraluminal inoculation, the uninjured cells showed an increase in number of CFU on both TLY and TLYD after 2 and 4 h of incubation. In contrast, the copper-injured cells did not show any significant change in TLY CFU count after 2 h, but TLYD log CFU per loop increased from 5.99 to 6.38, indicating recovery of the injured population. After 4 h, there was a further increase in the TLYD CFU count, although some increase in TLY CFU count was also observed. This indicated that a portion of the injured cell population recovered in the absence of cell multiplication during the initial 2-h period of incubation, while repair and multiplication occurred simultaneously during the period from 2 to 4 h.

Control segments inoculated with sterile PBS showed substantially lower counts (log CFU per loop = 3.1 ± 2.9, n = 14) on TLY plates. Since higher dilutions were used to enumerate the inoculated cells in the loops, the resident intestinal flora did not interfere with the count.

Survival and sodium deoxycholate tolerance of copper-injured cells in in vitro systems. Results shown in Fig. 4 indicate a slight decline in numbers of copper-injured H10407 and E7 cells when they were placed in PBS at 35°C for 4 h. However, with the addition of intestinal mucosal homogenate, there appeared to be no significant change.

Immediately after inoculation, only 5 to 12% of the injured cells were able to tolerate 0.1% sodium deoxycholate (Fig. 4) in the plating medium (88 to 95% injury). The percentage of such cells increased substantially with incubation time in the presence of intestinal mucosal homogenate but not in its absence. These data show that the cells of both strains recovered substantially within 4 h and started to grow in the presence of small amounts of intestinal mucosal homogenate.

FIG. 2. Percent injury (■) and percent lethality (□) in E. coli strains after exposure to different concentrations of chlorine for 10 min at 4°C. Error bars represent the standard deviations of the mean values of four trials.

FIG. 3. Comparison of growth of uninjured (■■■) and copper-injured (——) H10407 cells after intraluminal inoculation in mice. Symbols: ○, CFU on TLY; ●, CFU on TLYD.

FIG. 4. In vitro survival (log CFU/ml) and sodium deoxycholate tolerance [(CFU on TLYD/CFU on TLY) × 100] of copper-injured H10407 and E7 cells after incubation in PBS alone and in PBS containing 200 μg of mouse intestinal mucosal homogenate protein per ml incubated at 35°C. Symbols: ●, H10407 cells in PBS; ○, H10407 cells in PBS plus intestinal mucosal homogenate; ▲, E7 cells in PBS; ◇, E7 cells in PBS plus intestinal mucosal homogenate.
TABLE 1. Revival of copper- and chlorine-injured E. coli strains in ligated intestinal loops of mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Copper-injured cells</th>
<th>Chlorine-injured cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of cells with injury (mean ± SD) (n)* at:</td>
<td>Increase in cell no. a,b at:</td>
</tr>
<tr>
<td></td>
<td>0 h</td>
<td>4 h</td>
</tr>
<tr>
<td>H10407</td>
<td>82.5 ± 5.6 (6)</td>
<td>21.7 ± 3.6 (6)</td>
</tr>
<tr>
<td>TX432c</td>
<td>86.5 ± 4.8 (4)</td>
<td>17.5 ± 2.9 (4)</td>
</tr>
<tr>
<td>E6</td>
<td>91.5 ± 5.2 (4)</td>
<td>21.6 ± 9.6 (4)</td>
</tr>
<tr>
<td>E7</td>
<td>77.7 ± 7.0 (4)</td>
<td>26.5 ± 8.5 (4)</td>
</tr>
</tbody>
</table>

a Mean ± standard deviation of the mean (n is the number of replicates).
b CFU at 4 h/CFU at 0 h on TLY.
c TLYD containing 0.075% sodium deoxycholate was used to assess injury.

Revival of copper- and chlorine-induced injury of E. coli strains in ligated intestinal loops of mice. Results of preliminary experiments showed that the growth of chlorine-injured cells after 4 h of intraluminal incubation was substantially greater than that of copper-injured cells (data not shown) and suggest a faster revival and multiplication of these cells. Therefore, recovery of chlorine-injured cells was determined after 3 h instead of 4 h as with copper-injured cells. The results of revival of copper- and chlorine-injured cells in the gut of mice are shown in Table 1. Although there was substantial revival, none of the copper- or chlorine-injured strains showed a complete recovery to sodium deoxycholate tolerance in 4 h. After appropriate in vivo incubation, 17.5 to 26.5% of copper-injured cells and 20.3 to 27.6% of chlorine-injured cells were still unable to grow on TLY containing 0.1% sodium deoxycholate. However, revival appeared to be substantial before commencement of active multiplication.

Enterotoxigenic activity of copper- and chlorine-injured H10407 cells in rabbit ligated ileal loops. The rabbit ileal loop model was used to determine the effect of copper- and chlorine-induced injury on the in vivo enterotoxigenic activity of the test organisms. Fluid accumulation after intraluminal inoculations of copper-injured, uninjured, and chlorine-injured cells into ligated ileal loops is shown in Fig. 5A, C, and D, respectively. These figures do not show any significant difference in the values of fluid accumulation when an inoculum of 10^6 cells per loop was used. However, significant reduction in fluid accumulation (P < 0.01) compared with that of uninjured cells was observed when inocula of 10^7 and 10^8 copper-injured cells per loop were used. It was interesting that no significant difference (P = 0.05) was

![Graph A](https://example.com/graphA.png)  ![Graph B](https://example.com/graphB.png)  ![Graph C](https://example.com/graphC.png)  ![Graph D](https://example.com/graphD.png)

**FIG. 5.** Enterotoxigenic activity of uninjured, copper-injured, and chlorine-injured H10407 cells (contained in 1 ml of PBS) after intraluminal inoculation of rabbit ligated ileal loops. (A) Fluid accumulation by copper-injured cells; (B) fluid accumulation by copper-injured cells plus 0.1% sodium deoxycholate; (C) fluid accumulation by uninjured cells; (D) fluid accumulation by chlorine-injured cells.
observed in fluid accumulation caused by chlorine-injured cells compared with that of the control. Sodium deoxycholate is known to inhibit the in vitro growth of injured populations. However, the results of our in vivo experiments (Fig. 5B) with chlorine-injured cells did not indicate such inhibition. Fluid accumulation was comparable, regardless of the presence of 0.1% sodium deoxycholate in the inoculum.

**DISCUSSION**

Several agents are known to injure coliform bacteria in drinking water, but copper and chlorine appear to be the most significant factors in this process (2, 5, 12, 20). These agents have also been shown to cause injury in enteric pathogens like *Yersinia enterocolitica* and several strains of ETEC (13, 30, 31). The results of the present investigation show that different concentrations of copper and chlorine were lethal to some cells of the exposed *E. coli* strains but induced sublethal lesions in large proportions of the remaining population. An earlier in vitro study from this laboratory (31) demonstrated that copper-injured ETEC strains were able to recover when placed in a suitable medium and that they showed a slower rate of ST production. However, on extended incubation, enterotoxin levels were comparable to those produced by uninjured cultures. Somewhat similar studies on thermal injury and the process of repair in *Y. enterocolitica* (26), *Campylobacter jejuni* (24), *S. aureus* (3), and *Salmonella typhimurium* (36) have been reported. In these investigations, however, the revival process was studied in in vitro systems. Hence, there was no information on the revival of injured enteric pathogens in an in vivo system. Clearly, the recovery of coliform bacteria and subsequent expression of enteropathogenicity within the mammalian gut is of prime interest, since that is their focal site after being ingested.

In our study, copper-injured H10407 cells which were inoculated intraluminally showed substantial (but not complete) repair of injury after 2 h. Longer incubation allowed a proportion of the cells to proliferate, while the process of repair continued in the rest of the population (Fig. 5A). Simultaneous repair and proliferation of the injured population on extended incubation could be attributed to the varying severity of the sublethal lesions acquired by cells during the injury process. Thus, slightly injured cells would recover and start multiplying earlier than those with more severe injury (22).

The intestinal contents, including the mucosal layer, may assist the repair process by providing nutrients to the cells. The results of in vitro experiments indicate that injured cells regain their tolerance to sodium deoxycholate when incubated with small amounts of mucosal homogenate at 35°C but not in buffer alone. Further, this in vitro recovery appeared to be slower than that observed in the mouse intestinal lumen. Nutrient limitation could have been responsible for this difference, since it was reported previously that the recovery of copper-injured ETEC cells in a defined amino acid medium is slower than that observed in a nutrient-rich complex medium (31).

Chlorine-injured cells revived in a shorter time after intraluminal inoculation than did those injured by copper. The reason for the difference in the revival rate was not determined, but this discrepancy could have been due to differences in the nature of the sublethal lesion(s) caused by the two agents. However, the lumen of the small intestine appeared to be a suitable environment for recovery and growth of the injured pathogenic *E. coli* cells under the experimental conditions used in this study.

The effect of various stress factors on the pathogenic traits of different organisms has not been thoroughly investigated. Reduced virulence of copper- and chlorine-injured *Y. enterocolitica* cells in mice has been reported (13, 30) recently. Heat injury of *Agrobacterium tumefaciens* reduces it tumor-forming ability in the plant host (34). In contrast, injury caused by freeze-drying does not result in loss or reduction in virulence of *Y. pestis* (10), *S. gallinarum* (35), and *S. aureus* (7). Apparently, alteration(s) in the phenotypic expression of pathogenic traits of an injured organism is related to the nature of the stress factor and the severity of the sublethal lesion caused by it. This could explain why chlorine-injured H10407 cells recovered faster in the ligated ileal loops of rabbits and why they demonstrated undiminished enterotoxigenic activity (Fig. 5D) compared with that of uninjured cells (Fig. 5C). In contrast, the copper-injured cells appeared to be more severely injured, taking longer to recover and showing reduced fluid accumulation (Fig. 5A).

The apparent absence of coliform indicator bacteria in water may not be significant under all circumstances, since some pathogens may persist longer than the indicator organisms in a particular aquatic environment. Experiments with membrane chambers demonstrated wide variations in the survival rate of coliform organisms and enteric pathogens in well water (18). A somewhat similar variation in the extent of injury caused by chlorine in coliforms, *Salmonella typhimurium*, *Shigella dysenteriae*, and *Y. enterocolitica* has also been seen in our laboratory (unpublished observations). The results of this study also indicate variations in the sensitivity of various pathogens to copper- and chlorine-induced injury. Furthermore, the in vivo recovery process after chlorine injury was faster than that after copper injury, suggesting different modes of injury with different sources of stress.

In the experiments reported here, stressed *E. coli* strains of clinical origin recovered in both in vitro and in vivo systems. Injured cells were able to recover, grow, and demonstrate virulence within the mammalian gut after exposure to copper and chlorine under conditions that were similar to those in drinking water. These findings indicate that enteropathogenic strains of *E. coli* retain genotypic determinants required to cause pathogenesis after environmental stress and that such cells can recover and express enteropathogenicity in vivo. Hence, the ingestion of such bacteria in water and perhaps food represents a potential health hazard. Furthermore, the in vivo recovery process and expression of enterotoxigenic activity is greatly influenced by the nature of the environmental stress factor causing the injury.

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**LITERATURE CITED**


Escherichia coli is enteropathogenic to R. Water diluent. 


