Reduced Virulence of *Yersinia enterocolitica* by Copper-Induced Injury

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A sublethal concentration of copper (0.75 mg/liter) caused substantial injury (87 to 95%) of *Yersinia enterocolitica* serotype O:8 cells in 72 h at 4°C without producing extensive cell death. Copper-injured cells had a higher 50% lethal dose in mice (2,700 CFU) than uninjured cells (150 CFU). This reduced virulence correlated with more rapid clearance of the injured cells from the blood of mice after intravenous inoculation. A possible role of the liver in this process was shown by significant cell accumulation in mouse livers when copper-injured *Y. enterocolitica* cells were administered, compared with uninjured bacteria. In vitro studies with isolated mouse liver membranes showed higher titers of aggregation with copper-injured cells than control cells. The in vitro aggregation reaction and blood clearance activity in vivo were abolished by sugars that are known to interact with a hepatic lectin. Our data suggest that copper-induced injury reduces the virulence of *Y. enterocolitica* and that the liver may be involved in nonimmune rapid clearance of the injured cells, probably by interaction with a hepatic lectin(s).

Yersinia enterocolitica has been isolated from a variety of foods, such as raw milk (35, 38), cheese (36), raw meats (4, 37, 39), and vegetables (1), and from various water sources, such as pond and well water (9), drinking water (25), and streams and lakes (20). This organism is being increasingly recognized as the cause of a variety of infections in humans, including acute gastroenteritis, mesenteric lymphadenitis, septicemia, arthritis, and erythema nodosum (8, 42). Yersiniosis is regarded as a food- or waterborne disease because of the implication of food (3, 10, 21, 29, 46) or water (4, 14, 22, 25) in several outbreaks.

Y. enterocolitica may become physiologically injured from water treatment or the processing of foods. The resulting injured population shows increased sensitivity to selective agents in the recovery medium (16). Since injured organisms generally fail to multiply in selective media used for isolation or enumeration, they remain undetected. However, induced injury in the few pathogens that have been studied (11, 18, 41) did not alter their pathogenic characteristics. This was evident after they were recovered and grown in nonselective media.

In aquatic environments copper and other elements, even in trace amounts, present potentially important causes of bacterial injury. In a survey of the water supplies of the 100 largest cities of the United States, the copper concentrations ranged from 0.006 to 0.25 mg/liter (13). Other studies have indicated the prevalence of copper in household tap water samples in Dallas, Tex. (28), and in the drinking water distribution system of Bozeman, Mont. (12). Low levels of copper in a chlorine-free distribution system have been reported to cause substantial injury to coliform populations (12). However, copper injury of *Y. enterocolitica* has not been described previously, although injury from exposure to heat (34), radiation (16), and radiation and freezing (15, 19) has been recently reported.

In this paper we describe the copper-induced injury of *Y. enterocolitica* and the reduced ability of an injured popula-

tion to produce disease and lethality in mice. The role of the liver in the clearance of inoculated injured organisms in mice is also discussed.

MATERIALS AND METHODS

Test organism. *Y. enterocolitica* O:8 strain E661 was kindly supplied by D. A. Schiemann, Department of Microbiology, Montana State University, Bozeman. Samples of a stock culture were stored in 1% peptone water containing 40% glycerol at -70°C.

Preparation of cells and copper injury. A fresh inoculum of *Y. enterocolitica* was prepared from an overnight culture of the organism grown at 22°C in tryptic soy broth without glucose (Difco Laboratories, Detroit, Mich.) but supplemented with 1% lactose and 0.3% yeast extract (TLY broth). Fresh sterile TLY broth (100 ml) was inoculated with 100 µl of the inoculum and incubated at 22°C for 24 h without shaking. The cells were harvested by centrifugation at 6,000 × g for 20 min at 4°C, washed once with sterile reagent grade water (Milli Q Water System; Millipore Corp., Bedford, Mass.), and suspended in a 10-ml volume of water. The suspended cells were thoroughly shaken to disengage clumps and were then suspended in 90 ml of 1.68 × 10⁻³ M carbonate buffer (pH 7.0) (12) to an optical density at 600 nm of 0.6 (approximately 10⁹ cells per ml) in a glass-stoppered bottle. A copper sulfate solution (1 mg of Cu per ml) was then added to each cell suspension (4°C) to the desired final concentration. Samples were removed at different times to assess the extent of injury (27) by determining the numbers of differential CFU on nonselective TLY agar and a selective agar (TLY agar supplemented with 0.1% sodium deoxycholate). Decreases in viability were determined from the viable cell counts obtained with nonselective TLY agar before and after exposure of the cells to different concentrations of copper.

LD₅₀ determination. To determine 50% lethal doses (LD₅₀), we used 8- to 10-week-old female CD-1 mice (Charles River Breeding Laboratory, Inc., Wilmington, Mass.). Uninjured and injured cells were centrifuged and

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FIG. 1. Injury of *Y. enterocolitica* exposed to copper in 1.68×10^{-3} M carbonate buffer (pH 7.0). The cells (0.9×10^9 to 1.7×10^9 CFU/ml) were exposed to 0 (○), 0.5 (●), 0.75 (△), and 1.0 (▼) mg of Cu per liter. The bars represent the standard deviations of the means of three trials.

suspended in a sterile saline solution (0.9% NaCl), and a series of twofold dilutions was made. Appropriate dilutions were used to assess the viable cell counts. LD₅₀ were determined by intraperitoneal injection (0.5 ml) of different dilutions into mice (seven mice per dose). The animals had free access to food and water and were observed twice daily for 3 weeks. The organism was recovered by using *Yersinia* selective agar (Oxoid Ltd., London, England) from the livers, spleens, and hearts of all of the dead and terminally sick mice examined. The LD₅₀ were calculated by the method of Reed and Muench (33).

Blood clearance of bacteria in mice. Mice weighing 28 to 30 g were given 20 U of heparin (Sigma Chemical Co., St. Louis, Mo.) intraperitoneally 5 to 10 min before injection of 0.5 ml of saline (0.9% NaCl) containing approximately 10^7 cells into the tail vein of each anesthetized animal. When desired, 100 mg of sugar (L-rhamnose or methyl-D-mannoside) was added to the bacterial suspension. At specified times 50-μl samples of blood were drawn from the retroorbital venus plexus, diluted in saline, and plated onto TLY agar. A duplicate set of mice was used for each inoculation. The clearance rate per minute (*K*) was calculated by using the equation of Voltonen (44) as follows: $K = (\log C_1 - \log C_2)/(T_2 - T_1)$, where C_1 was the initial count, C_2 was the final count, and $T_2 - T_1$ was the time difference between final and initial counts.

Survival of *Y. enterocolitica* in fresh blood in vitro. The test mixture consisted of 1 ml of fresh mouse blood containing 50 U of heparin per ml and 0.1 ml of *Y. enterocolitica* (approximately 10^7 CFU/ml). This mixture was incubated at 35°C for 30 min with occasional shaking. At the end of the incubation period samples were diluted in phosphate-buffered saline (pH 7.4) and plated onto TLY agar to determine the number of viable cells.

Enumeration of viable bacteria in livers. Mice were killed by cervical dislocation 30 min after the administration of challenge inocula. A portion of each liver was excised,

weighed, and homogenized in 10 ml of sterile saline. Appropriate dilutions were plated onto TLY agar, and colonies were counted after incubation for 48 h at 35°C.

Preparation of mouse liver membranes. Liver membranes were prepared by using the method of Ray (32). Briefly, the livers of three or four mice killed by cervical dislocation were removed, washed, blotted dry, minced, and homogenized for 2 min in a laboratory homogenizer (Virtis Hi-Speed model 45 homogenizer; The Virtis Co., Inc., Gardiner, N.Y.) in 40 ml of 1.0 mM sodium bicarbonate buffer (pH 7.5) containing 0.5 mM CaCl₂ at 4°C. The resulting homogenate was sonicated in Biosonik IV sonicator (VWR Scientific, San Francisco, Calif.) for 2 min at 4°C and centrifuged for 20 min at $1,000 \times g$ to remove tissue particles. The supernatant obtained was centrifuged at $12,000 \times g$ for 5 min in a Sorvall model RC 2-B refrigerated centrifuge. The resulting pellet was suspended in 0.05 M Tris-hydrochloride (pH 7.5) containing 0.1 M NaCl, 0.01 M CaCl₂, and 1 mg of bovine serum albumin per ml. The protein concentration of the preparation was adjusted to 6 mg/ml; the preparation was stored at 4°C and used within 4 or 5 days.

Slide aggregation test. The method of Perry and Ofek (31) was slightly modified and used for slide aggregation of liver membranes by bacteria. A 20-μl portion of a liver membrane suspension (6 mg of protein per ml) was mixed with 20 μl of a serially diluted bacterial suspension (initial A_{600} , 1.0) on a glass slide. The slide was rocked gently for 3 to 4 min, and the aggregation reaction was noted. The reciprocal of the highest bacterial dilution capable of eliciting a positive liver membrane aggregation reaction represented the titer. To test the effect of sugars, 10 μl of a 100 mM solution of a sugar (methyl-D-mannoside, methyl-D-glucoside, L-rhamnose, or methyl-D-galactoside) was added to the liver membranes before mixing with the bacterial suspension. To test bacterial

FIG. 2. Decrease in viability of *Y. enterocolitica* exposed to copper in 1.68×10^{-3} M carbonate buffer (pH 7.0). The cells (0.9×10^9 to 1.7×10^9 CFU/ml) were exposed to 0 (○), 0.5 (●), 0.75 (△), and 1.0 (▼) mg of Cu per liter. The values represent the means of three trials $\pm$ standard deviation.

FIG. 3. In vivo clearance of intravenously inoculated *Y. enterocolitica* cells in mice. Averages of 0.7×10^7 CFU of uninjured (○) and 1.0×10^7 CFU of copper-injured (●) cells were used for injection (arrows). The bars represent the standard deviations of the means of seven trials. K_h and K_i are the clearance rates (CFU per minute) of uninjured and copper-injured cells, respectively.

aggregation caused by concanavalin A (Sigma), 20 μ l of concanavalin A (1 mg/ml) was mixed with 20 μ l of a bacterial suspension, and the reaction was noted as described above.

RESULTS

Bacterial injury and lethality induced by copper. The effects of different concentrations of copper on *Y. enterocolitica* are shown in Fig. 1. A small percentage (5%) of the population of a freshly grown and washed cell suspension (without the addition of copper) was not able to grow on TLY medium containing 0.1% sodium deoxycholate. More cells (about 12%) became nonviable on this medium after 24 h. However, no further reduction in the viability of the organism was observed during incubation for up to 4 days. The contact time needed to initiate the process of injury of *Y. enterocolitica* by copper appeared to depend upon the copper concentration. A higher copper concentration (1.0 mg/liter) caused substantial injury (30%) after 1 day, whereas a concentration of 0.5 mg/liter caused 36% injury in the exposed population after only 3 days. However, maximum injury (89%) was observed on day 3 with a copper concentration of 0.75 mg/liter.

Although the injury caused by the higher copper concentration (1.0 mg/liter) appeared to be substantial after 4 days (Fig. 1), this concentration resulted in a high rate of lethality (Fig. 2), ranging from 98% after 2 days to 99% after 4 days. Figure 2 also shows that 0.75 mg of copper per liter did not cause extensive cell death (about 30%) on day 3, compared

FIG. 4. Blood clearance of copper-injured *Y. enterocolitica* cells in saline (○), of cells plus 100 mg of L-rhamnose (●), and of cells plus 100 mg of methyl-D-mannoside (△). An average of 1.0×10^7 CFU (arrow) was used for intravenous inoculation in mice. K_c , K_r , and K_m are the clearance rates (in CFU per minute) of cells in saline, of cells plus L-rhamnose, and of cells plus methyl-D-mannoside, respectively.

with the cell death (12%) observed in the control (without copper) during the same time period.

In additional experiments copper-injured cells were obtained by exposing *Y. enterocolitica* to 0.75 mg of copper per liter for 3 days at 4°C, and the extent of injury varied from 87 to 95%.

Mouse virulence. The LD₅₀ of *Y. enterocolitica* cultures in mice are shown in Table 1. There was a marked increase (1.25 log₁₀ units) in the LD₅₀ of copper-injured cells (90% injury) compared with the controls. In both groups the external symptoms exhibited by infected mice were similar (i.e., lethargy, reduced intake of food and water, emaciation, and death). Internal lesions were prominent in the livers and spleens. Mesenteric lymph glands were also involved in several mice that were examined. Apparently death was caused by systemic infection since *Y. enterocolitica* was isolated from the livers, spleens, and hearts of dead and terminally ill mice.

In vivo bacterial clearance. The in vivo blood clearance rates of uninjured and copper-injured *Y. enterocolitica* were determined by using mice. Figure 3 shows that there was a significantly ($P < 0.005$) faster clearance rate for injured cells (K_i , 0.144 CFU/min) than for uninjured cells (K_h , 0.024 CFU/min) when the organisms were administered intravenously.

In vitro clearance. The results of five different trials indicated that there was no change in the number of viable *Y. enterocolitica* cells when the organisms were incubated with mouse blood. The average initial counts of uninjured and injured cells were 1.1×10^7 and 1.2×10^7 CFU/ml, respec-

TABLE 1. Mouse virulence of *Y. enterocolitica* cultures^a

Cell preparation ^b	% Injury	LD ₅₀ (CFU) ^c
Fresh culture	5.4	70
Culture suspended in a buffer and held at 4°C for 3 days	12.3	150
Culture suspended in a buffer containing 0.75 mg of copper per liter and held at 4°C for 3 days	90.4	2,700

^a All cultures were grown in TLY broth at 22°C.

^b All preparations for inoculation were made in 0.9% NaCl.

^c Determined by intraperitoneal inoculation.

TABLE 2. Viable counts in mouse livers 30 min after administration of uninjured and copper-injured *Y. enterocolitica* cells

Inoculum	Dose (log ₁₀) ^a	No. of cells per g of tissue (log ₁₀) ^a
Uninjured cells	7.04 ± 0.12	4.85 ± 0.29
Injured cells	7.11 ± 0.04	5.405 ± 0.06 ^b

^a Mean of four trials ± standard deviation.

^b $P < 0.025$.

TABLE 3. In vitro aggregation reaction

Reaction mixture	Reaction	
	Uninjured cells	Injured cells
Bacterial cells + liver membranes + saline ^a	+ ^b	+
Bacterial cells + concanavalin A + saline	+	+
Bacterial cells + liver membranes + methyl-D-mannoside	-	-
Bacterial cells + liver membranes + methyl-D-glucoside	-	-
Bacterial cells + liver membranes + L-rhamnose	+	+
Bacterial cells + liver membranes + methyl-D-galactoside	±	±

^a The bacterial cell preparation was a diluted (1:1) bacterial suspension that was originally adjusted to an optical density at 600 nm of 1.0.

^b +, Aggregation reaction positive; -, aggregation reaction negative; ±, aggregation reaction weak.

tively, and after 30 min of incubation at 35°C the values were 1.3×10^7 and 1.1×10^7 CFU/ml, respectively.

Effect of L-rhamnose and methyl-D-mannoside on in vivo clearance of injured cells. Figure 4 shows the blood clearance rates of injured cells in the absence and presence of L-rhamnose and methyl-D-mannoside. The presence of L-rhamnose did not appear to influence the clearance rate of injected cells, whereas methyl-D-mannoside significantly ($P < 0.05$) lowered the blood clearance rate.

Bacterial counts in livers. Viable bacterial counts in the livers of inoculated mice were determined to ascertain the role of this organ, if any, in the selective clearance of injured cells. Table 2 shows that the organisms accumulated in significantly higher numbers in livers when injured organisms were used than when uninjured cells were used.

Aggregation of liver membranes with *Y. enterocolitica*. Both uninjured and copper-injured *Y. enterocolitica* cells agglutinated liver cell homogenates. In the experiments which examined the effects of sugars on the aggregation reaction, 20 μ l of a liver membrane preparation and 10 μ l of a 100 mM solution of a sugar (Table 3) were placed on a glass slide and were mixed with 20 μ l of a diluted (1:1) cell suspension (original A_{600} , 1.0). In the controls 10 μ l of saline (0.9% NaCl) replaced the sugar. Our results (Table 3) indicate that the effects of sugars (methyl-D-mannoside, methyl-D-glucoside, L-rhamnose, and methyl-D-galactose) on the aggregation of liver membranes by uninjured and copper-injured cells are similar. Also, concanavalin A caused positive aggregation when it was mixed with uninjured or injured populations. Serial twofold dilutions of *Y. enterocolitica* cells were treated with a fixed amount of liver cell membranes to determine the aggregation titer. Table 4 shows that the injured population had a higher titer (4- to 16-fold) than the uninjured cells.

DISCUSSION

Metals are naturally present in trace amounts in drinking water. There could be several additional sources of metals, particularly copper, in distribution systems with copper material in pipes, valves, and appurtenances (28), or copper sulfate may be added to treat algal problems in reservoirs (2).

Most studies on the effects of heavy metals or other stress factors on microorganisms have been concerned with the inhibitory or lethal responses (6). Reports of injury of

pathogenic bacteria by sublethal concentrations of various stress factors and the associated effects of injury on the virulence of the organisms are few (11, 18, 30, 41, 45). Our results show that *Y. enterocolitica* cells are significantly injured within 3 days of exposure to copper (0.75 mg/liter) without showing extensive cell death. Injury caused by exposure to copper altered the virulence of the organism, as indicated by an increase in the LD₅₀ in mice. Reduced virulence in mice may be related to the rapid clearance of the cells from the blood observed after intravenous challenge with injured cells (Fig. 3).

Similar observations have been made by other workers on *Salmonella typhimurium* (5) and *Salmonella cholerae-suis* (40). An increased rate of blood clearance was observed by these investigators with rough strains compared with intermediate or smooth strains of the parent organisms. The faster rate of clearance was correlated with reduced virulence for mice and chickens.

The process of clearance appears to be independent of blood phagocytosis or serum bactericidal activity since the results of our in vitro study did not show any reduction in cell number during 30 min in mouse blood. Perry and Ofek (31) also reported that there was no reduction in the number of *Escherichia coli* cells in mouse blood during 60 min of in vitro incubation, whereas only 4.8% of the intravenously inoculated organisms were viable after 15 min.

It has long been known that the liver plays an important role in nonimmune clearance of invading bacteria (7, 17, 43) and that the process is primarily effected by trapping and endocytosis by the stationary macrophages present in the liver (7). However, the mechanism by which bacteria are recognized and trapped by livers and other organs remains unknown. Our data indicate that injured cells showing a faster clearance rate were trapped in the liver at significantly higher numbers than uninjured cells. It was suggested by Leunk and Moon (26) that the type 1 pili of *S. typhimurium* are a major factor in hepatic clearance of this organism. However, Perry and Ofek (31) observed that a hepatic lectin recognizes specific sugars on the surface of *E. coli* and that this compound was centrally involved in the nonimmune clearance of both piliated and nonpiliated strains from the blood of infected mice. It is unlikely that pili were involved in the hepatic clearance of *Y. enterocolitica* in our study. The results of electron microscopic examinations and hemagglutination reactions reported by several workers (23, 24; D. A. Schiemann and J. A. Devenish, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B54, p. 23) revealed that *Y. enterocolitica* cells grown at 22°C for up to 48 h do not exhibit pili.

However, it can be suggested that a mannose-specific liver lectin is involved since the rapid clearance of injured *Y. enterocolitica* cells was inhibited by methyl-D-mannoside but not by L-rhamnose. The specificity of the inhibition was

TABLE 4. In vitro titration of *Y. enterocolitica* with liver cell membranes^a

Trial no.	Titer of aggregation		Increased aggregation titer of injured cells (fold)
	Uninjured cells	Injured cells	
1	32	128	4
2	32	512	16
3	64	512	8

^a The initial cell density was adjusted to an optical density at 600 nm of 1.0.

further supported by the results of the in vitro aggregation reactions. The reaction was inhibited by methyl-D-mannoside or methyl-D-glucoside but not by L-rhamnose when these sugars were added to the liver membranes before mixing with bacterial suspensions. Partial inhibition of the in vitro reaction by methyl-D-galactoside suggests the involvement of a galactose-specific lectin of the liver, but this observation was not confirmed by in vivo experiments.

In general, the sugars that were shown to inhibit the aggregation reaction completely in vitro and blood clearance in vivo in our study have been reported to bind with a mannose-N-acetylglucosamine-specific lectin found in liver cell membranes (5). Since both uninjured and copper-injured *Y. enterocolitica* cells showed a positive aggregation reaction, an attempt was made to quantitate the reaction by titrating the diluted cell suspensions with a fixed amount of liver cell membranes. An increase (4- to 16-fold) in the titer of the copper-injured population could have been due to the increased interaction of bacteria with the liver membranes and perhaps can be related to the more rapid clearance of injured bacteria in vivo. The precise mechanism of the increased interaction of the injured cells with liver membranes in vitro and rapid clearance in vivo cannot be explained at present, but it is possible that the exposure of *Y. enterocolitica* to copper alters some of the surface properties of the cells, thereby affecting their virulence characteristics.

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