



Changes in the virulence of chlorine-injured *Yersinia enterocolitica* by Mark William LeChevallier
by Mark William LeChevallier

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in
Microbiology

Montana State University

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Abstract:

Experiments were designed to assess the effect of chlorine injury on virulence of waterborne enteropathogens. Higher chlorine doses (1.0-1.5 mg/l) were necessary to produce injured *Yersinia enterocolitica*, *Salmonella typhimurium* and *Shigella* spp. than enterotoxigenic *Escherichia coli* (ETEC) or coliform bacteria (0.5 mg/l) in the test system used. LD₅₀ experiments using mice showed that injured *enterocolitica* were 20 times less virulent than uninjured control cells (3300 and 160 cfu, respectively). This decrease in virulence was not related to reduced attachment to Henle 407 intestinal epithelial cells, but could be related to a loss of epithelial cell invasiveness. Loss of epithelial cell invasiveness was measured by four techniques: (i) the HeLa cell roller tube method, (ii) light microscopy on stained Henle cell monolayers, (iii) transmission electron microscopy of HeLa cells infected with *Y. enterocolitica*, and (iv) enumeration of intracellular bacteria in mouse intestines using fluorescent-antibody methods. In contrast, injured *S. typhimurium* and ETEC lost the ability to attach to Henle cells. Injured *Y. enterocolitica* showed reduced oxygen utilization, ATP synthesis and assimilation of radiolabeled thymidine, Uridine and methionine. Inhibition of RNA and protein synthesis by certain antimicrobics inhibited the invasiveness of *Y. enterocolitica*. Chlorine did not change the hydrophobicity or surface charge of injured pathogens. Preparations of membranes from untreated and antimicrobial treated *Y. enterocolitica* blocked the invasiveness of virulent *Yersinia*, while membranes from chlorinated cells were unable to block invasiveness. Electrophoresis of outer membrane proteins from chlorinated cells showed numerous changes as compared to untreated preparations. A theoretical model of how chlorine affected the invasiveness of injured *Y. enterocolitica* is presented.

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by

Mark William LeChevallier

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ABSTRACT

Experiments were designed to assess the effect of chlorine injury on virulence of waterborne enteropathogens. Higher chlorine doses (1.0-1.5 mg/l) were necessary to produce injured Yersinia enterocolitica, Salmonella typhimurium and Shigella spp. than enterotoxigenic Escherichia coli (ETEC) or coliform bacteria (0.5 mg/l) in the test system used. LD₅₀ experiments using mice showed that injured Y. enterocolitica were 20 times less virulent than uninjured control cells (3300 and 160 cfu, respectively). This decrease in virulence was not related to reduced attachment to Henle 407 intestinal epithelial cells, but could be related to a loss of epithelial cell invasiveness. Loss of epithelial cell invasiveness was measured by four techniques: (i) the HeLa cell roller tube method, (ii) light microscopy on stained Henle cell monolayers, (iii) transmission electron microscopy of HeLa cells infected with Y. enterocolitica, and (iv) enumeration of intracellular bacteria in mouse intestines using fluorescent-antibody methods. In contrast, injured S. typhimurium and ETEC lost the ability to attach to Henle cells. Injured Y. enterocolitica showed reduced oxygen utilization, ATP synthesis and assimilation of radiolabeled thymidine, uridine and methionine. Inhibition of RNA and protein synthesis by certain antimicrobics inhibited the invasiveness of Y. enterocolitica. Chlorine did not change the hydrophobicity or surface charge of injured pathogens. Preparations of membranes from untreated and antimicrobial treated Y. enterocolitica blocked the invasiveness of virulent Yersinia, while membranes from chlorinated cells were unable to block invasiveness. Electrophoresis of outer membrane proteins from chlorinated cells showed numerous changes as compared to untreated preparations. A theoretical model of how chlorine affected the invasiveness of injured Y. enterocolitica is presented.

INTRODUCTION

Much progress has been made during the past ten years towards understanding injury of bacteria in drinking water. Bissonnette et al. (17) in 1975 first described injury of bacteria in water while subsequent reports on the physiology of injury have been made by Camper and McFeters (42) Zaske et al. (251, 252) and Domek et al. (54). In addition, the influence of laboratory and environmental factors on the recovery of injured coliforms have been evaluated (16, 54, 133, 153). Recently, new methods for enumerating injured total and fecal coliforms in drinking water have been proposed (134). Despite this work, questions remain about the health significance of injured bacteria. Do indicator bacteria and pathogens injure in the same manner? Are injured pathogens still virulent? What is the physiological mechanism of reduced virulence?

The introduction to this study is divided into three parts. The first section reviews injury including sources of injury in water and factors that affect injury. Most of this work has been in relationship to coliform bacteria. The next part reviews the limited data regarding injury of pathogens. The last section covers Yersinia enterocolitica with special emphasis on the invasiveness of this pathogen.

Injury of Microorganisms in Water

Injury has been defined as the sublethal physiological consequence of exposure to stresses, which cause a loss in the ability of microorganisms to grow normally under conditions that are satisfactory for uninjured cells (15, 17, 42, 153). Injury has also been described as reversible; i.e., under proper conditions of temperature and nutrients, injured organisms can repair the cellular lesion and become indistinguishable from unstressed cells (15, 17, 42, 153). Injury has been observed in all genera of coliform bacteria. This sublethal damage has been observed as an inability of bacteria to proliferate on media containing selective agents that have no apparent inhibitory action on undamaged cells (15, 17, 97, 153, 183, 187). Therefore, most sublethal stresses will result in damaged cells incapable of growing on the selective media commonly used in aquatic microbiology.

Sources of Injury

Although the understanding of all potential sources of injury in water is incomplete, several parameters have been studied extensively.

Disinfectants

Chlorine has been the most widely recognized cause of injury to bacteria in drinking water (6, 16, 24, 42, 81, 83, 138, 140, 154, 157, 175, 177, 232, 249). Both the chlorine dose and the time of exposure have been shown to influence the degree of injury (42, 154). Most bacteria exposed to sodium hypochlorite are rapidly killed once the chlorine demand of the system is satisfied (134). However, a sizable portion of the survivors are injured in such a way that they fail to grow under the selective conditions commonly used to detect bacteria in drinking water. Because of a lack of adequate techniques to measure injury, the extent of bacterial stress in drinking water is largely unknown, although some reports estimate that coliforms are recovered with an efficiency of ≤ 10 percent in chlorinated aquatic environments (15, 16, 24, 42, 58, 152, 155).

Exactly how chlorine injures a microorganism has never been demonstrated experimentally (59). Baker in 1926 (8) theorized that chlorine destroys microorganisms by combining with proteins forming N-chloro compounds. This was extended by Green et al. (82) and others (122, 232) who showed that chlorine had powerful effects on sulfhydryl groups of proteins. Other evidence showed that hypochlorous acid (HOCl) converts several α -amino acids by

oxidation into a mixture of corresponding nitriles and aldehydes (177). The exact product of the reaction is dependent on chlorine concentration and pH (50, 51, 247). The action of chlorine on polypeptides can be understood in terms of electrophile-nucleophile interactions where electropositive chlorine reacts with electron-rich centers on the substrate (4). Functional groups in proteins that are susceptible to chlorine include: amino, imidazolium, guanidinium, sulfhydryl and indole residues; phenolic rings and the amide nitrogen of peptide bonds (38, 92, 220, 223). Stelmaszynska and Zgliczynski (220) showed with bovine albumin that ^{36}Cl was incorporated into N-terminal, ϵ -lysine groups and the amide nitrogen of peptide bonds. Other experiments have shown that cytochromes, iron-sulfur proteins and nucleotides are highly vulnerable to oxidative degradation by HOCl suggesting that chlorine causes physiological damage primarily to the bacterial cell membranes (4, 42, 83, 154, 232, 251, 252). Investigators have shown that respiration, glucose transport, and ATP levels all decrease in chlorine-injured populations (42, 83, 154, 232). Electron microscopy of injured cells has demonstrated morphological changes in the cell membrane (252). In addition, chlorination has been reported to cause death as a result of unbalanced metabolism (249), affect protein synthesis, (12, 177), and cause genetic

defects by modifying purine and pyrimidine bases (83, 99, 102, 175, 207).

Although few data exist that describe injury from other water disinfection agents such as ozone, chlorine dioxide, and ultraviolet irradiation, it has been suggested that these treatments also cause injury (15, 39, 188). Chlorine dioxide has been reported to inhibit membrane-bound enzymes and protein synthesis (12, 188). Ultraviolet irradiation is known to cause damage to nucleic acids as well as alter membrane functions (15).

Environmental Factors

Environmental factors such as heat, freezing, and sunlight are also known to cause injury (15, 39, 68, 100). Exposure to sunlight can inactivate 90 percent of the indicator bacteria in stream water within 13 to 32 minutes (68). Although these parameters are probably of little importance in water contained in distribution systems, they may affect bacteria in source waters and reservoirs. Changes in pH resulting from acid rain or acid mine drainage (15, 55) or from lime or soda additions in the treatment plant may also cause injury to indicator bacteria in surface water. pH is known to affect substrate transport across membranes (15) and the solubility of heavy metals.

The role of transition metals in the injury process in drinking water was recently described (54). A combination of laboratory studies and field data indicated that copper, at concentrations normally found in drinking water, could cause significant injury to Escherichia coli. Although other metals such as cadmium, lead, and zinc were also detected in drinking water samples, these metals or combinations of the metals were not as important in the injury process as copper alone. Copper has been detected in 65.2 percent of 380 finished drinking water supplies in the United States, with a mean concentration of 0.043 mg/L and a range of 0.001-1.060 mg/L (222). Experiments have indicated that 90 percent injury occurs in E. coli populations within two days at copper concentrations near the national average (54).

Statistical analysis of data from 30 drinking water samples suggested that pH, alkalinity, and temperature influenced the extent of injury by copper, presumably by altering the chemical species and activity of the dissolved copper. Other factors, such as total organic carbon levels and the species of bacteria present, are thought to influence injury by copper. Copper-injured cells show impaired respiratory activity and are unable to utilize oxygen during the repair process (54).

Sources of copper in drinking water include copper pipe material, brass, faucets, and other attachments

(222). Copper may also be introduced as copper sulfate, which is used to treat algal problems in reservoirs (76), and as trace contaminants in flocculation material. In addition, copper may enter water from geological sources. This process may be especially important in some areas, such as Montana, where copper is naturally abundant.

Biological Interactions

Another source of injury to indicator bacteria in drinking water is biological interactions (133). Some strains of heterotrophic or standard plate count (SPC) bacteria in drinking water have been shown to suppress coliform detection (74, 75, 101, 185, 243). It has been demonstrated that coliform suppression in municipal and rural water supplies can at least partly be attributed to elevated SPC numbers (3, 22, 75). For example, increased SPC densities have been noted prior to, or associated with, waterborne disease outbreaks (3, 75, 150, 162).

The presence of pathogens has also been documented in the absence of detectable coliforms in samples with high SPC numbers (3). Data from the National Community Water Supply Study (150) showed that the frequency of coliform detection decreased when SPC levels exceeded 500-1000 cfu/ml.

Interactions of SPC bacteria with coliforms may also cause injury. For example, experiments have shown that interactions between Pseudomonas maltophilia and E. coli

can produce 55 percent injury over a 7-day period (133). The decline in viable bacteria for the control and mixed coliform cultures was identical, but injury was observed only in the mixed culture. Injury did not occur until P. maltophilia levels reached 10^4 - 10^5 cfu/ml. The ratio of coliforms to P. maltophilia ($1:10^4$ - 10^5) in those experiments is similar to the ratios for coliforms and SPC bacteria in drinking water in which coliform suppression has been reported (coliforms--1-10 cfu/100ml; SPC-->500 cfu/ml) (48, 75, 163). Such interactions are important, particularly when samples are transported or stored. Previous studies have shown that coliform levels may vary significantly during sample transport (163, 164), but injury in these experiments was not controlled or examined.

Finally, the role in the injury process of the many toxic organic substances that may be dissolved in surface waters is completely unknown. As awareness of injured bacteria in drinking water increases, other causes of injury may become apparent.

Factors Affecting Injury

In addition to the primary causes of injury to bacteria in drinking water, a number of factors can influence the extent or severity of stress.

Environmental Factors

Environmental factors that influence the extent of injury include time and temperature of exposure of the bacteria in water. As mentioned previously, >90 percent injury can occur in an E. coli population within two days when exposed to 0.05 mg copper/L (54). Similarly, Bissonnette et al (17) showed that >90 percent of E. coli became injured when exposed for 2 days in mountain streams. Other investigators have reported 97 percent injury in E. coli populations exposed to reagent-grade water for 3 days (252). Results of experiments in which 230 membrane diffusion chambers containing bacteria were immersed in Bozeman, Montana drinking water indicate that 90 percent injury occurred in E. coli populations within 2-4 days (134). In general, as the temperature of the water increased, the time necessary to produce significant levels of injury decreased (7, 52, 187). Temperature was also shown to be an important variable in a computer model developed to predict injury in drinking water (54). These findings might be explained by the observation that higher water temperatures increase the activity of injurious agents such as chlorine, metals, and biological antagonists (7, 52, 54, 187). Other environmental factors that probably influence extent of injury include the bacterial strain, the physiological state of the organism, and the

concentration of dissolved nutrients, although little information regarding these factors is available.

Sample Handling

The way a water sample is collected and handled prior to examination greatly influences the viability of injured bacteria in that sample. Sodium thiosulfate is usually added to drinking water samples to neutralize any chlorine residual (6, 20, 72). A chelator such as EDTA is sometimes added to remove heavy metals, particularly if there is an extended period between sample collection and enumeration (20, 54, 72).

Laboratory manipulations involving exposure to diluents may cause underestimations of bacterial densities in drinking water (153). If diluents are maintained at approximately refrigerator temperature (around 4°C), their composition and exposure time are of minimal impact on enumeration efficiency. However, substantially lower recoveries of injured bacteria have been associated with use of diluents maintained at room temperature and with extended exposure times (153). The enrichment of diluents with low concentrations (0.05-0.1 percent) of organic material such as peptone, gelatin, tryptone, or milk has been demonstrated to be of value in the enumeration of stressed aquatic bacteria (15, 24, 40, 104, 120, 183, 221, 145, 246, 248). In addition, diluents (such as standard

methods buffer) that contain magnesium ($MgSO_4$ or $MgCl_2$) and phosphate (K_2HPO_4 or KH_2PO_4) (15) are known to help recover metabolically injured cells (72).

Method of Enumeration

The choice of enumeration methodology probably has the greatest impact on the recovery efficiency of injured bacteria. Important factors to be considered when choosing a method to enumerate injured cells include the surface pore morphology of the membrane filter, the enumeration technique and the composition of the selective medium (21, 47, 73, 88, 96, 98, 132, 134, 139, 182, 208, 214, 225, 226). The influence of these factors on coliform enumeration has been reviewed (134). Recently, new membrane filter techniques have been proposed for enumerating stressed total and fecal coliforms (134). A new medium (m-T7 agar) remains selective and at the same time minimizes the inhibition of stressed coliform bacteria (134).

Injury of Pathogens

The question concerning the significance of injured bacteria in water has been a problem not adequately answered. Presumably, conditions that stress coliforms would also injure waterborne pathogens, although there are no data to support this hypothesis. Some investigators

have examined the virulence of injured foodborne pathogens (49, 71, 209, 218). Sorrells et al. (218) found no difference in the virulence of Salmonella gallinarum for 6 week old chicks after injury by freezing. Similarly, Simon et al. (209) reported that the virulence for mice of three strains of Salmonella typhimurium did not change appreciably when samples were freeze-dried and stored for 1 or 2 years at 5°C. Fung and Vanden Bosch (71) and Collins-Thompson et al. (49) reported that Staphylococcus aureus regained the ability to produce enterotoxin B after resuscitation in a non-selective medium. Other pathogens including Yersinia enterocolitica, Campylobacter jejuni, and Pseudomonas aeruginosa have been injured by a variety of stresses (heat, freezing, chlorine and ionizing irradiation), but the virulence of the injured cells was not assessed (61, 62, 80, 142, 174, 199). E. coli injured by heating, freezing, drying or gamma irradiation became more sensitive to hydrophobic antibiotics (142). While these data indicate that pathogens are injured in foods and by food processing, the results may not be directly applicable to the aquatic environment.

Walsh and Bissonnette (237) studied the attachment of chlorine injured enterotoxigenic E. coli (ETEC) to human peripheral leukocytes. They concluded that reduced adhesive ability was due to the loss of surface structures

resulting from sublethal chlorination. While they did not test the in vivo virulence of chlorine-injured ETEC, Walsh and Bissonnette (237) did imply that a reduced ability to colonize the small intestine would result in an inability of this organism to initiate disease.

Recently, a "viable-but-nonculturable" stage of Salmonella enteritidis (189) and Vibrio cholerae (250) has been described. In this condition, cells exposed to the aquatic environment for prolonged periods of time become nonculturable by conventional techniques but could be resuscitated by additions of nutrients (189). Pathogens in this "viable-but-nonculturable" stage have been reported to remain virulent (Roszak, D.B., D.J. Grimes and R.R. Colwell, 1984, Abst. Annu. Mtg. Am. Soc. Microbiol., I 21, p. 125).

Yersinia enterocolitica

The genus Yersinia is currently comprised of seven Gram-negative coccobacillary species, Y. enterocolitica, Y. pseudotuberculosis, Y. pestis, Y. intermedia, Y. frederiksenii, Y. kristensenii and Y. ruckeri (14, 26, 27, 31, 230). Formerly classified as Pasteurella species, the genus is now named for the French bacteriologist, A. J. E. Yersin, who first isolated the causative agent of plague in 1894. The most notorious of the species, Y. pestis, has a "well defined heritage of human morbidity and mortality"

(29), while Y. enterocolitica has, until the late 1960's, remained virtually unknown. First described in 1939 (198), Y. enterocolitica has been etiologically incriminated in a broad range of clinical entities, the more frequent manifestations of which include: acute mesenteric lymphadenitis, terminal ileitis, gastroenteritis, septicemia, nonsuppurative arthritis, erythema nodosum and localized abscesses in liver and spleen (23, 93, 217).

Biochemical Characteristics

Y. enterocolitica has been characterized as "biochemically polydiverse and thermally perceptive" (23, 93, 119). The organism usually ferments glucose, sucrose, arabinose, galactose, sorbitol, trehalose, xylose and cellobiose at both 22° and 35°C within 48 h. Rhamnose, raffinose, dulcitol and adonitol are not utilized; acid from salicin and esculin hydrolysis is strain variable; and maltose is utilized at 22°C. Y. enterocolitica lacks enzymatic activity for gelatinase, oxidase, phenylalanine deaminase, citratase, lysine decarboxylase and arginine dihydrolase, but possesses catalase, B-galactosidase (except some strains), ornithine decarboxylase, nitrate reductase type B and urease activity. Although the rapidity of utilization of the aforementioned substrates may vary slightly with temperature, the tests for

acetylmethyl carbinol (Voges-Proskauer), growth in potassium cyanide and motility are temperature dependent and are expressed at 22°C but lacking at 35°C.

Growth temperature affects expression of genes on the 40- to 48-megadalton plasmid associated with virulence of Y. enterocolitica. Temperature affects calcium dependent growth, serum resistance, autoagglutination, synthesis of V and W antigens, and other polypeptides (18, 56, 90, 148, 178, 180, 211, 213). Growth temperature is known to affect the structure of Y. enterocolitica membranes including the O-specific sugars and fatty acid composition (11, 114, 239), surface properties of the bacterium (64, 124, 125), attachment to epithelial cells (148, 168, 172), production of fimbriae (64, 113, 168, 172) and sensitivity to certain bacteriophage (114, 115). While many genes in the chromosome and in the plasmid genome appear to be temperature dependent, their exact role in pathogenesis is not well understood. Some investigators (116) have presented evidence that lipopolysaccharide produced during growth in vivo resembles, antigenically, that produced during growth in vitro at 25°C. Clearly, Y. enterocolitica is an organism that can tolerate temperatures characteristic of both the aquatic environment and a warm blooded host.

Environmental Sources

It is well established that the animal kingdom serves as the major reservoir for Yersinia species (23, 45, 93). Y. enterocolitica of serotype and phage type identical to those recovered from human hosts have been isolated from animals, particularly the pig (23, 45, 93). Water, however, can serve as a vehicle for transmission of Y. enterocolitica to humans. There have been a number of reports of waterborne yersiniosis (60, 118, 131, 147) and many investigators have isolated Yersinia spp. from water (70, 94, 110, 111, 130, 156, 190, 197, 244), although usually these bacteria are avirulent. Keet (118) showed that Y. enterocolitica O:8 isolated from a mountain stream had the same phage and serotype as a strain recovered from a man suffering from septicemia after a hunting trip in the Adirondack Mountains of New York. He postulated that the water may have been contaminated by deer or other animal feces. Martin et al. (147) reported a family outbreak of yersiniosis in northern Saskatchewan, Canada, where the source of the etiologic strains could have been river water contaminated by a cow or her calf. Saari and Jansen (190) state that "an increasing number of atypical North American human Y. enterocolitica isolates suggest that water contamination may play an important role in the transmission of some forms of yersiniosis to man".

Virulence Determinants

While there are at least 50 serogroups of Y. enterocolitica (242), only a few serotypes are commonly associated with disease in man (45, 112, 117, 160, 195, 229). Human infections are most often associated with serotypes O:3, O:8, O:9, O:5,27, and O:13 (5, 45, 117, 160, 194, 195, 229). Virulence determinants associated with these serotypes include: autoagglutination, calcium dependency at 37°C, serum resistance, heat-stable enterotoxin production, tissue culture invasiveness, and the presence of V and W antigens (77, 103, 117, 136, 160, 170, 195, 203, 229).

A number of virulence determinants are associated with the 40- 48-megadalton plasmid. The plasmid appears to be essential for resistance of Y. enterocolitica against host defenses (148, 253). Strains that have lost the plasmid are avirulent. The virulence plasmids have been shown to code for at least 20 different polypeptides in minicells (181) of which one to four are associated with the outer membrane of the bacterium (19, 180, 181, 211). The plasmids of serotypes O:3 and O:9 share 90% homology with each other while the plasmids harbored by serotype O:8 have 75% sequence homology with those of O:3 and O:9 (89). The high degree of molecular relatedness of these plasmids is also reflected by the incompatibility phenomenon (10).

However, recently a mobilizable virulence plasmid from serotype O:8 was constructed and transferred to plasmid negative strains of O:3, O:5 and O:8 (91).

Calcium dependency for growth at 37°C is a plasmid associated trait that has received much attention (13, 28, 78, 109, 169). It has been speculated that calcium dependency reflects the ability of the bacteria to respond to their intracellular environment (28, 29, 169). Growth restriction occurs with the production of the V and W antigens when calcium levels are low, but growth resumes and the antigens are repressed when enough calcium is present to permit cell division (2.5 to 4.0 mM) (28, 29, 169). This calcium restriction reflects a normal metabolic step down characterized by shut off of stable RNA synthesis and decreased adenylate energy charge (28, 29).

Autoagglutination is another plasmid associated virulence determinant of Y. enterocolitica (112, 129). The bacteria produce a fluffy precipitate when grown in a tissue culture medium at 37°C but not at 25°C or when the plasmid has been lost (112, 129). It has been speculated that autoagglutination may be promoted by ancillary outer membrane proteins coded by the virulence plasmid (112).

Recently, Heesemann et al. (90) have presented evidence that the virulence plasmid also promotes attachment in some strains of Y. enterocolitica. Transconjugants that received the plasmid from serotypes

0:3 or 0:9 were tested for their ability to associate with HEp-2 cells. One strain (serotype 0:5) was able to interact with HEp-2 cells only after receipt of the cointegrate. However, strains of serotypes 0:3, 0:8 and 0:9 were able to interact with epithelial cells regardless of whether they contained the plasmid or not (90).

Invasiveness

Studies of the pathogenicity of Y. enterocolitica have revealed that the virulence of this organism is multifactorial consisting of both plasmid and chromosomal determinants (67, 215, 219). The ability of Y. enterocolitica to invade tissue is a key chromosomal virulence trait for this organism (146, 180, 194, 233).

Methods for evaluating bacterial attachment and invasiveness include light microscopy, viable plate counts and radiolabel assays (36, 53, 143, 151, 206). Light microscopy has the advantage of being able to estimate the percentage of cells colonized and/or infected but has the disadvantages of poor precision, is subjective and cannot distinguish live from dead cells (143). Recently, Nomarski differential interference contrast microscopy and UV incident light microscopy applied on the same microscope have been used to evaluate bacterial attachment and invasiveness (35, 36). The authors claim that the method is more accurate, sensitive, and simpler to use than

ordinary light microscopy (35, 36). Viable plate counts have the advantages of being objective, reproducible, and detect only viable bacteria (143). The accuracy of the method is limited by difficulty in obtaining homogeneous suspensions of individual bacteria (143). The roller tube method developed by Devenish and Schiemann (53) allows for the enumeration of intracellular Y. enterocolitica by killing extracellular bacteria with gentamicin. The system is simpler than microscopy, allows stricter control of multiplicity and provides a quantitative index for comparing the relative infection capability of Y. enterocolitica for HeLa cells (53). Other investigators have used different approaches to determine intracellular bacterial densities. Shaw et al. (206) used UV-irradiated bacteriophage to kill extracellular Yersinia and Shigella spp. in tissue culture infectivity assays. McCoubrey et al. (151) used incorporation of [¹⁴C]methionine into Y. enterocolitica in the presence of streptomycin and cycloheximide as a measure of intracellular bacterial levels. Since streptomycin does not cross the tissue cell membrane and cycloheximide inhibits eucaryotic protein synthesis, only the intracellular bacteria are active and able to incorporate the label. Radiolabel assays are objective and reproducible but generally are more expensive than the other test procedures (143).

A number of advantages are associated with the use of the in vitro tissue culture system for studying invasiveness. It is possible to choose cell lines from different tissue types and different animal species. The assays are simple, rapid and inexpensive to perform and the use of laboratory animals is avoided. Most importantly, in vitro assays permit attachment or invasiveness to be studied isolated from other factors that influence the result of an infection in vivo. Many investigators have noted strong correlations between tissue culture cell infectivity in vitro and the ability to penetrate epithelial cells in vivo (37, 145, 228). Une (227, 228) after studying the process of invasion of Y. enterocolitica both in vivo in rabbits and in vitro using HeLa cells, concluded that "the HeLa cell infection system well reflected the interaction between Yersinia and epithelial cells in vivo". However, since the precise biochemical nature of the invasion mechanism is not known, it cannot be stated for certain that the processes that occur with the engulfment of bacteria by HeLa cells (derived from a cervical carcinoma) are the same as human intestinal epithelial cells in vivo.

Since it is clear that the cell monolayer test for invasiveness does not completely reflect all of the virulence characteristics of enteropathogenic bacteria, use of in vivo tests are also necessary. One model used for

determining the in vivo virulence of enteric organisms is the Serény test (204). This test qualitatively measures the capacity to cause conjunctivitis in the eye of a guinea pig. A positive Serény test correlates well with virulence in Y. enterocolitica O:8 (65, 146, 193). Infection of intact intestinal epithelial linings has been used by some investigators to measure invasiveness (43, 44, 227). This method is more direct since intracellular bacteria can be observed using immunofluorescence techniques (67). Other virulence models include use of rabbits, mice, gerbils, and monkeys (43, 44, 45, 65, 193, 227).

Invasive Process

"Attachment" and "adhesion" are used here to denote the binding of a bacterium to a surface, while "invasion" is used to indicate the process of penetrating tissue. "Association" or "interaction" refers to the combined process of attachment and invasion where the two processes have not been delineated.

Investigators (85, 228, 253) have noted that when pathogenic strains of Yersinia and Shigella attached to HeLa cells in tissue culture a ruffling movement of the cell membrane stimulates a pinocytotic process by which the adherent bacteria become incorporated into the epithelial cell. Bacteria initiate this process by stimulating a phagocytic depression in the tissue culture cell

cytoplasmic membrane (79, 85, 123, 228, 253). The organisms are then incorporated into the cells by numerous fine appendages (microvilli). Modification of the epithelial cell surface by interferon, viruses, and chemicals can inhibit endocytosis of virulent bacteria (32, 33, 34, 85). Intracellular Y. enterocolitica are enclosed in phagocytic vesicles which accumulate near the nucleus (228).

Numerous investigators have studied the role of the bacterium in the invasive process (2, 30, 84, 86, 158, 171, 176, 191, 192, 216, 231, 234, 241, 242; also reviewed in 67, 87, 137, 215, 219). From these studies two requirements appear to be necessary for invasiveness; (i) the organism must be viable and metabolically active, and (ii) the organism must have certain surface components to initiate engulfment. Brunius et al. (30) showed that pretreatment of Y. pseudotuberculosis with formalin, glutaraldehyde or proteases inhibit its interaction with HeLa cells. Hale and Bonventre (84) have shown that virulent Shigella strains participate actively in the infection of tissue culture cells. They found that heating the organisms at 50°C for 2 min inhibited the infectivity of S. flexneri 2a for Henle cells and that a sublethal pulse of UV irradiation also diminished infectivity. Heat killed Y. enterocolitica have been reported by many authors to be noninvasive (168, 176, 216, 234). Okamoto et al.

(168) found Y. enterocolitica killed by UV irradiation or formalin lost their capacity to attach to HeLa cells. However, other researchers have indicated that formalin or UV killed bacteria still interacted with epithelial cells (176, 216, 234). The reason for this discrepancy is not clear, but it may have to do with the techniques employed to measure attachment and invasion. All of these investigators used microscopic methods which I have previously noted (page 19) as being imprecise.

A requirement for specific surface characteristics for invasiveness has been extensively studied with Shigella (2, 86, 171, 191, 192, 241, 242). Plasmids of Shigella flexneri, Shigella sonnei and enteroinvasive E. coli have been shown to code for specific outer membrane proteins, nine of which were similar in all three strains (86, 192). When the plasmid of S. flexneri was transferred to E. coli K-12 (normally avirulent and noninvasive) the recipient became invasive for HeLa cells (191). E. coli minicells containing the plasmid incorporated [³⁵S]methionine into 14 outer membrane proteins (86). Full pathogenicity for a variety of laboratory models including the Serény test and the rabbit ileal loop required the plasmid and three chromosomal segments (191). Hale et al. (86) have hypothesized that the outer membrane proteins encoded by the virulence plasmid bind to receptors on host enterocytes and stimulate engulfment. Maurelli et al. (149) showed

that restoration of invasiveness after growth at 30°C required protein synthesis. When shigellae were grown at 30°C and shifted to 37°C for 2 h in the presence of chloramphenicol, the bacteria remained noninvasive. Adamus and Romanowska (2) found significant quantitative differences in the outer membrane protein composition between virulent and avirulent strains of Shigella sonnei phase I. Other researchers have indicated that the composition of lipopolysaccharide in Shigella flexneri is important for the survival of the organism against host defenses and that glycolipids and lipoproteins are required to induce engulfment of the bacilli by epithelial cells (171, 173, 179). These data have been extended by others who have indicated that a small plasmid of Shigella dysenteriae is required to produce specific O-polysaccharide side chain structures of lipopolysaccharides which are necessary for invasiveness (241, 242). Similar reports have been made for Salmonella typhimurium indicating that the organism requires both plasmid-derived and lipopolysaccharide determinants to be adhesive and invasive in the HeLa test (105, 106, 158). The invasiveness of Y. pestis and Y. pseudotuberculosis has been related to the presence of chemical structures on the bacterial surface sensitive to the action of ether.

Certain surface characteristics have also been shown in Y. enterocolitica to be important for invasiveness (176,

216). It has been suggested that boiling cells destroys some heat-labile surface components essential for penetration of epithelial cells (176, 216). Hydrophobicity has been reported by some investigators to be important in the processes of attachment and invasion of tissues (124, 196). Schiemann and Swanz (196) found that all strains of Y. enterocolitica which were able to attach to Henle cells were also hydrophobic. Lachica and Zink (124, 125) correlated increased surface charge and hydrophobicity to the presence of the 40- to 48- megadalton plasmid. They suggested that hydrophobicity may be associated with the ability of the plasmid positive strains to colonize and survive in the gastrointestinal tract of mice. Hydrophobicity and electric charge have been shown to influence the phagocytosis of type 1 fimbriae-bearing E. coli (167, 205). One strain possessing a hydrophobic surface with weak negative charge was efficiently ingested while another strain which was hydrophilic and had an uncharged smooth lipopolysaccharide, resisted phagocytosis. The authors concluded that whether attached bacteria are ingested or not depends on their underlying physicochemical surface properties (167, 2050. Similar results have been noted for Salmonella (105, 107, 108). Hydrophobic interactions and electric charge were shown to be responsible for irreversible attachment and engulfment by HeLa cells.

Statement of Research Problem

The present study was conducted to investigate the changes in virulence of chlorine-injured Y. enterocolitica. This study had three objectives: (i) to compare the susceptibility of coliforms and pathogens to injury by chlorine; (ii) to evaluate with combinations of in vitro and in vivo tests the step(s) in the pathogenic process affected by chlorine; and (iii) to try to determine the physiological basis for reduced virulence.

MATERIALS AND METHODS

Bacterial Strains

The organism of primary interest in this study was a strain of Yersinia enterocolitica serotype O:8 (E661) donated by D. A. Schiemann, Montana State University. This organism was originally isolated from a human case of yersiniosis and has been previously characterized as autoagglutination positive, calcium dependent for growth at 37°C on magnesium oxlate agar(95, 180), invasive for HeLa cells, able to produce conjunctivitis in guinea pigs and lethal for mice (194, 195, 196). Biochemically, this serotype belongs to Knapp and Thal (121) biotype 2, Nilehn (165) biotype 2, Wauters (G. Wauters, Ph.D. thesis, Vander, Louvain, Belgium) biotype 1, Brenner et al. (25) biotype 1, and Schiemann and Devenish (194) biotype I (Table 1). The organism was resistant to a sulfamethoxyazole and trimethoprim combination (190 ug/ml:10 ug/ml), but sensitive to various levels of 8 other antimicrobics (Table 2).

In addition, several other Yersinia enterocolitica serotypes (also donated by D. A. Schiemann) were used in some experiments. These included: O:3 (E752), O:13 (E887; isolated from a monkey), O:20 (Em062), O:21 (E750), O:4,32 (E759), and O:5,27 (E771). All of the enteropathogens

(except E887) were originally isolated from humans. The virulence characteristics of most of these isolates have been published previously (194, 195, 196).

Table 1. Biochemical characteristics of Y. enterocolitica O:8 (E661).

Test	Incubation Temperature °C	Reaction
Kligler iron agar	35	K/A no gas, H ₂ S ⁻
Christensen's urea agar	35	+
Ornithine decarboxylase	35	+
Arginine decarboxylase	35	-
Lysine decarboxylase	35	-
Indole	30	+
Xylose	22	+
Salicin	35	-
Esculin	22	-
Sorbitol	35	+
Voges-Proskauer	22	+
ONPG	35 or 22	-
Sucrose	22	+
Sorbose	35	+
Lecithinase	22	+
Rhamnose	22	-
Raffinose	22	-
Melibiose	22	-
α-Methylglucoside	22	-
Citrate	22	-
Motility	22	+
Motility	35	-

Salmonella typhimurium isolates were obtained from the State Laboratory of Hygiene, Madison, Wisconsin and from H. Lior, Canadian Laboratory Center for Disease Control, Ottawa, Ontario. Two of the enterotoxigenic Escherichia coli (ETEC) cultures were provided by M. Levine, The Center

for Vaccine Development, University of Maryland School of Medicine, and a third ETEC isolate was obtained from R.A.

Table 2. Minimal inhibitory concentration (MIC) of various antimicrobics for Y. enterocolitica O:8.

Antimicrobial	Minimal Inhibitory Concentration (ug/ml)	
	at 22°C	at 35°C
Nalidixic acid	2 (2) ^a	2
Rifampicin	4 (8)	8
Chloramphenicol	8 (16)	8
Nitrofuratoin	50 (100)	50
Sulfamethoxyazole/ Trimethoprim (19:1)	4 (>200)	>200
Spectinomycin	16 (32)	16
Tetracycline	2 (4)	2
Penicillin G	16 (>32)	32
Gentamicin	1 (6)	1

a) Numbers in parentheses are 72 h MIC values. All other values are 24 h standard MIC results (240).

Wilson, E. coli Reference Center, Pennsylvania State University. All three isolates were reported by Wilson to be colonization factor antigen positive. Shigella flexneri and S. sonnei were obtained from the University of Washington Clinical Laboratories, Seattle, Washington. The S. dysenteriae isolate was part of the Montana State University stock culture collection. The coliforms were isolated in our laboratory from drinking water using the membrane filter technique (6), and included five strains of E. coli, two stains of Klebsiella oxytoca, two strains of

Enterobacter cloacae, and one strain each of Enterobacter aerogenes and Citrobacter freundii.

All cultures were stored at -70°C in a solution of 10% peptone and 40% glycerol. For each experiment, cultures were taken from the frozen stocks and subcultured only once. Therefore, changes in the virulence of the enteropathogens would not be expected to occur due to repeated passage of the culture.

Preparation of Injured Cultures

Bacterial cultures were grown 24 h in 100 ml of tryptic soy broth without dextrose supplemented with 1% lactose and 0.3% yeast extract (TLY broth). All cultures were grown at 35°C except the Y. enterocolitica isolates which were grown at 22°C . Cultures were diluted to 1×10^6 cells/ml in cold sterile reagent grade water and chlorinated with varying levels of free available chlorine (prepared daily from stock solutions of chlorine bleach; sodium hypochlorite). Cells were chlorinated for 10-30 min at 4°C and pH 6.5-7.0 in the dark. In this test system, carry over of nutrients from the TLY broth (1/1000 dilution) created a chlorine demand of 1.0 ppm in 5 min and 1.7 ppm within 10 min (measured by the N,N diethyl-p-phenylenediamine colorimetric method). Therefore, in most cases injury resulted from a combination of free and combined chlorine. The system was designed to reproducibly

injure bacteria and not intended to simulate the natural environment. Thus, the chlorine levels used in this study may not be directly compared to other reports. Samples were dechlorinated using sodium thiosulfate (final concentration 0.01%) (6) which was added to unchlorinated control cultures as well.

When high densities of injured cells were needed (approximately 5×10^8 cells/ml), cultures were washed twice in cold sterile reagent grade water to remove the culture medium and chlorinated as described above.

The level of chlorine injury was measured by the percent difference in plate counts between a non-selective medium (TLY agar) and a selective medium (TLY agar plus 0.1% deoxycholate; TLY-D (153)). TLY and TLY-D plates were incubated at 35°C for all bacteria.

LD₅₀ Determinations

Differences in virulence between healthy and injured *Y. enterocolitica* were determined using LD₅₀ assays in CD-1 mice (Charles River Breeding Laboratory Inc., Wilmington, MA). Cultures were injected intraperitoneally (seven mice per dose). Mice were observed for up to 21 days. LD₅₀ calculations were made according to Reed and Muench (184). Statistical analyses were made using a generalized linear interactive modeling program (GLIM) on natural logarithmic transformed data (9, 66).

Tissue Culture Attachment Assay

The procedures for measuring association between enteropathogens and epithelial cells have been described previously (196). Henle 407 intestinal epithelial cells (ATCC CCL-6) were maintained in Eagles's Basal Medium with Hanks' Balanced Salts (BME) containing 15% fetal bovine serum, penicillin G (50 IU/ml) and streptomycin (50 ug/ml). The epithelial cells were grown in tissue culture flasks until the monolayer was confluent. On the day prior to use, fresh medium without antimicrobial agents was provided. Before use, the cells were washed twice with Dulbecco's phosphate buffered saline without calcium (PBS) and harvested by scraping the monolayer off the flask wall. The cells were suspended in BME and the number of viable cells was determined microscopically by trypan blue exclusion. The cell density of the suspension was adjusted to 5.6×10^3 /ml by dilution with BME.

Bacteria were grown at room temperature (22°C) for 48 h (Y. enterocolitica) or at 35°C for 24 h (E. coli and S. typhimurium) in 0.13 M phosphate buffered salts medium (BSYE) containing 20 uCi/ml [^{35}S] methionine (196).

Bacteria were washed in cold sterile reagent grade water and adjusted to 5×10^8 /ml. Cells grown in this medium were injured by chlorine as described above. Aliquots of the bacterial suspension (0.2 ml) were added to 1.8 ml of

the Henle cells to give a multiplicity of $10^4:1$ bacteria to epithelial cells.

The mixture was incubated at 35°C for 5 min. This short incubation time was chosen so that only the process of bacterial attachment would be examined assuming that 5 min was too brief for engulfment to occur. The contents of the tube were transferred to 100 ml of PBS and filtered through a 5 μm pore size Nuclepore membrane (Nuclepore Corp., Pleasanton, CA) that had been pretreated with 50 ml of 1 mM disodium (ethylenedinitrilo)tetraacetate (EDTA) in 2% peptone (pH 8.2). After filtration under gravity, an additional 100 ml of PBS was added to rinse the membrane. Each test was performed in triplicate. To test for non-specific adhesion of bacteria to the 5.0 μm Nuclepore filters, control suspensions containing bacteria alone were prepared and filtered in a similar manner.

The specific activity of the radio-labeled bacteria was determined by filtering a known amount of bacteria through a 0.4 μm Nuclepore filter and determining the radioactivity. Membranes were placed in scintillation vials containing 10 ml of Aquasol (New England Nuclear, Boston, MA) and radioactivity was determined by scintillation counting in a Tri-Carb 460 Liquid Scintillation System (Packard Instrument Co. Inc, Downers Grove, IL). Counts were converted to disintegrations/min by reference to an efficiency curve based on carbon-14.

The radioactivity per bacterial cell was calculated and used to determine the average number of bacteria attached to each Henle cell.

Microscopic Evaluation of Attachment

Attachment of enteropathogens was also observed by direct microscopic observations. Henle cell monolayers grown on cover slips were infected with healthy and injured bacteria (5×10^8 cells/ml) for 30 min at 35°C . Infected monolayers were fixed in methanol, stained with May-Grunwald and Giemsa stains and examined using a light microscope. At least 50 Henle cells from each preparation were observed.

Tissue Culture Invasiveness

Procedures for the HeLa cell infection technique have been described elsewhere (53, 194), HeLa cells (ATCC CCL-2) were maintained in Minimal Essential Medium (Eagle) (MEM) with Earle Salts plus 10% fetal bovine serum and 50 IU of penicillin G and 50 ug of streptomycin per ml. Washed cells were recovered in antibiotic-free MEM and the density of recovered HeLa cells was determined by microscopic counting in a Fuchs-Rosenthal chamber. The concentration was adjusted to 2×10^5 cells/ml and one milliliter of this suspension was added to plastic 16 x 125

mm tissue culture tubes. Each experiment was performed in triplicate.

Healthy and injured Y. enterocolitica O:8 (E661) were prepared as described above. After chlorine injury, bacterial cells were centrifuged and resuspended in BME to a density of 2×10^6 /ml. One ml of the bacterial suspension was added to the tissue culture tube containing the HeLa cells giving an approximate multiplicity of 10 bacteria per epithelial cell. The mixture was placed on a roller apparatus (Labline Instruments Inc., Melrose Park, N.J.) and incubated at 35°C for 2 h. Resuscitation of injured cells did not occur to any appreciable extent during this time period. After 2 h infection time, 0.1 ml of gentamicin solution (2.5 mg/ml) was added to each tube and tubes were reincubated under the same conditions. Since gentamicin does not cross the epithelial cell membrane (53, 194), it killed only the extracellular bacteria and prevented further infection. After this period, the tube was centrifuged (5500 g, 10 min, 20°C) and the supernatant removed. One ml of a 1.0% solution of N-lauroylsarcosine in physiological saline was added to lyse the epithelial cells. The contents of the three tubes were combined and plated out in triplicate on TLY agar. The average colony count was used to calculate the invasiveness index (intracellular bacteria per epithelial cell). Since it was a concern that sarcosine may have a deleterious

effect on intracellular injured Y. enterocolitica, HeLa cells were also lysed by homogenization (Virtis Company, Gardiner, New York) at 25,000 rpm and plated out as described above.

The effectiveness of gentamicin on extracellular bacteria in the presence of tissue culture cells was also examined. HeLa cells were prepared as described above and chilled to 4°C in an ice bath. Since HeLa cells are incapable of engulfing bacteria at 4°C (30), all the Y. enterocolitica remained extracellular. After 2 h, gentamicin (2.5 mg/ml) was added to each tube and the suspensions were reincubated at 35°C for 1 h. The tubes were centrifuged, the supernatant withdrawn, the epithelial cells lysed using sarcosine, and the contents plated out as described above.

Microscopic Evaluation of Invasiveness

The invasion of epithelial cells by Y. enterocolitica was also assessed by direct microscopic observations. Cover slips containing HeLa cell monolayers (ca. 1.7×10^5 cells/cover slip) were infected with healthy and injured bacteria (ca. 3.5×10^6 cells) for 2 h at 35°C. Infected monolayers were vigorously washed with PBS, fixed in methanol, stained with May-Grunwald and Giemsa stains (for 10 and 20 min, respectively) and examined using a light microscope at 1250 x magnification. The experiment was

performed in triplicate and the number of intracellular bacteria in 100 HeLa cells from each preparation was recorded.

Infected HeLa cells for transmission electron microscopy (TEM) were prepared according to the HeLa cell invasiveness procedure described above. Cells were fixed using 3% glutaraldehyde in 0.1 M potassium-sodium phosphate buffer (PSPB) and post-fixed using 2% osmium tetroxide in PSPB. Samples were dehydrated with ethanol (50%, 70%, 95%, 100%; three changes, 10 min each) and embedded in Spurr's resin. Embedded samples were thin sectioned using an ultramicrotome and stained with uranyl acetate and Reynold's lead citrate. Samples were examined using a JEOL model JEM-100 CX scanning-transmission electron microscope.

In Vivo Model of Invasiveness

The invasiveness of healthy and injured Y. enterocolitica was also evaluated using an in vivo model. Healthy and injured Y. enterocolitica (ca. 1×10^9 cells) were injected into intestinal loops of CD-1 mice. The animals were fasted 24 h before the experiment. Methoxyflurane (Metofane; Northwest Veterinary Supply, Great Falls, MT) was used to anesthetize the mice. Intestinal loops were made by ligating the intestine 3 cm below the pylorus, washing down the intestinal contents with 0.5 ml saline, and then adding another tie 5 cm below

the first. Each ligation was made in duplicate using cotton thread. Controls included a noninvasive strain of Y. enterocolitica 0:5. After an appropriate incubation period, the mouse was sacrificed, the intestinal loops excised, fixed in buffered formalin and prepared for histological examination using the "Swiss roll" technique (159).

Tissues were stained with a Giemsa stain and by direct and indirect immunofluorescence (224). Antiserum used in the indirect procedure to serotype 0:8 was provided by D. A. Schiemann. Fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG was purchased from Sigma Chemical Co. (St. Louis, Mo). FITC conjugated antiserum to serotype 0:5 was provided by L. Mann. The number of intracellular bacteria in 100 villi of each preparation was recorded.

Assimilation of Radio-labeled Compounds

Y. enterocolitica was grown and injured as described above. Cells were pelleted by centrifugation and resuspended in 4 ml of Scheusner's minerals salts medium (per liter: 7.0 g K_2HPO_4 , 3.0 g KH_2PO_4 , 0.1 g sodium citrate, 1.0 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, and 10.0 g glucose). One ml of the cell suspension was added to 1 ml of mineral salts medium containing 100 uCi per ml of either [^{35}S]methionine, [3H]uridine or [3H]thymidine. Samples were incubated at room temperature (22°C) for 2 h. Samples (0.2 ml) were withdrawn and filtered through a 0.4 um

Nucleopore filter. Filters were washed with 100 ml of PBS and placed in a scintillation vial containing 1 ml lysis buffer (50 mM Trizma, pH 12; 4% SDS, 10 mM EDTA). After at least 10 min incubation in the lysis buffer, 10 ml of Aquasol was added and the vials were counted using a Tri-Carb 460 Liquid Scintillation System. The assimilation rate of the compounds were calculated as $\text{cpm}/5 \times 10^6$ bacteria/min.

ATP and Oxygen Measurements

Adenosine triphosphate (ATP) levels in healthy and injured bacteria were determined by the luciferin-luciferase method using a Turner Designs model 20e Luminometer and Turner Designs reagents (Mountain View, CA). Healthy and injured cells were suspended in Scheusners mineral salts medium. The amount of ATP per cell was calculated from triplicate experiments according to the manufacturer's instructions.

Oxygen uptake measurements were performed using a Gilson model KM oxygraph equipped with a YSI model 4004 Clark-type electrode. The chamber of the instrument was maintained at 32°C. Tryptic Soy Broth containing 1.0% glucose (1.8 ml) was added to the chamber and allowed to equilibrate for 2 min, followed by 0.2 ml of a 2×10^9 cell suspension. The oxygen uptake rates were determined

graphically from the slope of the lines from triplicate experiments (54).

Effect of Metabolic Inhibitors on Invasiveness

The invasiveness of Y. enterocolitica treated with various metabolic inhibitors was examined using the HeLa cell roller tube technique described above. The inhibitors (chloramphenicol, tetracycline, spectinomycin, nalidixic acid, nitrofurantoin or rifampicin; for concentrations used see Table 5) were added to the bacterial suspension just before the bacteria were added to the HeLa cells. No significant loss of bacterial or epithelial cell viability occurred, due to the inhibitors, during the 2 h incubation period. Changes in the invasiveness of Y. enterocolitica treated with the inhibitors was compared to an untreated control.

Measurement of Hydrophobicity and Electric Charge

Changes in surface hydrophobicity and electric charge of chlorine-treated Y. enterocolitica were determined by the methods used by Schiemann and Swanz (196). Cultures of Y. enterocolitica were grown in BSYE broth 48 h at 22°C. Bacteria were centrifuged and resuspended in 4 M NaCl in 10 mM phosphate buffer (pH 7.2) at concentrations about 10^9 cells/ml. Salmonella and ETEC isolates were grown on CFA agar (per liter: 1.5 g yeast extract, 0.05 g $MgSO_4$, 0.005 g

MnCl₂, 10.0 g casamino acids and 20.0 g agar; pH 7.0) at 35°C for 24 h. Bacteria were washed off the plate using 4 M NaCl and adjusted to a concentration of about 10⁶ cells/ml. Sepharose CL-4B and phenyl-Sepharose CL-4B (Pharmacia, Inc. Piscataway, NJ) were washed five times with equal volumes of 4 M NaCl and added to Pasteur pipettes plugged with glass wool to achieve a column of 25-30 mm after settling. The bacterial suspension (0.1 ml) was layered on top of the column and 5 ml of 4 M NaCl was added for elution. The number of bacteria added to the column and the number of bacteria recovered in the eluate were determined by plate counts on TLY agar and the percent adherent to the column was calculated. Since it was a concern that the high salt buffer might stress the bacteria, the same experiments were repeated using 10 mM Tris (Trizma) buffer.

Hydrophobicity of healthy, injured and chlorine-killed cells was also determined by partitioning in an aqueous-hydrocarbon mixture (196). Cultures were grown as described above, but were washed twice with 0.85 % NaCl. The density of each cell suspension was adjusted photometrically to an absorbance (400 nm; path length 1 cm) of 1.2 to 1.4 (DMS 80 Spectrophotometer, Varian Associates, Mulgrave, Australia). Four ml of the bacterial suspension were added to an acid-washed glass test tube and 0.5 ml of the hydrocarbon (hexadecane) was added. The tube contents

were mixed on a vortex mixer for 1 min and then allowed to stand for 15 min. The decrease in the absorbance of the aqueous phase at 400 nm was recorded.

Attachment of the bacteria to DEAE-Sepharose was used as a measure of surface charge. Protocols for these experiments were similar to those for the phenyl-Sepharose chromatography except DEAE-Sepharose CL-4B (Pharmacia, Inc.) and 10 mM Tris buffer were used.

Outer Membrane Studies

Isolation of cell membranes was similar to the procedure of Achtman et al. (1). Three 3-liter batches of Y. enterocolitica were grown for 48 h at 22°C in TLY broth and concentrated by tangential flow filtration (Pellicon Cassette System, Millipore Corp, Bedford, MA) to approximately 50 ml. The cells were washed twice by centrifugation in 0.85 % NaCl. The first batch was set aside as an untreated control. The second batch was treated with 1 mg chlorine per ml for 30 min at 22°C; while the last batch was treated with 32 ug tetracycline and 32 ug rifampicin for 2 h at 22°C. The cells were then centrifuged and resuspended in 25 ml of 20 mM Trizma (pH 7.2), 1 mM EDTA, 0.5 mM MgCl₂, 1 ug/ml DNase-I, 1 ug/ml RNase-A (bovine pancreas) and 0.02 mM phenyl methyl sulfonyl fluoride (a protease inhibitor). The cells were passed three times through a Sorvall model RM cell

fractionator and centrifuged at 3,000 x g for 10 min to remove whole cells. The membrane fractions were collected by centrifugation at 50,000 x g for 1 h and the pellet resuspended in 20 ml sterile reagent grade water. Fractions were examined under 1250 x phase contrast microscopy to confirm presence of the membranes and absence of whole cells. Membranes were stored at -20°C until use.

The effect of the membrane preparations on the ability of Y. enterocolitica to attach to epithelial cells was examined using the Henle cell attachment assay. Approximately 0.05 ml of each membrane preparation (75 ug/ml dry weight of membrane material, final concentration) was added to a 2 ml suspension of Henle cells (1×10^5 cells/ml) and bacteria (5×10^8 cells/ml). Differences in the levels of bacterial attachment in samples treated with various membrane preparations were compared to an unamended control.

Changes in the biological activity of outer membranes of Y. enterocolitica after chlorination were measured by the ability of the membranes to block invasiveness of Y. enterocolitica in the tissue culture system. Approximately 0.05 ml of each membrane preparation was added to a 2 ml suspension of HeLa cells and bacteria (final concentration: 75 ug/ml dry weight of membrane material). The effect of the membrane preparation on the viability of HeLa cells was

examined microscopically by trypan blue exclusion after the 2 h incubation time.

Electrophoresis of Outer Membrane Proteins

Equal amounts (4.5 mg dry weight) of the three membrane preparations were sedimented for 30 min in a microcentrifuge, and the supernatant fluid discarded. The sediment was suspended in 1.8 ml of Triton X-100 buffer (10 mM Trizma (pH 8.0), 5 mM MgCl₂, and 2% Triton X-100) and allowed to incubate 20 min at room temperature. The outer membranes were washed two additional times in Triton X-100 buffer with the last time being resuspended in 500 ul electrophoresis buffer (127, 128). Samples (50 ul) were subjected to SDS polyacrylamide gel electrophoresis in a 10% gel (a 5% stacking gel) using a modified Laemmli discontinuous buffer system (127, 128). The following purified proteins were used as molecular weight standards: thyroglobulin (200,000), B-galactosidase (107,000), phosphorylase B (97,400), bovine serum albumin (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000). Gels were stained with Coomassie brilliant blue and analyzed using a Gilford Multi Media Densitometer interfaced to a Gilford System 2600 for data manipulation and storage. Tracings were drawn using a Hewlett Packard model 7225B plotter.

Statistical Analysis

Statistical analyses were performed using the MSUSTAT interactive statistical package developed by R. E. Lund, Montana State University. The GLIM program was run with the help of Dr. M. A. Hamilton, Department of Mathematical Sciences, Montana State University.

Materials and Reagents

Tryptic soy broth without dextrose, casamino acids, and yeast extract were obtained from Difco Laboratories, Detroit, MI. Eagle's Basal Medium with Hanks' Balanced Salts was obtained from Irvine Scientific, Santa Ana, CA. Minimal Essential Medium with Earle Salts and Dulbecco's phosphate buffer saline were obtained through Gibco Laboratories, Grand Island, N. Y. [³⁵S]methionine (specific activity, 400 Ci/mM) was obtained from New England Nuclear, Boston, MA. [methyl-³H]thymidine (specific activity, 63 Ci/mM) and [5,6-³H]uridine (specific activity 39 Ci/mM) were purchased from ICN Radiochemicals, Irvine, CA. Fetal bovine serum, chloramphenicol, tetracycline, spectinomycin, nalidixic acid, nitrofuratoin, rifampicin, gentamicin, penicillin G, streptomycin, n-hexadecane, DNase, RNase, phenyl methyl sulfonyl fluoride, Trizma and sarcosine were obtained from Sigma Chemical Co., St. Louis, MO.

RESULTS

Chlorine Injury

Coliforms serve as indicators of potable water quality by indicating the possible presence of enteric pathogens. It has been suggested that conditions which stress coliforms would also injure waterborne pathogens. Because there are no data to support this hypothesis, I investigated the relative susceptibilities of coliforms and pathogens to injury by chlorine. Data presented in Figure 1 show that 10 strains of coliform bacteria became injured (approximately 90% injury) when exposed to 0.25 to 0.50 mg/l chlorine. Decreases in viability for coliforms in this range of chlorine concentrations were usually less than 0.5 log. At higher chlorine concentrations (>0.75 mg/l) the bacteria rapidly died. Figure 2 shows the susceptibility of three strains of ETEC to injury by chlorine. For comparison, the shaded area (in this figure and the three following) indicates the range of chlorine concentrations causing injury to the coliform bacteria. Figure 2 demonstrates that ETEC show the same response to chlorine as the coliform group. The sensitivity of seven strains of S. typhimurium to chlorine-injury is shown in Figure 3. Higher chlorine doses were required to injure

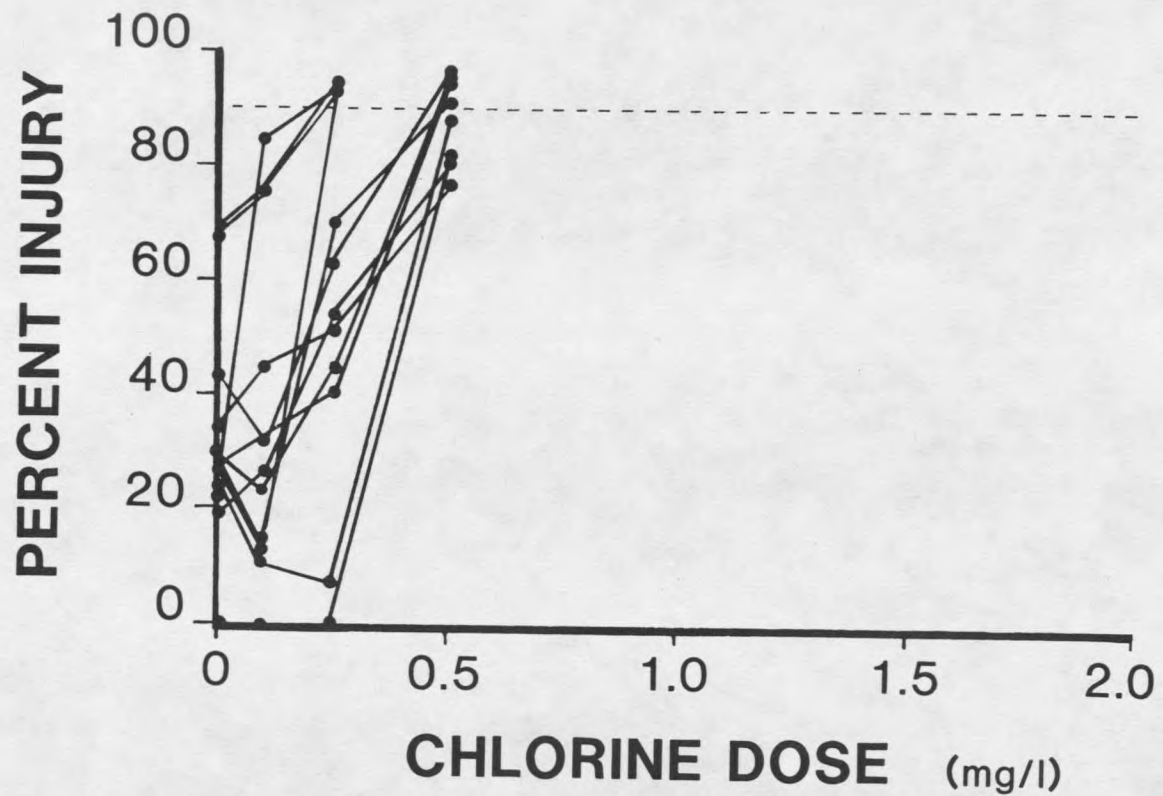


Figure 1. Susceptibility of 10 coliform strains to injury by chlorine. Bacteria were exposed to various levels of chlorine for 10 min at 4°C.

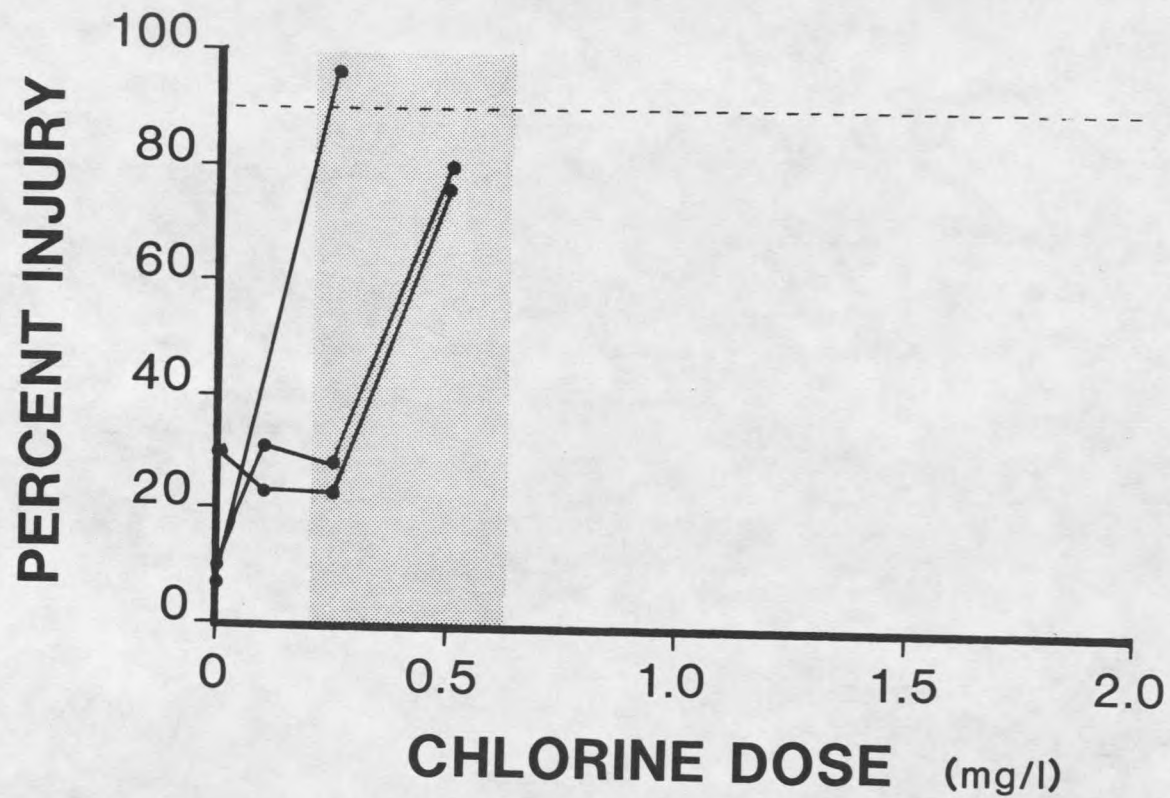


Figure 2. Susceptibility of three strains of enterotoxigenic *E. coli* (ETEC) to injury by chlorine. The shaded area represents the range of chlorine concentrations causing injury to coliform bacteria. Cells were exposed to various levels of chlorine for 10 min at 4°C.

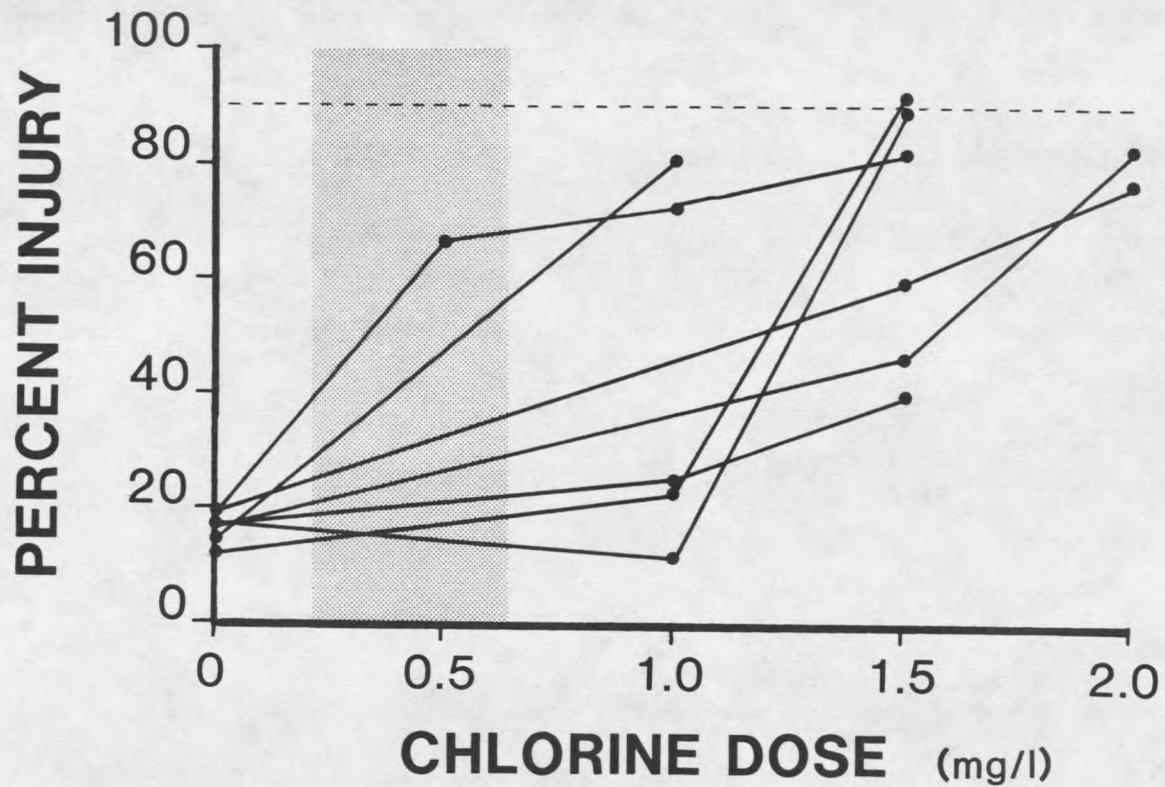


Figure 3. Susceptibility of seven strains of *S. typhimurium* to injury by chlorine. The shaded area represents the range of chlorine concentrations causing injury to coliform bacteria. Cells were exposed to various levels of chlorine for 10 min at 4°C.

Salmonella than the coliform bacteria. The most sensitive strain of S. typhimurium was more than twice as resistant to injury by chlorine than the most resistant coliform strain tested. A similar situation for Shigella is shown in Figure 4. One Shigella species (S. sonnei) was sensitive to TLY-D and exhibited nearly 70% injury without any treatment. This strain showed 89% injury when exposed to 1.5 mg/l chlorine for 10 min at 4°C with only a 0.6 log decrease in viability. Another species (S. flexneri) never demonstrated more than 41% injury (Figure 4). Although only three strains of Shigella were examined, the data indicate that these organisms are more resistant to chlorine-injury than are the coliform bacteria. Y. enterocolitica showed great variation in its susceptibility to chlorine-injury (Figure 5). Two strains (serotypes O:4,32 and O:5,27) were >90% injured at a chlorine dose of 0.5 mg/l. However, most of the Y. enterocolitica strains required greater than 1.0 mg/l chlorine to achieve the same level of injury (Figure 5).

Data from the previous figures were analyzed using the Mann-Whitney rank sum test for two independent samples, and the median, range and p statistics (for comparisons to the coliform group) are shown in Table 3. Significantly higher ($p < 0.05$) chlorine levels were required to injure populations of Y. enterocolitica, S. typhimurium and Shigella, (medians ranged from 1.00 to 1.50 mg/l chlorine)

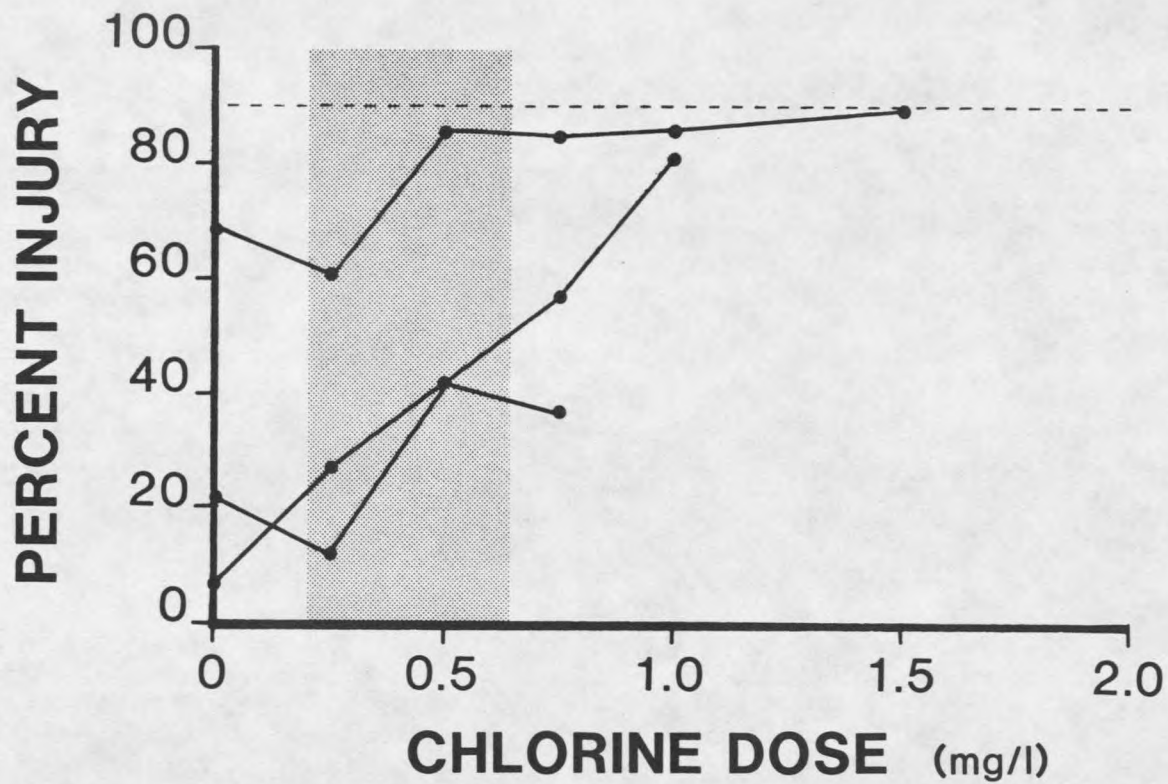


Figure 4. Susceptibility of three species of *Shigella* to injury by chlorine. The shaded area represents the range of chlorine concentrations causing injury to coliform bacteria. Cells were exposed to various levels of chlorine for 10 min at 4°C.

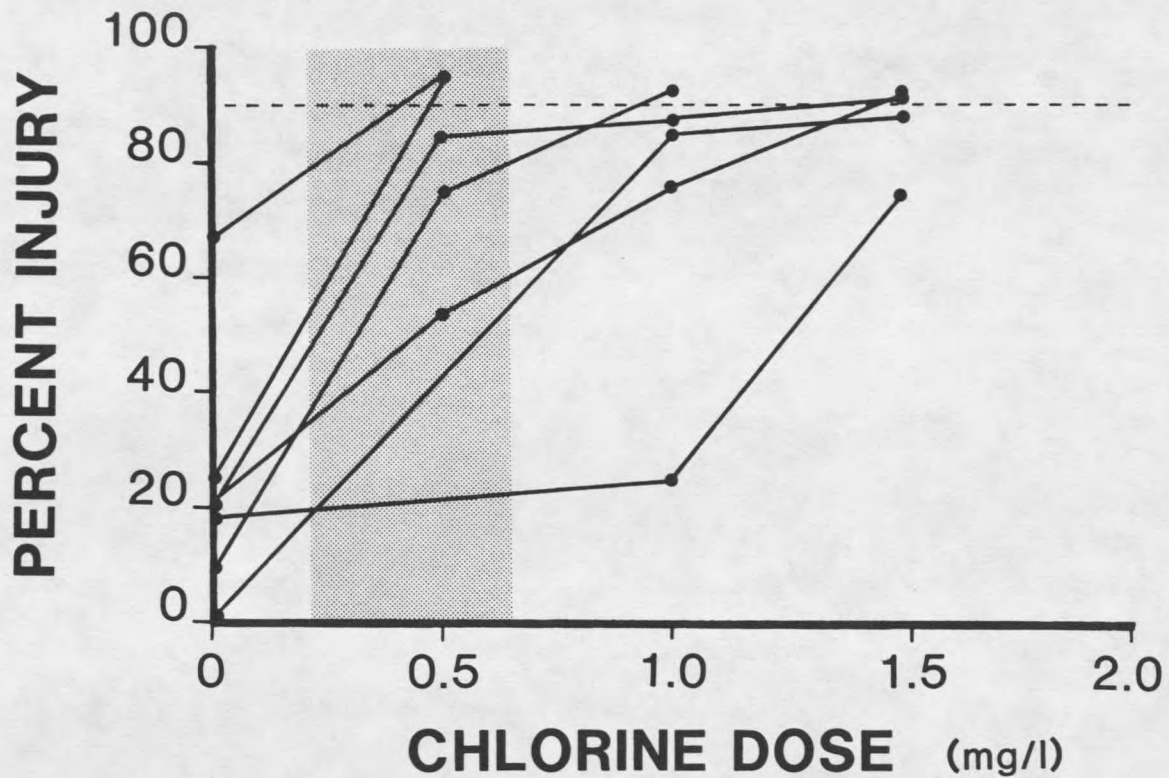


Figure 5. Susceptibility of seven strains of *Y. enterocolitica* to injury by chlorine. The shaded area represents the range of chlorine concentrations causing injury to coliform bacteria. Cells were exposed to various levels of chlorine for 10 min at 4°C.

Table 3. Susceptibility of Coliforms and Pathogens to Injury by Chlorine.

Organisms	# Of Strains Tested	Chlorine Concentration Causing Injury ^a (mg/l free chlorine)		p Value
		Median	Range	
Coliforms ^b	10	0.50	0.25-0.50	-----
ETEC ^c	3	0.50	0.25-0.50	0.540
<u>Y. enterocolitica</u> ^d	7	1.25	0.50-1.50	0.035
<u>S. typhimurium</u>	7	1.50	1.00-2.00	0.001
<u>Shigella</u> ^e	3	1.00	0.75-1.50	0.013

a) The concentration causing injury was calculated as the level of chlorine causing $\geq 90\%$ injury, or in cases where 90% injury was not achieved, the chlorine dose that caused the highest level of injury observed.

b) Coliform isolates included five strains of Escherichia coli, two strains of Klebsiella oxytoca, two strains of Enterobacter cloacae, and one strain each of Enterobacter aerogenes and Citrobacter freundii.

c) ETEC; enterotoxigenic Escherichia coli.

d) For a description of the serotypes used, see Materials and Methods.

e) Shigella species included S. dysenteriae, S. flexneri and S. sonnei.

than bacteria in the coliform group (median: 0.50 mg/l chlorine). The sensitivity of ETEC to injury by chlorine did not significantly differ ($p=0.540$) from that of the coliform bacteria. Coliform bacteria may be greater than 90% injured when Y. enterocolitica, S. typhimurium and shigellae are less than 20% stressed. These data indicate that coliforms may be injured (>90%) by chlorine while some pathogens remain unaffected.

LD₅₀ Assay

Comparison of the virulence for mice of healthy and injured Y. enterocolitica 0:8 (E661) is shown in Figure 6. Uninjured Yersinia had an LD₅₀ of 160 cells (95% confidence interval: 83-271), while chlorine-injured Y. enterocolitica (97% injured) showed an LD₅₀ 20 times higher (3300 bacteria; 95% confidence interval: 2150-5200). The curves were analyzed by a generalized interactive modeling program (GLIM) which showed that the slopes of the two lines were not significantly different (Figure 7). The GLIM program also showed that the two LD₅₀ values were significantly different ($p<0.001$). In fact, the deaths caused by the chlorine injured cells can be attributed to the 3% healthy cells in the population that were not injured (100 cells). These data suggest that injured Y. enterocolitica were avirulent in the mouse.

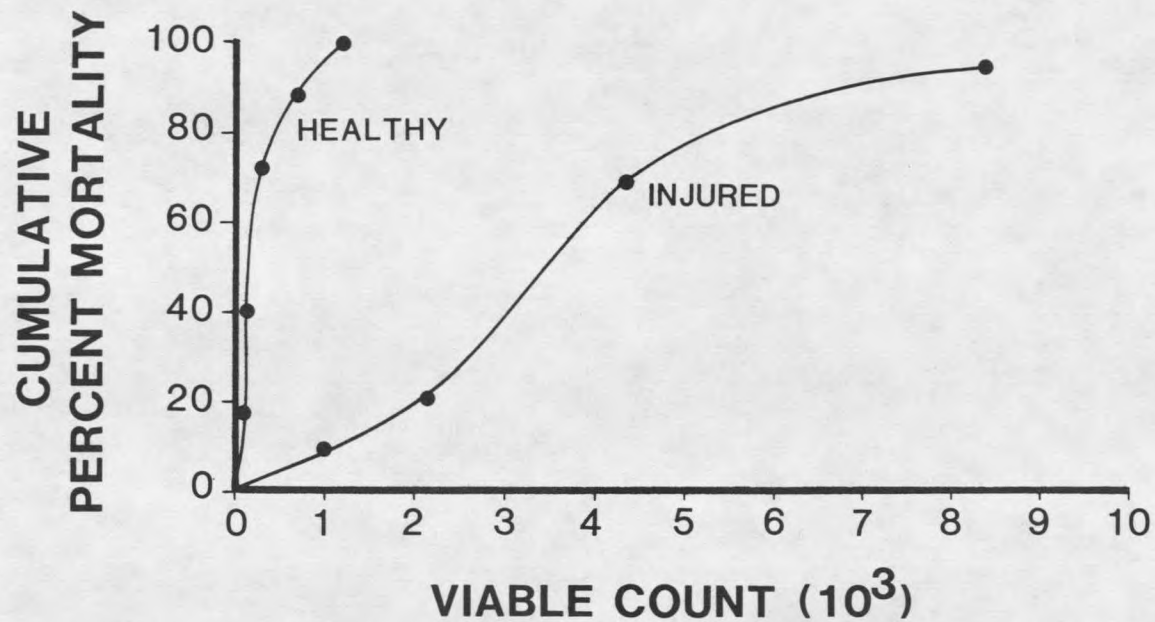


Figure 6. Comparison of the virulence of healthy and chlorine injured *Y. enterocolitica* O:8 (E661) in mice. The LD₅₀ value for healthy bacteria was 160 cells, while the LD₅₀ value for injured organisms was 3300 cells.

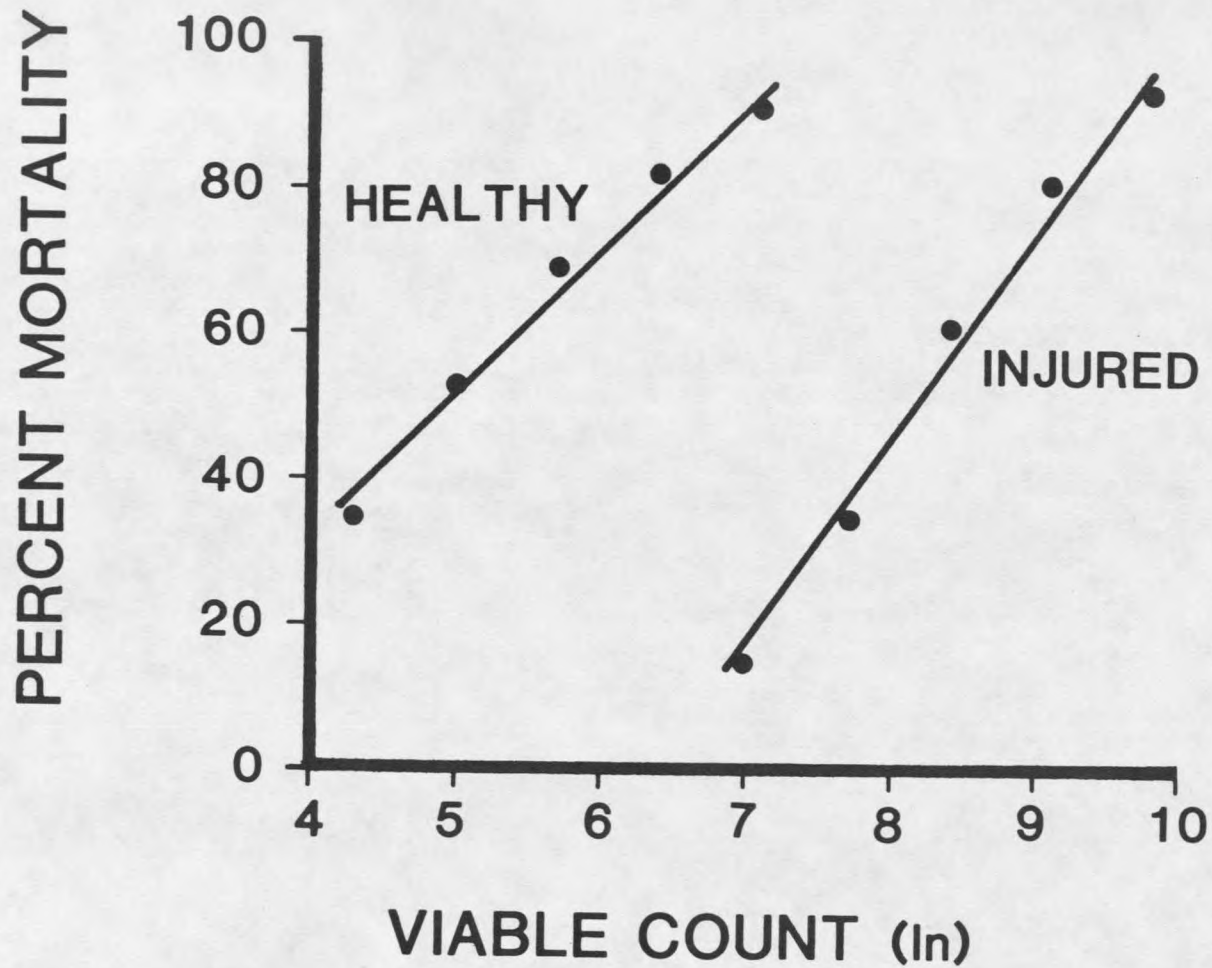


Figure 7. Evaluation of the LD₅₀ curves using a generalized interactive modeling program (GLIM). The slopes of the curves were not statistically different.

Y. enterocolitica 0:8 contains a virulence plasmid which is required for lethality for mice. No growth or pinpoint colonies on magnesium oxlate agar (MOX) at 35°C indicates calcium-dependency mediated by this plasmid. Chlorine injury did not alter the calcium-dependency of Y. enterocolitica 0:8 (>99% of the injured bacteria exhibited calcium-dependent growth on MOX agar). Thus, the decreased virulence of Y. enterocolitica could not be attributed to loss of the virulence plasmid.

Attachment Assay

Experiments were performed to determine which stage of the pathogenic process was affected by chlorine injury. Attachment to the host's epithelial cell surface is a requirement of all enteropathogenic bacteria. Data presented in Figure 8 indicate that injured Y. enterocolitica attached to Henle cells just as well as did healthy bacteria. Figure 8 also shows that chlorine-killed (column DC) Y. enterocolitica attached to epithelial cells at the same level as healthy bacteria. There were no significant differences between the control (0 mg/l chlorine) and any of the chlorinated samples.

These data are in contrast to results with healthy and injured ETEC and S. typhimurium (Figures 9 and 10). The figures show that with these enteropathogens, increasing levels of chlorine injury correlated with decreasing levels

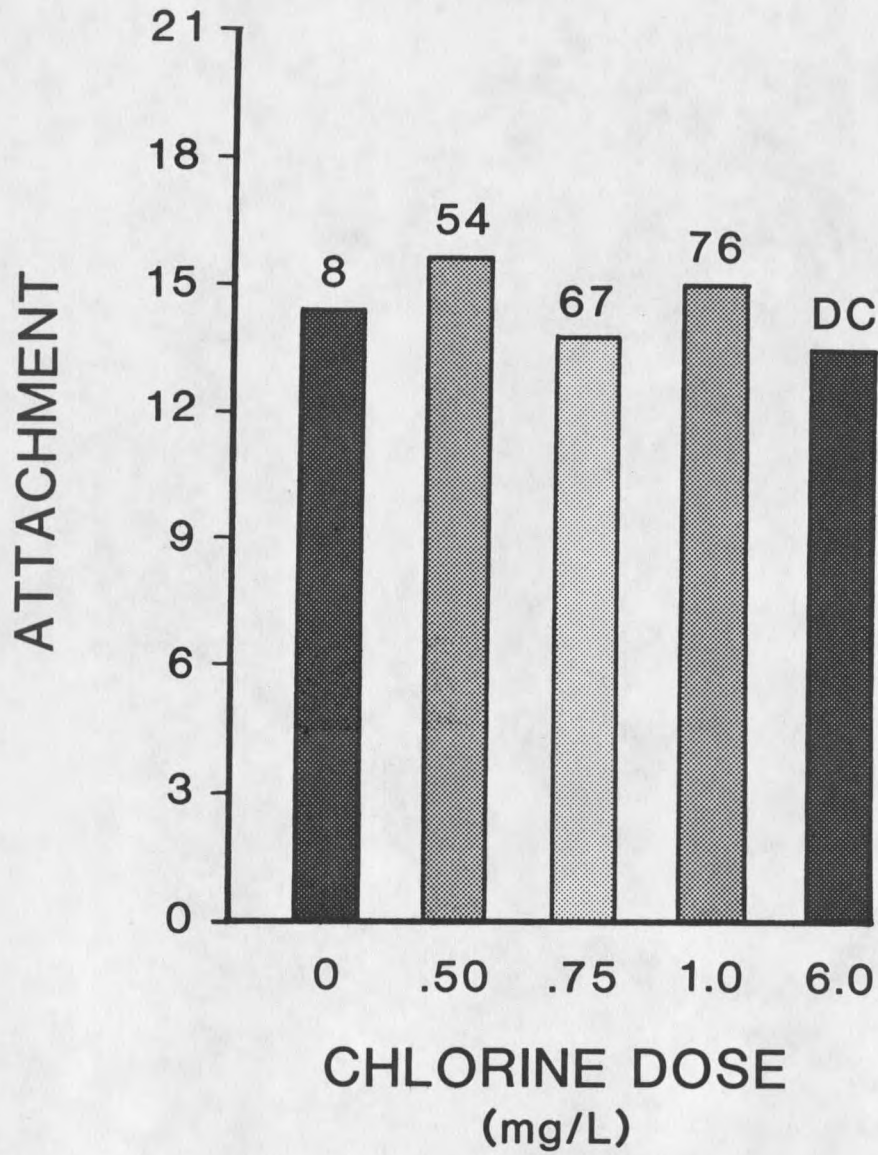


Figure 8. Attachment of *Y. enterocolitica* to Henle 407 intestinal epithelial cells at increasing chlorine doses. Attachment was measured as the average number of bacteria per Henle cell. Numbers at the top of the columns indicate percent injury. DC stands for dead cells.

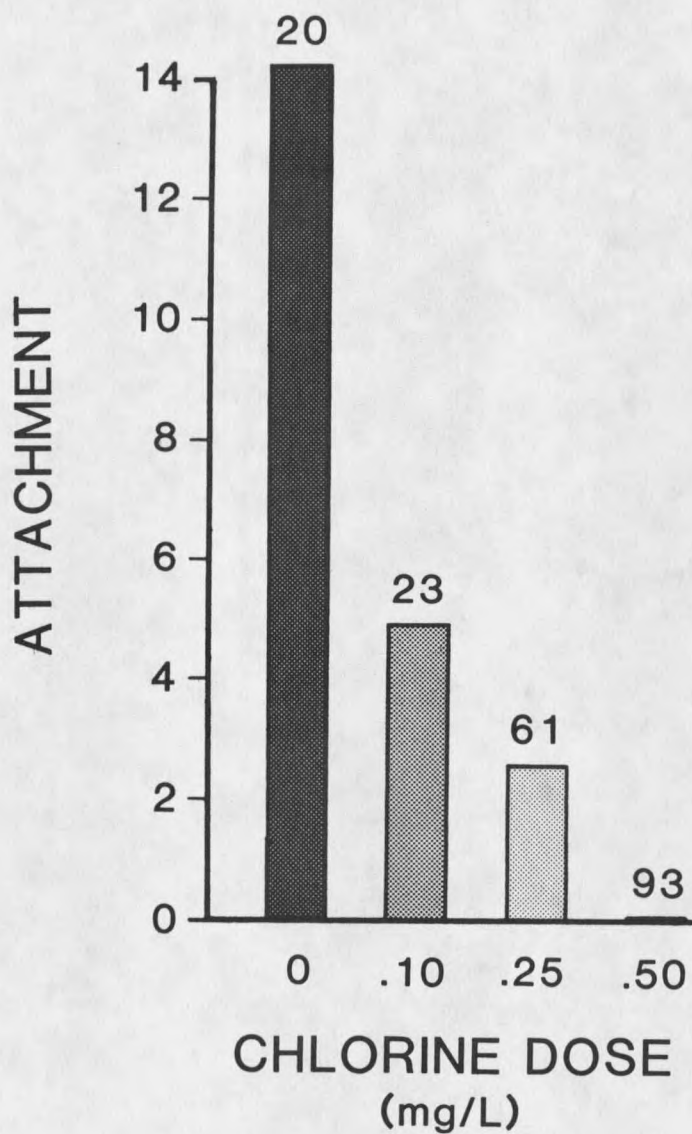


Figure 9. Attachment of enterotoxigenic *E. coli* (ETEC) to Henle 407 intestinal epithelial cells at increasing chlorine doses. Attachment was measured as the average number of bacteria per Henle cell. Numbers at the top of the columns indicate percent injury.

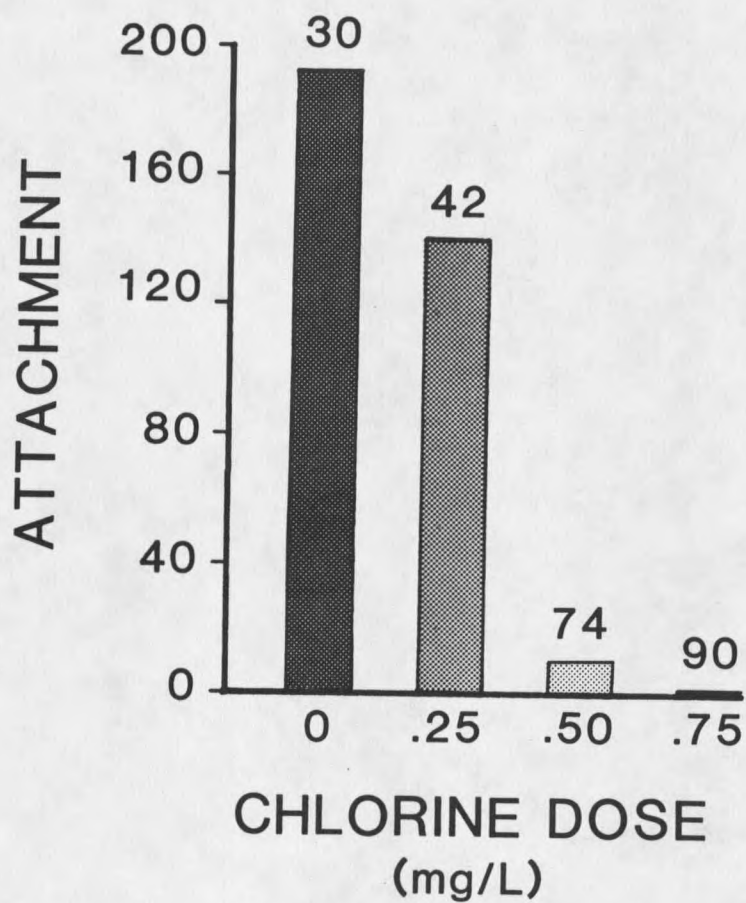


Figure 10. Attachment of *S. typhimurium* to Henle 407 intestinal epithelial cells at increasing chlorine doses. Attachment was measured as the average number of bacteria per Henle cell. Numbers at the top of the columns indicate the percent injury.

of attachment to Henle cells. In both instances when the pathogens were $\geq 90\%$ injured, no association was observed between bacteria and epithelial cells. Clearly, chlorine injury had a different effect on these two pathogens than that seen with Y. enterocolitica.

Examination of stained monolayers infected with healthy and injured ETEC and S. typhimurium confirmed the above observations (Figure 11). At least 50 cells were examined from each preparation. Uninjured cells attached at an average level of greater than 100 bacteria per epithelial cell (in many cases they were too numerous to count), while injured ETEC and S. typhimurium showed an average of 17 and 10 bacteria per Henle cell, respectively.

Invasiveness Assay

The second step required for virulence of Y. enterocolitica is the ability to invade epithelial cells. The interaction between pathogen and epithelial cell is dependent on multiplicity (the ratio of bacteria to HeLa cells). The effect of various multiplicities was examined using the roller tube system. A linear relationship between multiplicity and the number of intracellular bacteria was found for multiplicities between 2 and 10 (Figure 12). At values higher than 10 the curve began to plateau, indicating that the ability of the HeLa cells to

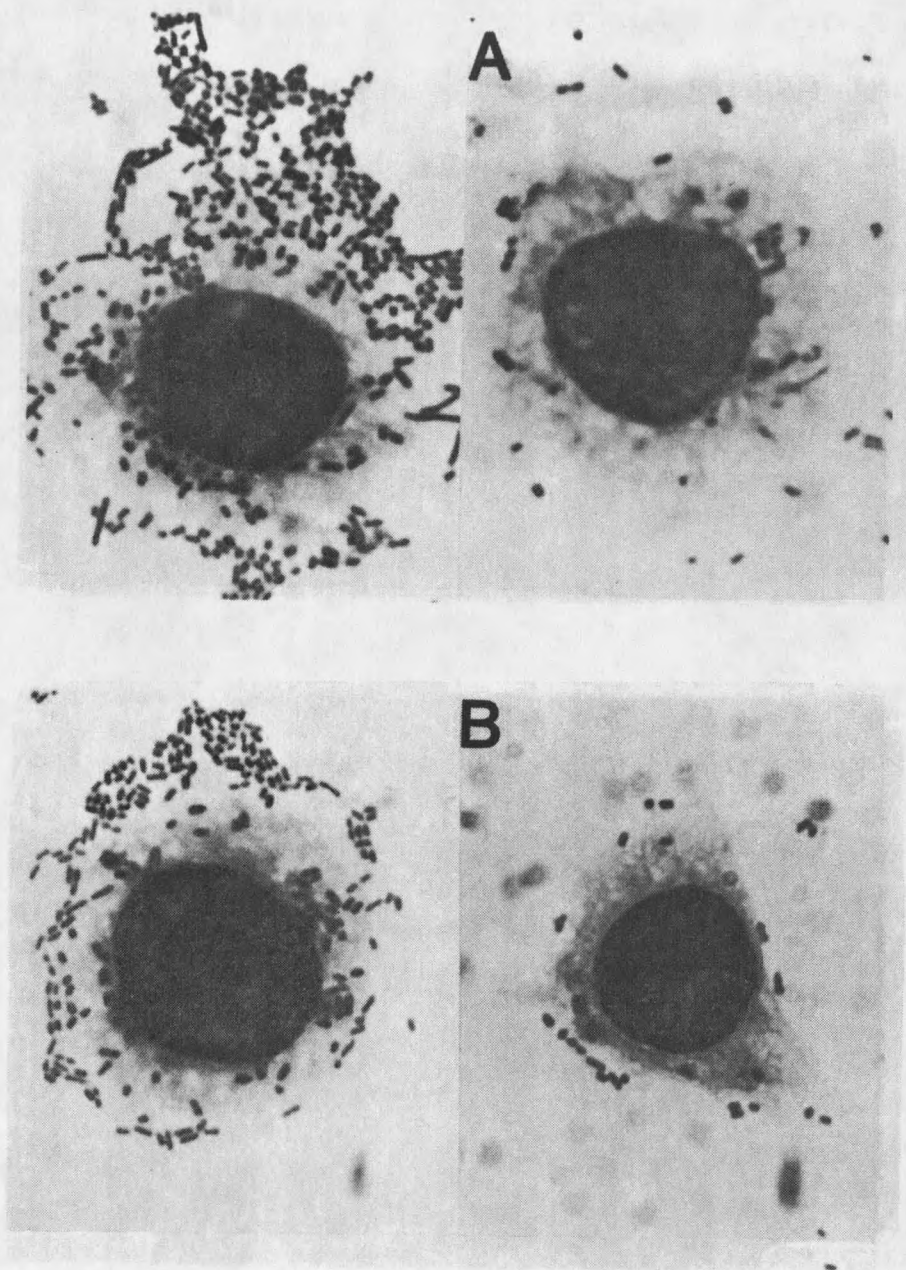


Figure 11. Photomicrographs of enterotoxigenic *E. coli* and *S. typhimurium* attached to Henle cells. (A) healthy (left) and injured (right) enterotoxigenic *E. coli* (B) healthy (left) and injured (right) *S. typhimurium*.

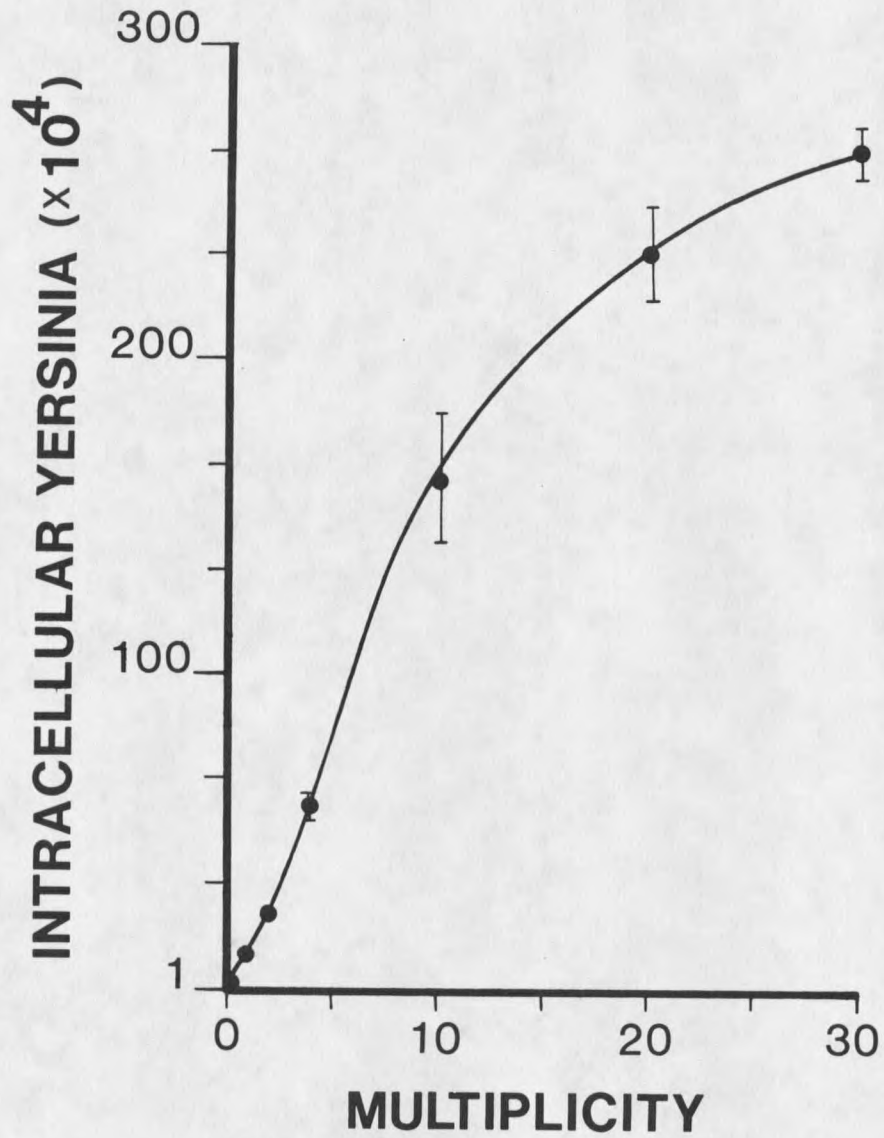


Figure 12. Effect of multiplicity on the invasiveness of *Y. enterocolitica* in HeLa cells. A constant number of HeLa cells (2×10^5) were used in each sample.

engulf bacteria had become saturated. These data indicate that a finite number of Y. enterocolitica 0:8 (approximately 30) can invade an epithelial cell under the conditions used.

The invasiveness of healthy and injured Y. enterocolitica 0:8 (93% injury) measured by the roller tube technique is compared in Figure 13. With this procedure, healthy Y. enterocolitica averaged 1.15 bacteria per HeLa cell, whereas injured cells showed an invasiveness index of only 0.01 bacteria per epithelial cell, a difference that was statistically significant ($p < 0.01$). Multiplicities for these experiments averaged 10.9 ± 3.4 for healthy cells, and 16.2 ± 6.2 for injured cells (5 replicate experiments each). The difference in invasiveness observed with injured Y. enterocolitica could not be attributed to multiplicity since multiplicities were higher for injured cells than for healthy bacteria.

Gentamicin effectively killed extracellular bacteria in the HeLa cell roller tube system, reducing background counts by 4.6 ± 0.3 logs. Experiments where the HeLa cells were chilled to prevent engulfment showed a reduction of 4.3 ± 0.9 logs. The effectiveness of gentamicin on Y. enterocolitica, therefore, was not influenced by the presence of epithelial cells.

During the injury procedure some bacteria died as a result of exposure to chlorine. Decreases in viability

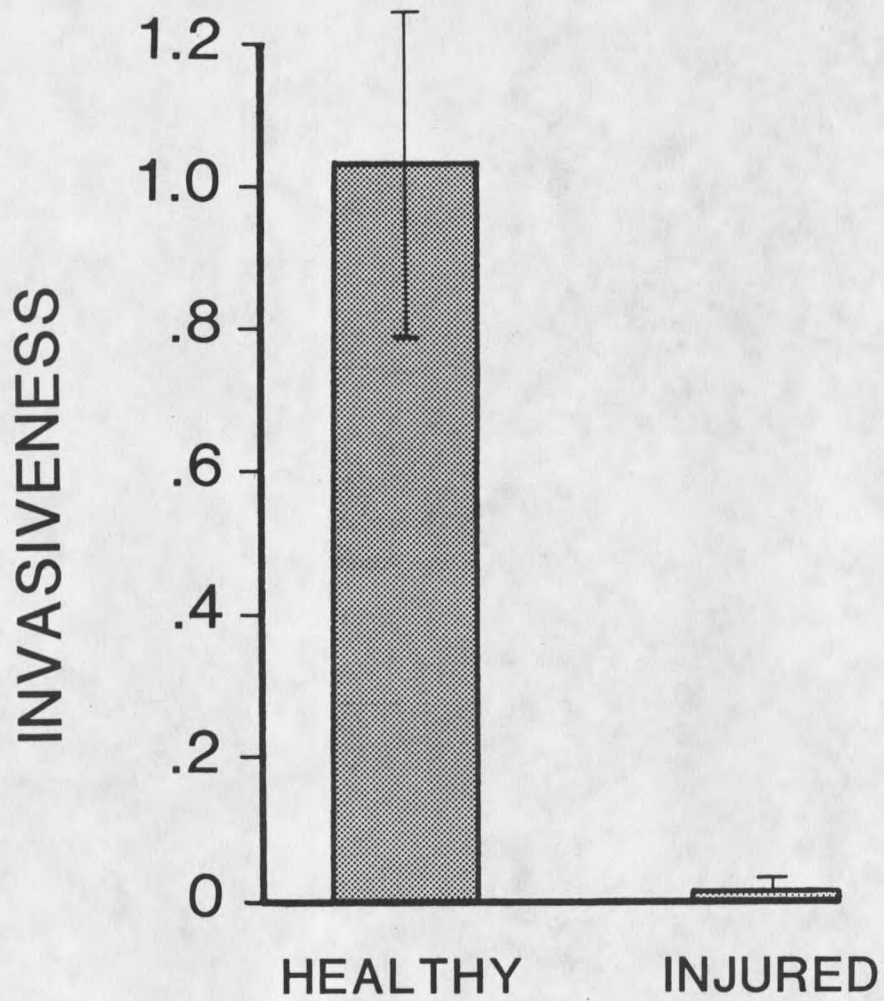


Figure 13. Invasiveness of healthy and injured *Y. enterocolitica* with HeLa cells. Invasiveness was measured as the average number of intracellular bacteria per HeLa cell.

were as high as 50% in some experiments. It was a concern that these dead cells might inhibit the infectivity of the injured population. Experiments were conducted using varying ratios of chlorine killed and uninjured Y. enterocolitica (ratios of 0.8:1, 1.6:1 and 8:1; dead to live cells). The results indicated that chlorine-killed bacteria did not interfere with the invasiveness of healthy cells in the concentration ranges used. The invasiveness index for this experiment was 6.6 for the uninjured control cells, 5.8 for a ratio of 0.8:1 (dead to live cells), 6.8 for 1.6:1 and 7.1 for a ratio of 8:1.

The invasiveness of Y. enterocolitica cells resuscitated in TLY broth after chlorine injury was also investigated. Cells were injured (99%) as described in the materials and methods and then resuscitated in TLY broth for 3 h at 22°C. Results of these experiments indicated that after the 3 h incubation, Y. enterocolitica repaired the chlorine induced lesion and regained their infectivity. The invasiveness indexes for healthy and resuscitated cells were 0.31 and 0.32, respectively.

Differences in the methods used to disrupt epithelial cells did not affect the results of the invasiveness experiments. Lysis of HeLa cells infected with injured Y. enterocolitica by homogenization or with sarcosine yielded intracellular bacteria at 1.4% and 0.4% (respectively) of the healthy cell level.

Microscopic Evaluation of Invasiveness

The invasion of epithelial cells by Y. enterocolitica was also assessed by direct light microscopic observations (Figure 14). Healthy bacteria infected 65% of the HeLa cells, while only 13% of the epithelial cells inoculated with chlorine-injured bacteria showed intracellular Y. enterocolitica. The average number of intracellular bacteria per HeLa cell was 2.02 and 0.17 for healthy and injured Y. enterocolitica, respectively.

Examination of HeLa cells by transmission electron microscopy showed that most of the cells infected by uninjured Y. enterocolitica contained intracellular bacteria while most of the preparations infected with injured cells (73% injury) lacked evidence of intracellular organisms (Figure 15). Six intracellular Y. enterocolitica can be observed in the cytoplasm of the HeLa cell in Figure 15a. Bacteria were never observed in the nucleus of the cells. The invasion process of Y. enterocolitica into HeLa cells was observed by transmission electron microscopy. Bacteria were shown to attach and be engulfed by microvilli of the HeLa cell (Figure 16). Eventually, the microvilli surrounded the bacterium and the pathogen was enclosed in a vacuole in the cytoplasm of the HeLa cell (Figure 17). Bacteria did not appear to be multiplying inside of the epithelial cells (Figure 17).

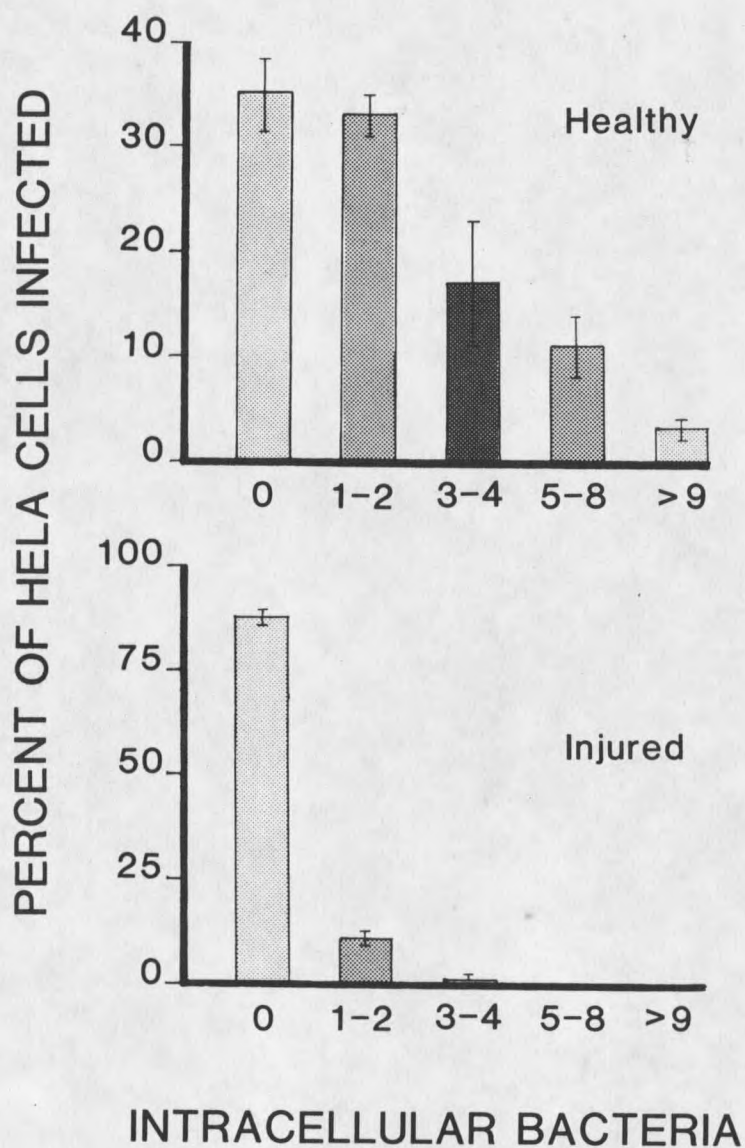


Figure 14. Microscopic evaluation of the invasiveness of *Y. enterocolitica* in a HeLa cells monolayer. The number of intracellular bacteria in 100 epithelial cells from triplicate experiments were recorded.

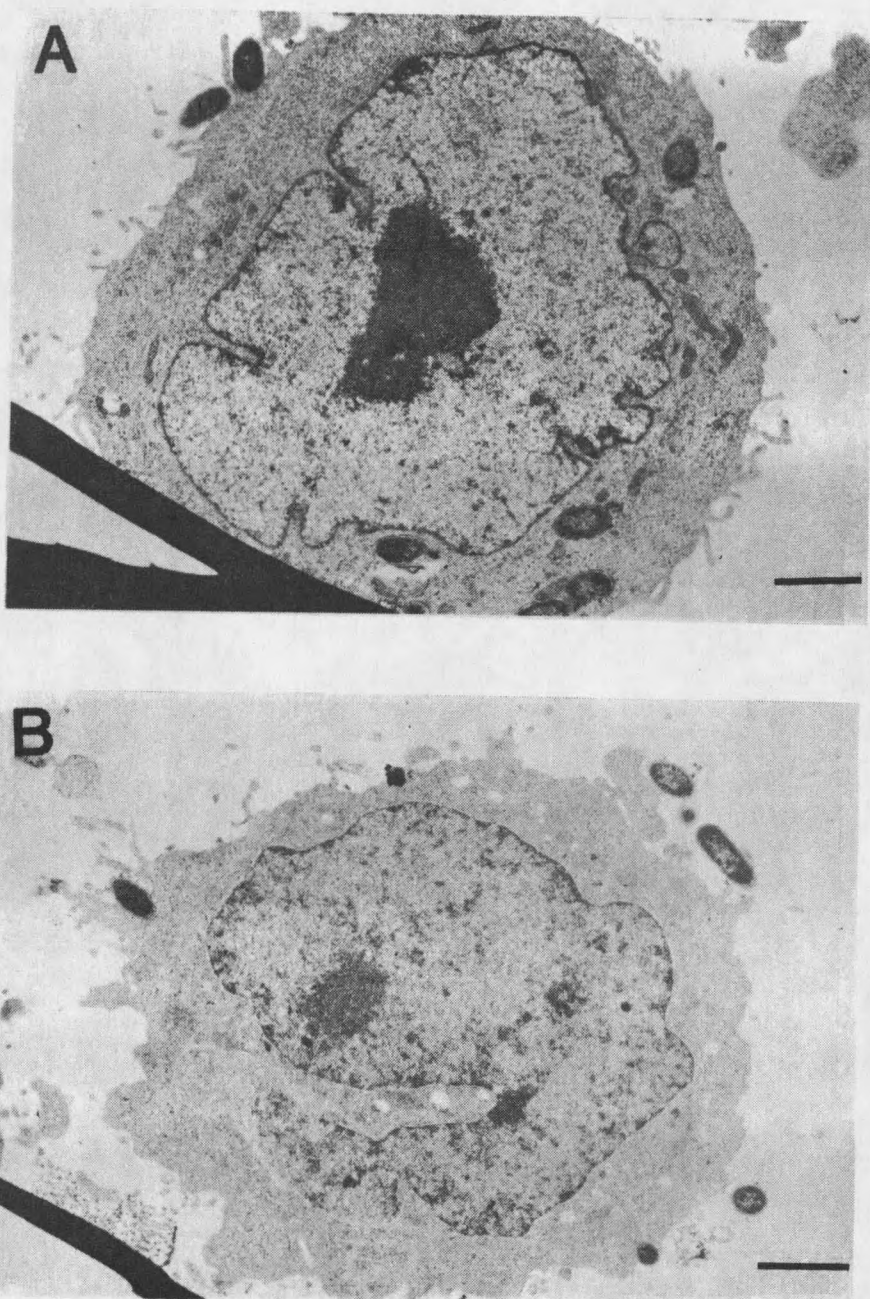


Figure 15. Transmission electron micrographs of HeLa cells infected with *Y. enterocolitica*. (A) healthy and (B) injured *Y. enterocolitica*. Bars indicate 2 μ m.

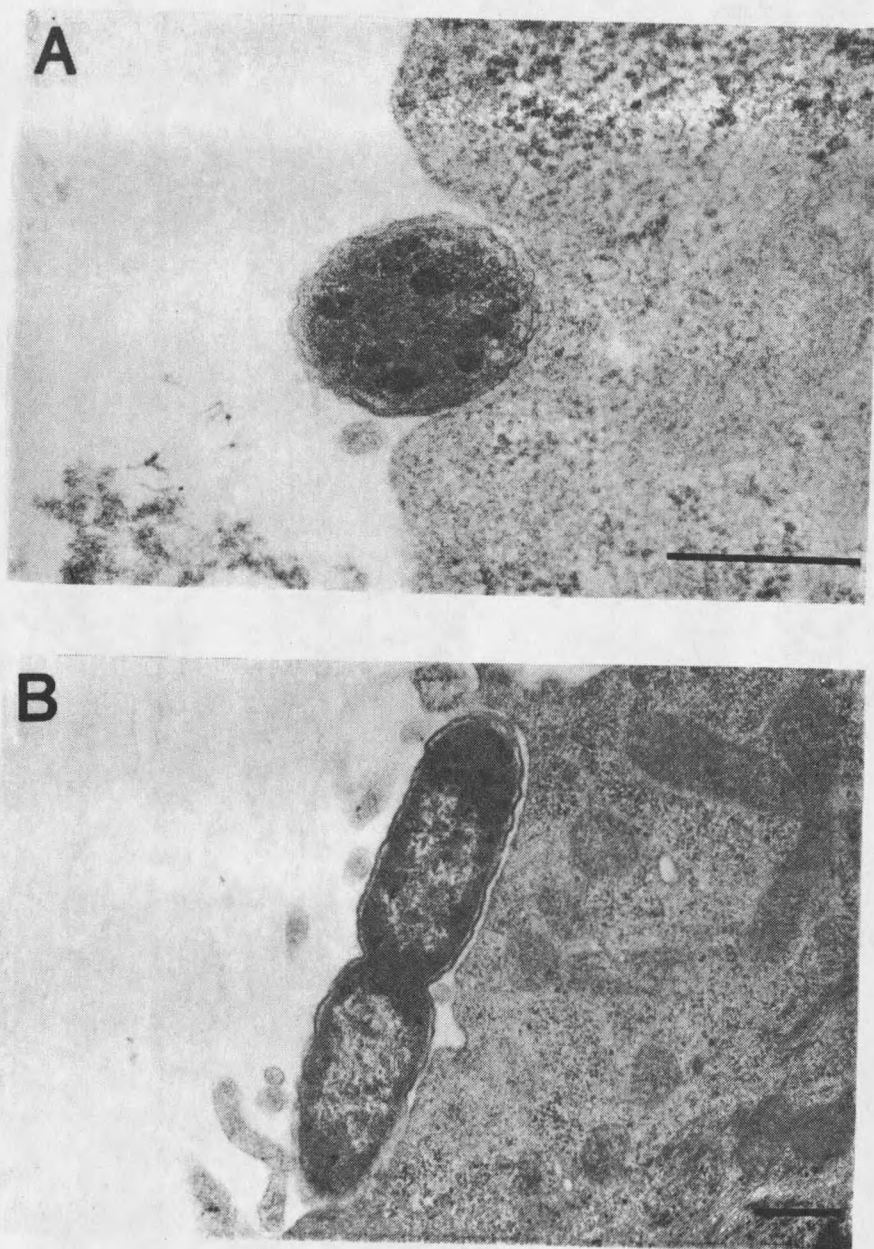


Figure 16. Initial phase of engulfment of *Y. enterocolitica* attached to HeLa cells. (A) A bacterium stimulates a depression in the cytoplasmic membrane of the tissue culture cell. (B) Microvilli start to engulf a bacterium. Bars indicate 0.5 μm .

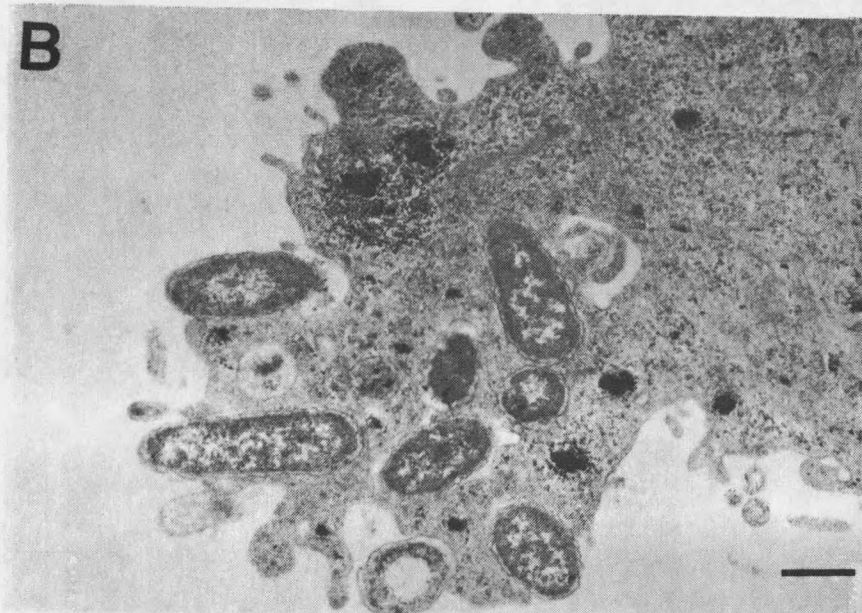
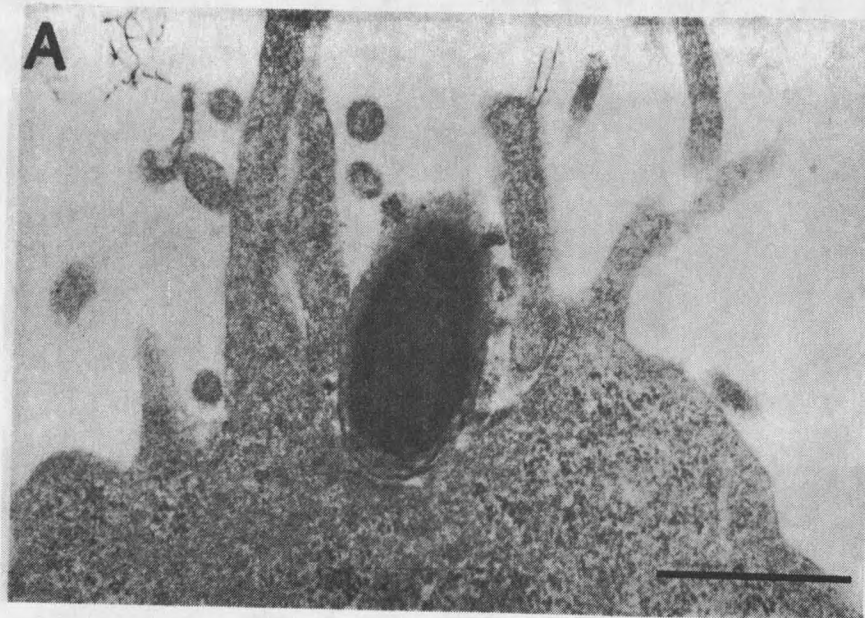


Figure 17. Engulfment of *Y. enterocolitica* by a HeLa cell. (A) A bacterium nearly engulfed by microvilli. (B) Vacuoles containing *Y. enterocolitica* in a HeLa cell. Bars indicate 1.0 μm .

In vivo Model of Invasiveness

The invasiveness of healthy and injured Y. enterocolitica was examined using an in vivo model. Cells were injected into intestinal loop segments of mice, incubated for various time intervals, fixed, mounted, stained using immunofluorescence techniques, and examined microscopically for intracellular bacteria. Because of the high cell densities used in these experiments, injury averaged only 50%. Data presented in Figure 18 indicate that intracellular healthy bacterial levels averaged 50% higher than the injured cells (significantly different at $p < 0.05$). A non-invasive strain of Y. enterocolitica showed low intracellular counts. Histologically, a greater immune response (accumulation of monocytes and neutrophils in the lamina propria, submucosa and musculature of the intestine) was seen in tissues infected with healthy cells than in tissues infected with injured cells. With tissues infected for 2 h, the majority of bacteria were located in vacuoles in epithelial cells of the villi. At 6 h, bacteria were still found in the villi, although Y. enterocolitica could be observed in the lamina propria and submucosa of the intestine. After 18 h post-infection, Y. enterocolitica were observed primarily in the submucosa, the musculature and Peyer's patches, with relatively few bacteria remaining in the villi.

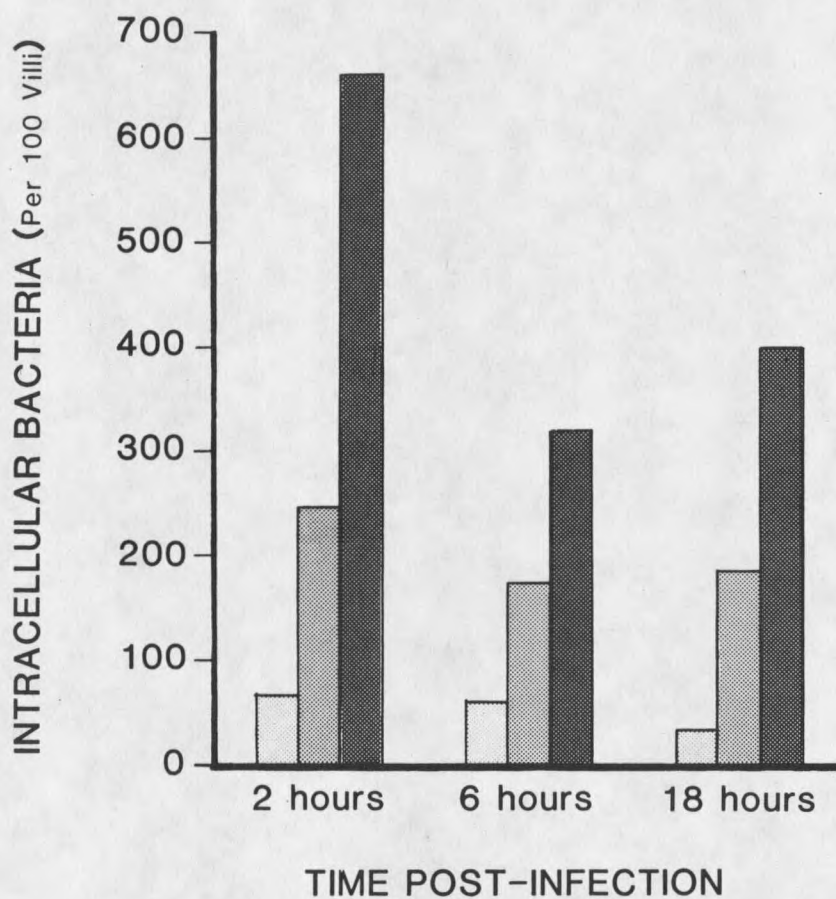


Figure 18. Enumeration of intracellular *Y. enterocolitica* in infected mouse intestine. The density of intracellular bacteria in 100 villi from each preparation was recorded. The number of intracellular *Y. enterocolitica* was significantly higher with healthy than injured bacteria. Lightly shaded column, non-invasive strain; medium shaded column, injured *Y. enterocolitica*; dark column, healthy cells.

Physiology of Chlorine-Injured *Y. enterocolitica*

Previous studies have shown that chlorine-injured coliforms have reduced metabolic and synthetic capabilities (42, 83, 154, 232). Experiments were initiated to determine if these studies could be extended to *Y. enterocolitica*. Adenosine triphosphate (ATP) levels in healthy and injured cells were determined by the luciferin-luciferase method. Injured *Y. enterocolitica* (85% injury) had ATP levels 91.7% lower than those found in uninjured cells (1.02×10^{-13} g ATP/cfu and 8.4×10^{-15} g ATP/cfu for healthy and injured *Y. enterocolitica*, respectively) (statistically significant at $p < 0.05$).

Oxygen uptake by healthy and injured *Y. enterocolitica* was measured using a Gilson oxygraph. Healthy bacteria respired at a rate of 1.11 ug O_2 /min/ 10^9 cells while injured *Yersinia* (96.7% injury) utilized 0.59 ug O_2 /min/ 10^9 cells (significantly different at $p < 0.01$)

The assimilation of radio-labeled thymidine, uridine and methionine, was used as an indicator of DNA, RNA and protein synthetic activity. Table 4 shows that injured *Y. enterocolitica* had significantly reduced assimilation of thymidine, uridine and methionine ($p < 0.05$) compared to uninjured bacteria. Cells were suspended in a glucose mineral salts medium (plus radio-label) during the 2 h time course of this experiment. Injured cells were incapable of

repairing the chlorine-induced cellular lesion(s) in this medium.

Table 4. Assimilation of various compounds by healthy and injured Y. enterocolitica.

Compound	Specific Activity (cpm/5 x 10 ⁶ cfu/min) ^a		
	Healthy	Injured ^b	% Difference ^c
Thymidine	43.0 ± 5.5	0.5 ± 0.1	99
Uridine	381 ± 0.1	26.3 ± 25.8	93
Methionine	5915 ± 900	351 ± 192	94

a) Values are ± standard deviation (SD).

b) Average injury caused by chlorination was 80%.

c) All values were significantly different (p < 0.05).

Effect of Metabolic Inhibitors on Invasiveness

The invasiveness of Y. enterocolitica treated with various metabolic inhibitors is shown in Table 5. Inhibitors of RNA (rifampicin) and protein synthesis (chloramphenicol, tetracycline, spectinomycin) significantly (p < 0.05) blocked the invasiveness of Y. enterocolitica into HeLa cells. Nalidixic acid significantly reduced the infectivity of Y. enterocolitica, but nitrofurantoin, also an inhibitor of DNA initiation, did not affect invasiveness. The concentration of antimicrobics used were above the MIC for Y. enterocolitica (see Table 2). These antimicrobics were chosen because

each is bacteriostatic, blocking a particular cellular process, and did not significantly affect bacterial or epithelial cell viability during the 2 h contact time. Bacterial densities before treatment with antimicrobics were $7.7 \pm 2.5 \times 10^5$, whereas viable counts after the 2 h contact time was $1.0 \pm 2.8 \times 10^6$. Epithelial cells grew equally well in tissue culture media with or without the antimicrobics.

Table 5. Effect of metabolic inhibitors on invasiveness of Y. enterocolitica.

Compound	Conc. per ml	Percent of Control Value \pm SD
<u>PROTEIN INHIBITORS</u>		
Chloramphenicol	32 ug	47 \pm 2.8*
Tetracycline	32 ug	24 \pm 1.6*
Spectinomycin	32 ug	21 \pm 4.9*
<u>DNA INHIBITORS</u>		
Nalidixic Acid	32 ug	38 \pm 11.8*
Nitrofuratoin	100 ug	100 \pm 10.2
<u>RNA INHIBITORS</u>		
Rifampicin	32 ug	15 \pm 9.2*

* Difference was statistically significant at $p < 0.05$.

Effect of Chlorination on Hydrophobicity and Electric Charge

Changes in surface hydrophobicity and electric charge of chlorine treated Y. enterocolitica were analyzed using

four approaches (Table 6). Partitioning in an aqueous-hydrocarbon mixture and attachment to phenyl-Sepharose (in 4 M NaCl or 10 mM Tris) were used to determine changes in surface hydrophobicity. A large decrease in absorbance of the aqueous phase (hexadecane method) or a large retention on the phenyl-Sepharose column indicates hydrophobic surface characteristics. Attachment to Sepharose alone was considered as an indication of non-specific adhesion. Results presented in Table 6 indicate that the hydrophobicity of Y. enterocolitica did not change in injured cells. However, chlorine-killed Y. enterocolitica did become significantly more hydrophobic. Healthy, injured and chlorine-killed S. typhimurium and ETEC showed no differences in hydrophobicity (Table 6). Some change was noted between healthy and injured S. typhimurium measured by phenyl-Sepharose 4 M NaCl chromatography, but this difference was thought to be due to the packing of the column and was inconsistent with the rest of the data for this organism.

Changes in the interaction of bacteria and DEAE-Sepharose are thought to be an indication of the surface charge of the organism (196). Once again, no difference in the attachment of healthy and injured pathogens to DEAE-Sepharose was observed (Table 6).

Table 6. Surface characteristics of healthy, injured, and chlorine-killed pathogens.

Organism	Condition	Hexadecane ^b	Percent Adsorption ^a				
			Phenyl Sephacrose 4 M NaCl	Sephacrose 4 M NaCl	Phenyl Sephacrose 10 mM Tris	DEAE Sephacrose 10 mM Tris	Sephacrose 10 mM Tris
<u>Y. enterocolitica</u>	Healthy	44.3 ± 5.3	91.6	20	70.9	99.6	12.5
	Injured	46.6 ± 2.8	90.3		79.5	99.9	
	Dead	82.4 ± 2.9					
<u>S. typhimurium</u>	Healthy	69.9 ± 7.1	37.6	6	99.7	97.0	21.7
	Injured	69.8 ± 13.7	74.4		99.7	97.1	
	Dead	76.8					
<u>E. coli</u> (ETEC)	Healthy	34.5 ± 5.1	78.3	0	97.8	99.8	77.4
	Injured	32.7 ± 5.8	62.0		99.4	99.6	
	Dead	33.4					

a) Percent of bacteria absorbed to different Sepharose columns.

b) Values are percent decrease in absorbance at 400 nm in the aqueous phase after separation of the aqueous-hexadecane mixture.

Outer Membrane Studies

Hydrophobic and electric charge experiments measure gross changes in surface characteristics. It was possible that chlorine-injury changed specific structures or functions of the bacterial membranes. To test this hypothesis, membranes from healthy, chlorinated and antimicrobial treated Y. enterocolitica were isolated and tested for their ability to block the invasiveness of normal cells.

Initially, various concentrations of membranes were screened for the one that gave optimal blocking of tissue culture invasiveness. This concentration was found to be 75 ug/ml dry weight (final concentration). The effect of this concentration of membranes on the ability of Y. enterocolitica to attach to epithelial cells is shown in Figure 19. No significant difference was seen between the unamended control and any of the samples treated with the membrane preparations.

Untreated membrane preparations blocked nearly 90% of the invasiveness of Y. enterocolitica in the HeLa cell roller tube system compared to controls in which no membranes were added (Figure 20), while chlorinated membranes showed only 29% blocking. Thus, chlorine changed the ability of the membranes to bind to the HeLa cells and block the receptor responsible for triggering engulfment.

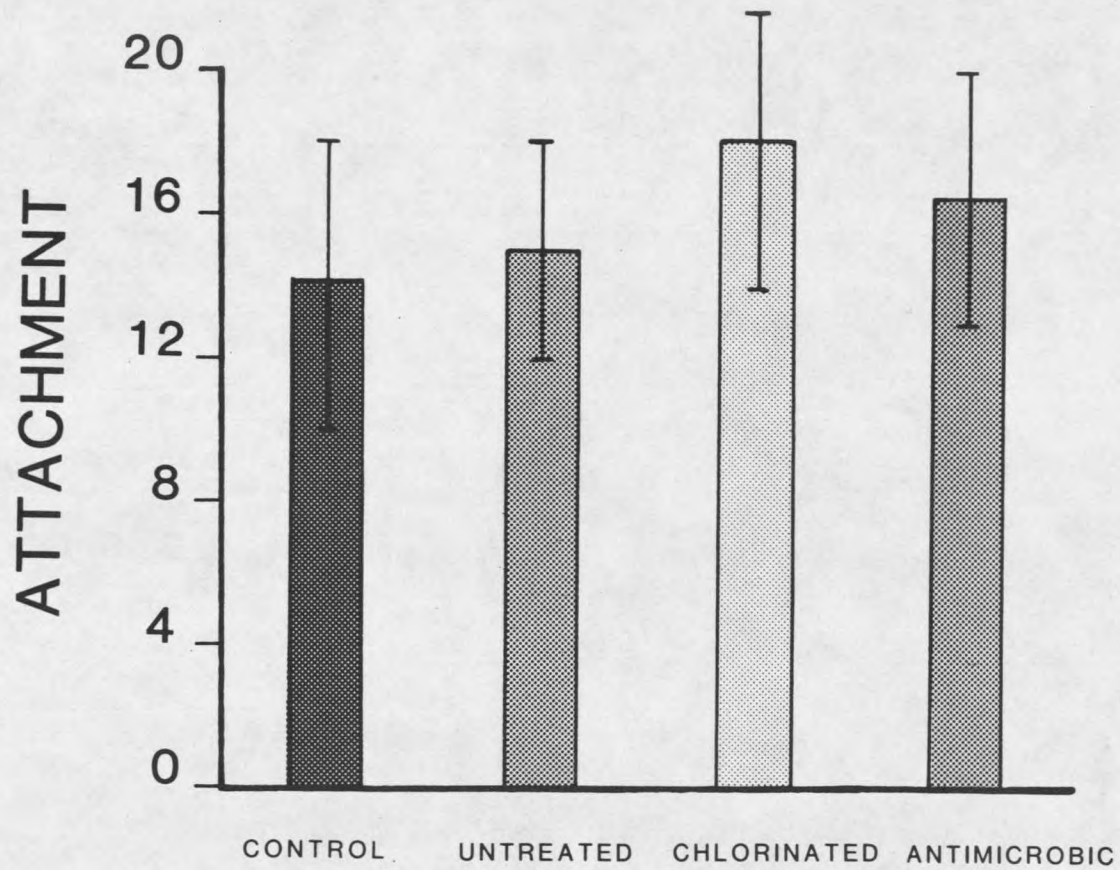


Figure 19. Effect of various membrane additions on the attachment of *Y. enterocolitica* to Henle cells. Attachment was measured as the average number of bacteria per epithelial cell.

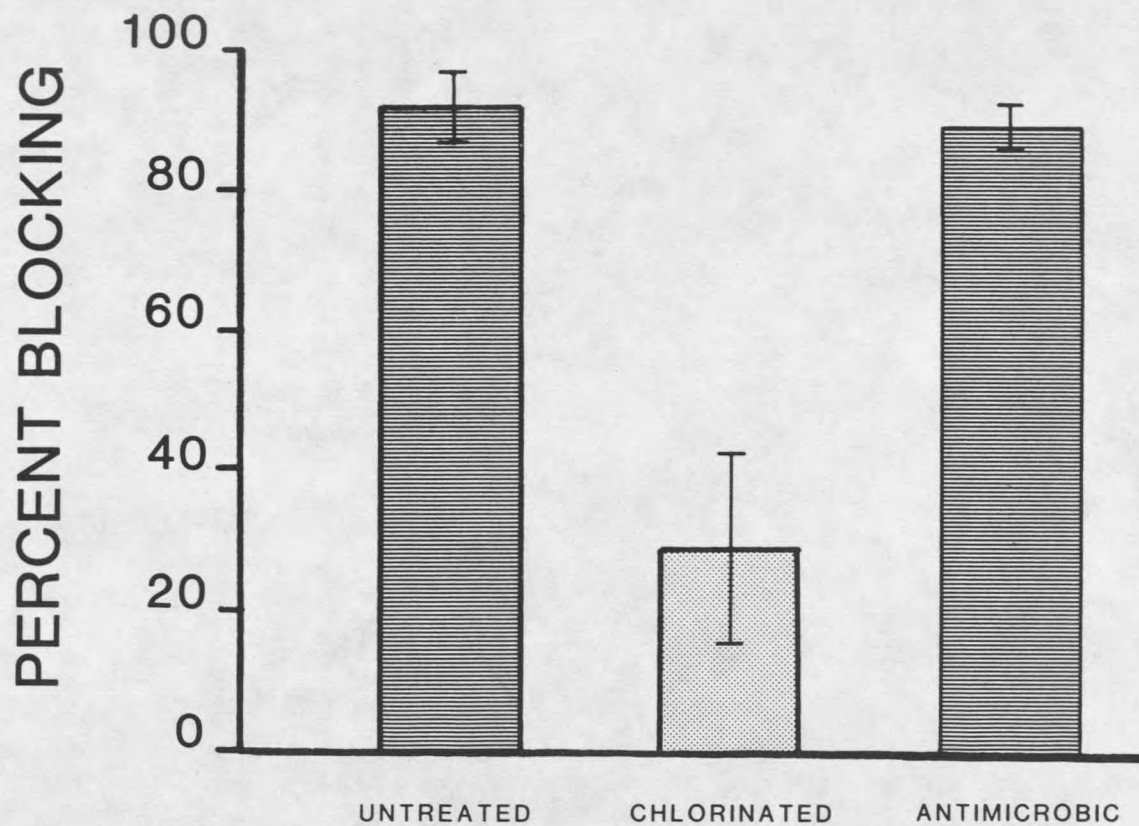


Figure 20. Effect of various membrane additions on the invasiveness of Y. enterocolitica.

(statistically significant at $p < 0.05$). Membranes from cells treated with a combination of tetracycline and rifampicin, however, showed no difference as compared to untreated membranes in their ability to block invasiveness (Figure 20).

Electrophoresis of Outer Membrane Proteins

Equal amounts of the membrane preparations were subjected to SDS polyacrylamide gel electrophoresis (SDS PAGE) using the modified Laemmli discontinuous buffer system. Results are shown in Figures 21 and 22. Numerous changes occurred in the outer membrane of chlorinated *Y. enterocolitica*. Two of the major outer membrane proteins (47,000 and 49,000 daltons) were reduced by 66%. Overall, the total amount of protein was reduced by 35%. Four protein bands were not apparent in the chlorinated cells (molecular weights 61,000, 34,400, 33,000 and 25,500 daltons), although this may be due to the low overall protein levels and the loading of the gel. Three new protein bands, however, (81,000 23,500 and 21,000 daltons) were observed.

Membranes from cells treated with antimicrobics also showed some changes in the outer membrane profiles (Figures 21 and 22). The total amount of protein was 20% lower than in the untreated membrane preparation. The 34,500 dalton protein band, present in the untreated membrane

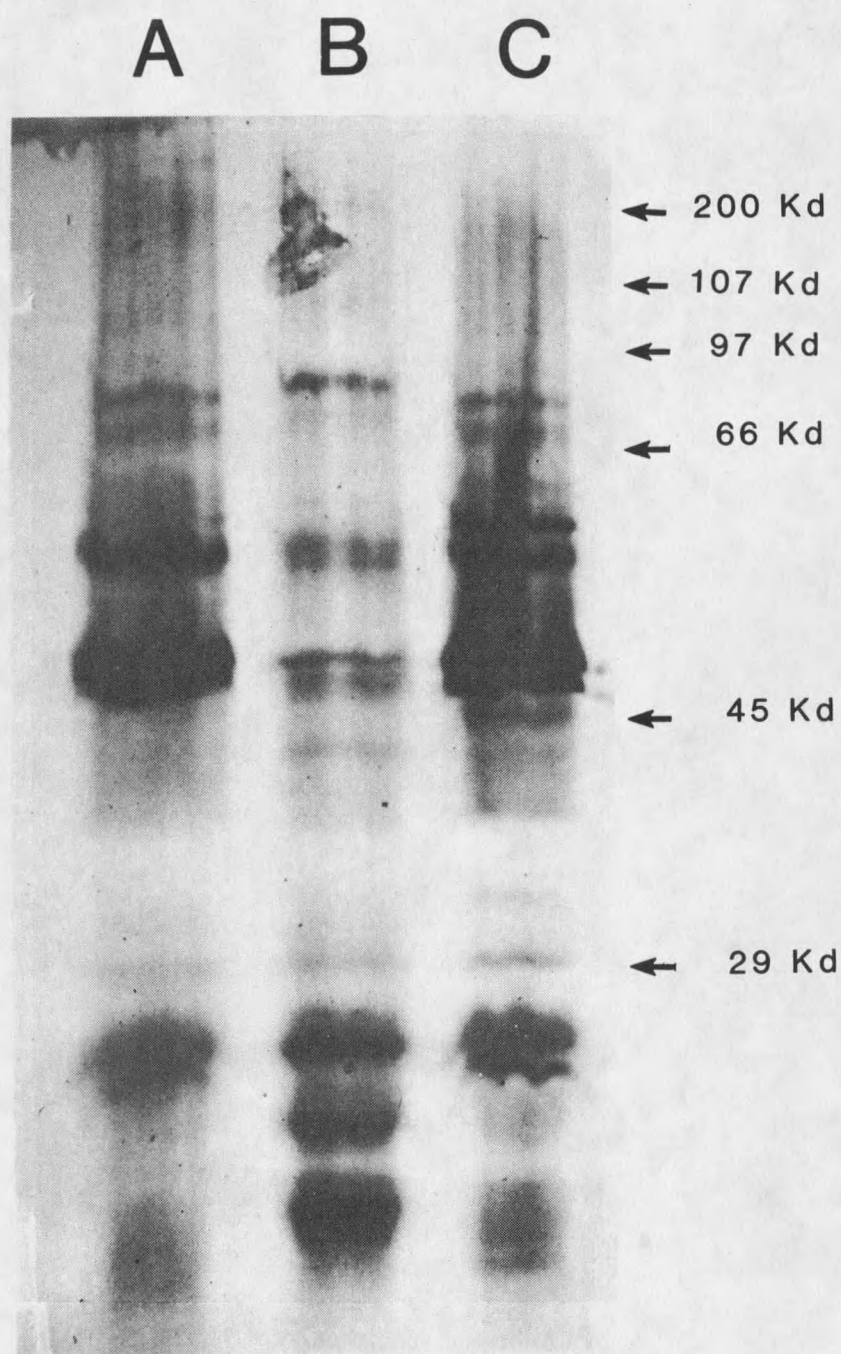


Figure 21. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membranes of *Y. enterocolitica*. (A) antimicrobic treated, (B) chlorinated and (C) untreated *Y. enterocolitica*.

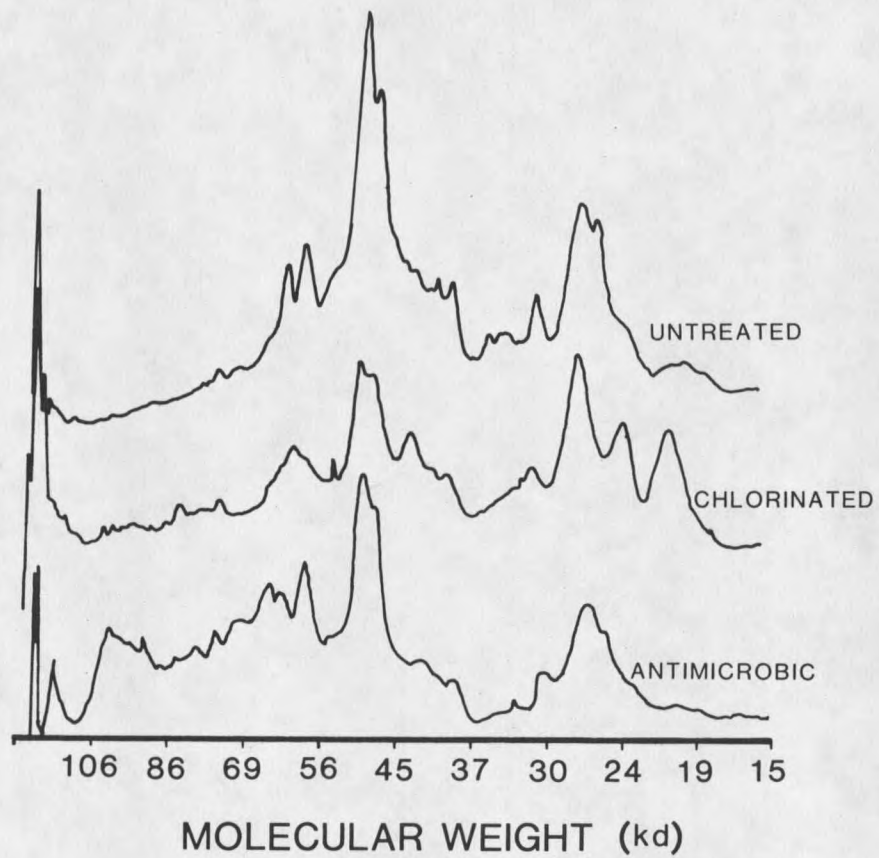


Figure 22. Densitometer tracing of gel shown in Figure 21.

preparation, was not detected possibly due to the overall lower protein levels and the loading of the gel. Two new bands showing molecular weights of 62,000 and 63,000 daltons appear on the densitometer tracing (Figure 22). However, these two peaks may be artifacts since examination of the gel (Figure 21) indicates only one band in this area. The "waviness" of the band may have caused the densitometer to read two peaks. The antimicrobial-treated membrane preparation contained the 61- 63,000, 25,500 and 33,000 dalton proteins apparently missing from the chlorinated sample, and does not show the new protein bands (23,500 and 21,000) that appeared after chlorination.

DISCUSSION

Relative Susceptibility of Coliforms and Pathogens
to Injury by Chlorine

The bacterial quality of potable water is presently assessed by an indirect method utilizing indicator bacteria collectively known as the coliform group. The premise underlying this test is that coliforms indicate the potential presence of pathogenic microorganisms. Recently, the use of coliform bacteria as indicators of potable water quality has been questioned in part because coliforms can become injured due to stresses in the aquatic environment and subsequently fail to grow on selective media commonly used for their enumeration. Various reports (97, 134, 135, 236) have indicated that as many as 90% of the indicator bacteria found in potable and surface water may not be recovered by standard techniques.

Presumably, conditions that stress coliform bacteria would also injure waterborne pathogens although there are no data to support this hypothesis. Results from this investigation indicate that coliforms are more susceptible to injury by chlorine than Y. enterocolitica, S. typhimurium and the shigellae (Table 3). The most sensitive S. typhimurium strain was twice as resistant to

injury by chlorine as any of the coliform bacteria (1.0 and 0.5 mg/l chlorine, respectively). The indicator bacteria may show greater than 90% injury when the pathogens are unaffected.

The susceptibility of coliform bacteria to chlorine injury did correlate with the sensitivity of ETEC to this disinfectant (Figure 2). It is not known, at present, what makes an organism sensitive or resistant to chlorine, but it appears that pathogenic E. coli do not differ from non-pathogenic E. coli in their susceptibility to chlorine-injury.

Data from this study have important implications regarding the public health significance of injured coliform bacteria. The results show the importance of enumerating injured coliforms since some pathogens are more resistant to chlorine-injury than the indicator bacteria. Failure to recover injured coliforms could result in water deemed "potable", which would actually contain virulent pathogens. Several outbreaks of salmonella and poliovirus have been reported in drinking water which had no or low coliform levels, possibly because injured coliforms were not detected by standard techniques (22, 141, 161, 202). In one example (22), over 16,000 residents of Riverside, California became ill after drinking municipal water containing S. typhimurium. Coliform densities (0.14 coliform/100 ml) were below accepted levels. However,

coliforms were enumerated using the multiple fermentation tube (MPN) technique, a method with a number of problems (63, 201), including an inability to detect injured coliforms (134). Data reported here suggest that enumeration of injured coliforms could provide a more encompassing and sensitive indicator system capable of detecting potential public health threats in contaminated water.

Differences in Chlorine Susceptibility Among Pathogens

Results presented in Figures 2-5 indicate that different pathogens have varying sensitivities to injury by chlorine. Significantly more chlorine was required to injure Y. enterocolitica, S. typhimurium and the shigellae than ETEC (Table 3). The susceptibility of Y. enterocolitica and S. typhimurium to injury by chlorine was also significantly different ($p < 0.05$). Because of the different susceptibilities to chlorine-injury observed in this study, extrapolation of these data to other pathogenic bacteria would not be reliable. For example, S. typhimurium has been shown to be more sensitive to injury by copper than is Y. enterocolitica (A. Singh, Montana State University, personal communication). Further research on other enteric pathogens and different aquatic stresses must be done before a comprehensive picture is possible.

Differences between the pathogens studied are also reflected in the virulence determinants affected by chlorine. Injured Y. enterocolitica were able to attach to epithelial cells (Figure 8) while injured ETEC and S. typhimurium lost this capacity (Figures 9 and 10). The results of this report support the conclusions of Walsh and Bissonnette (237) who reported that chlorine-injured ETEC lost the ability to attach to peripheral leukocytes. It is possible that chlorine damaged the fimbriae responsible for the interaction between ETEC and S. typhimurium and epithelial cells. Since Y. enterocolitica do not produce fimbriae when grown for short periods at 22°C (126, 136, 194, 195, 212, 229) attachment of this pathogen to epithelial cells is mediated in a different manner. The precise mechanism of attachment, however, is unknown. More research on pathogens exhibiting a variety of virulence determinants is necessary to gain a complete understanding of how chlorine affects the pathogenicity of these organisms.

Reduction in Virulence of Y. enterocolitica by Chlorine-Injury

Results presented in Figure 6 indicate that chlorine-injured Y. enterocolitica are avirulent when tested by the mouse LD₅₀ assay. The LD₅₀ value for healthy bacteria was 160 cells (95% confidence interval: 83-271), while the LD₅₀

value for injured organisms was 3300 cells (95% confidence interval 2150-5200). The deaths observed with the injured suspension can be attributed to the 3% percent of uninjured healthy bacteria (LD₅₀ of 100 cells) remaining in the population. These data suggest that injured Y. enterocolitica were avirulent in the mouse.

These results are in contrast to those reported by other investigators (49, 71, 209, 218). Sorrells et al. (218) found no difference in the virulence of Salmonella gallinarum for 6 week old chicks after injury by freezing. Similarly, Simon et al. (209) reported that the virulence for mice of three strains of S. typhimurium did not change appreciably when samples were freeze-dried and stored for 1 or 2 years at 5°C. Other investigators (49, 71) have indicated that heat or freeze-dried injured Staphylococcus aureus regained the ability to produce enterotoxin B after resuscitation in a non-selective medium. Pathogens in the "viable-but-nonculturable" stage have been reported to remain virulent (Roszak, D.B., D.J. Grimes and R.R. Colwell, 1984, Abst. Annu. Mtg. Am. Soc. Microbiol. I-21, p.125). However, these types of stresses may cause different physiological responses than those produced by chlorine-injury.

Walsh and Bissonnette (237) indicated that ETEC injured by chlorine had a reduced ability to attach to peripheral leukocytes. These authors concluded that the

reduced adhesive ability of chlorine-injured pathogens would result in an inability to colonize the small intestine and initiate disease. This conclusion is true provided injured pathogens cannot repair the cellular lesion caused by chlorine. In this study, injured Y. enterocolitica regained invasiveness for epithelial cells after resuscitation in TLY broth for 3 h at 22°C. Injured cells that repaired the chlorine-induced damage (able to grow on TLY-D agar) were indistinguishable in the HeLa cell invasiveness test from untreated healthy cells. Injured Y. enterocolitica apparently did not become resuscitated in vivo when inoculated intraperitoneally in mice (Figure 6). The close correlation between injury (the inability to grow on a selective medium) and virulence (as measured by the mouse LD₅₀) indicated that stressed cells did not recover from the chlorine induced injury inside the mouse peritoneum. Singh et al. (210) have shown using mice that Y. enterocolitica injured by copper was cleared from the blood stream by the liver faster than were uninjured cells.

The reduced virulence of Y. enterocolitica was not associated with inability to attach to Henle cells (Figure 8), but could be related to a loss of epithelial cell invasiveness. Since invasiveness has been characterized as a key virulence requirement for this organism (37, 53, 136, 145, 146, 180, 194, 195, 228, 238), it is likely that the lack of invasiveness demonstrated by injured cells is

responsible, at least in part, for the decreased virulence observed in the mouse LD₅₀ assay. The reduction in invasiveness of injured Y. enterocolitica was determined by four different approaches. (i) The HeLa cell roller tube technique showed that healthy Y. enterocolitica averaged 1.15 intracellular bacteria per HeLa cell, while injured cells averaged only 0.01 bacteria per epithelial cell, a 99% decrease (Figure 13). This method enumerated only viable intracellular bacteria since gentamicin effectively killed extracellular bacteria, even in the presence of epithelial cells. (ii) Direct light microscopic observations also indicated that Y. enterocolitica injured by chlorine had reduced tissue culture cell invasiveness (Figure 14). Healthy Y. enterocolitica infected 65% of the HeLa cells, while 87% of the epithelial cells inoculated with chlorine-injured bacteria lacked intracellular organisms. The average number of intracellular bacteria per HeLa cell was 2.02 and 0.17 for healthy and injured Y. enterocolitica, respectively. These results are in close agreement with those obtained using the HeLa cell roller tube study, considering the multiplicity was higher (20.6). (iii) Transmission electron microscopy (TEM) is one method to conclusively show intracellular bacteria in infected HeLa cells. Most of the preparations inoculated with chlorine-injured Y. enterocolitica lacked evidence of intracellular bacteria, while most of the cells infected

with healthy organisms contained intracellular bacteria (Figure 15). Quantization of TEM data is difficult since successive slices of the same preparation can yield erroneous results (ie. counts of the same bacterium in the same epithelial cell). However, the data gained from the TEM experiments support the general conclusion that chlorine-injured Y. enterocolitica are less invasive than did healthy bacteria. (iv) The invasiveness of healthy and injured Y. enterocolitica was also examined using an in vivo model. Injured cells injected into intestinal loops of mice showed significantly lower intracellular levels than healthy bacteria (Figure 18). Histologically, a greater immune response was observed in tissues infected with healthy bacteria. A greater accumulation of monocytes and neutrophils was probably in response to the higher intracellular bacterial levels. Similar histological observations have been recorded by other investigators (43, 69, 186, 227). In the early stages of infection, the organisms were detected in vesicles formed in the cytoplasmic space of epithelial cells (227). The bacteria then progressed to the lamina propria and Peyer's patches. From the Peyer's patches the bacteria spread to the mesenteric lymph nodes and eventually become systemic (43, 69, 227). Primary lesions are characterized by massive infiltration of monocytes and neutrophils (43, 69, 186, 227).

The results of this study also show that the mechanisms of attachment and invasion are two separate processes in epithelial cell association. Chlorine affected the invasiveness of Y. enterocolitica, but did not interfere with attachment of the bacteria to epithelial cells. The data suggest that the process of invasion is more than simple association of the bacteria with the epithelial cell surface, but involves a specific mechanism, a communication, with the epithelial cell which induces endocytosis. Healthy Y. enterocolitica were able to stimulate engulfment, while chlorine damaged this mechanism in injured cells. Possible reasons for reduced invasiveness are discussed below.

Reasons for Reduced Invasiveness

The physiological reason(s) why injured Y. enterocolitica lose the ability to invade epithelial cells is an intriguing question with important implications concerning the pathogenicity of this organism. Two requirements appear to be necessary for invasiveness: (i) The organism must be viable and metabolically active; and (ii) the organism must have certain surface components to initiate engulfment.

Role of Metabolism in Invasiveness

Hale and Bonventre (84) have shown that enteroinvasive shigellae do not act as inert particles, but participate actively in the infection of tissue culture cells. Other investigators have found Yersinia killed by heat, UV irradiation, formalin or glutaraldehyde were noninvasive for tissue culture cells (30, 168, 176, 216, 234). The results of this study indicate that viable, but chlorine injured, Y. enterocolitica have reduced invasiveness in vitro and in vivo.

Previous studies have shown that chlorine causes damage to the bacterial cell membrane of E. coli and other bacteria (4, 42, 83, 154, 232, 251, 252). Investigators have shown that respiration, glucose transport and ATP levels all decrease in injured E. coli populations (42, 54, 83, 154, 232). Electron microscopy of injured cells has demonstrated morphological changes in the cell membrane. Data presented in this report indicate that chlorine-injured Y. enterocolitica show many of the same physiological changes described for injured coliform populations. Adenosine triphosphate (ATP) levels were 91.7% lower in injured Y. enterocolitica than in healthy cells (1.02×10^{-13} g ATP/cfu and 8.4×10^{-15} g ATP/cfu for healthy and injured Y. enterocolitica, respectively). Respiration rates of healthy and injured cells were

significantly different. Healthy bacteria respired at a rate of 1.11 ug O₂/min/10⁹ cells, while injured Yersinia utilized 0.59 ug O₂/min/10⁹ cells. These rates compared favorably to data presented by Domek et al. (54) for healthy and injured E. coli growing on glucose. The assimilation of thymidine, uridine and methionine was also significantly reduced in injured Y. enterocolitica (Table 4). It is likely that both the process of uptake and utilization of these compounds in DNA, RNA and protein synthesis were affected by chlorine since these mechanisms require ATP. The results of these studies indicate that chlorine-injured Y. enterocolitica has reduced metabolic capabilities.

Experiments were performed to determine the influence of metabolic activity on the invasiveness of Y. enterocolitica in the HeLa cell roller tube system. Inhibitors of RNA and protein synthesis (chloramphenicol, tetracycline, spectinomycin and rifampicin) significantly reduced the invasiveness of Y. enterocolitica into HeLa cells (Table 5). The data suggest that RNA and subsequent protein synthesis are required for invasiveness. It is possible that proteins are necessary to mediate the invasive signal (2, 86, 149). Nalidixic acid significantly reduced the infectivity of Y. enterocolitica, but nitrofurantoin, also an inhibitor of the initiation of DNA replication, did not affect invasiveness (Table 5).

Nalidixic acid has other effects on the bacterial cell in addition to inhibiting gyrase activity (57). Blockage of DNA replication by nalidixic acid leads to induction of the SOS regulatory pathway, and as a result, proteins that participate in DNA repair increase in abundance. Nalidixic acid may not decrease the amount of proteins synthesized, it may alter the types of proteins produced so that structures responsible for stimulating engulfment are not made. While a similar argument may be made for unknown side effects of the other antimicrobics used, the data do suggest that some metabolic activity is required on the part of the bacterium to induce endocytosis and that the reduced invasiveness of chlorine-injured Y. enterocolitica may be due, in part, to the reduced metabolic activity observed with these cells.

Role of Surface Components in Invasiveness

Hydrophobicity and surface electric charge have been reported by some investigators to be important in the processes of attachment and invasion of tissues (124, 125, 167, 196, 205). Lachica and Zink (124, 125) correlated increased surface charge and hydrophobicity to the presence of the 40- to 48-megadalton plasmid and suggested that hydrophobicity may be associated with the ability of plasmid-positive strains to colonize and survive in the gastrointestinal tract. Hydrophobicity and electric charge

have been shown to influence the phagocytosis of type 1 fimbriae-bearing E. coli (167, 205).

Results presented in this study show that no differences in hydrophobicity or surface charge were observed between healthy and injured pathogens (Table 6). An increase in hydrophobicity, however, was seen with chlorine killed Y. enterocolitica. The lack of difference in hydrophobicity between healthy and injured S. typhimurium and ETEC was surprising. Hydrophobicity has been associated with the presence of fimbriae in these bacteria (41, 105, 144, 235). Since chlorine affected the attachment of injured S. typhimurium and ETEC, it was expected that chlorine would also affect their hydrophobicity. It may be possible that chlorine damaged the activity of the fimbriae without affecting hydrophobicity. Additional research on the effects of chlorine on the attachment mechanisms of these bacteria is necessary.

Chlorine changed specific structures or functions in membranes of Y. enterocolitica. Untreated membrane preparations blocked nearly 90% of the invasiveness of Y. enterocolitica in the HeLa cell roller tube system (Figure 20), while chlorinated membrane preparations showed only 29% blocking. The inability of chlorinated membrane to block invasiveness was thought to be due to an inability to associate to the HeLa cells and block the receptor

responsible for triggering engulfment. Curiously, antimicrobial treated membranes retained the ability to block invasiveness (Figure 20). The difference between the activities of the chlorinated and antimicrobial treated membranes may be due to one of three possibilities: (i) cells may have repaired the effects of the antimicrobics between the time the antimicrobics were removed and the time the cells were fractionated; (ii) antimicrobics may not destroy the component that binds to the HeLa cell receptor to the same extent that chlorine does, so that a noticeable difference may not be apparent at the concentrations used (note that chlorine reduced invasiveness by 99% in the HeLa cells roller tube technique, but tetracycline and rifampicin reduced invasiveness by an average of 80%); or (iii) antimicrobics and chlorine may affect invasiveness by different mechanisms.

A requirement for specific surface characteristics for invasiveness has been observed in Shigella, Salmonella, enteroinvasive E. coli and Yersinia (86, 171, 176, 192, 216, 231, 242). Plasmids of Shigella flexneri, Shigella sonnei and enteroinvasive E. coli have been shown to code for specific outer membrane proteins (86, 192). When the plasmid of Shigella flexneri was transferred to E. coli K-12 the recipients became invasive for HeLa cells (192). E. coli minicells containing the plasmid incorporated

[³⁵S]methionine into 14 outer membrane proteins (86). Hale et al. (86) hypothesized that the outer membrane proteins encoded by the virulence plasmid bind to receptors on the host enterocytes and stimulate engulfment. Other investigators have shown that protein synthesis is required to restore invasiveness when shigellae are shifted from 30°C to 37°C (149). Heat-labile surface components have been shown to be essential for the penetration of Y. enterocolitica into epithelial cells (176, 216). These reports suggest that a bacterial surface structure (probably proteinaceous in nature) is responsible for triggering engulfment by epithelial cells in vitro.

The outer membranes of normal, chlorinated and antimicrobial-treated Y. enterocolitica were subjected to SDS-polyacrylamide gel electrophoresis using a modified Laemmli discontinuous buffer system (Figures 21 and 22). Numerous changes were observed in the outer membrane of chlorinated Y. enterocolitica. Two of the major outer membrane proteins were reduced by 66%, four protein bands present in untreated cells were not observed, while three new protein bands were observed (Figures 21 and 22). The antimicrobial treated membranes showed one protein band apparently missing and the total amount of protein reduced by 20%. The missing bands from the chlorinated and antimicrobial treated membranes may be due to differences in the protein loading of the gels. These proteins may be

present, but at reduced levels. It was the intent of these experiments to show changes in the levels of outer membrane proteins that may be responsible for the differences seen in the membrane blocking studies. The fact that chlorine reduced some proteins (47,000 and 49,000 daltons) by 66%, while the overall protein level was reduced by 35%, indicates that chlorine did affect certain proteins. The new low molecular weight proteins may be degradation products of larger protein structures.

These data demonstrate changes in the outer membranes of chlorine and antimicrobial treated Y. enterocolitica, but it cannot be determined if these changes are related to invasiveness until the mechanism of invasiveness in Y. enterocolitica is understood and the protein(s) responsible for invasiveness identified.

Theoretical Model of Invasiveness

A theoretical model, consistent with existing information, of how chlorine affected the invasiveness of Y. enterocolitica is shown in Figure 23. The model does not explain the specific mechanism of Y. enterocolitica attachment to the epithelial cell surface, nor does it represent the details of phagocytosis (205). However, the model implies that there may be two receptors, one responsible for attachment, the other involved in invasiveness. The model assumes that there is a surface

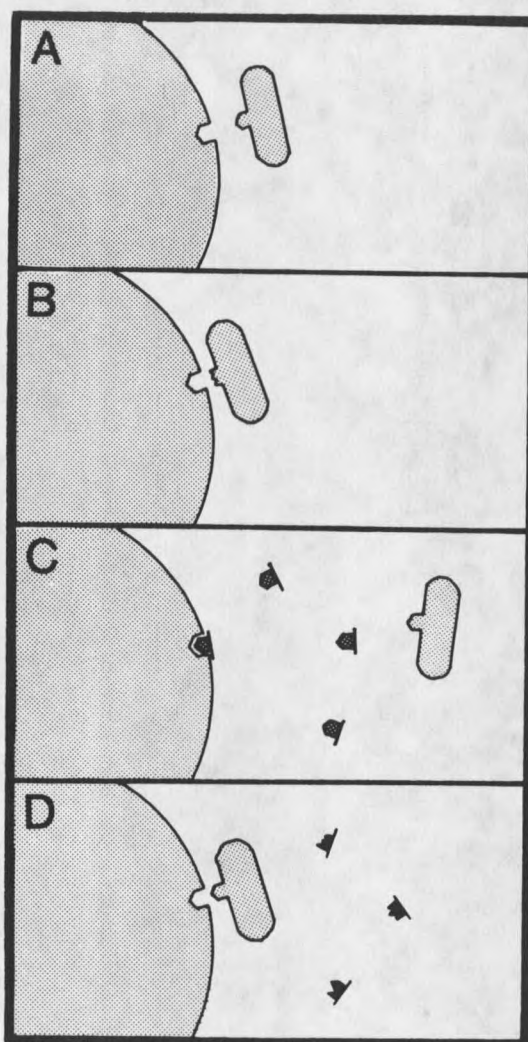


Figure 23. Theoretical model of how chlorine affects the invasiveness of *Y. enterocolitica*. The model is not intended to show the mechanism of attachment. A) Virulent *Y. enterocolitica* with surface component interact specifically with receptor on HeLa cell to stimulate engulfment. B) Chlorine-injured *Y. enterocolitica* attach to HeLa cells but cannot stimulate engulfment because the trigger has been damaged. C) Membranes from untreated *Yersinia* associate with the receptor on HeLa cells and inhibit engulfment. However, bacteria can still attach to the epithelial cell surface. D) Membranes from chlorinated *Yersinia* contain damaged trigger and cannot block the invasiveness of virulent *Y. enterocolitica*.

component of Y. enterocolitica that stimulates the engulfment of the bacteria by epithelial cells. Stimulation may involve active metabolism on the part of the bacterium. The trigger is thought to be composed, at least in part, of protein (ie. protein, lipoprotein or a protein-lipoplysaccharide complex). When pathogenic Y. enterocolitica attach to an epithelial cell, the trigger associates in a specific manner with a receptor on the epithelial cell to initiate endocytosis (Figure 23a). Chlorine damages this trigger so that invasiveness is inhibited (Figure 23b). Membranes from untreated Yersinia are added to the HeLa cell roller tube system and interact specifically with the receptors on the epithelial cells to block invasiveness of virulent Y. enterocolitica (Figure 23c). The inability of chlorinated membranes to block the invasiveness of Y. enterocolitica may indicate that chlorine damaged the trigger making it unable to associate in a specific manner with the receptor. Chlorine, however, did not change the membranes to the extent that hydrophobicity or surface charge of the cell was altered, but did change its biological activity. Because chlorine-damaged triggers were not occupying the receptors on the epithelial cells, virulent Y. enterocolitica were free to attach and invade the tissue culture cells (Figure 23d).

Verification of this model awaits the understanding of the mechanism of invasiveness and identification of the

trigger protein. At present, neither the mechanism of invasiveness nor the structure(s) involved are understood. Development of specific antisera to the trigger, identification of the trigger by Western blot techniques and production of noninvasive mutants of Y. enterocolitica are some of the experiments that need to be done to answer important questions regarding the pathogenicity of this organism.

Conclusions

The following conclusions can be made from data presented in this report:

1. Higher chlorine doses were required to produce injured Y. enterocolitica, S. typhimurium and Shigella spp. than ETEC and coliform bacteria in the test system used.
2. LD₅₀ experiments using mice showed that chlorine-injured Y. enterocolitica were 20 times less virulent than uninjured control cells.
3. Virulence determinants affected by chlorine may vary from one pathogen to another. Y. enterocolitica showed reduced invasiveness, while ETEC and S. typhimurium had a reduced ability to attach to epithelial cells.
4. The processes of attachment and invasion for Y. enterocolitica are two separate mechanisms. Invasion is more than simple association of the bacterium with the

epithelial cell, but involves a specific trigger to stimulate engulfment.

5. Two requirements appear to be necessary for invasiveness: (i) The organism must be viable and metabolically active; and (ii) the organism must have certain surface components to initiate engulfment.

6. Chlorine-injury did not change the hydrophobicity or surface electric charge of Y. enterocolitica, but did alter the ability of the membranes to block the invasiveness of virulent cells.

7. Chlorine caused numerous changes in the outer membrane proteins of Y. enterocolitica. Whether these changes are related to the decreased invasiveness of injured cells awaits a more complete understanding of the invasive process of this organism.

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