Modification of Bacterial Serum Susceptibility by Rifampin

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A subinhibitory concentration of rifampin converted a strain of *Escherichia coli* from serum resistant to serum susceptible. When continually cultured in nutrient broth containing 1.5 μ g of rifampin per ml, this strain of *E. coli* became susceptible to killing by both normal human serum and normal rabbit serum. Compared to the original strain, the rifampin-treated *E. coli* displayed no detectable change in adherence capability, but appeared less virulent in the rabbit model of endocarditis. A rifampin-resistant mutant of the *E. coli* strain was not found to undergo conversion to serum susceptibility upon culture in rifampin.

Many gram-negative enteric bacilli causing bacteremia demonstrate resistance to the bactericidal action of human serum (9, 17, 19, 22). The property of serum resistance possessed by smooth, encapsulated enterobacteria has been viewed as an important virulence factor in human infectious diseases (9, 12) as well as in animal models of infection (1, 7, 10, 18).

Conversion of serum-resistant *Escherichia coli* to a serum-susceptible state has been observed after treatment with diphenylamine in concentrations less than those required for complete inhibition of bacterial growth (4). The antibiotic polymyxin B is able to induce surface changes in some gram-negative bacteria, leading to serum susceptibility (19). Subinhibitory concentrations of the antimicrobial agents tetracycline and streptomycin have also been shown to increase bacterial susceptibility to killing by serum (3).

In this study we describe the conversion of a serum-resistant *E. coli* to serum susceptibility by passage in a subinhibitory concentration of rifampin. In association with this increase in serum susceptibility we observed decreased virulence of the converted *E. coli* in the rabbit endocarditis model.

MATERIALS AND METHODS

Bacteria. A strain of serum-resistant *E. coli* (designated D8 strain) was isolated from a fecal specimen in the Diagnostic Microbiology Laboratory of Children's Hospital, Birmingham, Ala., and was used in these studies. It was agglutinated by antiserum to O128 antigen (BBL Microbiology Systems, Cockeysville, Maryland). After streaking for purity on MacConkey agar, several colonies were selected and inoculated to nutrient agar slants, incubated at 37°C overnight, and thereafter stored at 25°C. *E. coli* D8 was inhibited by 12.5 μ g of rifampin per ml, determined by standard broth dilution methods.

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Passage of *E. coli* in subinhibitory concentrations of rifampin. Rifampin (3-[4-methylpiperazinyliminomethyl] rifamycin SV) was purchased from Sigma Chemical Co., St. Louis, Mo. For each experiment, a stock of rifampin was freshly prepared in methanol. When added to nutrient broth, the final methanol concentration was well below 0.1%, a concentration that did not influence the serum susceptibility of *E. coli* D8. The nutrient broth used in these studies was Lennox broth (LB), constituted as previously described (8). Rifampin was added to LB to yield a final concentration of 1.5 μ g/ml, and this solution was designated LB-R. Two or three colonies of *E. coli* D8 were inoculated into 5.0 ml of either LB or LB-R and incubated at 37°C until a cell density of 10^9 /ml was present (usually 14 h). Approximately 10^4 bacteria were then removed and inoculated into a new 5.0-ml sample of the same preparation as initially (LB or LB-R), and the total process was repeated at least 10 times. *E. coli* D8 passaged in broth containing the subinhibitory concentration of rifampin was designated *E. coli* D8-R.

Test serum. Serum from five healthy volunteers was pooled and used in all experiments requiring normal human serum (NHS). Venous blood drawn aseptically was allowed to clot, and the serum was separated by centrifugation, pooled, and frozen in small aliquots at -70°C. Serum was used within 8 weeks of collection and thawed only once, just before the start of each experiment. Blood collected from five healthy New Zealand white rabbits was used as the source of normal rabbit serum (NRS), which was pooled and stored at -70°C.

Test for bactericidal action of serum. Determination of susceptibility of *E. coli* D8 and *E. coli* D8-R to NHS and NRS was performed using a modification of the procedure described by Vosti and Randall (22). Dilutions of the cells were made in Veronal-buffered glucose with calcium, magnesium, and 0.1% gelatin, prepared as described by Rapp and Borsos (13), and 0.1-ml samples were incubated with 0.9 ml of the test serum (90%, vol/vol) at 37°C in a standing water bath. Serum was diluted in either Veronal-buffered glucose or Hanks balanced salt solution (Microbiological Associates, Walkersville, Md.). At intervals during the incubation period, duplicate 0.05-ml samples of the

incubation mixture were withdrawn, serially diluted in Hanks balanced salt solution and spread on nutrient agar. After overnight incubation, colonies were counted, and curves representing cell survival over time were constructed.

Preparation and infection of rabbits. The experimental animals used were random-bred New Zealand white rabbits weighing from 1.8 to 2.5 kg. A polyethylene catheter (Clay Adams, Parsippany, N.J.) was fixed in the left heart as previously described (2) under anesthesia with intramuscular acepromazine (10 mg), ketamine (50 mg), and scopolamine (0.25 mg) (11). After 36 to 48 h, each rabbit was injected with either *E. coli* D8 or *E. coli* D8-R via an ear vein. Before injection, bacteria were washed once in Hanks balanced salt solution, sedimented, resuspended, and diluted 1:10 in Hanks balanced salt solution. For *E. coli* D8 the inoculum contained $2.4 \pm 0.3 \times 10^8$ colony-forming units ($n = 12$). The inoculum of *E. coli* D8-R was $1.3 \pm 0.4 \times 10^8$ colony-forming units ($n = 27$).

Culture of endocardial vegetations. Rabbits were killed by intravenous injection of pentobarbital at various intervals after inoculation of bacteria, and hearts were removed and opened using aseptic precautions. Vegetations were excised and homogenized in 0.3 to 0.5 ml of LB using tissue grinders (Scientific Products, McGaw Park, Ill.), and the homogenates were spread on agar plates. Identification of *E. coli* from excised vegetations was made by noting the presence or absence of growth after overnight incubation. The identity of bacteria thus recovered was confirmed with *E. coli* O128 antiserum.

Determination of adherence to canine aortic valve tissue. Aortic valve leaflets from mongrel dogs sacrificed for other purposes were removed with sterile technique from the hearts and placed in sterile phosphate-buffered saline solution. As previously described (6), circular sections of tissue were obtained using a 2-mm punch biopsy instrument, and these sections were placed in phosphate-buffered saline. Four valve sections were then placed in 3.0-ml samples of suspensions of either *E. coli* D8 or *E. coli* D8-R, prepared as described above, in phosphate-buffered saline contained in small petri dishes. Tissue sections and bacterial suspensions were agitated at 160 cycles per min on a rotary shaker at 25°C. After 1 h, the tissue sections were removed and individually washed three times by swirling in 3.0-ml samples of phosphate-buffered saline for 15-s periods. Each valve section was placed into a mortar, along with 3.0 ml of phosphate-buffered saline and a small quantity of sterile sand, and was homogenized with a pestle. Quantitation of cells adhering to valve tissue was performed by culture and enumeration on agar plates of bacteria recovered from the homogenized suspensions. The "adherence ratio" (6) was determined as the proportion of bacteria in the original suspension that was recovered from the homogenized valve tissue. The number of bacteria recovered from valve tissue represented the mean number adherent to four valve sections.

RESULTS

Modification of serum susceptibility of *E. coli* D8 by rifampin passage. As originally isolated and maintained, *E. coli* D8 grew as a

smooth strain and was resistant to killing by pooled NHS. In experiments comparing the behavior of *E. coli* D8 and *E. coli* D8-R in NHS, *E. coli* D8 multiplied 10-fold when incubated for 60 min in NHS (Fig. 1). *E. coli* D8-R, the rifampin-treated strain, demonstrated a 2-log decrease in titer during 60 min of incubation in NHS. The bactericidal effect of NHS on *E. coli* D8-R was abolished by prior heating of the serum at 56°C for 30 min (data not shown). In analogous experiments using pooled NRS in place of NHS, serum-resistant *E. coli* D8 grew well in 25% NRS (Fig. 2). In contrast, *E. coli* D8-R was killed by this concentration of NRS. Heat inactivation of 25% NRS abolished its bactericidal activity against the modified *E. coli* D8-R (data not shown).

A rifampin-resistant mutant of *E. coli* D8

FIG. 1. Survival curves for serum-resistant *E. coli* D8 (▼) and rifampin-treated, serum-susceptible *E. coli* D8-R (○) during incubation in 90% pooled NHS at 37°C.

FIG. 2. Survival curves for serum-resistant *E. coli* D8 (▼) and rifampin-treated, serum-susceptible *E. coli* D8-R (○) during incubation in 25% pooled NRS at 37°C. A rifampin-resistant mutant of *E. coli* D8 (△) was not serum susceptible after passage in a subinhibitory concentration of rifampin.

(minimal inhibitory concentration $> 640 \mu\text{g/ml}$) was selected on agar containing rifampin (frequency, 10^{-9}). After preparation in LB-R in a manner identical to that described for the parent strain, this mutant demonstrated no change in its serum resistance (Fig. 2).

Studies utilizing the rabbit endocarditis mode. After induction of vegetations at the aortic valve, 12 rabbits were inoculated with serum-resistant *E. coli* D8, and 27 rabbits received serum-susceptible *E. coli* D8-R prepared by passage in LB-R, as described. The results of cultures of endocardial vegetations removed at 4, 10, and between 24 and 72 h are shown (Table 1). Eleven of 12 rabbits inoculated with serum-resistant *E. coli* D8 had infected vegetations when examined. A comparison of results obtained 24 to 72 h after inoculation attained statistical significance ($P = 0.04$, Fisher's exact test).

Adherence studies utilizing canine aortic valves. The unmodified *E. coli* D8 and the rifampin-treated D8-R strain were compared with regard to their ability to adhere to excised canine aortic valve tissue (Table 2). Adherence ratios for both strains did not differ significantly.

DISCUSSION

Rifampin, a derivative of rifamycin SV, inhibits deoxyribonucleic acid-dependent-ribonucleic acid polymerase by binding to the beta-subunit of this enzyme. Protein synthesis is inhibited due to a block in synthesis of messenger ribonucleic acid (23).

Growth of bacteria in the presence of subinhibitory concentrations of tetracycline and

streptomycin has been shown to decrease serum resistance (3). To our knowledge, a similar effect has not been described due to rifampin. Riva et al. (16) reported that passage through subinhibitory concentrations of rifampin would cure F plasmids from *E. coli*, but changes in serum susceptibility were not examined. Plasmids have recently been thought to be responsible for small degrees of serum resistance in *E. coli* and other enterobacteria (5, 14, 15, 21). However, curing by rifampin of plasmid-mediated serum resistance is unlikely to explain the observations we have described. We noted that when the serum-susceptible strain was recultured in broth lacking rifampin, the bacteria reverted to their previous serum-resistant state over 24 h. This behavior, of course, is not characteristic of a strain from which a plasmid has been eliminated. Rather, we believe that rifampin may reversibly inhibit synthesis of one or more cell surface components critical to serum resistance in this strain.

The serum resistance possessed by a mutant *E. coli* D8 highly resistant to rifampin was not affected by subinhibitory concentrations of rifampin. This finding suggests that rifampin does not induce serum susceptibility in *E. coli* D8 by direct interaction with the cell surface, which might facilitate the action of bactericidal factors present in that serum.

We observed that the conversion of *E. coli* D8 to serum susceptibility by rifampin appeared to be associated with decreased virulence in the rabbit endocarditis model. Durack and Beeson (1) have previously studied the induction of endocarditis in rabbits by using serum-susceptible *E. coli*. They found that although bacteria adhered to vegetative tissue soon after inoculation, serum-susceptible *E. coli* did not cause persistent intracardiac infection. In the present study, cultures of endocardial vegetations were also usually positive if obtained a short time after rabbits were inoculated with either serum-resistant or serum-susceptible *E. coli*. Rifampin-treated serum-susceptible bacteria adherent to vegetations might be expected to revert to serum resistance, as had been observed in the present study in *in vitro* cultures. If reversion occurred, it was not sufficiently prompt to affect the ultimate bacteriological status of most vegetations examined at 24 to 72 h. Durack and Beeson recovered serum-susceptible *E. coli* at up to 6 h after inoculation (1). Additionally, we found that *E. coli* D8 and *E. coli* D8-R demonstrated equal ability to adhere to dog heart valve tissue. Therefore, taken together, our results support the conclusion of Durack and Beeson that susceptible *E. coli* adherent to vegetative tissue may be killed thereon by serum.

TABLE 1. Progress of *E. coli* endocarditis in rabbits

Time (h)	Infected/examined		P ^a
	<i>E. coli</i> D8-R ^b	<i>E. coli</i> D8 ^c	
4	4/5	3/3	NS
10	4/5	3/3	NS
24-72	5/17	5/6	0.04

^a Fisher's exact test. NS, Not statistically significant.

^b Serum susceptible.

^c Serum resistant.

TABLE 2. Comparison of bacterial adherence to canine aortic valve tissue in phosphate-buffered saline

Strain	Serum resistance	Adherence ratio ($\times 10^5$) ^a
<i>E. coli</i> D8	Resistant	530
<i>E. coli</i> D8-R	Susceptible	610

^a Calculated as described in the text.

The prevalence of strains of *Enterobacteriaceae* that may undergo significant conversion to serum susceptibility as a result of the action of rifampin or other antibiotics has not been formally investigated. We have examined a number of *E. coli* isolated from human infectious diseases and noted that approximately 20 to 25% exhibited variable degrees of increased susceptibility to serum when cultured in subinhibitory concentrations of rifampin (unpublished data).

In 1969, Feingold (4) suggested that the development of agents that could sensitize bacterial pathogens to the bactericidal activity of serum might represent an important mode of antimicrobial therapy.

However, the clinical relevance of our present studies with rifampin is unknown. If investigations can identify more precisely the mechanisms responsible for virulence properties among bacteria, agents selective in their effect on such properties may be developed and may be of clinical benefit.

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