INFECTION AND IMMUNITY, June 1980, p. 837-841
0019-9567/80/06-0837/05$02.00/0

Vol. 28, No. 3

Bactericidal Activity of Human Serum Against *Escherichia coli* χ1776

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Escherichia coli χ1776 is a host strain providing the EK2 level of biological containment. Laboratories equipped to use this strain may be located near medical care facilities where patients prone to infection may be found. We have therefore documented the marked susceptibility of χ1776 to killing by human serum from normal as well as ill individuals.

The EK2 host χ1776, a descendant of *Escherichia coli* K-12, was constructed as a safe bacterial strain for use with plasmid cloning vectors in studies employing recombinant deoxyribonucleic acid technology (3; R. Curtiss III, D. A. Pereira, J. E. Clark-Curtiss, J. C. Hsu, R. Goldschmidt, S. I. Hull, R. Moody, L. J. Maturin, Sr., M. Inoue, and L. S. Alexander, submitted for publication). The laboratories in which these experiments are performed are often located at medical centers in populous urban areas. Therefore, in close proximity to such facilities are many persons with normal resistance to infection as well as hospitalized persons with diseases associated with altered or deficient host immunity to infection. It was therefore of interest to determine whether χ1776 might survive certain host defenses. Normal serum possesses a bactericidal capacity mediated via the complement system for many gram-negative bacteria (25). Although in humans this system constitutes only one of several host defenses against infection, its importance has been emphasized (1, 9, 13, 23). We sought to determine whether χ1776 could survive in normal human serum or in sera from persons with hereditary and acquired conditions associated with increased susceptibility to infection.

MATERIALS AND METHODS

Bacterial strains. The strains of bacteria used in this study are listed in Table 1. *E. coli* χ1776 and wild-type χ1833 are K-12 strains which have not previously been examined for susceptibility to human serum. *E. coli* Lilly is a rough strain previously found to be susceptible to the bactericidal activity of human serum (31). *E. coli* 75R is a smooth strain, highly resistant to killing by human serum, and was originally recovered from blood culture from a patient at the University of Alabama Medical Center.

Test sera. Individual sera from six patients and pooled normal human serum (NHS) from four healthy volunteers were employed (Table 2). Venous blood was drawn aseptically and allowed to clot, and the serum was separated by centrifugation. Individuals from whom serum was obtained had not received any antimicrobial therapy during the preceding 2 weeks. Serum was either used immediately or frozen in small samples at −70°C. Frozen sera for these experiments were used within 8 weeks of collection and thawed only once, just before the start of an experiment. All sera were found to be bacteriologically sterile. The hemolytic activity of complement in the NHS was 244 50% hemolytic units per ml, determined by using a modification of the method of Kent and Fife (10). Complement-dependent hemolytic activity of the C6-deficient human serum (C6DHS) was previously determined to be absent (30). Classic pathway hemolytic complement activity of the C2DHS was known to be below normal (15). Total hemolytic complement activity of the sera from the agammaglobulinemic individual and the immunocompromised patients was normal. Complement was inactivated by heating the serum for 30 min at 56°C. When serum was diluted, Veronal-buffered glucose with calcium, magnesium, and 0.1% gelatin, prepared as described by Rapp and Borsos (20), was used. Approval for collection and use of patients' sera in this study was granted by an Institutional Review Board.

Test for bacterial susceptibility to serum. Bacteria were grown at 37°C in Lennox nutrient broth (11) to an absorbance of 0.4 at 600 nm, which corresponded to approximately 2×10^8 cells per ml. Cells were sedimented by centrifugation at 8,000 rpm in an SS34 rotor in a Sorvall centrifuge for 10 min and suspended in one-fifth the original volume of Veronal-buffered glucose. A 0.1-ml sample of the bacterial cell suspension was added to 0.9 ml of serum-Veronal-buffered glucose mixture and incubated in a standing water bath at 37°C. Survival of 100% was equivalent to 10^8 cells per ml in all cases. Duplicate 0.05-ml samples were withdrawn after 5, 10, 20, 30, 60, and in some cases 120 min of incubation, diluted in buffered saline with gelatin solution (Curtiss et al., submitted for publication), and plated by the soft agar overlay method on Penassay agar (Difco Laboratories, Detroit, Mich.). Sera, diluents, and agar used with *E. coli* χ1776 were supplemented with 100 μg of diaminopi-

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TABLE 1. *Bacterial strains*

Strain	Genotype[a]	Derivation
χ1776	F⁻ *tonA53 dapD8 minA1 supE42* Δ40[*gal-uvrB*] λ⁻ *minB2 rfb-2 nalA25 thyA142 oms-2 metC65 oms-1* Δ29[*bioH-asd*] *cycB2 cycA1 hsdR2*	χ1849 (Curtiss et al., submitted for publication)
χ1833	F⁻ *supE42* λ⁻ *nalA27*	(Curtiss et al., submitted for publication
Lilly (ECL3-6777)	Wild-type prototroph	(31)
75R	Wild-type prototroph	R. A. Bobo

[a] Allele numbers have been assigned by the Coli Genetic Stock Center.

TABLE 2. *Human sera*

Serum	Source		
	Age (yrs)	Sex	Comments
Agammaglobulinemic human serum[a]	12	M	
C2DHS	7	M	See (15)
C6DHS	6	F	See (30)
Serum "T"	24	F	Systemic lupus erythematosus
Serum "F"	23	M	Renal transplant recipient; corticosteroid and azathiaprine therapy
Serum "C"	67	M	Hodgkin's lymphoma and disseminated varicella virus infection
Pooled NHS			Four healthy adults

[a] Contains less than 1 mg of immunoglobulin per ml.

melic acid per ml and 20 μg of thymidine per ml. Agar plates were incubated for 24 or 48 h at 37°C, and colonies were enumerated.

RESULTS

Effect of NHS on *E. coli* χ1776. *E. coli* χ1776 showed a decrease in titer of 4, 5.5, and 7 logs after 30 min in 6.3, 12.5, and 25% NHS, respectively (Fig. 1). Heat-inactivated NHS was not bactericidal over 30 min for χ1776 when diaminopimelic acid and thymidine were supplied. When this experiment was performed using heat-inactivated NHS supplemented only with thymidine, *E. coli* χ1776 underwent diaminopimelic acid-less death at the rate of 1 log per 24 h (data not shown).

Effect of NHS and agammaglobulinemic human serum on *E. coli* strains. *E. coli* χ1776 was found to be slightly more susceptible to 25% NHS over time than either *E. coli* Lilly or *E. coli* χ1833 (Fig. 2a). However, none of the serum-susceptible strains tested in this fashion yielded survivors after 60 min in NHS. A 25% concentration of agammaglobulinemic serum caused a 6-log fall in titer of χ1776 after 60 min (Fig. 2b). Corresponding to the results with NHS, *E. coli* χ1776 and *E. coli* Lilly tested in agammaglobulinemic serum were both more susceptible than *E. coli* χ1833. Serum-resistant *E. coli* 75R increased in titer in both NHS and agammaglobulinemic human serum during each assay period.

Effect of C2DHS and C6DHS on *E. coli* χ1776. *E. coli* χ1776 showed a slightly greater than 2-log decrease in titer after 60 min in 25% C2DHS (Fig. 3). Thus, C2DHS was much less

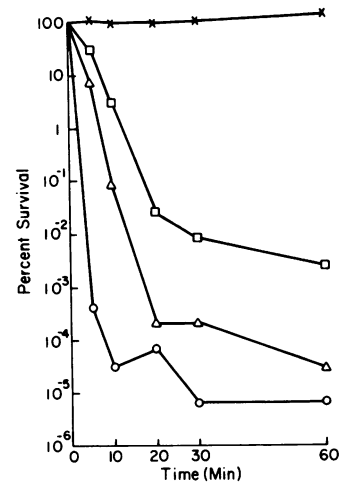

FIG. 1. *Effect of NHS on the survival of E. coli χ1776. Heat-inactivated NHS (×), 6.3% NHS (□), 12.5% NHS (△), and 25% NHS (○).*

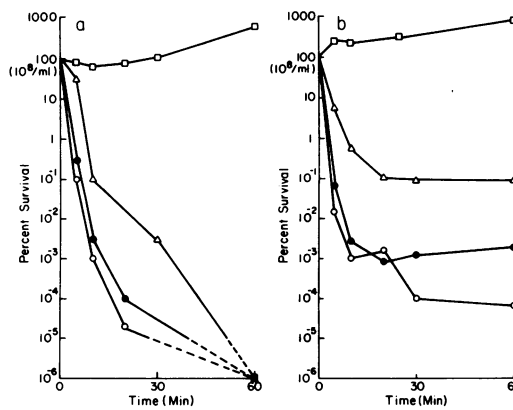

FIG. 2. *Comparison of survival of four E. coli strains in 25% NHS (a) and in 25% agammaglobulinemic human serum (b): serum-resistant E. coli strain 75R (□), E. coli χ1833 (△), E. coli Lilly (●), and E. coli χ1776 (○).*

bactericidal for χ1776 over this time period than was NHS (Fig. 1). C6DHS completely lacked bactericidal activity against χ1776 over 30 min. When heat-inactivated NHS was added to C6DHS, prompt killing of the strain was observed.

Effect of sera from immunocompromised patients on *E. coli* χ1776. Sera from three patients with immunodeficiency states (Hodgkin's lymphoma, systemic lupus erythematosus, and corticosteroid therapy for renal transplantation) were tested individually and found to be bactericidal for both *E. coli* χ1776 and *E. coli* Lilly (Fig. 4). Both strains demonstrated a mean 6-log decrease in titer after 30 min in each patient serum tested.

Effect of enrichment and mutagenesis on the serum susceptibility of *E. coli* χ1833. An attempt was made to select mutant derivatives of *E. coli* χ1833 which might be more serum resistant after testing in NHS (Fig. 5). After the first incubation in NHS was performed, three surviving colonies were picked from the last time of sampling that yielded survivors (usually the 60-min sample), placed in Lennox broth, and grown at 37°C to a titer of 2×10^8 cells per ml. The incubation in 25% NHS was then repeated using this inoculum, followed by incubation, selection of three surviving colonies, and repetition of the procedure. We found no evidence that four such cycles would select for isolation of χ1833 cells that were more resistant to serum. Mutagenesis of χ1833 using either nitrosoguanidine or ultraviolet light also failed to produce cells with increased resistance to serum (data not shown).

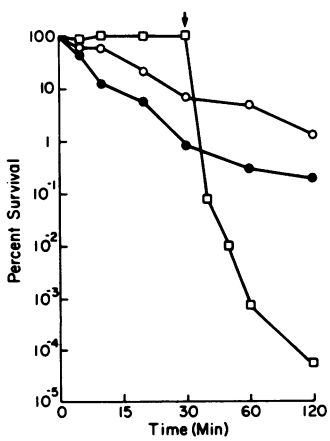

FIG. 3. *Effect of C2DHS and C6DHS on the survival of E. coli χ1776: 12.5% (○) and 25% (●) C2DHS and 12.5% C6DHS (□). At 30 min (arrow), an equal volume of heat-inactivated NHS was added to the assay mixture employing C6DHS.*

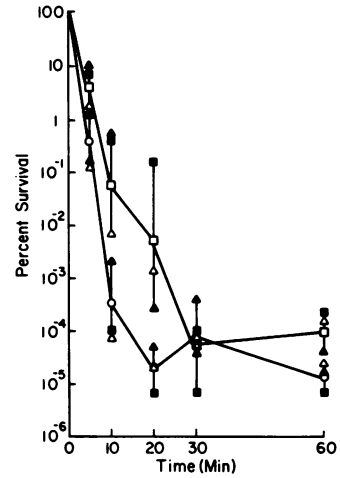

FIG. 4. *Effect of serum from three immunocompromised patients on survival of E. coli χ1776 (○) and E. coli Lilly (□). Points used for survival curves reflect geometric mean titers of bacteria surviving at each sampling time of sera from the patient with systemic lupus erythematosus (△), the renal transplant recipient (▲), and the patient with Hodgkin's lymphoma and disseminated varicella infection (■).*

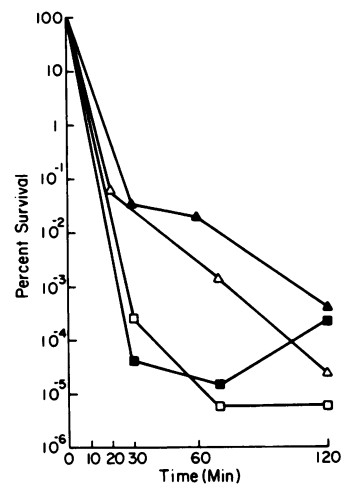

FIG. 5. *Effect of enrichment for serum-resistant derivatives of E. coli χ1833. Survival of survivors from the first (▲), second (△), third (■), and fourth (□) cycle of killing by 25% NHS. See text for other details.*

DISCUSSION

Experimental evidence (4, 8, 14, 24) and clinical observations (5, 13, 16, 23) suggest that serum resistance is an important virulence factor for some gram-negative bacteria. The future uses of recombinant DNA technology make it desirable for the bacterial strains used for such purposes to be avirulent and to constitute no

hazard to laboratory personnel or patients in medical care facilities. Enteric bacteria are able to resist the bactericidal activity of NHS via mechanisms which have been extensively studied (2, 6–8, 13, 22, 27, 28), but remain incompletely understood. Taylor and Hughes (28) recently suggested that the serum resistance of gram-negative enteric bacteria was multifactorial in origin and in any one individual strain might be due to several cell surface-associated components. The serum resistance of some *E. coli* strains appears to depend in part on the presence of lipopolysaccharide side chains in the cell wall. *E. coli* K-12, which completely lacks lipopolysaccharide side chains because of defects in the required *rfb* genes (17, 26), has been found to be serum susceptible in several studies (7, 28). *E. coli* K-12 strains carrying the R1 or R100 plasmids were thought by Reynard and Beck (22) to demonstrate significant levels of serum resistance. This analysis was not supported, however, by Taylor and Hughes (28), who performed similar experiments. That plasmids may increase levels of serum resistance in non-K-12 *E. coli* has been shown by Taylor and Hughes (28) and Fietta et al. (7). Using the anti-K-12 antiserum, Akiyama and Inoue (2) were able to select for complement-resistant K-12 derivatives. These isolates, however, were not stable upon passage and therefore apparently had adapted to the experimental conditions rather than having undergone stable genotypic change to complement resistance.

Complement, when activated either by the classical (9, 25) or the alternative pathway (29), empowers NHS with a bactericidal system which is lytic for many gram-negative bacteria. Further evidence for the importance of the complement system in lysis of serum-susceptible *E. coli* K-12 strains is presented in this study. Serum lysozyme, which may play only a minor role in bactericidal activity, may be of increased importance in patients with hematological disorders accompanied by hyperlysozymemia (19). Normal levels of serum antibodies are not required for lysis of *E. coli* K-12 strains, since we noted that serum from an individual with agammaglobulinemia was rapidly bactericidal for these strains.

Persons with deficiencies of individual late-acting complement components C5 through C9 are often susceptible to recurrent infections caused by encapsulated pyogenic bacteria (1, 9, 18). In most instances, the biological functions usually mediated by the early complement components—opsonic activity, immune adherence, and chemotactic factor generation—are normal. However, sera from such individuals are almost always deficient in hemolytic complement activity and bactericidal activity. As expected, serum from a young female with hereditary absence of C6 (30) was not bactericidal for χ1776 or other serum-susceptible *E. coli* strains. However, bactericidal activity against χ1776 was restored to the test C6DHS when heated NHS was added, presumably providing C6 which is considered heat stable (12).

Deficiency of the second component of complement is one of the more common complement deficiency states, and affected individuals rarely experience recurrent infection (1, 15). We found that C2DHS killed χ1776 at a slower rate than did NHS. Similar kinetics of killing, presumably mediated by the alternate pathway of complement activation, have been described by Reed and Albright in the killing of a *Shigella* strain by C2DHS (21).

We conclude that *E. coli* χ1776 is susceptible to human serum from normal individuals and patients with increased susceptibility to infection. These studies suggest that χ1776 has diminished potential for pathogenicity in humans in circumstances in which the bacterium might interact with human serum or other body fluids possessing an activated complement system.

ACKNOWLEDGMENTS

We thank Robert Stroud, Elliott Pearl, and Larry Vogler for providing serum used in this study. We thank Pat Pierce and Sue Merrell for assistance in preparing the manuscript.

This research was supported by Public Health Service grants (AI-07041 and AI-11456) and a Public Health Service contract (AI-62508) from the National Institutes of Health. W.J.A. was supported by a Public Health Service postdoctoral traineeship (T32-AI-07041) from the National Institutes of Health.

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