

Inhibition of Serum Bactericidal Reaction by Lipopolysaccharide

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An Rc-mutant of *Escherichia coli* that lacks UDPgalactose 4-epimerase grows normally without galactose but makes lipopolysaccharide lacking most of its carbohydrate. Exogenous galactose overrides the mutation and results in the formation of a complete lipopolysaccharide, thereby producing a smooth phenocopy. The smooth phenocopy was much more resistant to the bactericidal activity of normal human serum than was the rough phenotype. More complement was utilized by the rough mutant in the bactericidal process than by the smooth phenocopy. An antiserum was prepared in rabbits to a specific outer membrane protein in the mutant bacterium, the lambda receptor, whose expression could be suppressed by the addition of 10 mM maltose. The effect of the O-antigen in the lipopolysaccharide produced by the smooth phenocopy on the binding of antibody to the lambda receptor was determined. The smooth phenocopy exhibited significantly less binding of antibody than did the rough phenocopy. In addition, expression of the lambda receptor had little effect on the binding of antibody to the lambda receptor in the smooth phenocopy but caused significantly increased binding in the rough mutant. The results suggest that the increased resistance to the lethal action of normal human serum shown by the smooth phenocopy may be due to the blocking of antibody binding sites by the O-antigen of lipopolysaccharide, thereby preventing activation of the classical pathway of complement.

The bactericidal effect of normal human serum plays an important role in host defense against bacterial infection. This phenomenon has been widely noted and studied since the late 1800s (2, 11) and has been shown to be complement mediated (6). Bacterial resistance to this natural host defense mechanism has drawn a great deal of interest as an explanation for the underlying etiologies for many clinically apparent situations ever since Roantree and Rantz provided such a scenario in 1959 (13). Although serum resistance of gram-negative bacteria probably has a multifactorial basis *in vivo*, the outer membrane clearly is involved as the most peripheral component of the bacterial cell envelope. Lipopolysaccharide (LPS), a unique component of the gram-negative outer membrane, has been scrutinized for its possible role in serum resistance, particularly because of its O-side chain, which extends out from the lipid bilayer.

Investigation has shown that the incorporation of LPS into the outer membrane of rough, serum-sensitive, gram-negative bacteria that lack the peripheral O-side chain renders them more resistant to the lethal action of complement (1, 16). It has also been demonstrated that the quantity of LPS in the outer membrane of smooth, gram-negative bacteria, which are able to synthesize a complete LPS molecule with O-side chains, is also an important factor in determining their serum resistance (17).

However, the role of LPS in the serum resistance exhibited by smooth organisms remains unclear. Differences in membrane fluidity have been noted to affect the final expression of complement action (9). In addition, other membrane differences between smooth and rough organisms may play a role in serum resistance by preventing certain complement components from remaining surface bound (7, 8).

We set out to investigate the effect of LPS on the cellular binding of antibody to a specific cell envelope protein, the lambda receptor. The lambda receptor is an outer membrane protein present in some members of the *Enterobacteriaceae*, most notably *Escherichia coli* (14), which functions not only

as a receptor for the phage lambda but also as the maltose transport protein in these organisms (5, 15). Specific killing by antibody and complement of *E. coli* K-12 strains bearing the lambda receptor has been previously reported (4).

In this report we present evidence showing that bactericidal activity not only parallels complement utilization but also that LPS plays a vital role in preventing the attachment of an antibody to a specific cell surface protein, the lambda receptor.

MATERIALS AND METHODS

Bacterial strains and media. A mutant of *E. coli* (O111:B4), designated J-5 (3), was a gift from Loretta Leive of the Laboratory of Biochemical Pharmacology, National Institutes of Health, Bethesda, Md. This organism normally grows as a rough phenocopy, lacking O-side chains, in the absence of galactose. When galactose is exogenously supplied the organism is able to make a complete LPS and assumes a smooth phenotypic appearance (3). The cells were grown in M-9 minimal medium (10) supplemented with Casamino Acids. Galactose at a final concentration of 1 mM was added to the growth medium to generate production of smooth phenocopies. Expression of the lambda receptor was suppressed by addition of 10 mM maltose (5). Overnight and log-phase cultures were grown at 37°C with shaking and aeration. Organisms were plated on Penassay agar (Difco).

E. coli LE 392, which was used as the host strain for the lambda phage plaque inhibition assay, was obtained from Philip LoVerde, Department of Microbiology, State University of New York, Buffalo.

Sera. Human serum was collected from a normal healthy donor and stored at -70°C until used. Antiserum was prepared against the lambda receptor by inoculating rabbits intraperitoneally with 0.6 ml of heat-killed *E. coli* J-5 cells (10⁶ cells per ml) expressing the lambda receptor. Subsequent immunizations were given intravenously with 0.3 ml of the bacterial suspension twice a week for 8 weeks. The collected serum was adsorbed with *E. coli* J-5, which did not express the lambda receptor, until there was no visible

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TABLE 1. Relationship between bacterial killing and complement utilization for rough and smooth phenocopies

No. of cells/ml	% Survival ^a for <i>E. coli</i> J-5:		% Complement activity remaining ^a for <i>E. coli</i> J-5:	
	Rough	Smooth	Rough	Smooth
10 ⁸	0.6 ± 0.3	165.4 ± 68.1	0.3 ± 0.3	62.6 ± 9.9
10 ⁷	0.2 ± 0.1	133.9 ± 21.6	9.9 ± 6.2	67.9 ± 10.8
10 ⁶	0.1 ± 0.1	18.7 ± 3.3	76.9 ± 9.7	81.7 ± 7.5

^a Cells were grown without added maltose (i.e., receptor expressed). Results are means and standard deviations.

clumping of the adsorbing bacteria as determined by a slide agglutination procedure. The adsorbed antiserum was stored at -70°C until used. Specific *E. coli* O-antiserum was a gift from William Bartholomew of the Erie County Medical Center, Buffalo, N.Y.

Isotope. ¹²⁵I-labeled staphylococcal protein A was generously donated by Murray Stinson of the Department of Microbiology, State University of New York, Buffalo. The labeled protein A had a specific activity of 2.27 × 10⁻⁶ mCi/ng.

Assay of bacterial killing. Freshly harvested log-phase bacteria were washed three times with 0.15 M NaCl and suspended in the assay buffer, which was Veronal-buffered saline with added Mg²⁺, Ca²⁺, and gelatin (VBS-G) (12). The suspension was spectrophotometrically adjusted to an absorbance of 0.50 at 600 nm to yield approximately 4 × 10⁸ bacteria per ml. The suspension was then diluted 1:100 in VBS-G, and equal volumes of cells were mixed with serum that had been appropriately diluted with VBS-G. The assay mixture was incubated in a 37°C water bath for 60 min. Viable counts were made with serially diluted samples spread on Penassay agar plates, followed by overnight incubation at 37°C. Results were expressed as percent survival based on colony counts at time zero and after 60 min.

Complement fixation assay. Serum from the bactericidal assay was tested for its complement-fixing ability (12). Results (Table 1) are expressed as percent complement activity remaining based on absorbance readings obtained at time zero and after 60 min of incubation. Results are expressed as absorbance at 413 nm in Fig. 1 and 2. Controls in all experiments contained the complete reaction mixture except for bacterial cells.

Lambda phage plaque inhibition assay. Lambda phage were generously donated by Philip LoVerde. The lambda phage were diluted in buffer containing 10 mM Tris (pH 7.5), 10 mM MgCl₂, and 0.1 M NaCl. They were stored over a few drops of chloroform in the Tris-Mg²⁺ buffer with 0.1% gelatin added. The plaque assay was preceded by an adsorption phase in which 1.0 ml of diluted lambda phage was mixed with 2.0 ml of adsorption solution and 1.0 ml of *E. coli* J-5 in which the lambda receptor was either expressed or suppressed. The adsorption incubation was facilitated by an adsorption solution containing 10 mM MgCl₂ and 10 mM CaCl₂. The mixture was incubated in a shaking water bath at 37°C. The adsorption mixture was then filtered through a membrane filter and serially diluted. The plaque assay consisted of adding 10 ml of serially diluted lambda phage to 250 ml of *E. coli* LE 392 host bacterial cells and 250 ml of adsorption solution.

The assay mixture was also incubated in a shaking water bath at 37°C for 20 min. After incubation, 2.5 ml of top agar (0.7% agar and NZY broth) was added and mixed. The

mixture was poured onto NZY agar plates and incubated overnight at 37°C.

Radioassay procedures. An overnight culture of *E. coli* J-5 was washed three times in 0.15 M NaCl and suspended in VBS-G to its original volume before incubation of 1.0 ml of bacteria with 1.0 ml of absorbed rabbit serum at 37°C for 30 min. After centrifugation and removal of the supernatant by aspiration, 1.0 ml of radiolabeled protein A was added, and the mixture was vortexed before being incubated at 37°C for 30 min. The mixture was then centrifuged, and the pellet was washed twice with 0.15 M NaCl. Radioactivity in the pellet was then measured in a Beckman 4000 gamma counter for 1 min. Results are expressed as counts per minute. Differences noted were tested for significance by using analysis of covariance.

RESULTS

Phenotypic expression of bacteria in the presence and absence of galactose. *E. coli* J-5 cells were tested for their ability to make complete LPS molecules in the presence and absence of galactose. The criteria used to judge the phenotype of the organism were (i) colonial morphology on nutrient agar; (ii) agglutination with acriflavine dye, which non-specifically agglutinates rough cells; and (iii) agglutination with specific *E. coli* O-antiserum. The addition of galactose to the growth medium of *E. coli* J-5 produced smooth cells, as illustrated by the classical smooth, moist colonial appearance on nutrient agar, agglutination by the specific *E. coli* O-antiserum, and negative reaction with the acriflavine dye. Conversely, *E. coli* J-5 grown in the absence of galactose produced wrinkled, dry colonies on nutrient agar, no agglutination with the specific *E. coli* O-antigen antiserum, and positive reaction with the acriflavine dye, all characteristic of rough cells.

Bacterial killing and complement utilization. Smooth and rough *E. coli* J-5 cells were compared for their ability to withstand the bactericidal effects of normal human serum at a final dilution of 1:48 at three cell concentrations: 10⁶, 10⁷, and 10⁸ cells per ml. In addition, the amount of complement remaining after the bactericidal process was measured at the same cell concentrations by complement fixation (Table 1). The smooth phenocopy of *E. coli* J-5 was much more resistant to the bactericidal properties of normal human

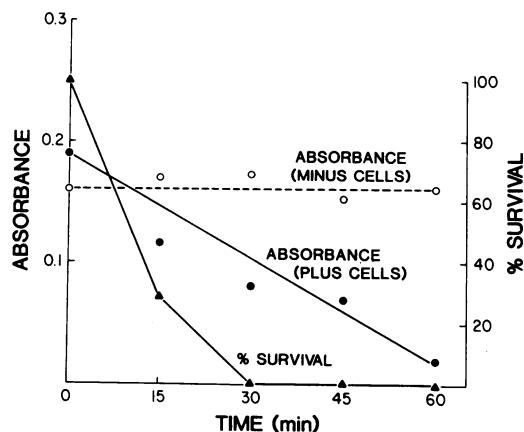


FIG. 1. Time course study showing the depletion of complement from normal human serum by the rough mutant and bacterial killing over a 60-min period.

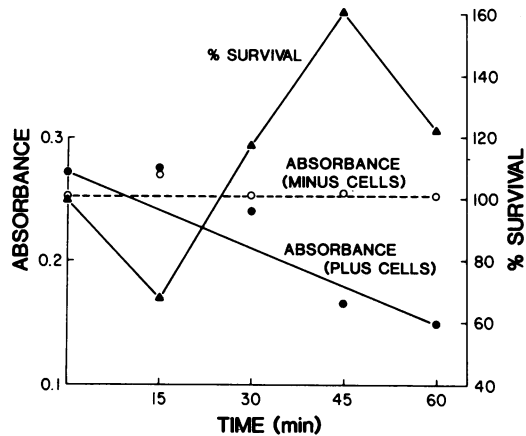


FIG. 2. Time course study showing the depletion of complement from normal human serum by the smooth phenocopy and bacterial killing over a 60-min period.

serum than was the rough phenocopy at all cell concentrations tested. Utilization of complement by smooth and rough cells was similar at 10^6 cells per ml, but a progressively larger difference between the two cell types at 10^7 and 10^8 cells per ml, respectively, was noted.

In single experiments, the time of bactericidal activity and complement utilization for both rough and smooth cells at 10^7 cells per ml were measured (Fig. 1 and 2). Rough cells showed a very high sensitivity to the bactericidal properties of normal human serum, whereby the percent survival dropped to 48% within the first 15 min of incubation with serum. Increased complement utilization followed the drop in percent survival throughout the entire time course. Smooth cells exhibited a marked resistance to the bactericidal effect of the serum over the entire 60-min time course, with percent survival reaching 122% (growth) at the end of the incubation. Complement utilization also increased over the 60-min period but did not reach the extreme noted with the rough organisms. In other experiments (data not shown) it was found that the presence or absence of the lambda receptor did not affect killing of either rough or smooth organisms by normal human serum.

Expression of the lambda receptor. That expression of the lambda receptor could be controlled by the concentration of maltose in the growth medium was tested with a plaque-inhibition assay (Table 2). Pre-adsorption of the virus with *E. coli* J-5 grown in the absence of maltose resulted in an inhibition of lambda phage infection and plaque formation in the *E. coli* LE 392 host cells. Conversely, pre-adsorption of the virus with *E. coli* J-5 grown with maltose resulted in host

TABLE 2. Lambda phage plaque inhibition assay for detection of lambda receptor

Dilution	No. of plaques ^a for:		
	<i>E. coli</i> J-5 with maltose	<i>E. coli</i> J-5 without maltose	Control (no cells)
10^{-5}	500	0	500
10^{-6}	58	2	56
10^{-7}	3	1	3
10^{-8}	1	0	0

^a The results with phage dilutions of 10^{-6} and 10^{-7} are the means of two experiments. The results with phage dilutions of 10^{-5} and 10^{-8} are from single experiments.

TABLE 3. Effect of LPS on the binding of anti-lambda receptor antibody

<i>E. coli</i> strain	Amt of protein A (cpm) bound by cells:		Difference (B - A) ^a
	Without lambda receptor (A)	With lambda receptor (B)	
J-5 rough	46,506	58,331	11,825
J-5 smooth	28,279	30,525	1,246

^a The difference between B and A for rough and for smooth cells is significant at $P < 0.05$ from analysis of variance.

cell plaque formation. The results are consistent over a range of virus dilutions.

Effect of LPS on binding of lambda receptor antibody. Smooth and rough phenocopies of *E. coli* J-5 both expressing and lacking the lambda receptor by manipulating the growth environment with respect to galactose and maltose were compared for their ability to bind rabbit anti-lambda receptor antibody as detected by radiolabeled protein A. The results are the means and standard deviations of four experiments (Table 3). The rough cells exhibited a significantly greater difference in protein A binding in the presence and absence of the lambda receptor than did the smooth phenocopy ($P < 0.05$ from analysis of covariance).

DISCUSSION

E. coli J-5 is an Rc-mutant that lacks the enzyme UDPgalactose 4-epimerase and therefore cannot synthesize O-side chains to make complete LPS molecules unless galactose is exogenously supplied. Rough and smooth phenocopies can thus be predetermined by manipulating the availability of galactose in the growth medium.

The smooth phenocopy was much more resistant to the bactericidal effect of normal human serum than was the rough mutant at all concentrations of cells tested. Since the bacteria are presumably genetically identical, the difference can be directly attributable to the presence of O-side chains in the LPS of the smooth phenocopy.

Studies were done to determine whether the O-side chains on the LPS of the smooth cells were acting to deplete the serum of its complement at a distance from the cell surface, thereby minimizing complement deposition on the outer membrane. The results showed that at all cell concentrations tested, the smooth phenocopy utilized less complement in the bactericidal process than did the rough mutant cells, particularly when greater cell concentrations were used. It therefore appears that the LPS of the smooth phenocopy does not protect the cell by depleting complement far from the cell surface.

Antibody studies were then done to determine whether the LPS of the smooth phenocopy could be blocking the accessibility of cell surface antigens to antibody, a necessary first step in the classical complement sequence. The phage lambda receptor, which also serves in the maltose transport system, was chosen as the cell surface antigen against which antibodies were developed. The ability to manipulate the expression of the lambda receptor by maltose availability in the surrounding growth environment was checked by a lambda phage plaque-inhibition assay. The results showed that *E. coli* J-5 possessed the lambda receptor and that its expression could be suppressed by the addition of maltose to the growth medium. Since expression of the lambda receptor could be controlled, it was possible to produce cells without

the antigen to be used for adsorbing all other nonspecific antibodies from the rabbit immune serum.

The effect of LPS on the binding of antibody to the lambda receptor was tested by using radiolabeled protein A as the indicator of bound antibody. The results showed that the rough mutant bound almost twice as much of the radiolabel as did the smooth phenocopy. They also showed that the difference in binding in the presence and absence of the lambda receptor was significantly greater in the rough mutant than in the smooth phenocopy, suggesting that antibody binding was being blocked by the O-side chains of the LPS of the smooth organisms.

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