The Surface Antigens and Phage Receptors in *Escherichia coli* B* (33031)

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Studies of cell walls of gram-negative bacteria have revealed a three-layered structure consisting of lipoprotein, lipopolysaccharide, and mucopeptide (1). Substantial progress has been made in chemical characterization of the lipopolysaccharide and the mucopeptide, but the nature of the lipoprotein component has remained obscure. The surface structures of the gram-negative bacteria have been found to be highly antigenic. The polysaccharidic component is the most clearly identifiable antigen (2), but the immunogenicity of the lipoprotein has not been demonstrated heretofore. The cell surface of the bacteria also provides the sites for viral attachment and in some species of Enterobacteriaceae (i.e., Salmonella) a fairly good correlation has been established between the possession of certain antigenic determinants and sensitivity to bacterial viruses (3). These data indicated a close relationship between phage receptors and surface antigens. In the present study we investigated the relationship between the receptors for T phages and surface antigens of *Escherichia coli* B and attempted to identify antibodies directed against these structures.

Materials and Methods. Phage attachment to its specific receptor-site was measured by two techniques: (i) It is known that phage attachment to isolated receptor substance leads to the self-destruction of the phage. Purified soluble phage receptor containing substances were mixed with a known number of phage particles. Following incubation of the mixtures for 3 hours at 37°C the number of phage particles in the supernatant were determined by plating aliquots in soft agar in the presence of an *E. coli* B inoculum. Phage handling techniques used in our experimental work are described in detail by Adams (4). (ii) The ability of the antibacterial sera to prevent phage adsorption was tested by incubating serum dilutions with intact bacteria or solutions of purified receptor substances at 37°C for 1 hour prior to addition of phage particles to the mixtures. In the presence of antibodies directed against phage-receptor sites, the phage particles could not interact with the receptor sites, and the number of phage particles in the mixtures remained unchanged throughout the experiment.

The lipopolysaccharidic substance was prepared by the method of Westphal *et al.* (5). Cell wall suspensions of *E. coli* B were extracted with a mixture of phenol and water (45:55) at 68°C for 30 min. After cooling, the upper aqueous phase was separated, and this phase contained the water-soluble lipopolysaccharide. The polysaccharide was purified by ultracentrifugation, deoxyribonuclease trypsin digestion, and repeated alcohol precipitation. The lipoprotein substance was also prepared from cell walls of *E. coli* B. The cell wall suspensions were first solublized by lysozyme and EDTA. The soluble lipopolysaccharide–protein complex was dissociated by 2 *M* urea and the protein fraction separated by gel electrophoresis. The lipopolysaccharide substance was found to be heat-resistant (1 hour at 80°C) and unaffected by proteolytic enzymes. The lipoprotein substance was heat labile (inactivated by heating for 30 min at 80°C) and its antiphage activity abolished by treatment with trypsin, papain, or lipase. The lipopolysaccharide was found to be composed of 80% polysaccharide and 20% lipid. The lipoprotein component was made up of 95% protein, 3% lipid, and 2% sugar. Thus, most of the lipid was associated with the polysaccharide. The antibacterial serum was prepared by immunizing rabbits intravenously with multiple doses of live cells of *E. coli* B. Animals were injected with the bacteria

* This study was initiated at the House of the Good Samaritan, Children's Hospital Medical Center, Boston, Massachusetts, and supported by PHS Training Grant 5T1 GM 1255.

[1] NIH Research Career Development Awardee (7K03 HE25302).

TABLE I. Inactivation of T-Phages by Different Preparations of *E. coli* B.[a]

Preparation tested	Percentage inactivation of phage tested			
	T_2	T_3	T_6	T_7
Heated *E. coli* B (5)[b]	90	95	85	98
Lipopolysaccharide (0.5)	0	96	0	90
Lipoprotein (0.2)	89	0	94	0

[a] Phage inocula (2×10^8 particles/mixture).
[b] Dosage (mg) given in parentheses.

twice weekly; each immunizing dose consisted of 10^9 cells.

Oligosaccharides were prepared by acid hydrolysis of purified lipopolysaccharide. A solution of 0.1 *M* acetic acid was used for hydrolysis which was carried out at 100°C. The oligosaccharides were separated on a Sephadex G-25 column. The size and composition of the oligosaccharides were determined by paper chromatography.

Results. The specificity of the antiphage activity of the purified receptor substances was tested by incubating 2×10^3 phage particles per tube (T_2, T_3, T_6 and T_7 phages were used) with solutions of the receptor substances.

As shown in Table I, the lipopolysaccharide neutralized phages T_3 and T_7 and lipoprotein substance inactivated phages T_2 and T_6. Intact heat-killed *E. coli* B cells possessed receptors for all four phage types. In further experiments the agglutinating and phage-absorption preventing properties of antibacterial sera of rabbits immunized with *E. coli* B were determined. Figure 1 illustrates the rises in antibody titers in the course of immunization. It was found that the titers of agglutinins and the antireceptor activity of the serum for phages T_3 and T_7 increased in a strikingly similar pattern. The antireceptor activity of the serum for phages T_2 and T_6 developed in a different, independent pattern. This marked difference in the development of antireceptor serum activity indicated that the antiserum may contain two types of antibodies directed against different antigenic determinants. To test this possibility, antisera containing high antireceptor activities for all phages were absorbed either with the lipopolysaccharide or with lipoprotein preparations and the absorbed sera were tested for their antireceptor activities. As shown in Tables II and III, antiserum which was absorbed with the lipopolysaccharide lost its capacity to neutralize receptors for phages T_3 and T_7 but still inactivated receptors for phages T_2 and T_6. Antiserum absorbed with the lipoprotein preparation lost its capacity to neutralize receptors for phages T_2 and T_6, but still inactivated receptors for phages T_3 and T_7. Heat-killed cells of *E. coli* B removed the serum antireceptors activity for all four tested phages. Antiserum absorbed with the lipopolysaccharide lost its agglutinating activity; absorption of the antiserum with the lipoprotein fraction had no detectable effect on the agglutination of bacteria by the serum.

The results provided evidence that the antiserum indeed contained two types of antibodies, one directed against the antigenic determinants in the lipopolysaccharide and the other against the determinants in the lipoprotein substance of the cell wall. Immunodiffusion experiments in Ouchterlony plates revealed precipitin formation between the polysaccharidic antigen and the antiserum. No visible precipitation occurred between the protein substance and the antiserum. The close relationship between the phage receptors for phages T_3 and T_7 and the polysaccharidic antigen was further confirmed in the immune reaction inhibition experiments. Oligosaccharides prepared by mild hydrolysis

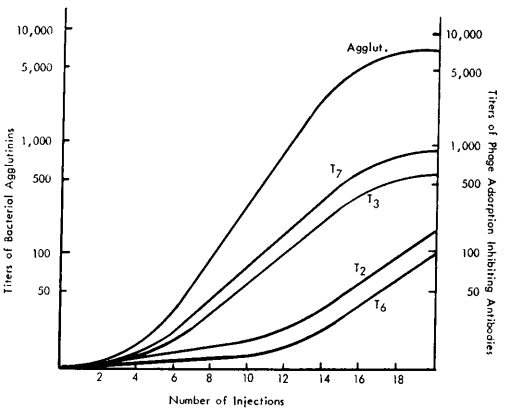

FIG. 1. Titers of agglutinins and phage-adsorption inhibiting antibodies.

TABLE II. Effect of Antibacterial Antiserum on the Inactivation of T-Phages by the Protein Preparations.[a]

Preparation tested	Percentage inactivation of phage tested	
	T_2	T_6
Lipoprotein (conc 0.1 mg)	93	100
Lipoprotein plus anticoli antiserum (dil 1:10)	0	0
Lipoprotein plus anticoli antiserum abs. with whole cell	90	94
Lipoprotein plus antiserum abs. with lipopolysaccharide	0	0
Lipoprotein plus anticoli antiserum abs. with lipoprotein	91	96

[a] Phage inocula (2×10^8 particles/mixture).

(0.1 *M* acetic acid) of the polysaccharide were used as inhibitors of the precipitin reaction and of the serum antireceptor activity. The oligosaccharides were separated on a Sephadex G-25 column. It was found that a polymer of 6 sugars (hexosaccharide) in amounts of 10 μg/mixture inhibited effectively both the precipitin reaction and the serum antireceptor effect. The oligosaccharide was composed of glucose, glucosamine, and heptose. Results of the experiments concerned with the inhibitory effect of the oligosaccharides are shown in Table IV. It is interesting to note that as shown in Table V the phage inactivating capacity of the lipopolysaccharide was lost more rapidly than its antiserum precipitating activity. These results indicated that although the phage receptor and the somatic antigen may share a common determinant, they still may differ in their chemical structure.

Discussion. Our studies confirmed Weidel's contention that in *E. coli* B, receptor-sites for different phages are located in different layers of the cell wall (6). Weidel suggested a mosaic-like structure of phage receptors based upon solubility of the cell wall in 90% phenol. Weidel believed that the phenol soluble substance, which contained receptors for T_2 and T_6 phages, was made up of lipoprotein and that the phenol insoluble part, containing receptors for T_3 and T_7 phages, was made up of lipopolysaccharide. The close link between the somatic polysaccharidic antigen and the phage receptors in enteric bacteria was also investigated and demonstrated by Goebel and Jesaitis (7). These investigators showed that the somatic antigen of phage-resistant *Shigella* is different in its chemical structure from the antigen of the parent phage-sensitive bacterium. The ability of antibacterial antiserum to prevent phage adsorption was described in the past by several investigators and it was also shown that this antibacterial antiserum has no direct antiphage activity (8–11). Utilizing the antireceptor activity of the bacterial antiserum, data were obtained suggesting existence of two types of antibodies, both directed against the surface antigens of *E. coli* B. Antibody

TABLE III. Effect of Antibacterial Antiserum on the Inactivation of T-Phages by the Lipopolysaccharide.

Preparation tested	Percentage inactivation of phage tested	
	T_3	T_7
Lipopolysaccharide (conc 0.1 mg)	96	99
Lipopolysaccharide plus anticoli antiserum (dil 1:10)	0	0
Lipopolysaccharide plus anticoli antiserum abs. with whole cells	92	93
Lipopolysaccharide plus anticoli antiserum abs. with lipopolysaccharide	95	97
Lipopolysaccharide plus anticoli antiserum abs. with lipoprotein	0	0

TABLE IV. Inhibition of Antiserum Activity by Oligosaccharide.[a]

Preparation tested	Percentage inactivation of phage tested	
	T_3	T_7
Lipopolysaccharide (conc 0.1 mg)	97	100
Lipopolysaccharide plus antiserum (dil 1:10)	0	0
Lipopolysaccharide plus antiserum plus oligosaccharide (10 μg)	91	93
Oligosaccharide control (10 μg)	0	0
Antiserum control (dil 1:10)	0	0

[a] Phage inocula (2×10^3 mixture). Antiserum prepared against live *E. coli* B.

directed against the lipopolysaccharidic component of the cell wall prevented adsorption of phages T_3 and T_7 and these antibodies were also responsible for the agglutinating and precipitating properties of the antibacterial serum. Antibodies which prevented adsorption of phages T_2 and T_6 were directed against the lipoprotein component of the cell wall. To our knowledge this is the first report describing antibodies directed against the protein structure of the cell wall in gram-negative bacteria. Thus far, no information on specific biological or biophysical properties of this antibody is available. *E. coli* B is a nonpathogenic bacterium, but all gram-negative bacteria including the pathogens are assumed to have a similar three-layer structure of the cell wall. The discovery of immunogenicity of the cell wall protein enables us to postulate that the antibodies produced against this type of antigen may have some role in defense against infections by gram-negative bacteria. In fact, in many gram-negative pathogens, antigens responsible for induction of resistance to infection are not well defined. Antibodies produced against the lipoprotein fraction do not seem to cause agglutination of bacteria and do not precipitate with the protein antigen. Thus, the inhibition of phage adsorption seems at present to be the only method for their detection.

TABLE V. Effect of Acid Hydrolysis on Phage Receptor and Antigenic Activity of Lipopolysaccharide.[a]

Time of hydrolysis (min)	Percentage inactivation T_3	Degree of precipitation with *E. coli* antiserum
0	99	+++
5	98	+++
10	98	+++
15	65	+++
20	0	+++
25	0	+++
30	0	+++
35	0	+++
40	0	++
45	0	++
50	0	++
55	0	+
60	0	+

[a] Lipopolysaccharide (conc 0.1 mg/ml). Hydrolysis performed with 0.1 *N* acetic acid.

Summary. Two types of phage-receptor substances were isolated from the cell wall of *E. coli* B. The receptors for T_3 and T_7 were closely related to the somatic antigen. Antibacterial antiserum prevented adsorption of all four phages of the receptor sites. Antibodies which prevented adsorption of phages T_3 and T_7 were directed against the somatic antigen of the bacterium. Antibodies which prevented adsorption of phages T_2 and T_6 were directed against the lipoprotein moiety of the cell wall.

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Received Jan. 22, 1968. P.S.E.B.M., 1968, Vol. 128.

Immunological Studies of Duck Myoglobin* (33032)

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Myoglobin, the oxygen binding, heme protein of muscle, is widely found throughout the animal kingdom from molluscs (1) to man. Its concentration in muscle tissue of man is related to age, or maturity (2,3). It is present in both cardiac and skeletal muscle, but is deficient in smooth muscle (4,5). In some birds, certain skeletal muscles (e.g., the pectoral muscles of the chicken) may be pale in color, and deficient in myoglobin, while other muscles of the same bird may contain myoglobin. This report describes the production of a specific antiserum for an avian (duck) myoglobin, and its use in measuring age-related variations in muscle myoglobin content. Relationships between several bird myoglobins, and some physicochemical and immunologic characteristics of duck myoglobin are presented.

Materials and Methods. Myoglobin, concentrated from duck skeletal muscle by ammonium sulfate precipitation and gel filtration as previously described for that of man (3,6), was used as starting material for further chromatographic separation. Immunological precipitin reactions, and measurement of antigen content by radial diffusion in gels containing solutions of specific antibody have been described (3). Supernatant fluids obtained after centrifugation, at approximately 1500*g* for 30 min, of 30%, 0.15 *M* saline suspensions of muscle homogenates, were used as crude tissue extracts.

Results. 1. *Preparation of purified duck myoglobin by ion-exchange chromatography, for use as antigen.* Concentrated myoglobin, prepared from skeletal muscle of an adult muscovy drake by ammonium sulfate precipitation, was chromatographed on columns of DEAE-cellulose, at pH 8.0, with a variable ionic gradient (Fig. 1). Three components were resolved from the preparation. B, which eluted at low ionic strength, represented most of the heme protein content. The ratio of optical densities at 415 mμ and 280 mμ, for this component, was greater than 4. Two other heme-containing components were also

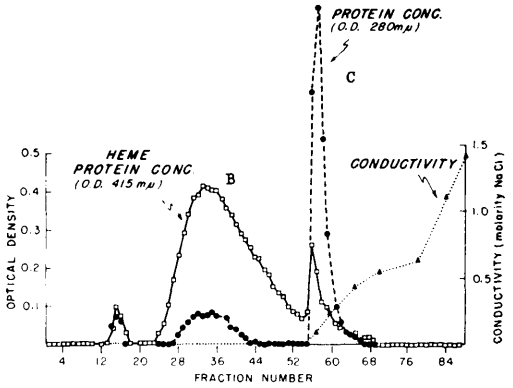

FIG. 1. DEAE-cellulose chromatographic elution diagram of partially purified duck myoglobin. Three heme protein containing components (A,B,C) are resolved. Starting buffer: 0.005 *M* phosphate, pH 7.5; final buffer: 0.005 *M* phosphate + 1 *M* NaCl, pH 7.5. Ionic gradient applied from a three-chambered Varigrad device. Percentage final buffer in each chamber: (1) 0, (2) 50, and (3) 33.3. Column 33 × 3 cm (18 gm of DEAE-cellulose, 0.89 meq/gm).

* Supported in part by grant from the John A. Hartford Foundation, New York, N. Y.