Studies of a Receptor for Felix 0–1 Phage in *Salmonella minnesota*

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SUMMARY

Isolated lipopolysaccharide from a rough mutant of *Salmonella minnesota* was shown to act as a receptor for Felix 0–1 bacteriophage. The isolated receptor structure could be dissociated by sodium deoxycholate with subsequent loss of receptor activity. When the deoxycholate was removed by dialysis the receptor activity was restored. Ultrasonic treatment destroyed the receptor activity and electron micrographs indicated that the ultrasonic treatment had altered the structure of the lipopolysaccharide. The experiments suggest that the inactivation of phages can be used as a test of the biological activity of a lipopolysaccharide. The interaction between the 0–1 phage and the receptor structure was also studied with the aid of electron microscopy.

INTRODUCTION

The somatic polysaccharide antigens in the cell wall of enterobacteria were shown to act as receptors for bacteriophages by Levine & Frisch (1933*a, b*, 1934) and Burnet (1934). Weidel (1951) and Hotchin, Dawson & Elford (1952) observed the direct attachment of phages to isolated cell walls by examination in the electron microscope. Since then, the binding of phages to isolated cell walls as well as to purified receptor structures has been observed for a variety of Gram-positive and Gram-negative bacteria.

Felix 0–1 phage (Felix & Callow, 1943) lyses 96–99·5 % of *Salmonella* strains studied (Cherry, Davis, Edwards & Hogan, 1954; Thal & Kallings, 1955; Seidel, 1956). Some details of the particular relationship between the 0–1 phage and salmonellas have been investigated (Kallings, 1967; Kallings & Lindberg, 1967). The 0–1 phage adsorbs to and lyses the rough mutant *Salmonella minnesota* R60, while it does not adsorb to or lyse another rough mutant *S. minnesota* R345. According to chemical analysis (Lüderitz *et al.* 1965) the difference between these two rough organisms is that the polysaccharide of strain R60 contains 2-keto-3-deoxyoctonate (KDO), heptose, galactose, glucose and glucosamine, whereas the polysaccharide of strain R345 contains only KDO, heptose, galactose and glucose.

As shown by Oroszlan & Mora (1963) and Ribi *et al.* (1966) surface-active agents such as sodium laurylsulphate and sodium deoxycholate have the ability to dissociate endotoxins. The polysaccharide and lipopolysaccharide complexes are dissociated into subunits with concomitant loss of biological activity. When the agent is removed the subunits reaggregate to macromolecular complexes and the biological activity reappears.

The present paper forms part of the studies on phage o-I and *Salmonella* initiated by Dr L. O. Kallings, and describes the isolation from *Salmonella minnesota* of a lipopolysaccharide receptor structure for phage o-I. The receptor activity of the isolated material was measured by its inactivation of the o-I phage. The depolymerization of the receptor structure by sodium deoxycholate was found to be correlated with a decrease in the o-I phage-inactivating capacity of the preparation. Upon removal of the surface-active agent the o-I phage-inactivating ability was partly restored. The effect of ultrasonic treatment of the receptor structure was examined and the interaction between the o-I phage and the receptor structure studied by electron microscopy.

METHODS

Bacteria. *Salmonella minnesota* strains R60 and R345 were obtained from Dr L. O. Lüderitz (Max-Planck-Institut für Immunbiologie, Freiburg, Germany).

Phage. Felix o-I phage was propagated on *Salmonella paratyphi* strain B76 by the agar layer method (Adams, 1959). The phage was purified and concentrated by alternate cycles in a Christ Omega preparative ultracentrifuge at 5000 g to sediment debris, and at 30,000 g to sediment phage. The phage pellet was suspended in 0.01 M-phosphate buffer (pH 7.2). The purified stock had a titre of 2.6×10^{10} plaque-forming units (p.f.u./ml.). The phage suspension was stored at $+4^\circ$.

Media. The defined glucose+salts medium M9 Adams (1959) was used for continuous cultivation of salmonella strains. This medium without glucose was used in the o-I phage inactivation experiments and as a diluent. Nutrient broth (Difco) was used for the growth of *Salmonella paratyphi* B76.

Preparation of lipopolysaccharide. Acetone-killed and dried bacteria were extracted by a modification of the phenol+water method of Westphal, Lüderitz & Bister (1952). The collected aqueous layers were washed five times with chilled ether to remove residual phenol. Nitrogen was passed through the solution to remove ether. The opalescent solution was freeze-dried. A 2% (w/v) suspension of the material in distilled water was centrifuged at 105,000 g for 4 hr in a Christ Omega preparative ultracentrifuge. The sediment was washed twice with water and recentrifuged. The pellet was freeze-dried. This lipopolysaccharide preparation (LPS) was used in the experiments.

Inactivation of phage o-I by the lipopolysaccharide. The LPS was diluted in medium M9 in twofold dilution steps. The o-I phage was diluted in medium M9 to 2×10^8 p.f.u./ml., which was the concentration used for inactivation experiments. One ml. of each LPS dilution + 1.0 ml. o-I phage suspension were mixed and incubated in a water bath at 37° for 60 min. Assay of unadsorbed o-I phage was performed by adding 1.0 ml. of the LPS+phage mixture to 8.5 ml. melted 0.6 (w/v) agar and 0.5 ml. of a 4 hr broth culture of the indicator strain (*Salmonella paratyphi* B76). Two ml. of this mixture was poured on agar plates. After overnight incubation at 37° , the plaque numbers were recorded. The experiments were done in duplicate. The inactivation of the o-I phage was determined by comparing with controls where the LPS was replaced by the defined M9 medium. The activity of the lipopolysaccharide was expressed as the concentration of LPS (dry wt/volume) causing 50% inhibition of the o-I phage under the experimental conditions (PhI50; Beumer & Dirkx, 1960).

Treatment of lipopolysaccharide with sodium deoxycholate (SDC). The LPS was

dissolved as completely as possible in 0.1 M-tris + HCl buffer (pH 8.0). SDC dissolved in 0.1 M-tris buffer was added to the LPS solution to a final concentration of 0.5 %. The mixture was kept at 4° overnight. The LPS + SDC suspension was dialysed three times against 0.1 M-tris-buffer (pH 8.0) followed by dialysis five times against medium M 9 (pH 7.4 at 4°).

Ultrasonic treatment of lipopolysaccharide. The LPS was dissolved as completely as possible in distilled water to give a 0.8 % (w/v) suspension. The suspension was kept in an ice-water bath and was treated in a water-cooled M.S.E. (Measuring & Scientific Equipment Ltd, London) 500 W. ultrasonic disintegrator at 20 kc./s. for 5 min. During this procedure the temperature did not rise above 46°. A sample was removed and tested immediately for 0-1 phage inactivating ability and examined in the electron microscope.

Electron microscopy. The negative-staining technique (Brenner & Horne, 1959) with 2 % (w/v) sodium tungstic silicate (STS; pH 7.2) as the staining medium was used. For increasing the spreading quality of the suspension 0.1 % (w/v) bovine serum albumin was added to the STS solution. A thin layer of carbon was evaporated on to the surface of 400-mesh Formvar-coated specimen grids, from which the Formvar was subsequently dissolved away with amyl acetate. The phage and lipopolysaccharide materials were mixed with STS and applied as a drop on to the specimen grid. The solution remained on the grids for 60-120 sec. before the excess liquid was withdrawn, leaving a thin film. The specimens were examined with a JEM-5 Y electron microscope at a magnification of $\times 40,000$.

RESULTS

Lipopolysaccharide preparations from *Salmonella minnesota* strains R60 and R 345 were tested for their inactivating capacity towards phage 0-1. The experiments were made in a salt solution (M 9 medium without glucose) which gave a maximum adsorption of the 0-1 phage. Fig. 1 shows that the concentration of LPS from strain R60 necessary to inactivate 50 % of the phage (PhI₅₀) varied from 0.4 to 0.9 µg. LPS/ml. for different preparations. Repeated experiments with LPS from strain R 345 showed no inactivation of the 0-1 phage, even in suspensions containing 1.0 mg. LPS/ml. These experiments suggest that LPS from strain R60 acted as a receptor for the 0-1 phage while LPS from strain R 345 did not.

The interaction between the phage and receptor was observed with the electron microscope. Plate 1, figs. 1, 2 show the 0-1 phage with a polyhedral head, 60 mµ in diameter, a tail 90-100 mµ length with a base plate 25 mµ wide with 6 tail fibres, generally extended. Plate 1, fig. 3 shows the 0-1 phage adsorbed on to LPS of strain R60. The LPS is seen as long filaments several microns in length and 60-90 Å wide. The 0-1 phage head is empty, clearly showing its polyhedral shape, the tail sheaths have contracted to a length of about 50 mµ. The contracted sheaths display longitudinal striations in contrast to the cross striations seen in extended tail sheaths. No tail fibres are visible. Plate 2, fig. 4 shows the 0-1 phage and LPS from strain R 345. The LPS from R 345 shows the same modal pattern as the LPS of R60, with long filaments of a rather uniform width. None of the phages has been adsorbed on to the LPS; they are seen with uncontracted tail sheaths.

Fig. 1. The o-1 phage inactivating capacity of different samples of phenol+water-extracted lipopolysaccharides from *Salmonella minnesota* strain R60. The activity of the lipopolysaccharide (LPS) is expressed as the concentration of LPS (dry wt./vol.) necessary to inactivate 50 % of the phage (PhI₅₀). x-x, PhI₅₀ = 0.91 μg. LPS/ml.; O-O, PhI₅₀ = 0.73 μg. LPS/ml.; ●-●, PhI₅₀ = 0.51 μg. LPS/ml.; Δ-Δ, PhI₅₀ = 0.36 μg. LPS/ml.

Fig. 2. Action of sodium deoxycholate on lipopolysaccharide (LPS) from *Salmonella minnesota* strain R60. The o-1 phage inactivating capacity of the LPS is expressed as PhI₅₀. O-O, LPS of strain R60 treated with deoxycholate, PhI₅₀ = 2750 μg. LPS/ml. When the deoxycholate concentration was 0.06 % (corresponding to LPS 1375 μg./ml.) no inactivation of the o-1 phage was observed. After dilution of deoxycholate to less than 0.03 % the phage inactivating ability was restored. ●-●, LPS of strain R60 where the deoxycholate was removed by dialysis; PhI₅₀ = 110 μg. LPS/ml.; Δ-Δ, LPS of strain R60 after further treatment by phenol+water extraction, PhI₅₀ = 17 μg. LPS/ml.

Treatment of lipopolysaccharide from strain R60 with sodium deoxycholate (SDC)

Lyophilized LPS from strain R60 was dissolved in 0.1 M-tris-buffer (pH 8.0) and SDC added to a final concentration of 0.5 % (w/v). After addition of SDC the opalescence of the LPS suspension disappeared almost completely. The PhI_{50} of the LPS from strain R60 preparation was 0.5 $\mu\text{g./ml.}$ Addition of SDC resulted in a dramatic decrease in the O-1 phage inactivating ability to a PhI_{50} value corresponding to 2750 $\mu\text{g. LPS/ml.}$ (Fig. 2). When the concentration of SDC was diluted with M 9 medium to less than 0.03% the receptor activity of the LPS was restored. No decrease in the infectivity of the O-1 phage was observed in control experiments with 0.5 % (w/v) SDC, nor did this concentration interfere with the adsorption of the phage to sensitive bacteria.

Fig. 3. Effect of ultrasonic treatment of lipopolysaccharide from *Salmonella minnesota* strain R60. The O-1 phage inactivating capacity of the LPS is expressed as PhI_{50} . ●—●, LPS of strain R60 before ultrasonic treatment; $\text{PhI}_{50} = 0.35 \mu\text{g. LPS/ml.}$; △—△, LPS of strain R60 after 5 min. ultrasonic treatment, $\text{PhI}_{50} = 35 \mu\text{g. LPS/ml.}$

The deoxycholate was removed by dialysis against 0.1 M-tris buffer and against the defined medium M 9. The dialysis against medium M 9 restored the optimal conditions for O-1 phage adsorption. The inactivating capacity of the LPS suspension increased after dialysis to a PhI_{50} corresponding to 110 $\mu\text{g. LPS/ml.}$ After treatment of the dialysed LPS suspension with phenol, the inactivating ability was further increased to a PhI_{50} value corresponding to 17 $\mu\text{g. LPS/ml.}$

The processes were directly observed by electron microscopy. Intact LPS of R60 appeared as long filaments with a uniform width (Pl. 1, fig. 3). When this LPS of R60 was treated with 0.5 % SDC the filaments dissociated into elements which could not be seen with the electron microscope, i.e. the deoxycholate which had dried on the grid covered them. However, when the deoxycholate was removed by dialysis the elements re-associated into units which could be observed as a population of short rods, spherical and irregular particles (Pl. 2, fig. 5). The size varied from 100 to 400 Å.

The dissociated R60 lipopolysaccharide was further aggregated when the suspension

was treated by phenol+water extraction. Plate 2, fig. 6 shows a representative field; some filaments are more than 1000 Å long and there are rods from 300 to 500 Å long. The width of the filaments varied from 60 to 100 Å. There were also many spherical particles measuring around 250 Å in diameter. The smallest observed units of lipopolysaccharide R60 which adsorbed phages measured from 200 to 300 Å.

Ultrasonic treatment of lipopolysaccharide of strain R60

Ultrasonic treatment of LPS of strain R60 decreased its O-1 phage inactivating capacity to about 1 % of that of the original preparation (Fig. 3). The PhI_{50} value increased from 0.4 µg. LPS/ml. to 35 µg. LPS/ml.

Electron micrographs of preparations before and after ultrasonic treatment showed great variations. The filaments had disintegrated into rods and spherical particles. Plate 2, fig. 7 is an electron micrograph showing a representative field with some rods from 600 to 900 Å long. The majority of particles were rods from 150 to 240 Å long and from 60 to 80 Å wide. Spherical particles of diameter about 100 Å could be seen.

DISCUSSION

Investigations carried out in many laboratories (Beckmann *et al.* 1964; Nikaido *et al.* 1964; Osborn *et al.* 1964; Lüderitz *et al.* 1965) led to the hypothesis first proposed by Davies (1960), Kauffmann *et al.* (1960) and Kauffmann *et al.* (1961) that the O antigen in *Salmonella* contains a central core of lipopolysaccharide common to all the salmonellas examined so far. Antigenic determinants attached to this core give the different *Salmonella* species their serological specificity. The O-1 phage, which adsorbs to both S forms and to a certain class of R-mutants, probably attaches to a cell wall structure which is common in *Salmonella*. As shown here in the inactivation experiments and the electron micrographs the isolated lipopolysaccharide from *Salmonella minnesota* strain R60 acts as a receptor structure for the O-1 phage.

The non-reducing *N*-acetyl-D-glucosamine unit which probably terminates the polysaccharide core in *Salmonella minnesota* R60 may determine the specificity of the O-1 phage receptor. The only difference between *S. minnesota* R60 and strain R345 so far detected is that R345 lacks the glucosamine unit in the polysaccharide (Lüderitz *et al.* 1965). As suggested by the inactivation experiments and seen with the electron microscope the lipopolysaccharide of strain R345 does not cause contraction of the tail sheath of the O-1 phage. The terminal *N*-acetyl-D-glucosamine unit may in fact mediate the adsorption of the O-1 phage to the lipopolysaccharide. Taylor (1966) found that the adsorption of Vi-phage II to the Vi-polysaccharide of *S. typhi* was followed by an enzymic de-acetylation of the polysaccharide. The enzyme, situated on the tip of the Vi-phage tail, split *O*-acetyl as well as *N*-acetyl groups by hydrolysis.

Ribi *et al.* (1966) have found that sodium deoxycholate can dissociate lipopolysaccharide endotoxins from Gram-negative bacteria into subunits with molecular weights of about 20,000, with a subsequent loss of ability to produce fever in rabbits. The biological activity reappeared when the deoxycholate was removed. Their findings suggested another way of testing how the integrity of the lipopolysaccharide structure of *Salmonella minnesota* R60 is related to O-1 phage receptor activity. By treatment of the receptor structure with deoxycholate the phage inactivating capacity decreased to less than 0.1 % of that of the original lipopolysaccharide suspension (Fig. 2). The

failure of the subunits to adsorb the O-I phage can be explained in at least two different ways: (i) the adsorption of the phage to the subunits is blocked by the presence of deoxycholate; (ii) the subunits are too small to contain a single complete site of attachment to the phage. When the concentration of deoxycholate was diluted to less than 0.03 % the phage inactivating capacity was restored. This concentration may be too low either to block adsorption of phage or to keep the subunits dissociated. When the deoxycholate was removed by dialysis the subunits recombined to form complete receptor structures. The phage inactivating capacity could be further increased by phenol treatment. The experiments suggest that the inactivation of phages can be used as a simple test of the biological activity of a lipopolysaccharide along with other methods, e.g. toxicity and fever response in rabbits.

The smallest units which were found to cause ejection of the O-I phage nucleic acid and contraction of its tail sheath measured from 200 to 300 Å of length. Ribi *et al.* (1966) have found particles of the same size to have molecular weights ranging from 500,000 to 1,000,000. As a comparison, the soluble lipopolysaccharide receptor structure for coliphage T4 in *Shigella sonnei* varied from 50 to 100 Å in size (Jesaitis & Goebel, 1953). The *Escherichia coli* B receptor structure of phage T5, which contained a core of lipopolysaccharide covered with lipoprotein, has a spherical shape around 315 Å (Weidel & Kellenberger, 1955).

Ultrasonic treatment caused a decrease in the O-I inactivating capacity of *Salmonella minnesota* R60 lipopolysaccharide. The observed gross morphological changes of the lipopolysaccharide of strain R60 after ultrasonic treatment might explain the difference in O-I phage inactivating capacity of the two suspensions. Whereas untreated lipopolysaccharide offered a receptor structure for the phage, ultrasonic treatment disintegrated the lipopolysaccharide into smaller units with no inactivating capacity. The residual inactivating capacity after disintegration might be explained by the presence of the remaining rods still retaining intact receptor sites.

Investigations on the O-I phage receptor have been made by the author on some of the *Salmonella typhimurium* mutant strains used by Subbaiah & Stocker (1964). Isolated lipopolysaccharides from strains containing the terminal acetyl-glucosamine unit inactivated the O-I phage, whereas lipopolysaccharides from strains lacking the amino sugar did not. Thus, strains containing the central core in *S. minnesota* and *S. typhimurium* offer a receptor for the O-I phage provided that the terminal acetyl-glucosamine unit is present. If the central core in *Salmonella* represents a receptor for the O-I phage, the chemical configuration of the lipopolysaccharides of most enterobacteria must differ from that of *Salmonella*. Alternatively the cell wall of Enterobacteriaceae may contain the receptor which may be blocked by other surface components. Experiments are in progress to investigate the O-I phage inactivating capacity of isolated lipopolysaccharides from other *Salmonella* and enterobacteria strains.

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EXPLANATION OF PLATES

The magnification in figs. 5-10 is $\times 100,000$ and in fig. 4 $\times 400,000$. The 0-1 phage and the lipopolysaccharide were negatively stained with sodium tungstic silicate (STS).

PLATE 1

Figs. 1, 2. Felix 0-1 phage.

Fig. 3. Lipopolysaccharide isolated from *Salmonella minnesota* strain R 60 with adsorbed 0-1 phage particles. The tail sheaths (indicated by an arrow) are contracted.

PLATE 2

Fig. 4. Lipopolysaccharide isolated from *Salmonella minnesota* strain R 345 and 0-1 phage. No phage adsorbed on to the lipopolysaccharide.

Fig. 5. Lipopolysaccharide of strain R 60 after treatment with sodium deoxycholate and subsequent dialysis. Two of the 0-1 particles are seen with contracted tail sheaths, one has an uncontracted tail sheath (indicated by an arrow).

Fig. 6. The sodium deoxycholate treated lipopolysaccharide of strain R 60 after dialysis and phenol + water extraction. The 0-1 particles are adsorbed on to the LPS. Some detached contracted tail sheaths (indicated by an arrow) are also seen.

Fig. 7. Lipopolysaccharide from *Salmonella minnesota* strain R 60 after 5 min. ultrasonic treatment.