A PROTEIN FACTOR ASSOCIATED WITH SERUM RESISTANCE IN *ESCHERICHIA COLI*

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PLATES XV AND XVI

It has long been known that many strains of gram-negative bacteria are insensitive to the bactericidal action of normal human serum despite the presence in the serum of bactericidal antibody and complement (Georgevitch, 1926; Mackie and Finkelstein, 1931); the nature of this resistance remains obscure. Serum resistance in *Escherichia coli* has been variously ascribed to the amount of cell-wall lipopolysaccharide (LPS), the length and number of LPS O-side chains and to both the amount and haemagglutination-inhibiting activity of acidic polysaccharide K antigen (see review by Inoue, 1972). In a recent study (Taylor, 1975), it was found that none of these factors was able to explain the behaviour of serum-resistant mutants derived from smooth, *E. coli* strains isolated from the urinary tract, and it was suggested that these mutants synthesised an additional surface component, possibly protein in nature, which was responsible for serum resistance.

The present study concerns the analysis of one of the serum-resistant mutants and the corresponding parent strain by gel-diffusion techniques and by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis of cell-envelope proteins in an attempt to determine the basis of the mutation to serum resistance. Colonially rough mutants isolated from cultures of the serum-resistant mutant were also examined.

MATERIALS AND METHODS

Bacterial strains. *E. coli* no. LP729 is a smooth strain of serotype O9 which is rapidly killed by human serum after a delay of 1 h; *E. coli* no. I7 is a serum-resistant mutant derived from strain LP729 by mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (Adelberg, Mandel and Chen, 1965). Both strains produce negligible amounts of K antigen, are stable in 0.2% acriflavine and synthesise LPS containing large amounts of mannose, a sugar confined to the O-side chain of LPS from *E. coli* O9 strains. *E. coli* nos. I7fa and I7gb are colonially rough mutants plated from an aged (4-week) nutrient-broth culture of strain I7. *E. coli* no. I7fa is rapidly killed by serum and is a *rfb* mutant (Schmidt, Jann and Jann, 1969) with a complete LPS R2 core but defective in the synthesis of the LPS O9-side chain. Strain I7gb possesses a full complement of LPS O-side chains and interacts with serum in an identical way to strain LP729. Both colonially rough forms spontaneously agglutinate in 0.2% acriflavine. Whereas strains LP729 and I7 are able to cause kidney infection in an experimental rat model, strains I7fa and I7gb are unable to do so (Taylor and Koutsaimanis, 1975). These strains are described in detail by Taylor (1975).

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Ouchterlony gel diffusion. Rabbit antisera against live and heat-killed (100°C; 1 h) bacteria were prepared according to the scheme of Schlecht and Westphal (1967). For the preparation of antigens, cells were cultivated in 2½-litre conical flasks containing 700 ml of Nutrient Broth no. 2 (Oxoid) at 37°C in an orbital incubator (120 orbits per min.), washed with water and extracted with 10 ml of 0.2M LiCl for 2 h in a reciprocating waterbath. It has recently been shown by Frascch and Gotschlich (1974) that extraction of gram-negative cells with 0.2M LiCl results in the efficient release of protein surface-antigens without significant cell lysis. Gel diffusion was carried out according to Grados and Ewing (1969).

Protease treatment. Proteolytic digestion of LiCl-extracted antigens was performed with trypsin (BDH Chemicals Ltd, Poole, Dorset) and pronase (BDH) at an enzyme-to-antigen-protein ratio of 1 : 10 and the buffer systems described by Mescher, Strominger and Watson (1974). Mixtures were incubated at 37°C for up to 72 h.

Gel filtration. Washed cells from a 15-litre nutrient-broth batch-culture (37°C; 16 h) were extracted with 100 ml of 0.2M LiCl for 2 h at 60°C in a reciprocating waterbath. After extensive dialysis against water at 4°C, the extract was freeze-dried, suspended in 10 ml of 0.15M NaCl + 0.01M Tris-HCl, pH 7.5, and applied to a column (2.5 cm × 60 cm) of Sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden). The column was eluted with 0.15M NaCl + 0.01M Tris-HCl, pH 7.5, at a flow rate of 20 ml per h and 20-min. fractions were collected. The E_{280} of each fraction was determined, and the sugar content of each fraction measured by the phenol-H₂SO₄ technique (Dubois *et al.*, 1956).

Envelope preparations. Cells were cultivated in 1-litre volumes of Nutrient Broth no. 2 (Oxoid) in 2-litre flanged conical flasks at 37°C in an orbital incubator (100 orbits per min.). After 4 h, when cultures of all four strains had reached the late exponential phase, the cells were collected by centrifugation at 4°C. Packed cells were resuspended in saline and, after freezing, were broken by three passages through an LKB X-press. After thawing, the suspensions were diluted with saline at 4°C, mixed thoroughly with a syringe fitted with a 23-gauge needle and centrifuged at 100 000 *g* for 30 min. The resulting pellets were thoroughly dispersed in distilled water and recentrifuged. The deposits obtained were again suspended in water and centrifuged in a bench centrifuge for 10 min. to remove unbroken cells and debris. The envelope fractions were then collected by centrifugation at 100 000 *g* for 1 h, dispersed in small volumes of water and frozen until required.

Electrophoresis. Slab gel electrophoresis with a discontinuous SDS buffer system was based on the method of Laemmli (1970) as modified by Ames (1974).

Separating and stacking gels contained 11% and 5% of acrylamide respectively. To dissolve the envelopes, a 0.5-ml sample containing 2 mg of envelope protein was added to 0.5 ml of 0.125M Tris-HCl buffer, pH 6.8, containing 4% of SDS, 10% of β-mercaptoethanol, 20% of glycerol and 0.002% of bromophenol blue, and the mixture was heated to 100°C for 5 min. After electrophoresis, protein bands were stained with Coomassie Brilliant Blue R250 (B.D.H. Ltd) by the method of Weber and Osborn (1969). Carbohydrate-containing bands were stained by the periodic acid-Schiff (PAS) procedure according to Zacharius *et al.* (1969) as modified by Huang and Evans (1973). A full description of the electrophoretic procedures is given by Parton (1975).

RESULTS

Gel diffusion of LiCl extracts

It was previously suggested (Taylor, 1975) that the serum resistance of *E. coli* strain I7 was due to the presence of a surface component not found in the parent strain LP729. Therefore, LiCl extracts of the various strains were used in gel-diffusion studies with anti-I7 antisera. In initial experiments, bacteria were extracted with LiCl at 60°C. With extracts from strains I7, I7gb and I7fa, a precipitin line was consistently found close to the antiserum well when whole-cell antiserum was used; this line did not form against extracts

from strain LP729 (fig. 1). The precipitin lines that formed near the antigen wells contained the O-antigen component. When the temperature of LiCl extraction was lowered to 45°C, the inner precipitin line was evident only with extracts from strains I7 and I7gb. However, when the extraction temperature was raised to 80°C, all extracts formed the inner precipitin line with whole-cell antiserum against strain I7. Gel-diffusion patterns identical with those found with antisera against live and heat-killed strain-I7 cells were obtained with antisera produced against live and heat-killed strain-LP729 cells. The gel-diffusion experiments were repeated with preparations obtained by extraction with 0.2M NaCl at 45°C, 60°C and 80°C; results were identical with those found with 0.2M LiCl extracts.

These results suggested that the mutation to serum resistance had resulted in a modification of the cell surface that enabled a particular antigen to be extracted from the cell mass at a lower temperature. Alternatively, the serum-resistant mutant produced greater quantities of the antigen, and the absence of a precipitin line with strain LP729 was due to inadequate amounts of the antigen in the 45°C and 60°C extracts. The 60°C extracts from strain LP729 were concentrated up to 16-fold and examined by gel-diffusion with whole-cell antisera against strains I7 and LP729 in the usual way. With both antisera, a precipitin line, which formed a line of identity with the inner precipitin line in strain-I7 extracts, was found in 4-, 8- and 16-fold concentrates but was absent from 2-fold concentrates. This indicated that unconcentrated 60°C extracts of strain LP729 contained the antigen but in insufficient quantities to form a precipitin line.

The inner precipitin line did not form with 60°C extracts of strain I7 after

FIG. 2.—Fractionation of 0.2M LiCl (60°C; 2 h) extract of *E. coli* strain I7 on Sepharose 4B. Fractions were examined by the phenol-H₂SO₄ technique and the absorbance at 490 nm (O—O) measured. Fractions were also examined for absorbance at 280 nm (●—●).

treatment for 24 h with trypsin, but was still evident when extracts treated for 72 h with pronase were used. The antigen did not form lines of identity with material obtained by cetavlon precipitation of phenol-water extracts (Westphal and Jann, 1965) of strain I7 or strain LP729, and was non-dialysable. The antigen does not, therefore, represent the acidic polysaccharide K antigen of the cell, but is a protein.

Gel filtration of 60°C LiCl extract

An attempt was made to investigate further the nature of the antigen responsible for the inner precipitin line by subjecting a 60°C LiCl extract of strain I7 to fractionation on a column of Sepharose 4B. Three major peaks were obtained (fig. 2). Fractions 14–40, comprising the first two peaks, reacted with O antiserum against strain I7 in gel-diffusion tests. The first peak, which was rich in E₂₈₀ absorbing material, eluted with the void volume. Gel diffusion with whole-cell antiserum against strain I7 indicated that the antigen corresponding to the inner precipitin line appeared in fractions 43–50 and was the only detectable antigen present in fractions 43–46 (fig. 3). Precipitin lines from these fractions formed lines of identity with the inner precipitin line from *E. coli* I7 60°C LiCl extracts of strain I7.

Column fractions from the various peaks were pooled, dialysed against water and examined by SDS-polyacrylamide gel electrophoresis. Fig. 4 shows the protein compositions of fractions 13–20, 21–39 and 43–46 in comparison with the envelope-protein profile of strain I7. Fractions 43–46 contained one major band with an estimated molecular weight of 46 000 daltons (46k) and only traces of other components. PAS staining failed to reveal the presence of carbohydrate-containing bands in the sample, so the 46k polypeptide component presumably represents the inner precipitin line antigen of the LiCl extract.

Fractions 13–20 and 21–39 contained several major polypeptide components including a 46k band (fig. 4). This and certain other bands both in the column fractions and in the envelope fraction were somewhat distorted. PAS staining of identical gels revealed the presence of several carbohydrate-containing bands in the same regions of the gels as the distorted polypeptide bands. Since the 46k polypeptide band in fractions 43–46 was not distorted, and no carbohydrate-containing bands were detected in this sample, the presence of the carbohydrate bands in the other samples may have been responsible in some way for the distortion of the polypeptide bands.

Although fractions 13–20 and particularly fractions 21–39 apparently contained the 46k component, which was also found in fractions 43–46, they did not form the inner precipitin line in gel-diffusion tests against whole-cell antiserum. They did, however, react with O antiserum in these tests. Thus, in these fractions, the 46k polypeptide was presumably complexed with other components such as LPS and so was unable to form a discrete precipitin line. Evidence for such a complex was obtained by gel-diffusion studies of fractions 13–20 and 21–39 after heat treatment at 100°C for varying periods of time. With both pools the inner precipitin line appeared after heating for 5 min.

and increased in intensity as the heating period was extended to 30 min. Two additional precipitating antigens not found in the original 60°C LiCl extracts were also evident in both pools after 30 min. at 100°C.

Comparison of envelope-protein profiles

The envelope-protein profiles of all four *E. coli* strains are shown in fig. 5. Distorted bands were repeatedly obtained in the profiles of strains LP729, I7 and I7gb. PAS staining revealed carbohydrate-staining material in the same region of the gel and this, as suggested above, may have interfered in some way with the migration of the polypeptides during electrophoresis. This was also suggested by the finding that the protein profile of strain I7fa was not distorted and no carbohydrate-staining material was detected in the gel.

Despite the problem of band distortion, the protein profiles of strains LP729 and I7 were indistinguishable. There were no apparent differences in envelope-protein composition that could account for the difference in serum sensitivity between the two organisms. Strain I7gb, which is morphologically rough but has complete LPS O-side chains, had a similar profile but possessed an extra band at 41k and showed other minor differences. Strain I7fa, the rough strain that lacks the LPS O-side chains, appeared to be quite different from the other three strains though this may in part be due to the lack of band distortion in this profile. One obvious difference, however, was that I7fa envelopes lacked a prominent band at 31k which was present in all of the other three strains.

It should be noted from fig. 5 that the envelope of all four strains contained approximately equal amounts of the 46k polypeptide band, the only significant band found in column fractions 43–46.

DISCUSSION

Previous studies with these strains had demonstrated that no gross changes in the lipopolysaccharide or acidic polysaccharide components of the bacterial wall had occurred to account for the mutation to serum resistance, although it did appear that lipopolysaccharide O-side chains were responsible for a delay in the serum killing of the parent strain LP729 (Taylor, 1975). The immunogel-diffusion studies were therefore instigated in an attempt to determine whether the serum-resistant mutant produced antigens not found in strain LP729. The 60°C LiCl extracts of the serum-resistant mutant I7 gave rise to a precipitin line not found with extracts of the parent strain LP729, although further experiments indicated that LP729 extracts contained the antigen but in insufficient quantity to form lines of precipitation. One interpretation of these results is that strain I7 produces greater quantities of the protein antigen than strain LP729, and we suggest that this protein is responsible for the serum resistance of strain I7. The present results do not, however, exclude the possibility that a subtle modification in the arrangement of surface components is responsible for the differences in extractibility of the antigen and for differences in serum sensitivity. If the 46k envelope-protein band represents the same polypeptide

in all samples, the antigen responsible for the inner line of precipitation appears to be present in approximately equal amount in the envelopes of all four strains (fig. 5). The composition of cell-envelope proteins, however, may not be a true reflexion of the composition of the cell surface; for example, surface material may be washed off during envelope preparation. In addition, the 46k antigen band responsible for the inner precipitation line may have coincided with a more prominent envelope band; changes in the quantity of the antigen might not, therefore, be easily detected in the envelope profiles.

No direct evidence was obtained for the position of the antigen within the bacterial cell, but the fact that it was able to evoke a strong antibody response after inoculation of live cells into rabbits and that it could be extracted from the cell mass under relatively mild conditions suggests that it is present at or near the cell surface. The presence of such a component would accord with the generally held view that serum resistance is due to the presence of adequate amounts of structures which impede the attachment or subsequent activity of antibody and complement (Wardlaw, 1964; Glynn and Howard, 1970). It is clear that the presence of the antigen in amounts comparable with those found in strain I7 is not alone sufficient to render a strain resistant to serum. Strains I7gb and I7fa appear to contain more of the antigen in a readily extractable form than strain LP729 but are serum sensitive, indicating that the presence of other cell-surface components, such as lipopolysaccharide O-side chains, is essential for expression of full serum resistance. The only major component of fractions 43-46 obtained by Sepharose 4B chromatography (figs. 2 and 3) was a protein with an apparent molecular weight of 46k daltons, and it is likely that this protein represents the antigen of the inner precipitation line in strain-I7 LiCl extracts.

Although it has been previously suggested that serum resistance is due to the presence of abundant amounts of LPS O-side-chain material (Feingold, 1969) and to the amount of acidic polysaccharide K antigen associated with the cell (Glynn and Howard, 1970; Howard and Glynn, 1971) these theories fail to explain a number of observations. Nelson and Roantree (1967) isolated serum-sensitive mutants from serum-resistant strains of *Salmonella typhimurium* and *S. enteritidis*. The majority were rough or part rough, but sensitive mutants that were found to be culturally and serologically smooth had LPS with ratios of O-side-chain sugar to core sugar identical with those of the LPS of the parent strains. Current theories were also unable to explain the serum reactivity of 28 smooth strains of *E. coli* isolated from patients with urinary-tract infections (Taylor, 1976). Taylor (1975) found that recombinants that had inherited a full complement of O8-specific LPS side chains from a K-negative, serum-resistant, Hfr *E. coli* donor strain were sensitive to serum. Many gram-negative bacteria synthesise no K antigen and yet are resistant to serum. *Klebsiella* strains are frequently found to be sensitive to serum (Fierer, Finley and Braude, 1972) even though most strains of this genus produce copious amounts of hexuronic-acid-containing acidic polysaccharide (Sutherland, 1972).

Recent findings have shown that smooth-to-rough variation involves changes in envelope-protein composition as well as in LPS composition (Ames,

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FIG. 1 (top left).—Immunogel-diffusion patterns obtained with 0.2M LiCl extracts (60°C; 2 h) of *Escherichia coli* strains (1) LP729, (2) I7, (3) I7gb and (4) I7fa. The centre well contained anti-serum (4:1 concentration) prepared against live cells of strain I7.

FIG. 3.—Immunogel-diffusion patterns of fractions 13–60 obtained by chromatography of 0.2M LiCl (60°C; 2 h) extract of *E. coli* strain I7 on Sepharose 4B. The centre wells contained anti-serum (4:1 concentration) prepared against live cells of the same strain.

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FIG. 4 (left).—Protein compositions of (a) column fractions 13–20, (b) fractions 21–39 and (d) fractions 43–46; for comparison, (c) envelope-protein profile of strain 17 by SDS-polyacrylamide gel electrophoresis.

FIG. 5 (right).—Envelope-protein profiles of *E. coli* strains (a) LP729, (b) 17, (c) 17gb, (d) 17fa. Molecular weight (k-daltons) of various bands are given on the right.

Spudich and Nikaido, 1974; Koplow and Goldfine, 1974; Parton, 1975). Changes in sensitivity to antibiotics, dyes, etc., and in serum sensitivity during smooth-to-rough variation have been attributed in the past to changes in LPS composition. It is now evident that further investigation of the role of envelope proteins is likely to lead to a more complete understanding of these phenomena.

Of interest was the observation that O-antigen-containing components of the 60°C LiCl extract of strain I7 could be resolved into two distinct fractions by chromatography on Sepharose 4B (fig. 2). The first, high-molecular-weight peak contained large amounts of E₂₈₀ absorbing material, presumably protein, and relatively small amounts of carbohydrate, and was excluded from the gel. The second peak, which was retarded by the gel, contained greater amounts of carbohydrate and less of protein. Gel electrophoresis indicated that the two peaks also had a slightly different protein composition (fig. 4).

It was previously suggested (Taylor, 1975; Taylor and Koutsaimanis, 1975) that the rough characteristics of strain I7gb, and its failure to cause kidney infection in experimentally infected rats, might be due to the loss of a single protein factor from the cell surface. The results of gel electrophoresis of envelope proteins (fig. 5) indicate that the explanation is likely to be more complex. A number of minor differences were found between the envelope profiles of strains I7 and I7gb, including the appearance of an extra band in the I7gb profile. It is not known whether these differences are related in any way to the other differences between the strains.

SUMMARY

Immunogel-diffusion studies showed that 60°C LiCl extracts of the smooth serum-resistant mutant *Escherichia coli* strain I7 contained greater amounts of a protein antigen than did extracts of the parent strain LP729. An extract of strain I7 was fractionated on Sepharose 4B and the protein antigen was found as the only detectable antigen in a number of fractions; sodium dodecyl sulphate-polyacrylamide gel electrophoresis indicated that these fractions contained one major polypeptide band with a molecular weight of 46 000 daltons. We suggest that this protein antigen may be partly responsible for the serum resistance of strain I7 though its presence in other serum-sensitive strains suggests that additional factors are essential for full serum resistance.

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