Antigenic Cross-reactivity of Major Outer Membrane Proteins in Enterobacteriaceae Species

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The protein constituents in the outer membrane (OM) of several serotypes of *Escherichia coli* and some other Enterobacteriaceae cross-reacted antigenically. Solubilized OM preparations of these bacteria were applied in interfacial precipitin tests to antisera elicited in rabbits against whole bacterial cells, absorbed with their appropriate lipopolysaccharide before testing. The resulting immunocomplexes were analysed on polyacrylamide gels. Protein profiles of the immunoprecipitates showed a considerable antigenic cross-reactivity of outer membrane proteins between most *E. coli* serotypes. Cross-reactivity, though substantially lower, was also found with OM from three other Enterobacteriaceae species, but was not detectable with *Pseudomonas aeruginosa* OM. When OM preparations were solubilized at room temperature, the peptidoglycan-bound proteins in the molecular weight range 37000 to 41000 predominated in the protein profiles of the immunocomplexes. In profiles of immunocomplexes obtained with boiled OM preparations, a heat-modifiable protein (mol. wt 33000) predominated. The major OM proteins of the Gram-negative bacterium may therefore play a role as common surface antigens of the family of Enterobacteriaceae.

INTRODUCTION

The outermost envelope of Gram-negative bacteria consists of the outer membrane and lipopolysaccharide (LPS). The immunology of the LPS of the Enterobacteriaceae has been thoroughly investigated, especially the somatic or O antigen. Antibodies against this heat-stable antigen have agglutinating properties and give type-specific protection (Tate *et al.*, 1966; Ziegler *et al.*, 1973). Another antigenic structure found in most Gram-negative bacteria is the flagellar or H antigen. In some organisms, like *Escherichia coli* and *Klebsiella pneumoniae*, there is a third antigen, K antigen, which represents a polysaccharide capsule exterior to the outer membrane. Antibodies against the K antigen protect only against homologous organisms (Kaijser & Ahlstedt, 1977). Since antisera against these diverse antigens provide only strain-specific protection, workers have searched for cross-reacting bacterial antigens. The common antigen described by Kunin *et al.* (1962) is well known, but immune response to this antigen differs in patients with various enterobacterial infections (Neter *et al.*, 1973), and McCabe & Greely (1973) failed to demonstrate protective activity of antibodies to this antigen in experimental infections. Another antigen shared by most Gram-negative rods is the so-called inner core, which links the O antigen of the LPS to the lipid A moiety buried in the outer membrane layer. The protective value of antibodies against the inner core of LPS is controversial. Chedid *et al.* (1968), Ziegler *et al.* (1973) and McCabe *et al.* (1977) demonstrated their beneficial effect against infections caused by different Gram-negative strains. However, Ng *et al.* (1976) found no protection in mice challenged with heterologous strains, probably due to inaccessibility of the immunodeter-

minants. Young *et al.* (1975) concluded that antibodies against this shared structure act primarily as antitoxins rather than as opsonins and that they provide no enhancement of blood stream clearance. Antibodies against lipid A, which has a similar structure in most Gram-negative rods, have been described (Galanos *et al.*, 1971), but they lack protective activity (Mullan *et al.*, 1974; McCabe *et al.*, 1977). One of the major protein components of the outer membrane of Gram-negative bacteria has been described as a common antigen by Braun *et al.* (1976). The protective value of this antigen is questionable since in wild-type strains it may be shielded by other membrane components. The immunogenicity of the other proteins of the outer membrane has been reported (Dankert & Hofstra, 1978). Comparison of polyacrylamide gel electrophoretic protein profiles of the outer membranes of some Enterobacteriaceae species reveals certain similarities (Schnaitman, 1970; Lugtenberg *et al.*, 1977) suggesting that outer membrane proteins may form common antigens.

In this study, we have investigated the antigenic cross-reactivity of outer membrane proteins in some *E. coli* serotypes, three related Enterobacteriaceae species and *Pseudomonas aeruginosa*.

METHODS

Bacterial strains and growth conditions. *Escherichia coli*, serotypes O1 K⁻, O4 K2, O26 K60, O55 K59 and O75 K⁻, were isolated in our laboratory. *Escherichia coli* O111 K58 and its galactose epimerase-deficient mutant strain J5 were a gift from Dr B. Witholt, Laboratory of Biochemistry, State University, Groningen. All strains of *E. coli* and the *Salmonella typhimurium* strain were serotyped by the Rijksinstituut voor de Volksgezondheid, Bilthoven, The Netherlands. *Klebsiella pneumoniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa* were isolated and identified in our laboratory according to Cowan & Steel (1965). Bacteria were grown in 10 l flasks under vigorous aeration at 37 °C in a liquid medium containing (g l⁻¹ in deionized water): Na₂HPO₄, 7.5; KH₂PO₄, 3; NaCl, 0.5; NH₄Cl, 0.1; MgSO₄·7H₂O, 0.25; CaCl₂·2H₂O, 0.0015; yeast extract, 10; and glucose, 5. The pH was adjusted to 7.4. *Escherichia coli* strain J5 was grown on essential salts medium (Vogel & Bonner, 1956) with glycerol as a carbon source.

Isolation of outer membrane. Bacteria were grown for 3 h to late-exponential phase and harvested by centrifugation (8000 g, 15 min, 4 °C). They were sheared in a homogenizer (MSE) for 1 min in 0.05 M-Tris/HCl buffer (pH 7.8) containing 1 mM-EDTA to remove extracellular materials, and then disintegrated by repeated passage through an X-press (Nike, Eskilstuna, Sweden). Outer membranes (OM) were obtained after Triton X-100 (Sigma) extraction of the crude membrane fraction (Schnaitman, 1971).

Isolation of lipopolysaccharides. Bacteria were grown overnight in glucose broth, harvested (8000 g, 15 min) and dried with acetone. LPS was prepared by phenol/water extraction (Westphal *et al.*, 1952). The aqueous phase was dialysed against demineralized water (48 h, 4 °C), concentrated to approximately 50 ml in a rotary evaporator (Bühler, Tübingen, Germany) and finally lyophilized. Preparations were stored at 4 °C until used. Protein content was estimated according to Lowry *et al.* (1951). 2-Keto-3-deoxy-octonate was determined by the thiobarbituric acid method (Ellwood, 1970).

Preparation of antisera. For each bacterial strain, two rabbits were injected intravenously with formaldehyde-fixed cells. Each rabbit received four doses, increasing from 0.5 × 10⁹ to 2 × 10⁹ cells, over 2 weeks. Rabbits were bled 1 week after the final injection. Samples (10 ml) of the sera were stored without preservative at -20 °C.

Absorption of antisera. Antibodies directed against bacterial LPS were removed from the sera by step-wise addition of a concentrated solution containing the appropriate crude LPS (10 mg ml⁻¹; not freed from nucleic acids; protein content less than 5 % (w/w) in the Lowry assay, OM proteins not detectable by polyacrylamide gel electrophoresis). Usually about 1.5 mg was added to 1 ml antiserum. The absence of antibodies against LPS was tested by interfacial immunoprecipitation tests with the antigen dissolved in 0.9 % (w/v) NaCl (Kwapinski, 1972) and by immunodiffusion in agarose.

Agglutination tests. These were done as previously described (Dankert & Hofstra, 1978).

Immunoprecipitin tests. Antigenic solutions were prepared by solubilization of OM in sodium dodecyl sulphate (SDS) at room temperature or at 100 °C. At room temperature, OM samples were dispersed in phosphate-buffered saline (PBS) containing 0.2 % (w/v) SDS followed by sonication (10 to 20 s, 50 Hz; Branson Ultrasonics, Soest, The Netherlands). In the other procedure, samples were boiled for 5 min in PBS containing 2 % (w/v) SDS, then diluted with PBS to 0.2 % (w/v) SDS. The final OM concentration in both preparations was 1 mg ml⁻¹. The cross-reactivity of SDS-solubilized OM proteins as antigenic components of the Gram-negative envelope was determined in interfacial immunoprecipitin tests with antiserum against *E. coli* O26 K60 (Dankert & Hofstra, 1978). In addition, OM of *E. coli* O26 K60 was

reacted with each of the antisera elicited against the bacterial strains used in this study. Pre-immunization sera of all rabbits used for the preparation of antisera were applied as controls. All antisera were absorbed with their specific LPS before use in immunoprecipitin tests to exclude the possibility of cross-reacting LPS. SDS was added to the antisera to a final concentration of 0.15 % (w/v). Immunoprecipitates were sedimented (20 min, 10000 g, 4 °C), washed three times with 0.9 % (w/v) NaCl containing 0.15 % (w/v) SDS and stored at -20 °C until analysed on polyacrylamide gels.

SDS-polyacrylamide gel electrophoresis. This was carried out according to Laemmli (1970) on slab gels as described by Ames (1974). Staining, destaining and photography of the gels, as well as the application of standard proteins for molecular weight estimation, were described by Dankert & Hofstra (1978).

RESULTS AND DISCUSSION

Outer membrane proteins of several Gram-negative bacteria

The major protein complex of the outer membranes (OM) of seven strains of *E. coli* was resolved by polyacrylamide gel electrophoresis (PAGE) into distinct protein bands with apparent molecular weights ranging from 33000 to 41000 (Fig. 1, tracks 1 to 7). Three or, sometimes, four bands could be seen in this region. Lugtenberg *et al.* (1975) were able to resolve the major OM proteins of *E. coli* K12 into four bands (a, b, c and d). Bands b and c represent protein I of Henning *et al.* (1973), while band d is the heat-modifiable protein, described as protein II* by Garten *et al.* (1975). A band moving like protein a, Schnaitman's protein 3B (Manning & Reeves, 1977), was seen in the profiles of strain J5 and serotypes O111 K58, O4 K2 and O75 K-. The protein b band was usually rather faint, though visible in all strains when OM preparations were applied to the gels in small amounts. Protein c was present in the PAGE profiles of all strains, while protein d was visible as a dense band in all profiles except that from *E. coli* O1 K-. The profile of this serotype showed a protein band (mol. wt about 35000) which was not observed in the profiles of the other strains. Proteins III (mol. wt 16000) and IV (mol. wt 7500) of Henning *et al.* (1973) were present in the OM fractions of all strains, but the bands were very weak in the profile of strain J5.

Profiles of OM fractions of strains of *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa* are also shown in Fig. 1 (tracks 8 to 11). The profile of *S. typhimurium* OM (Fig. 1, track 8) was similar to that described by Ames (1974). The electrophoretic mobility of the most pronounced band was similar to that of band b in the *E. coli* strains. In the major protein region of the profile of OM from *K. pneumoniae* (Fig. 1, track 9) two bands with apparent molecular weights of 39000 and 34000 were seen. These bands had similar electrophoretic mobilities to proteins b and d of *E. coli*. The OM profile of *Proteus vulgaris* showed bands corresponding to molecular weights of 38000 and 41000 (Fig. 1, track 10) coinciding with proteins c and a of *E. coli*. A weaker band moved like the 35000 dalton protein band of *E. coli* O1 K-. The profiles of *S. typhimurium* and *K. pneumoniae* OM showed a protein band in the molecular weight region of 15000 to 16000 possibly resembling protein III in the *E. coli* patterns. Lipoprotein was not detected in amounts comparable to those in most *E. coli* strains.

Thus OM protein profiles of different enterobacterial strains had common features, as described by Schnaitman (1970) and Lugtenberg *et al.* (1977). However, the similarity was much more evident between the strains of one species (*E. coli*) than between strains of different species in the family of Enterobacteriaceae. In membrane fractions of *Pseudomonas aeruginosa*, extracted by Triton X-100 and analysed by PAGE, at least six proteins with molecular weights ranging from 18000 to 50000 were seen (Fig. 1, track 11). This profile was unlike any of the enterobacterial OM protein patterns.

Fig. 1. Polyacrylamide gel electrophoresis patterns of outer membrane preparations of *E. coli* serotypes O1 K⁻ (1), O26 K60 (2), O111 K58 (3), strain J5 (4), O4 K2 (5), O55 K59 (6), O75 K⁻ (7), *Salmonella typhimurium* (8), *Klebsiella pneumoniae* (9), *Proteus vulgaris* (10), *Pseudomonas aeruginosa* (11). On the left, proteins are designated I to IV after Henning *et al.* (1973) and a, b, c and d after Lugtenberg *et al.* (1975). Molecular weights are indicated on the right.

Polyacrylamide gel electrophoresis of immunocomplexes from antiserum against E. coli O26 K60 and outer membrane preparations of Gram-negative bacteria

Cross-reactivity between OM proteins was determined in interfacial precipitin tests with OM fractions, solubilized in SDS at room temperature or at 100 °C, and rabbit antiserum prepared against *E. coli* O26 K60, absorbed with its appropriate LPS. After LPS absorption the bacterial agglutination titre of this antiserum had decreased from 1:8000 to 1:64. OM preparations of most *E. coli* serotypes gave clearly visible precipitin rings resembling those observed with *E. coli* O26 K60 OM, though those for *K. pneumoniae* and *Proteus vulgaris* were weak. OM of *E. coli* O1 K⁻, *S. typhimurium* and *Pseudomonas aeruginosa* gave insignificant precipitin rings.

PAGE profiles of repeatedly washed immunoprecipitates formed with OM preparations solubilized at room temperature showed the presence of albumin, the heavy and light chains of rabbit immunoglobulins and some of the major OM proteins (Fig. 2). Cross-reactivity with the OM proteins of the *E. coli* strains (except O1 K⁻) was greater than with the three other Enterobacteriaceae species and there was no apparent cross-reactivity with *P. aeruginosa* OM. Peptidoglycan-associated proteins b and c were more evident than the heat-modifiable protein d (Fig. 2, tracks 3 to 8). In the immunocomplexes obtained with O4 K2 and O75 K⁻ OM, the 41000 dalton protein gave a dense band. The 16000 dalton protein and lipoprotein (mol. wt 7500) were visible in the PAGE profiles of immunocomplexes of most *E. coli* strains when gels were freshly stained.

Fig. 2. Polyacrylamide gel electrophoresis patterns of immunoprecipitates obtained in interfacial precipitin tests carried out with antiserum against *E. coli* O26 K60 (absorbed with LPS O26) containing 0.15 % (w/v) SDS and outer membrane preparations of several Gram-negative bacteria, solubilized in 0.2 % (w/v) SDS at room temperature: *E. coli* serotypes O1 K⁻ (2), O26 K60 (3), O111 K58 (4), strain J5 (5), O4 K2 (6), O55 K59 (7), O75 K⁻ (8); *Salmonella typhimurium* (9); *Klebsiella pneumoniae* (10); *Proteus vulgaris* (11); *Pseudomonas aeruginosa* (12). A control gel of the OM of *E. coli* O26 K60 is shown on track 1 (protein designations as in Fig. 1). Non-microbial bands in the immunoprecipitates correspond to heavy and light chains of rabbit immunoglobulins and albumin, as indicated by a control gel (13): IgM-H, heavy chains of IgM; Alb, albumin; IgG-H, heavy chains of IgG; L, light chains.

Figure 3 shows protein profiles of immunocomplexes formed after reaction of OM preparations solubilized in boiling SDS, with anti-O26 K60 serum. Rabbit immunoglobulin bands were present in all profiles, although for *E. coli* O1 K⁻ and *Pseudomonas aeruginosa* these were very weak. Bands of OM proteins could be seen in most profiles. The precipitate resulting from the reaction between OM of *E. coli* O26 K60 and the antiserum against this strain contained the OM proteins with a marked predominance of protein d over proteins b and c (Fig. 3, track 3). This predominance was also seen in most other profiles (Fig. 3, tracks 4 to 8 and 10). Bands in the molecular weight range of proteins a, b and c of *E. coli* OM (36000 to 41000) were seen in most profiles, but were only visible for a short time after gel-destaining.

The results presented in Figs 2 and 3 indicated cross-reactivity of the major OM proteins of the *E. coli* strains and of three related Enterobacteriaceae species. The difference between protein profiles of immunoprecipitates obtained with antigens solubilized at room temperature (Fig. 2) and at 100 °C (Fig. 3) was probably caused by the degree to which the OM components were solubilized as polymolecular structures or as separate molecules. At room temperature, 0.2 to 2% (w/v) SDS only solubilizes protein d and part of proteins III and IV, while proteins b and c and part of proteins III and IV remain associated with the peptidoglycan (Lugtenberg *et al.*, 1977; Hindennach & Henning, 1975). These peptidoglycan-associated membrane constituents may be precipitated when antibodies to at least one of the composing subunits are present in the antiserum. When such immuno-

Fig. 3. Polyacrylamide gel electrophoresis patterns of immunoprecipitates obtained in interfacial precipitin tests carried out with antiserum against *E. coli* O26 K60 (absorbed with LPS O26) containing 0.15 % (w/v) SDS and outer membrane preparations of several Gram-negative bacteria, solubilized by boiling in 2 % (w/v) SDS, followed by dilution to 0.2 % (w/v) SDS: *E. coli* serotypes O1 K⁻ (2), O26 K60 (3), O111 K58 (4), strain J5 (5), O4 K2 (6), O55 K59 (7), O75 K⁻ (8); *Salmonella typhimurium* (9); *Klebsiella pneumoniae* (10); *Proteus vulgaris* (11); *Pseudomonas aeruginosa* (12). Control gels (1 and 13) as in Fig. 2.

precipitates are boiled in SDS before PAGE analysis the protein molecules composing the original complex are separated and will then be detected in the PAGE profiles as distinct bands, suggesting that these proteins had participated in the precipitin reaction as individual antigens. Such an explanation may account for some of the apparent anomalies in Fig. 2. For instance, in profiles 6 and 8 the 41 000 dalton protein of *E. coli* O4 K2 and O75 K⁻ was detected in immunoprecipitates obtained with room temperature-solubilized OM of these serotypes and antiserum against type O26 K60, although OM of the latter does not contain this protein (see Fig. 1, track 2). Solubilization of the OM in SDS at 100 °C separates all the constituent molecules except a part of the lipoprotein (protein IV) which is covalently bound to the peptidoglycan layer (Braun, 1975). The appearance of OM proteins in PAGE profiles of immunoprecipitates obtained after application of boiled OM to antisera therefore shows the participation of these OM proteins as separate molecules in the formation of immune complexes. Under these circumstances most *E. coli* OM proteins in the 33 000 to 41 000 dalton range were seen in the PAGE profiles of the immunoprecipitates with a strong predominance of protein d over proteins b and c (Fig. 3). This suggests that protein d may be the dominant common surface antigen, particularly since this protein is shared by most strains studied (see Fig. 1). However, the effect of boiling and denaturation by SDS on the antigenicity of the proteins cannot be determined properly, because these drastic methods have to be employed to achieve their separation into single molecules.

Fig. 4. Polyacrylamide gel electrophoresis patterns of immunoprecipitates obtained in interfacial immunoprecipitin tests carried out with outer membrane preparations of *E. coli* O26 K60, solubilized in 0.2% (w/v) SDS at room temperature, and rabbit antisera containing 0.15% (w/v) SDS. Antisera were raised against *E. coli* serotypes O1 K⁻ (2), O26 K60 (3), O111 K58 (4), strain J5 (5), O4 K2 (6), O55 K59 (7), O75 K⁻ (8), *Salmonella typhimurium* (9), *Klebsiella pneumoniae* (10), *Proteus vulgaris* (11), *Pseudomonas aeruginosa* (12). All sera were absorbed with their appropriate LPS. Control gels (1 and 13) as in Fig. 2.

Polyacrylamide gel electrophoresis of immunecomplexes from E. coli O26 K60 outer membrane preparations and antisera against Gram-negative bacteria

Figure 4 shows PAGE profiles of immunoprecipitates formed in the interface between antisera elicited against various bacteria and the OM of *E. coli* O26 K60 suspended at room temperature in 0.2% (w/v) SDS. The antisera were absorbed with their appropriate LPS; this caused a decrease in bacterial agglutination titres to values below 1:128. The amounts of OM proteins found in the immunecomplexes were closely related to the amounts of immunoglobulins. The profile of the immunoprecipitate formed with the antiserum against *E. coli* O26 K60 and the OM of the same serotype showed the major OM proteins in the 33000 to 41000 dalton range (Fig. 4, track 3). The bands of proteins b and c were much stronger than the protein d band in this profile. When the OM of *E. coli* O26 K60 solubilized at room temperature was reacted with antisera against the other strains, proteins b and c were seen in all profiles, while protein d was only apparent in the immunecomplexes formed with antisera against some of the *E. coli* serotypes.

The results of similar experiments using *E. coli* O26 K60 OM solubilized at 100 °C are shown in Fig. 5. The protein pattern of immunoprecipitates from antiserum against *E. coli* O26 K60 and OM of the same serotype (Fig. 5, track 3) showed the major OM proteins in the 33000 to 41000 dalton range and the 16000 dalton protein. In contrast to the results obtained with OM solubilized at room temperature (Fig. 4), the application of boiled OM gave a predominance of protein d compared with proteins b and c. Protein d was detected in all profiles except those obtained after application of *K. pneumoniae*, *Proteus vulgaris* or *Pseudomonas aeruginosa* antisera (Fig. 5, tracks 10, 11 and 12), while proteins b and c

Fig. 5. Polyacrylamide gel electrophoresis patterns of immunoprecipitates obtained in interfacial precipitin tests carried out with outer membrane preparations of *E. coli* O26 K60, solubilized by boiling in 2% (w/v) SDS followed by dilution to 0.2% (w/v) SDS, and rabbit antisera containing 0.15% (w/v) SDS. Antisera were raised against *E. coli* serotypes O1 K⁻ (2), O26 K60 (3), O111 K58 (4), strain J5 (5), O4 K2 (6), O55 K59 (7), O75 K⁻ (8), *Salmonella typhimurium* (9), *Klebsiella pneumoniae* (10), *Proteus vulgaris* (11), *Pseudomonas aeruginosa* (12). All sera were absorbed with their appropriate LPS. Control gels (1 and 13) as in Fig. 2.

were only visible in the profiles obtained with some *E. coli* antisera (Fig. 5, tracks 3 to 6 and 8).

From the results shown in Figs 2 and 3 we could only obtain an impression of the degree of cross-reactivity since it was not possible to standardize the amounts of all the antigens applied. However the results shown in Figs 4 and 5 were obtained by reacting equal quantities of *E. coli* O26 K60 OM (solubilized at room temperature or 100 °C) with antisera against all bacteria (absorbed with their appropriate LPS). Using room temperature-solubilized OM of *E. coli* O26 K60 (Fig. 4), the strongest cross-reactivity was found with *E. coli* O111 K58 and its rough mutant J5; these strains closely resemble serotype O26 K60 in their protein profiles (Fig. 1). Applying boiled OM of *E. coli* O26 K60 (Fig. 5), a high degree of cross-reactivity was found again with *E. coli* O111 K58 and J5 and with serotypes O4 K2 and O75 K⁻. Serotype O75 K⁻ differs markedly from serotype O26 K60 in its OM profile but both have protein d, which is the major antigen when boiled OM is applied. The strong cross-reactivity of protein d in these serotypes was also seen when anti-O26 K60 serum was reacted with boiled OM of serotype O75 K⁻ (Fig. 3, track 8). Cross-reactivity was very weak with serotype O1 K⁻ or *Pseudomonas aeruginosa*. The OM protein profiles of both these organisms showed little similarity to that of *E. coli* O26 K60.

To test the significance of our observations, cross-absorptions with OM preparations were carried out to remove cross-reacting antibodies from some of the sera. OM of *E. coli* O26 K60 was used to absorb antisera against *E. coli* O75 K⁻ and *Proteus vulgaris*, while

antiserum against *E. coli* O26 K60 was absorbed with *E. coli* O1 K⁻ OM. After the absorption, immunoprecipitation tests were carried out with the original OM preparations (solubilized at room temperature or 100 °C). Absorption of anti-*E. coli* O75 K⁻ serum with OM of *E. coli* O26 K60 removed all reactivity against the OM of the original serotype, although theoretically antibodies against the 41 000 dalton protein should have been left in the serum. Absorption of antiserum against *Proteus vulgaris* with OM of *E. coli* O26 K60 removed hardly any of the activity of this serum against its appropriate OM. Finally, absorption with OM of *E. coli* O1 K⁻ did not affect the activity of anti-*E. coli* O26 K60 serum against OM of this serotype. These results confirmed the general conclusions about cross-reactivity of OM proteins stated above, although the specificity of the absorption was not as high as would have been expected if all OM proteins had acted as independent antigens. For proteins a, b and c, in particular, other methods need to be applied in future to achieve a better definition of these proteins as separate antigens.

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