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Modification of the Lipid Moiety of the Enterobacterial Common Antigen by the "*Pseudomonas* Factor"

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Pseudomonas aeruginosa produces a factor (PF) which affects the enterobacterial common antigen (ECA), resulting in failure of the antigen to modify erythrocytes for hemagglutination by ECA antibodies. In the present study the nature of PF was determined. Pronase treatment abolished its activity, indicating the protein nature of PF. PF-treated ECA no longer coated erythrocytes but still reacted with ECA antibodies in immunoelectrophoresis tests with monospecific antiserum to ECA, although differences were noted between the precipitation patterns of PF-treated and untreated ECA. Therefore, PF does not significantly affect the antigenic determinant of ECA but rather affects its lipid carrier, an L-glycerophosphatide. Accordingly, differences in the sugar chain could not be detected by high-voltage paper electrophoretic examinations of partial hydrolysates of PF-treated and untreated ECA. PF liberates all fatty acids from ECA, similarly to commercial lipases, as evidenced by the liberation of unsubstituted glycerol upon HF degradation at 0°C of PF-treated ECA. The lipase activity of PF is indicated also by the observation that a strain of *P. aeruginosa* with reduced lipase production and an exolipase-negative strain affect ECA either less or not at all. We conclude that PF is a lipase acting on the lipid moiety of ECA, which is responsible for the coating of erythrocytes, but not significantly on the serological determinant, the amino sugar chain.

The enterobacterial common antigen (ECA), first described by Kunin (3, 4), represents a bacterial surface antigen shared by almost all *Enterobacteriaceae*. The free, non-immunogenic form of ECA is produced by both smooth and rough strains, whereas some rough mutants with complete core of the R1 or R4 type also produce immunogenic ECA bound to the lipopolysaccharide (LPS) core. The free form of ECA was isolated recently by two independent methods, and its major sugar components were determined to be *N*-acetylglucosamine and *N*-acetylmannosaminuronic acid (5, 6). ECA, as an amphipathic molecule, is composed of a hydrophilic amino sugar chain and a hydrophobic L-glycerophosphatide part. The latter was identified on the basis of its reactivity with phospholipase A_2 and by means of mass spectrometric characterization of the diglyceride obtained by treatment of ECA with hydrogen fluoride (H.-M.Kuhn, Ph.D. thesis, University of Freiburg, Freiburg, West Germany, 1981; H.-M. Kuhn, A. Marx, and H. Mayer. Abstr. German Branch Meet. Am. Soc. Microbiol. Mainz, West Germany, abstr. no. 34, 1981). The amino sugar chain carries the serological specificity of ECA, whereas the physicochemical properties of the molecule (e.g., micelle formation and attachment to cell surfaces) depend on the lipid part.

In culture fluids of *Pseudomonas aeruginosa* and a strain of psychrophilic *Pseudomonas*, a factor selectively destroying ECA was found by chance (12, 13). Treatment of ECA with this factor, assumed to be an enzyme, results in a loss of reactivity with ECA antibodies in passive hemagglutination tests. The nature of this factor, referred to as *Pseudomonas* factor (PF), and its action on ECA are the subject of this communication.

MATERIALS AND METHODS

Strains of *P. aeruginosa* types 4, 6, and 10 were obtained from the Laboratory of Bacteriology, Children's Hospital of Buffalo, Buffalo, N.Y.; the mutant B132 of *P. aeruginosa* ATCC 9027, with reduced lipase production was obtained from U. Winkler, Bochum, West Germany; and the lipase-negative mutant was obtained from G. Döring, Tübingen, West Germany. All other strains were derived from the culture collection of the Max-Planck-Institut, Freiburg. The enriched exolipase preparation was a gift of G. Döring, Tübingen.

ECA was isolated from *Salmonella montevideo*

SH94 according to the method of Männel and Mayer (7) by means of the combined phenol-water-phenol chloroform petroleum ether extraction procedure.

PF was prepared as described by Whang and Neter (12). The strains were grown in petri dishes (diameter, 14 cm) on D1.5 agar at 37°C for 72 h; the resulting growth was harvested with 10 ml of saline per plate. The suspension was centrifuged at 7,000 × *g* for 20 min, and the supernatant fluid was subjected to membrane filtration (Millex, 0.22 μm; Millipore Corp., Bedford, Mass.). The filtrate was stored at −20°C or lyophilized.

Treatment of ECA with PF. ECA (1 mg) was suspended in 10 ml of PF and incubated at 56°C for 72 h (12). After being freeze-dried, the material was suspended in 1 ml of water (ECA = 0.1%).

PF treatment of whole cells. *S. montevideo* SH94 (growth of one plate) was incubated with PF obtained from 10 plates at 56°C for 48 h. After centrifugation (7,000 × *g* for 10 min) and washing with saline, the sediment was suspended in 3 ml of saline and boiled for 1 h. After another centrifugation, 100 μl of supernatant fluid was used to coat erythrocytes for hemagglutination tests. For control purposes the growth of one plate was incubated with saline instead of PF and treated in the same manner.

ECA antiserum was prepared in New Zealand White rabbits by intravenous immunization with *Escherichia coli* O14 (strain F2387). To eliminate antibodies other than those directed against ECA, the serum was absorbed with the isogenic ECA-negative mutant F1327 (8).

Passive hemagglutination and hemagglutination inhibition tests were performed in microtiter plates, using the Takatsy microdiluter system (7) (both from Dynatech Laboratories, Inc., Alexandria, Va.). Coated human erythrocytes were added to antiserum in twofold serial dilutions for passive hemagglutination or to a preincubated mixture of antiserum and inhibitor (in twofold serial dilutions of the inhibitor) for inhibition tests.

Immunoelectrophoresis (9) was carried out in agarose (Litex, Glostrup, Denmark) (1% in barbital buffer [pH 8.6]) on glass slides in a chamber (CAMAG, Berlin, West Germany) at 10 V/cm.

High-voltage electrophoresis was carried out on paper (2043a; Schleicher & Schüll, Dassel, West Germany) at 45 V/cm for 1 h, using pyridine-formic acid-acetic acid-water buffer (pH 2.8). The dried paper was stained with silver nitrate (11).

Thin-layer chromatograms (on precoated silica gel plates; Merck 60, Darmstadt, West Germany) were developed three times in a solvent system of butanol-water (9:1, vol/vol) and stained with benzidine–Na *meta*-periodate (Merck) for the detection of glycerol.

Amino acid analysis was carried out in an automatic amino acid analyzer (D-500; Durrum, Palo Alto, Calif.).

Free fatty acids were converted to their methyl esters by diazomethane and identified by gas-liquid chromatography on a CPSil-5 capillar column (Chrompack, Berlin) of 25-m length and 0.25-mm inner diameter at a 175°C isotherm with a flow rate of 1 ml of N_2 per s.

For HF treatment, 0.5 ml of 48% aqueous HF (Merck) was added to 5 mg of PF-treated ECA in a polyethylene tube fitted with a cap. After 48 h at 0°C, the material was lyophilized over KOH, resolved in water, and again lyophilized.

Pronase treatment was done by incubating 1 mg of ECA or PF-treated ECA with 20 μg of pronase E from *Streptomyces griseus* (Serva, Heidelberg, West Germany) in 1 ml of 0.1 M Tris buffer (pH 7.7), at 37°C overnight.

For treatment with esterase or lipase, 1 mg of ECA was dissolved in 1 ml of 0.1 M Tris buffer (pH 7.7) and incubated for 48 h at 37°C with 200 μl of esterase from pig liver (Boehringer, Mannheim, West Germany) or with 5 mg of lipase from *Candida cylindracea* (Sigma Chemical Co., Munich, West Germany).

De-O-acetylation was performed by incubating 1 mg of ECA in 100 μl of 5% triethylamine (EGA, Steinheim, West Germany) for 25 min at 50°C. The material was lyophilized and dissolved in 1 ml of water (1).

RESULTS

The activity of PF was checked by testing PF-treated ECA in the passive hemagglutination test. Untreated ECA readily coated erythrocytes, which then were agglutinated in the presence of ECA antiserum. In accordance with the observation of Whang and Neter (13), PF-treated ECA no longer reacted in this system. The activity of PF was affected only slightly by lyophilization of PF before treatment of ECA. PF treatment of whole bacterial cells failed to show any effect on ECA, since supernatant fluid from cells boiled after PF treatment coated erythrocytes similarly to supernatants of untreated cells for agglutination by ECA antibodies.

In the following experiments PF of *P. aeruginosa* type 4 was used, and the activity of each preparation was checked. To confirm its presumed protein nature, PF was incubated with pronase E at 37°C overnight and then used to modify ECA. Passive hemagglutination tests showed that pronase-treated PF had lost its activity on ECA (Table 1). Complexing of ECA with basic and hydrophobic proteins has been described recently (2). To prove that the loss in reactivity of PF-treated ECA was not due to complexing of ECA with PF, ECA was incubated with PF and then treated with pronase E at 37°C overnight. Pronase did not restore the ECA activity of PF-treated antigen (Table 1), suggest-

TABLE 1. Effect of pronase E treatment of PF on ECA

ECA preincubated with:	ECA hemagglutinin titer
Untreated	5,120
PF	<10
Pronase-treated PF	5,120
PF[a]	<10

[a] After incubation with PF, the ECA was treated with pronase.

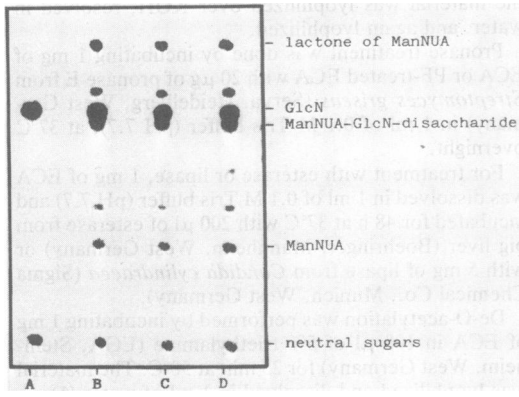

FIG. 1. High-voltage electrophoresis of hydrolysates on ECA with and without treatment with PF (pH 2.8, 45 min, 45 V/cm). Lanes: A, standards of glucose and *N*-acetylglucosamine (GlcN)(10 μg each); B–D, hydrolysates (150 μg each, 4 N HCl, 100°C, 1 h) of ECA treated with boiled (10 min) PF (B), ECA treated with PF (C), and untreated ECA (D). ManNUA, *N*-acetylmannosaminuronic acid.

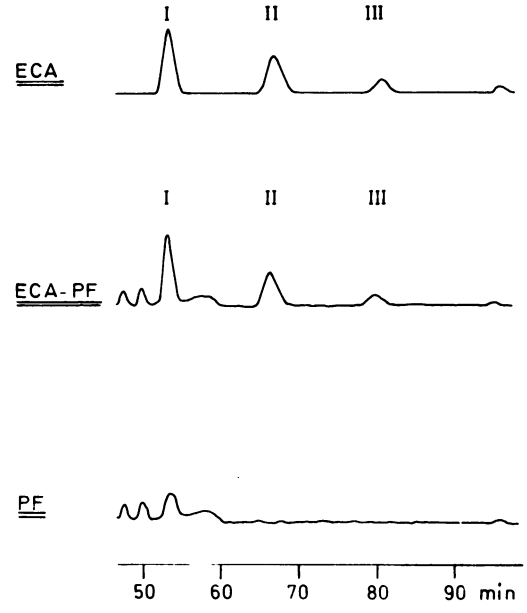

FIG. 2. Elution profile of hydrolysates (50 μg each, 4 N HCl, 100°C, 1 h) of untreated ECA, PF-treated ECA (ECA-PF), and (PF) alone on a Durrum (D-500) automatic amino acid analyzer. Peaks: I, *N*-acetylglucosamine; II, *N*-acetylmannosaminuronic acid–α1.4-*N*-acetylglucosamine disaccharide; III, lactone of *N*-acetylmannosaminuronic acid.

ing the enzymatic nature of PF action.

In view of these results it was of interest to determine whether, as suggested by Whang and Neter (12), PF affects the antigenic determinant, namely, the amino sugar part of the ECA molecule. To this end, the following experiments were carried out. When partial hydrolysates (4 N HCl, 1 h, 100°C) of PF-treated and untreated ECA were tested by high-voltage electrophoresis, no differences were noted regarding the number of mono- and oligosaccharides (Fig. 1). Similar results were obtained on the automatic amino acid analyzer: partial hydrolysates of PF-treated ECA showed the typical elution profile of ECA (H.-M. Kuhn, Ph.D. thesis), consisting of glucosamine, mannosaminuronic acid-glucosamine disaccharide, and the lactone of mannosaminuronic acid (Fig. 2). No changes in the amino sugar part were detectable after PF treatment.

Hemagglutination inhibition tests revealed that PF-treated ECA neutralized antibodies, although less effectively than did untreated antigen. In one particular experiment, the minimal inhibitory amount preventing hemagglutination completely was 0.97 μg of PF-treated ECA per ml as compared with 0.24 μg/ml for the untreated antigen. With monospecific antiserum in immunolectrophoresis tests, PF-treated ECA yielded precipitation; however, only a single precipitation arc was observed with the treated preparation, instead of the birdwing pattern of untreated ECA (Fig. 3). This change may be due to loss of micelle formation, as has been observed after alkali or phospholipase A_2 treatment of ECA (H.-M. Kuhn, Ph.D. thesis; Kuhn et al., Abstr. German Branch Meet. Am. Soc. Microbiol. Mainz, abstr. no. 34, 1981).

The fact that PF treatment results in loss of micelle formation but does not abolish serological activity of ECA indicates that PF acts on the lipid part of the molecule, presumably by splitting off fatty acids. Accordingly, PF-treated ECA was subjected to chloroform-methanol-water extraction, and the chloroform phase was analyzed for liberated fatty acids. It was found that PF liberates fatty acids from ECA. By comparing the amounts of liberated palmitic acid, it is evident that PF differs in its action from phospholipase A_2. The latter liberates only fatty acids located in C-2 position of the glycerophosphatide, whereas PF, like alkali, liberates the entire amount of fatty acids from both the C-

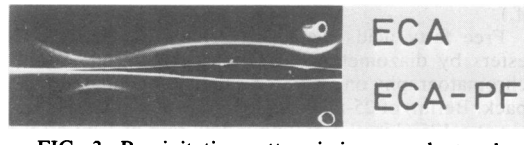

FIG. 3. Precipitation pattern in immunoelectrophoresis with a 1% solution of ECA or PF-treated ECA (ECA-PF) and antiserum against *E. coli* O14, absorbed by strain F1327 (an *E. coli* O14 ECA-negative mutant).

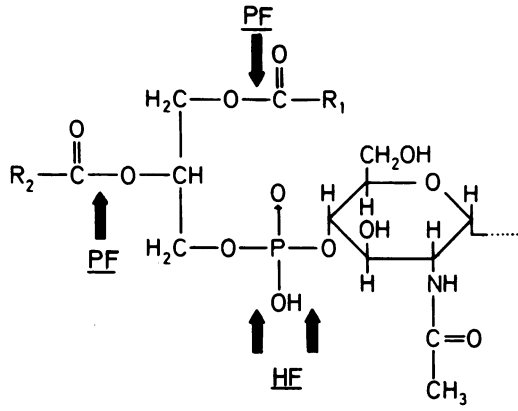

FIG. 4. Proposed sites of attack on lipid part of ECA by PF and HF.

1 and the C-2 positions. To provide further proof, PF-treated ECA was subjected to HF degradation at 0°C. It is known that HF cleaves phosphomono- and diester linkages. A diglyceride was obtained by HF treatment of ECA, whereas, as expected, unsubstituted glycerol was obtained from PF-treated ECA (Fig. 4).

Modification of ECA with a preparation rich in exolipase of *P. aeruginosa* gave the same results as did modification with PF: loss of ECA titer in passive hemagglutination tests while the inhibitory capacity was maintained, change of birdwing pattern to a single arc in immunoelectrophoresis, and liberation of fatty acids from both the C-1 and C-2 positions. In contrast, culture fluids, prepared in the same way as PF, of a strain with reduced exolipase production (strain B132) and an exolipase-negative mutant of *P. aeruginosa* affected ECA either less or not at all (Table 2), thus confirming the lipase activity of PF.

With commercially available enzymes, lipase treatment of ECA also led to loss of ECA titer in passive hemagglutination tests, whereas esterase treatment resulted only in a slight reduction of the antibody titer. Esterase treatment released only 10% of fatty acids, probably derived from nonaggregated ECA. Treatment with either lipase or esterase reduced the inhibitory capacity of ECA by two dilution steps, from 0.24 μg/ml in the case of untreated ECA to 0.97 μg/ml in the case of the modified antigen.

DISCUSSION

Recent studies on the chemical structure of ECA have revealed that it is composed of a sugar chain which is attached to a lipid moiety via a phosphodiester bridge. The serological specificity is located in the sugar strand, but a number of physicochemical properties, such as micelle formation and adhesion to surfaces, are dependent on the unmodified lipid moiety. When one fatty acid is split off by treatment with phospholipase A_2 or when both fatty acids are split off by alkali treatment, ECA loses its coating capacity but retains its antigenicity to a significant extent, as shown by hemagglutination inhibition and by agar gel precipitation.

Observations by Whang and Neter (12) revealed that supernatants of *P. aeruginosa* (PF) alter ECA in such a way that treated ECA no longer modifies erythrocytes for hemagglutination by ECA antibodies. In the present study we have shown that the active factor in the culture fluids of *P. aeruginosa* is an enzyme. Analytical results indicated that, compared with the untreated control, all sugars and oligosaccharides are present in partial hydrolysates of PF-treated antigen. Gel precipitation and hemagglutination inhibition tests showed that the antigenicity of the molecule is largely retained. Thus, PF does not significantly affect the sugar chain of ECA, which carries the serological specificity. This is in accord with the observation that PF has no effect on ethanol-insoluble ECA derived from immunogenic *E. coli* O14 (13): the ethanol-insoluble ECA differs from ethanol-soluble ECA since the sugar strand is bound to the LPS core in the former. So far as the lipid moiety of ECA is concerned, it was recognized that PF modifies this part of the molecule in a way similar to that of alkali or commercial lipase. This was revealed by analytical studies which showed that the fatty acids are eliminated from both positions of the ECA glycerophosphatide. Additionally, a preparation enriched in *P. aeruginosa* exolipase acts on ECA in a way identical to that of PF, whereas culture fluids of mutants which fail to produce exolipase or produce lesser amounts either fail to show any effect or have a reduced effect on ECA, suggesting the possible identity of PF and exolipase of *P. aeruginosa*.

PF also affects LPS, as was shown by the release of fatty acids on incubation with PF (unpublished data). In contrast to ECA, loss of fatty acids does not destroy the coating capacity of LPS. Thus, the action of PF on LPS is not detectable by the passive hemagglutination test.

TABLE 2. PF activity of *P. aeruginosa* strains with reduced (strain B132) or absent exolipase production

ECA preincubated with *P. aeruginosa*	ECA hemagglutinin titer
	1,280
Type 4	<10
Strain B132	160
Lipase-negative mutant	1,280

This led Whang and Neter (12) to the assumption of an "ECA selectivity of PF," not confirmed in the present study. PF also eliminates fatty acids from commercially available phospholipids (unpublished data), but it is important to keep in mind that PF represents a crude culture fluid. Thus, the question of whether both activities on ECA and phospholipids are identical remains to be elucidated.

PF-treated ECA shows a somewhat reduced antibody-neutralizing capacity compared with untreated ECA. It is not known whether this is due to a higher reactivity of the micellar form of untreated ECA as compared with the deacylated form, which is unable to form micelles, or whether a small, as yet undetected modification of the sugar strand is responsible. ECA contains some O-acetyl groups which are assumed to be attached to the sugar chain. It was found recently that in the case of Vi antigen of *Salmonella typhi*, which also is substituted by O-acetyl groups, the O-acetyl moiety plays a significant role in one of the two antigenic determinants (10). Preliminary results obtained by treatment of ECA with an esterase or with 5% triethylamine, which should de-O-acetylate selectively, also showed a slightly reduced antibody-neutralizing capacity of ECA, whereas the erythrocyte-coating capacity, in contrast to that of PF-treated ECA, was only slightly reduced or even unaffected. Determination of the O-acetyl content of ECA after PF, esterase, and triethylamine treatment remains to be done. Thus, the question remains of whether the loss of O-acetyl groups is responsible for the slightly reduced antibody-neutralizing capacity of PF-treated ECA. However, it is clear that the release of fatty acids is responsible for the loss of erythrocyte-coating capacity. An initial attempt to investigate the effect of PF on whole cells was not informative. Thus, it remains to be determined whether PF can modify ECA incorporated in the outer membrane of the enterobacterial cell envelope and thus modify surface properties of the bacteria.

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