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Enterobacterial Common Antigen-Tetanus Toxoid Conjugate as Immunogen

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The methods of limited periodate oxidation and reductive amination were used to obtain covalently linked enterobacterial common antigen (ECA) with tetanus toxoid. This procedure is simple and gives a good yield of the conjugate with high ECA content (molecular ratio of ECA to tetanus toxoid, 6:1). The ECA-tetanus toxoid conjugate is immunogenic in rabbits, in contrast to free ECA or a mixture of ECA with proteins. This conjugate produces high levels of ECA-specific immunoglobulin G antibodies, which can be used as a standard serum.

Enterobacterial common antigen (ECA), first described by Kunin et al. (20), is a glycophospholipid present in the outer membrane of the cell envelope of bacteria from the *Enterobacteriaceae* family (24, 27). ECA exists in two different forms: a non-immunogenic free form and a lipopolysaccharide-linked immunogenic form. The first form is present in almost all wild enterobacterial strains, whereas the latter can be found in a few rough mutants of *Escherichia coli* or *Shigella* sp. with a complete core of the R_1 or R_4 type (16, 27).

Two different methods of isolation and purification of ECA have been independently elaborated (23, 25). Pure ECA preparations isolated by these methods proved to be non-immunogenic (26) or slightly immunogenic (23) after intravenous administration into rabbits, but their immunogenicity can be raised after complexing with proteins (19).

Rabbit antiserum obtained by intravenous injection with ECA immunogenic bacterial strains contained mainly immunoglobulin M (IgM) antibodies (32). The level of IgG antibodies was very low. Similar ratios of IgM to IgG antibodies in antisera obtained against ECA-protein complexes after intravenous administration were observed (19).

It has been shown (3, 6, 31, 34) that covalent coupling of polysaccharides to protein carriers enhances their immunogenicity. Many different methods of conjugation of sugar antigens to proteins have been elaborated (1, 3, 6, 13, 31, 34, 36). These conjugates quite often contained carcinogenic substances such as aromatic amines. Recently, Jennings and Ługowski (14) have applied the technique of Schwartz and Gray (35) to the preparation of highly immunogenic conjugates of meningococcal capsular group A and group C polysaccharides with tetanus toxoid (TT).

The conjugate obtained contains a reduced Schiff base which has already been identified in animal tissue (28). The protein chosen as a carrier was TT because it is approved universally as a human vaccine. It creates the possibility for potential use of these artificial antigens as human vaccines.

MATERIALS AND METHODS

Bacterial strains. *Shigella sonnei* 9773 phase I was obtained from the Dysentery Reference Laboratory (London, England), and phase II was obtained by spontaneous mutation of phase I. *E. coli* 014 was kindly provided by F. Ørskov (Copenhagen, Denmark). The bacteria were grown in liquid medium as described previously (33).

Protein and ECA preparations. Crude TT preparation was obtained from Wytwórnia Surowic i Szczepionek (Warszawa, Poland). The outer membrane protein preparation of *S. sonnei* phase II was kindly supplied by G. Adamus (this laboratory), and ECA from *Salmonella montevideo* was supplied by H. Mayer (Max-Planck-Institut für Immunbiologie, Freiburg, Germany). ECA from *S. sonnei* phase I or II was isolated and purified by the procedure of Ługowski and Romanowska (23).

Analytical methods. Protein was determined by the method of Lowry et al. (21), and hexosamine was determined by the method of Ludowieg and Benmaman (22).

De-*O*-acetylation of ECA. ECA was de-*O*-acetylated with 0.25 M NaOH a 56°C for 1 h. The product was neutralized with 1 M HCl.

Conjugation of ECA with TT and purification of the conjugate by gel filtration. The ECA-TT conjugate was prepared by the method of Jennings and Ługowski (14). *S. sonnei* phase II ECA preparation (40 mg) was oxidized with 4 ml of 0.1 M $NaSO_4$ solution (pH 4.7) at room temperature in the dark. The excess of periodate was destroyed by the addition of ethylene glycol. The

reaction mixture was separated on a Sephadex G-25 column (2.6 by 90 cm). The fraction eluted with the void volume containing ECA was freeze-dried. The oxidized ECA (36 mg) was dissolved in 1 ml of 0.5 M K_2HPO_4 (pH 9.0). The TT (3 mg), sodium cyanoborohydride (40 mg), and 2 drops of toluene were added to the ECA solution. The reaction mixture was kept in a sealed vial for 14 days at 37°C and then applied to a Sephadex G-200 column (1.6 by 100 cm) equilibrated with phosphate-buffered saline. The ECA-TT conjugate was usually eluted at 80 to 100 ml of effluent volume. This fraction (20 ml) was concentrated by ultrafiltration. The yield of the conjugate amounted to 3 mg (about 90%).

Immunization procedure. Rabbits were immunized in the footpads with 50 μg of the antigens suspended in Freund complete adjuvant at days 0 and 21. The animals were bled 14 days after the second injection. Anti-*E. coli* 014 serum obtained by intravenous immunization of rabbits with acetone-dried bacterial cells 8239 was used as a standard anti-ECA serum.

Serological methods. (i) Passive hemagglutination test. The passive hemagglutination test was done as described previously (23). Horse erythrocytes coated with ECA from *S. sonnei* phase I or II preparations were used.

(ii) Quantitative microprecipitin test. The quantitative microprecipitin test was carried out essentially by the method of Kabat and Mayer (15). The reaction mixture contained in addition 2% polyethylene glycol 6000. The protein in the precipitates was determined by the method of Lowry et al. (21).

(iii) ELISA. An enzyme-linked immunosorbent assay (ELISA) was performed by a modification of the method of Voller et al. (37) in polystyrene microtiter plates (Linbro Chemical Co.). The following reagents were prepared. Rabbit IgG was purified from normal serum by precipitation with ammonium sulfate, followed by chromatography on a DEAE-cellulose column (12). Specific goat anti-rabbit IgG antibody was obtained by affinity chromatography of goat anti-rabbit IgG (Fc) serum (Nordic) on a Sepharose 4B-rabbit IgC column (7). The specific antibody was eluted from this column with 3 M KCNS (pH 7.3). Pure antibody was conjugated with alkaline phosphatase by the addition of glutaraldehyde (2) by the method of Engvall et al. (11). The conjugate was purified by gel chromatography on a Sepharose 6B column. The fraction containing both enzyme and antibody activities was concentrated by ultrafiltration and stored at 4°C in the presence of 0.05% NaN_3 and 2% human serum albumin. The test was carried out as described in detail in a previous paper (14), using microtiter plates freshly coated with ECA preparation at pH 8.6.

(iv) Double immunodiffusion. Double immunodiffusion was performed in a 1% agarose gel in phosphate-buffered saline containing 2% polyethylene glycol 6000 (30).

(v) Immunoelectrophoresis. Immunoelectrophoresis was carried out by the methods listed below with a 1% agarose gel in 0.02 M barbital buffer (pH 8.6) containing 2% polyethylene glycol 6000. The thickness of the gel layer was 2 mm. The antibody gel contained 10% of the appropriate antiserum. Crossed electrophoresis and tandem crossed electrophoresis were performed as described by Weeke (38) and Krøll (17), respectively; rocket-line immunoelectrophoresis was performed by the method of Krøll (18). After electrophoresis, gels were washed free of excess reagent and photographed directly or after staining with 1% amino black 10B.

Inactivation of IgM antibodies. IgM antibodies were inactivated by treatment of whole immune sera with dithiothreitol by the method of Olson et al. (29).

RESULTS

Immunochemical characteristics of the ECA-TT conjugate. The ECA-TT conjugate (3 mg) separated from the reaction mixture by Sephadex G-200 column chromatography was examined for sugar and protein content. Based on the results of hexosamine and protein determinations, the molar ratio of ECA to TT in the conjugate was about 6:1. The preparation was homogeneous in agarose gel electrophoresis and column chromatography on Sephadex G-200; it was eluted as a symmetrical peak (distribution coefficient K_{AV_1}, 0.15). The conjugate (1 mg/ml) reacted strongly with anti-*E. coli* 014 as well as with anti-TT sera in an agarose double immunodiffusion test.

Immunogenicity of the ECA-TT conjugate. The immunogenicity of the conjugate in comparison with free ECA and mixtures of ECA with proteins was determined in rabbits. The levels of anti-ECA antibodies in immune sera determined by passive hemagglutination are summarized in Table 1. It is evident that only anti-ECA-TT conjugate and anti-*E. coli* 014 sera were active. These data were confirmed by ELISA, a very sensitive and specific method for measuring low concentrations of antibodies. Even in such a sensitive test as the ELISA, antiserum against the mixture of ECA and TT appeared to be almost inactive in the reaction with ECA.

The total amount of antibodies in the active sera was measured by a quantitative microprecipitin test. In the equivalence point, ECA precipitated from anti-ECA-TT conjugate and anti-*E. coli* 014 sera 2.8 and 0.55 mg of antibody per ml of serum, respectively.

TABLE 1. Comparison of anti-ECA antibody titers in rabbit immune sera as determined by passive hemagglutination and ELISA

Immunogen	Anti-ECA reciprocal titer	
	Passive hemagglutination	ELISA[a]
ECA-TT	10,240	4.80×10^6
ECA	10	ND[b]
ECA + TT	10–20	0.61×10^2
ECA + outer membrane protein	10–20	ND
E. coli 014 cells	5,120–10,240	0.96×10^6

[a] The ELISA titer was defined as the highest serum dilution which gave an absorbance of 1.0 in 100 min at 400 nm.

[b] ND, Not determined.

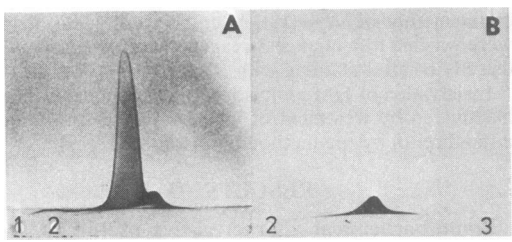

FIG. 1. Immunoelectrophoretic analysis of anti-ECA-TT conjugate serum. (A) Tandem crossed immunoelectrophoresis; (B) crossed immunoelectrophoresis. Antigens: 1, ECA (10 μg); 2, ECA-TT (20 μg). After the first-dimension electrophoresis, 10 μg of TT was applied to well 3. The first dimension was done at 10 V/cm for 1 h and 15 min (anode to the right); the second dimension was done at 10 V/cm for 10 h (anode at top).

The active sera were treated with dithiothreitol, which selectively destroys 19S antibodies (disulfite bond reduction). After incubation with dithiothreitol, the immune sera were examined in a passive hemagglutination test. The titer of anti-ECA-TT conjugate serum was unchanged, whereas the anti-*E. coli* 014 serum titer decreased fourfold. This fact indicates that the latter antiserum contained mainly anti-ECA IgM antibodies.

Immunospecificity of anti-ECA sera. The quantitative precipitin analysis of anti-ECA-TT conjugate serum treated with different antigens, such as ECA-TT, native ECA, de-*O*-acetylated ECA, and TT, indicated that all antigens except free TT reacted strongly with this antiserum. In the equivalence point, native ECA precipitated 2.8 mg of antibody per ml of serum. ECA (de-*O*-acetylated) precipitated 3.4 mg of antibody per ml, and the ECA-TT conjugate percipitated 3.55 mg of antibody per ml. The stronger reaction of anti-ECA-TT serum with the ECA-TT conjugate than with free ECA preparations and the absence of reaction with free TT can be explained by the presence of additional antibodies against the linkage site between the conjugate components.

Anti-*E. coli* 014 serum reacted fairly strong with the native ECA and much more weakly with the de-*O*-acetylated ECA (in the equivalence point, native ECA precipitated 0.56 mg of antibody per ml, and de-*O*-acetylated ECA precipitated 0.1 mg of antibody per ml). These results are consistent with the presence of *O*-acetyl substituents of ECA on the *E. coli* 014 cells. In contrast, the de-*O*-acetylated ECA precipitated more antibodies (3.4 mg/ml of serum) from anti-ECA-TT serum than did the native ECA. This reversal, when compared with the results obtained with anti-*E. coli* 014 serum, can be best explained by the fact that the ECA-TT conjugate contained almost no *O*-acetyl substituents. These groups, being base labile, were probably lost during preparation of the conjugate.

The above results were confirmed by using immunoelectrophoresis techniques. The anti-ECA-TT conjugate serum reacted with the ECA-TT conjugate and with free ECA but not with TT in crossed immunoelectrophoresis (Fig. 1). The reaction of partial identity between free ECA and the ECA conjugate was probably due to the presence of additional anticonjugate antibodies having neither ECA nor tetanus activities.

A reaction of total identity between native and de-*O*-acetylated ECA in rocket-line immunoelectrophoresis was observed when anti-ECA-TT conjugate serum was used (Fig. 2).

Serum obtained after immunization with the mixture of ECA and TT (not containing anti-ECA antibodies; Table 1) was tested by immunoelectrophoresis with TT as the antigen. The antiserum reacted with free and conjugate-linked TT (Fig. 3). Additionally, it was shown that the conjugate did not contain free toxoid, because it formed the single precipitin peak, whereas the mixture of ECA-TT and TT is separated during electrophoresis.

Cross-reactions of anti-ECA-TT serum. The conjugate used as an immunogen contained ECA derived from ECA-immunogenic *S. sonnei* phase II. To determine whether ECA obtained from ECA-non-immunogenic *S. sonnei* phase I is serologically different from phase II ECA, tandem crossed immunoelectrophoresis was carried out. A reaction of total identity of these antigens running into anti-ECA-TT conjugate serum was observed (Fig. 4A).

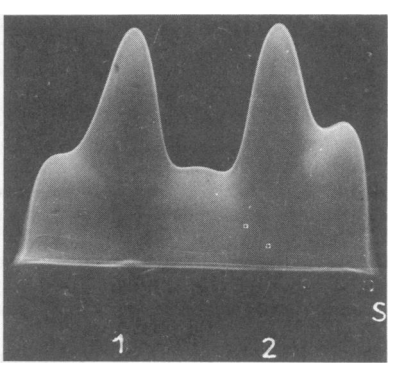

FIG. 2. Rocket-line immunoelectrophoresis comparison of native and modified ECA preparations against anti-ECA-TT serum. Sample gel S contained 5 μg of ECA. Native ECA preparation (1 μg) was placed in well 1, and de-*O*-acetylated ECA (1 μg) was placed in well 2. Electrophoresis was carried out at 5 V/cm for 18 h (anode at the top).

The same method was used to compare the antigenic relationship between two ECA preparations derived from different genera of *Enterobacteriaceae*: *S. sonnei* phase I and *S. montevideo* (Fig. 4B). The main component of the ECA preparation of *S. montevideo* gave a reaction of total identity with the ECA from *S. sonnei* phase I.

DISCUSSION

Monospecific anti-ECA serum is essential for immunochemical studies of ECA. To obtain such a serum was the aim of many earlier studies. To increase the immunogenicity of free ECA, it was mixed with proteins (bovine serum albumin, outer membrane protein, protamine sulfate). This experiment, carried out in our laboratory and Mayer's laboratories, was only partially successful: the antigen was immunogenic, but antibodies obtained in rabbits after intravenous injection were mainly of the IgM class (19). The use of such an antiserum in serological tests is limited. We tried to increase the IgG antibody level by changing the route of antigen administration from intravenous to footpad. Unfortunately, footpad immunization was unsuccessful because neither free ECA nor the mixture of ECA with protein was immunogenic. This result is probably due to the absence of covalent linkages between ECA and the protein carrier.

The use of periodate oxidation and reductive amination methods made it possible to obtain ECA covalently linked with TT. The method is relatively simple and gives a high degree of ECA substitution on protein carrier: the molar ratio of ECA to TT amounted to 6:1. This artificial conjugate (in contrast to the mixture of its components) produced mainly IgG antibodies in rabbits that precipitated strongly with ECA.

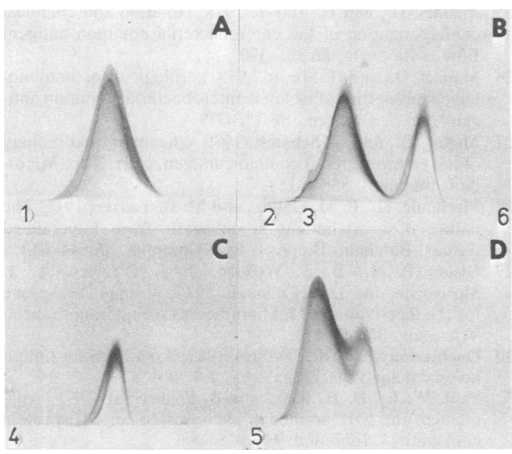

FIG. 3. Immunoelectrophoretic analysis of antiserum against the mixture of ECA and TT. Antigens: 1, 17 μg of TT; 2, 17 μg of TT; 3, 17 μg of ECA-TT; 4, 20 μg of ECA-TT; 5, 10 μg of ECA-TT plus 10 μg of TT. After the first-dimension electrophoresis, 5 μg of ECA was applied to well 6. For the conditions of electrophoresis, see the legend to Fig. 1.

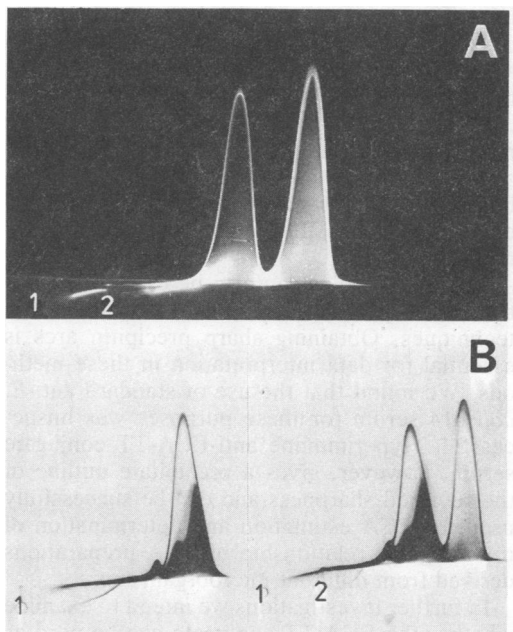

FIG. 4. Comparison of ECA preparations originating from various bacterial strains run against anti-ECA-TT serum by tandem crossed immunoelectrophoresis. (A) ECA from *S. sonnei* phase II (1 to 10 μg) and ECA from *S. sonnei* phase I (2 to 10 μg). (B) ECA from *S. montevideo* (1 to 10 μg) and ECA from *S. sonnei* phase I (2 to 5 μg). For the conditions of electrophoresis, see the legend to Fig. 1.

The difference in immune response to both forms of antigens may be due to involvement of helper T-cells in antibody production against the ECA-TT conjugate. Their cooperation is essential for the induction of IgG antibodies and memory cells (5, 8, 9). For this reason, a thymus-dependent antibody response is desirable for vaccine-induced long-term protection against infectious diseases (4).

Classical standard anti-ECA serum is obtained after immunization of rabbits with *E. coli* 014 cells, which contain *O*-acetylated ECA molecules. This antiserum reacts with different intensities with native (*O*-acetylated) and de-*O*-acetylated ECA preparations. Many problems arise in the interpretation of serological reaction data, because various ECA preparations obtained by different methods (or even by the same method) are non-homogeneous in *O*-acetyl group content. The reaction of anti-ECA serum obtained after immunization of rabbits with the

ECA-TT conjugate with ECA does not depend on the *O*-acetyl group content. Because this antiserum contains no antibodies against enterobacterial cell wall components except ECA, it can be used as a standard anti-ECA serum. The possibility of obtaining such a monospecific antiserum containing predominantly IgG (7S) antibodies is of importance to ECA localization experiments with the immunoperoxidase technique.

These antibodies can also be used in qualitative and quantitative immunoelectrophoresis techniques. Obtaining sharp precipitin arcs is essential for data interpretation in these methods. We found that the use of standard anti-*E. coli* 014 serum for these purposes was unsuccessful. Hyperimmune anti-ECA-TT conjugate serum, however, gives a precipitate outline of the required sharpness and can be successfully used for ECA estimation and determination of the antigenic relationship of ECA preparations derived from different microorganisms.

In further investigations we intend to examine whether the ECA-TT conjugate can be used as an effective, nontoxic enterobacterial vaccine in humans.

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