Vaccine Preparations Used for Immunization of Eel *Anguilla japonica* against *Edwardsiella tarda* Infection

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To clarify the protective properties of *Edwardsiella tarda* polysaccharide reported previously, and to confirm the supposition that the elimination of lipid A from the immunogen can increase the immune protection of the preparation in the eels against experimental challenge with *E. tarda* EF-1, crude lipopolysaccharide (LPS), purified polysaccharide and whole cell without lipid were injected intramuscularly in the eels.

The highest survival was recorded in eels immunized with crude LPS preparation and good resistance was shown also in the eels immunized with the whole cell without lipid. Eels immunized with purified polysaccharide did not show protection after challenge. Chemical analysis showed a high degree of purification for the polysaccharide preparation and perhaps this was the reason for the loss of immunogenicity and effectiveness of the preparation. This may have shown the importance of protein and nucleic acid as haptens or carriers in the preparations. The results also suggested that the elimination of the lipidic part from the whole cell may be an easy way to obtain an effective vaccine preparation against *E. tarda* infection.

Edwardsiellosis is a septicemic disease of eel caused by the bacterium *Edwardsiella tarda*. Attempts have been made to immunologically control this disease, but heat or formalin killed whole cells were not effective.¹⁻³⁾ Immunization with *E. tarda* crude lipopolysaccharide (LPS) and crude polysaccharide (PS), was an effective immunogen producing high antibody titers and higher protection than whole cell preparation in eel when experimentally challenged with live *E. tarda* EF-1.^{4,5)} Immunization of eels with *E. tarda* EF-1 crude PS preparation gave the highest resistance against experimental challenge. These results suggested that the polysaccharidic part of the LPS stimulate protective immune response in the eel. But it was not clear if this preparation worked as protective antigen because the preparation included small quantities of nucleic acids and proteins which may have some role in the protection of eel.

This research was performed to understand the role and the importance of polysaccharide as determinant of immune protection in eel using a highly purified PS preparation. Because the results of previous studies also suggested that the elimination of the lipid A may increase the immunogenicity of the preparation in the eel,⁵⁾ the immunogenicity of whole cells without lipid, was examined as well as purified polysaccharide.

Materials and Methods

Experimental Animals and Aquaria

Ninety to ninety-five gram eels (*Anguilla japonica*, Temminck et Schlegel) obtained from an eel farm in Kochi Prefecture, Japan were used. The eels were kept at 25°C in 60 l tanks with recirculating, aerated and filtered well water. The eels were in the tanks a few days before the start of the experiment to accustom them to the new conditions. The fish were not fed during the experiment.

Microorganism

Edwardsiella tarda strain EF-1 was originally isolated in the June '79 from the kidney of diseased eel from Japanese eel ponds in Fukuroi, Shizuoka Prefecture, Japan. The organism has been passed by injection in eel to maintain virulence.

Preparation of Antigens

Lyophilized EF-1 was precultured in Brain Heart Infusion (BHI, Difco, Detroit, MI) at 30°C with shaking for 18 h, then inoculated in the same medium and incubated at 30°C for 48 h with shaking. The broth culture was centrifuged at 10,000 × g for 30 min. The pellet was washed by centrifugation with distilled water. The harvested cells were subjected to enzyme digestion.⁶⁾ In

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brief, the cells were placed in 50 mM sodium phosphate buffer, pH 7.0, containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 0.05% (w/v) sodium azide and stirred for 1 min. Egg white lysozyme (0.1 g; P-L Biochemicals, Inc., Milwaukee, Wis.) was added, and the suspension was stirred at 4°C for 16 h. The preparation was placed at 37°C for 20 min, and then stirred for 3 or 4 min. Four volumes of 20 mM MgCl₂ and bovine pancreas ribonuclease and deoxyribonuclease (P-L Biochemicals, Inc., Milwaukee, Wis.) at final concentration of 1 µg/ml each was added. The suspension was incubated 10 min at 37°C and then for 10 min at 60°C.

The LPS was extracted by Westphal's phenol-water method⁷⁾ from the washed enzyme treated cells of *E. tarda* EF-1. This preparation and further purification were made as previously described.⁵⁾ The purified LPS was then degraded by mild acid hydrolysis to obtain the polysaccharide fraction. The LPS in 1% acetic acid was heated at 100°C for 90 min. After lyophilization the reaction mixture was dissolved in water and centrifuged at 1,000 × g for 20 min to separate the lipid A as sediment. Polysaccharide obtained from the supernatant by lyophilization was purified by repeating precipitation with ethanol (six times) and the final purification was performed with alternate precipitations using 1 and 6 volumes of ethanol.⁸⁾ Finally, the solution was dialyzed against distilled water, lyophilized and stored at 5°C until used.

To determine the purity of the polysaccharide preparation, protein was analyzed by the Lowry method⁹⁾ and the nucleic acid were detected by measuring adsorption at 260 nm.¹⁰⁾

The vaccine preparation called "whole cell without lipid" (CWL) was obtained extracting the lipid by SOXHLET method,¹¹⁾ for 20 h using ethyl ether. The pellet obtained was suspended in sterile physiological saline at a concentration of 100 mg/ml and stored at 5°C until used.

Immunization of Eels

The eels were injected intramuscularly with 0.1 ml of 1 mg crude LPS, 0.04 mg purified PS and 10 mg of CWL. The fish were reimmunized with the same quantity of antigen seven days after the first immunization. Control subjects were injected with 0.1 ml sterile physiological saline.

Challenge

Eels were injected intramuscularly with 0.05 ml of a serial dilution of EF-1 broth culture twenty-

five days after the second immunization. The number of the injected live cells was determined by plate count method. Groups of 20 vaccinated and non-vaccinated eels were challenged with 1.1×10^7 cells/fish. Necropsy and isolation of *E. tarda* EF-1 on TSI (Eiken Chemical Co., Ltd., Tokyo) at 37°C was performed.

Titration of Serum Antibody

Five eels from each group were bled 21 days after the second immunization to determine agglutination antibody titer against formalin killed whole cells of *E. tarda* EF-1. Serum was collected also from surviving eels 21 days after challenge and titrated.

Immunodiffusion

For comparison of antigenicity among crude LPS, CWL and purified PS, Ouchterlony immunodiffusion with rabbit anti-EF-1 serum was performed.

Results

High antibody titers were shown in all immunized groups, particularly in the group where the CWL preparation was used as the immunogen (Table 1).

Table 1. Antibody titer in eel immunized with crude lipopolysaccharide (LPS), purified polysaccharide (PS) and whole cell without lipid (CWL) from *Edwardsiella tarda* EF-1

Immunization Group	Agglutination Titer (1:)	
Crude LPS	1,024	
	1,024	
	512	(676)*
	512	
	512	
Purified PS	512	
	512	
	512	(388)
	256	
	256	
CWL	8,192	
	4,096	
	4,096	(4,705)
	4,096	
	4,096	
Control	<4	
	<4	
	<4	(<4)
	<4	
	<4	

* Figures in parentheses indicate geometric mean of titer.

Table 2. Protective immunogenicity of crude lipopolysaccharide (LPS), purified polysaccharide (PS) and whole cell without lipid (CWL) preparations against challenge with 1.1×10^7 cells/eel of *Edwardsiella tarda* EF-1

Immunization Group	Number of Fish Immunized and Challenged* ¹	Number of Fish Survived	Survival (%)	RPS* ²	Probability Factor* ³
Crude LPS	16	14	87.5	82.1	$p=0.01$
Purified PS	19	9	47.4	24.8	n.s.* ⁴
CWL	19	13	68.4	54.8	$p=0.05$
Control	20	6	30.0		

*¹ Eel were injected intramuscularly with *E. tarda* EF-1 viable cells 25 days after the second immunization.

*² Relative Percent Survival = $\left(1 - \frac{\% \text{ mortality of vaccinated fish}}{\% \text{ mortality of controls}}\right) \times 100$

*³ $\chi^2 (n=1, p=0.05) = 3.841$, significantly different.

*⁴ n.s. = not significant.

Table 3. Agglutination titer in eels surviving challenge with *Edwardsiella tarda* EF-1

Immunization Group	Number of Fish	Agglutination Titer (1:)	Geometric Mean
Crude lipopolysaccharide	14	2,048–8,192	3,360
Purified polysaccharide	9	256–512	332
Whole cell without lipid	13	4,096–16,384	6,889
Control	6	32–64	57

Fig. 1. Ouchterlony immunodiffusion analysis of crude lipopolysaccharide (LPS), whole cell without lipid (CWL) and purified polysaccharide (PS) of *Edwardsiella tarda* EF-1.

Peripheral wells: 1 and 2, crude LPS; 3 and 4, purified PS; 5 and 6, CWL.

Central well: anti-EF-1 serum.

The results of the experimental challenge are shown in Table 2. Because some eels were lost by accident after immunization, the groups, each original 20 subjects, became from 16 to 19 eels. The survival in the control group was significantly lower than those in the groups immunized with crude LPS and CWL preparations. The best protection was obtained by immunization with

crude LPS preparation ($p \leq 0.01$). The CWL gave relatively high protection ($0.02 \leq p \leq 0.05$), showing a survival of 68.4% (Relative Survival of 54.8%). No significant protection with purified PS was shown.

The results of agglutination test using serum from the eels surviving challenge showed an increased titer following challenge except for the purified PS immunization group which showed almost the same titer as before challenge (Table 3).

The chemical analysis of the purified PS showed 2.7% protein and 1.1% nucleic acids still present in this preparation. Ouchterlony immunodiffusion showed 4 precipitin lines for the CWL and only one precipitin line for the crude LPS preparation which was common between crude LPS and CWL but was not identical because a spur was observed with CWL and not with LPS (Fig. 1). No precipitin line for the purified PS was shown.

Discussion

This experiment was performed to examine the protective properties of immunogenic substances of *E. tarda* cells, particularly the polysaccharide of the LPS and the effect of lipid A suppressing the immune response.

The results of agglutination test showed that the antibody titers do not always correlate to the protection obtained. Vaccination with the crude

LPS preparation increased agglutination antibody titer, but the highest titer was obtained by immunization with CWL and the lowest with the purified PS preparation. In contrast, the protection against experimental challenge differed among the preparations: the crude LPS was the most effective and the purified PS was less effective. The agglutination antibody titers increased in the immunized eels surviving challenge, and an increase in titer was also shown in the control subjects surviving challenge. It was shown in previous research that immunization with the crude PS preparation gave high resistance to experimental challenge,⁵⁾ but eels immunized with purified PS preparation did not show high resistance and nor increase of antibody titer after challenge. The difference between these two PS was the amount of protein and nucleic acid left in the preparations. In this study, the content of protein and nucleic acid were reduced from 5.0% to 2.7% and from 9.3% to 1.1% respectively. This fact suggests the protein or the nucleic acid may play an important role in protection against *E. tarda* infection. The PS alone may not induce immune response in eel for protection. The polysaccharides show differences in immune response: the PS of *Pneumococcus* produce antibody formation in man and mouse but not in rabbit.¹²⁾ There is no data about the antibody production against PS in the eels. The presumption of the low immunogenicity of polysaccharides in the eels may be confirmed from the results of low antibody titer after challenge and low antigenicity observed in the immunodiffusion where the PS failed to produce precipitin line.

Recently, TANAMOTO *et al.*¹³⁾ showed immune suppression using an anticomplement test by non-hydroxylated fatty acids. Chemical analysis of *E. tarda* EF-1 LPS showed the lipid A of this organism is composed mainly of non-hydroxylated fatty acids.¹⁴⁾ Therefore, the lipids which include the lipid A, were removed from the whole cells of *E. tarda* EF-1. High antibody titer and protection of the eels immunized with CWL supports the suppressive effect of the lipid A. Results of the challenge tests presume the main protective antigen would exist in the crude LPS or the crude PS fractions, but not in the purified preparations. This indicated the remaining protein or nucleic acid in the crude LPS and PS preparations had an immunogenic effect. The reason why the crude LPS resulted higher protection than the CWL may be due to the large amount of LPS in the

crude LPS preparation. Many of the antigens observed in the immunodiffusion of the CWL may not play an important role in the immunization of eel with *E. tarda* vaccine, but may increase the agglutination titer that does not correlate to the protection level of eels immunized using these vaccines.

The results of this study confirm that the crude LPS vaccine increased the survival rates, but also showed the elimination of the lipid from whole cell gave good protection in eel against experimental challenge with *E. tarda* EF-1. Protein and nucleic acid may play a very important role in the protective properties of these immunogens. But little attention has been paid to the immunogenicity of proteins and nucleic acids detected in various *Enterobacteriaceae*. More studies are required to clarify the immunogenicity of the proteins and the nucleic acids included in the LPS of *E. tarda*.

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