Immunogenicity Of Conjugate Based On Detoxified LPS Antigen Of *Vibrio Cholerae* O139

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INTRODUCTION

Cholera, an acute dehydrating diarrheal disease is caused by a Gram-negative bacterium *Vibrio cholerae*, belonging to O1 or O139 serogroups. Attack by a new *V. cholerae* O139 strain, was an unprecedented change in the history of cholera. Since its appearance in southern India and Bangladesh in late 1992, O139 strains have been continually isolated from environmental samples in Bangladesh, India and China[1,2,3,4] . Also for this reason, *V. cholerae* O139 still poses a serious threat to developing countries, therefore vaccine against this novel strain is required. LPS, the major constituent of the outer membrane of Gram-negative bacteria[5], is the dominant protective antigen of *V. cholerae* and represents the main area of interest in cholera subcellular vaccine design[6]. The aim of the study was to prepare a glycoconjugate vaccine against *V. cholerae* O139. The immunogenicities of conjugate, induced IgM and IgG antibodies as well as levels of selected cytokines (IFN-gamma, TNF-alpha, IL-4, IL-6, IL-10 and IL-12) were determined by enzyme-linked immunosorbent assay.

METHODS

1. Conjugate Preparation and Characterization

Bovine serum albumin (BSA) was treated with succinic acid anhydride in mild alkali solution to convert all reactive aminogroups of BSA to carboxyls. Carbohydrate-protein conjugate was prepared by reaction of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride activated CBSA (carboxylated BSA) with hydrazine treated LPS (dLPS). Prepared dLPS–CBSA conjugate was characterized by MALDI-TOF MS. Number of dLPS per molecule of protein (n) was calculated from $\Delta m/z$ of $[M + H]^+$ peaks of conjugate (dLPS–CBSA) and CBSA.

2. Experimental animals

Mice (BALB/c, female, 6 weeks old, Research Institute of Animal Production, Velaz, Prague, Czech Republic) were used as host animals in all immunization studies. Mice groups (each containing 10 mice) were injected subcutaneously, without any adjuvant, with 2.5 μg of dLPS alone or as conjugate per dose. Blood samples were collected on days 0, 14, 28, 42 and 77.

3. Limulus amebocyte lysate (LAL) test

Limulus amebocyte lysate test kit (E-TOXATE Kit, Sigma, USA) was used for the detection and semiquantitation of endotoxin (LPS) in the sample of dLPS used for preparation of conjugate vaccine. The result is judged as negative if the sample contains less than 0.015 EU/mL.

4. Anti-LPS Abs response of mice

The production of mice anti-*V. cholerae* O139 LPS IgG, IgM and IgA antibodies in mice sera were determinated by ELISA test. The amount of Igs was measured at 630 nm. For the exact expression of antibody levels, we used an appropriate calibration curve based on reference mouse serum (Mouse Reference Serum, Bethyl Laboratories, Inc.) for immunoglobulin quantification (in ng/mL). Antibody levels were quantified using optimal sera dilution (1:100 vol.) to fit into a linear range of dilution curve for all tested sera. All data were expressed as mean SD of 10 mice per group.

5. Quantitative detection of cytokines

Cytokines in sera were measured in duplicate using specific ELISA kits for IL-4, IL-6, IFNγ, TNFα, and IL-10 (BioLegend, Belgium). The conversion of measured absorbance values (MRX photometer for microtiter plates, 630 nm) to pg/mL was conducted on the calibration curve.

RESULTS

1. Characterization of dLPS-CBSA conjugate (Fig. 1)

- The number of conjugated dLPS per molecule of CBSA was 2–3 (Fig. 1).
- Potential endotoxic activity of dLPS and dLPS-CBSA conjugate were tested by LAL test. Our prepared antigens were not pyrogenic (<0.015 endotoxin unit per mL) and were suitable for a subcutaneous application.

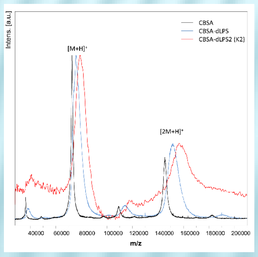

Fig. 1. Positive-ion MALDI-TOF mass spectra of the CBSA and conjugate dLPS–CBSA after 24 h at r.t. ($n \approx 1$) and extra 8h at 35 C recorded in linear mode with M(dLPS–CBSA) ≈79500 Da, which correspond to $n \approx 2–3$.

References

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RESULTS

2. Antibody response induced by dLPS-CBSA conjugate

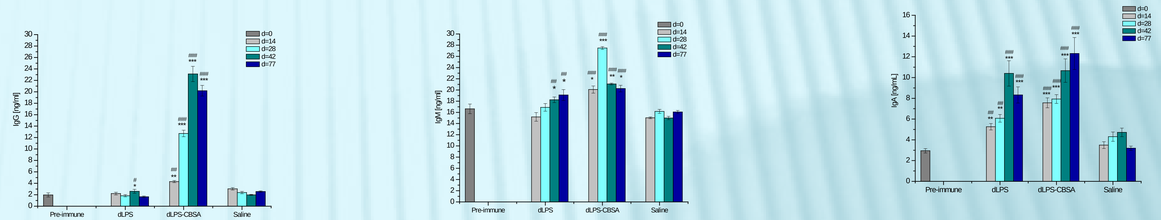

Fig. 2. Induction of specific anti-dLPS antibodies IgG, IgM and IgA following immunization with dLPS and dLPS–CBSA conjugate. Comparisons of all results from vaccinated groups with the pre-immune (*) and control (#) reference results were performed using ANOVA. The experimental data are expressed as geometric means SD. Levels of significance: ***/###, 0.000 < P < 0.001; **/##, 0.001 < P < 0.01, */#, 0.01 < P < 0.05. Differences were considered significant where P < 0.05.

3. Conjugate dLPS-CBSA stimulates production of T_H1 , T_H2 cytokines in serum

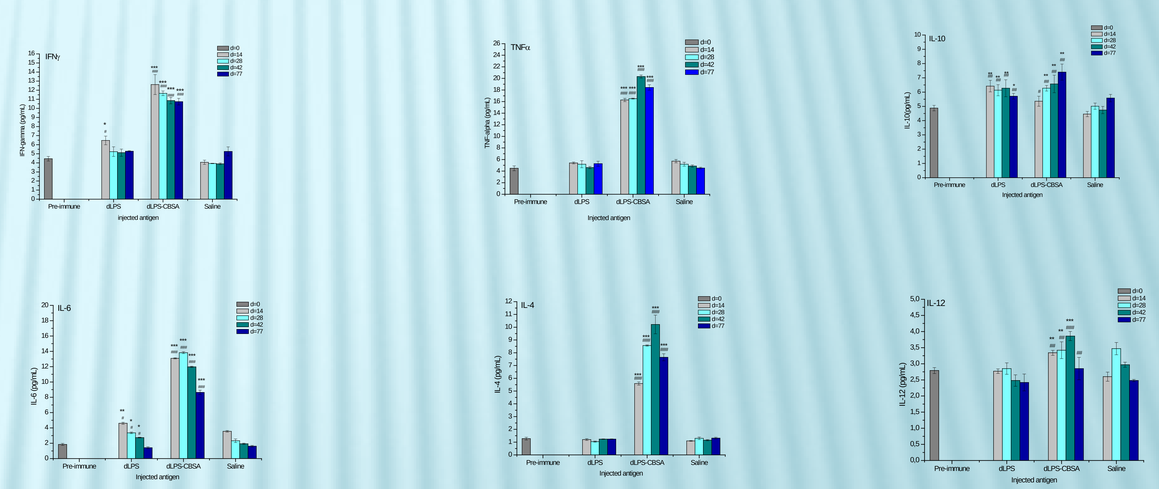

Fig. 3. Cytokine detection in serum from dLPS-CBSA-, dLPS- and saline-vaccinated mice. Each bar represents the mean SD. Comparisons of dLPS-CBSA- and dLPS-vaccinated groups were done with the saline control sera (#) and pre-immune sera (*). Levels of significance: ***/###, 0.000 < P < 0.001; **/##, 0.001 < P < 0.01, */#, 0.01 < P < 0.05. Differences were considered significant where 0.01 < P < 0.05.

Conclusions

According our previous experiments, the way of attachment and size of the epitope is a crucial for effective activation of the immune system, enhancing the immunogenicity of the conjugate. The amount of dLPSs coupled to CBSA in our coupling procedure, was lower than we expect. Nevertheless, the polysaccharide/protein ratio (2–3 mol/mol) obtained herein was sufficient for induction IgG, IgM and IgA response in immunized mice. The unconjugated dLPS elicited a moderate IgM response and very low IgG response comparable to pre-immune and saline control sera. Conjugate induced the significant increasing amounts (P < 0.001) of specific anti–LPS IgM antibodies only after 2nd dose (d=28). In contrast, the level of specific anti-LPS IgG and IgA antibodies, induced with conjugate progressively increased during the course of immunization. After the 2nd (d=28) and 3rd dose (d=42), conjugate elicited a significant level of specific IgG (P < 0.001) and IgA (P < 0.001) compared to pre-immune and saline control sera, lasting at least 35 days (P < 0.001). This could suggest that dLPS was functionally converted, due to the protein carrier effect, into a T-dependent antigen. Efficient T-cell dependent response is manifested by the effective switch of specific IgM isotype antibodies to IgG and IgA, and presumably long-term B-cell memory function. Conjugate elicited high levels of both, T_H1(IFN-gamma, TNF-alpha) and T_H2 (IL – 4 , IL – 6) cytokines.

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