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Vaccine 24S2 (2006) S2/74–S2/75
Vaccine
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Preparation and evaluation of *Vibrio cholerae* O1 EL Tor Ogawa lipopolysaccharide–tetanus toxoid conjugates

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Available online 16 February 2005

Abstract

The lipopolysaccharide (LPS) of *Vibrio cholerae* is considered one of the most important antigens from the point of view of immunogenicity in these bacteria. We have undertaken detoxification of this LPS by basic hydrolysis and the resultant amine groups were used for their conjugation to tetanus toxoid as carrier protein using carbodiimide-mediated coupling. The resulting conjugates were inoculated in Balb/c mice for immunogenicity studies. The anti-LPS IgG and vibriocidal antibodies were measured in serum. The antigenicity of this conjugated was evaluated by ELISA, with serums of humans vaccinated with a strain genetically modified. The conjugated elicited: high titers of IgG anti-LPS, high titers of vibriocidal antibodies and there was recognition of LPS by antibodies from cholerae immunised human serum. These results show that the conjugated LPS obtained by us, could be evaluated like a potential vaccine for human use.

Keywords: LPS; Detoxification; *Vibrio cholerae*; Conjugate vaccine

1. Introduction

The lipopolysaccharide (LPS) of *Vibrio cholerae* is considered the principal immunogenic surface antigen. In cholera, a direct relationship between levels of anti-LPS antibodies, vibriocidal titers and protection against disease has been demonstrated [1]. Three types of cholera vaccine have been developed: whole bacterial cells inactivated by heat and administered subcutaneously; killed bacterial cells of *V. cholerae* O1 combined with the purified recombinant B subunit of cholera toxin; and live attenuated vaccine containing the genetically manipulated *V. cholerae* O1 strain, CVD 103-HgR. These cholera vaccines have several drawbacks: the duration of the protection induced by inactivated oral vaccine is too short in infants; there are safety concerns associated with the live attenuated vaccine; they are less effective in malnourished individuals and in people with vibriocidal antibodies, and the reproducibility of batches of live vaccines is low [2]. Considering the potential advantages of a conjugate vaccine including increase of immunogenicity in infant and young children and lower reactogenicity, we proposed to obtain immunogenic conjugates from LPS of *V. cholerae* O1 EL Tor Ogawa and tetanus toxoid as a carrier protein.

2. Materials and methods

2.1. LPS conjugates to TT

LPS (5 mg mL^{-1}) in 0.5 M NaOH was put in an oven at 100 °C for 3 h, was cooled to room temperature and brought to pH 7.0 by the addition of 0.1 M HCl. It was passed through a Superose 6 column in 0.15 M NaCl. The fraction corresponding to 0.66 Kav was sterile filtered and assayed for LPS and amine group contents. After that, TT (25 mg mL^{-1}) was treated with 0.1 M of EDAC by 30 min at 4 °C and activated LPS was added (1 mg mL^{-1}) and maintained by 4 h to 4 °C

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doi:10.1016/j.vaccine.2005.01.130

with the pH between 5 and 5.6, after that, it was ultrafiltered 0.15 M NaCl, was sterile filtered, and assayed for LPS and protein contents, and antigenicity.

2.2. Immunization

Groups of eight Balb/c mice (CENPALAB, Cuba), 18–20 g each at the beginning of the experiments were injected intramuscularly with 2 μg of LPS alone or conjugated on 0 and 28 days. Sera were taken before each injection and 7 and 14 days after the second injection [3].

2.3. Biological assay

Complement mediated vibriocidal antibodies were measured against *V. cholerae* VC 12 (biotype classical serotype Ogawa) strain using human serum as a source of complement [4].

2.4. Serological assays

Anti-LPS IgG were determined by ELISA, using LPS (25 μg/mL) as coated antigen and hyperimmune sera as a reference [5]. Identity of LPS after conjugated was verified through indirect ELISA using unconjugated or conjugated LPS (25 μg/mL) as coated antigen and positive and negative sera from human volunteers inoculated with the strain 638 *V. cholerae* O1 El Tor Ogawa.

2.5. Statistical analysis

Antibody levels are expressed as the geometric mean and were defined as the reciprocal of the higher dilution of serum that were positive and have an OD over 0.4. For processing the data of inverse titer with logarithmic transformation varian analysis and *t*-test ($p < 0.05$) were used.

3. Results and discussion

3.1. Composition of the conjugates

The activated LPS had an average content of 2.24 μmol of amine groups per milligram of LPS. The ratio of those conjugates obtained in all experiments ($n = 5$) were similar, obtaining them with an average LPS/TT ratio of 6.07. The yields for all the conjugates were between 60 and 70%. The identity of LPS by an indirect ELISA showed that the conjugated and unconjugated LPS were recognized for the anti-LPS antibodies present in serum from human volunteers inoculated with the strain 638 *V. cholerae* O1 El Tor Ogawa.

3.2. LPS-specific IgG antibodies

The kinetic production of IgG antibodies was measured. It showed that the conjugates prepared elicited statistically significant rises ($p < 0.05$) in the levels of anti-LPS IgG antibodies after the second and third doses compared with unconjugated LPS (OD = 1.072 for LPS-TT versus 0.332 for LPS).

Gupta et al. [6] showed that detoxified *V. cholerae* LPS bound to cholera toxin elicited LPS antibodies with vibriocidal activity in mice. Gu et al. [7] reported that LOS conjugated to TT were immunogenic in both mice and rabbits, and the sera showed bactericidal activity against both homologous and heterologous strains.

3.3. Vibriocidal antibodies

Vibriocidal activity in sera of Balb/c immunized with LPS–TT conjugates or native LPS was not observed after first injection. However, after the second dose there was an increase in the vibriocidal activity in animals immunized with the conjugate while this was not observed with unconjugated LPS (LPS–TT = 4063.75 versus LPS = 485.03).

The complement mediated bactericidal activities of the LPS-induced antisera were examined with homologous Ogawa strain. Most mouse antisera showed vibriocidal activities against the homologous strain, but all derivatives elicited higher vibriocidal antibody levels after 28 days. Vibriocidal activities of sera from Balb/c mice immunized with LPS conjugates were similar to their LPS pattern and also the higher levels were obtained after 28 days of first injection. These results correspond with others reported by some authors for immunization schemes with LPSm and cellular vaccines. The whole cell vaccine elicited earlier and a higher titer the vibriocidal activity, but after 28 days, the vibriocidal levels elicited by both immunogens were similar [6]. Lastly, these results show that the conjugated LPS obtained by us could be evaluated as a potential vaccine for the human use.

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