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Retinoic acid pre-treatment down regulates *V. cholerae* outer membrane vesicles induced acute inflammation and enhances mucosal immunity

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ABSTRACT

Bacterial outer membrane vesicles have been extensively investigated and considered as a next generation vaccine. Recently, we have demonstrated that the cholera pentavalent outer membrane vesicles (CPMVs) immunogen induced adaptive immunity and had a strong protective efficacy against the circulating *V. cholerae* strains in a mouse model. In this present study, we are mainly focusing on reducing outer membrane vesicle (OMV) -mediated toxicity without altering its antigenic property. Therefore, we have selected All-trans Retinoic Acid (ATRA), active metabolites of vitamin A, which have both anti-inflammatory and mucosal adjuvant properties. Pre-treatment of ATRA significantly reduced CPMVs induced TLR2 mediated pro-inflammatory responses *in vitro* and *in vivo*. Furthermore, we also found ATRA pre-treatment significantly induced mucosal immune response and protective efficacy after two doses of oral immunization with CPMVs (75 μg). This study can help to reduce OMV based vaccine toxicity and induce better protective immunity where children and men suffered from malnutrition mainly in developing countries.

1. Introduction

Vibrio cholerae causes acute watery diarrhoea in many developing countries where outbreaks occur perennially due to poor drinking water quality and lack of sanitation [1]. Globally, the cholera burden is estimated to affect 3–5 million people and kill 120,000 people annually [2]. Due to the emergence of more virulent and multidrug resistant *V. cholerae*, the management of cholera is getting complicated [3–5]. Dukoral and Shancol are the two licensed oral cholera vaccines (OCV), which contain heat-killed *V. cholerae* O1/O139 serogroups. Due to the emergence of several variants of *V. cholerae* O1 and O139 strains in the cholera endemic regions, the formulations of these oral vaccines are debateable and it was shown that two doses of OCV vaccine are adequate to induce the protective immune responses [6]. Considering their short protective efficacy and need of a cold chain during delivery, scientists and academician are exploring for a suitable cholera vaccine.

Several studies showed that the outer membrane vesicles (OMVs) of Gram-negative bacteria, including *V. cholerae* are now considered as a novel next generation vaccine candidate [7]. OMVs of *V. cholerae* are effective thermo-stable immunogens, can induce a significant protective immune response and block transmission by inhibiting the motility of *V. cholerae* [8–11]. OMVs of *V. cholerae* are formed spontaneously mainly with lipopolysaccharides, outer membrane proteins and active secretive proteases [12]. These OMVs can activate different pattern recognition receptors (PRRs) such as TLR4, TLR2, which causes the generation of acute inflammation [13–15]. Several studies demonstrated that OMVs of *V. cholerae* induce pro-inflammatory responses and cytotoxic effects in intestinal epithelial cells [16]. Hence, the toxicity is considered as a major concern in OMV based vaccines. For safety reasons, the licensed OMV based meningococcal vaccine has been detergent treated to reduce LPS-mediated endotoxicity [7]. LPS derived "O" antigen is a major protective antigen of *V. cholerae*. However, detergent treatment or genetic modification of LPS is not appropriate for *V. cholerae* OMV based vaccine. For this reason, we have made an attempt to develop an alternative strategy to reduce *V. cholerae* OMVs toxicity without altering its antigenic property. We have selected ATRA, an active metabolite of Vitamin A having immuno-modulatory properties to suppress OMVs mediated toxicity. Recent studies showed that ATRA treatment reduces

Abbreviations: ATRA, All-Trans Retinoic Acid; OMVs, outer membrane vesicles; CPMVs, cholera pentavalent outer membrane vesicles; DMSO, dimethyl Sulfoxide; sIgA, secretory IgA; *V.cholerae*, Vibrio cholerae.

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LPS-specific pro-inflammatory cytokine production *in vitro* and *in vivo* [17–20]. Previously, we also found that the ATRA treatment down regulate *Salmonella enterica serovar* Typhimurium mediated colitis, intestinal pro-inflammatory cytokine production and tissue damage [21]. ATRA is now considered as a potent mucosal adjuvant as it induces gut homing behaviour of the lymphocytes [17].

Recently, we have formulated a new pentavalent antigen, which was derived from five different serogroups and serotypes of *V. cholerae* OMVs (CPMVs). After four doses of oral immunization, the CPMVs induce a long term adaptive and protective immunity against the currently predominant *V. cholerae* strains in both adult and suckling mouse model [22].

In this present study, we observed that two doses of oral immunization with CPMVs induce protective immune responses in ketamine treated adult mouse model. We also found that CPMVs induce TLR2 mediated pro-inflammatory cytokine secretion *in vitro* and acute inflammation *in vivo*. Furthermore, pretreatment with ATRA suppressed *V. cholerae* OMVs mediated acute inflammation without inhibiting the protective immune response by OMVs in the mouse model.

2. Materials and methods

2.1. Bacterial strains

OMV antigens were prepared from *V. cholerae* strains O395 (O1, classical, Ogawa), N16961 (O1, El Tor, Inaba), NLC8 (O1, El Tor, Ogawa, variant), KII575 (O139) and SRO6 (O6). MAK757 (O1, El Tor, Ogawa), AM157 (O1, El Tor, Ogawa, variant), SG24 (O139) and SR O6 (O6) were used for the challenge study [22]. All strains were stored in 15% glycerol with brain heart infusion broth (BHIB, Difco, USA) at −80 °C. Prior to experimentation, each strain was grown in tryptic soy agar (TSA; Difco) and tryptic soy broth (TSB; Difco).

2.2. Preparation of CPMVs

Outer membrane vesicles (OMVs) were isolated from five *V. cholerae* strains as previously described [22]. Protein and LPS were determined by the modified Lowry protein assay kit (Pierce, USA) and Kdo method [11]. Isolated OMVs from each strain were mixed in an equal proportion to make the different concentration of 25, 50, 75 μg/200 μl of PBS. This cocktail of OMVs was considered as CPMVs, which were stored at −80 °C.

2.3. Oral immunization

Three groups of female BALB/c mice (n = 5) were immunized orally on day 0th and 14th with different concentrations (25, 50 and 75 μg) of purified CPMVs in 200 μL of PBS as previously described (Fig. 1a) and similarly another set of immunized mice were left for measuring long term immune response up to 56 days. In another experiment, three groups of adult mice (n = 10) and neonatal mice (age 5 days, n = 10) were taken. One group of adult mice was orally administered with ATRA (37.5 μg/100 μl in corn oil) 48 h prior to CPMVs immunization [23,24]. The second group received only corn oil as a medium control (Fig. 5a). On day 0, first and second groups were immunized with CPMVs as previously described and ATRA treatment was continued for three days. A third group of mice were immunized with PBS as non-immunized control. All the animal experiments were conducted following the standard operating procedure as defined by Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), Ministry of environment and forest, Government of India and the Institutional Animal Ethical Committee of NICED (Registration No. 68/1999/CPCSEA dated 11-03-1999; Approval No.: Apro/77/24/11/2010, Ref. No. NICED/CPCSEA (AW) 215/2009-2015).

2.4. ELISA

Blood and intestinal lavage were collected from different time intervals on 0, 14th, 28th, 42nd and 56th day after first oral immunization. *V. cholerae* LPS-specific immunoglobulins, IgG1, IgG2a, IgA, and IgM in serum and secretory IgA in intestinal lavages, of immunized and non–immunized mice were measured by ELISA as previously described [22]. LPS of different serogroups and serotype of *V. cholerae* were isolated by phenol chloroform method [8].

2.5. Adult mice model for protection study

Immunized and non-immunized mice were challenged orally with 200 μl of bacterial suspension containing 10^9 cells of wild type *V. cholerae* as previously described [25] and after 24 h of infection, CFU/gm of intestine of each mouse were measured following the standard protocol [25].

2.6. Cell culture

RAW 264.7 mouse macrophage cells were grown in RPMI 1645 medium with 10% fetal bovine serum and penicillin–streptomycin-amphotericin B (Fungizone). Confluent cells were harvested and seeded into 12-well plates at a concentration of 5×10^6 cells/well. CPMVs (10 μg/ml), proteinase-K treated CPMVs (10 μg/ml) and polymixin-B (50 μg/ml) pre-conditioned CPMVs was added to each well. After 6 h, different pro-inflammatory cytokine responses were measured. siRNA Knockdown—the expression of TLR2 and TLR4 were blocked by transfection with siRNA (Santa Cruz Biotechnology) using the manufacturer's protocol. The RAW264.7 macrophages were transiently transfected with TLR2 and TLR4 siRNA for 48 h. The transfected cells were then used in the subsequent assays.

In another experiment, ATRA (Sigma Aldrich; 1 μM dissolved in DMSO) and DMSO only treatment as a vehicle control were treated for 16 h before CPMVs (10 μg/ml) stimulation in macrophage cells.

2.7. TLR2 and TLR4 expression by RT-PCR and Flow cytrometry

Total RNA was extracted from CPMVs treated and untreated macrophage by RNA Aqueous-4 PCR kit (Ambion Inc.) at different time incubation times (2 h and 6 h). The RNA was reverse transcribed using RETRO script kit (Ambion Inc.) and amplified by PCR with gene-specific primers for GAPDH, TLR2 and TLR4 using the mouse specific primers as previously described [26].

For the TLR2 and TLR4 surface expression of macrophages, CPMVs treated and non-treated cells were stained with FITC-conjugated anti-mouse TLR2 and TLR4 (BD Bioscience) (at 1:200 dilution) and isotype control were stained with FITC-conjugate anti-rat IgG (at 1:1000 dilution, BD Bioscience) as previously described [26]. The stained cells were examined on a FACS Calibur and results were analyzed using the Cell Quest software (Becton Dickinson).

2.8. Preparation of whole-cell, nuclear and cytoplasmic extracts and immunoblotting

Macrophage cells were incubated with CPMVs (10 μg/ml) for 60 min. Whole-cell lysates were prepared by lysing the cells in ice cold lysis buffer containing 20 mM Tris-HCl (pH-8.3), 150 mMNaCl, 1 mM EDTA, 1% Nonidet P-40, and protease and phosphatase inhibitors (5 mg/ml pepstatin A, 1 mM PMSF, 0.5 mMiodoacetamide, 1 mM sodium metavanadate, 10 mM sodium