

## Preliminary Studies on a Derivative Verotoxin as Oral Adjuvant

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### Abstract

Most vaccines are still delivered by injection. Mucosal vaccination would increase compliance and decrease the risk of spread of infectious diseases due to contaminated syringes. However, most antigens are unable to induce immune responses when administered mucosally and require the use of strong adjuvant or effective delivery systems. *Vibrio cholerae* toxin (CT) is a powerful mucosal adjuvants when co-administered with soluble antigens, but present important drawbacks such as residual toxicity. In the current report, a recombinant verotoxin, rVTX1 from *Escherichia coli* O157 has been tested to be used as oral adjuvant. A common antigen, BSA (bovine serum albumin), was orally co-administered with the toxoid rVTX1 in BALB/c mice. Commercial CT was used as a reference adjuvant. In this study, the specific antibody response was determined in sera (IgG1, IgG2a, IgA and IgE) and in fecal samples (IgA). In addition, the oral toxicity of the new adjuvant candidate was studied in mice. Results indicated that rVTX1 possesses a higher mucosal adjuvant activity than CT when administered orally without inducing any toxic symptoms. These preliminary results support further experiments to demonstrate the potential applications of this protein in oral vaccine development.

**Keywords:** Oral adjuvante; Cholera toxin; Verotoxin; Immunogenicity

### Introduction

Nowadays, the vast majority of vaccines in use are administered by injection, but protective mucosal immune responses are more effectively induced by mucosal immunization such as the oral one [1]. Thus, in contrast to parenteral vaccines, mucosal vaccines induce immunity in both, systemically as well as in mucosal tissues. In addition, mucosal vaccines offer the easiness for administration with the compliance of patients and avoid the use of needles and its subsequent inconveniences. For instance, according to OMS, each year healthcare staff suffers 2 million accidental needle sticks, which 2.5% results in infection [2]. Despite the many attractive features of oral vaccination, there are some difficulties to be considered: i) chemical barriers such as low pH, digestive enzymes, and surfactant activity by bile salts; ii) physical barriers, such as mucus and epithelial tight junctions [3] and (iii) immune barriers. Certainly, the most difficult problem to solve is the immune tolerance. The mucosal immune system is naturally predisposed to exhibit immune tolerance to the encountered antigens or strange substances in order to prevent unwanted and excessive inflammatory responses [4]. Consequently, potent mucosal adjuvants or antigen delivery systems are needed to overcome natural mucosal tolerance and, then, to promote the induction of mucosal immunity.

Many diverse classes of compounds have been tested as mucosal adjuvants including emulsions, cytokines, polymers, particulate delivery systems and microbial products [5-9]. Nowadays, only six of the vaccines that are currently approved for human use are administered mucosally. These include the live oral vaccines against poliovirus (live attenuated poliovirus strains), *Salmonella enterica* var.

Typhi (live attenuated *S. Typhi*), or rotavirus (pentavalent attenuated reassortant rotavirus) [10], and the non-living bacteria of *Vibrio cholerae* adjuvanted with CT (cholera toxin subunit B), Dukoral® [11]. CT has demonstrated adjuvant properties in mucosal vaccines against a variety of diseases [12-17]. However, the clinical use of CT reports several adverse effects such as severe diarrhea after oral administration and the undesirable side effects involving their entry into the central nervous system when given by the nasal route [18]. The CTB subunit lacks the toxicity associated with the CTA subunit, and present strong immunoadjuvant properties. However, this property is reduced when administered orally [19-21].

The benefits derive from adjuvant incorporation into any vaccine formulation have to be balanced with the risk of adverse reactions and must be justified if its inclusion in the vaccines or therapeutic product [22,23]. Thus, efforts are now focused on the development of new mucosal adjuvants that can offer high adjuvant activity with null toxicity. *Escherichia coli* Shiga toxin strains produce two forms of verotoxin, VTX1 and VTX2. The structure of VTX is described as AB<sub>5</sub> (similar to CT), containing one A subunit (the toxic domain) which possess an enzymatic activity, and five B subunits (receptor-binding domain) [24]. The B moiety play a role in the translocation of the A moiety into the cytosol [4].

The aim of this study was to determine if a recombinant form of VTX1 may be used as oral adjuvant. The performed toxicity and immunogenicity studies in BALB/c mice indicated that rVTX1 possesses a higher mucosal adjuvant activity than CT when administered orally without inducing any toxic symptoms.

## Material and Methods

### Production of toxins and recombinant toxoid

**The cholera toxin was obtained from Sigma-Aldrich (Barcelona, Spain):** Native VTX was obtained from a clinical isolated of *Escherichia coli* O157 after induction of prophages encoding toxin with a quinolone [25,26]. Briefly, *E. coli* O157 was subcultured in TSB for 4 h and then treated with 10 µg/ml norfloxacin for prophage induction for 4 h. After inactivation with BEI-FA [27] cells were harvested (6,000 g, 30 min) and the packed cells resuspended in polymyxin B sulfate solution (0.1 mg/ml, 37°C, 30 min) to release cell bound toxins, and then filtered using 0.2 µm pore size filter. The culture supernatant was precipitated with 70% ammonium sulfate (12 h, 4°C, under stirring), centrifuged (16,000 g, 10 min) and the pellet was resuspended in PBS and dialyzed against 10 mM Tris (pH 7.4) for 48 h.

The recombinant VTX1 (rVTX) was cloned and expressed in a pET-30a vector. Briefly, the VTX1 (type strain C600 [H19]) gene was ligated into the pET-30a expression vector and transformed in BL21 (DE3) strain of *E. coli* as per standard protocol. Positive clones were selected on LB agar plates containing kanamycin (50 µg/ml). PCR using forward and reverse primers to VTX-1 was used to detect the presence of recombinant plasmid. Positive clones were further confirmed by sequencing analysis before expression of recombinant protein. In order to express the VTX-1, the selected clone was inoculated in 500 ml of LB broth containing kanamycin (50 µg/ml) and incubated at 37°C, 210 rpm. The expression of the recombinant protein was induced by addition of Isopropylβ-D-1-Thiogalactopyranoside (IPTG) to a final concentration of 1 mM, during 4 h. The recombinant protein was purified from the supernatant by affinity chromatography (HisTrap™ HP columns prepacked with precharged Ni Sepharose™ using the ÄKTAprime™ automated liquid chromatography system, GE Healthcare).

**SDS-PAGE and Immunoblotting analysis:** SDS-PAGE was performed using gels of 12% acrylamide (Criterion™ XT precast gel BIO-RAD). Native and recombinant VTX (2 µg/mL) were treated in sample buffer (10% SDS, β-mercaptoethanol) and boiled 10 min at 100°C. The gels were stained with Coomassie brilliant blue (BIO-RAD). The apparent molecular weight of the proteins was determined by comparison with standard molecular weight markers (Rainbow, RPN756, Amersham Pharmacia Biotech).

Unstained proteins separated electrophoretically were transferred from the gel to a nitrocellulose membrane (Whatman® Protran®; pore size 0.45 µm) using a semidry blotting system at 0.8 mA/cm<sup>2</sup> for 30 min (Trans-Blot® SD Transfer Cell, BIO-RAD). After blocking of protein-binding sites (PBS with 5% skimmed milk, overnight, RT), the membranes were washed 3 times with PBS-T and then incubated with sera from rabbits immunized with VTX1+VTX2 (Certest Biotech, Zaragoza, Spain) and diluted 1:1,000, 3 h at 4°C. The membranes were washed again 3 times with PBS-T and then peroxidase (PO)-conjugated secondary antibody (GAR/IgG Fc-PO, 1:1,000) was added for 60 min at RT. Finally, membranes were extensively washed with PBS-T and the antibody-antigen complexes were visualized after addition of a substrate/chromogen solution (H<sub>2</sub>O<sub>2</sub>/cloro α-naftol).

**Proteomic analysis:** Samples were treated with 1.6 µg/mL of trypsin in 50 mM ammonium bicarbonate at 37°C overnight. Tryptic peptides were applied to a MALDI-TOF MS plate in a solution of 10 mg/mL

alpha-cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid and 50% acetonitrile. Each digested and desalted sample was resuspended in 10 µL of Buffer A (95% water, 5% acetonitrile, 0.1% formic acid) and spotted into a LC-MS/MS system. Peptide mass fingerprints were analysed and searched against theoretical VTX spectra using the Mascot Daemon software package (Matrix Science, London, United Kingdom). The following search parameters were applied: mass accuracy, ± 0.25 Da; fixed modification of methionine oxidation; and a maximum of one tryptic missed cleavage. All MALDI-TOF MS identifications were performed at least in duplicate. A protein was considered to be identified if the scores from the database searches clearly exceeded the algorithm's significance threshold ( $P < 0.05$ ).

**Toxicity assays:** Experiments were performed in compliance with the regulations of the Ethics Committee of the University of Navarra in line with the European legislation on animal experiments. All experiments were carried out in BALB/c female mice, 8-12 weeks old at the onset of the corresponding study.

**In vivo mice oral inoculation:** Female BALB/c mice (6-8 week old; Harlan, Spain) were fasted overnight before inoculation but allowed access to water up to 1 h before the start of the experiments. Six mice were orally challenged with 40 µg of rVTX1 and monitored for 5 days.

**Ligated ileal loop assay:** BALB/c mice (6-8 week old; Harlan, Spain) were fasted overnight before inoculation but allowed access to water up to 1 h before the start of the experiments. The animals were anesthetized by the intraperitoneal administration of 0.2 ml solution of 150 mg/Kg of Ketamine (Imalgene 1000, ©Merial, Barcelona), and 10 mg/Kg (Rompum®, Bayer Hispania, Barcelona). The abdomen of each mouse was disinfected an antiseptic solution (iodine, Betadine) immediately before surgery. Then, the intestine was exteriorized through a midline incision maintaining a strict aseptic condition. Three intestinal loops (1 cm long) per mouse were prepared by a double ligation of the intestine. Care was taken to avoid interfering with the blood supply. rVTX1 (1 µg, 3 µg, or 5 µg, in 50 µl PBS) was injected into the loop by using a 27-gauge needle oblique to the intestinal lumen. As a reference, 1 µg of CT (in 50 µl PBS) was inoculated in different loops and animals. Control untreated group was inoculated with 50 µl PBS. After inoculation, the incision in the peritoneum, abdominal muscles, and skin was closed in one plane by using super glue. The surgical procedure lasted approximately 10 min per animal. The animals recovered 30 min later and they were euthanized 2 h after surgery. The ligated ileal loops were collected and weighed. Then, the samples were fixed by immersion in formalin for 24 h before being processed to obtain thin sections (8 µm) which were stained with Hematoxylin and eosin for histological analysis.

### Oral immunization

Experiments were performed in compliance with the regulations of the Ethics Committee of the University of Navarra in line with the European legislation on animal experiments. All experiments were carried out in BALB/c female mice, 8-12 weeks old at the onset of the priming immunization.

Female BALB/c mice were randomly divided ( $n=6$ ). Animals were orally immunized with either free BSA (Sigma-Aldrich, ref. A2153) (80 µg or 20 µg) or coadministered with 10 µg of either CT or rVTX. The oral immunization was carried out in a total volume of 0.2 ml of PBS by cannula. All animals were sacrificed five weeks after initial immunization. Blood and fecal samples were obtained at weekly intervals after initial immunization until the end of the experiment.

Serum samples were stored at -80°C until analyzed. Fecal pellets were processed for IgA determination. Briefly, 100 mg of pellets were mixed with PBS containing 0.05% Tween 20 (PBS-T) plus 3% non-fatty milk (Sigma-Aldrich). After centrifugation (10,000 x g, 10 min), supernatants were mixed with protease inhibitor cocktail (Sigma-Aldrich) and stored at -80°C until analyzed.

Specific antibodies against BSA or the corresponding toxoid in serum and fecal extracts were determined by indirect ELISA. Briefly, microplate wells (Immuno-Maxisorp, Nunc®, Roskilde, Denmark) were coated with 10 µg/ml of either BSA or toxoid diluted in sodium carbonate-bicarbonate buffer (0.05M, pH 9.6), and incubated overnight at 4°C. In case of determination of antibodies against the toxoid, the plates were blocked with PBS containing 3% BSA for 1 h, RT. Serum or fecal samples (see above) were diluted in PBS (1:80) in triplicates (1 h, RT). After intense washing with PBS Tween 20 (PBS-T) buffer, the PO-goat anti mouse (classes IgG1, IgG2a, IgA or IgE) (Sigma) was added for 1 h at 37°C. The detection reaction was performed by incubating the sample with ABTS substrate for 20 min at room temperature. Absorbance was measured with an ELISA reader at 405 nm. (Sunrise remote; Tecan-Austria).

### Statistical analysis

All analyses were performed using SPSS® statistical software for windows. Analysis of variance (ANOVA) was employed to analyze data and, when it indicates a significant difference, the Tukey post hoc test was used to assess the difference between groups. P-values < 0.05 were considered statistically significant.

## RESULTS

### Structural studies of rVTX

The protein profile of rVTX1 was compared with native VTX (nVTX) by SDS-PAGE followed by immunoblotting. The protein staining revealed the presence of a band of 72 kDa which correspond to the holotoxin, and also the subunit A (31-32 kDa), subunit A2 (5 kDa) and the subunit B (7.7 kDa). Immunoblotting analysis performed with specific sera corroborated the identity of A subunits. The calculated AMW for subunit A in the nVTX1 was 31 kDa, whereas in rVTX1 was 30 kDa. The proteomic analysis revealed a deletion in the subunit A1 of rVTX1 of 6 aminoacids (MHHHHH) equivalent to 820 Da.

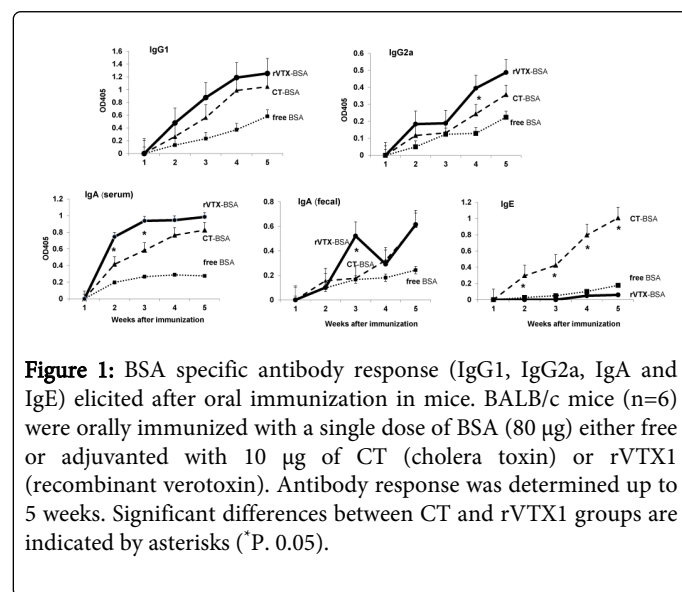
### Toxicity of VTX1-R and CT toxoids after oral administration

The mice orally inoculated with 40 µg the VTX1-R did not present any symptoms during the follow-up. One of the biological activities of VTX1 and CT is the induction of fluid accumulation in the intestine. Thus a ligated ileal loop assay was carried out in order to measure the toxicity of rVTX1 and CT by measuring the volume and weight of toxin-induced fluid accumulation in the ligated ileal loops at 3 h after inoculation. There was a marked fluid accumulation and distention in CT (1 µg) treated loops, with an increase of 65 µl with respect the PBS control group. In contrast, rVTX1 treated loops showed no fluid accumulation with any concentration (Table 1). In order to confirm the results observe from macroscopically analysis, histological studies and subsequent staining was performed. Thin sections of ileal loops 3 h-post inoculation were stained and analyzed under the microscope. Negative control loops were inoculated with PBS, showing normal full-length intestinal villi with a well-preserved epithelium and lamina

propria, and no cellular infiltration. Results revealed that 5 µg of the rVTX1 did not show any cellular infiltration in villi. However, loops inoculated with 1 µg of CT exhibited substantial diffuse cellular infiltration of the mucosa and villi (results not shown).

	PBS	CT (1 µg)	rVTX1		
			(1 µg)	(3 µg)	(5 µg)
Weight increase (mg)	2.7 ± 7.4	55.0 ± 10.5*	-2.7 ± 1.1	-2.5 ± 4.9	6.0 ± 4.2
Volume increase (µL)	-12.5 ± 5.0	53.3 ± 23.1*	-15.0 ± 5.0	-12.5 ± 3.5	-7.5 ± 3.5

**Table 1:** Toxicity of rVTX1 in ligated ileal loop in mice. Intestinal ligated ileal loop were practiced in BALB/c mice (n=3) which were inoculated with 50 µl of three different concentrations (1 µg, 3 µg or 5 µg) of rVTX1. The data correspond with the increase values compared with untreated loops (ileal loop's weight and volume, 3 h after the inoculation). CT (1 µg) was used as positive control, and PBS as negative control. Significant differences between treatments are indicated by asterisks (\*p. 0.05).



**Figure 1:** BSA specific antibody response (IgG1, IgG2a, IgA and IgE) elicited after oral immunization in mice. BALB/c mice (n=6) were orally immunized with a single dose of BSA (80 µg) either free or adjuvanted with 10 µg of CT (cholera toxin) or rVTX1 (recombinant verotoxin). Antibody response was determined up to 5 weeks. Significant differences between CT and rVTX1 groups are indicated by asterisks (\*P. 0.05).

### Evaluation of adjuvant activity after oral administration

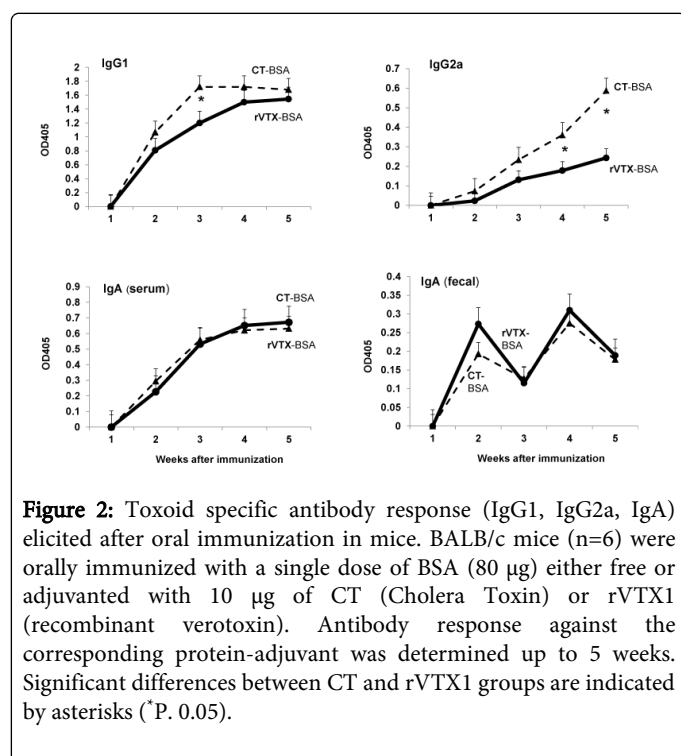
In order to study the adjuvant activity of rVTX1, the protein BSA was used as a model antigen. BALB/c mice were orally immunized with a single dose of BSA (80 µg or 20 µg) in the presence or absence of the adjuvant CT or rVTX1, and the specific antibody responses evaluated by ELISA. First, we will describe the BSA-specific antibody response, and, later, against the toxoid used as adjuvant.

**BSA specific response:** The evolution of the serum (IgG1, IgG2a, IgA, IgE) specific antibodies and mucosal (IgA) responses was determined by indirect ELISA. High levels of IgG2a and IgG1 isotypes, were induced after oral immunization with any of the three toxoids. rVTX1 elicited the highest levels and was also the most effective in inducing specific IgA in serum, as well as in mucosal surfaces (Figure 1). There was observed a dose-response effect (80 µg vs. 20 µg) but with a similar kinetic pattern (not shown). Concerning a potential allergenic effect, IgE levels were also measured in serum (Figure 1).



Free BSA or co-administered with rVTX1 did not induce a BSA-specific IgE response. In contrast, CT induced the highest levels of IgE in serum, being statistically significant comparing with the rest of the groups.

**Toxoid specific response:** The neutralization of the adjuvanticity of the toxoids by pre-formed antibodies is a problem that could abrogate the adjuvant effect. Thus, the antibody response elicited against the own toxoid was also measured. The results showed that the toxoid antibody response in sera (IgG1, IgG2a, IgA) or feces (IgA) were low (Figure 2). The highest levels of antibodies IgG1 and IgG2A isotypes (2.A) correspond to the commercial adjuvant CT. There were significant differences in the IgG2a levels between CT and rVTX1.



**Figure 2:** Toxoid specific antibody response (IgG1, IgG2a, IgA) elicited after oral immunization in mice. BALB/c mice (n=6) were orally immunized with a single dose of BSA (80 µg) either free or adjuvanted with 10 µg of CT (Cholera Toxin) or rVTX1 (recombinant verotoxin). Antibody response against the corresponding protein-adjuvant was determined up to 5 weeks. Significant differences between CT and rVTX1 groups are indicated by asterisks (\*P. 0.05).

## Discussion

Vaccines delivered through mucosal surfaces are increasingly studied because of their properties to effectively induce both systemic and mucosal immune responses, as well. In addition, they are cost-less, safer, easily administrable and suitable for mass vaccinations [28]. The main obstacle is to reach the inductive sites of the gut and to break the natural immune tolerance of those mucosal sites. This challenge may be overcome by using adjuvants [29]. Bacterial toxoids, such as CT and LT have been used as oral adjuvants for 20 years. Even though its adjuvanticity, these toxoids present some problems related to toxicity [18] and allergenicity to bystander antigens [2]. Many diverse compounds have been tested as alternative mucosal adjuvants, such as microbial products, cytokines and particulate delivery systems, among others [30]. Bacterial flagellin, being recognized by TLR5 on antigen presenting cells and enterocytes, has been successful used as mucosal adjuvant [3] however, it may be ineffective under certain circumstances [31]. DNA adjuvants, some cytokines and chemokines have also been studied as mucosal adjuvants [4], however, are costly and may induce mutations [3]. Particle delivery systems, such as nanoparticles, have been studied extensively as adjuvants [5,7-9],

however, the current high production costs may limit a large-scale production [4,5].

We present here the preliminary results obtained with a new recombinant bacterial toxin (rVTX1) with respect toxicity and oral-adjuvanticity. First of all, the absence of toxicity findings after oral inoculated mice (above 40 µg) and in the ligated ileal loop assays (above 5 µg), support the use of rVTX1 as adjuvant. In contrast, CT (1 µg) induced inflammation in the ligated ileal loops with a significant luminal fluid accumulation, massive cellular infiltrates and damage in the intestinal villi. In contrast, the intestinal villi of the rVTX1 samples appeared with no signs of induced toxicity.

Although the B subunits of the CT and VTX have the same protein fold and function as lectins for glycolipids [24], they differ in the number and structure of the carbohydrates that they recognize: cholera toxin B-pentamer (CT-B) has one binding site per subunit to the branched pentasaccharide of ganglioside GM1, while the verotoxin B-pentamer (VTX-B) has three binding sites per subunit to the trisaccharide portion of globotriaosyl ceramide (Gb3), [32,33]. On the other hand, there are two forms of VTX (VTX1, VTX2) that share structure but with different toxicity, thus, it has been found that VTX-2 is 400 times more toxic (as quantified by LD50 in mice) than VTX-1 [33]. VTX1 consist of an enzymatically active A subunit, and pentamers of B subunits, responsible for their binding to host cells [24]. The SDS-PAGE and immunoblotting analysis of the rVTX1 revealed a subunit A with lower AMW with respect native VTX1 (30 kDa vs. 31 kDa, respectively). Comparing both sequences in gene bank data bases, it was revealed a deletion in the subunit A1 of 6 aminoacids (MHHHHH) equivalent to 820 Da, approximately, confirming the 1 kDa AMW difference. Several mutant exotoxins have been described elsewhere with diminished enterotoxic properties but retaining immunoadjuvant capacity [34]. For instance, it has been reported that minor mutations in the A subunit may reduce its toxicity. Thus, Bosworth et al., by a site directed mutagenesis of the A subunit gene, produced a mutant that differed from the parent toxin in just one aminoacid. This product was safely tested as a potential vaccine demonstrating that the immune response and adjuvant effect induced in mice are not dependent on the toxic activity of the A subunit [35]. Accordingly, we hypothesized that the "toxoid" nature of the here described VTX1-R adjuvant candidate could reside in the observed deletion of subunit A [36,37]. However, further studies must be done to verify this hypothesis.

When rVTX1 was tested as oral adjuvant when co-administered with BSA, strong specific serum and mucosal antibody responses were induced. The levels of IgG1, IgG2a and IgA were even higher than those obtained with CT adjuvant, which have been reported to induce good antibody responses at mucosal surfaces [3,4]. The induction of IgG2a, TH1 marker in mice, is very significant as being very difficult to achieve after oral immunization [17].

Results also confirm the previously reported drawback for CT as adjuvant since increases the allergenicity, IgE response, of the co-administered antigen [37]. In contrast, oral rVTX1-BSA inoculation did not elicit specific IgE, adding an extra-value of confidence for the use of this new oral adjuvant candidate.

Concerning the elicited response against the own toxoid, our results indicate that, although rVTX1 elicited antibodies to itself, the levels were similar to those induced by CT against itself but lower in the case of the IgG2a isotype. This is an important concern about the use of immunogenic proteins as adjuvants due to neutralization by pre-

existing antibodies [38]. This is particularly relevant when the protein-adjuvant is used in several doses and in different vaccines [3]. In any case, the effect of those hypothetical neutralizing properties should be tested.

Finally, due to the lack of toxicity and immunogenic properties of rVTX1 another prospect is to use this toxoid as a vaccine component against related enterotoxic pathogens, such as *Shigella* [39].

In summary, the results presented here suggest the feasibility of using rVTX1 as a mucosal adjuvant, since it induces a high antibody response against the co-administered antigen without IgE induction, and has no toxicity under the reported experimental conditions. However, further studies are required in order to assess the lack of toxicity under different host conditions, the resistance to degradation in acidic and surfactant conditions, or the capacity to induce and elicit cellular immune response at the inductive tissues like Peyer patches.

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