ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
June 2008; 7(2): 53-60

Immune Responses of Mice Immunized with Active Recombinant Shiga Toxin and Its Derivatives

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Received: 10 September 2007; Received in revised form: 26 November 2007; Accepted: 28 December 2007

ABSTRACT

Bacterial protein toxins have been exploited as therapeutic agents and as vaccines. An issue of deserving interest is development of new generations of vaccines and immune adjuvants.

In this study an active assembled recombinant Shiga toxin of *Escherichia coli* (rStx1) and its derivatives, recombinant A and B subunits (Stx1-A and Stx1-B), were used to immunize mice. The elicited antibody responses were compared with and without using adjuvant. Protection against intraperitoneal lethal dose of rStx challenge was observed by immunization with sublethal dose of rStx1, rStx-A and rStx-B subunits.

The immunological studies on toxin subunits can be used for immunization against systemic shiga toxin mediated disease and also subunits as a vector for antigen presentation in immunotherapeutic approaches. In our experiment, while stimulation of the immune system by A and B subunits were different, both subunits produced neutralizing antibodies. Regarding B subunit the amount of specific IgG1/IgG2a antibody ratio was higher than A subunit.

In addition B subunit stimulated proliferation of immune cells with IFNγ production the same as rStx1, suggesting that B subunit can be used as an immunomodulator to stimulate the immune response in conjunction with other recombinant proteins.

Key words: A and B subunits; Immunomodulation; Protection; Shiga toxin

INTRODUCTION

Shiga toxins (Stx), which are also known as shiga-Like toxins or Vero toxins, are produced by the enteric pathogens *Shigella dysenteriae* and enterohemorrhagic *Escherichia coli* (EHEC).

Shiga toxins are A-B holotoxins including one enzymatically active A subunit associated non-covalently to five identical receptors binding B subunits. The A subunit is approximately 32 kDa and the B subunits each has a molecular mass of 7.7 kDa, making the holotoxin approximately 70 kDa. [1] Stx is a member of the AB5 class of bacterial protein toxins and related toxins such as heat-labile toxins (LT) and cholera toxin (CT) from *Vibrio cholerae*.[2] Despite their structural similarity as AB5 family toxins, these different toxins have different immunological properties.[3] CT or LT, or their B subunits, have been applied either as adjuvant or as coupling agents or genetic fusion partners for peptides derived from different antigens.[4] Route of administration of these

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toxins, as antigens or coupled to toxins is another factor which can affect the immune response and have protective potential against a variety of infections.[4] However, application of the toxins or their subunits as adjuvant for human use requires an understanding of their mode of action and the separation of their desirable immunomodulatory properties from their toxicity.[4] It has been shown that the adjuvant action is not critically dependent upon the enzymatic activity of the A subunit, and that the isolated B subunit may exert different effects on cells of the immune system than do the intact toxins.[5] However immunomodulatory effects of enterotoxin and its subunits can result in the enhancement of immune responses and thus can be considered as immunoregulatory agents.[5] Immunomodulatory mechanism mediated by each subunit of holotoxin give better understanding of toxin effect on the immune system and for application of these subunits as vaccine candidate, adjuvant or vector.

So far experiments have been shown that the holotoxin with B subunit mutants of verotoxin 1 administered intraperitoneally without adjuvant; stimulated a strong antibody response in BALB/c mice and the immune sera neutralized the activity of VT1 *in vitro*.[6]

On the other hand, protection against Stx-1 challenge was reported by immunization of mice with purified mutant Stx-1. The mutant toxins were nontoxic to Vero cells *in vitro* and to mice in vivo and induced the immunoglobulin G antibody against the wild-type Stx1, which neutralized the cytotoxicity of Stx1. The induced antibody titers depended on the mutation at position 170 of the A subunit.[7] However, it has been shown that the mutants can produce antibody against both A and B subunits while immunization could be protective. [8] However, more detailed studies are needed for each subunit to determine their exact role in protection and stimulation of immune responses. In this study, importance of each subunit in provoking immune response and protection against shiga toxin-producing *E. coli* were assessed separately with and without using adjuvant.

MATERIALS AND METHODS

Expression of Constructs

Different constructs have been made in order to express A subunit (pBAD-A), B subunit (pBAD-B) and AB subunits by using gene III secretion signal (pBAD-AB) in periplasmic space of Top10 transformed bacteria. The expression of the cloned genes was induced by different concentrations of L-arabinose (0.00002%-0.2%) under the control of ara promoter of pBAD vector.[9]

Preparation of Crude Toxin and Subunits by Periplasmic Extraction and Purification

Pellet of the induced clones were used for purification of the expressed protein. The bacterial cells from pellet were suspended in 0.1% polymyxine B in Phosphate-buffered saline (PBS) (pH 7.4) as described previously[9]. The purified protein concentration was estimated by absorbance at 280 nm with bovine serum albumin as the control (Protein assay Kit, Bio-Rad).

Mouse Lethality

BALB/c mice of age 7 weeks were administered intraperitoneal doses of recombinant Stx. Survival of the mice was observed for 4 days, and the 50% lethal dose (LD_{50}) was calculated.[10]

Animal Immunization Protocols

Female BALB/c mice of age 6-7 weeks were subcutaneously administered 50 µg of antigens for each group (A, B, AB5) consisting five mice per group. The mice were injected with an equal amount of Complete Freund's Adjuvant (CFA) (Sigma) as A+, B+, AB+ and booster dose with the same dose of the antigens with an equal volume of Freund's incomplete adjuvant (Sigma) on the days of 21 and 35. Sera were collected 2 weeks after the last immunization. The immunized mice were used for the Stx1 challenge experiment. In another set of experiment, the immunization was performed with the same amount of the antigens without using adjuvant as A-, B-, AB-.[7]

Challenge Experiment

Two weeks after the last immunization, the immunized mice were challenged intraperitoneally with the toxic amount of recombinant Stx1 and observed for 4 days, and LD_{50} values were calculated.

Analysis of Antibody Response

Enzyme-Linked immunosorbant assays (ELISA) were used for detection of immunoglobulin G (IgG), IgG1 and IgG2a antibodies to Stx1. The purified Stx1 derivatives of A and B subunit was dispensed to a 96-well plate (10µg in PBS/well) and incubated at 4ºC for 16 h. After four times washings with PBS/Tween®20,

wells were blocked for 1 h with 2% skim milk (Merck). Serial dilutions of serum samples were added and incubated at room temperature for 2 h. Peroxidase-conjugated goat anti-mouse IgG (Sigma) IgG1 and IgG2a (Zymed Laboratories Inc.) were added as the secondary antibody. Biotinylated rat monoclonal antibody to mouse IgG1 and IgG2a (Zymed Laboratories Inc. CA USA) which specifically bind to HRP-streptavidin conjugate (1.25 mg/ml) were used for IgG1 and IgG2a detection. For total IgG, 1:500 dilutions and for IgG1 and IgG2a, 1:2000 dilutions were used (100 µl/ well). After four more times washing development was done with single component TMB peroxidase substrate Kit (Bio-Rad Laboratories). O.D. at 450 nm was measured by ELISA reader (Awareness Technology Inc.). Furthermore IgG antibodies from mice were examined by western blotting assay. The purified derivatives were applied to SDS-PAGE and electro transferred to a PVDF nylon membrane (Roche) after electrophoresis. Protein bands were detected by the sera prepared from immunized mice. The sera and the secondary antibody were diluted 100 and 1000 times respectively before use. The sera from PBS and vector alone injected mice were considered as control.

***In vitro* Cytotoxicity and Neutralization Assay**

HeLa cell (National Cell Bank, Pasteur Institute of Iran) cytotoxicity of the periplasmic extracts of rStx, A and B subunits were checked. HeLa cells (10^5 per well) were grown in 6-well microtiter plates (Nunc) for 24 h in RPMI medium (Biosera), pH 7.4, supplemented with 10% heat inactivated fetal bovine serum (Biosera), 100 unit/ml penicillin-streptomycin (Biosera) at 37ºC in a 5% CO2 atmosphere. Briefly, 100 µl of the periplasmic sterilized sample solution was incubated with 10^5 HeLa cells in 2 ml medium at 37ºC.For cytotoxicity assay, rStx was prepared in PBS (pH 7.5) then serial 10-fold dilutions of rStx were added to HeLa cells, incubated overnight and observed microscopically. An average percentage of cell death calculated from three separate experiments. Moreover neutralization of the cytotoxicity was done by incubation of antibody raised against A, B, AB5 with rStx to inhibit the toxicity. In 5% FBS-RPMI 5µl of serum from immunized mice was mixed with different dilutions of rStx and incubated at 37ºC for 30 min. The mixture of rStx and the sera were dispensed to HeLa cells (1.2×10^5/ml) in a 96-well micro titer plate and the plate were incubated at 37ºC for 48 h. The cell viability was measured by Neutral Red (Merck) assay. [11] Briefly after 3 h incubation with neutral red solution, viable cells were fixed with 1% CaCl2 in 0.5% formaldehyde for 3-5 min. After that, washing cells were lysed with 1% acetic acid in 50% ethanol. The absorbance was measured by ELISA reader (Awareness Technology Inc.) at 540 nm.

Lymphocyte Proliferation Assay

Mice were sacrificed three weeks after the last immunization. Spleens of three f the mice were separated and cultured at 2×10^6 viable cells per ml in 2 ml volumes in 24 well plates. Cultures were established in the presence of antigens (20µg/ml) and cell proliferation was assessed. [12] After incubation at 37ºC in 5% CO2 humidified atmosphere, cells were pulsed with 0.5 µCi [^{3}H] thymidine (Amersham, UK), incubated for 18h and were harvested. The thymidine incorporation was determined by liquid scintillation by counting in a β liquid scintillation counter (Pharmacia). Data are expressed as stimulation index (SI) representing the ratio of count per minute (cpm) of the triplicate stimulated cultures to the cpm of control culture.

Cytokine Assay

Supernatants of above cultures were pooled after 72h incubation and stored at -70°C for cytokine assay. Cytokines were assessed by using commercial IFNγ and IL4 capture enzyme-Linked immunosorbent (ELISA) assays. Mouse IFNγ and IL4 Module Set from Bender MedSystems, Inc. were used for IFNγ and IL4 assay following manufacturer's instructions. All tests were performed in triplicate for three mice.

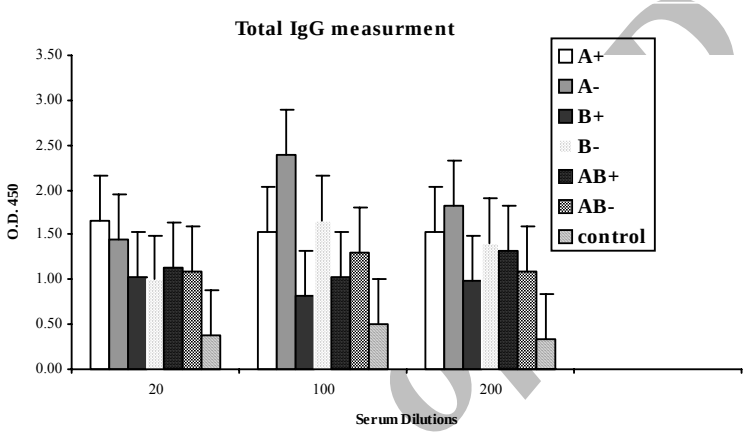
RESULTS

A and B subunits expression is confirmed by western blotting with rabbit antibodies which was already raised against purified eluted form of A and B subunits in our previous work.[9]

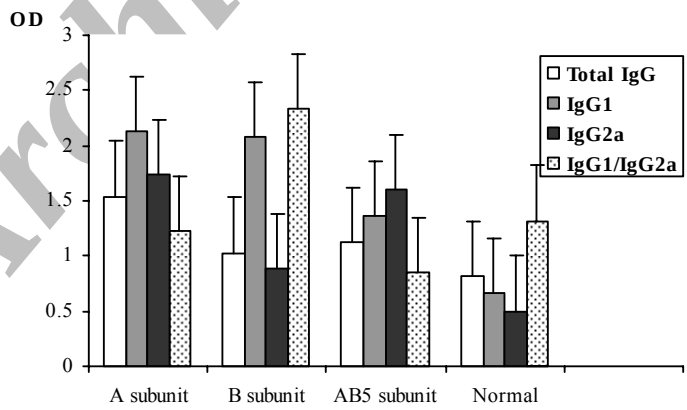
Antibody Response to Immunization

The ability of A and B subunits in modulating immune system was assessed by measuring total IgG response. Moreover to compare the ability of each subunit itself, they were used with and without adjuvant (A+, A-, B+, and B- respectively).

In this regard, serum pools of immunized mice were collected from each group. Antibodies against A and B subunits were detected by ELISA in sera of all immunized mice. An anti-A and anti-B, specific IgG antibody response to each subunit after mice immunization was indicated. A subunit was raised the highest amount of specific total IgG production with and without using adjuvant (Figure 1a). However immunization with AB5 holotoxin with and without adjuvant (AB+, AB- respectively) revealed that its stimulation of immune response is almost similar to B subunit. On the other hand, subunit could raise much better response while comparing the IgG production by AB5 holotoxin. Furthermore production of IgG1 and IgG2a and IgG1/IgG2a ratio was measured for each group to compare the specificity of mice humoral immune responses in different groups. While the plates were coated with A, B subunits and AB5 holotoxin respectively, high titer of IgG1 level was detected by A and B subunit. The highest amount of IgG1/IgG2a ratio corresponding to the specificity of response was displayed by B subunit (Figure 1b).



a



b

Figure 1. a) Graphic presentation of total IgG antibody production after immunization with A, B, AB5 holotoxin with and without adjuvant, different dilutions of the serum were used for optimum reaction
b) Graphic presentation of total IgG, IgG1, IgG2a antibody production against A, B, AB5 holotoxin and IgG1/IgG2a ratio after immunization with adjuvant

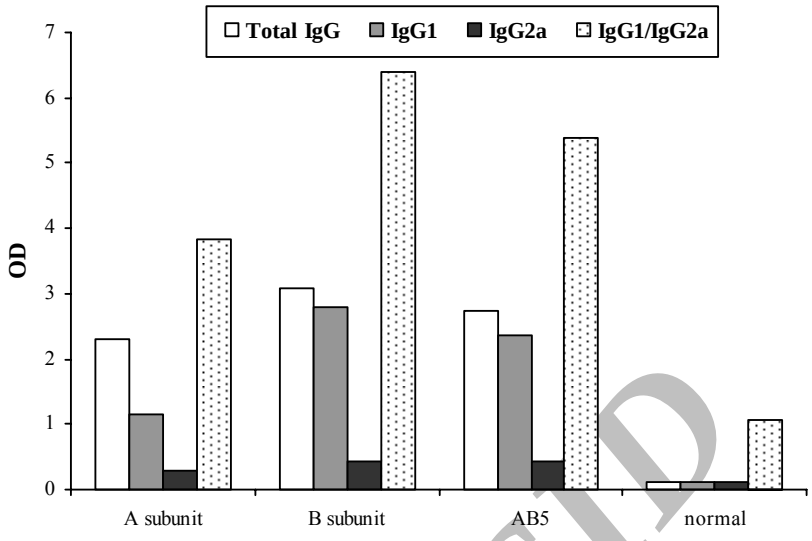


Figure 2. Graphic presentation of produced total IgG, IgG1, IgG2a antibody against holotoxin

Moreover similar results were shown when ELISA plate was coated with rStx (Figure 2). In this way the highest amounts of total IgG and IgG1 levels were shown for B subunit, when it was compared to other groups.

Western-blotting experiment revealed specific response to the subunits of Stx by the sera from immunized mice. Raised antibodies from immunized mice were detected A and B subunits (Figure 3). By western blotting experiment A and B subunits were separately detected by A, B and AB specific antibodies raised in mice.

Splenocyte Proliferation and Cytokine Production

To investigate immune response induction in splenocytes from immunized mice, the proliferation assay was analyzed by AB5, A and B subunits (with and without using adjuvant).

The strongest proliferation was displayed by B-subunit without using adjuvant as shown by the level of stimulation index (Figure 4a). The capacity of the cytokine production in the splenocytes of the immunized mice was measured by the levels of IFNγ and IL4 production (Figure 4b). In the collected supernatant of cells from mice without using adjuvant significant level of IFNγ production was observed by recombinant holotoxin (Figure 4b).

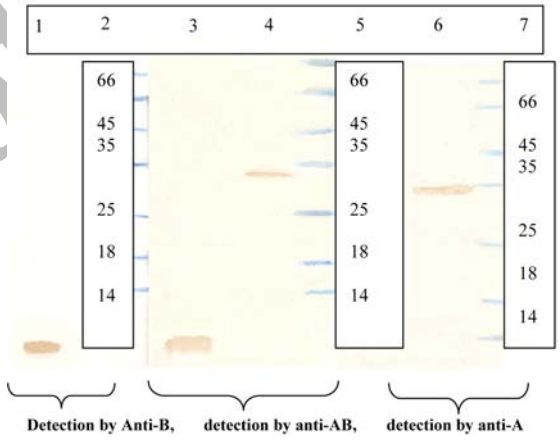
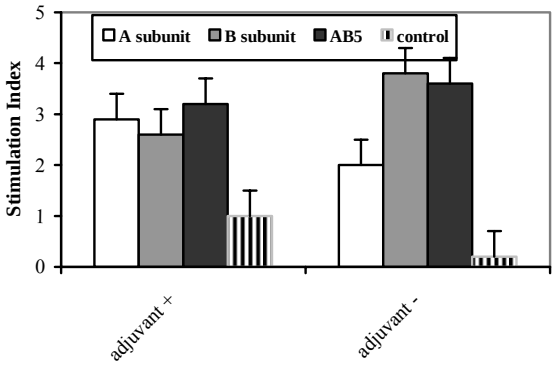


Figure 3. Western Blot analysis of antibody raised against A, B, and AB5 From Left to right: B subunit detected by anti-B, A and B subunit detected by anti-AB, A subunit detected by anti-A; Lanes: 1, B subunit, 2, MWM, 3 and 4, AB holotoxin, 5, MWM, 6, A subunit, 7, MWM

Cytotoxicity and Neutralization of the Cytotoxicity

Lethality of recombinant AB5 (rStx-1) was assessed *in vitro* and *in vivo*. HeLa cell cytotoxicity by recombinant holotoxin, AB5 was observed while it was also lethal in vivo in BALB/c mice. Percent of toxicity was measured and it was shown that the A and B subunits with the same dilutions did not show any toxicity on the cells. The 50% cytotoxic effect of AB5 was determined using different dilutions on the HeLa cells.



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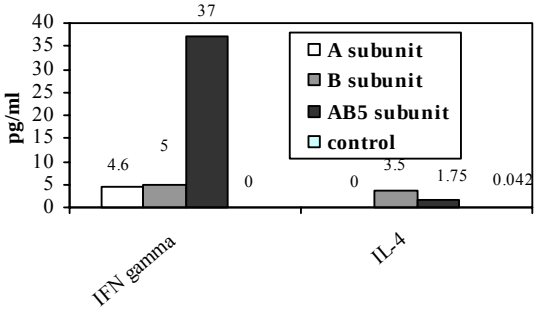

b

Figure 4. a) The Stimulation Indexes of rStx, A and B subunits with and without adjuvant, b) Cytokine production in supernatants of cell culture of mice immunized without adjuvant, The results are representative of two independent experiments

On the other hand inhibition of the toxicity was achieved with antibody against A, B subunits and rStx-1 (Figure 5). The toxicity was neutralized by AB5 and B subunit antiserum and the highest level of inhibition with different dilutions of toxin was obtained by B subunit (Figure 5).

Protection of Immunized Mice against Lethal Toxin Challenge

Mice immunized with A, B and AB5 holotoxin were challenged with lethal dose of recombinant toxin two weeks after the last boost [7]. Two times more than LD_{50} of rStx was used for challenge. All of PBS immunized mice died by day 4, while the immunized mice survived (Table 1).

DISCUSSION

To control outbreaks caused by Enterohemorrhagic *E. coli* (EHEC) which produces Stx and to reduce the mortality due to hemolytic-uremic syndrome, a safe and effective vaccine is required.[7, 13-15] So far many efforts have been made by several research groups to develop different kinds of vaccines i.e. a live vaccine, [16] cell component vaccine,[17] polysaccharide-conjugated vaccine,[18] and B subunit or toxoid vaccine of Stx.[19, 20] In 1991, immunogenicity of shiga toxin B subunit, its fragments and its protective humoral responses against the action of shiga toxins was shown.[14]

In addition it was shown that intraperitoneally immunization by VT1 without using adjuvant stimulated a strong antibody response in BALB/c mice and the immune sera neutralized its activity.[6] However, more detailed studies are needed for each subunit to determine its exact role in protection and stimulation of immune responses. In this study the effect of each subunit of rStx was assessed and compared with holotoxin. Subunits of holotoxin were all immunogenic and induced IgG antibody. In our study it was shown that adjuvant action was not critically dependent upon the A subunit, since the isolated B subunit might exert different effects on cells of the immune system than do the intact toxin. While the results of Ishikawa *et al.* [7] indicated that the neutralization of the cytotoxicity was mainly seems to be due to the antibody directed to A subunit. On the other hand an association between the cytotoxicity of the protein and its ability to induce cytokine release has already been suggested.[6]

Table 1. Protection experiment of immunized mice challenged with recombinant holotoxin.

Dose /µg	No. of surviving mice/total of immunized mice with Mice immunized with						
	A+	A-	B+	B-	AB+	AB-	PBS
50	5/5	5/5	5/5	5/5	5/5	5/5	5/5
100	5/5	5/5	5/5	5/5	5/5	5/5	3/5
150	5/5	4/5	5/5	5/5	5/5	5/5	0/5
200	5/5	2/5	5/5	5/5	5/5	5/5	0/5

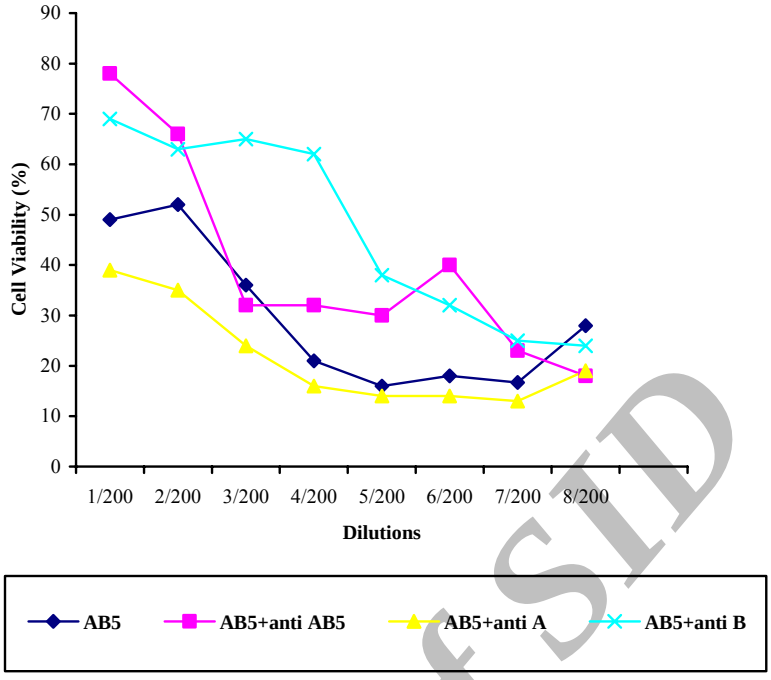

Figure 5. Recombinant AB5 toxicity and inhibition of toxicity in HeLa cells measured by Neutral red assay, different dilutions of anti-A, anti-B and anti-AB5 were used.

In our experiment, B subunit with and without using adjuvant promoted specific antibody response by higher amount of IgG1/IgG2a ratio. Enterotoxins, especially CT and LT or their non-toxic derivatives have been used as mucosally applied adjuvant for numerous antigens and experimental vaccines against a wide range of infections. [21] However the nature of the used adjuvant varies greatly and although adjuvant provide enhanced immune responses their use can also elicit adverse side effects, as shown for CFA.[22] Therefore, subunits can be used as vaccines or as adjuvant defined as materials co-administered with antigens in vaccines to enhance the desired immune responses. It is important to study the effect of each subunit on stimulating immune responses and comparing the potential protection induced after immunization with and without using adjuvant.

In our experiment, the expressed recombinant holotoxin and its subunits induced antibody titers that mostly were IgG1. Considering higher IgG1/IgG2a ratio by B subunit, it was suggested that immunization with recombinant holotoxin was mostly dependent on B subunit. Regarding cytokine production both IFNγ and IL-4 were detected in the supernatants from cell cultures of mice without using adjuvant by B subunit as confirmed by stimulation index. In our experiment INFγ production was observed by rStx, while regarding B subunit, the same result was shown by using adjuvant. While using adjuvant, the amount of IFNγ for B subunit was increased similar to rStx holotoxin. This result suggests that B subunit of shiga toxin, like Stx could be effective adjuvant for induction of mixed Th-type as it was suggested that they can augment antigen specific immune responses via dendritic cells.[23] In addition despite specific antibody production by A subunit, B subunit was more potent for neutralization of cytotoxicity by holotoxin. Moreover complete protection has been shown by B subunit in vivo. All data presented here suggested that the B subunit could be a stimulator of specific and protective antibody response. So far, studies focused on either mutant form of holotoxin while in the present study the recombinant active holotoxin has been used for immunization and its modulatory effect was compared with those of A and B subunits for the first time. Furthermore our results suggest that the B subunit without toxicity can induce cytokine production and can be used as an immunomodulator or an adjuvant for developing recombinant protein vaccines.

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