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Humoral immune response to *Diphtheria* and *Tetanus* toxoids by intranasal administration

Mohammad pour-dounighi*, N., Zolfagharian, H.

Department of Purification of Toxoids & Antisera, Razi Vaccine and Serum Research Institute, Karaj. Iran.

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ABSTRACT

The immunogenicity of ten different formulations of intranasal diphtheria and tetanus vaccines which containing different absorption enhancers, adjuvants and other excipients were determined in guinea pigs by the serum neutralization (SN) method. From these ten formulations, it was selected four formulations which gave significant immunogenicity in guinea pigs. In order to design the "*final formulation*" composition of these four formulations investigated properly and final formulations designed accordingly and tested in human volunteers. The parenteral and intranasal diphtheria and tetanus toxoids (DT) vaccines were tested in two groups of human volunteers, and serological responses were estimated in both groups (The parenteral DT vaccine containing aluminum phosphates as an adjuvant). Our results showed very good serological responses ($p < 0.01$) in both groups of human volunteers. It can be concluded that the intranasal vaccination can be a good alternate in the field of vaccination.

***Keywords*:** Nasal vaccine, Diphtheria, Tetanus

INTRODUCTION

Although universal use of the traditional parenterally diphtheria and tetanus vaccines have significantly reduced the incidence of human diseases. There is a need for the development of new generation and more refined vaccines, including those that can be administered by mucosal routes. There is an increasing trend to replace the parenteral routes of vaccination by mucosal immunization via respiratory system. The current diphtheria and tetanus vaccine, which were developed in the 1920s, based on formaldehyde-detoxified *diphtheria* and *tetanus* toxins are in low purity and have been associated with some undesired side effects. A number of studies on intranasally delivered vaccines have reported (Aggerbeck *et al* 1997, Heritage *et al* 1998 and Gluck *et al* 1999). They have been demonstrated significantly enhance the systemic and mucosal immune responses after mucosal vaccination. Recently Yoko and his coworkers have reported (2003) anti-tetanus toxoid (anti-TT), anti- diphtheria toxoid (anti-DT) serum and mucosal antibody responses induced by intranasal immunization. They showed that anti-TT and anti-DT serum and mucosal

* Author for correspondence.E-mail:n.mohammadpour@rvsri.com

antibody responses induced by repeated intranasal immunization using rCTB adjuvant lasted for a long period and that, for improving the affectivity of vaccination, different rCTB-containing vaccines should be administered at appropriate intervals. There are few more reports on nasal vaccination (Yoko *et al* 2003, Jerry *et al* 1992). These reports showed the delivery systems on different adjuvants for mucosal immunization of tetanus and diphtheria. The synthesis of specific IgG is stimulated and induction of systemic tolerance from a mucosal antigen exposure is prevented.

The current studies were performed to investigate the specific antitoxin responses and safety on animal (Phase 0) and human healthy volunteers (Phase I) by nasal immunization with purified diphtheria and tetanus toxoids incorporated in different drop formulations contains absorption enhancers, adjuvants and other excipients are discussed and compared with a parenteral immunization. It is interesting to mention that for human study we have taken medical ethic from our ministry of health.

MATERIALS AND METHODS

Diphtheria* and *Tetanus toxoids. *Diphtheria* toxin was produced by the method described by Holt (1950), using a semi-synthetic medium based on an acid hydrolysate of casein together with salts, maltose, yeast extract and Muller II growth factors. The titer of toxin of 6-7 days culture was 100-120 lime of flocculation (Lf)/ml. The toxoid was purified by ultra filtration, followed by salting out by ammonium sulfate and column gel (Sephadex G-25) chromatography. The titer of the final product varied from 1600-1900 Lf/mg. Tetanus toxin was prepared by the method which was described (Latham *et al* 1962), using an enzymatic digest of casein (N-Z case TT, Sheffield Chemical Company, USA). The toxoid was purified by salting out with ammonium sulfate as outlined by Levine and Stone (1951) followed by column gel (Sephadex G-25) fractionation. The final purity of tetanus toxoid was 1200-1500 Lf/mg. The quality controls for diphtheria and tetanus toxoids were performed according to the minimum requirements of the World Health Organization (1990) and United State Pharmacopoeia (2005). Both toxoids were free from beef or other animal proteins.

Absorption enhancers. In order to increase of absorption and avoid rapid catabolism of refined fluid diphtheria toxoid (DT) and tetanus toxoid (TT) antigens in the mucosal surface, absorption enhancers were incorporated in the vaccine formulations. Absorption enhancers used in the present study include surfactants (cationic, anionic and nonionic) and bile acids (Merck Co. Germany). The amount of surfactants to be incorporated into the vaccine composition suitably ranges from 0.01 to 10 % by weight, preferably 0.1 to 5% by weight on the basis of the total weight of the composition (Toshihiko *et al* 1994).

Adjuvants. For enhance the stimulation of mucosal immune system and preparation of sustained release formulation an oily form adjuvant was mixed with vaccine composition. The adjuvants used in this study include higher fatty acids prepared from Merck Chemical Company Germany (Toshihiko *et al* 1994).

Humectants, Preservatives and Binders. These agents in order to physicochemical and microbial standardization of vaccines formulations were incorporated in vaccines compositions. Humectants used in this study include xylite, glycerin and sorbite (Merck Chemical Company, Germany). Benzoic acid and parahydroxy benzoic acid derivatives were formulated in compositions of vaccines as preservatives (Fluka Chemical Company, UK). Formulations of nasal vaccine used in this research project contain binders such as hydroxy ethyl cellulose, hydroxy methyl cellulose, methy cellulose (Merck Chemical Company, Germany) and sodium polyacrylate (Fluka Chemical Company, UK) (Boylan 1986, Collet 1990).

Animals. Equal numbers of female and male short hair guinea pigs, weighing about 300-350 g, were used both for immunization purposes and for the titration of diphtheria antitoxin (DA) by the SN test (European Pharmacopoeia 2002). NIH mice, weighing about 18-20g were used for the titration of tetanus antitoxin (TA) by the SN test (Gupta *et al* 1985). All of animals used were obtained from the Razi institute small Animal Facility and during of study all animals kept at good husbandry practice conditions.

Preparation of vaccine. To the purified DT and TT were added diluents and other additives such as absorption enhancers, adjuvants, preservatives, humectants and binders in order to obtain desirable pharmaceutical preparations used as a nasal drop. The toxoids used were supplied by the section for production of bacterial vaccines at Razi vaccine and sera research institute. Stock of purified fluid DT (1500 Lf/ml) and TT (1500 Lf/ml) was stored at +4 Ċ. Phosphate- buffered saline (PBS) at PH 6.8-7.0 used as a diluents. Compositions listed (table 1) in the formulation of vaccines (exception of DT and TT) were dissolved in 40 ml PBS at pH 6.8-7.0 and the solution was slowly agitated for 20 min at room temperature. Then required amounts of DT and TT were mixed with this solution which was again agitated for 20 min at room temperature.

Measurement of anti DT and anti TT Levels. For measurement of diphtheria and tetanus antitoxins, the in-vivo serum neutralization test (European pharmacopeia 2002, Gupta *et al* 1985) was performed as follows: The SN test for diphtheria antitoxin titration was performed following the recommendations of European Pharmacopoeia (2002). Briefly, four two- fold serum dilutions of the immune guinea pig were incubated with the diphtheria toxin for 30 min in darkness at room temperature. A volume of 0.2 ml of each mixture injected intracutaneously into shaven flanks of 2 guinea pigs, the animals were observed during 2 days and erythematous effects were recorded. Control group of guinea pigs injected with diphtheria toxin mixed with defined amount of standard diphtheria antitoxin (SDA) were included in each assay and the results were used to confirm the test - dose of the toxin and to correct the antitoxin value obtained. Antibody titers elicited by the diphtheria component of vaccines were calculated by the Speerman-Karber method (Finney 1964) and expressed in IU/ml. For measurement of tetanus antitoxin by SN method, the toxin neutralization ability of serum in mice estimated (Gupta *et al* 1985).

Schedule of nasal immunization of animals (Phase 0). Ten vaccine formulations with different compositions were prepared as nasal drops (Table 1). Twenty groups of guinea pigs (each comprising 3 animals) were selected. Groups 1 to 10 with blank samples and groups 11 to 20 with vaccine formulations number 1 to 10 (respectively) were immunized by intranasally administering of these solutions, two times at four weeks intervals. Above mentioned formulations were contained DT and TT as well as absorption enhancers and other excipients. The amount of vaccine used for immunizations of guinea pigs was one drop per dose (approx. 0.05 ml, DT 2.5 Lf and TT 5 Lf).

Volunteers and schedule of nasal immunization (Phase I). Thirty healthy volunteers with a mean age of 27 years (rang 23- 35 years), conducted to evaluate side effects and serological responses to nasal vaccine (Final formulation). Volunteers were divided to three 10 person groups (group 1: test, group 2: positive control and group 3: negative control). Each volunteer of group one was given four drop (approximately 0.2 ml, DT 10 Lf & TT 20 Lf) of final formulation intransally, on two occasions with a 4 week interval. The second group was similarly treated with the all composition mixture of final formulation without DT and TT. The third group of healthy volunteers was immunized by intramuscular rout with conventional combined diphtheria and tetanus adult vaccine.

Collection of sera. All of human and animal groups were bled before administration of vaccines (day 0) and 4 week after of last vaccination. Blood was obtained from animals and human volunteers by venous puncture. The sera were stored frozen at -20 ºC.

Statistical analysis. The significance of difference between SN titers of animals and human volunteers before and 4 weeks after of last vaccination was established by the paired t-test (Armitage 1977).

RESULTS

Immunization of guinea pigs with nasal formulations DT and TT. The immunogenicity of diphtheria and tetanus component of ten nasal vaccine formulations (Table 1) were determined by calculating the mean of anti DT and anti TT antibodies levels measured in the sera of guinea pigs immunized with each vaccine formulation. Among of these different formulations significant increase of TA and DA resulted by formulations F2, F8, F9 and F10. Negative control groups were not showed detectable increase of DA and TA titers. These observation confirms the virtue of stimulation of the immune responses by absorption enhancers and oily adjuvants when mixed with DT and TT and the enhanced toxoids absorption and sustained releasing of toxoids (Table 2). Therefore, the immune system exposed with efficient amount of antigens in longer time. With combination of formulations F2, F8, F9 and F10 final formulation was prepared as nasal drop, in order to examination on healthy human volunteers.

Immunization of human healthy volunteers. The final formulation for intranasal administration were prepared by dissolving DT and TT (50 and 100Lf/ml, respectively) in vehicles having nonionic surfactant, bile acid derivative, oily adjuvant, glycerin and benzoic acid derivative. Results obtained from determination of serological responses

Table 1. Compositions of nasal vaccine formulations 1 to 10

Compound	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
DT(Lf/ml)	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00
TT(Lf/ml)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
CS(%W/V)	0.10	-	-	-	0.80	-	0.10	-	-	-
NS(%W/V)	-	0.40	-	-	-	-	-	-	-	0.40
AS(%W/V)	-	-	-	-	-	1.00	-	-	1.00	-
OA(%W/V)	-	-	0.50	-	-	0.50	0.50	0.50	-	-
Glycerin(%W/V)	-	-	-	0.80	-	0.80	-	0.80	-	2.00
Xylite(%W/V)	-	0.80	-	-	0.50	-	-	-	-	-
HMC(%W/V)	-	0.80	-	0.80	0.80	-	-	-	-	-
HEC(%W/V)	0.80	-	-	-	-	-	-	-	-	-
MC(%W/V)	-	-	0.60	-	-	-	-	-	-	-
SP(%W/V)	-	-	-	-	-	-	0.20	-	-	-
Sorbite(%W/V)	-	-	0.60	-	-	-	-	-	2.00	-
BAD(%W/V)	-	0.10	-	0.10	0.02	-	-	-	0.10	0.02
Bad(%W/V)	-	-	-	-	-	-	-	0.10	0.20	1.00

DT: Diphtheria Toxoid, TT: Tetanus Toxoid, CS: Cationic Surfactant, NS: Nonionic Surfactant, AS: Anionic Surfactant, OA :Oily Adjuant, HMC: Hydroxy Methyl Cellulose, HEC: Hydroxy Ethyl Cellulose, MC: Methyl Cellulose, SP: Sodium Polyacrylate, BAD: Benzoic Acid Derivative, Bad: Bile acid derivatives.

Table 2. SN anti DT & anti TT titers elicited by intranasal immunization in guinea pigs (n=3)

Animal groups	4-weeks after last immunization	
	Anti DT SN titer IU/ml	Anti TT SN titer IU/ml
1	0.01 ± 0.000	0.01 ± 0.000
2	0.30 ± 0.100	0.20 ± 0.020
3	0.01 ± 0.005	0.01 ± 0.005
4	nd	0.005 ± 0.001
5	0.006 ± 0.001	0.004 ± 0.001
6	0.004 ± 0.001	nd
7	0.006 ± 0.001	0.005 ± 0.001
8	0.40 ± 0.100	0.30 ± 0.050
9	0.30 ± 0.050	0.10 ± 0.050
10	0.30 ± 0.030	0.40 ± 0.000

nd= not determined

Table 3. SN anti DT & anti TT titers elicited by intranasal immunization in human volunteers [human volunteers group 1] (n=3)

Human volunteers	Anti DT SN titer IU/ml		Anti TT SN titer IU/ml	
	Before immunization	4 weeks after last immunization	Before immunization	4 weeks after last immunization
1	0.03 ± 0.005	0.60 ± 0.1 00	0.04 ± 0.010	0.40 ± 0.010
2	nd	0.50 ± 0.100	0.04 ± 0.010	0.30 ± 0.010
3	0.04 ± 0.010	0.60 ± 0.000	nd	0.70 ± 0.100
4	0.10 ± 0.020	1.00 ± 0.200	0.10 ± 0.020	0.70 ± 0.020
5	0.02 ± 0.000	0.80 ± 0.100	0.10 ± 0.010	0.60 ± 0.020
6	0.01 ± 0.000	nd	0.03 ± 0.005	0.90 ± 0.100
7	0.02 ± 0.005	0.90 ± 0.100	0.02 ± 0.000	0.90 ± 0.100
8	0.01 ± 0.000	0.50 ± 0.100	0.01 ± 0.000	0.10 ± 0.020
9	0.04 ± 0.010	0.70 ± 0.200	0.03 ± 0.005	nd
10	0.01 ± 0.000	0.20 ± 0.010	0.06 ± 0.020	0.30 ± 0.040

nd= not determined

Table 4. SN anti DT & anti TT titers elicited by parenteral administration of conventional vaccine in human volunteers [Human volunteers group 2] (n=3)

Human volunteers	Anti DT SN titer IU/ml		Anti TT SN titer IU/ml	
	Before immunization	4 weeks after last immunization	Before immunization	4 weeks after last immunization
1	nd	0.80 ± 0.100	0.02 ± 0.000	2.00 ± 0.300
2	0.30 ± 0.050	4.00 ± 0.100	0.06 ± 0.005	1.50 ± 0.400
3	0.02 ± 0.000	1.20 ± 0.300	0.00 ± 0.000	0.80 ± 0.200
4	0.03 ± 0.005	nd	0.05 ± 0.010	2.00 ± 0.100
5	0.03 ± 0.010	1.10 ± 0.200	0.06 ± 0.010	1.30 ± 0.100
6	0.04 ± 0.005	5.00 ± 0.200	nd	0.70 ± 0.100
7	0.01 ± 0.000	0.90 ± 0.200	0.02 ± 0.005	0.80 ± 0.200
8	0.10 ± 0.020	3.50 ± 0.400	0.04 ± 0.010	0.80 ± 0.000
9	nd	4.10 ± 0.300	0.04 ± 0.010	nd
10	0.04 ± 0.010	2.80 ± 0.400	0.03 ± 0.000	3.02 ± 0.400

nd= not determined

Results obtained from determination of serological responses (Table 3) illustrate the responses for each person and give useful evidence of nasal immunization by two doses of antigens incorporated in formulation contains absorption enhancers, adjuvants and other excipients. For obtain a significant rise of specific antitoxins, use of absorption enhancer agents and adjuvants in nasal drop formulation of vaccine seems to be essential. Also, commercial aluminum phosphate adsorbed DT vaccine used as a parenteral control vaccine was enhanced Anti DT and anti TT levels significantly (Table 4). Our results showed that there was a significant ($p<0.01$) increase in the SN titers of healthy humans after injection of DT vaccine and second intranasally administration of selected nasal vaccine (Table 3 & 4). In serological responses of second group of human volunteers (negative control) were not observed a significant variation than 0 day.

Follow-up for adverse effects. Volunteers were asked to be cautions about the possible side effects such as allergic or hypersensitivity reactions occur following intranasally administration of final formulation. No severe side effects were noted in the volunteers, following vaccine given two times 4 week intervals, in the days and weeks after nasal immunization and after parentral immunization. Ten percent (10%) of volunteers experienced unpleasant stinging lasting 10 min after the intranasal vaccination and 20 percent of volunteers experienced pain and redness in injection site after parentral vaccination.

DISCUSSION

Vaccination via a mucosal rout is logical and a natural means for immunization, because initial defense is available at the mucosal site against most microbial pathogens. The mucosal route has advantages over intramuscular and subcutaneous injection. It is an easy and safe rout of administration. At the present time, there is the urgent need for a new generation vaccines to prevent many pathogens of the great causes of morbidity of humans from infecting mucosal sites [the GI tract, respiratory and urogenital tracts *etc.*] (Spier 1993). The mucosal surface represent a tremendous surface area, over 400 m² in humans (Eldridge *et al* 1989), and this fact has been used extensively in recent years to achieve immunogenic absorption and penetration. The mucosal immune system is considered by many to be a "new world "in the area of immunology and has numerous unique features compared to the classical systemic immune compartment. The mucosal immune system consists of specialized IgA inductive and effecter sites, as well as unique cell trafficking patterns that underlie the common mucosal immune system (CMIS). Furthermore, epithelial cells that line mucosal surfaces are themselves an integrated component of the mucosal immune system and provide signals important for initiation of the mucosal inflammatory response and key cell- cell communication between epithelial cells and mucosal lymphoid cells. The concept of CMIS has provided a rational basis for the clinical development of mucosal vaccines for prevention of infectious diseases (Martin *et al* 1996). It is now well established that environmental antigens, which are most often encountered by inhalation, can be taken up into specialized lymphoreticular tissues in the upper respiratory tract (URT) (Rudzik *et al* 1975). Specialized antigen - transporting cells, termed M cells, are found overlying mucosal associated lymphoid folicles. Similar cells are found overlying lymphoid tissues in the nasopharynx and bronchia. In humans, the major nasopharyngeal sampling sites are tonsils and related lymphoid tissues, which together are known as waldeyer's ring. Antigen uptake across nasal epithellium not associated with lymphoid folicles may also be important for stimulation of immunity. Intranasal administration of antigen is now well established as a method for stimulating systemic and secretory antibody responses in mice and humans

(Weltzin *et al* 1997). Diphtheria and tetanus purified toxoids have similar structure to insulin (Hashemi 1992). Simultaneous nasal administration of insulin with absorption enhancers as a standard formulation contains required other excipients lead to efficient absorption of insulin and decrease in glucose level (Lisbeth & Stanley 1992). Yoko and his coworkers (2003) studied on rCTB as adjuvant in tetanus and diphtheria toxoid vaccines. They suggested that the anti TT and DT serum and mucosal antibody responses induced by repeated intranasal immunization. In another study it was shown negatively charged liposomes as immunological adjuvant for tetanus and diphtheria toxoids. They have found that negatively charged liposomes enhance the immune effects of the combination of the tetanus and diphtheria vaccines (Popescu *et al* 1998). Bramwell and his research group (2003) also introduced melittin as adjuvant for intranasal immunization of tetanus and diphtheria toxoids via the nasal route. McNeela and his coworkers (2000) studied on mucosal diphtheria vaccine. They have formulated CRM 197 of diphtheria toxin with chitosan and enhanced local and systemic antibody and the responses by nasal delivery (McNeela *et al* 2000). All above mentioned research works which was conducted in the different research centers are agree with our results and our observations confirmed them. Our preliminary experiments on guinea pigs indicated that some of absorption enhancers and oily adjuvants (agents incorporated in formulations no 2, 8, 9 and 10) were induced better serological responses. Final formulation was designed with combination above noted formulations as nasal drop and examined on healthy human volunteers. This nasal formulation of DT and TT was induced a significant serological response. We do not yet what immune mechanism the toxoids mixed with absorption enhancers and adjuvants are taken up from nasal mucose to systemic immune system. These limited observations indicate that formulations contain absorption enhancers and oily adjuvants could be used as a means of antigen delivery for nasal immunization generating a significant antibody response against the DT and TT toxoids after two administrations. The antibody titer of antigens studied in present project can effectively be increased by the addition of a surfactant, a bile acid and oily adjuvant to the composition for nasal drops. Among the surfactants, preferred are nonionic surfactants such as poly oxyethylene octyl ether, stearyl ether, acetyl ether, octylphenyl ether and Triton X100, whose average molar number of added ethylene oxide ranges from 5 to 30, anionic surfactants such as sodium lauryl sulfate and potassium lauryl sulfate (Toshihiko 1994). Among the derivatives of bile acid, preferred are amide compounds of bile acids with amino or amino sulfonic acids. The protective vaccine composition for diphtheria and tetanus infections should comprise a fat- soluble adjuvant such as oleic acid, stearic acid and palmitic acid, humectants such as glycerin, sorbite, xylite and PEG, preservatives such as benzoic acid and thereof derivatives and binders such as cellulose derivatives.

The diphtheria vaccine specific for babies is not usually used to immunize people over 10 years of age, because can be produce sever allergic reactions. Therefore, these recipients were vaccinated with 2 Lf of diphtheria toxoid only. In spite of the prominent allergic responses normally observed in all mature vaccine the nasal administration of two doses of DT and TT did not produce any noticeable side effects. The vaccine composition of the present study make it possible to intransally immunize a subject. Exploitation of the mucosal immune system offers several advantages includes the efficacy of currently available vaccine can be enhanced by vaccination procedures to achieve both mucosal and systemic immunity, safety and minimization of adverse effects, the lake of need for experienced personnel and equipment for administration, the increase of persons compliance, possibility of increase in vaccine effectiveness in the elderly, facility of eradication of some diseases and low cost of final product and

vaccination by mucosal vaccines (Richard 1994). There are few reports on intranasal immunization or mucosal vaccination (Berstad *et al* 2000). The ability of chitosan microparticles to enhance both the systemic and local immune responses against DT after oral and nasal administration in mice was investigated. They demonstrated that when DT associated to chitosan microparticles results in protective systemic response after oral vaccination and in significant enhancement of IgG production after nasal administration. Therefore, these in-vivo experiments demonstrate that chitosan microparticles are very promising mucosal vaccine delivery systems. There have been few attempts to demonstrate that mucosally delivered vaccines (Aggerbeck *et al* 1997, Gluck *et al* 1999, Yoko *et al* 2003) can generate cellular immune responses in man (Berstad *et al* 2000). Berstad and his colleagues showed induction of antigen specific T cell responses in human volunteers after intranasal immunization with a whole cell pertussis vaccine. This study for first time demonstrated a nasally delivered subunit vaccine, can enhance systemic T cell responses in human (Berstad *et al* 2000).

In the present study, we also found highly significant serological responses in human volunteers as well as in animal, after administration of diphtheria and tetanus toxoid by intranasal route.

However we observed significant acceptable serological responses in human volunteers intranasally vaccinated with DT when compared with those parenteral vaccinated.

In conclusion, the present study introduced a suitable nasal vaccine formulation as a nasal drop, which can be used for immunization against diphtheria and tetanus diseases.

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