

ORIGINAL ARTICLE

Toward an Alum Free Mono-Component Monovalent Pertussis Vaccine: A Cytokine Response Assay

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ABSTRACT

Background: Current evidence indicates the resurgence of whooping cough despite high coverage of whole-cell (wP) and acellular (aP) pertussis vaccines. **Objective:** To investigate the cytokine response to a genetically inactivated protein containing the S1 subunit of pertussis toxin (PTS1) with and without the Listeriolysin O (LLO-PTS1), in comparison with current wP and aP vaccines in the mice model. **Methods:** Thirty-six female NMRI mice aged 8 to 12 weeks (25 ± 5 g) were divided into six groups, including control (n=6) and five treated groups (n=6/each). Treated groups received intraperitoneal injection of recombinant PTS1, recombinant fusion LLO-PTS1, aP, wP, and sham (phosphate-buffered saline), whereas the control group did not receive anything. After 60 days, the serum levels of IFN- γ , IL-4, and IL-17 cytokines were evaluated by ELISA method. **Results:** Our findings showed LLO-PTS1 significantly increased IL-17 and IL-4 cytokines compared with wP and aP vaccines. IFN- γ failed to increase substantially in the LLO-PTS1 group compared to others, but it was non-inferior to standard vaccines. **Conclusion:** Our alum free mono-component monovalent recombinant fusion protein (LLO-PTS1) could bear the capacity to stimulate the release of IFN- γ similar to wP and aP vaccines in the mouse model. Besides, it showed better results in stimulating the release of IL-17 and IL-4 response. This study can be regarded as a platform for further probes in booster pertussis vaccine development.

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INTRODUCTION

Prior to vaccines availability, pertussis (P) caused serious complications and even death in communities, especially in infants and young children. Combination vaccines that were effective in controlling diphtheria, tetanus and pertussis (DTwP) become accessible around 90 years ago hence lowering the prevalence rate of the diseases from 157 to 1 in 100000 in the 1970s (1). It followed that several countries discontinued or markedly decreased the use of this vaccine due to the severe reactions (encephalopathy and death) it showed (2). Primarily, in 1980 Margaret Pittman described pertussis as a toxin-mediated disease, the researcher whose perspective first time contributed to less-reactogenic acellular vaccines in Japan. In the developed countries, major pertussis epidemics have recently occurred, and several studies have identified some of the disadvantages of DTaP vaccines comprising the small number of antigens, (3-5) in DTaP vaccines versus >3000 in DTwP vaccines, and the type of cellular immune response (Th-2) as to showing less efficacy and shorter duration of protection (3, 4). Pertussis toxin (PT), as the main antigen, as well as pertactin and fimbriae, play a role in protecting against pertussis, but filamentous hemagglutinin alone fails to provide protection (5). Further, as asserted by a Swedish cohort vaccine study, low or undetectable levels of IgG-anti-PT is assumed to be the best predictor of susceptibility to reinfection (6). Moreover, the Gothenburg study reported the induction of anti-PT induced good protection utilizing a PT-only vaccine (7). Seroepidemiologic studies on humans have also shown pertactin and filamentous hemagglutinin antibodies arising independently of pertussis infection and not inhibiting pertussis (8). Present findings, however, assert that PT, particularly detoxified genetically, represents the main antigen that ascertains protection against disease even if it is not associated with infection. Another finding demonstrated the optimal pertussis vaccine as being the one triggering both mucosal and systemic responses similar to those occurring under natural infection, thus contributing to a long-term protection from both disease and infection (9). For vaccinology, PT inactivation and stabilization method appears to be important for the reason that altering the molecular structure of PT, via chemical modifications, heat or genetically, has the potential to alter its antigenicity. Due to the fact that the epitopes of PT are conserved in the genetically modified PT, as opposed to being destroyed in the chemical inactivation process, today, genetically inactivated PT has gained preference over its chemically inactivated type. This inactivation method triggers the use of lower antigen doses, saving both the antigenic effect and immunogenicity and 3D structure of protein configuration (10). Regardless of the problems around the production of combination vaccines, some concerns may emanate from possible interferences between antigens. For the reason that the safety and effectiveness of the combination vaccines may be more complicated to monitor and regulate compared with single-component vaccines, regulatory concerns have arisen from their use (11). As has been suggested by the immunologic theory, the simultaneous exposure of the immune system to multiple conjugate antigens bears the capacity to induce either elevated or suppressed immune responses. The assumption for the occurrence of suppression lies in giving specific carrier for a polysaccharide more than once, a phenomenon which is called carrier-induced epitopic suppression. It has also been suggested the need for sufficient post-marketing surveillance plans are changed from non-combined to combined vaccines (12). Concerning its high efficacy, pertussis toxin (PT-only) vaccine has been utilized in Denmark for 15 years. On balance, combination vaccines, when compared to the

injection of their antigens separately, bear slightly more local side-effects (13). Being a T helper-2 (Th-2) cell activator, aluminum adjuvant (known as alum) secretes interleukin 4 (IL-4) cytokine that triggers B-cells to generate neutralizing antibodies; however, it is rather ineffective against pathogens like pertussis which require Th-1 cell-mediated immunity (14). Given the limitations of aluminum adjuvants to elicit cell-mediated immune responses, particularly for bacterial diseases in which cell-mediated immune responses are critical for treatment or prevention, a need to develop alternative vaccine adjuvants against specific antigens is felt (15). Adding microbial components, in order to stimulate strong immune responses, into subunit vaccine formulations is regarded as one such approach for generating new immunopotentiators. Known as pathogen-associated molecular patterns (PAMPs), these molecules, e.g., Toll-like receptors (TLRs), are generally conserved components from pathogens (e.g. nucleic acids, flagellin, and cell wall components) which fail to develop in humans (16). A host of probes has reported the fusion of antigens with TLRs to generate significantly higher potent immune responses compared to antigen-adjuvant simple mixtures (17-20). This arises from the fact that antigen-adjuvant conjugation helps to ensure the delivery and uptake of both components by the same antigen-presenting cell (20-22), ensuring the antigen and adjuvant presence in the same phagosome; this promotes optimal major histocompatibility complex (MHC) class II presentation of antigens and stimulation of Th cell responses (23,24). Antigen-presenting cells (APCs) are better stimulated in vaccines infused with both the antigen and adjuvant, and provide a higher immune response than non-fusion (mixture) vaccines accordingly reducing the dose of these components and thus the risk of side effects (25). In this study, therefore, efforts were made to explore the cytokine response to a genetically inactivated protein containing the S1 subunit of PT (PTS1) with and without the Listeriolysin O (LLO-PTS1) as a TLR-4 agonist (26), compared with current wP and aP vaccines in the mouse model.

MATERIALS AND METHODS

Animals. Thirty-six female Balb/C, inbred mice aged 8 to 12 weeks (25 ± 5 g) were obtained from Pasture Institute in Tehran, Iran, and cared for in the Animal Care facilities at Yazd Reproductive Sciences Institute in Yazd, Iran. Pellet chow and tap water were provided for the *ad libitum*. All animals were housed in polypropylene cages, temperature ($21 \pm 2^\circ\text{C}$), relative humidity (30-40%), and 12-h/12-h light-dark cycle. In addition, all protocols were conducted according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

After ordering, gene cloning, expression of the S1 subunit of PT in the *Shuffle T7* strain of *E. coli* and purification, the MTT assay was done to ensure the safety of the synthesized protein developed by the researchers (the authors of this study) in two types of PTS1 and LLO+PTS1(LLO-PTS1). A report of the first phase of this study is being published (27). The present report describes the second phase of our study to investigate the immunogenicity of these proteins in the mice model. The mice were randomly divided into six groups (n=6/each), control group did not receive anything, Sham group received Phosphate Buffered Solution (PBS), PTS1 group received PTS1 protein (35 μ l; 0.1mg/ml) + 35 μ l complete ferund adjuvants (CFA) for first injection and 35 μ l incomplete ferund adjuvants (IFA) for next injections, LLO-PTS1 group received LLO-PTS1 protein (35 μ l; 0.05mg/ml) + 35 μ l CFA for the first injection and 35 μ l IFA for

next injections, aP group received aP vaccine (3.5 μ g PT=70 μ l, Infanrix, GSK, USA), wP group received wP vaccine (0.5 IU:70 μ l, DTwP-Pasteur, Iran). Injections were done intraperitoneally three times with three-week intervals. Two months after the first injection, mice were anesthetized with a mixture of Ketamine (10 mg/ml) and Xylazine (0.1 mg/ml) and blood samples were collected from the heart cavity. After clotting, the serum was separated by centrifugation at 3000 g for 10 min in a refrigerated centrifuge. The serum levels of interleukin 17 (IL-17), IL-4, and Interferon-gamma (INF- γ) cytokines were evaluated by mouse R&D systems® ELISA Kit.

Ethical Considerations. All ethical considerations related to working with laboratory animals in this study were considered and followed in accordance with the national ethics protocol. The research proposal was reviewed and approved by the Ethics Committee of Yazd Shahid Sadoughi University of Medical Sciences in Yazd, Iran (Code: IR.IUMS.REC.1394.25818).

Statistical Analysis. Data were analyzed using Statistical Package for the Social Sciences (SPSS) software version 21. Mean and standard deviation of cytokines were used to evaluate the efficacy of standard vaccines (aP and wP), as well as researcher-made proteins (PTS1 and LLO-PTS1). Data were analyzed using One-way ANOVA test and Post hoc (LSD) analysis. P-value < 0.05 was considered statistically significant. Standard vaccines indices were used as a comparison arm to compare the vaccine efficacy. Superiority approach analysis was first performed after which non-inferiority analysis was considered. By definition, a non-inferiority trial aims to confirm the test drug or vaccine as being not worse than the comparator by more than a small pre-specified amount. This amount is known as the non-inferiority margin. If the difference between the new vaccine and the standard vaccine does not exceed this prespecified margin, non-inferiority can be confirmed. Non-inferiority analysis should be based on clinical and statistical methods. Three methods are frequently used to analyze non-inferiority: The fixed-margin, point-estimate and the synthesis method. We analyzed non-inferiority in this trial with the point-estimate method using the immune biomarker difference (28).

RESULTS

Totally, 36 mice were studied in six groups: PTS1, LLO-PTS1, aP vaccine, wP vaccine, control, and sham. Based on the results, immunization with LLO-PTS1 contributed to a statistically significant increase in IL-4 and IL-17 compared to the other groups (superiority) but PTS1 could not stimulate the secretion of any of these cytokines compared to aP, wP vaccines. In addition, INF- γ did not significantly increase in the LLO-PTS1 group compared to others, but it was non-inferior to standard vaccines (non-inferiority) (Table 1-3).

As for the data under analysis, one can calculate a lower limit of CI of standard vaccine and sham group difference of immune biomarker of INF- γ (M1; -38.9) and an alternative non-inferiority margin (M2;-18.29) based on 50% preserved fraction of the point estimate of M1 (Figure 1). The Δ or non-inferior margin was considered using the lower limit of a standard deviation of INF- γ changes compared to the control group. Non-inferiority analysis showed that INF- γ was in the Δ interval in LLO-PTS1 group and was non-inferior (Table 4).

Table 1. Comparison of the mean of IL-4, IL-17, and IFN- γ in the study groups.

Cytokines	Case groups				Control groups		P-value
	PTS1 (n=6)	LLO-PTS1 (n=6)	wP (n=6)	aP (n=6)	Sham (n=6)	Control (n=6)	
IL-4	23.38 \pm 8.37	44.88 \pm 13.98	26.83 \pm 9.22	24.33 \pm 13.47	28.51 \pm 12.63	24.38 \pm 7.96	0.02
IL-17	19.24 \pm 7.02	36.95 \pm 19.75	18.72 \pm 6.98	20.37 \pm 8.61	21.84 \pm 10.50	18.20 \pm 5.93	0.05
IFN-γ	29.50 \pm 7.52	25.84 \pm 5.82	26.85 \pm 7.25	30.25 \pm 11.02	28.74 \pm 9.40	25.21 \pm 2.58	0.83

One-way ANOVA test, PTS1: S1 subunit of pertussis toxin; LLO-PTS1: PTS1 with Listeriolysin O; wP: Whole-cell pertussis vaccine; aP: Acellular vaccine, IL: Interleukin; IFN- γ : Interferon-gamma

Table 2. Mean Difference (I-J) \pm SE of IL-4, IL-17, and IFN- γ in LLO+PTS1 group in comparison with other study groups.

LLO-PTS1	Other groups	IL-4	p-value	IL-17	p-value	IFN- γ	p-value
	PTS1	21.50* \pm 6.85	0.004	17.71* \pm 6.72	0.014	-3.65 \pm 4.66	0.440
	wP	18.05* \pm 6.85	0.014	18.23* \pm 6.72	0.012	-1.00 \pm 4.66	0.830
	aP	20.56* \pm 6.53	0.004	16.58* \pm 6.41	0.015	-4.41 \pm 4.44	0.330
	Control	20.50* \pm 6.53	0.004	18.75* \pm 6.41	0.007	0.63 \pm 4.44	0.888
	Sham	16.37* \pm 6.85	0.024	15.10* \pm 6.72	0.033	-2.89 \pm 4.66	0.539

One-way ANOVA and Post-hoc test, PTS1: S1 subunit of pertussis toxin; LLO-PTS1: PTS1 with Listeriolysin O; wP: Whole cell vaccine; aP: Acellular vaccine, IL: Interleukin; IFN- γ : Interferon-gamma

Table 3. Mean Difference (I-J) \pm SE of IL-4, IL-17, and INF- γ in PTS1 group in comparison with other study groups.

PTS1	Other groups	IL-4	p-value	IL-17	p-value	IFN- γ	p-value
	LLO-PTS1	-21.50* \pm 6.85	0.004*	17.71* \pm 6.72	0.014	3.65 \pm 4.66	0.440
	wP	-3.45 \pm 7.16	0.633	0.52 \pm 7.02	0.942	2.64 \pm 4.86	0.591
	aP	-0.942 \pm 6.85	0.892	-1.12 \pm 6.72	0.868	-0.75 \pm 4.66	0.872
	Control	-0.99 \pm 6.85	0.885	1.04 \pm 6.72	0.878	4.28 \pm 4.66	0.366
	Sham	-5.12 \pm 7.16	0.480	-2.60 \pm 7.02	0.714	0.75 \pm 4.86	0.878

One-way ANOVA and Post-hoc test, PTS1: S1 subunit of pertussis toxin; LLO-PTS1: PTS1 with Listeriolysin O; wP: Whole-cell vaccine; aP: Acellular vaccine, IL: Interleukin; IFN- γ : Interferon-gamma

DISCUSSION

Pertussis is still considered a serious threat to public health in developing countries with high morbidity in the elderly and serious complications even death in infants and children (29). Routine whooping cough vaccines can neither be used until 6 to 8 weeks of age, nor they provide lasting immunity in people aged over 7 years, such that we can witness a rapid decrease in immunity in people vaccinated in the community. Accordingly, this heightens the number of carriers, the circulation of bacteria in the

community as well as its transmission to sensitive groups. A need, therefore, is sensed to produce a new vaccine that can be used at all ages and as a booster. Serum antibody titers are, on the other hand, inadequate to provide significant immunity, and serum levels of virulence-neutralizing antibodies wane rapidly following infection or vaccination against pertussis (5,30,31).

Table 4. IFN- γ Changes in aP and wP groups in comparison of the sham group.

Groups	IFN- γ changes \pm SD	Δ
aP vs. sham	1.51 \pm 19.8	-18.29
wP vs. sham	-1.9 \pm 10.8	-8.9

All data present as Mean \pm SD, IFN- γ : Interferon-gamma; wP: Whole cell vaccine; aP: Acellular vaccine

In this study, therefore, we made an attempt to examine the cell-mediated immune response of a genetically inactivated protein-containing PTS1 alone and in combination with LLO (LLO-PTS1), developed by researchers (the authors of this study), to compare with current wP and aP vaccines in the mouse model.

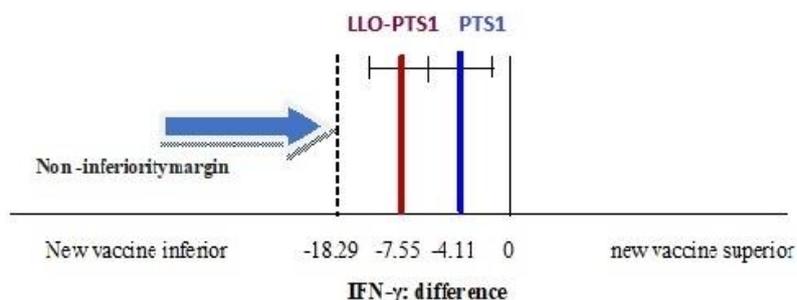


Figure 1. Non-inferiority margin M2 in IFN- γ . Non-inferiority margin based on 50% preserved a fraction of the point estimate of M1 was (M2; -18.29) about LLO-PTS1 and PTS1. The Δ or non-inferior margin was considered using the lower limit of a standard deviation of IFN- γ changes compared to the control group.

Upgrading current pertussis vaccines can be initiated in two steps; eliminating unnecessary components of the vaccine (3) and, improving the quality of the main component of the vaccine, i.e. PT, including a genetically nondenatured, detoxified mutant (13); this has been shown to stimulate the immune system better than chemically inactivated PT with a lower dose (one-fifth) (8). Evidence from this research reveals LLO-PTS1 significantly increased IL-17 and IL-4 in comparison to PTS1 and routine pertussis vaccines including wP and aP. Moreover, although IFN- γ in the LLO-PTS1 group did not increase significantly in comparison with others, it was non-inferior to standard vaccines. Note that immunity is achieved via wP vaccines by Th-17 (IL-17) and Th-1 (IFN- γ) or by cells that activate neutrophils and macrophages. The aP vaccines commonly used in the modern world to contain 3-5 pertussis antigens have been

successful in stimulating Th-17 and Th-2 cells, but their weakness is in stimulating Th-1 cells and providing long-term immunity (32). Of course, the fusion protein designed by the researchers in this study (LLO-PTS1) exhibited a higher immune response compared with routine wP and aP vaccines. Moreover, our results illustrated that the PTS1 protein lacking adjuvant stimulates the humoral immune system (IL-4) similar to standard vaccines; however, the IL-4 means in the LLO-PTS1 group compared to others and even the wP group stood significantly higher. This represents the superiority of this protein for stimulating the humoral system compared to routine vaccines. IFN- γ increased in the PTS1 as in the aP group and was not weaker compared to the wP group. Further, although the LLO-PTS1 fusion protein failed to significantly boost IFN- γ compared to wP and aP vaccines, in comparison with standard vaccines it was not inferior (non-inferiority). On balance, approximately 20 years of research on mice models have espoused the key role of Th-1 and Th-17 cells in immunogenicity against pertussis while the role of Th-2 is less frequently discussed (33). In the last decade, a study has revealed that wP vaccines stimulate Th-1 / Th-17. IFN- γ also greatly triggers immunity by wP vaccines, however, with a smaller role of Th-17 cells. On the contrary, aP vaccines stimulate Th-2 / Th-17 in which IL-17 plays a key role whereas IL-4 was not essential for bacterial removal (34). Importantly, a critical difference in T-cell immunity has been identified: wP-vaccinated animals, similar to infected baboons, had their own Th-17 and Th-1 cells specific to *Bordetella Pertussis*(BP) while vaccination with aP vaccines stimulated Th-1/Th-2 response; this is in agreement with the studies on mice. Interestingly, baboons vaccinated with aP vaccines are also protected against the disease but not against bacterial colonization. Transmission of BP to unvaccinated contacts occurred in those animals that were unable to induce a rapid clearance of infection. Conversely, wP vaccination cleared the lungs rapidly. Therefore, it has been hypothesized that the failure of aP vaccination to contain bacterial colonization in the airways and its subsequent transmission could be a possible explanation for the resurgence of pertussis in a world of high-coverage vaccine (35). Numerous studies have attempted to assess the immunity decline in a wide range of 2 to 30 years by determining the duration of protection (36-39). Studies on vaccine-induced T-cell stimulation have shown the pivotal role of natural booster infection for maintaining protection despite the reduction in humoral response (40,41). Today, it can be hypothesized that a decrease in bacterial circulation contributes to a reduction in the natural booster effect in high-coverage vaccinated populations. For pertussis, some new vaccines are developed and supported, but whether we really need to replace current vaccines or whether a modification is sufficient is still controversial (42,43). An in-depth knowledge around the immune response of T-cells to BP may clearly assist in finding a response to this critical issue. Although consensus exists as to generating novel vaccines to stimulate an immune response by a mixture of Th-1 and Th-17, the path to this goal is not yet known. It is not clear at this point how to shun tissue damage resulting from severe stimulation of Th-1 and Th-17. Further investigations are needed to regulate T-cells response to BP. There are several suggestions most of which underline the use of new adjuvants such as a TLR4-agonist like LLO that activate dendritic cells and this interaction activates Th-1 response. Nowadays, alum is prescribed as an adjuvant in aP vaccines, and although it has a long background as to its safety in humans, it stimulates Th-2 cells and antibodies in the human body. Recent studies have identified that wP or natural infection vaccines, which provide relatively high levels of immunity, stimulating Th-1 and Th-17 responses whereas aP triggers Th-

17 and Th-2 cells in mice (32). Therefore, the introduction and replacement of new adjuvants stimulating Th-1 cells can be successful in creating better protection from pertussis than an alum-formulated vaccine. In the present study, we could accomplish this by replacing alum with LLO as an adjuvant. Although our results demonstrated better stimulation of Th-2 and Th-17 through the researcher-made protein and similar results were obtained in Th-1 stimulation compared to a mice-model vaccine, this alum free mono-component monovalent recombinant fusion protein may enjoy the potential to generate better outcomes in Th-2 and Th-17 stimulation compared to routine vaccines by containing lower vaccine doses and antigen levels. In conclusion, our alum free mono-component monovalent recombinant fusion protein (LLO-PTS1) was able to stimulate the Th-1 response similar to wP and aP vaccines (non-inferiority) in mice model. In addition, it had better results in Th-17 and Th-2 response (superiority). This study can be regarded as a springboard for further probes in booster pertussis vaccine development.

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