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Novel adjuvants derived from attenuated lipopolysaccharides and lipid As of purple non-sulfur photosynthetic bacteria

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ABSTRACT

Adjuvants are substances that enhance adaptive immune response to antigen. Development of a safe and effective immunostimulant adjuvant is essential for the efficacy of a vaccine to protect against infectious pathogens. Purple non-sulfur photosynthetic bacteria exhibited nontoxic natural lipid A variants that are distinct in their chemical structures from that of the *Escherichia coli*-type lipid A. In this study, the adjuvant efficacy of attenuated lipid A variants and their corresponding lipopolysaccharides (LPSs), derived from purple photosynthetic bacteria (*Rhodocyclus tenuis* and *Rhodobacter sphaeroides*) were evaluated. LPS was extracted using modified phenol-chloroform-petroleum ether method and lipid A was separated by mild acid hydrolysis. Trinitrophenol (TNP) was conjugated to hen egg albumin (TNP-HEA) and used as haptenic antigen. The LPS and lipid A adjuvant candidates were formulated in oil-in-water emulsion (OIWE) and evaluated to elicit anti-TNP IgG against TNP-HEA conjugate in BALB/*c* female mice. The anti-TNP IgG titers were measured using ELISA. The intact LPS-based adjuvants present in OIWE formulation showed significantly higher efficacy to elicit anti-TNP IgG titers against TNP-HEA conjugate compared to their corresponding lipid A-based adjuvants. As expected, the OIWE formulations of all LPS- and lipid A-based adjuvant candidates showed higher activities compared to the aqueous formulations. Slow reduction in the levels of anti-TNP IgG antibodies in the serum was observed over 4 months after immunization using the LPS- and lipid A-based adjuvant candidates which may provide a long protection against pathogens. The attenuated LPSs and lipid A's from the photosynthetic bacteria showed promising results to develop novel safe and effective adjuvants that can evoke the immune response. The most promising adjuvant candidate was the LPS-based adjuvant from *R. tenuis*.

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1. Introduction

The efficiency of a vaccine to protect against disease depends on its ability to induce an appropriate immune response of sufficient intensity. Adjuvant is an essential component in the vaccine formulation that can stimulate antibody production (humoral immunity) and/or activate T lymphocytes (cell mediated immunity). Several adjuvants are available for animal research and human use such as alum (aluminum salts), complete Freund's (CFA), CpG DNA, Quil A, O-deacylated lipopolysaccharide (O-deacyl-LPS), monophosphoryl lipid A (MPL), and Adjuvant Systems (AS) group [1–6]. To improve the efficacy of the adjuvant, the adjuvant might be combined with another immunostimulatory agent such as MPL/trehalose dicorynomycolate (TDM) [7], MPL/aluminum salts that are present in AS04 [8], and MPL/QS-21 which present in AS01 and AS02 [2,8].

LPSs are the major cellular components of cell walls of almost all Gram-negative bacteria [9,10]. They are present in the outer leaflet of the outer membranes. The enterobacterial LPSs are potent adjuvants for protein antigens. Nevertheless, their toxicity prevents their use as adjuvant [11]. LPS is generally composed of three distinct parts: lipid A, core region, and O-specific chain. Lipid A is embedded in the outer membrane of Gram-negative bacteria responsible for the endotoxic properties. Core region is composed of oligosaccharide and connecting lipid A to O-specific chain, while O-specific chain extends from the surface of the bacteria and is

Abbreviations: Kdo, 2-keto-3-deoxyoctylosonic acid; LPS, lipopolysaccharide; OS, oligosaccharide; MPL, monophosphoryl lipid A; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; FA, Freund's adjuvant; CFA, complete Freund's adjuvant; AS, adjuvant systems; PCP, phenol-chloroform-petroleum ether; ELISA, enzyme linked immunosorbant assay; TDM, trehalose dicorynomycolate; S-TDCM, synthetic trehalose dicorynomycolate; TNBS, 2,3,4-trinitrobenzenesulfonic acid; TNP, trinitrophenol; HEA, hen egg albumin; BSA, bovine serum albumin; TNP-HEA, trinitrophenol-hen egg albumin conjugate; TNP-BSA, trinitrophenol-bovine serum albumin conjugate; PBS, phosphate buffered saline; OIWE, oil in water emulsion; BBS, borate buffer saline.

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responsible for antigenic properties. The *Escherichia coli*-type lipid A structure represents a highly toxic form of bacterial lipid As. Mild acid hydrolysis cleaves the glycosidic phosphate of the reducing glucosamine of lipid A moiety [12], leading to production of an artificially modified non-toxic lipid A (MPL) that can be used as a potent immunostimulant [11]. Activity of MPL is attributed to its ability to activate antigen presenting cells and to induce synthesis of cytokines such as IFN-γ and TNF-α, and IL-2 [11,13]. Different structures from those of *E. coli*-type lipid A (lipid A variants) have been reported in lipid As of some phototrophic and chemotrophic bacteria [9]. *Rhodocyclus tenuis* lipid A has an extra (non-acylated) glucosamine substituting the free hydroxyl group at the 4 position of the reducing glucosamine [14], while, *Rhodobacter sphaeroides* lipid A has 3-oxo-fatty acid attached to the amide-linked group at position 2 of the reducing glucosamine [15]. These natural lipid A variants as well as their corresponding LPSs, showed attenuated toxicity [9].

Development of a new novel safe adjuvant is essential for vaccine efficacy to protect against infectious diseases [16]. Several new adjuvants were evaluated for their ability to increase the immunity of experimental vaccines in animal model. Some adjuvants appear safe and superior to alum [5]. For example, MPL, artificially modified lipid A derived from *Salmonella minnesota* R595, showed a reduced toxicity and was used as immunostimulant adjuvant [6,7,11]. Nevertheless, it is only approved to be used in human for specific cases [6,16].

In this study, the adjuvant-efficacy of the attenuated lipid A variants and their corresponding LPSs derived from *R. tenuis* and *R. sphaeroides* were evaluated in an attempt to find new adjuvant candidates that are safe and efficient to immunostimulants. The adjuvant candidates were evaluated in combination with synthetic trehalose dicorynomycolate (S-TDCM) present in oil-in-water emulsion (OIWE) and compared to the aqueous formulation for production of antibodies against haptenic trinitrophenol conjugated to hen egg albumin (TNP-HEA).

2. Materials and methods

2.1. Organisms and growth conditions

Bacterial strains (*R. tenuis* DSM 109 and *R. sphaeroides* DSM 158) were purchased from the German Collection of Microorganisms and Cell Culture (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)) (Braunschweig, Germany). Bacterial strains were heterotrophically cultivated in large scale (20 L each) using culture medium recommended by the supplier, under tungsten light and anaerobic conditions at 25 °C.

2.2. LPS extraction and lipid A preparation

LPS was isolated by a modified phenol-chloroform-petroleum ether (PCP) extraction method [17], and purified by repeated ultracentrifugation (105,000 g, 4 °C, 5 h). Lipid A was obtained from LPS by mild acid hydrolysis as described earlier [18]. Lipid A and LPS were converted to their monovalent cation salts by electrodialysis [19]. The electrodialyzed sample was neutralized with 0.1 M triethylamine and then freeze dried. MPL was prepared as described earlier [12].

2.3. Preparation of standard TNP-protein conjugates

TNP-HEA and TNP-BSA conjugates were prepared as described by Garvey *et. al.* [20]. The protein concentration in the dry sample was determined by Bradford method [21]. Stock solution of the conjugate (TNP-HEA conjugate) was prepared by dissolving an equivalent amount of 1 mg protein in 1 ml sterile saline and stored in dark at 4 °C.

2.4. Adjuvant preparation and mice immunization

Stock solution of OIWE was prepared by suspending 1.0 mg S-TDCM, 0.08 ml squalene (hexamethyl-tetracosahexane), and 0.008 monooleate (Tween 80) in 2 ml sterile saline, heated and mixed at 65 °C for 15 min. The OIWE-lipid A adjuvant from photosynthetic bacteria was prepared in a concentration of 0.5 mg of lipid A/1.0 ml OIWE stock solution, while the OIWE-LPS adjuvant was prepared in a concentration of 1.0 mg LPS/1.0 ml OIWE stock solution (ca. equivalent to 0.5 mg lipid A/1 ml OIWE stock solution). The TNP-HEA conjugate solution was emulsified in 1:1 ratio with the adjuvants (OIWE-lipid A, OIWE-LPS, Freunds, RIBI, OIWE alone, and alum), and shaken at 4 °C overnight [22]. Aqueous adjuvant formulations [13] were prepared in the same way except the adjuvants (attenuated lipid A, attenuated LPS, and MPL of *S. typhimurium*) were suspended in saline. Control treatments were also prepared by mixing the TNP-HEA conjugate solution with the same volume of saline. Complete Freund adjuvant was used in immunization and incomplete Freund adjuvant was used in booster injections. Groups of six (6–8 weeks old) female BALB/*c* mice were immunized on day 0 with the prepared TNP-HEA conjugate emulsified in one of the prepared adjuvant formulations as described above (200 µl/mouse were injected intraperitoneally (i.p.) at two sites (100 µl each). Mice were boosted on days 21 and 42 after immunization, bled on days 52, 82, 112, 142, and 172, and sera were collected and stored at −20 °C.

2.5. Enzyme linked immunosorbant assay (ELISA)

The sera titers raised against the TNP-HEA conjugates were measured by ELISA technique as described earlier [12,23], and TNP-BSA conjugate was used as coating antigen. The reciprocal dilution of serum producing an absorbance of 0.75 was defined as the titer.

2.6. Statistical analysis

The experimental data were analyzed using the software programs Prism 5 (Graph Pad). The statistical difference between groups were determined by student *t*-test (for two groups) or by one way ANOVA followed by Tukey's test (for more than two groups) [6]. The data were expressed as mean ± SE. A *P*-value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. LPS extraction and lipid A preparation

The photosynthetic bacterial strains were cultivated using culture medium as recommended by DSMZ under tungsten light and anaerobic conditions at 25 °C. The LPS samples were isolated in yields of 2–3% from dried bacterial cells using the modified PCP extraction method [17]. Partial acid hydrolysis of LPS samples afforded insoluble lipid As in yields of 20–30% and soluble oligosaccharides. Electrodialysis of LPS and lipid A samples afforded 80–90% of uniform salted forms with improved solubility.

3.2. Adjuvant activities

The attenuated LPS- and lipid A-based adjuvants derived from the photosynthetic bacteria were evaluated to stimulate the production of anti-TNP IgG antibodies in mice 10 days after the second

boost with TNP-HEA conjugate. The OIWE-LPS adjuvant derived from *R. tenuis* elicited significantly the highest level of anti-TNP IgG titer than all other treatments (Fig. 1). Followed by the OIWE-LPS adjuvants derived from *R. sphaeroides*. Interestingly, the OIWE-lipid A adjuvants from *R. tenuis* and *R. sphaeroides* elicited similar levels of anti-TNP IgG titers to Freund's adjuvants but higher than that of commercially available RIBI adjuvant (MPL derived from *Salmonella minnesota* R595 in OIWE formulation).

The activities of OIWE-LPS and OIWE-lipid A adjuvants from *R. tenuis* and *R. sphaeroides* to elicit anti-TNP IgG in plasma against TNP-HEA conjugate were compared to the commercially available adjuvants over 4 months after immunization. The levels of anti-TNP IgG titers elicited using OIWE-LPSs and Freund's adjuvants showed higher titers than that measured using RIBI and alum (Fig. 2). As expected, the lowest levels of anti-TNP IgG titers were reported for control saline and OIWE alone treatments.

3.3. Comparison of LPS and lipid A based adjuvants

The levels of anti-TNP IgG titers in plasma elicited against TNP-HEA conjugate vaccine emulsified with the OIWE-LPS adjuvants from *R. tenuis* or *R. sphaeroides* were compared to their homologous OIWE-lipid A adjuvants over 4 months after immunization. The levels of anti-TNP IgG titers elicited using OIWE-LPS adjuvant of *R. tenuis* showed significantly higher titers than its homologous OIWE-lipid A adjuvant (Fig. 3A). Nevertheless, no significant differences were observed in the levels of anti-TNP IgG titers between OIWE-LPS adjuvant and its homologous OIWE-lipid A adjuvant of *R. sphaeroides* (Fig. 3B).

3.4. Comparison of OIWE and aqueous formulations

The activities of the OIWE-LPS and OIWE-lipid A formulations, derived from *R. tenuis* and *R. sphaeroide*, were compared to their

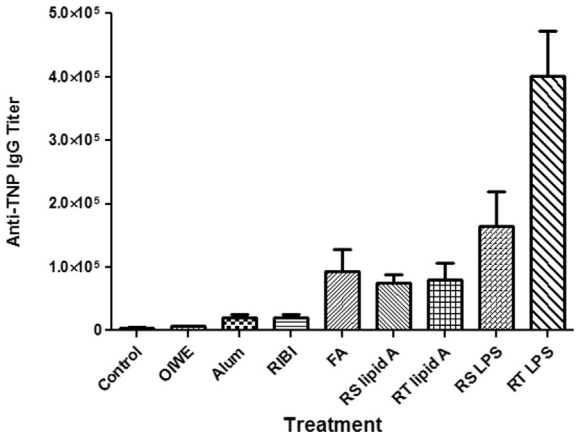

Fig. 1. ELISA of anti-TNP IgG titers elicited in BALB/*c* mice against TNP-HEA conjugate vaccine emulsified with OIWE-LPS or OIWE-lipid A adjuvants, prepared by mixing OIWE with one of the attenuated LPS or lipid A samples derived from *R. tenuis* or *R. sphaeroides*, and compared to the commercially available adjuvants alum, RIBI, and Freund's. Control: conjugate in PBS as a negative control; OIWE: conjugate emulsified with OIWE alone; Alum: conjugate emulsified with alum; RIBI: conjugate emulsified with commercial RIBI; FA: conjugate emulsified with Freund's adjuvant; RT LPS or RT lipid A: conjugates emulsified with OIWE-LPS or OIWE-lipid A from *R. tenuis*, respectively; and RS LPS or RS lipid A: conjugate emulsified with OIWE-LPS or OIWE-lipid A from *R. sphaeroides*, respectively. Mice were immunized on day 0, boosted on days 21, and 42, and bled on day 52. The titer level is depicted as A_{405}, the reciprocal dilution of serum producing an absorbance of 0.75 was defined as the titer. Each point represents the ± standard error for 6 mice sera.

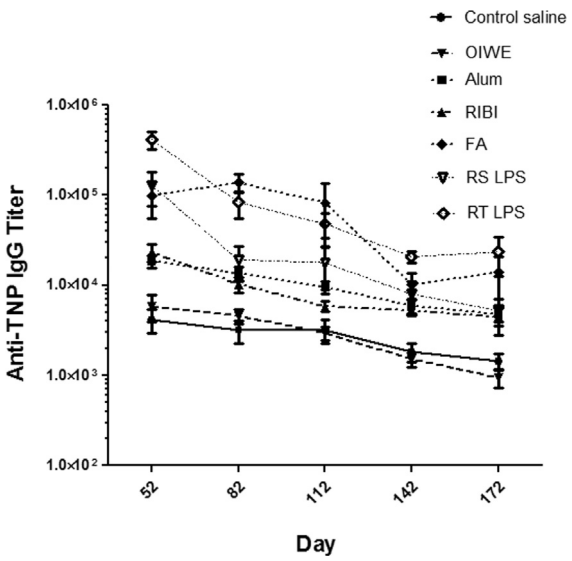

Fig. 2. ELISA of anti-TNP IgG titers elicited in BALB/*c* mice against TNP-HEA conjugate vaccine emulsified with OIWE-LPS adjuvants derived from *R. tenuis* or *R. sphaeroides*, and compared to that of the commercially available adjuvants alum, RIBI and Freund's. Mice were bled on days 52, 82, 112, 142, and 172. Abbreviations, immunization, and assay measurements are the same as in Fig. 1.

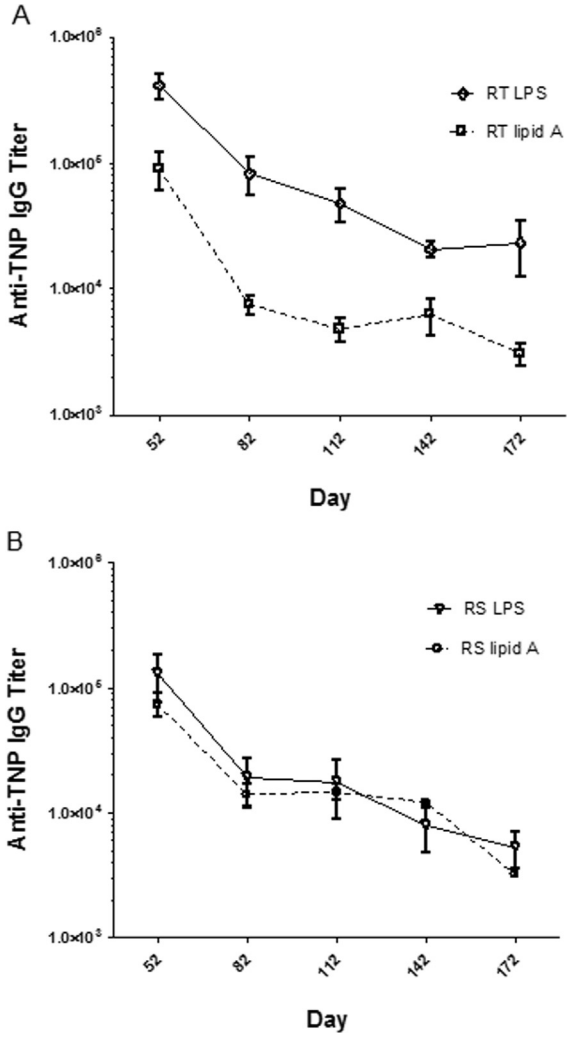

Fig. 3. Comparison the activities of OIWE-LPS and OIWE-lipid A adjuvants derived from *R. tenuis* (A) and *R. sphaeroides* (B), to elicit anti-TNP IgG titers in BALB/*c* mice against TNP-HEA conjugate vaccine. Abbreviations, immunization, and assay measurements are the same as in Fig. 1, bleeding is the same as in Fig. 2.

corresponding aqueous formulations. All OIWE adjuvant formulations showed significantly higher anti-TNP IgG titers, over 4 months after immunization with TNP-HEA conjugate, compared to their aqueous formulations (Fig. 4).

4. Discussion

Vaccines are the most effective way to protect against infectious diseases. Efficacy of protective vaccine depends on its ability to

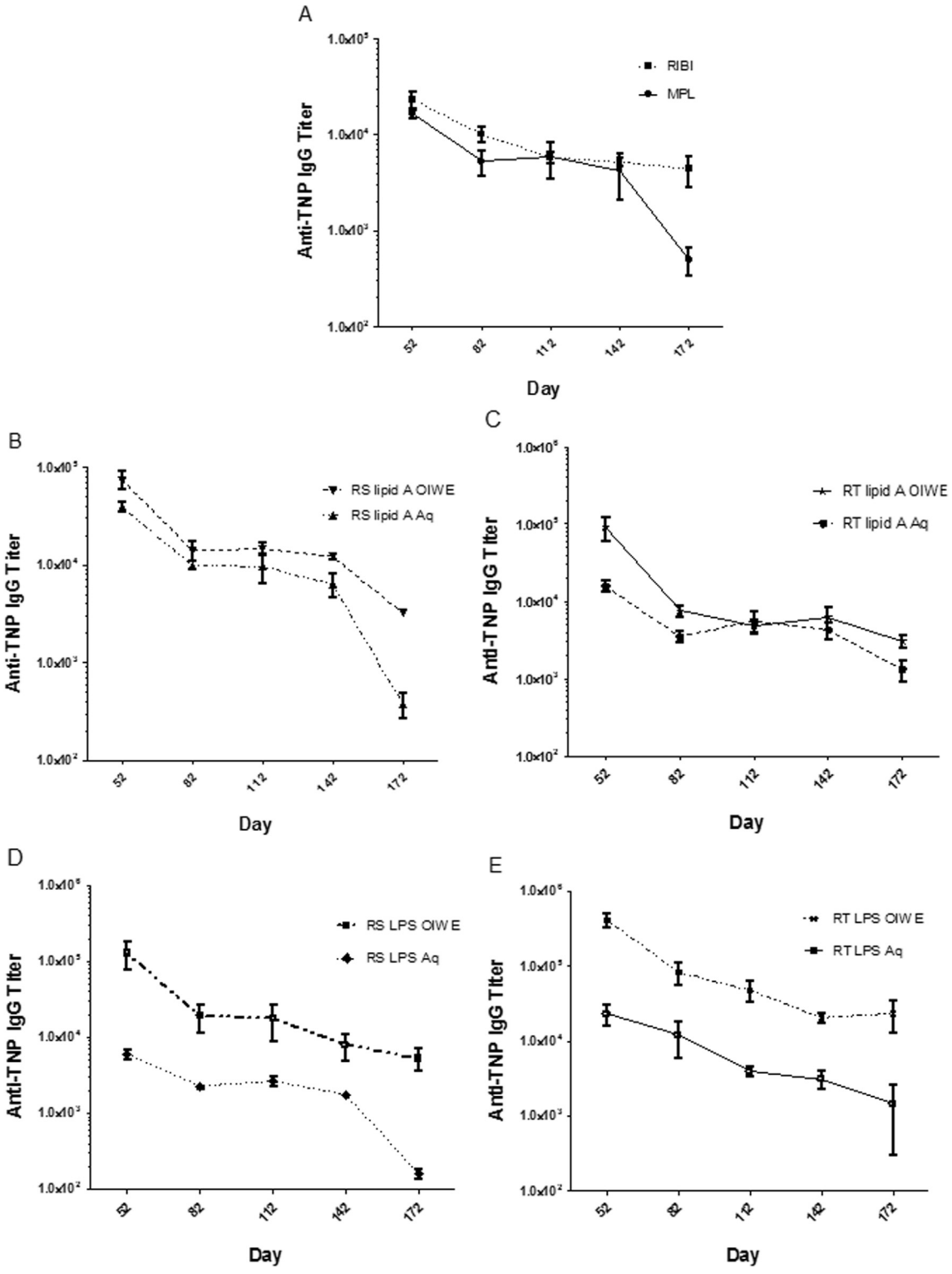

Fig. 4. Comparison the activities of the OIWE and aqueous formulations to elicit anti-TNP IgG titers in BALB/*c* mice against TNP-HEA conjugate vaccines. RIBI and MPL adjuvants (A), OIWE-lipid A and aqueous-lipid A adjuvants of *R. sphaeroides* (B), OIWE-lipid A and aqueous-lipid A adjuvants of *R. tenuis* (C), OIWE-LPS and aqueous-LPS adjuvants of *R. sphaeroides* (D), and OIWE-LPS and aqueous-LPS adjuvants of *R. tenuis* (E). Abbreviations, immunization, and assay measurements are the same as in Fig. 1, bleeding is the same as in Fig. 2.

stimulate robust and sustained immune responses. Such responses require both a suitable antigen and an appropriate adjuvant that stimulate the immune response of sufficient intensity. Several commercially available adjuvants that vary in their chemical nature and mechanism of action have been used on experimental animals, but their toxicities preclude human use [1]. Alum (aluminum salts) is the adjuvant that is approved by Food and Drug Administration (FDA) for use in humans. However, alum is less efficient to stimulate humoral and cell-mediated immunity compared to other commercially available adjuvants [5,12]. Several new adjuvants have been evaluated of which some (such as MPL) are now approved for selective use in humans in a few countries [6,16]. To improve immunological responses, MPL was combined with another immunostimulatory agent such as TDM [7], or with aluminum salts which is present in AS04 [8], or with QS-21 which is present in a liposome formulation such as AS01 or in emulsion-based formulations such as AS02 [2,8].

In this study, attenuated LPSs and their corresponding lipid A's from purple non-sulfur photosynthetic bacteria (*R. tenuis* and *R. sphaeroides*) were evaluated in an attempt to find potent adjuvants that are safe and effective to stimulate humoral immune response in mice. The adjuvant candidates were evaluated using OIWE formulation and compared with aqueous formulations, because various formulations significantly influence the type of the immunological response [8,13].

The LPS-based adjuvants derived from *R. tenuis* and *R. sphaeroides* in OIWE formulation showed high efficacy to stimulate the immune responses compared to the lipid A-based adjuvants. These results could be attributed to the presence of the inner-core in the intact LPS that triggers a signal to stimulate the immune system [24]. In addition, the saccharide moiety in the LPS increases hydrophilicity and might improve the solubility of the molecule. Previous studies have shown that intact non-toxic LPS had *in vitro* immuno-stimulatory activity, while no stimulatory activity was observed using chemically detoxified LPS [25]. The OIWE-LPS adjuvant candidate derived from *R. tenuis* was observed to be the most efficient adjuvant candidate to activate the immune system. This could be attributed to the chemical structure of the LPS, where the lipid A of *R. tenuis* has an extra (non-acylated) glucosamine substituting the free hydroxyl group at the 4 position of the reducing glucosamine [14], while *R. sphaeroides* lipid A has 3-oxo-fatty acids attached to the amide-linked group at position 2 of the reducing glucosamine [15]. In addition, Lebbar *et al.* [24] have reported that the minimum structure of the inner-core that induces IL-1 secretion is the hep-(1 → 5)-Kdo disaccharide which is present in the inner-core of *R. tenuis* [26] but not in the inner core of *R. sphaeroides* [27]. The lipid A-based adjuvants from *R. tenuis* and *R. sphaeroides* showed higher activity to stimulate the immune system than MPL-based adjuvant, which could be attributed to the removal of the glycosidic phosphate from the lipid A derived from *S. minnesota* R595 in MPL.

Slow reduction in the anti-TNP IgG titers, over 4 months after immunization, was observed using either LPS- or lipid A-based adjuvants. Similar results were observed using TiterMax adjuvant, where the duration of the antibody response has been related to the persistence of the antigen [1]. This indicates that the LPS- and lipid A-based adjuvants, used in this study, may provide prolonged high level of anti-TNP IgG antibodies in the serum, indicative of sustained protective response.

As expected, the OIWE formulations of LPS- and lipid A-based adjuvants derived from *R. tenuis* and *R. sphaeroides*, showed higher activities to elicit anti-TNP IgG compared to the aqueous formulations, which could be attributed to the synergistic interaction between the adjuvant candidate (attenuated LPS or lipid A) and the immunostimulatory agent (S-TDCM) [13]. This is in agreement with previous studies where MPL/TDM combination enhanced the adaptive response for production of antibodies [7]. Didierlaurent *et al.* [2] reported that AS01 activity depends on synergistic effect between QS-21 and MPL.

Attenuated LPSs and lipid A's from photosynthetic bacteria (*R. tenuis* and *R. sphaeroides*) showed promising results for adjuvant candidates that are safe and effective to evoke immune robust and prolonged responses in the development of protective vaccines. The most promising adjuvant candidate was the LPS of *R. tenuis* present in OIWE formulation. Further studies are needed to evaluate the optimal concentration of the LPS- and the lipid A-based adjuvant candidates, and also to evaluate their response with various vaccine candidates to stimulate protective immune responses against specific diseases. Large number of photosynthetic- and chemolithotrophic- bacteria have natural lipid A variants that are distinct in their chemical structures from that of *E. coli*-type lipid A [9]. Such lipid As (and their corresponding LPSs) are non-toxic and can be evaluated as potent adjuvant candidates.

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Conflict of interest

There was no conflict of interest to declare.

Ethics in publishing

All procedures performed in this study involving laboratory animals were in accordance with the ethical standards of the national and the University of Jordan guidelines for the care and use of animals. This work does not contain any studies on human.

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