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Novel Mannosylated Synthetic Saponin as Vaccine Adjuvant for Recombinant Japanese Encephalitis Antigen

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Abstract

A novel mannosylated trisaccharide based synthetic saponin, bearing a trisaccharide cluster linked to olanaldehyde was used as vaccine adjuvant. The novel saponin glycolipid was found to be non-hemolytic as compared to QS 21. The effectiveness of the synthesized molecule in terms of targeting dendritic cells was confirmed using mannose inhibition assay. Adjuvanting ability of the molecule was quantified using recombinant Japanese encephalitis antigen in BALB/c mice model. The *in vivo* immunological evaluation of the molecule has elicited a potent humoral (20 folds compared to alum) and cellular response (enhanced $CD8^+$ response). The overall data from *in vivo* experimentation suggests the effectiveness of the synthesized molecule in terms of less toxic, dose sparing without compromising in the immune response and in addition eliciting Th1 response together with cell mediated immunity (CTL) - the most essential immune response traits of a good vaccine candidate against viral diseases.

Keywords: Mannosylated adjuvant; Receptor targeting; Japanese encephalitis antigen; Th1 response; Cell mediated immunity; Humoral response

Abbreviations

WHO: World Health Organisation; JEV: Japanese Encephalitis Virus; TMB: 3,3',5,5'-Tetramethylbenzidine; IL: Interleukin; Ig: Immunoglobulin; EDTA: Ethylene Diamine Tetra Acetic Acid; BSA: Bovine Serum Albumin; APC: Antigen Presenting Cell; DC: Dendritic Cell; PRR: Pattern Recognizing Receptors

Introduction

Japanese encephalitis virus (JEV) is an important arboviral infection belonging to Flaviviridae family, and related to dengue, yellow fever, West Nile viruses etc, highly prevalent in Asia [1]. Human along with other mammals serve as natural host and are the main cause of viral encephalitis. It is transmitted to humans through infected *Culex* species mosquitoes. Once humans are infected, they do not develop sufficient viraemia to infect mosquitoes. Rather the virus exists in a transmission cycle between mosquitoes, pigs and/or birds that is why this disease is largely found in rural settings [2]. According to WHO an estimated 68,000 clinical cases are reported every year. Mostly infections are mild with fever and headache or with no symptoms, but 1 in 250 results in severe clinical illness [2]. Though it's rare but permanent neurologic or psychiatric sequela occurs with 30-50% fatality. The major issue in Japanese encephalitis is that there is no cure and the treatment is focused on reducing clinical symptoms, so that the infection can be kept under control. Moreover, as the highest case record is from Asian countries where the best method to eradicate, such viral infections would be through mass immunization. Especially for people who plan to travel to Japanese encephalitis virus endemic areas, vaccination becomes a boon [3].

There are 4 different vaccines currently in use [4,5] and the major drawbacks of each has been listed in Table-1. Thus, development of safer and efficacious vaccines are encouraged in the current scenario [6]. Moreover, it is always good to be prepared for the outbreak which generally occurs approximately once in fifteen years. Considering the economic status of endemic area which largely constitutes developing and underdeveloped countries, developing affordable vaccines using appropriate adjuvants meant for antigen dose sparing together with increasing the efficacy to derive qualitatively specific immune response is essential. Choosing a right combination of antigen and adjuvant would be mandatory to develop effective vaccines against Japanese encephalitis. Antigen

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Table 1: Existing Japanese encephalitis vaccines and their drawbacks[5].

S.No	Type of vaccines	Drawbacks	Reference
1	Inactivated mouse brain-derived vaccines	Inadequate duration of protection, needs multiple doses, some countries the price per dose is high	WHO http://www.who.int/biologicals/areas/vaccines/jap_encephalitis/en/
2	Inactivated Vero cell-derived vaccines	Side effects reported – fever, loss of appetite, ecchymosis, nausea	[6]
3	Live attenuated vaccines	Anaphylaxis, arthus reaction, angioedema, idiopathic thrombocytopenic and henoch-schonlein purpura, urticarial, etc.	
4	Live recombinant vaccines	Diarrhea, nasal congestion, sickness, injection site pain and erythema, pyalgia, myalgia, nausea	

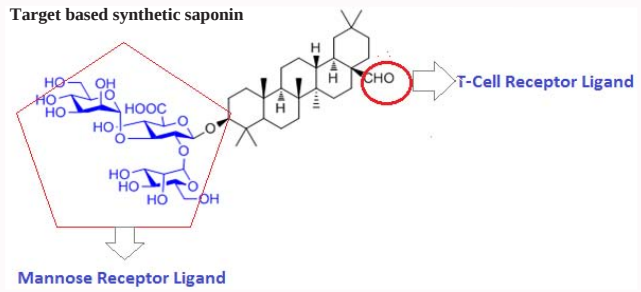

Figure 1: Structure of novel mannosyl trisaccharide based synthetic saponin (**MS**).

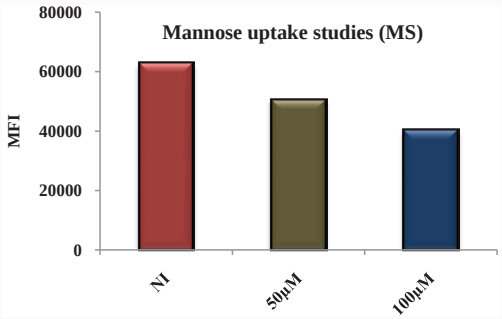

Figure 2: Mannose inhibition assay performed on dendritic cells. Quantification of the up take was performed using FACS. The test sample **MS** tagged with FITC suspended in medium containing various concentrations (no inhibition (NI), 50 and 100 µM) of free mannose were added to the cells. After 6 h, the uptake inhibition was quantified which is directly related to the mean fluorescence intensity of the samples analyzed.

delivery into antigen presenting cells (APCs) is a vital feature for an adjuvant. In this context, herein we report a combination of recombinant JE antigen together with a novel designer glycolipid adjuvant as vaccine composition. The design of adjuvant structure is inspired by the trisaccharide moiety of QS-21. It is composed of a β-D-glucuronic acid residue connected at 2 and 3-position with α-linked mannopyranosyl residues instead of β-D-galactose and β-D-xylose respectively as found in QS21. Lectins have been crucial players in mediating cell adhesion and migration [7]. Selection of mannose in the adjuvant design would mean targeting mannose receptor (lectins) located on the DCs leading to rapid internalization of antigen-adjuvant combination which is the primary goal of modern vaccine adjuvant development [8]. This trisaccharide moiety is linked β-glycosidically to the 3-position of a triterpene (oleonaldehyde) that encompass a aldehyde moiety as found in quillaic acid residue of QS21 which is responsible for interacting with T-cell receptor amine group through a Schiff's base to evoke cellular response. The overall design of the adjuvant structure is inspired by minimal structural component of QS-21, a clinically used adjuvant. This is an elegant structural design with an objective to target specific pattern recognizing receptors (PRR) on DCs and T-cells. Immunopharmacological and *in vitro* toxicological evaluation of a novel vaccine composition encompassing the oleonaldehyde based synthetic glycolipid adjuvant (compound MS) together with a recombinant JE antigen is discussed here (Figure 1).

Methods and Materials

Hemolysis

Blood was obtained from healthy mice by collecting it in k3 EDTA vacutainer (BD). The blood was transferred into Dulbecco's phosphate-buffered saline (DPBS) and centrifuged at 2000rpm for 10min, this washing step was repeated three times. Test compounds at 1000 µg mixed with DPBS were introduced into eppendorf tubes. Aliquots of 25 µL of erythrocyte suspension were added to the tubes and the volume was adjusted to 1ml with PBS. The tubes were then incubated at room temperature for 10min with constant rotation. After incubation, the tubes were centrifuged (2min at 5000rpm) and finally the percentage of hemolysis was determined by comparing the absorbance (λ = 577nm) of the supernatant with that of control samples hemolyzed with QS-21. From the hemolysis results, the concentration that induces 50% hemolysis of the erythrocytes (HD50) was subsequently calculated [11].

Mannose inhibition assay

Splenic dendritic cells were isolated using magnetic cell isolation kit (Pan DC enrichment Kit) and cultured using requirements in RPMI 1640 media for three days. $2x10^5$cells/well were cultured overnight in a 12-well plate, the medium was then aspirated and test sample **MS** tagged with FITC suspended in medium containing various concentrations (50 and 100 µM) of free mannose were added to the cells. After 6 h, the cells were washed carefully and hooked to BD FACS to quantify the uptake inhibition which is directly related to the mean fluorescence intensity of the samples analyzed [12].

In-vivo studies

The lead compound (**MS**) was studied in detail further using BALB/c mice. Female BALB/c mice (6-8 weeks) were purchased from Centre for Cellular and Molecular Biology (CCMB), Hyderabad. All animal experiments were approved by the ethical committee and animals were kept in accordance with the *Committee for the Purpose of Control and Supervision of Experiments on Animals* (*CPCSEA*) guidelines with an IAEC No *IICT/66/2016*. They were immunized at three different concentrations (2, 10 and 50 µg/dose) along with recombinant Japanese Encephalitis antigen 1 µg/dose, this dose optimization of the antigen was performed in-house [13] and alum (200µg) was given along with antigen as a positive control. A booster dose was given on 14th day and blood from the immunized mice was collected for serum and mice were sacrificed on 28th day.

Antibody titre by indirect ELISA (IgG, IgG1 and IgG2a)

Antigen specific antibody titers of the immunized mice were

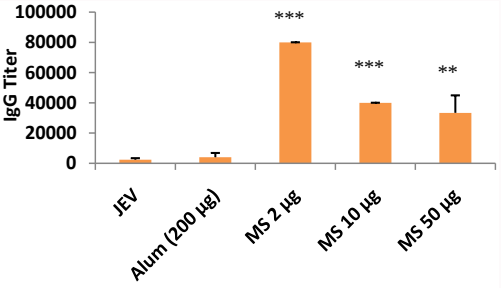

Figure 3A: Antibody titter of the *in vivo* immunized mice on 28th day using Indirect ELISA. The compound **MS** in comparison with that of the antigen alone and standard alum has elicited 20, 10 and 8.3 fold higher titer at 2 µg; 10 µg and 50 µg/dose respectively. *** = $p \leq 0.0005$, ** = $p \leq 0.005$, were considered statistically significant. Error bars represent mean ± SD (n = 4).

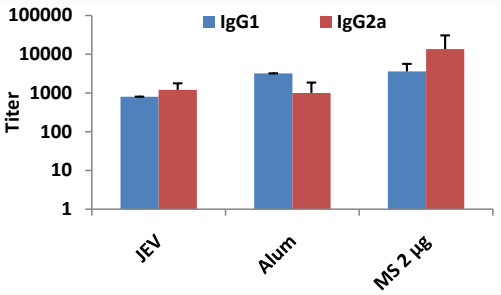

Figure 3B: Isotype titers of MS analogue compared with the standards elicited Th1 (IgG2a) response. Error bars represent mean ±SD. (n = 4).

estimated using the serum samples. 96 well micro titer plates were coated with JEV antigen recombinant (1µg/ml) in sodium carbonate buffer (pH 9.4) and left over night at 4°C. Wells were blocked with 1% BSA in PBS and incubated for 1h at room temperature (RT) for reducing non-specific binding. Then plates were washed with PBS and Tween 20 (0.05%) (Wash buffer) three to four times, followed by addition of two fold serially diluted serum samples. After incubation for 2hr at 37°C, plates were washed, added with horseradish peroxidase (HRP) conjugated anti mouse IgG, IgG1 and IgG2a and incubated for 1hr at RT. After incubation, plates were washed thrice, TMB substrate was added. Plates were incubated at RT for 4-30min, reaction was stopped by adding stop solution (2N H_2SO_4) and absorbance was determined at 450nm with multimode reader (Infinite pro - TECAN) [14].

Immunophenotyping

Single cell suspension from the spleens was prepared from the immunized and control mice after euthanization. Approximately $3x10^5$ cells were aliquoted into each FACS tube for staining. Staining was as per the manufacturer's protocol and acquisition was carried out using BD FACSVerse flow cytometer. Compensation was established using BD Biosciences compensation beads. Post acquisition flow cytometry analysis was performed using FACS Suite software [15].

Cytokine estimation

Sandwich ELISA was carried out by BioLegend capture and detection antibodies. Briefly, 96-Well plates were coated with capture antibody (1µg/mL) dissolved in coating buffer (carbonate buffer pH 9.4) per well incubated overnight at 4°C. Wells were blocked with 1% BSA for 1h at RT. After blocking, 100µL/well of supernatant of the restimulated splenocytes was added and incubated for 3h. After

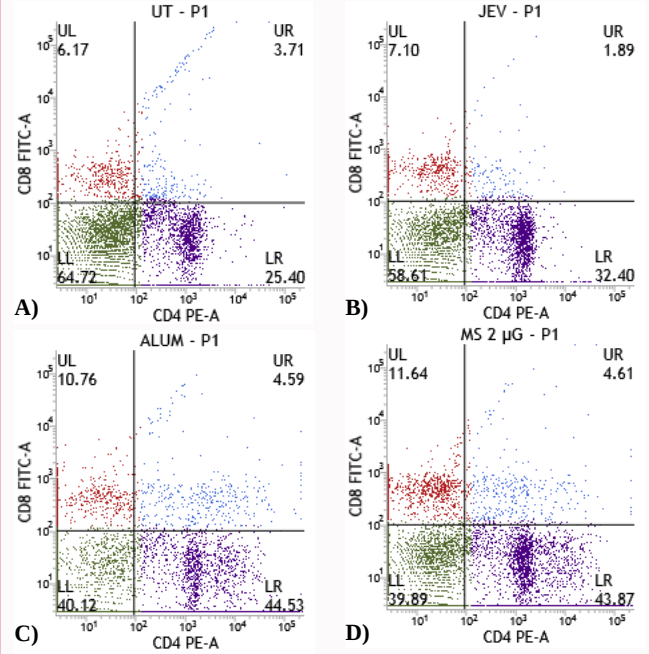

Figure 4: Immunophenotyping of the immunized mice splenocytes for quantification of $CD4^+$ and $CD8^+$ positive cells. Compared to antigen and standard alum, compound expressed enhanced $CD8^+$ (Cytotoxic T lymphocytes) response.

washing, biotinylated secondary antibody (1µg/mL) was added along with enzyme. Plates were incubated for 1h at RT. Then plates were washed and TMB substrate solution was added. The reaction was stopped after 30 min with a stopping solution. Absorbance was measured at 450 nm with plate reader [16].

Safety studies

We have examined for possible adverse reaction of effective dose. No weight loss was observed in mice when recorded on days 7, 14 and 28 days post injections (0th day). Mice were kept under observation for 28 days post-injection for any toxic manifestation and no visible symptoms such as inflammation, allergic response's or any other undesirable effects like granuloma, induration, edema etc. were observed after the treatment of saponin conjugates at various concentrations.

Statistical analysis

The statistical significance of the experiment was determined by two-tailed student's test in Excel. *** = $p \leq 0.0005$, ** = $p \leq 0.005$, were considered statistically significant. Error bars represent mean ± SD (n = 4).

Results and Discussion

Preliminary evaluation

Preliminary toxicity evaluation of the synthetic saponin analogue by hemolysis gives us a hint regarding safety of the molecule. Hemolysis of MS at 1000µg/mL was estimated, along with a positive control – QS-21 at 20µg/mL. It is very evident that MS has HD 50 of 637.5µg which is extremely non-toxic compared to QS-21 expressing at 12.85 µg. Receptor targeting studies as supportive information for our hypothesis was also performed (Figure 2) for the target specificity of the molecule. Mannose receptors on the dendritic cells were targeted with the analogue along with free mannose (at two different

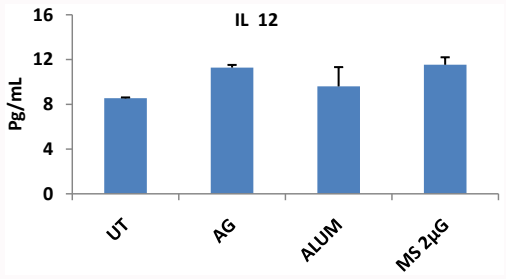

Figure 5: IL-12 Quantification from supernatant of immunized mice splenocytes after antigen restimulation where compound **MS** expressed IL-12 cytokine release. Error bars represent mean ± SD (n = 4).

concentrations) in a competitive inhibition assay and the result obtained proved that the molecule was taken up by DC's through the mannose receptor. MS was taken up for immunoprofiling studies on BALB/c mice at 2µg, 10µg and 50µg/dose with, recombinant Japanese encephalitis antigen (1µg/dose) along with standard alum (200µg/dose).

In-vivo evaluation

To evaluate the efficacy of the compound as an adjuvant quantification of its antibody titer is the preliminary requisition. The (Figure 3A) shows IgG titer of the compound MS (2µg, 10µg and 50µg/dose) in comparison with that of the antigen alone at (1 µg/dose) and standard alum (200 µg/dose). Our analogue MS has elicited 20, 10 and 8.3 fold higher titer at 2µg; 10µg and 50µg/dose respectively compared to the standard alum. MS analogue elicited a Th1 response (Figure 3B) evident from the isotype quantification, where IgG2a (Th1) was more profoundly expressed compared to IgG1 (Th2).

Additionally, cells from the treated mice spleen give us an insight of the surface markers such as $CD4^+$ and $CD8^+$. Compared to antigen and standard alum, compound expressed enhanced $CD8^+$ response (Cytotoxic T lymphocytes) and CTL cell activation is made evident with it in MS [10] (Figure 4). Similarly, spleen cells were also used to check the cytokine estimation by ELISA. IL-12 otherwise known as T cell stimulating factor has shown an enhancement in comparison to antigen and alum groups (Figure 5).

During the period of immunization mice were periodically observed for any granuloma, induration or edema, but none of the symptoms were observed physically till 28th day of sacrifice.

Conclusion

In conclusion, we have demonstrated for the first time, the usefulness of a novel oleonaldehyde based trisaccharide saponin glycosides containing β-linked glucuronopyranosyl and α-linked mannopyranosyl residues as vaccine adjuvant. *In vitro* haemolytic assay clearly indicated that mannosylated saponins are less toxic and the target specificity has also been proved. 20 fold higher antibody titer and optimal cytokine production indicated the high suitability of oleonaldehyde based saponin MS at the lowest concentration *i.e.* at 2 µg/dose as vaccine adjuvant. The observed higher efficacy of the new adjuvant entity compared to alum, may be attributed to the improved receptor targeting by the ligand (both phagocytic mannose receptor and T-cell receptors) effecting rapid uptake of antigens by DCs. These results provide a valuable entry to less expensive, easy to prepare analogues of mannosylated saponins as minimal structural QS-21 mimics with considerably less toxicity and enhanced immunological response ($CD4^+$ and $CD8^+$). The data shown above, gives clue regarding the potentiality of this molecule as vaccine adjuvant suitable for viral antigens and intracellular pathogens which require a potent Th1and cell mediated immune response.

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