Effects of a chemically derived homo zwitterionic polysaccharide on immune activation in mice

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In this study, a chemically modified homo zwitterionic polysaccharide (ZPS), sulfated chitosan, was used to examine its effects on murine immune response. The results showed that homoZPS could markedly promote the proliferation of both splenic T/B cells and adhesive cells. In particular, flow cytometry assay demonstrated that the sulfated chitosan could non-specifically activate CD3$^+$ and CD8$^+$ T cells proliferation in vitro. The effectiveness of sulfated chitosan as adjuvant was tested using bovine serum albumin (BSA) and diphtheria toxin (DT) as antigens and compared with that of aluminum hydroxide. The levels of specific antibodies to BSA and DT significantly increased after homoZPS vaccination. Both homoZPS and aluminum hydroxide adjuvants could protect mice against the attack of DT from edemas of spleen and tail. The present findings demonstrated the chemically derived homoZPS could be a potential candidate in the development of T-lymphocyte dependent vaccine adjuvants.

Keywords chemically derived homoZPS; sulfated chitosan; immune response; splenic cells; adjuvant

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Introduction

Carbohydrates in the form of capsular polysaccharide and/or lipopolysaccharide (LPS) on the cell surface are the main virulence factors in many bacteria isolated from infected persons, and the immune response to these components could confer protection to humans against the invasion of pathogens [1,2]. However, the development of vaccines based on polysaccharides is difficult due to several problems involved. First, most bacterial polysaccharides are T-lymphocyte independent antigens. Children below 2 years of age and the elders respond poorly to polysaccharide antigens because of immaturity or aging of the immune system [3]. Second, adaptive MHCII-mediated CD4$^+$ T cell activation has been considered to be strictly limited to protein antigens [4]. When presented to T cells by MHCII, peptide antigens generally elicit a T cell-dependent immune response typified by the production of Th1 or Th2 cytokines as well as IgG and the induction of immunologic memory. But the T-lymphocyte independent nature of a polysaccharide antigen may be overcome by conjugating polysaccharides to a protein carrier [5,6]. Such conjugates have been proven to be efficient in inducing T-lymphocyte dependent immunity as well as protecting infants and the elderly from infection [7].

Several researches reported that some polysaccharides can also activate CD4$^+$ T cells in vivo and in vitro in rodents [8–10]. Despite the difference in their chemical compositions and monosaccharide sequences, these polysaccharides that can activate T cells share a striking structural feature, that is, they are zwitterionic polymers that display a high density of both positive and negative charges. The presence of a zwitterionic charge motif within the repeating unit is a necessary characteristic for polysaccharides to activate T cells because these polysaccharides would not activate T cells when eliminating the alternating charge character of the carbohydrate. Conversely, neutral or negatively charged polysaccharides showed the ability to activate T cells or facilitate abscess formation when they are chemically altered to be zwitterionic [8]. It was demonstrated that zwitterionic polysaccharide (ZPS) could be presented by MHCII in B cells to engage T cells via interaction with αβTCRs after oxidation by nitric oxide, acidification, and processing in vivo [11]. These studies collectively suggest that ZPS is a T cell-dependent antigen.
All reported ZPSs that showed special immune characteristics belonged to hetero-polysaccharides from bacterial capsule, and few researches proved whether the homoZPS composition of monosaccharide residues had a similar effect on immunogenicity as heteroZPS. Though Tzianabos et al. [8] reported that chemically modified zwitterionic charge capsular polysaccharide from Bacteroides fragilis could modulate abscess formation in rat, there are few studies on whether modified ZPS could be used as a potential adjuvant candidate to increase antibody titers and induce prolonged response with accompanying memory.

In this study, we used homoZPS that was chemically modified from a positive polysaccharide, chitosan, to examine the influence of chemically modified homoZPS on immune response. To evaluate the possibility for homoZPS to be a vaccine adjuvant, we compared the immune responses to protein antigen in mice administered with homoZPS and aluminum hydroxide, respectively.

**Materials and Methods**

**Sulfation**

Sulfation of chitosan was done as previously described [12]. Briefly, the sulfating agent was prepared using dry pyridine and chlorosulfonic acid as the report described. Two hundred milligrams of chitosan (with 95% purity and 90% deacetylation degree, obtained from Nicechem, Shanghai, China) were suspended in 20 ml of dry formamide at room temperature with stirring for 30 min, followed by addition of the sulfating reagent. After the reaction was completed, the mixture was cooled naturally and neutralized with 10% (w/v) NaOH solution. The product was isolated by extensive dialysis against distilled water for 3 days. The dialyzate was concentrated to 245 ml and freeze-dried to produce chitosan sulfate (homoZPS). HomoZPS was identified by Fourier-transform infrared spectra and NMR. Fourier-transform infrared spectra were recorded on a Nicolet Impact410 spectrometer (Thermo Nicolet, Madison, USA) with the scanned wave number ranging from 4000 to 400 cm\(^{-1}\). The sulfur and nitrogen contents in the sulfated sample were determined by the Vario EL III elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany), and the degree of substitution, which refers to the average number of sulfate residues on each monosaccharide residue, was calculated [13]. \(^{1}\)H-NMR spectra for 5 mg/ml homoZPS solution in D\(_2\)O were recorded in a Bruker avance AV-300 spectrometer (Bruker, Kalkar, Switzerland) at room temperature (data not shown). It was shown that the molecular ration of N:C in sulfated chitosan was about 1:1.

**Animals and cells**

Male C57BL/6 mice (3 weeks old) were obtained from the Shanghai Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China) and maintained at an animal facility under pathogen-free conditions. The handling of mice and experimental procedures were conducted in accordance with experimental animal guidelines.

Spleens were removed aseptically, and splenic T/B lymphocytes and adhesive cells were isolated according to the standard methods [14]. Erythrocytes were lysed using red blood cell-lysing buffer (0.15 M NH\(_4\)Cl, 1 M KHCO\(_3\), 0.1 mM Na\(_2\)EDTA, pH 7.2) for 5 min and washed in RPMI 1640 medium containing 10% fetal calf serum (JRH Bioscience, Brooklyn, VIC, Australia). Cells at 1 x 10\(^7\) cells/ml were incubated for 1 h at 37.7 ± 0.8°C to separate adhesive cells from suspended T/B lymphocytes. Enriched adhesive and T/B cell populations were used in the following experiments. All cells were cultured in RPMI 1640 medium containing 10% fetal calf serum in a humidified incubator containing 5% CO\(_2\) at 37°C. Adhesive cells, such as macrophages and spleen cells, were incubated in Petri dishes (growth area: 9.6 cm\(^2\)) at 4 x 10\(^6\) cells/ml.

**Stimulating proliferation test**

In homoZPS-stimulated murine splenic lymphocyte proliferation test *in vitro*, lipopolysaccharide (LPS; Sigma, St. Louis, USA), and concanavalin A (ConA; Sigma) was used as control stimulators.

The cell growth rate was detected by MTT method. Spleenic cells were incubated in collagen-coated 24-well culture plate for attachment for about 3 h, and then were treated with PBS (as control), LPS (activator of B cells), homoZPS, chitosan, and ConA (activator of T cells) with a range of 10–250 \(\mu\)g/ml, respectively (LPS, homoZPS, chitosan and ConA dissolved in PBS). The wells with medium only were used as blank group. Twenty four hours after the treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) was added to all wells to the final concentration of 1 mg/ml, and the plates were incubated in an atmosphere of 5% CO\(_2\) at 37°C for an additional 4 h. The reaction was stopped by the addition of 100 \(\mu\)l of isopropanol. Optical density was read at 570 nm by an
Flow cytometry
The surface phenotypes of cells were identified using monoclonal antibodies. FITC-labeled anti-CD3 antibody and PE-labeled anti-CD8 antibody were purchased from Jingmei Biotech (Shenzhen, China). Carboxyfluorescein succinimidyl ester (CFSE) flow cytometric test was used for measuring specific homoZPS-induced CD3⁺, CD8⁺ T-lymphocytes proliferation according to instructions of CFSE cell proliferation kit (Invitrogen, Grand Island, NY, USA). Fluorescence staining was performed at 4°C in 200 μl Hanks’ balanced salt solution after treatment of cells with labeled anti-CD3, CD8 antibodies (Jingmei), respectively, in staining buffer (Jingmei) according to manufacturer’s recommendations. The stained cells were then fixed with 1% paraformaldehyde dissolved in PBS and analyzed by flow cytometry (FCM) on EPICS ELITE with logarithmic amplifier (Coulter Corp., Hialeah, FL, USA). Lymphoid cells were gated by the forward and side scatter gating method for the analysis of lymphocyte population [15].

Effect of different adjuvants on immune response of bovine serum albumin
Four groups of mice (each group contained 12 mice, 15 ± 1 g) were used for the experiment. Mice were immunized intraperitoneally (i.p.) with PBS (0.188 M Na₂HPO₄, 0.012 M NaH₂PO₄, 0.3 M NaCl, pH 7.8), 20 μg/kg bovine serum albumin (BSA) (Sigma) (each mouse was injected with 0.5 ml PBS containing 0.3 μg BSA), or BSA formulated with 40 μg/kg of homoZPS (each mouse was injected with 0.5 ml PBS containing 0.3 μg BSA and 0.6 μg homoZPS) or 0.5 mg/ml aluminum hydroxide (each mouse was injected with 0.5 ml aluminum hydroxide solution with 0.3 μg BSA; Superfos Biosector, Frederikssund, Denmark), respectively, according to the pharmacologic protocols. The group that received PBS was used as placebo. Twenty-one days later, these animals were re-immunized with the same procedure. The level of antibody IgG specific to BSA (collecting blood from tail vein, about 10 μl blood from each mouse) was monitored by enzyme-linked immunosorbent assay (ELISA) every 7 days after each immunization.

Effect of different adjuvants on protection against diphtheria toxin
Four groups of mice (each group contained six mice, 15 ± 1 g) were used for this experiment. Mice were immunized with 40 μg/kg diphtheria toxin (DT) (0.5 ml PBS containing 0.6 μg DT), DT formulated with 40 μg/kg of homoZPS (0.5 ml PBS containing 0.6 μg DT and 0.6 μg homoZPS), or 0.5 mg/ml aluminum hydroxide (0.5 ml aluminum hydroxide solution with 0.6 μg DT) i.p. according to the pharmacologic protocols. A group that received PBS was used as placebo. Twenty-one days later, the animals were immunized again with the same procedure. Mice were sacrificed 2 weeks after the last injection, and sera were collected for antibody level measurement. HRP-labeled anti-murine IgG, IgM, IgG1, IgG2a, IgG3 antibodies (WAKO, Shanghai, China) were used as secondary antibodies to monitor the level of major antibody subclasses specific for DT by ELISA. The tail head and tail end of mice were measured with vernier calipers. Measurement points located at 0.5 cm from the tail end and tail head of mice, respectively.

To evaluate the effects of homoZPS on NO production in mice, another four groups of mice (each group contained 12 mice) were immunized with 40 μg/kg DT in PBS (0.5 ml PBS containing 0.6 μg DT), DT formulated with 40 μg/kg of homoZPS (0.5 ml PBS containing 0.6 μg DT and 0.6 μg homoZPS), or 0.5 mg/ml aluminum hydroxide (0.5 ml aluminum hydroxide solution with 0.6 μg DT) i.p. On day 7, 14, and 21 after the immunization, four mice each were selected randomly and sacrificed for NO concentration detection with the NO assay kit (Keygen, Nanjing, China) and spleens were weighed, respectively.

Enzyme-linked immunosorbent assay
ELISA assay was carried out as previously described [16,17]. To determine specific serum antibody titers, 96-well polystyrene microtiter plates were activated with 2 μg/ml of poly-L-lysine in carbonate buffer (pH 9.6) at 37°C for 2 h. The plates were washed three times with double distilled water, and then coated with 10 μg/ml corresponding antigens diluted in PBS (pH 7.6) at 4°C overnight. After coating, the plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (T-PBS) and blocked with 1% skim milk in T-PBS. Two-fold serum dilutions (dilutions starting from 1:100) were prepared in T-PBS and were incubated at 37°C for 2 h in the plates. After three washes with T-PBS, the plates were incubated with anti-mouse IgG-HRP
antibody at room temperature for 2 h. Finally, the plates were washed three times with T-PBS, followed by addition of 100 μl substrate of HRP (ABTS substrate kit for HRP, obtained from Maixin_Bio, Fuzhou, China). Chromogenic reaction was chemically stopped with 50 μl of 50% sulfuric acid after 15 min and absorbance at 405 nm was read using an ELISA reader (DNA expert, TECAN, Männedorf, Switzerland).

**Statistical analysis**

Data were analyzed using the statistical software program Systat 10 (SPSS, Chicago, IL, USA). All data are presented as mean ± SD of three independent experiments. P < 0.05 was considered statistically significant.

**Results**

**Effect of homoZPS on murine splenic lymphocyte proliferation**

To test whether homoZPS could elicit the proliferation of spleen cells, the splenic T/B lymphocytes were treated with homoZPS for 24 h. Figure 1 shows the stimulation effects of different concentrations of homoZPS on the mixed splenic lymphocyte incubated in RPMI 1640 medium in vitro. The results showed that homoZPS activated spleen T/B cells proliferation significantly in a dose-dependent manner. In particular, 100 μg/ml sulfated chitosan could induce cell proliferation four times more than the PBS group. In the parallel experiment, LPS and ConA were used because they are effective in stimulating lymphocyte proliferation in murine strains. Chitosan (non-modified ZPS) was used as an alternative control. At 24 h of incubation, LPS, chitosan, and ConA stimulated splenic T/B cell proliferation by about 1 fold.

In animals, splenic adhesive cells (SACs), such as dendritic cells and macrophages, appear to be the major antigen presenting cells (APCs) subtypes in immune protection. A broad range of APCs including B cells, monocytes, and dendritic cells can facilitate this immune activity of ZPS [8,18,19]. In this study, we also investigated whether homoZPS could stimulate in vitro proliferation of normal SACs cells. As shown in Fig. 2, sulfated chitosan caused significant proliferation of SACs obtained from the spleen in comparison to those stimulated with the medium alone, chitosan or LPS. These results indicated a broad-spectrum mitogenic effect of homoZPS on T, B, and APCs.

**Effect of ZPS on the proliferation of different subtypes of splenic T cells**

Previous study indicated that heteroZPS stimulation in vitro enhances the survival of T cells and suggested that heteroZPS interacts directly with T cells to promote abscess formation [9]. Natural heteroZPS was shown to activate CD4+ T cells both in vitro and in vivo, which as a process requires available MCHII molecules on APCs [11].

Our results also showed that homoZPS could activate the CD4+ T cells proliferation effectively (data not shown). We further investigated whether other subtype T cells except CD4+ cells could be activated in splenocytes by homoZPS. It was shown that the number of...
CD3<sup>+</sup> cells increased by about 80% after a 3-day culture with a high concentration of homoZPS (100 μg/ml) [Fig. 3(A)]. Meanwhile, no obvious increase was obtained when homoZPS was decreased to 20 μg/ml [Fig. 3(B)].

Interestingly, no CD8<sup>+</sup> cells were detected in the culture when the splenocytes were cultured in RPMI-1640 medium containing 10% calf serum, while significant CD8<sup>+</sup> cell proliferation was observed after addition of homoZPS. The proliferation of CD8<sup>+</sup> cells was enhanced by about two times when the concentration of ZPS increased from 20 μg/ml to 100 μg/ml in the medium [Fig. 3(C,D)].

Contrasting immune response of BSA aided with homoZPS and aluminum hydroxide

In this study, we evaluated the immunogenic effects of BSA when using homoZPS and aluminum hydroxide as adjuvants in mice, respectively. No significant specific antibody to BSA was detected in placebo group mice. Following secondary immunization, the effects of adjuvants on production of antibody specific to BSA were shown in Fig. 4(A). The immune response to BSA is potentiated-assisted with both adjuvants compared with the BSA in PBS group (P < 0.01). The humoral immune response to BSA was enhanced significantly by both homoZPS and aluminum hydroxide. The results indicate that the experimental adjuvant based on homoZPS is very effective in potentiating the immunogenic activity to protein. The responses of IgG elicited by BSA formulated with aluminum hydroxide were in the same level as those induced by BSA formulated with ZPS. Fourteen days after second vaccination the highest antibody levels induced by BSA with help of homoZPS for BSA reached 1 x 10<sup>6</sup>.

Contrasting immune response of DT aided with ZPS and aluminum hydroxide

DT is toxic to most eukaryotic cells and species, including man, and the negative effect of this toxin on cells is to inhibit protein synthesis. It can damage lymphatic-vessel...
and other tissues permanently [20]. We further investigated the feasibility of homoZPS as adjuvant when mice were immunized with DT. C57BL/6 mice were immunized with DT alone (DT dissolved in PBS) or with adjuvants. Aluminum hydroxide was used as positive control. Mice sera were tested for IgM, total IgG, and its three subtypes, IgG1, IgG2a, and IgG3, against DT.

The concentrations of induced specific antibodies to DT are shown in Figure 4(B). Both ZPS and aluminum hydroxide could enhance mice immune response to DT effectively. Both IgM and IgG antibody responses in the ZPS group and aluminum hydroxide group were significantly higher than those in the DT alone group. Furthermore, homoZPS elicited statistically significantly higher responses than aluminum hydroxide for IgM and IgG2a ($P < 0.01$). The result that the immune responses elicited by ZPS were significant in IgG2a suggested that homoZPS could help to activate the Th cell in the vaccination process.

Figure 5 showed that spleen weight of the mice immunized with DT alone was twice heavier than those that received DT formulated with either aluminum hydroxide ($P < 0.05$) or ZPS ($P < 0.01$). There was no obvious difference in the average spleen weight of each mouse between the homoZPS group and the control group. It was shown that homoZPS treatment provided significant protection against the attack of DT to mice.

After the second immunization with DT alone, we also observed serious edema of mice tails and many blood clots on mice tails, and the diameter of tail head and tail end is about two and four times larger than those in the other three groups, respectively (Figure 6). DT could attack the blood vessels of all control group mice and result in blood effusion and edema symptom on mouse skin. No edema symptom and blood clots was observed on tails of the two groups treated with DT with ZPS or with aluminum hydroxide during all experimental phases.

Figure 7 showed that nitric oxide concentration in mice blood could be enhanced by 2 fold after DT treat-
ment alone. Both homoZPS and aluminum hydroxide adjuvants could decrease the concentrations of NO induced by DT to a basal level.

Discussion

A number of researches have shown that heteroZPS could effectively stimulate T-lymphocyte proliferation [11–13,19,20]. In this work, we study the immunocompetence of homoZPS prepared in our laboratory which harbored SO$_4^{2-}$ and NH$_3^+$ on each residue in mice. HomoZPS induced an obvious dose-dependent proliferation of spleen cells within 24 h. Compared with the stimulating effects of LPS, ZPS, ConA, and chitosan on spleen adhesive cells and lymphocytes in vitro, the homoZPS could elicit the proliferation of both spleen T/B lymphocytes and the adhesive cells more effectively and quickly. Cultured with 50 μg/ml ZPS for 24 h, the proliferation rate of lymphocytes reached three times; meanwhile, the proliferation rate of adhesive cells, most of which were macrophages, reached up to 62.3% when stimulated by 100 μg/ml homoZPS for 24 h.

The proliferation of the T-lymphocyte subclass detected by FCM showed that homoZPS could notably improve the proliferation of different subtypes of T-lymphocytes including CD3, CD8 cells, the attractive phenomenon is worthy of further study. To detect the effects of homoZPS on the mice immune systems in vivo, the mice were immunized with the antigen BSA and DT formulated with homoZPS or Al (OH)$_3$ adjuvant, respectively. It indicated that homoZPS could help affinity maturation of the antibody. The anti-BSA IgG antibody level was markedly increased after the second immunization [Fig. 4(A)]. The titers of IgG, IgG1, IgM, and IgG2a of the mice to DT immunized twice by homoZPS adjuvant at the 14th day are 3.75E+06, 4.39E+05, 2.33E+05, and 1.36E+05 each [Fig. 4(B)]. HomoZPS could greatly stimulate the expression of antibodies within a short time. Consequently, the antibodies could clean out the toxins rapidly to prevent mice from blood hypoosmolality or intumesce of the spleen. The results showed that homoZPS treatment provided significant protection for mice against the attack of DT. Immune enhancement of ZPS in mice is perhaps different from the protective mechanism of aluminum hydroxide. Compared with the protective characteristics of DT-dependent sustained release via aluminum hydroxide colloid, ZPS might protect mouse against DT attack through strong and quick immune response. Eliminating DT quickly in mice through high-level specific IgG2a to DT and increasing antigenic response might be the main protective mechanism of homoZPS. Inflammatory disease is associated with increased production of NO and activation of the inducible nitric oxide synthase pathway, and decrease NO concentrations could reduce inflammation obviously [21]. Decreased NO concentrations in mice blood resulted from adjuvants might also prevent edema in mice (Fig. 7).

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