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Original article

Mucoadjuvant properties of lipo- and glycoconjugated derivatives of oligochitosans

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ABSTRACT

Chitosan, (1-4)-2-amino-2-deoxy-beta-D-glucan, is a deacetylated form of chitin, an abundant biodegradable, positively charged natural polysaccharide. Chitosan is used for antigen delivery through mucosal barrier due to its ability to disrupt tight junctions. Here we produced new water-soluble low-molecular weight chitosan (LMW-Chi) lipid derivatives and compared their ability to stimulate humoral response with the effect of unmodified LMW-Chi or its oligosaccharide derivatives. LMW-Chi effectively penetrated into macrophage-like, lymphoid and epithelial cells. It also stimulated in mice IgG production to model proteins delivered either by subcutaneous or intranasal routes. Adjuvant effect of chitosan derivatives was comparable to or lower than that of unmodified LMW-Chi. Thus, it is possible that adjuvant effect is induced by unmodified glucosamine units of chitosan.

1. Introduction

Vaccines represent one of the most successful strategies in medical science. Modern vaccines target not only infectious diseases, but also autoimmune disorders, allergies, chronic inflammatory diseases and cancer. Development of new effective and safe prophylactic and therapeutic vaccines requires new adjuvants containing immunomodulators specifically adapted to the antigen and the target population. Past approaches have been largely empirical and generally used a single type of adjuvant, such as aluminium salts or emulsions, which stimulated mostly humoral immunity. Recent advances in basic immunology have led to the design and development of more specific and focused adjuvants, which induce a strong T-cell response essential, for example, for antiviral protection [1]. Still other efforts are directed for developing alternatives to traditional vaccine delivery, including non-parenteral (i.e., mucosal or transcutaneous) immunization. Mucosally or transcutaneously delivered vaccines offer a number of possible advantages over traditional vaccines, especially elimination of needles and the need for specially trained healthcare specialists to administer vaccines. A major limiting factor for the development of mucosal vaccines is the availability of safe and effective adjuvants. Most known mucosal adjuvants include cholera toxin, the heatlabile enterotoxin, unmethylated CpG dinucleotides, and monophosphoryl lipid A [2–5].

During the past decade, much attention has been devoted to the use of chitosan and its derivatives for the mucosal delivery of vaccines in experimental animal models and in humans [6–8]. Chitosan is considered to be a mucoadhesive polymer able to penetrate epithelial barrier via tight junctions and activate resident macrophages through binding to mannose receptor [9–11]. Chitosan structure and positive charge favor the production of various high molecular weight chitosans (HMW-Chi) able to form polyplexes, nano-, and microparticles [12–14]. Several chitosan derivatives such as trimethyl derivative, 5-methyl-pyrrolidinone chitosan, chitosan hydroglutamate, and others were used as effective adjuvants for protein and DNA delivery via mucosal barrier [15–17]. However, most studies on chitosan included only a limited number of derivatives, making it difficult to conclude which of chitosan modifications serves best to its adjuvant properties.

This study was aimed to obtain water-soluble LMW chitosans with improved mucoadhesive properties. To this end, new water-soluble lipid LMW-Chi derivatives: palmitoylchitosan and oleyl-β-alanylchitosan were obtained. To increase their solubility at neutral pH their succinoyl analogues: succinoylpalmitoylchitosan and

Abbreviations: DD, deacetylation degree; Gal, galactose; Glc, glucose; Man, mannose; Glc-NH_2, glucosamine; LB, lactobionic acid; LMW-Chi, low molecular weight chitosan; HMW-Chi, high molecular weight chitosan.

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succinoyl(oleyl-β-alanyl)chitosan, were also prepared. Known oligosaccharide LMW-Chi derivatives as well as unmodified LMW-Chi and HMW-Chi were used as a control. Adjuvant effect of chitosans on humoral IgG response was estimated in two strains of mice and to two different exogenous protein antigens to obtain more reliable data. Model protein antigens Asp f 2 from *Aspergillus fumigatus* fungi and ovalbumin (OVA) delivered either by subcutaneous injections or via mucosal route were used in *in vivo* studies. The effects of molecular weight and degree of deacetylation of LMW-Chi were compared. Most chitosans studied stimulated IgG responses, however, there were no significant differences between LMW-Chi and its derivatives. HMW-Chi derivatives were less effective in augmentation of humoral response than LMW-Chi. Thus, it is likely that adjuvant effect was induced by unmodified glucosamine units of chitosan. However, this conclusion must be verified in further studies.

2. Results

2.1. Synthesis and NMR characterization of lipoconjugates

Succinoyl- and palmitoyl-derivatives of chitosan were synthesized by anhydride method according to the procedures described for HMW-Chi [18,19]. The oleinic acid residues were introduced into chitosan oligomers as oleyl-β-alanyl intermediates preactivated with HBTU in the presence of HOBt (see Section 4). In all cases the substitution degree was controlled by using the varied matrix/ligand proportions to produce water soluble compounds. All lipid conjugates produced by Hirano et al. [18] with HMW-Chi were insoluble possibly due to high weight of chitosan used for the reaction. When fat acids were conjugated to low-molecular chitosan the solubility of lipid derivatives depended on the substitution degree. As a result water-soluble succinoylpalmitoylchitosan contained 25 succinoyl residues and 5 palmitoyl residues per 100 monosaccharides' residues, i.e. degree of N-acylation/carboxyacylation was estimated as 5%/25%, respectively. The content of substituents per 100 monosaccharides' residues in conjugates used in this study was the following: 6.4 palmitoyl residues in palmitoylchitosan; 20 oleyl residues in oleyl-β-alanylchitosan; 40 succinoyl and 20 oleyl residues in succinoyl(oleyl-β-alanyl)chitosan.

The ^{1}H NMR spectrum of succinoylpalmitoylchitosan as a representative example is shown in Fig. 1. The resonances at 1.21, 2.04, and 2.54 ppm are assigned to *N*-palmitoyl ($(-CH_2-)_{11}$), *N*-acetyl, and *N*-succinoyl ($-CH_2-CH_2-$) protons, accordingly. The degree of N-acylation/carboxyacylation was calculated from the ratio of the integral intensity of H-1 (4.56 ppm) to that of *N*-succinoyl and *N*-palmitoyl protons. The ^{1}H NMR spectra of other lipid derivatives were analogous.

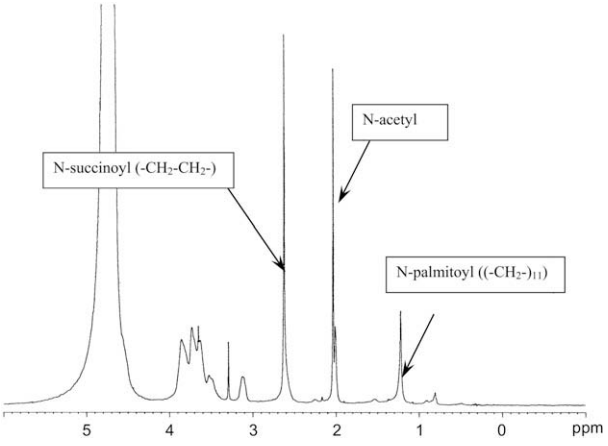

Fig. 1. The ^{1}H NMR spectrum of succinoylpalmitoylchitosan. The resonances at 1.21, 2.04, and 2.54 ppm are assigned to *N*-palmitoyl $(-CH_2-)_{11}$, *N*-acetyl, and *N*-succinoyl ($-CH_2-CH_2-$) protons, accordingly.

2.2. Chitosan penetrates into cells

To estimate whether LMW-Chi penetrates into the cells we labeled it with FITC and co-incubated LMW-Chi–FITC conjugate with macrophage (RAW 264.7), lymphoid (BYRB lymphoma) or epithelial (HeLa, HaCaT) cell lines for 1 h (Fig. 2). RAW cells effectively engulfed the conjugate and 100% of cells were FITC positive, while among lymphoma and epithelial cells only 30–60% were FITC positive demonstrating some selectivity in chitosan penetration. LMW-Chi–FITC conjugate was not toxic for RAW or lymphoma cells while it decreased viability of epithelial cells from 80 to 70% possibly because cells became more sensitive to trypsinization used for cell detachment.

Earlier it was shown that oligochitosan internalization by RAW cells is mediated by a macrophage lectin receptor with mannose specificity [9,10]. The mechanisms of BYRB, HeLa, and HaCaT staining by Chi–FITC are unknown. To visualize whether Chi–FITC penetrates into these cells we used confocal microscopy and showed that Chi–FITC was able to both bind epithelial (HaCaT) and lymphoid (BYRB) cell membranes and penetrate into these cells by unknown mechanism (Fig. 2, bottom panel).

2.3. Effect of molecular weight and degree of deacetylation of chitosans on humoral response to proteins in vivo

To study the *in vivo* effect of chitosan molecular weight and deacetylation degree we prepared two panels of LMW-Chi: one with different molecular weights (150, 30, 22, 17, 7 and 3.5 kDa) and fixed DD = 0.85 and the other one with different DD (0.85, 0.77, 0.67, and 0.42) and fixed molecular weight of chitosan (22 kDa). Mice were immunized subcutaneously daily for 5 days with a mixture of a model protein Asp f 2 and LMW-Chi at 1:5 ratio, accordingly, in PBS. For the exception of 3.5 kDa all other LMW-Chi preparations with DD = 0.85 effectively stimulated IgG response estimated 2 weeks post immunization (Fig. 3A). On the contrary, stimulating activity severely depended on DD and was the highest in samples with DD = 0.85 (Fig. 3B).

2.4. LMW-Chi and its glycoconjugates possess similar stimulating activity

Chitosans conjugated with oligosaccharides are used for DNA delivery or dendritic cell targeting, however, their ability to stimulate humoral response to exogenous proteins was not studied earlier. We prepared LMW-Chi conjugates with mannose, galactose, glucose, lactobionic acid and glucosamine and studied their adjuvant activity. The content of saccharide residues was determined by conductometry of amino groups before and after the conjugation of Chi with mono- or disaccharide residues. It varied from 4 to 23%. Mice were immunized as before with a mixture of Asp f 2 and LMW-Chi glycoconjugates at 1:5 ratio, accordingly. The results demonstrated that the effect of chitosan conjugates on IgG response to Asp f 2 was either comparable or less than induced by LMW-Chi itself (Fig. 4). Conjugates of LMW-Chi with glucose and galactose were less effective than the ones with lactobionic acid, mannose, or glucose-amine (Fig. 4).

2.5. LMW-Chi and its lipoconjugates as mucosal adjuvants

Chitosan lipoconjugates are potential candidates for mucosal delivery systems due to increased mucoadhesive properties. To study their adjuvant activity we obtained LMW-Chi conjugates

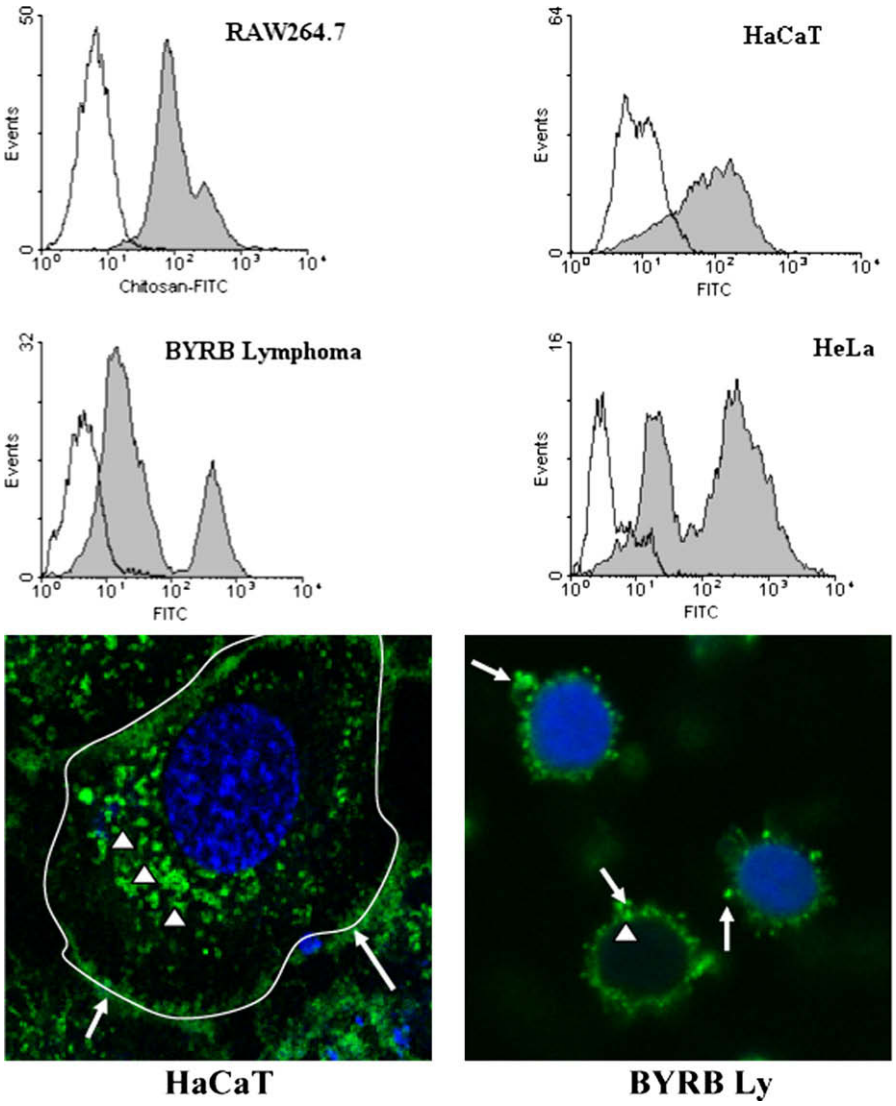

Fig. 2. Penetration of LMW-Chi–FITC into the cells of different origins. Upper panels: cells from murine macrophage cell line RAW264.7 and BYRB lymphoma, or human epithelial cells HaCaT or HeLa were incubated with LMW-Chi–FITC (gray areas) or with control FITC solution in medium (solid lines) for 1 h and studied by flow cytometry. Lower panel: confocal microscopy of HaCaT (left) and BYRB (right) cells. HaCaT epithelial cell membrane is drawn. Membrane associated FITC staining is shown with arrows while intracellular FITC accumulation – with arrowheads. Nuclei are stained with DAPI. Magnification ×3000.

with oleic and palmitic acids. To increase their solubility we also produced succinyl derivatives of these molecules. Because lipoconjugates are designed for mucosal delivery we immunized mice i.n. with a mixture of LMW-Chi lipoconjugates and OVA using earlier published protocol [20]. To optimize immune response in preliminary experiments mice were immunized i.n. with different doses of OVA. Detectable IgG production was found only when mice were immunized with more than 20 μg/mouse/day and 5 daily instillations. So, to estimate mucoadjuvant activity of LMW-Chi lipoconjugates 25 μg of OVA per mouse/day and 5 daily instillations were used. In this case we also mixed OVA and LMW-Chi lipoconjugates at 1:5 ratio, accordingly. Humoral response to OVA induced by i.n. immunization was significantly less pronounced than the one induced by s.c. protocol. The titers of antigen specific IgG varied from 400 to 4000 for i.n. immunization (total dose of antigen 125 μg/mouse) and from 3000 to 15,000 (total dose – 50 μg/mouse) for s.c. protocol (data not shown). This more than two order difference in the antigen recognition threshold is evidently a result of mucosal barrier function. Combination of OVA with LMW-Chi or its lipoconjugates for i.n. immunization increased the IgG titers in BALB/c mice (Fig. 5). There was no difference between adjuvant activity of LMW-Chi and its oleic and palmitic conjugates while succinic derivatives were unexpectedly significantly less active (Fig. 5). This experiment was repeated twice with close results and a representative example is shown.

2.6. Mucoadjuvant activity of HMW-Chi and LMW-Chi

HMW-Chi and chitosan microparticles are also used for intranasal delivery of proteins. Thus, we compared the activity of LMW-Chi with chitosan microparticles and high molecular weight carboxymethylchitosan (700 kDa) in BALB/c and CBA mice immunized i.n. with OVA as described above. Because s.c. experiments with LMW-Chi were fulfilled with both CBA and BALB/c mice while i.n. only with BALB/c we wanted to see whether CBA strain is also responsive to adjuvant effect of LMW-Chi in i.n. protocol. We also included in this study C57BL/6 strain, however, the letter responded neither to antigen alone not to a combination of antigen with chitosans possibly due to a better functioning of mucosal barrier. As before LMW-Chi significantly increased OVA specific IgG production in BALB/c mice as well as in CBA while chitosan microparticles were less effective in both strains (Fig. 6). Carboxymethylchitosan

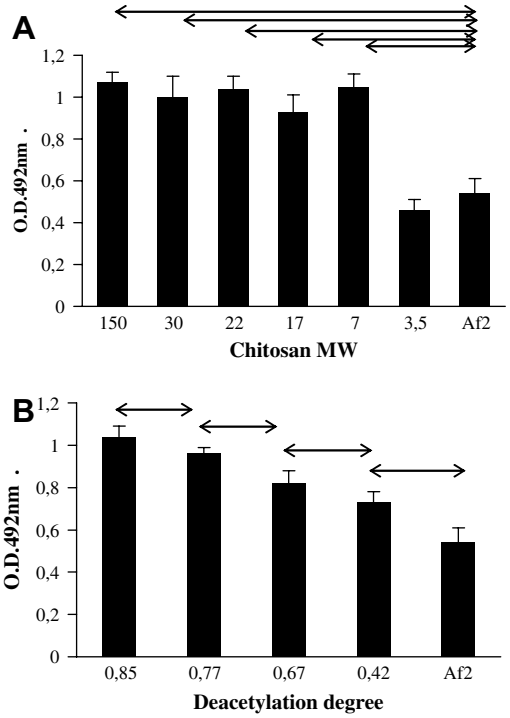

Fig. 3. Induction of IgG to Asp f 2 in CBA mice immunized by 5× s.c. injections with or without chitosan of different MWs (A) and DD (B). Asp f 2 (10 μg per injection) and chitosan (100 μg per injection) were mixed and injected in 100 μl per mouse. Serum was obtained 2 weeks after immunization and diluted 1:500. The results here and in the next figures are presented as mean of optical densities (O.D.) after subtracting the background ± SD. O.D. value for control intact mouse serum was <0.05. Statistically significant differences (t-test, <0.05) are shown with arrows.

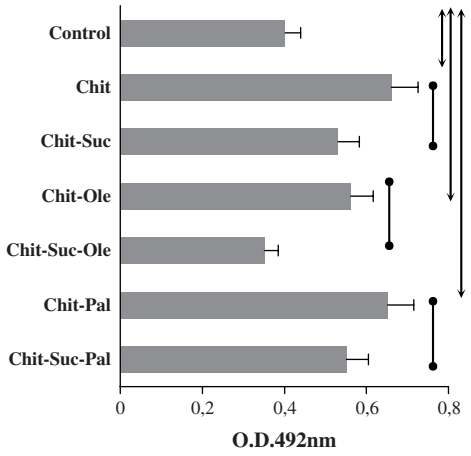

Fig. 5. Induction of IgG to OVA in BALB/c mice immunized 5 times i.n. with 25 μg per instillation OVA with 125 μg/instillation of negatively charged or lipophilic derivatives: succinyl (Chi-Suc), oleil (Chi-Ole), succinyl and oleil (Chi-Suc-Ole), palmitoil (Chi-Pal), or succinyl and palmitoil (Chi-Suc-Pal) derivatives. Sera were diluted 1:10. O.D. value for control intact mouse serum was <0.08. Statistically significant differences (t-test, <0.05) are shown with arrow bars (OVA vs Chi derivatives) or ballet bars (Chi derivatives vs succinyl ones).

did not show any effect in comparison with immunization of mice with OVA only.

3. Discussion

Adjuvants and vehicles which are used for vaccines must answer several criteria such as they should be biodegradable; contain charged and/or lipophilic groups which increase tropism to cell membranes; should protect antigens from proteolysis in body fluids; and also they should prevent antigen dilution before it reaches antigen-presenting cells. Synthetic polymers polyvinylpyrrolidone, polyacrylamide, polyethylene glycol, polyoxidonium, and others answer only some of these properties [21–23]. Chitosan, (1-4)-2-amino-2-deoxy-beta-D-glucan, a deacetylated form of chitin, an abundant, biodegradable, nontoxic, positively charged natural polysaccharide, qualifies most well to all the necessary requirements both as DNA and protein delivery vehicle. In the body chitosan is degraded either by chitinases secreted by intestinal cells or present in plant food either it can partially be degraded by lysozyme [24].

Chemical properties of chitosan are determined by its molecular weight and deacetylation degree. Here we showed that molecular weight of chitosan did not affect its adjuvant properties, while DD was more important. These data are also supported by the study of Lavertu et al. [25], who tested chitosan/DNA complexes for gene transfection in HEK 293 cells *in vitro*. Several formulations with high DD and low MW produced high levels of transgene expression comparable with commercial transfection

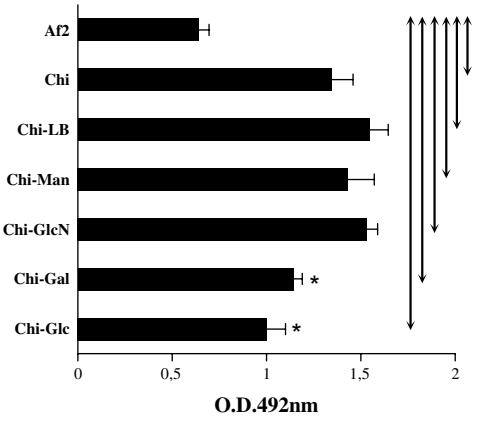

Fig. 4. Induction of IgG to Asp f 2 in BALB/c mice immunized by 5× s.c. injections with LMW-Chi modified by carbohydrate residues: lactobionic acid (Chi-LB), mannose (Chi-Man), glucose-amine (Chi-GlcN), galactose (Chi-Gal), and glucose (Chi-Glc). Statistically significant differences between Af2 and Af2 + chitosan derivatives (t-test, <0.05) are shown with arrows. Statistically significant differences between Chi-Gal, Chi-Glc and other chitosan carbohydrate derivatives (t-test, <0.05) are shown with asterisks.

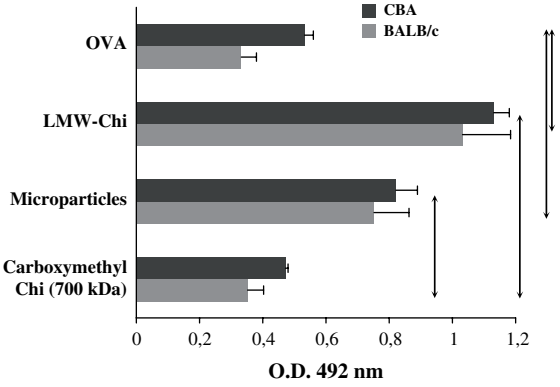

Fig. 6. Induction of IgG to OVA in BALB/c and CBA mice immunized 5 times i.n. with 25 μg per instillation of OVA mixed with 125 μg of high-molecular weight chitosans: chitosan microparticles or carboxymethylchitosan 700 kDa. Sera were diluted 1:10. O.D. value for control intact mouse serum was <0.07. Statistically significant differences (t-test, <0.05) are shown with arrow bars.

reagents. Deacetylation of chitosan increases the summary charge by this mean increasing the ability to form electrostatic complexes with proteins. On the other hand it is known that DD decreases biodegradability of chitosan [26]. Hypothetically, endosomal escape of chitosan-based complexes occurs less readily with high DD chitosans [27]. This can lead to a better antigen presentation of proteins.

High molecular weight chitosan obtained by traditional method is poorly soluble in water and thus is of a limited usage in practice. Chemical modifications – *N*-trimethylchitosan chloride, mono-*N*-carboxymethylchitosan and others are used to overcome this disadvantage [7]. In this work we obtained low-molecular weight chitosan by proteolitic hydrolysis. This LMW-Chi possessed all the advantages of chitin such as biodegradability, nontoxicity, positive charge of the natural polysaccharide. At the same time it became water soluble when dissolved in 50 mM HCl and then neutralized by sodium hydroxide to reach pH 7.2–7.4. Succinylation increased solubility and made LMW-Chi soluble in water without previous acidification. However, succinyl derivatives unexpectedly demonstrated decreased adjuvant properties of lipophilic LMW-Chi.

LMW-Chi effectively penetrates into the cells of different origins. Earlier it was shown that chitosan interacts with murine macrophage cell line RAW264 via mannose-like receptor [10,11]. So, we compared the effects of LMW-Chi on RAW264 cells, which express mannose receptor, and on murine T-cell lymphoma BYRB, and human epithelial cell lines HaCaT and HeLa which do not express this receptor. Non-phagocytic cells were also stained with Chi–FITC, however, we found two different subpopulations in HeLa and BYRB cells showing that some subpopulations were responsive to chitosan, while others did not bind it. It could be hypothesized that FITC positive cells also express mannose receptor. Normally these receptors behave as antigen uptake/processing receptors and are highly expressed on professional antigen presenting cells such as dendritic cells and macrophages, however, they are also found at moderate levels on B-cells, at low levels on T- and NK cells [28], as well as on epidermal cells [29]. On the other hand it can be hypothesized that other mechanisms different from mannose receptor binding could be involved in LMW-Chi penetration into mammalian cells.

LMW-Chi is an ideal adjuvant because it can be easily modified to prepare conjugates with desired properties. Earlier it was shown that oligosaccharides target chitosan conjugates to dendritic cells, which are the major antigen presenting cells in the body. Chitosan modified by fat acids on the other hand sorbs better to mucosal tissues and by this mean enhances hydrophobic molecules delivery via mucosal layers [7,30]. Technically it is possible to conjugate chitosan with any anchor residues such as oligosaccharides and/or lipophilic compounds and with antigenic proteins of interest. In this work we obtained LMW-Chi conjugated with various carbohydrates and fat acids. As the standard procedures were not applicable for incorporation of oleic acid in chitosan for this purpose we used β-alanine as a special linker. First, we prepared oleyl-β-alanine, which was coupled with chitosan by HBTU-method and the conjugate received was further modified with succinic anhydride.

However, all the LMW-Chi-conjugates studied in this work were not more active than unmodified LMW-Chi. We hypothesized that unmodified glucosamine units of LMW-Chi are responsible for the adjuvant effect on humoral immune response induced in mice.

Adjuvant effect of LMW-Chi was studied *in vivo* using two strains of mice, two antigens and two routes of immunization to diversify the results. In all cases the enhancement of IgG response by chitosan derivatives was comparable to or lower than unmodified LMW-Chi. Thus, water-soluble, unmodified, and thus cheap LMW-Chi can be used as adjuvant in parental and mucosal vaccines.

4. Experimental protocols

4.1. Antigens and chemicals

D-galactose, D-mannose, D-glucosamine D-glucose, lactobionic acid, *N*-hydroxysuccinimide, mono-chloroacetic acid, and hydroxylamine were obtained from Fluka, Switzerland; EDC, NHS, MES-(2-[*N*-morpholino]ethanesulfonic acid), sodium-acetate, sodium hydroxide, 2-propanol, mono-chloroacetic acid, ovalbumin, and bovine albumin were purchased from Sigma Co, USA; celloviridine G20x and crab chitosan were obtained from Berdsk and "BioProgress", accordingly (Russia). Recombinant Asp f 2 from *A. fumigatus* fungi was a gift of Dr.V.P. Kurup, (USA).

4.2. Chitosan oligosaccharides

LMW water-soluble chitosan was obtained from HMW crab chitosan by hydrolysis using Celloviridine G20x as described [31]. The reaction was performed for 0.5–2 h in sodium-acetate buffer (pH 5.2) at 55 °C and 1:400 enzyme/substrate ratio, and terminated by 1 M sodium hydroxide. The precipitate was isolated by centrifugation at 5000 g for 15 min, resuspended in water and extensively dialyzed using Spectra/Por membrane (Cole-Parmer, USA) against distilled water. This protocol was used throughout all final purifications. The yield of lyophilized LMW-Chi was 80%. MW of LMW-Chi depended on the time of digestion. Chitosan with various degrees of acetylation was produced by reacetylation of the original chitosan (methanol/2% acetic acid at 54:51 v/v ratio). The amount of acetic anhydride was in the range 0.1–2.0 mmol per 1 g of chitosan [32]. The degree of chitosan deacetylation (DD) was estimated by conductometric titration [33]. A panel of LMW-Chi with different MWs and DD (MW in kDa/DD) was prepared: 150/0.85; 30/0.85; 22/0.85; 17/0.85; 7/0.85; 5/0.85; 22/0.77; 22/0.67; 22/0.42. Intrinsic viscosity was determined at 25.0 ± 0.5 °C in Ubbelohde viscometer using 0.2 m sodium-acetate and 2% acetic acid (at ratio 1:1 v/v) as a solvent. The viscosity-average MW was calculated according to the Mark-Houwink equation: $\eta = k \times M^{\alpha}$. Molecular weights of chitosan samples were determined by high-performance liquid chromatography. The weight-average MW (M_w), the number-average MW (M_n) and polydispersion M_w/M_n for chitosan were determined using Ultrahydrogel 500 column 7.8 × 300 mm (Waters, USA) in 0.15 M ammonium acetate, 0.05 M acetic acid, pH 5.2 and the elution rate 0.5 ml/min.

4.2.1. Chitosan-mannose, chitosan-galactose, chitosan-glucose and chitosan-glucosamine

Chitosan-mannose (Chi-Man), chitosan-galactose (Chi-Gal), chitosan-glucose (Chi-Glc) and chitosan-glucosamine (Chi-GlcN) were synthesized as described in Ref. [34] with minor modifications [35]. LMW-Chi (MW 15–24 kDa, DD = 0.85) 0.5 g was dissolved in 0.02 M aqueous acetic acid at 1% (w/v). After that, monosaccharide (mannose, glucose, galactose or glucosamine) was dissolved in chitosan solution to a final monosaccharide concentration of 1% (w/v). Every 24 h aliquots were collected for the absorbance analysis at 420 nm. Reaction was conducted at 65 °C for 5 days. After that the solution was centrifuged, dialyzed and then lyophilized as described above. The yield of water-soluble chitosan-saccharide derivatives was 55–60%. Substitution degree was determined using N-alkylated monosaccharide chitosan derivatives. To 5 ml aliquots of 10 mg/ml chitosan derivatives in 0.02 M CH_3COOH, (pH 5.3–5.9) 5 mg of $NaCNBH_3$ was added. Reaction was conducted for 18–24 h. The yield of lyophilized N-alkylated monosaccharide chitosan derivatives was 80–85%. Substitution degree varied from 5 to 8%.

4.2.2. Chitosan-lactobionic acid (Chi-LB)

Chi-LB was synthesized as described in Ref. [36]. LMW-Chi (15–24 kDa, DD = 0.85) was coupled with LB via active ester intermediate using EDC and NHS in 0.1 M MES buffer. Briefly, LMW-Chi (0.25 g) was dissolved in 25 ml of MES buffer (25 mM, pH 6.0). The carboxyl group of LB (0.19 g) was activated by the NHS/EDC dissolved in 10 ml of MES buffer. EDC was 4-fold molar excess over LB and NHS/EDC molar ratio was 1:1. The activated LB solution was added to the chitosan solution and the mixture was stored under stirring for 24 h at room temperature. The reaction was quenched by adding hydroxylamine to final concentration of 10 mM, and the pH of the reaction was brought to 8.0. The resulting product was dialyzed and lyophilized as above. The yield was 60%. Free amino groups were determined by conductometric titration [33]. Substitution degree varied from 8 to 23%.

4.2.3. Carboxymethylchitosan (CMC)

CMC was prepared according to Ref. [37] with minor modifications. Chitosan 5 g (0.03 M) with MW 700 kDa and DD = 0.85 suspended in 15 ml 40% sodium hydroxide solution was kept at 20 °C overnight. The alkaline chitosan was transferred to 70 ml 2-propanol, and 5.7 g (0.06 M) mono-chloroacetic acid was added in portions. After stirring at room temperature for 2 h, heat was applied to bring the reaction mixture to 60 °C for another 2 h. Then acetic acid was added to the mixture to adjust pH to 7.0. CMC salt was filtered, washed with ethanol, dialyzed, and vacuum dried at room temperature. The yield of CMC was 89%, degree of substitution was 0.42 as determined by elemental analysis. IR spectra: 1594 cm^{-1} (COO^- ion), 1651 cm^{-1}, 1559 cm^{-1}, 1320 cm^{-1} (amides I, II and III, accordingly).

4.2.4. Succynoylchitosan

Succynoylchitosan was conjugated from LMW-Chi (28 kDa, DD = 0.85) as described in [18]. Chitosan (84 mg, 3 μmol) was dissolved in 3 ml of 0.1% acetic acid and 9 ml of methanol was added, followed by the addition of succinic anhydride (13.5 mg, 135 μmol) in 3 ml of methanol and the mixture was stored under stirring at room temperature overnight. Methanol was removed by evaporation, and 5 volumes of cold acetone were added to the residual solution. The mixture was kept at 4 °C for 10 min. The precipitate was isolated by centrifugation (3000 rpm, 15 min), dried on open air, suspended in 1 ml of 0.5% acetic acid and lyophilized. The yield was 74 mg (76.6%).

4.2.5. Palmitoylchitosan

LMW-palmitoylchitosan was received according to the procedure published for HMW-palmitoylchitosan [19]. Briefly, 170 mg of chitosan (17 kDa, DD = 0.85), was dissolved in 2 ml of 0.5% acetic acid and 2 ml of methanol was added to the solution. Then fresh palmitic anhydride (66.9 mg, 135.5 μmol) in 8 ml of methanol was added dropwise and the mixture was left under stirring at room temperature overnight. Methanol was removed by evaporation, 10 ml of diethyl ether was added, the aqueous phase was separated, diethyl ether was evaporated and the precipitate was dried on open air and further dissolved in the minimal amount of 0.5% acetic acid. The obtained palmitoylchitosan was lyophilized and kept at room temperature until use. The yield was 120.5 mg (71%). The solubility of LMW-palmitoylchitosan depended on the numbers of fat acids' substitutions (see Section 2.1).

4.2.6. Oleyl-β-alanylchitosan

Oleyl-β-alanylchitosan was prepared in two steps: firstly, oleyl-β-alanine was obtained using *N*-hydroxysuccinimide ester of oleinic acid and, secondly, oleyl-β-alanylchitosan was synthesized according to Ref. [38].

1. *N*-Hydroxysuccinimide ester of oleinic acid. Oleinic acid (280.5 mg, 1 mmol) was added to the solution of NHS (115 mg, 1 mmol) in dry ethyl acetate (5 ml). To this, dicyclohexylcarbodiimide (206 mg, 1 mmol) in dry ethyl acetate (3 ml) was added and the reaction mixture was left at room temperature overnight. Dicyclohexylurea was removed by filtration and solvent was removed by evaporation to afford *N*-hydroxysuccinimide ester of oleinic acid as oil, which stored in cold under nitrogen. *N*-oxysuccinimide ester of oleinic acid was dissolved in 1 ml of ethanol and cold to −4 °C. The residual volume of ethanol was removed at room temperature, and the solution was evaporated to form clear oil (432 mg, 52%).

2. Oleyl-β-alanine. *N*-hydroxysuccinimide ester of oleinic acid (269 mg, 0.71 mmol) in THF (10 ml) was added to a solution of β-alanine (3.19 mg, 0.71 mmol) and sodium bicarbonate (61.06 mg, 0.71 mmol) in water (7.1 ml). After 12 h the solution was acidified to pH 2 with 2 N hydrochloric acid and THF was removed by evaporation. The precipitate was filtered, washed with distilled water (5 × 7.1 ml), and crystallized from chloroform/petroleum ether to afford oleyl-β-alanine as a white solid (171 mg, 65% yield); mp 77 °C.

3. To the solution of oleyl-β-alanine (31.6 mg, 90 μmol) in 1 ml of DMF were added HBTU (34.11 mg, 90 μmol) and HOBt (12.15 mg, 90 μmol). In a separate flask 34 mg (2 μmol) of LMW-Chi (17 kDa, DD = 0.85) was dissolved in 1 ml of 0.5% acetic acid and 0.04 ml of *N*-ethyldiisopropylamine and 10 mg of ionole were added. Two solutions were mixed immediately. The reaction mixture was blown with nitrogen and then was stirred at room temperature for 4 h. 10 ml of cold acetone was added to this solution, the precipitate was separated by centrifugation (3000 rpm, 15 min), dried on open air, dissolved in minimal volume of 0.5% acetic acid and lyophilized to afford the title product (32.5 mg, 95%) as a white solid.

4.2.7. Succinoylpalmitoylchitosan

To obtain succinoylpalmitoylchitosan, palmitoylchitosan (120.5 mg, 7 μmol) was dissolved in 4 ml of 0.5% acetic acid and 16 ml of methanol; succinic anhydride (28 mg, 280 μmol) was added and the mixture was stirred at room temperature overnight. Methanol was evaporated and the obtained aqueous solution was washed with diethyl ether (3 times with 5 ml). The aqueous phase was separated and lyophilized (80 mg, 66.5%).

4.2.8. Succinoyl(oleyl-β-alanyl)chitosan

To the solution of oleyl-β-alanylchitosan (30 mg, 1.76 μmol) in 1 ml 0.5% acetic acid and 5 ml of methanol succinic anhydride (5 mg, 50 μmol) were added and the mixture was stirred at room temperature overnight. Further, methanol was evaporated and to the residue 7 ml of cold acetone was added. The product was isolated by centrifugation (3000 rpm, 15 min), dried on open air, dissolved in minimal volume of 0.5% acetic acid and lyophilized (14.8 mg, 50%).

4.2.9. LMW-Chi water-soluble stock solutions

LMW-Chi derivatives produced from 17–28 kDa oligochitosan, DD = 0.85, were dissolved in 50 mM HCl at 10 mg/ml. All solutions were neutralized by sodium hydroxide to reach pH 7.2–7.4, further diluted to 2 mg/ml in saline, divided in aliquots and kept at −20 °C until use. All synthesized LMW-Chi derivatives were water-soluble under pointed conditions and were dissolved in water for all experiments.

4.2.10. Preparation of microparticles

To prepare microparticles by salt fractionation, 0.25% chitosan solution in 3.75% acetic acid was stirred in the presence of 1.0% (w/w) Tween 80 for 1 h. The obtained solution was supplemented with

10% Na_2SO_4 to a final concentration of 1% (v/v), and the resulting mixture was incubated under stirring for another 20 min. The formed microparticles were separated by centrifugation at 3000 rpm for 30 min. The pellet was resuspended in MilliQ water to wash the microparticles and centrifuged again. This washing procedure was repeated twice. The chitosan microparticles were 0.85–1.70 μm in size and positively charged (38.1 ± 0.1 mV). They were stored in colloidal solution (20 mg/ml) in the cold. The yield of microparticles, determined by drying aliquots of the resulted solution, was 70–90%. Before protein loading the microparticles were resuspended in 0.1 M sodium-phosphate buffer, pH 7.2. The maximum adsorption efficiency of model antigen (interferon-alpha) was 88%, the capacity of microparticles was 11.8–12.7 μg/mg [39].

4.3. NMR spectroscopy

All acylchitosan derivatives were analyzed by NMR spectroscopy. ^1H NMR spectra were recorded on Bruker DRX 500 spectrometer (500 MHz) in D_2O (0.63 ml) + DCl (1 M 0.3 ml) at 30 °C. All samples were prepared in 5 mm diameter tubes at concentration 2 mg/ml. The chemical shifts are given on the δ scale relative Me_4Si.

4.4. Tracking of FITC-labeled chitosan

LMW-Chi was labeled with FITC using standard method [40]. To analyze cell penetration LMW-Chi–FITC conjugate was incubated with mouse macrophage cell line RAW 264.7, mouse lymphoma BYRB and human epithelial cells' lines HaCaT, and HeLa (grown to confluent layer for all adhesive cells or at 10^6/ml for BYRB) at 100 μg/ml for 1 h at 37 °C. After 3 washes with PBS cells were analyzed by flow cytometry. For confocal imaging DAPI (Pierce, Rockford, Illinois, USA), 5 μg/ml, was added for the last 15 min. After washing 3 times with PBS cells were fixed with 4% *para*-formaldehyde for 1 h at RT, washed again, mounted in Vectashield medium (Vector Labs, USA) on glass slides and immediately observed using a confocal microscope. For flow cytometry HaCaT and HeLa cells after incubation with LMW-Chi were trypsinized, washed in FACS buffer and studied.

4.5. Flow cytometry and confocal imaging

For the FACS analysis cells were transferred to FACS buffer (PBS, 1% bovine serum albumin, 0.05% NaN_3). Two-color flow cytometry was performed using FACScan and CellQuest softwares (BD Biosciences). Live events (5000–10,000) were acquired with propidium iodide exclusion of dead cells. Nikon TE 2000 confocal microscope with Nikon-EZ-C1 program was used.

4.6. Animals

Ten–twelve weeks old inbred BALB/c (H-2^d) and CBA (H-2^k) mice purchased at the Central Farm, Moscow, were used in this study. Institutional Animal Use and Care Committee approved the experimental protocol followed in this research.

4.7. Chitosan/protein mixtures

All chitosan derivatives, both LMW and HMW, dissolved in water (see Section 4.2.9) and protein antigens dissolved in PBS were mechanically mixed at concentrations indicated below, left for 1 h at room temperature at slow stirring and then used for *in vivo* and *in vitro* assays.

4.8. Subcutaneous immunization

BALB/c and CBA mice (5 mice per group) were immunized s.c. at the tail base with the mixture of 50 μg of chitosan or chitosan derivatives and 10 μg Asp f 2 in PBS in 100 μl/mouse. Mice were immunized daily for 5 days. Sera from immunized animals were collected two weeks after the last immunization. Animals were bled from tail vein and sera were collected, pooled, and stored frozen until use.

4.9. Intranasal immunization

BALB/c and CBA mice (5 mice per group) were immunized intranasally (i.n.) with mixtures of 125 μg chitosan or chitosan derivatives and 25 μg of OVA in PBS in 30 μl/mouse. For i.n. immunization, mice were anesthetized by ether. Mice were immunized daily for 5 days by slow instillation of protein–chitosan mixtures altering the side of instillation each day. Sera from immunized animals were collected in the same way as above two weeks after the last immunization and stored frozen until use.

4.10. Enzyme linked immunosorbent assay (ELISA)

Asp f 2 and OVA specific IgG was determined according to the instructions of the manufacturer. In brief, 100 μl of Asp f 2 or OVA (5 μg/ml) per well were coated on ELISA plates overnight (Costar). All dilutions were made in 1% of BSA in PBS and incubations were made at room temperature. Dilutions 1:10 or 1:500 were used for sera obtained from i.n. or s.c. immunized mice, accordingly. Plates were incubated for 2 h and washed three times with 0.05% Tween 20 in PBS. Anti-mouse IgG–HRP conjugate (Bio-Rad) was then added at working dilution and incubated for 1 h. After incubation and washing the color was developed using *ortho*-phenylene diamine as the substrate. The O.D. was measured at 492 nm on ELISA reader. The data are presented as means of net O.D. values after subtracting the blanks ± standard deviation (SD).

4.11. Statistics

The means and SD were calculated for each group, and the parameters were compared by t-test analysis using Excell Statistic Program.

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