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Mannosylated Liposomes with Built-in Peptidoglycan Based Immunomodulators for Subunit Vaccine Formulations

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THIS PAPER IS DEDICATED TO PROF. MLADEN ŽINIĆ ON THE OCCASION OF HIS 70TH BIRTHDAY

Abstract: The aim of the present study is preparation of mannosylated liposomes with built-in small molecule immunopotentiators for targeted, receptors mediated, delivery of antigens. The liposomes were mannosylated in two ways, by covalent attachment of *p*-aminophenyl-α-D-mannopyranoside to the preformed liposomes and by incorporation of synthetic mono-, di- and tetramannosyl-lipoconjugates into the lipid bilayer of liposomes. Four different mannosylated liposome formulations with incorporated model antigen, ovalbumin (OVA), and immunomodulators, PGM and Ad_2TP2, were prepared and characterized. The influence of mannosylated liposome formulations on the antigen-specific humoral immune response was investigated. It has been shown that mannosylated liposomal formulations did not enhance the humoral immune response and production of anti-OVA antibodies but they significantly affected the type of OVA specific immune reaction and directed it towards Th1 type.

Keywords: mannosylated liposomes, targeted delivery, peptidoglycan monomer, adamantyltripeptide, adjuvants, subunit vaccines.

INTRODUCTION

MODERN vaccine development in the 21st century is based on the recombinant subunit strategy.[1–3] Subunit vaccines contain selected antigens that carry specific pathogen epitopes necessary for efficacious anti-pathogen specific immune response. The problem with highly purified recombinant proteins or synthetic antigens used, is their lower immunogenicity in comparison with attenuated or killed whole organism in conventional vaccine formulations.[4] In order to overcome low immunogenicity, powerful adjuvants are being developed.[5,6] The liposomes are recognized as excellent drug (or antigen) delivery vehicles since they are biocompatible, nontoxic, biodegradable and capable of site-specific drug delivery.[7] Liposomes with built-in small molecule immunopotentiators represent potent adjuvant formulations which can transport the antigen from the injection site to the lymphoid tissue, ensure protection of antigen from the action of hydrolytic enzyme as well as to enhance production of specific cytokines and augment the immune response.[8,9] Mannosylated liposomes have been considered particularly attractive drug delivery carriers because of their ability to target mannose receptors (MR), which have very important role in a large variety of cellular processes.[10,11] MRs expressed on macrophages and antigen presenting cells, APCs mediate endocytosis and actively cooperate in antigen capture and presentation. MRs recognize carbohydrate moieties of many pathogens therefore targeting of glycosylated antigens or carrier systems to MRs is a method to enhance vaccine or drug activity.[12] MRs specifically recognize terminal mannose, fucose and *N*-acetylglucosamine carbohydrate.[13,14] Carbohydrate–protein interactions between individual monosaccharide units (ligands) and protein (receptor) are usually weak in affinities but it can be overcome throught multivalency. The enhanced binding affinity resulting from simultaneous interaction of multiple determinant sugar residues with several binding sites of a multiple receptor is known as ''glycoside cluster effect''.[15,16]

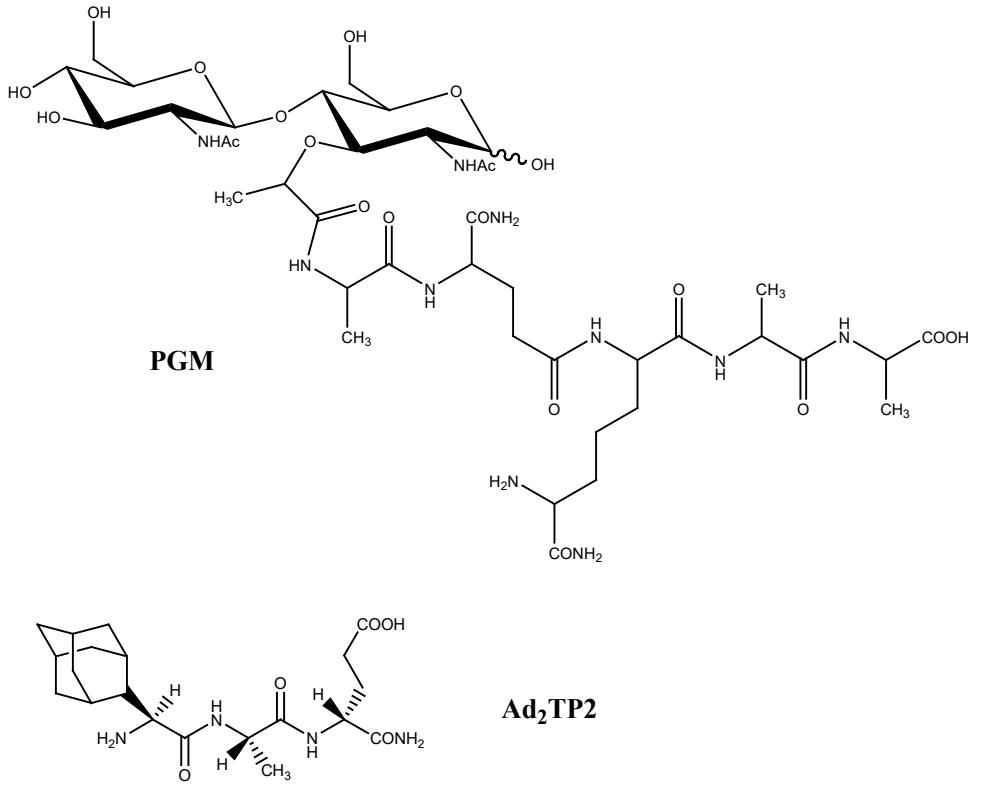

Figure 1. The chemical structures of peptidoglycan monomer (β-D-GlcNAc-(1→4)-D-MurNAc-L-Ala-D-*iso*Gln-*meso*DAP(εNH$_2$)-D-Ala-D-Ala, PGM) and adamantyltripeptide L-(adamant-2-yl)-Gly-L-Ala-D-*iso*Gln (Ad$_2$TP2).

Various strategies have been developed to achive adequate surface decoration of liposomes with the mannose ligands in order to obtain efficient recognition by MRs.[17] It was shown that mannose density on the surface of liposomes as well as stereochemical arrangement of mannosyl epitopes, valency and type of linker used, significantly affect recognition and binding by MRs.[18–20] Efficiency of mannosylated liposomes in targeted drug delivery has been demonstrated in numerous studies.[21–27] Our previous studies have been focused on the synthesis of novel immunomodulators and potent adjuvant formulations with improved activity.[28–30] In recently published paper we described the synthesis of mono-, di- and tetramannosyl-lipoconjugate, used for mannosylation of liposomes, and studies of interaction of prepared mannosylated liposomes with model lectine, concanavaline A (Con A) by quartz crystal microbalance (QCM).[31]

Here we report preparation of mannosylated liposomes with built-in peptidoglycan based immunomodulators, peptidoglycan monomer (β-D-GlcNAc-(1→4)-D-MurNAc-L-Ala-D-*iso*Gln-*meso*DAP(εNH$_2$)-D-Ala-D-Ala, PGM), adamantyltripeptide L-(adamant-2-yl)-Gly-L-Ala-D-*iso*Gln (Ad$_2$TP2) (Figure 1), and ovalbumin (OVA) as a model antigen. Previously synthesized mono-, di- and tetramannosyl-lipoconjugates (Figure 2) were incorporated into lipid bilayer. Additionally, in order to compare different ways of liposome mannosylation to their *in vivo* effect, preformed liposomes were mannosylated by covalent attachment of *p*-aminophenyl-α-D-mannopyranoside on liposomes' surface. Mannosylated liposome formulations of antigen with built-in immunomodulators were characterized and their adjuvant effect was tested. Furthermore, the ability of liposome formulations with structuraly different mannosyl ligands on the surface, to modulate production of total anti-OVA IgG antibodies, IgG1 and IgG2a subclasses was tested.

EXPERIMENTAL

Materials and Methods

1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino-(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG-NH$_2$) and L-α-phosphatidylcholine, type XI-E: from fresh egg yolk (egg-PC) were from Avanti Polar Lipids. Cholesterol from porcine liver, *p*-aminophenyl-α-D-mannopyranoside, 2,4,6-trinitrobenzene sulfonic acid (TNBSA), *o*-phenylenediamine dihydrochloride (OPD), bovine serum albumin (BSA), Tween 20, monoclonal anti-chicken egg albumin (clone OVA-14, mouse IgG1 isotype), avidin-peroxidase

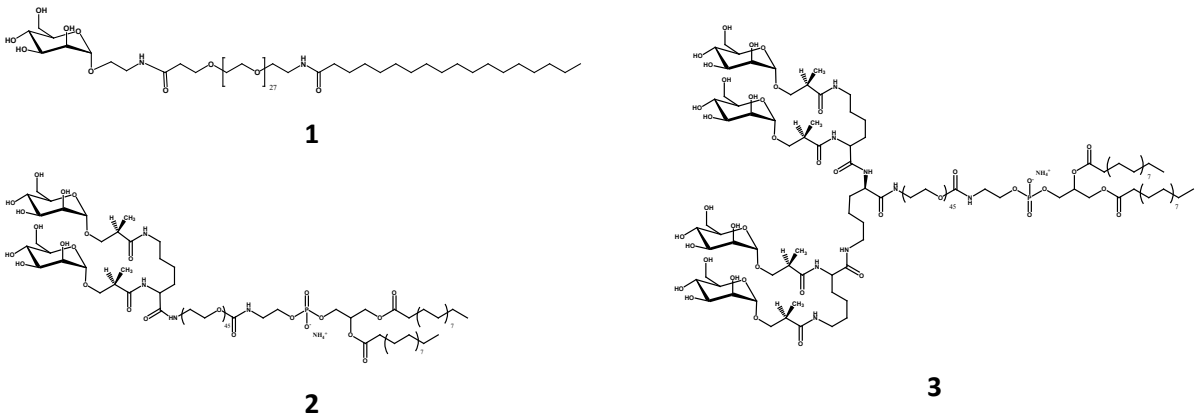

Figure 2. The chemical structures of (**1**) mono-, (**2**) di- and (**3**) tetravalent mannosyl-lipoconjugates.

were obtained from Sigma-Aldrich. Ovalbumin (OVA) was purchased from Serva. Horseradish peroxidase conjugated goat anti-mouse IgG (HRP-anti-mouse IgG) was from BioRad Laboratories. Biotin-conjugated rat anti-mouse IgG1 and anti-mouse IgG2a monoclonal antibodies and streptavidin-peroxidase were purchased from BD PharMingen.

Acetonitrile and trifluoroacetic acid (TFA) were of HPLC grade from Merck (Darmstadt, Germany). A daily supply of water was obtained from Millipore Simplicity–Personal ultra-pure water system (Bedford, MA, USA).

Chemicals for buffers and solutions were from Kemika, unless stated otherwise. Peptidoglycan monomer (PGM) was prepared in PLIVA, Chemical and Pharmaceutical Works (Zagreb, Croatia), according to the previously described procedure.[32] L-(adamant-2-yl)glycyl-L-alanyl-D-isoglutamine (Ad_2TP2) was prepared at the Institute of Immunology (Zagreb, Croatia), as described earlier.[33] Monovalent, **1**, divalent, **2**, and tetravalent, **3**, mannosyl-lipoconjugates were prepared as previously described (Figure 2).[31]

Chromatographic analysis were carried out using the Waters HPLC System equipped with 2996 PDA detector and Empower software (Milford, MA, USA). LiChrosorb RP-18 (5 µm), 4 × 250 mm (Merck) and TSK-gel G300SW 7,5 mm × 60 cm (Tosoh Biosciences), columns for HPLC analyses were used.

Preparation of Mannosylated Liposomes Formulations

Multilamellar liposomes were prepared by thin lipid films following previously described methods.[31,34] Briefly, egg-phosphatidylcholine, cholesterol and mannosyl-lypoconjugates (**1**, **2** or **3**) in a molar ratio of 7 : 5 : 0,5 were dissolved in chloroform : methanol (2 : 1). The chloroform and methanol were evaporated to dryness under vacuum on a rotary evaporator, and then lipid film was hydrated with OVA in saline, concentration 1 mg/mL by gentle hand shaking. The concentration of amphiphiles in liposome suspension was 4 mg/mL. For incorporation PGM or Ad_2TP2 into liposomes, the methanol solution of PGM or ethanol solution of Ad_2TP2 was added to the lipid solution. The concentration of immunomodulators, PGM and Ad_2TP2, in liposome suspension was 5 mg/mL. The liposome suspension was left overnight at 4 °C to swell and stabilize. Liposomes were separated from non-encapsulated material by ultracentrifugation. The liposome suspensions were placed into polycarbonate centrifugation tubes and centrifuged in the ultracentrifuge (Beckman model L5-65, Beckman Coulter TM, USA) for 1 h at 55 000 × g. After centrifugation the supernatants were separated from the pellets and submitted to HPLC analysis. The pellets were resuspended in saline and used for further investigations. Liposome size was reduced by repeated extrusion through polycarbonate membranes with the pore size of 800 and 200 nm using the 0,5 mL extruder (LiposoFast, Avestin Inc., Canada).

Liposomes were prepared under sterile conditions, using sterile glassware and saline while chemicals and solvents were of high purity.

Mannosylation of Preformed Liposomes with *p*-Aminophenyl-α-D-Mannopyranoside

Preformed liposomes were mannosylated by covalent attachment of *p*-aminophenyl-α-D-mannopyranoside to DSPE-PEG2000-NH_2 of the liposomes using glutaraldehyde as a coupling agent following previously described method.[35] Briefly, prepared phosphatidylcholine, cholesterol and DSPE-PEG2000-NH_2 liposomes in a molar ratio of 7 : 5 : 0,5 (4 mg/mL) with encapsulated antigen and immunomodulators was mixed with *p*-aminophenyl-α-D-mannopyranoside (10 equivalent of DSPE-PEG2000-NH_2) and 20 % solution of glutaraldehyde (140 equivalent). Suspensions were mixed 5 min and then mannosylated liposome suspensions were separeted from uncoupled *p*-aminophenyl-α-D-mannopyranoside and glutaraldehyde by ultracentrifugation. The liposome suspensions were placed

into polycarbonate centrifugation tubes and centrifuged in the ultracentrifuge (Beckman model L5-65, Beckman Coulter TM, USA) for 1 h at 55 000 × g. After centrifugation the supernatants were separated from the pellets and the pellets were resuspended in saline. The liposome suspensions were extruded through polycarbonate membranes with the pore size of 800 and 200 nm using the 0,5 mL extruder.

To estimate the coupling efficiency of *p*-aminophenyl-α-D-mannopyranoside, the uncoupled amino groups of DSPE-PEG2000-NH_2 on the liposomes were measured spectrophotometrically as described in literature.[35] 100 µL of 4 % $NaHCO_3$ and 100 µL 10 % SDS were added to 100 µL liposome suspension coupled (or uncoupled) with *p*-aminophenyl-α-D-mannopyranoside. The liposome suspension was incubated at 30 °C and after 20 min 100 µL 0,1 % TNBSA solution was added. The resulting solution was kept at 40 ° C for 2 h. The reaction was terminated by adding 50 µL of 1 M HCl. The absorbance of the final solution was read at 335 nm against blank sample prepared as above with 100 µL of saline instead of the liposome suspensions. The coupling efficiency (CE) was calculated indirectly in accordance to the formula, $CE = (1 - A_{uncoupled} / A_{total}) \times 100$ %, where the CE is the coupling efficiency of *p*-aminophenyl-α-D-mannopyranoside, $A_{uncoupled}$ is absorbance of uncoupled amino groups of DSPE-PEG2000-NH_2 on the liposomes after mannosylation with *p*-aminophenyl-α-D-mannopyranoside and A_{total} is the absorbance of amino groups of DSPE-PEG2000-NH_2 on the liposomes prior to conjugation with *p*-aminophenyl-α-D-mannopyranoside.

Determination of the Entrapment Efficiency

The entrapment efficiency of OVA, PGM and Ad_2TP2 in mannosylated liposomes was determined by HPLC in the supernatants following ultracentrifugation, as described previously.[36,37] All analyses were performed at a flow rate of 1,0 mL/min at room temperature. The eluate of OVA was monitored at 280 nm and eluates of PGM and Ad_2TP2 were monitored at 214 nm.[38] The isocratic system A was 0,1 M phosphate buffer with 0,1 M sulphate buffer and 0,05 % NaN_3, pH = 6,6, prepared with an ultra pure water (daily supplied from a Simplicity – Personal ultra pure water system, Millipore, USA). Two gradient systems were used for the tested compounds. The gradient solvent systems used were made of acetonitrile containing 0,035 % TFA and water containing 0,05 % TFA. Each system contained a different percentage of acetonitrile and the amount of acetonitrile was changed at the indicated running times. For system B with a running time 25 min, the percentage of acetonitrile at 0, 20 and 25 min was 3, 17 and 3, respectively. For system C with running time 20 min, the percentage of acetonitrile at 0, 15 and 20 min was 10, 30 and 10, respectively. System A was used for analyses of OVA, system B for analyses of PGM and system C for analyses Ad_2TP2. Samples (100 µL OVA, 50 µL PGM and 20 µL Ad_2TP2) were injected onto column using autosampler. The standard curves of tested compounds were constructed for each compound prior to the analyses of supernatants from the liposome preparations. The amounts of the nonentrapped compounds were determined using standard curve and the amounts of the entrapped compounds was calculated by subtracting the obtained value from the total amount of the compound used for liposome preparation.

In vivo Testing of Immunostimulating Activity

All experiments were carried out using NIH/OlaHsd female adult mice. During the experimental period animals were housed in the Animal Facility of the Institute of Immunology. Food and water were provided ad libitum. All animal work was performed in line with a national legislative of animal welfare (NN 135/06). Animals were immunized and boosted two times at 21-days intervals. Mice were anaesthetized with i.p. application of ketamine/xylazine (25 mg/kg each) and blood was collected from axillary's plexus on the 7th day after second booster. Individual sera were decomplemented at 56 °C for 30 min and stored at –20 °C until tested. Mice were immunized subcutaneously in the tail base with 0,1 mL (10 µg OVA) of appropriate immunisation solution. The amount of lipids administered to each mouse was 400 µg.

ELISA for Qualitative and Quantitative Determination of Anti-OVA IgG

Anti-OVA antibodies were determined by previously described ELISA modified as follows.[36,39] Flat-bottomed high binding ELISA plates (Costar, USA) were coated with 100 µL of 15 µg/mL OVA solution in carbonate buffer, pH = 9,2, overnight at room temperature (RT). Non-specific antibody binding was blocked by incubation with 0,5 % (*w*/*v*) BSA in PBS–T (0,05 % (*v*/*v*) Tween 20 in PBS) buffer (200 µL per well) for 2 h at 37 °C. Standard antibody preparation and mouse sera to be tested (100 µL per well) were added in serial two-fold dilutions and incubated overnight at RT. All samples were analysed in duplicates. Plates were washed and 100 µL of goat HRP–anti-mouse IgG (4 000 × diluted) was added and incubated 2 h at 37 °C. After washing, the substrate solution consisting of 0,6 mg/mL OPD solution in citrate–phosphate buffer, pH = 5,0, with 0,5 µL 30 % H_2O_2 /mL was added (100 µL per well) and incubated for 30 min at RT in the dark. The enzymatic reaction was stopped with 50 µL per well 12,5 % H_2SO_4 and absorbency at 492 nm was measured using a microplate reader (Reader 530; Organon Teknika, The Netherlands). All washings (three times after each step) were done with PBS–T buffer in microplate washer (Multiwash; Labsystems, Finland).

Quantitative determination of anti-OVA IgGs was done by parallel line analysis comparing each serum to the standard - monoclonal anti-OVA IgG, to which we voluntarily assigned 20 000 arbitrary units per mL (AU/mL).

For quantification of OVA specific immunoglobulin G subclass, IgG1 and IgG2a, plates were coated with OVA as described above and incubated with sera and standard antibody preparations (IgG1 or IgG2a). Plates were washed and 100 µL of biotinylated rat anti-mouse IgG1 at 0,05 µg/mL or biotinylated rat anti-mouse IgG2a at 0,5 µg/mL were added to each well and incubated 2 h at 37 °C. After washing, avidin-HRP (50 000 × diluted) was added for determination of IgG1, while streptavidin-HRP (100 000 × diluted) was used for IgG2a determination (100 µL per well) and incubated 2 h at 37 °C. Plates were washed and substrate solution was added as describe above. The enzymatic reaction was stopped with 12,5 % H_2SO_4 and absorbency at 492 nm was measured using a microplate reader.

The relative quantities of antibody subtypes were determined by parallel line assay using appropriate standard preparation. The monoclonal anti-OVA IgG1 was a standard for relative quantification of anti-OVA IgG1 to which 400 000 AU/mL was assigned, while polyclonal mouse serum containing high levels of anti-OVA IgG2a was used as a standard for relative quantification of IgG2a specific antibodies with voluntarily assigned 5 000 AU/mL.

Statistical Analysis

Statistical analyses were performed using Statistica 6.0 for Windows, StatSoft Inc. The significant difference between experimental groups was determined by Mann-Whitney U-nonparametric tests. Probability values less than 0,05 ($p < 0,05$) were considered significant.

RESULTS AND DISCUSSION

Preparation of Mannosylated Liposomes

Mannosylated liposomes have been studied as promising carriers for targeted drug delivery because of their ability to target MRs, which are considered to be pattern-recognition receptors.[12,40] The glycosylation as well as mannosylation of liposome surface can be accomplished in several ways.[17] The most used methods encompasses adsorption of different glycopolimers or a chemical reaction between the glycosylconjugate and liposome.[41] Although simple, these methods are constrained regarding the stability of liposomes in the reaction mixture. Incorporation of synthesized glycosyl lipoconjugates into lipid bilayer is the most elegant but also the most challenging method. Chemical synthesis, purification and characterization of such amphiphilic compounds is a very complex process.[17] Synthesis of new glycosyl lipoconjugates provides many advantages with respect to the design of carbohydrate ligands having specific structural characteristics and their binding on different lipophilic anchors such as fatty acids[31] and cholesterol.[42] In our previous research, we have shown how adamantane can be used as a membrane anchor in lipid bilayer for different carbohydrate molecules which are exposed on liposome surface.[30,43] The obtained results have confirmed that Con A successfully recognized mannosyl ligands exposed on liposome surface and exhibits higher affinity to multivalent mannosyl ligands.[31]

Here, we report preparation and characterization of mannosylated liposomes with/without built-in peptidoglycan based immunomodulators, PGM or Ad_2TP2, and with entrapped model antigen, ovalbumin. The mannosylated liposomes were prepared in two ways, by incorporation of synthetic mono-, di- and tetramannosyl-lipoconjugates (**1**, **2**, **3**) into the lipid bilayer of liposomes or by covalent attachment of *p*-aminophenyl-α-D-mannopyranoside to the preformed liposomes. Immunomodulators were incorporated into multilamellar large liposomes bearing structurally different mono- and multimannosyl-ligands on the surface using modified film method. Ethanol/methanol solutions of PGM or Ad_2TP2 were added into organic phase together with phospholipids and mannosyl-lipoconjugate and prepared thin lipid films were hydrated with 1 mg/mL OVA in saline.

Post-modification of preformed liposomes was carried out by covalent attachment of *p*-aminophenyl-α-D-mannopyranoside to DSPE-PEG2000-NH_2 incorporated in liposomes made of phosphatidylcholine : cholesterol : DSPE-PEG2000-NH_2 = 7 : 5 : 0,5 using glutaraldehyde as the coupling agent in accordance with the previously published procedure[35] (Figure 3). Excessive amount of glutaraldehyde and *p*-aminophenyl-α-D-mannopyranoside after chemical reactions were removed by ultracentrifugation. Coupling efficiency of *p*-aminophenyl-α-D-mannopyranoside and liposome was measured spectrophotometrically by adding the visualization reagent, TNBSA. The calculated coupling efficiencies of *p*-aminophenyl-α-D-mannopyranoside and preformed liposomes are presented in Table 1.

Coupling efficiency of *p*-aminophenyl-α-D-mannopyranoside on empty liposomes and liposomes with encapsulated OVA were 28,06 and 28,73 % respectively, and are in accordance with the published data.[35] It was demonstrated that OVA did not affect the coupling efficiency of *p*-aminophenyl-α-D-mannopyranoside and preformed liposomes. Increase in coupling efficiency was observed for liposomes with entrapped PGM and particularly for liposomes with entrapped Ad_2TP2. A possible explanation lies in the abovementioned ability of adamantane to accommodate itself in the lipid bilayer while the polar part of the molecule

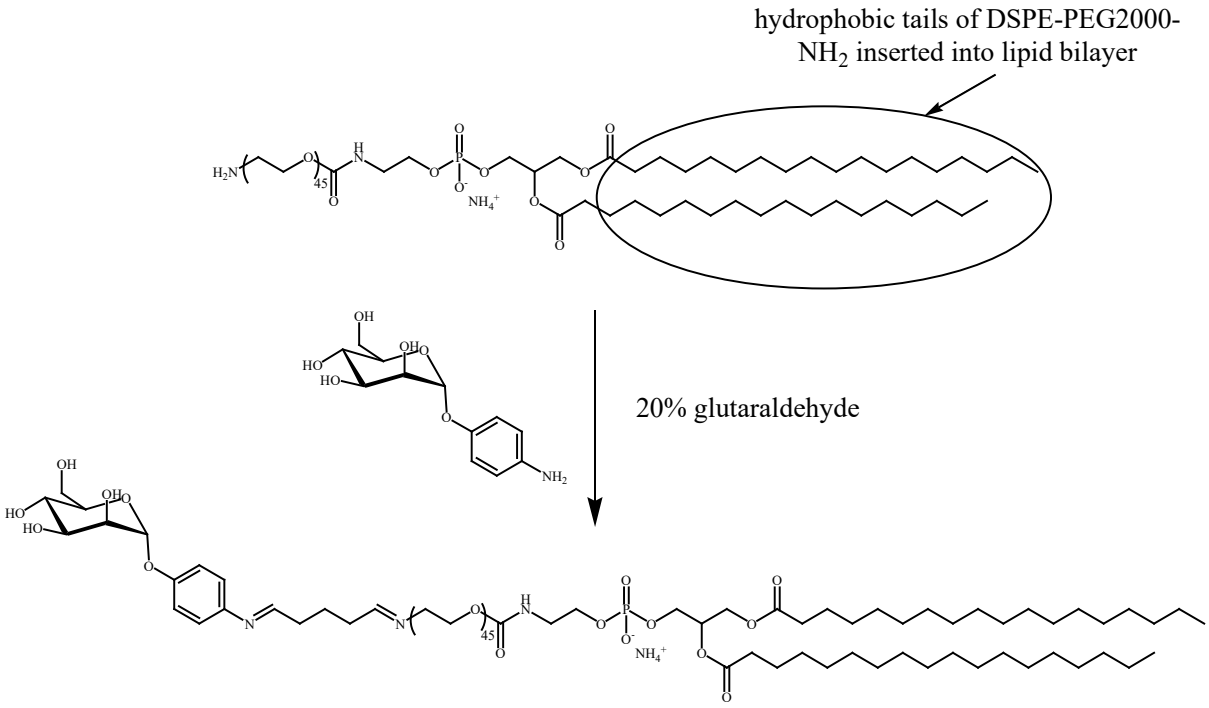

Figure 3. Post-modification of preformed liposomes and covalent attachment of *p*-aminophenyl-α-D-mannopyranoside to the DSPE-PEG2000-NH_2 in MLV liposomes.

Table 1. Coupling efficiency of *p*-aminophenyl-α-D-mannopyranoside and preformed liposomes. The results are expressed as an average value ± standard deviation of three different liposome preparations in duplicates

Sample	Coupling efficiency / %
Empty liposomes	28,06 ± 4,77
Liposomes with OVA	28,73 ± 9,83
Liposomes with OVA + PGM	41,24 ± 6,49
Liposomes with OVA + Ad_2TP2	73,79 ± 2,39

is exposed on liposome surface. While Ad_2TP2 can be entrapped in the aqueos phase of liposomes, it is also partialy incorporated in lipid bilayer of liposomes.[44] The peptide part of Ad_2TP2 is near the liposome surface and its free amino group is available for reaction with glutaraldehyde and *p*-aminophenyl-α-D-mannopyranoside. Since the PGM is a hydrophilic molecule and is not embedded in the lipid bilayer of a liposome we presume that PGM could be non-specifically adsorbed on hydrophylic polyethylene glycol in DSPE-PEG2000-NH_2 molecule incorporated in liposome bilayer. PGM possesses a reactive amino group on diaminopimelic acid in the peptide part of the molecule that could react with glutaraldehyde and thus potentiate the binding of *p*-aminophenyl-α-D-mannopyranoside. The obtained results have confirmed that covalent attachment of *p*-aminophenyl-α-D-mannopyranoside to preformed liposome has successfully conducted with high coupling efficiency.

Determination of the Entrapment Efficiency of OVA and Immunomodulators, PGM and Ad_2TP2

In order to perform *in vivo* evaluation of prepared mannosylated liposome formulations, it is necessary to determine the entrapment efficiency of antigen and built-in immunomodulators. The entrapment efficiency of OVA, PGM and Ad_2TP2 in mannosylated liposomes was determined in accordance with previously developed methods.[34,36] The results of entrapment efficiency of OVA, PGM and Ad_2TP2 into mannosylated liposomes are presented in Table 2.

It was shown that the entrapment efficiency for OVA and immunomodulators is highest in preformed liposomes which underwent post-modification by covalent attachment of *p*-aminophenyl-α-D-mannopyranoside. The entrapment efficiency of OVA, PGM and Ad_2TP2, is about 30 % in preformed mannosylated liposomes and it is in accordance with the method and buffer used. Previously, we have shown that the amount of OVA, PGM and Ad_2TP2 entrapped into non-mannosylated liposomes mostly depends on the ionic strength of buffer and the type of phospholipids used.[36,37]

The amount of OVA, PGM and Ad_2TP2 entrapped into mannosylated liposomes containing PC and cholesterol together with mono-, di- and tetra mannosyl-lipoconjugates varies greatly. Synthesized mannosyl-lipoconjugates containing DSPE-PEG2000-NH_2 and branched carbohydrate ligand bound to the linker, are very complex molecules and they strongly affect the formation of lipid bilayer of liposomes and incorporation of compounds into liposomes. The increase of number of mannose molecules in di- and tetramannosyl-lipoconjugate, incorporated into liposomes causes increased branching of bulky moieties on the inner and outer sides of liposome leaflets. Consequently, the entrapped aqueous volume with examined compounds is smaller, resulting in the decrease of entrapment efficiency of OVA, PGM and Ad_2TP2 into di- and tetramannosylated liposomes in comparison with entrapment efficiency into preformed monomannosylated liposomes. Therefore, entrapment of compounds into tetramannosylated liposomes was lowest because of multivalency and branching of mannose residues of tetramannosyl-lipoconjugate **3** incorporated into lipid bilayer of liposomes. It is known that incorporation of DSPE-PEG into liposomes reduces the internal aqueous volume by bulky PEG chains which cover inner and outer liposome leaflets, resulting in the decrease of entrapment efficiency.[45,46] The influence of bulky multivalent mannosyl lipoconjugates on incorporation, is best exemplified Ad_2TP2, where the incorporation efficiency is decreased in dimannosylated and particularly in tetramannosylated liposomes compared with monomannosylated liposomes. The amount of entrapped Ad_2TP2 is the highest in the monomannosylated preformed liposomes and lowest in tetramannosylated liposomes since the adamantane except to the aqueous phase can also be incorporated into the lipid bilayer. The decrease of entrapment efficieny of OVA and PGM in di- and tetramannosylated liposomes compared with monomannosylated preformed liposomes is also observed. Here, the entrapment efficiency of OVA into monomannosylated liposomes containing monomannosyl-lipoconjugate, Man-PEG-palmitamide **1** is the lowest. This result can be explained by electrostatic repulsion of amphiphile and OVA during rehydratation of lipid film and liposome formation. OVA is negatively charged at pH = 7.4 (pI = 4.6).[31] PC-liposomes at pH = 7,4 have slightly negative charge and PEGylation of liposomes decreases the zeta potential because a hydrophilic coat on the surface of liposomes is formed. Incorporation of Man-PEG-palmitamide **1**, into lipid bilayer causes less shading of negative charge due to smaller size of the polymer chain of PEG molecule in comparison with PEG molecule in tetramannosyl conjugate, as we have shown previously.[31]

The Influence of Different Mannosylated Liposome Formulations of OVA and Immunomodulators on Anti-OVA Immune Response *in vivo*

Adjuvant property of liposomes was first reported in 1974[47] and since then the liposomes have been intensively studied in terms of delivery of different antigens.[43,44] Liposomes can be excellent antigen carriers and adjuvants of a new generation due to their biocompatibility and their physicochemical properties which can be tailored to obtain different immunological effects. The binding of specific ligands at liposome surface for targeted pathogen recognition receptors (PRRs) is one of the convenient ways to activate the entire immune response. PRRs are molecules on cell surface that recognize pathogens and can affect the strength and direction of the immune response.[48,49] MRs are group of PRRs molecules and C-type lectins which are expressed in mice and humans and show mannose–binding ability but their role in host defence is still not clearly understood. The participation of MRs in phagocytosis, antigen presentation and intracellular signalling have been shown.[50,51] This explains why the design and synthesis of a new drug delivery systems using mannosyl ligands for targeting MRs represents a promising model to achieve strong and specific immune response.[10]

In present study, we have prepared mannosylated liposomes with built-in small molecule immunopotentiators, PGM and Ad_2TP2, for targeted delivery of OVA antigen and evaluated their influence on production of specific anti-OVA IgGs, particularly IgG subclasses. Increased production

Table 2. Entrapment efficiency of OVA, PGM and Ad_2TP2 in mannosylated liposomes. The results are expressed as an average value ± standard deviation of three different liposome preparations in duplicates

Sample	Entrapment efficiency / %		
	OVA	PGM	Ad_2TP2
Preformed monomannosylated liposomes	36,46 ± 7,42	31,30 ± 6,49	36,00 ± 9,34
Monomannosylated liposomes	8,24 ± 2,07	19,05 ± 9,26	27,22 ±8,32
Dimannosylated liposomes	27,18 ± 7,42	25,31 ± 3,84	16,30 ± 0,71
Tetramannosylated liposomes	10,42 ± 1,78	17,76 ± 3,26	6,12 ± 1,84

of IgG2a in mice is an indicator of activation of a Th1 immune response and the ratio IgG1/IgG2a can be used as an indirect marker for Th type of immune response.[52] Our study addressed the effects of mannose valency of synthesized ligands exposed on the liposome surface, on the recognition by MRs as well as effects of prepared liposome formulations on the specific anti-OVA immune response. Adjuvant activity of mannosylated liposome formulations *in vivo,* was evaluated in NIH/OlaHsd mice according to our previously described procedure.[36,39] OVA was chosen as an antigen of low immunogenicity in accordance with recommendations for adjuvant testing.[53] Production of total anti-OVA IgG, as well as anti-OVA IgG1 and anti-OVA IgG2a subclasses was determined by ELISA in the mice sera after the second booster. The parallel line assay using appropriate standard antibody preparation for relative quantities of antibodies determination was used. Results are expressed in arbitrary units per milliliter (AU/mL). Results of total anti-OVA IgG production in mice immunized with mono-, di- and tetramannosylated liposome formulations with OVA and immunomodulators, are presented in a box-and-whisker plot in Figure 4. Encapsulation of OVA into mannosylated liposomes overall decreased the amount of produced specific IgGs in comparison with amount of IgGs produced in experimental group of mice where antigen was applied in saline. Incorporation of PGM and Ad_2TP2 in mannosylated liposomes did not significantly affect the total anti-OVA IgG production in comparison to mannosylated liposomes with antigen alone with the exception of dimannosylated liposomes. The possible explanation could be low OVA availability since significantly lower entrapment of OVA, PGM and Ad_2TP2 into mannosylated liposomes was observed (Table 2).

Adjuvants can express their activity through several mechanisms of action: the depot effect, increased antigen presentation effect, antigen targeting effect, immunomodulation effect and CTL induction effect.[54] As we mentioned above, adjuvants can modulate the Th1/Th2 ratio of induced immune response.[55,56] In order to investigate how the incorporation of OVA, PGM and Ad_2TP2 into mannosylated liposome can modulate Th type of specific immune response, the levels of antibody subclasses were tested. The amounts of specific anti-OVA IgG1 and anti-OVA IgG2a, indicators of Th2 and Th1 type of immune responses are presented in Figure 5 and Figure 6, respectively. In all experimental groups the amount of anti-OVA IgG1 antibodies production closely resembled those of total anti-OVA IgG levels. Significant decrease of anti-OVA IgG1 antibody levels was found in mice immunized with mannosylated liposome formulations with or without immunomodulators in comparison to group treated with OVA in saline alone (Figure 5). Exception was found for a group of mice immunized with antigen and immunomodulators encapsulated into dimannosylated liposomes.

Trend of increased anti-OVA IgG2a antibodies production in mice immunized with preformed monomannosylated and dimannosylated liposome formulations of OVA in comparison to control group of OVA in saline, was found (Figure 6 a and 6 c). Experimental group immunized with monomannosylated and tetramannosylated liposome formulations of OVA with or without PGM and Ad_2TP2 had lower anti-OVA IgG2a levels in comparison with group immunized with OVA in saline (Figure 6 b and 6 d). This could be a result of extremely low entrapment efficiency of OVA. However, in the case of tetramannosylated liposomes addition of Ad_2TP2 and even PGM (despite low entrapment efficiency) increased anti-OVA IgG2a level in comparison with group of mice that received only antigen encapsulated in tetramannosylated liposomes (Figure 6 d).

The ratio of anti-OVA IgG1/IgG2a levels was calculated for each serum separately and is presented in a box-and-whisker plot in Figure 7. A significant difference in anti-OVA IgG1/IgG2a ratio was found for monomannosylated and dimannosylated liposome formulations of OVA in comparison to control group of mice received OVA in saline, indicating that mannosylated liposome formulations could shift the immune reaction to Th1 type. Addition of immunomodulators, PGM and Ad_2TP2 into the mannosylated liposomes, reversed anti-OVA IgG1/IgG2a ratio back to mixed Th type. The obtained results are in accordance with our previous investigations of encapsulation of PGM into conventional PC : CHOL : DCP liposomes and their influence on specific immune response.[36]

Several research groups have reported synthesis of antigen delivery systems that targeted MRs and successfully modulate the immune response. Shimizu at al. have shown that the administration of soluble leishmanial antigen (SLA) encapsulated in liposomes coated with neoglycolipds containing oligomannose residues, Man5-DPPE and Man3-DPPE, induces a specific Th1 immune response in mice and protection against *Leishmania major* infection.[57,58] Hattori et al. studied potency of mannosylated cationic liposomes for DNA vaccination using OVA-encoding pDNA (pCMV-OVA).[59] They have shown that mannosylated cationic liposomes enhanced gene expression of an antigen and enhanced Th1 response in comparison to naked pCMV-OVA and pCMV-OVA complexed with non-mannosylated liposomes. The importance of valency and structural features of mannose ligands for immunomodulation of specific immune response is undeniable but has not yet been completely understood. It has been demonstrated that the affinity of MRs was enhanced with mannose valencies, increasing from 2 to 6 terminal mannose residues in synthesized series of lysine-based cluster oligomannosides.[60] Espuelas et al. studied interaction of plain liposomes and mannosylated liposomes with immature human dendritic cells (iDC).[61,62] They have observed that multibranched

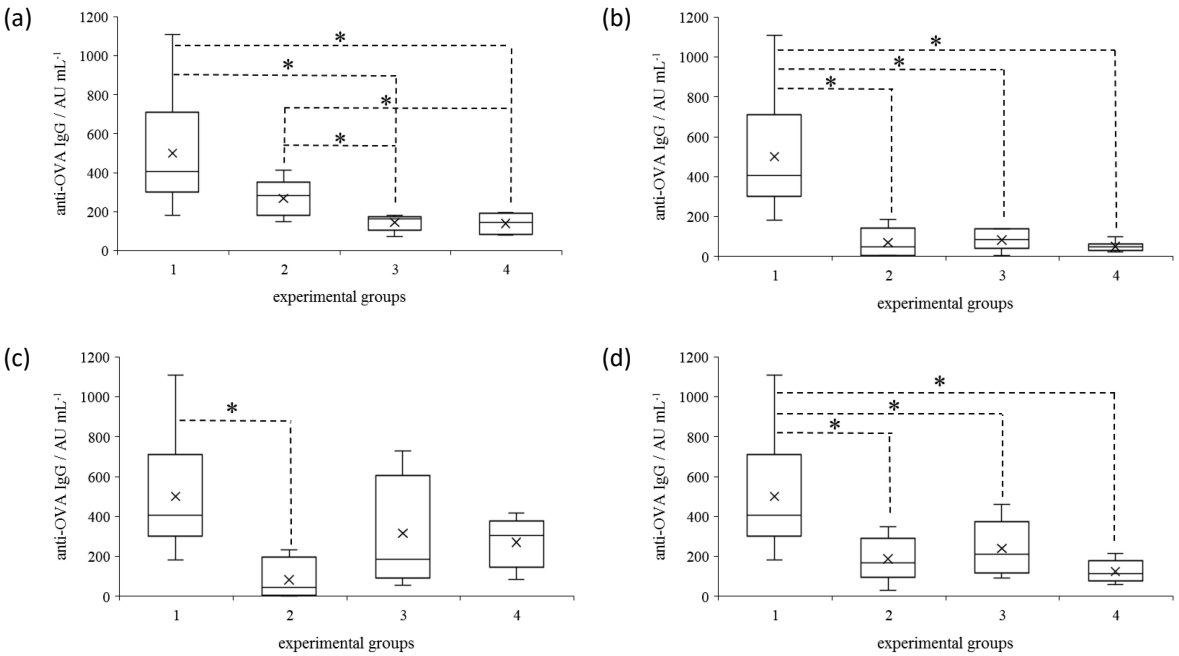

Figure 4. Anti-OVA IgG antibodies levels raised in mice after immunization and two boosters with mannosylated liposome formulations of OVA, PGM and Ad_2TP2: preformed monomannosylated liposomes (a), monomannosylated liposomes (b), dimannosylated liposomes (c), and tetramannosylated liposomes (d). Experimental group: 1. OVA in saline, 2. OVA in mannosylated liposomes, 3. (OVA + PGM) in mannosylated liposomes, 4. (OVA + Ad_2TP2) in mannosylated liposomes. × group mean value; – median group value; ∗ statistically significant difference between experimental groups ($p < 0,05$).

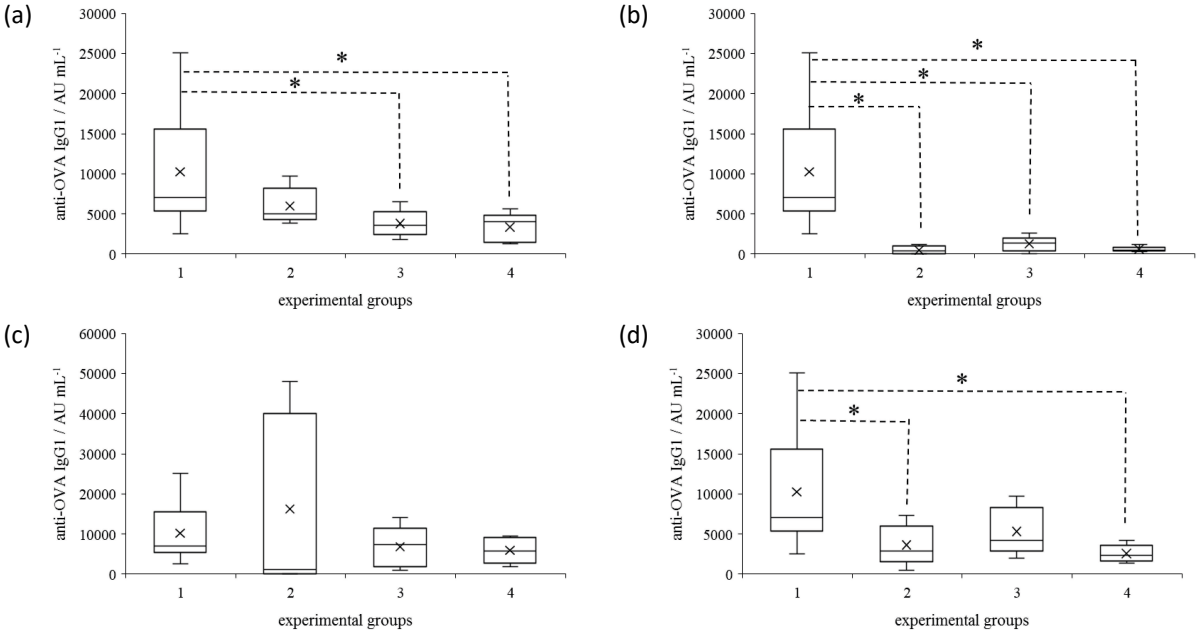

Figure 5. Anti-OVA IgG1 antibodies levels raised in mice after immunization and two boosters with mannosylated liposome formulations of OVA, PGM and Ad_2TP2: preformed monomannosylated liposome (a), monomannosylated liposome (b), dimannosylated liposome (c), and tetramannosylated liposome (d). Experimental group: 1. OVA in saline, 2. OVA in mannosylated liposomes, 3. (OVA + PGM) in mannosylated liposomes, 4. (OVA + Ad_2TP2) in mannosylated liposomes. × group mean value; – median group value; ∗ statistically significant difference between experimental groups ($p < 0,05$).

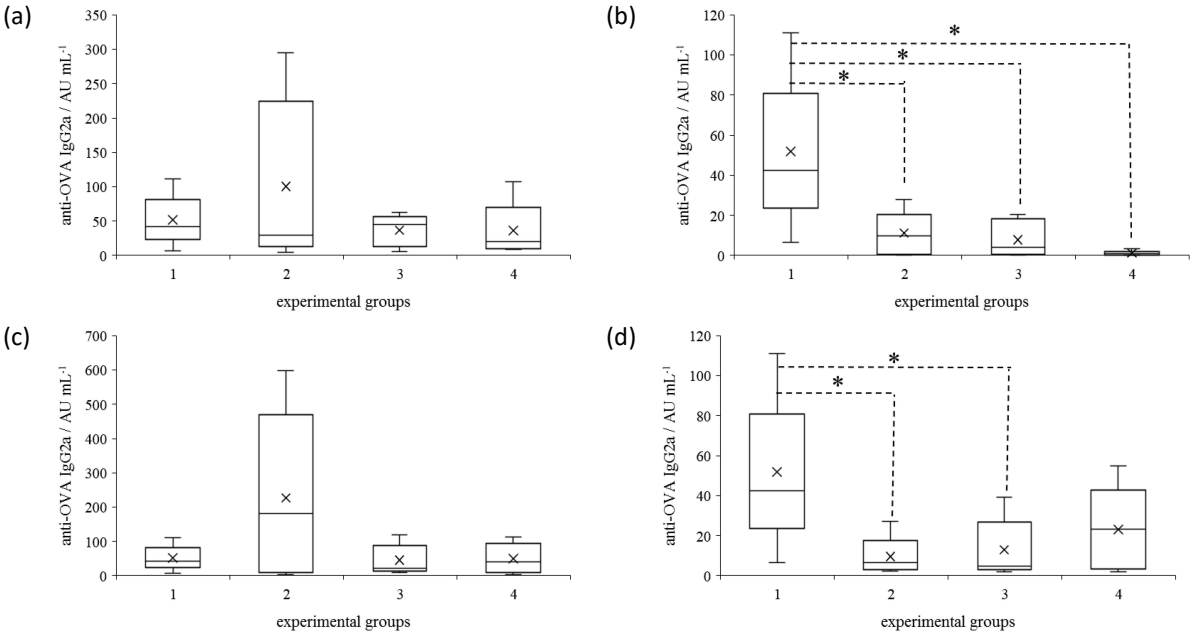

Figure 6. Anti-OVA IgG2a antibodies levels raised in mice after immunization and two boosters with mannosylated liposome formulations of OVA, PGM and Ad_2TP2: preformed monomannosylated liposome (a), monomannosylated liposome (b), dimannosylated liposome (c), and tetramannosylated liposome (d). Experimental group: 1. OVA in saline, 2. OVA in mannosylated liposomes, 3. (OVA + PGM) in mannosylated liposomes, 4. (OVA + Ad_2TP2) in mannosylated liposomes. × group mean value; – median group value; ∗ statistically significant difference between experimental groups ($p < 0,05$).

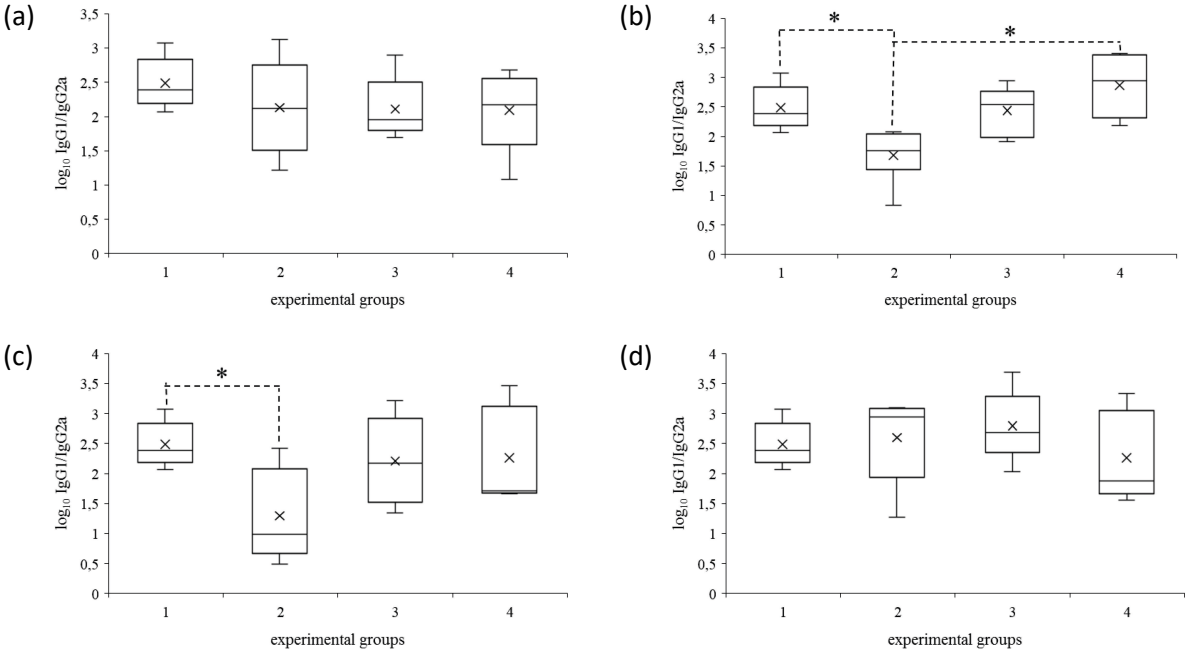

Figure 7. The ratio of anti-OVA IgG1 and anti-OVA IgG2a levels developed in NIH/OlaHsd (H-2q) mice after immunization and two boosters with mannosylated liposome formulations of OVA, PGM and Ad_2TP2: preformed monomannosylated liposomes (a), monomannosylated liposomes (b), dimannosylated liposomes (c) and tetramannosylated liposomes (d). Experimental group: 1. OVA in saline, 2. OVA in mannosylated liposomes, 3. (OVA + PGM) in mannosylated liposomes, 4. (OVA + Ad_2TP2) in mannosylated liposomes. For each mouse serum, obtained after second booster, $\log_{10}$ (IgG1/IgG2a) was calculated; × group mean value; – median group value; ∗ statistically significant difference between experimental groups ($p < 0,05$).

mannosylated lipids incorporated into liposomes were more efficiently targeted to iDC than monomannosylated lipids.

Our results are in accordance with published data and we confirmed that in defined conditions for *in vivo* experiments, dimannosylated liposome formulations were as efficient as tetramannosylated liposome formulations. Also, we have found that the preformed monomannosylated liposomes have similar, sometimes even better, effect on modulating of specific immune response than monomannosylated liposomes with incorporated monomannosyl lipoconjugate.

Although the mannosylated liposomal formulations of OVA, together with PGM and Ad_2TP2 did not show enhanced immunostimulation of humoral immune response, they significantly affected the type of specific immune reaction to applied OVA as antigen and directed it towards Th1 type. A significant difference in anti-OVA IgG1/IgG2a ratios was found for monomannosylated and dimannosylated liposome formulations of OVA in comparison to control group of mice receiving OVA in saline. Until now, no mannosylated liposomal formulations of OVA together with PGM or Ad_2TP2 have been described, therefore the obtained results are valuble contribution to the development of new adjuvants.

CONCLUSIONS

In this work mannosylated liposome formulations of OVA and either PGM or Ad_2TP2 were prepared and characterized. The liposomes were mannosylated in two ways, by covalent attachment of *p*-aminophenyl-α-D-mannopyranoside to the preformed liposomes and by incorporation of synthetic mono-, di- and tetramannosyl-lipoconjugates into the lipid bilayer of liposomes. The entrapment efficiency of OVA, PGM and Ad_2TP2 was calculated and the obtained results show that entrapment efficiency is highest in monomannosylated preformed liposomes, while in liposomes mannosylated with synthetic mono-, di- and tetramannosyl-lipoconjugates it depends on the structure and valency of carbohydrate epitope. The lowest entrapment efficiency of OVA was found in monomannosylated liposomes. Furthermore, only 6,1 % of Ad_2TP2 was encapsulated in tetramannosylated liposomes due to steric hindrance during the encapsulation.

Preliminary *in vivo* evaluation of adjuvant ability of prepared mannosylated liposomes with built-in PGM and Ad_2TP2, was performed and the influence on production of specific anti-OVA IgGs was tested. The specific anti-OVA IgG1 and anti-OVA IgG2a, indicators of Th2 and Th1 type of immune responses were measured. Significant decrease of anti-OVA IgG1 antibody levels was found in mice immunized with liposome formulations both with and without immunomodulators in comparison to group treated with antigen in saline alone. The ratio of anti-OVA IgG1/IgG2a levels was calculated and it reveals that monomannosylated and dimannosylated liposome formulations of OVA significantly decrease this ratio, in comparison to control group of mice which received OVA in saline. The results demonstrated that mannosylated liposomal formulations did not enhance the humoral immune response and production of anti-OVA antibodies but they significantly affected the type of OVA specific immune reaction, causing polarization to Th1, a cellular type of immune response.

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Conflict of Interest. The authors declare no conflict of interest.

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