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Morphological and immunological characterization of immunostimulatory complexes based on glycoglycerolipids from *Laminaria japonica*✪

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Some physicochemical properties of glycoglycerolipids (monogalactosyldiacylglycerol, digalactosyldiacylglycerol and sulfoquinovosyldiacylglycerol) from the sea algae *Laminaria japonica*, as well as their ability to become incorporate into immunostimulating complexes (ISCOMs), used as a delivery system of microbial and tumor antigens in vesicular form, were studied. These glycolipids were found to differ essentially in fatty acid composition, unsaturation index and thermotropic behavior. The possibility of ISCOM modification by embedding the glycolipids studied instead of a phospholipid component in vesicles was shown. A preliminary research of the immunogenicity of the pore-forming protein from *Yersinia pseudotuberculosis* in modified (by monogalactosyldiacylglycerol) and typical (egg phosphatidylcholine) ISCOMs did not reveal a significant enhancement of immune response in comparison with that of isolated protein.

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Abbreviations: Chol, cholesterol; Con A, concanavaline-A; DGDG, digalactosyldiacylglycerol; FCS, fetal calf serum; ISCOM, immunostimulating complex; LPS, lipopolysaccharide; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PHA, phytohemagglutinin; SQDG, sulfoquinovosyldiacylglycerol.

The use of isolated antigens in vaccine construction allows to cause highly specific immune response to bacterial and virus pathogens and tumor cells. However, most of highly purified antigens have insufficient immunogenicity. In this connection, significant efforts are made to search for effective adjuvants and new techniques of antigen presentation to immunocompetent cells (Morein & Hu, 2000).

During last years, much attention has been devoted to the multimeric form of antigen presentation by immunostimulating complexes (ISCOMs) (Kersten & Crommelin, 1995; 2003), which represent submicron non-living particles, composed of saponin, cholesterol and phospholipids. In experimental animals ISCOMs induced protection against various pathogenic microorganisms, such as *Neisseria gonorrhoeae* (Kersten *et al.*, 1988), herpes simplex virus (Mohamedi *et al.*, 2001), rotavirus (van Pinxteren *et al.*, 1999) etc. Immunization with antigens included in such structures, in comparison with antigens in liposomes or as killed virus particles, enhanced the specific antibody response 10–100-fold or more (Morein *et al.*, 1984). Antigens presented to the immune system in ISCOMs can elicit a wide range of immune activity, included Th1-like and Th2-like immune responses (Ennis *et al.*, 1989; Mohamedi *et al.*, 2001).

The various antigenic carriers and adjuvants not only enhance the immune response of the macroorganism to an antigen, but also possess immunomodulating properties (Morein & Hu, 2000). They can influence the type and character of the induced immune reactions to the point of activation of inadequate immune response, which can aggravate the clinical course, raise sensitivity of the host organism to infection or cause autoimmune reaction. The induction gear of the immune response can differ depending on the structure and physicochemical parameters of the antigenic carriers and adjuvants. In this connection, the use of natural compounds with different structures and physicochemical properties to design ISCOMs with various immunomodulating activities seems to be urgent. In this context we attempted to modify lipid composition of ISCOMs by incorporation of glycoglycerolipids, such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerols (DGDG) and sulfoquinovosyldiacylglycerol (SQDG), isolated from the marine macrophyte *Laminaria japonica*.

The biological properties of these major polar lipids of plants are poorly understood, although it is known that MGDG from green algae show anti-tumor-promoting effect (Morimoto *et al.*, 1995). SQDG from marine algae inhibit DNA-polymerase and HIV-reverse transcriptase (Gustafson *et al.*, 1998; Loya *et al.*, 1998; Ohta *et al.*, 1998). Nothing is known about their adjuvant properties, though some glycolipids reveal such activity and can be used as adjuvants (Fujii *et al.*, 2003; Matsumoto *et al.*, 2003). On the other hand, the biological activity of marine algae and grasses is connected with polyunsaturated fatty acids, which are abundant components of their glycoglycerolipids. In turn, the phase state of polar lipids influences the immunogenicity and immunospecificity of lipid–protein complexes (Bakouche & Gerlier, 1986). Bearing in mind these facts, we have undertaken a study of the relationship between physicochemical properties of polar lipid constituents and ISCOM immunological activity.

The pore-forming protein from *Yersinia pseudotuberculosis*, being a species-specific antigen of *Yersinia* (Novikova *et al.*, 1996), was chosen as the antigen in ISCOM vaccines. The outer membrane porins of Gram-negative microorganisms are immunodominant antigens. They are capable of inducing production of protective, bactericidal and opsonizing antibodies. As components of artificial vaccines, porins are successfully applied to prevent infectious diseases caused by the following pathogens: *Pseudomonas aeruginosa, Neisseria meningitides, N. gonorrhoeae, Salmonella typhimurium, Proteus mirabilis* and some

representatives of *Shigella* (Adamus *et al.*, 1980; Buchanan & Arko, 1977; Karch & Nixdorff, 1981).

In the present work physicochemical properties and ISCOM-forming capacity of different glycoglycerolipids isolated from *L. japonica* were investigated, as well as a preliminary immunological study of MGDG-modified ISCOM with porin from *Y. pseudotuberculosis* was carried out.

MATERIALS AND METHODS

Egg yolk phosphatidylcholine (PC) was isolated according to (Singleton *et al.*, 1965); cholesterol (Chol), saponins from *Quillaja saponaria*, octylglucoside, fetal calf serum (FCS), RPMI-1640 medium, 199 medium, lipopolysaccharide (LPS), phytohemagglutinin (PHA), concanavaline-A (Con A), peroxidase-coupled anti-mouse Ig (M+G) were obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.); commercial zymosan from *Saccharomyces cerevisiae*, produced by Russia; [^{3}H]thymidine was purchased from "Isotop" (Russia).

Glycoglycerolipid isolation and analysis. Glycoglycerolipids – MGDG, DGDG and SQDG, were isolated from brown algae *Laminaria japonica*, Aresch (Phaeophyta) as described in (Sanina *et al.*, 2003). The algae were harvested in Possiet Bay (the Sea of Japan) in the summer from seawater at a temperature of 20–23°C. Freshly collected algae were thoroughly cleaned to remove epiphytes, small invertebrates and sand particles and then heated for 2–3 min in boiling water to inactivate enzymes. Total lipid extracts from about 10 kg of algae were obtained according to (Folch *et al.*, 1957) immediately after the collection and the following homogenization of the algae. Extracted lipids were taken to dryness at 60°C under vacuum and solubilized in $CHCl_3$. Extracts were stabilized with 0.1– 0.05% 2,6-di-tert-butyl-*n*-cresol and stored at –20°C until required.

Crude glycoglycerolipids were isolated from total lipid extract by column chromatography on silica gel by gradient elution with acetone/chloroform (1:1, v/v), acetone, acetone/benzene/acetic acid/water (200:30:3:10, by vol.). Then, glycoglycerolipids were purified by preparative thin-layer chromatography (TLC) on silica using acetone/benzene/acetic acid/water (200:30:3:10, by vol).

The purity of lipids was checked by two-dimensional silica TLC with chloroform/acetone/methanol/acetic acid/water (100:40:20:20:8, by vol.) in the first direction and acetone/benzene/acetic acid/water (200:30:3:10, by vol.) in the second direction (Vaskovsky & Khotimchenko, 1982). Chromatographically pure glycoglycerolipids were solubilized in chloroform for use for ISCOM preparation, as well as for analysis of glycolipid phase transitions and fatty acid composition. Phase transitions of glycoglycerolipids were analyzed as described in (Sanina *et al.*, 2003). Glycolipids were introduced into standard aluminium pans. Vacuum-dried samples of approximately 10 mg were sealed into pans and placed in a DSM-2M differential scanning calorimeter (Puschino, Russia). Samples were either heated or cooled at 16°C/min in a temperature range between 100°C and 80°C at a sensitivity of 5 mW. The peak in the plot of heat capacity *versus* temperature was recorded as the phase transition temperature, t_{max}. The temperature range was calibrated by using naphthalene, mercury and indium. Analysis of acyl chains linked to glycolipids was carried out by gas-liquid chromatography (GLC) as described in (Khotimchenko, 1993).

ISCOM preparation and analysis. The membrane pore-forming protein from the human pathogen *Yersinia pseudotuberculosis* was isolated and purified using a simple, efficient and non-denaturing method (Novikova *et al.*, 1989). ISCOMs were prepared by the dialysis method (Lövgren & Morein, 1988). Briefly, 50 μg pore protein in 0.25% SDS were mixed with 1 mg saponin and 500 μg PC and Chol. Lipids (PC/Chol or MGDG/Chol or

DGDG/Chol or SQDG/Chol) were previously dissolved in 4% octylglucoside to a final concentration of 10 mg/ml. Phosphate-buffered saline (PBS), pH 7.2, was added to adjust lipid concentration to 1 mg/ml. The mixture was sonicated for 15 min in a sonicator bath and extensively dialyzed against PBS. During the first 6 h, the dialysis was carried out at room temperature and the following 12 h at 4°C.

ISCOM particles formation was observed in a JEM-7A electron microscopy (Jeol, Japan) using 2% phosphotungstic acid solution as contrasting agent. Sedimentation coefficient was determined a K32M ultracentrifuge (Russia) through 10–60% (w/w) sucrose in PBS at 200 000 × ***g*** for 18 h at 4°C. The ISCOM samples were cleaned of sucrose by dialysis against PBS for 24 h. The determination of lipids (PC or glycolipids) in ISCOMs was by using silica TLC. The protein pellet was solubilized in 5% SDS (w/v) and the protein content was determined according to (Lowry *et al.*, 1951).

Immunological study. Three groups of 10 BALB/c mice, 18–20 g, were immunized twice (in four weeks) into the peritoneal cavity (i.p.) either with 5 μg MGDG-ISCOMs or with 5 μg PC-ISCOMs, both containing porin, or with 50 μg porin in PBS (200 μl). The mice were bled by retroorbital plexus puncture at the third week after the first immunization and the second week after booster. Lymphatic organs (thymus and spleens) were aseptically removed.

Serum samples were analyzed to define total antibody response by enzyme-linked immunosorbent assay (ELISA). ELISA was carried out in polystyrene microplates (Dynatech microElisa, Finland). The plates were coated with porin protein as antigen at 10 μg/ml. Antigen was diluted in sodium carbonate/bicarbonate buffer (pH 9.6) and 100 μl solution was added to the plates, which were incubated for 2 h at 37°C. The plates were washed three times with PBS (pH 7.4), containing 0.05% (v/v) Tween 20, and drip dried. To prevent non-specific binding, 250 μl of PBS with 0.5% Tween 20 (contact buffer) was added to the wells, and the plates were held overnight at 4°C. Then the wells were washed three times as described above and drip dried.

Aliquots of 100 μl mouse serum of each sample initially diluted from 1:200 to 1:3200 with PBS buffer, were distributed into the wells and incubated for 2 h at 37°C. Sera from non-immunized mice served as negative control. Then plates were washed as described above, and 100 μl aliquots of peroxidase-coupled anti-mouse Ig (M+G), diluted 1:1000 in contact buffer, were added. The plates were incubated for 1 h at 37°C, washed and dried. Substrate (70 μl) (0.05% *o*-phenylendiamine, 0.012% H_2O_2 in phosphate/citrate buffer, pH 5.0) was added to the wells and incubated for 20 min at room temperature in dark. Enzyme reaction was stopped by addition of 30 μl 0.25 M H_2SO_4. The specific antibody activity was monitored at 492 nm by multichannel spectrophotometer. Nonimmunized mouse sera served as negative control.

To determine lymphocyte functional activity, spleens were gently homogenized in 199 medium. The supernatant containing single cells was centrifuged (250 × ***g*** for 10 min at 4°C) and suspended at density 1×10^6 cells/ml in FCS-RPMI. Aliquots (180 μl) of the cell suspension were placed in a flat-bottomed 96-well tissue culture plate and cultured for 72 h at 37°C with LPS (50 μg/ml) or Con A (20 μg/ml) or PHA (25 μg/ml) in humidified atmosphere of 5% CO_2. Control wells received distilled water without mitogen. The three-day cultures were pulsed, in triplicate, with 1 μCi [^{3}H]thymidine for 6 h before harvesting. At the end of incubation the cells were harvested and radioactivity was measured in a liquid scintillation counter (Mark III, U.S.A.).

To estimate bactericidal activity 10 μl of heparinized blood was mixed with 10 μl opsonized zymosan particles (150 μg/ml) and 10 μl NBT in PBS, pH 7.2. After 30 min incubation smears were made and stained with

methylene green. Neutrophiles with dark blue granules of formazan were counted under the microscope.

All values are the arithmetic mean ±S.D. Student's unpaired *t*-test was used to determine statistical significance.

RESULTS AND DISCUSSION

The drastic increase of the antigen presentation efficiency of ISCOMs to immunocompetent cells makes it reasonable to consider these complexes as potential antigen carriers in various vaccines. Spontaneous incorporation of membrane proteins into ISCOM structure is possible when phospholipids are used as an additional component. Usually phosphatidylcholine and phosphatidylethanolamine are applied for these purposes. In the present study, we tried to obtain ISCOMs, consisting of marine algae glycoglycerolipids, *Quillaja* saponins, cholesterol and of *Y. pseudotuberculosis* porin as antigen. The original ratio of glycoglycerolipid/Chol/*Quillaja* saponins/protein for ISCOM preparation was 1:1:4:0.5 (by weight). Earlier, this ratio was shown (Lövgren & Morein, 1988) to be optimum for PC-ISCOM formation.

The three major glycoglycerolipids of the brown alga *L. japonica* essentially differ in physicochemical properties. The analysis of fatty acid composition showed that the content of polyunsaturated fatty acids was decrease, and the percentage of saturated fatty acids increased in the following sequence: MGDG–DGDG–SQDG (Fig. 1). As a result, the unsaturation index of the most saturated glycolipid, SQDG, was about 5 and 1.5 times lower than one of MGDG and DGDG, respectively. MGDG comprised a wide spectrum of both C_{18} and C_{20} polyunsaturated fatty acids, the major of which were 18:4n-3, 20:5n-3, 20:4n-6, 18:3n-3, 18:2n-6,18:3n-6. DGDG and, especially, SQDG contained noticeably less polyunsaturated acids. Their major fatty acids were the saturated palmitic and myristic acids, and the unsaturated oleic and linoleic fatty acids.

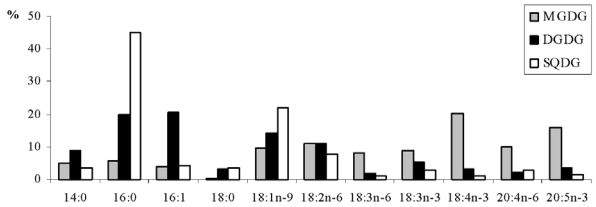

Figure 1. Fatty acid composition of glycoglycerolipids from *L. japonica* harvested in summer (% of total fatty acids).

According to the revealed distribution of fatty acid residues between the glycoglycerolipids, thermal transitions of the most unsaturated glycolipid, MGDG, mainly occurred at the lowest temperature range from –78 to 4°C (Fig. 2). Thermograms of both DGDG and SQDG were shifted to higher temperatures and situated between about –50 and 60°C,

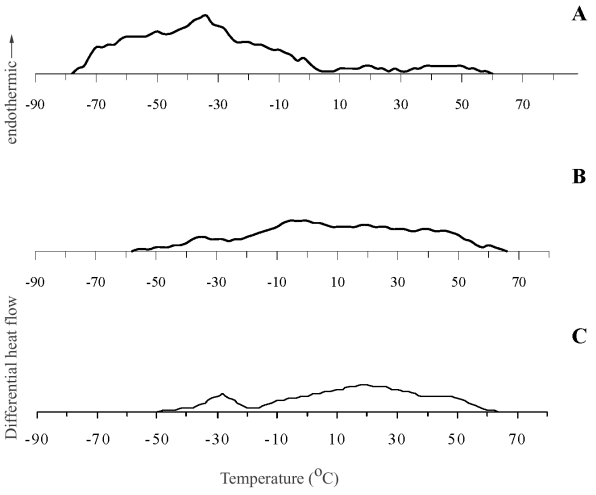

Figure 2. DSC thermograms of major glycoglycerolipids isolated from *L. japonica*.

MGDG (A), DGDG (B) and SQDG (C). Vertical bar represents 0.5 mW. Scanning rate, 16°C/min. Sample weight, 10 mg

while t_{max} of DGDG was substantially lower in comparison with SQDG (–6–0°C and 20°C,

respectively), which coincided with the different degree of its fatty acid unsaturation.

Abundant ISCOMs particles with characteristic cage-like morphology were found in MGDG-modified ISCOM preparation (Fig. 3). Thus, MGDG inclusion in ISCOMs did not change the classical morphology of the vesicles (Kersten & Crommelin, 1995). The particles formed had a ring-shaped form (Fig. 3) with pores and a sedimentation coefficient (19 S), typical for ISCOMs. However, the modified ISCOMs showed significant size heterogeneity, varying from 16.5 nm to 33 nm. One may conclude that MGDG has a weaker ISCOM-forming ability than phospholipids (PC or PE).

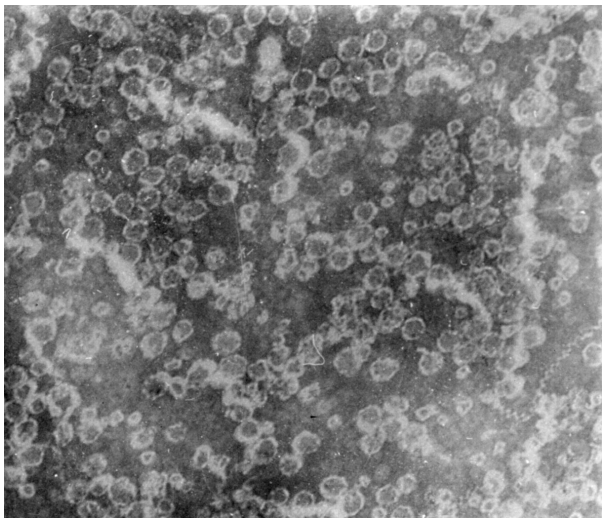

Figure 3. Negative staining electron micrograph (100 000 ×) MGDG-ISCOMs.

Bar is 100 nm.

As a result of DGDG incorporation, two types of structures were formed: 11–15 nm ISCOMs and spirals, from which the vesicles seemed to form (not shown). SQDG was not able to form ISCOM particles.

The comparative biochemical analysis of ISCOM components revealed, that the inclusion of glycolipids varied in the preparations. MGDG content was higher in comparison with DGDG and virtually the same as PC (by weight).

Beyond the different fatty acid composition and thermal transitions, these hydrated polar lipids are characterized by different superstructures: MGDG – hexagonal, SQDG – lamellar, and DGDG – globular (isotropic) phases. The hydration number decreases in the following sequence: SQDG–DGDG–MGDG. Also, the number of bound water molecules is suggested to depend on the chemical structure of the sugar head groups (Sanina *et al.*, 2002). The type of lipid superstructure is important for the ISCOM-forming ability of the polar lipid component. Thus, more effective incorporation of MGDG may be explained by its capacity to form hexagonal mesophase and by comparatively lower hydration, i.e. higher hydrophobity of this glycolipid.

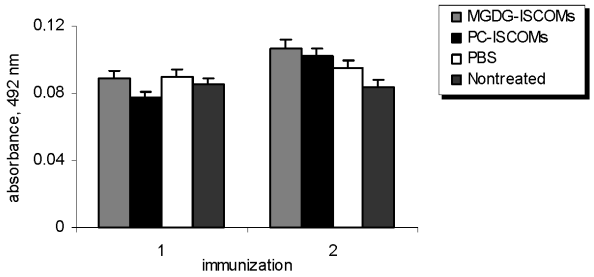

Figure 4. Antibody level analysis in mouse sera 3 weeks after first immunization (1) and 2 weeks after booster (2).

Experiments were performed in duplicate, and values are means ±S.D. from at least three mice; $P < 0.05$.

In our study the trimeric molecular form of the pore-forming protein from *Y. pseudotuberculosis* used as antigen was determined in all the ISCOM preparations tested. However, we did not observe an enhancement of the antibody response by PC-ISCOMs or MGDG-ISCOMs containing bacterial porin in comparison with isolated porin trimer even after reboost (Fig. 4). The functional activity of spleen lymphocytes from mice immunized with ISCOM vaccines (MGDG-ISCOM or PC-ISCOM) to B-cell mitogen (LPS) did not differ from that of control animals (Fig. 5C). A relative high proliferation rate was observed under stimulation of lymphocytes from mice

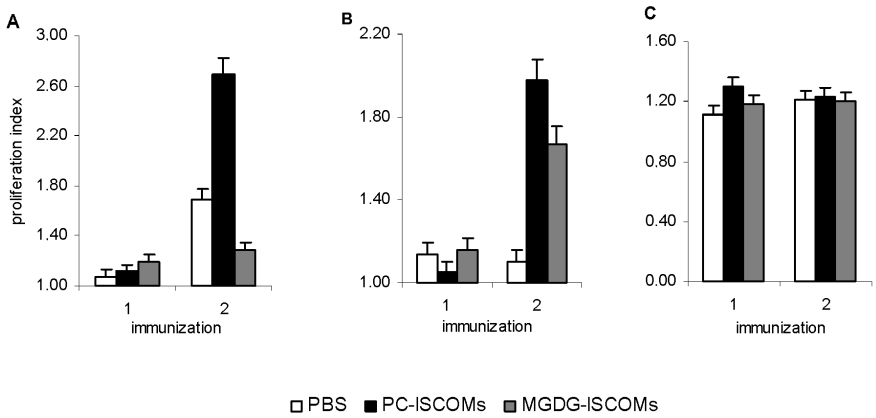

Figure 5. Mouse spleen lymphocyte blastogenesis in the presence of mitogens: (A) PHA; (B) Con A; (C) LPS.

The analysis was carried out 3 weeks after the first immunization (1) and 2 weeks after booster (2). The proliferation index was determined from the ratio of mean c.p.m. of test culture to c.p.m. of control ones. Experiments were performed in duplicate and values are means ±S.D. from at least three mice. The significance in cell proliferation compared to the corresponding controls was determined by the Student's *t*-test: $P < 0.05$.

given PC-ISCOM with T-cell mitogens (PHA or Con A) (Fig. 5A, B). An enhancing effect of MGDG-ISCOM vaccine was observed only in the case of mitogenic stimulation with Con A, which was less than PC-ISCOM effect (Fig. 5B). Such responses of lymphocytes to stimulation by T-cell mitogens are presumed to reflect the status of the cell-mediated immunity in the animals immunized with porin-containing vaccines. Although the general effectiveness of both ISCOM preparations was moderate, it would see that lipid modification had immunomodulatory effect on spleen cell potential to T-cell mitogens.

The investigation of bactericidal activity of blood neutrophiles did not reveal authentic distinctions between three groups tested (not shown). These findings indicate the probable absence of a nonspecific stimulation of the immune system to protein antigen by ISCOM vaccines.

The total decrease of the level of blood leucocytes (Fig. 6), lymphoid organs weight reduction (Fig. 7) in combination with high hemolytic activity of the pore-forming protein (ED_{50} 3 μg/ml) *in vitro* obviously testify to the immunodepressive properties of the antigen. At the same time, ISCOM vaccination did not exert such negative effects in the animals in comparison with free protein administration. Probably the ISCOM vesicles deposit the antigen and decrease its toxicity, although they are not highly immunologically effective. So, native trimeric pore-forming protein can not be considered an acceptable antigen for vaccine development against *Y. pseudotuberculosis*.

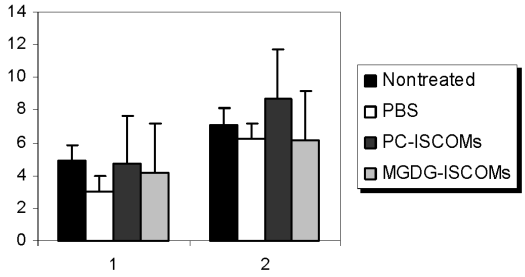

Figure 6. Total number of leukocytes in mouse peripheral blood after first (1) and second (2) immunization.

The results are expressed as arithmetic mean (thousands of cells in μl) ±S.D. of three mice; $P < 0.05$.

This study showed the possibility of ISCOM composition modification by using of plant glycolipids instead of a phospholipid component in the vesicles. The absence of a marked immunostimulatory activity of modified and classical ISCOMs to the porin from *Y. pseudotuberculosis* confirms that the choice of adjuvant must be determined by specific antigen

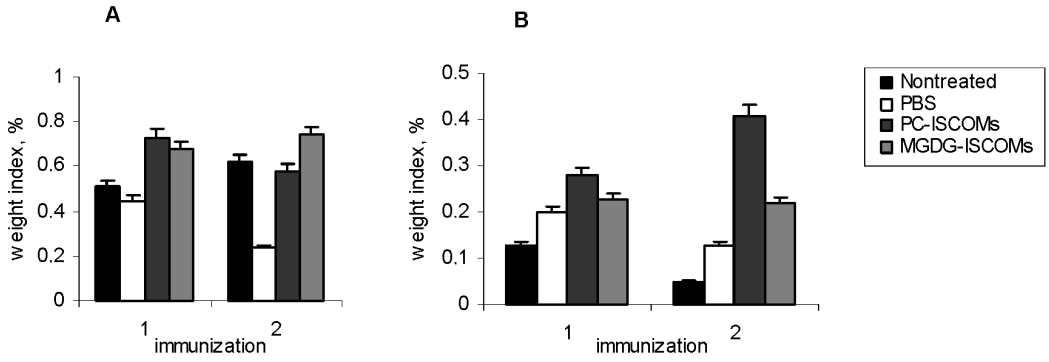

Figure 7. Weight indexes of mouse lymphoid organs (A, spleen; B, thymus) after primary and secondary immunization.

The weight indexes were calculated as the percentage ratio between organ weight and body weight. Experiments were performed in duplicate and values are means ±S.D. from at least three mice; $P < 0.05$.

properties used for vaccine designing. In this connection, the main direction of further research may be the choice of suitable molecular form of porin. Earlier it was shown (Portnyagina *et al.*, 1999) that heat-modified monomer of porin from *Y. pseudotuberculosis* lost the pore-forming activity and had higher immunogenic properties than its native trimeric form (Lychatskaya *et al.*, 1985).

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