## BIOCHEMISTRY

# Physicochemical and Immune Properties of Glycoglycerolipids from *Laminaria japonica* in Immunostimulating Complexes (ISCOMs)

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**Abstract**—Certain physicochemical properties of glycoglycerolipids from marine alga *Laminaria japonica* (monogalactosyldiacylglycerol, digalactosyldiacylglycerol, and sulfoquinovosyldiacylglycerol) and their ability to be incorporated into immunostimulating complexes (ISCOMs) used for delivery of microbial and tumor antigens in vesicular form were comparatively described. These glycolipids proved to considerably differ by fatty acid composition, degree of unsaturation, and phase transition temperatures. Production of modified ISCOMs through incorporation of these glycolipids into the vesicle instead of the glycolipid component was demonstrated. Preliminary data demonstrated no significant increase in immune response to *Yersinia pseudotuberculosis* porin in the modified (with monogalactosyldiacylglycerol) and classical (with phosphatidylcholine) ISCOMs as compared to pure porin.

## INTRODUCTION

Development of a new generation of vaccines is based on application of isolated antigens providing for a highly specific immune response to bacterial and viral pathogens as well as to tumor cells. However, high-purity antigens often have insufficient immunogenicity substantiating considerable efforts taken to searching efficient adjuvants and new ways to present antigen to immune system cells (Morein and Hu, 2000).

A considerable number of recent publications deal with a multimeric form of antigen delivery in immunostimulating complexes (ISCOMs) (Kersten and Crommelin, 1995, 2003). ISCOMs are artificial submicron particles composed of saponin, cholesterol, and phospholipids. Immunization with antigens included in such structures increases formation of the corresponding specific antibodies by a factor of tens as compared to antigens encapsulated in liposomes or dead viral particles (Lövgren and Morein, 1988). Antigens incorporated into ISCOMs can be delivered by various antigen-presenting cells (dendritic cells, B cells, and peritoneal cells) that consequently stimulate proliferation of Tx cells secreting both Tx1 and Tx2 type cytokines (Bungener *et al.*, 2002).

Sjolander *et al.* (1997) demonstrated that various carriers and adjuvants both enhanced the immune response of the organism to antigen and had an immunomodulating activity. They affected the type and pattern of induced immune responses up to activation of an inappropriate immune response that can aggravate disease development, increase sensitivity to infection, or induce an autoimmune response. The mechanism of immune response induction depends on the structure and physicochemical properties of the used carrier and/or adjuvant. In this context, application of natural compounds with physicochemical properties similar to the components of classical ISCOMs seems relevant for obtaining vesicles with different immunomodulating activity. Within the frames of this approach, we decided on changing the composition of lipid component of ISCOMs through replacing phospholipids with plant glycoglycerolipids from marine algae.

Medical and biological properties of the main plant polar lipids remain poorly explored, although monogalactosyldiacylglycerol (MGDG) from green algae proved to have antitumor activity (Morimoto *et al.*, 1995). Sulfoquinovosyldiacylglycerol (SQDG) from marine algae inhibits DNA polymerase and reverse transcriptase of HIV (Gustafson *et al.*, 1998; Loya *et al.*, 1998; Ohta *et al.*, 1998). Biological activity of marine glycoglycerolipids is explained by both the structure of their carbohydrate component and the presence of considerable quantities of polyunsaturated fatty acids. Bakouche and Gerlier (1986) demonstrated that phase state of polar lipids affected immunogenic and

immunospecific properties of lipid–protein complexes. Considering this fact we tried to study the relationship between immune activities of ISCOM and physicochemical properties of its lipid component.

We choose a pore-forming protein from *Yersinia pseudotuberculosis*, a species- and genus-specific antigen of yersiniae (Novikova *et al.*, 1996), as an antigen for incorporation into ISCOMs. Outer membrane porins of Gram-negative microorganisms are highly immunogenic surface antigens. They can induce formation of protective, bactericidal, and opsonic antibodies. As components of synthetic vaccines, porins are successfully used to prevent infections induced by pathogenic *Pseudomonas aeruginosa, Neisseria meningitides, N. gonorrhoeae, Salmonella typhimurium, Proteus mirabilis* and certain representatives of *Shigella* genus (Buchanan and Arko, 1977; Adamus *et al.*, 1980; Karch and Nixdorff, 1981).

Here we studied physicochemical properties and ISCOM-forming capacity of glycoglycerolipids of different structure isolated from *Laminaria japonica* and performed preliminary tests for the effect of modified ISCOMs on immunogenic properties of the membrane pore-forming protein from *Y. pseudotuberculosis* after replacement of the phospholipid (egg phosphatidylcholine) with MGDG.

## MATERIALS AND METHODS

Glycoglycerolipids were isolated from brown alga *Laminaria japonica* Aresch (Phaeophyta) as described by Sanina *et al.* (2003). Plant material was collected in Posiet Bay (Sea of Japan) in summer at water temperature of 20–23°C. Total lipid extracts (about 10 kg) were obtained as described by Folch *et al.* (1957) immediately after homogenizing and combining the material. Raw glycoglycerolipids were isolated from total lipid extracts by silica gel column chromatography. The purity of lipids was controlled by two-dimensional TLC (Vaskovsky and Khotimchenko, 1982). Determination of the temperature of phase transition ($T_{max}$) of glycoglycerolipids and analysis of their acyl chains were carried out as described by Sanina *et al.* (2003) and Khotimchenko (1993), respectively. Porin trimer was isolated from *Y. pseudotuberculosis* as described by Novikova *et al.* (1989) while egg phosphatidylcholine (PC) was isolated according to Singleton *et al.* (1965). Hemolytic activity of porin was determined on mouse blood cells after a 1-h incubation at 37°C.

Chromatographically pure glycoglycerolipids were used to prepare ISCOMs as described by Lövgren and Morein (1988). Lipid mixtures (PC/cholesterol or MGDG/cholesterol) were solubilized in 4% aqueous solution of octyl glucoside to final concentration of 10 mg/ml. The lipid mixture was complemented with 50 µg of porin in 0.25% dodecyl sulfate and 1 mg saponin. Lipid content was adjusted to 1 mg/ml by phosphate buffered saline (PBS), pH 7.2. The mixture was

sonicated for 15 min and dialyzed against PBS for 6 h at room temperature and 12 h at 4°C.

Forming ISCOM particles were negatively contrasted with 2% phosphotungstic acid (pH 7.0) and observed under an electron microscope JEM-7A (Jeol, Japan). Sedimentation coefficient was determined by centrifugation on a K32M preparative ultracentrifuge (Russia) in sucrose gradient (10–60% by weight) at 200 000*g* and 4°C for 18 h. ISCOM samples were washed from sucrose by dialysis for 2 h against PBS. The lipids included in ISCOM were identified by TLC on silica gel. Protein was quantified according to Lowry *et al.* (1951) after ISCOM solubilization by sodium dodecyl sulfate (5% w/v).

Three groups of BALB/c mice (10 animals each) were twice intraperitoneally immunized (at a 4-week interval) with 5 µg protein antigen in MGDG–ISCOMs and PC–ISCOMs or with 50 µg protein in 200 µl PBS. Analysis of the blood and lymphoid organs was performed 3 weeks after the first immunization and 2 weeks after reimmunization. Total rate of antibody formation in serum was determined by enzyme linked immunosorbent assay (ELISA) using peroxidase-conjugated antimouse Ig(M+G). Sera of unimmunized animals served as a negative control.

Functional activity of splenocytes was determined by lymphocyte transformation test (LTT) in the presence of polyclonal mitogens LPS (50 µg/ml), Con A (20 µg/ml), or PHA (25 µg/ml). Proliferation rate was monitored by incorporation of [³H]-thymidine introduced into three-day-old cultures 6 h prior to the end of cultivation. Distilled water was added to the control wells instead of mitogens. Radioactivity of the incorporated level was determined on paper filters using a liquid scintillation counter Mark III (United States).

For evaluation of bactericidal activity of neutrophils, 10 µl heparinized blood was mixed with 10 µl zymosan (150 µg/ml) and 10 µl 0.2% nitroblue tetrazolium in PBS, pH 7.2. After a 30-min incubation, smears were prepared and stained with methylene green. The number of functionally active neutrophils was counted under a microscope.

The obtained data were statistically processed using the Student's *t*-test.

## RESULTS AND DISCUSSION

The presence of a phospholipid (PC or PE) is required for spontaneous incorporation of membrane proteins into ISCOM structure. Here we studied possible modification of ISCOM by replacement of phospholipids with glycoglycerolipids of various structure (MGDG, DGDG, and SQDG) from *L. japonica* and using a pore-forming protein from *Y. pseudotuberculosis* as a model antigen.

First, we determined qualitative and quantitative fatty acid composition of plant glycolipids and described their physicochemical properties. All studied
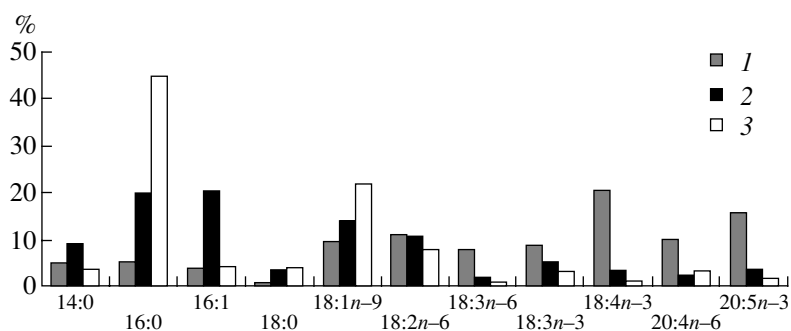
**Fig. 1.** Fatty acid composition of glycoglycerolipids MGDG (*1*), DGDG (*2*), and SQDG (*3*) from *L. japonica* per cent of total fatty acid content.

glycolipids had palmitic and myristic acids as the major saturated fatty acids and oleic as well as linoleic acids as the major unsaturated acids. Figure 1 demonstrates that MGDG included a wide spectrum of $C_{18}$ and $C_{20}$ polyunsaturated fatty acids with 18:4n-3, 20:5n-3, 20:4n-6, 18:3n-3, 18:2n-6, and 18:3n-6 as the major components. DGDG and, particularly, SQDG featured a notably lower number of unsaturated acids. The content of polyunsaturated fatty acids decreased in the series of glycoglycerolipids MGDG > DGDG > SQDG. According to our calculations, the unsaturation index of the most saturated glycolipid, SQDG, is 1.5 and 5 times lower than that of DGDG and MGDG, respectively.

We studied thermal phase transitions of these glycolipids by differential scanning calorimetry (Fig. 2). The least unsaturated MGDG proved to have the lowest transition range from –78 to 4°C. Transition ranges of DGDG and SQDG shifted towards high temperature ($T_{min}$ ranged from –50 to 60°C). At the same time, $T_{max}$ of DGDG was considerably lower as compared to SQDG (from –6 to 0°C and 20°C, respectively). The obtained data point to a correlation between the transi-

tion temperature and the degree of fatty acid unsaturation of glycolipids: a high degree of unsaturation seem to correspond to low transition temperature. Previously Sanina *et al.* (2002) demonstrated that these glycolipids differ by the degree of hydration decreasing in the series SQDG > DGDG > MGDG. Clearly, the amount of bound water increases directly with polarity of carbohydrate groups.

Hence, the studied plant glycoglycerolipids from brown alga *L. japonica* considerably differ by fatty acid composition, degree of unsaturation, and transition temperatures.

Second, these glycoglycerolipids were used as a lipid component of modified ISCOMs. The used component weight ratio of 1:1:4:0.25 (cholesterol:glycolipid:saponins:protein) is optimal for vesicle formation (Lövgren and Morein, 1988) controlled by electron microscopy and ultracentrifugation in sucrose gradient.

Comparative biochemical analysis of ISCOM components demonstrated different incorporation of glycolipids into vesicles as compared to PC. Monogalactosyl and digalactosyldiacylglycerols efficiently formed ISCOMs and the content of MGDG in the correspond-
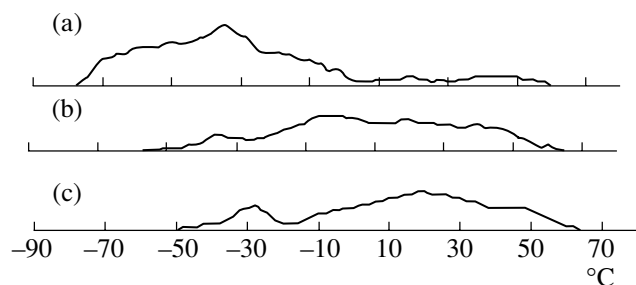


**Fig. 2.** Thermograms of the major glycoglycerolipids, MGDG (a), DGDG (b), and SQDG (c) isolated from *L. japonica* (differential scanning calorimetry); ordinate: heat absorption, °C; scanning rate, 16°C/min; sample weight, 10 mg.
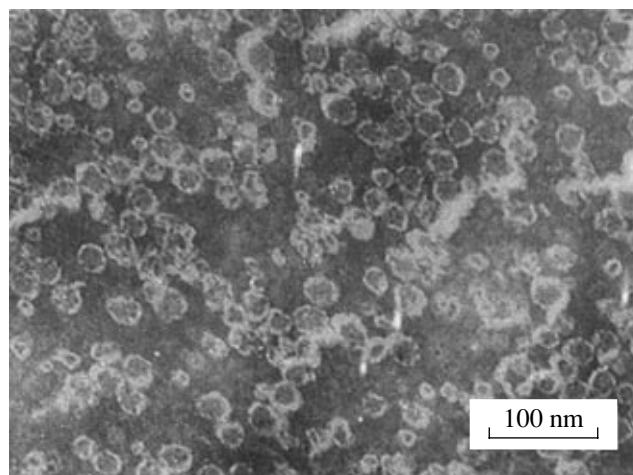


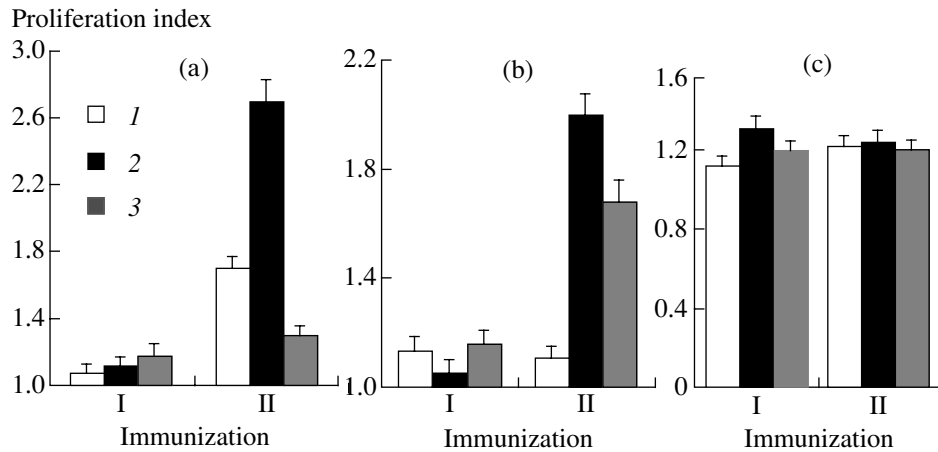**Fig. 3.** Electron micrographs of MGDG–ISCOM vesicles.

**Fig. 4.** Blastogenesis of spleen lymphocytes of mice immunized with PBS (*1*), PC–ISCOM (*2*), or MGDG–ISCOM (*3*) in the presence of mitogens PHA (a), Con A (b), or LPS (c); the assay was carried out 3 weeks after the first immunization (I) or 2 weeks after reimmunization (II); proliferation indices were calculated from incorporation ratio between the studied and control cultures.

ing vesicles was similar to that of PC and higher than that of DGDG. In contrast to the galactoglycerolipids, SQDG was not observed in the vesicles. Incorporation of MGDG into ISCOMs did not alter the classical vesicle morphology (Kersten and Crommelin, 1995). The obtained micrographs demonstrated numerous ring-shaped vesicles with the number and arrangement of pores similar to typical ISCOMs (Fig. 3). Note that the modified ISCOMs featured a considerable heterogeneity, their size varied from 16.5 to 33 nm. It is possible that this heterogeneity is due to irregular arrangement of micellae composing the vesicles. The sedimentation coefficient of the obtained particles was 18–19S.

Two types of structures were observed after DGDG incorporation into the vesicles (data not shown): 11–15 nm ISCOMs and spirals; formation of the latter can hardly be explained without knowing their biochemical composition.
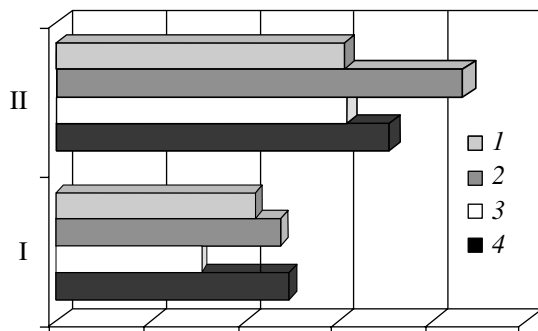
The studied hydrated polar lipids featured different superstructures apart from different fatty acid composition and transition temperatures: hexagonal (MGDG), lamellar (SQDG), and globular (DGDG). Apparently, the pattern of lipid superstructure depends on ISCOM-forming properties of polar lipid components. Hence, a more efficient incorporation of MGDG can be explained by formation of hexagonal mesophase and relatively high hydrophobicity of this glycolipid.

Porin proved to be efficiently incorporated into all ISCOM types. However, no significant increase was observed in immune response of mice immunized by the protein in PC–ISCOM or MGDG–ISCOM relative to those immunized by pure porin. These specimens induced neither B cell proliferation on LPS (Fig. 4c) nor synthesis of specific antibodies. The level of T cell proliferation induced by PHA and Con A also proved insignificant (Figs. 4a, 4b). An insignificant glycogen-induced increase in proliferative activity of immuno-
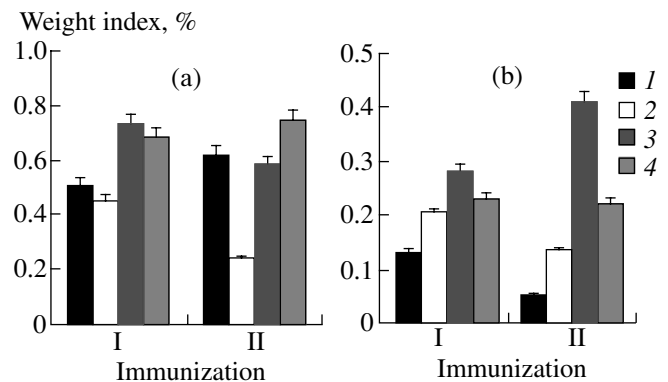


**Fig. 5.** Total number of lymphocytes in the peripheral blood of immunized and intact mice 3 weeks after the first immunization (I) and 2 weeks after reimmunization (II); animal groups: *1*, MGDG–ISCOM; *2*, PC–ISCOM; *3*, PBS; *4*, intact mice; abscissa: number of leukocytes, thousand/ml blood.



**Fig. 6.** Weight indices of lymphoid organs, spleen (a) and thymus (b), after the first (I) and second (II) immunization; animal groups: *1*, intact mice; *2*, PBS; *3*, PC–ISCOM; and *4*, MGDG–ISCOM; the indices were calculated as organs we per cent of body weight.

competent cells as well as low levels of humoral response and bactericidal activity of neutrophils point to the absence of both unspecific and specific stimulation of the immune system.

A decreased level of total leukocytes (Fig. 5) and weight of lymphoid organs (Fig. 6) coupled with high hemolytic activity ($ED_{50}$ = 3 μg/ml) *in vitro* clearly indicate immunodepressive activity of the protein antigen. At the same time, administration of ISCOM specimens induced no negative changes in the animals as compared to administration of pure porin. One can propose that these changes are due to storage properties of ISCOM, that nevertheless did not cancel immunodepressive effect of the used antigen. Thus, porin in a three-dimensional form cannot be considered as a convenient antigen for developing ISCOM vaccines for prevention of pseudotuberculosis infection.

The obtained data demonstrate the possibility of modification of ISCOM composition by introduction of plant glycolipids. However, the absence of immunostimulating activity in the modified as well as classical ISCOMs for *Y. pseudotuberculosis* porin suggests a correlation between antigen and adjuvant properties and confirms the current opinion that immunostimulant selection depends on the properties of a particular antigen used for vaccine development. In this context, selection of a molecular form of porin can become an important trend in further studies. Previously Portnyagina *et al.* (1999) demonstrated that heat-denatured porin monomer loses the pore-forming activity and has a higher immunogenic activity.

## ACKNOWLEDGMENTS

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