

# Enhancement of the immune response to poorly immunogenic gangliosides after incorporation into very small size proteoliposomes (VSSP)

Francisco Estevez<sup>a,\*</sup>, Adriana Carr<sup>b</sup>, Leobaldo Solorzano<sup>a</sup>, Oscar Valiente<sup>b</sup>, Circe Mesa<sup>b</sup>, Osquel Barroso<sup>b</sup>, Gustavo Victoriano Sierra<sup>a</sup>, Luis Enrique Fernandez<sup>b</sup>

<sup>a</sup>Finlay Institute, Ave. 27 No. 19805, La Lisa, P.O. Box 16 017, C. de La Habana, Cuba

<sup>b</sup>Center of Molecular Immunology, Calle 216 esq. a 15, Atabey, Playa, P.O. Box 16 040, C. de La Habana, Cuba

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## Abstract

Certain gangliosides are tumor-associated antigens that constitute potential targets for cancer immunotherapy. A major drawback in the design of ganglioside-based cancer vaccines, however, is the poor immunogenicity of these glycolipids. Here we report the immunological and physicochemical properties of very small size proteoliposomes (VSSP) obtained by using anionic detergents to incorporate gangliosides into the outer membrane protein complex (OMPC) of *N. meningitidis*. VSSP of three different gangliosides, GM3, NGcGM3 and GD3, were tested. These gangliosides differ in level of expression in normal tissues and in immunogenicity in different animal species. We show that the immunization with VSSP in an oil adjuvant consistently induced both IgM and IgG anti-ganglioside antibodies. In the mouse, the anti-ganglioside IgG fraction was not restricted to the typical T-independent isotype IgG3. Unexpectedly, significant levels of the T-dependent IgG1, IgG2a and particularly IgG2b were also found. VSSP-mediated enhancement of the immunogenicity was not restricted to the relatively immunogenic ganglioside GD3, satisfactory immune responses against highly tolerated GM3 and NGcGM3 were also obtained. Similar results were achieved in chickens and monkeys. No reactogenicity was observed even when self-gangliosides were used for immunization. VSSP overcame natural tolerance to gangliosides in an adjuvant dependent fashion. © 1999 Published by Elsevier Science Ltd. All rights reserved.

**Keywords:** Cancer vaccines; Proteosomes; Gangliosides; Proteoliposomes

## 1. Introduction

Gangliosides are sialylated glycosphingolipids present on the plasma membrane of mammalian cells. In tumors, however, they are overexpressed or display abnormal glycosylation patterns [1,2]. Sometimes, free gangliosides are also shed into the blood stream of patients suffering certain malignancies [3,4].

It has been observed that cancer patients who

develop antibodies against certain gangliosides have better prognosis and prolonged survival compared to those that are antibody-negative [5]. Similarly, antibodies against different gangliosides have been shown to mediate protection in animal models against challenge with ganglioside-expressing tumors [6,7]. These findings, among others, point to gangliosides as promising targets for the active specific immunotherapy of cancer [8]. However, a major drawback of ganglioside vaccines is the poor immunogenicity of these glycolipids. Gangliosides are generally autoantigens tolerated immunologically to a greater or lesser degree. Furthermore, they are categorized as T-cell-independent antigens, i.e. they are not recognized by T lymphocytes, which therefore do not provide help

\* Corresponding author. Present address: Center of Molecular Immunology, Calle 216 esq. a 15, Atabey, Playa, A. Postal 16040, C. de La Habana, Cuba. Tel.: +53-7-335049; fax: +53-7-333509.

E-mail address: frank@ict.cim.sld.cu (F. Estevez)

(cytokines) to drive the maturation of the immune response.

To augment ganglioside immunogenicity, both a protein carrier and a strong adjuvant must be used. Different approaches have been studied [9,10], including the use of carrier proteins capable to non-covalent bind gangliosides. As no chemical modification is needed to bring carrier protein and ganglioside together, the full antigenic structure of the latter remains unchanged, making achievement of success in immunization more likely.

In a previous work, Portoukalian et al. [11] immunizing with 9-*O*-acetyl-GD3 ganglioside adsorbed onto VLDL (very low density lipoproteins) could induce both IgM and IgG antibodies in mice. Another work by Livingston et al. [12] showed that the addition of GD3 to purified preparations of meningococcal outer membrane proteins (proteosomes) generated conjugates that consistently induced anti-GD3 IgM antibodies in mice.

These results, however, have been obtained only for very low-expressed gangliosides, which are known to be fairly good immunogens [13]. Previous work in our laboratory has shown that VLDL-based preparations do not induce antibodies against highly expressed gangliosides (GM3 in chicken, GM3 and NGcGM3 in mice, etc.) even when very strong adjuvants (complete Freund's adjuvant, CFA; incomplete Freund's adjuvant, IFA; or Montanide ISA 51) are used (unpublished results). On the other hand, the immune response obtained with proteosome-based ganglioside preparations has been, until now, mostly restricted to the IgM isotype.

We have observed, however, that proteosome-based preparations can be optimized in such a way that they can render immunogenic even highly tolerated gangliosides. Here we report the physicochemical properties and the immunogenicity of very small size proteoliposomes (VSSP) obtained by the incorporation of gangliosides into the outer membrane protein complex (OMPC) of *N. meningitidis* using anionic detergents. VSSP of three different gangliosides, GM3, NGcGM3 and GD3, were tested. These gangliosides are present at different levels in the normal tissues of the three animal species studied. The immune response induced by these preparations was evaluated in terms of a time course profile of antibody production, isotype/subclass distribution and antibody specificity. Some results are compared among the different animal species.

## 2. Materials and methods

### 2.1. Gangliosides

GM3 and *N*-glycolylneuraminic acid-containing

GM3 (NGcGM3) were extracted from dog and horse erythrocytes respectively by a modification of the method described by Folch [14]. The solvent partition step was replaced by a mild base treatment followed by a solvent extraction with hexane. Further purification was achieved by Silica Gel 60 (Merck Darmstadt, Germany) chromatography in chloroform–methanol–ammonia 2.5 M (65:25:4).

GD3 ganglioside was obtained from bovine butter-milk powder and purified as previously described [15]. GM1, GD1a and GT1b were isolated from bovine brain [16] and NGcGM2 from BALB/c mice livers [17].

Purity was monitored by HPLC [18] or HPTLC as described below and referred to standard gangliosides.

### 2.2. Animals and immunization schedule

Three different animal models were used: 10–12 week-old outbred chickens, line B4 (LABIOFAM, Cuba); BALB/c female mice, 7–8 weeks of age, purchased from CENPALAB (Havana, Cuba); and young non-human primates (*Macacas fascicularis*), which were maintained in the animal house facility of CENPALAB (Havana, Cuba).

Immunization was carried out by the intramuscular route as specified for each experiment. Immunogens were prepared either by mixing equal volumes of vaccine and a 2 mg/ml solution of alum (Alhydrogel; Superfos Biosector, Denmark) or by emulsifying equal volumes of vaccine and an oil adjuvant. Two different oil adjuvants were used: CFA (Sigma, St. Louis) and incomplete Freund adjuvant (Montanide ISA 51) (Seppic, France). The latter is a highly purified form of IFA in which the irritant emulsifying agent Alarcel A has been replaced with mannide monooleate (Montanide 80). This form was preferred because it is less toxic than IFA and has been extensively used in human trials of contraceptive, cancer and AIDS vaccines [19]. Each dose contained 120 µg of ganglioside in a total volume of 0.1 ml. Animals were bled before each immunization and 14 d after the last one, unless otherwise stated. Sera were stored at –20°C.

Previously to immunization and blood collection, monkeys were anesthetized with 1 mg of Ketamina (Lab. Reig Jofre, S.A., Barcelona) per kg of weight by the intramuscular route. All the animals were treated according to the Cuban National Laboratory Animal Use Guidelines.

### 2.3. Preparation of vaccines

The outer membrane proteins complex (OMPC) was purified at the Finlay Institute (Havana, Cuba) as previously described [20]. Group B *N. meningitidis* strain 385 (B4 P1.15) was cultivated and the culture centri-

fuged to obtain 500 g (wet weight) of biomass. This biomass was resuspended in 25 l of buffer containing 0.09% sodium deoxycholate (DOC), 50 mM Tris-HCl, pH 8.5, and 2 mM EDTA. The extraction process was carried out at 4°C for 2.5 h. During this period, 10 treatments of 30 s each were given in an ultrasonic bath (Ultraware, Radleys, UK), alternating with magnetic stirring at 250 rpm.

Cell debris was separated by centrifugation at 10,000g and the supernatant treated with DNase and RNase (5 mg/l each) at 37°C. The extract was centrifuged at 100,000g for 2 h and the pellet resuspended in 200 ml 5% sodium deoxycholate/(50 mM) Tris-EDTA buffer, pH 9. Gel filtration on Sephacryl S-300 HR (Pharmacia Biotech, Sweden) was carried out using 1% sodium deoxycholate as eluant. The first eluted peak was the basic material containing the vesicles as revealed by electron microscopy following negative staining with uranyl acetate. The OMPC was precipitated with ethanol, washed with the same solvent, and stored at -80°C in 40 mg portions.

To incorporate gangliosides into the OMPC (VSSP preparation procedure), 40 mg aliquots of OMPC were dissolved to a final concentration of 1 mg/ml in 0.01 M Tris-HCl buffer, pH 8.5, containing 12 mM DOC and 1 mM sodium dodecylsulfate (SDS). Forty mg ganglioside was then added and the mixture stirred. The resultant solution was dialyzed for 14 d against 0.01 M Tris-HCl buffer, pH 8.5, using a 3.5 KDa cut-off membrane (Spectra/Por, Spectrum Medical Industries, Houston, Texas) to remove detergents. The dialyzed preparation was centrifuged for 1 h at 100,000g, pellets were discarded and supernatants assayed for protein and ganglioside contents. Finally, the VSSP solution obtained was filter-sterilized (0.2 µm cellulose acetate membranes; Sartorius, Germany) and stored at 4°C.

Vaccine lots NGcGM3/VSSP, GM3/VSSP and GD3/VSSP were prepared with the respective ganglioside and the OMPC. The lot designated OMPC was prepared in the same way but without the addition of any ganglioside, and the lot designated GM3 was prepared with the ganglioside alone.

#### 2.4. Physical and chemical characterization of vaccines

Protein assay was performed by a modification of the method of Lowry [21]. Gangliosides were identified by HPTLC as described below and their concentration determined by the resorcinol method [22].

For the electron microscopy study, samples were adsorbed 5 min onto carbon-coated Formvar grids and negatively stained with 1% phosphotungstic acid, pH 7.0, or 2% uranyl acetate. Grids were examined at 20–80,000-fold magnification at 60–80 kV.

Gel filtration chromatography on Sepharose CL-4B (Pharmacia Biotech, Sweden) was carried out in a C10/40 column equilibrated with 0.01 M Tris-HCl buffer, pH 8.5, containing 0.15 M NaCl. Fractions of 1 ml were collected and assayed for protein and ganglioside concentration. The partition coefficient  $K_{av}$  was done as described by Sofer and Nystrom [23]. Standard proteins were used as molecular weight markers in the same conditions as the samples. A calibration curve,  $K_{av}$  vs. MW, was established.

CsCl equilibrium density gradient analysis of the vaccine was performed with an initial one-step gradient with a density of 1.525 g/ml in the lower half and 1.005 g/ml in the upper half. Tris-HCl buffer (0.01 M, pH 8.5) was present throughout the gradient, and the vaccine was initially uniformly distributed in the upper solution. Centrifugation was performed in a Kontron TFT 70.13 angle head rotor at 55,000 rpm (211,000g) and 10°C for 48 h. Fractions were collected, and density, sialic acid content and protein concentration determined for each fraction.

#### 2.5. ELISA

PolySorp 96-well plates (Nunc, Denmark) were coated with 0.16 nmol/well of ganglioside dissolved in methanol and dried at 37°C for 1.5 h. To reduce background, plates were washed with PBS containing Tween 20 (0.05%, v/v) plus 0.2 M extra of NaCl and then blocked with skimmed milk (2%, w/v) in PBS. Serum samples, biotinylated goat anti-IgM or anti-IgG antibodies specific for each animal species and streptavidin-conjugated alkaline phosphatase (Jackson, West Grove, PA) were diluted in blocking buffer and sequentially incubated on the plates for 2 h at room temperature with extensive washing between each incubation. The enzymatic reaction was visualized with *p*-nitrophenyl phosphate (PNPP), 1 mg/ml, dissolved in 1M diethanolamine buffer, pH 9.6, plus 1 mM MgCl<sub>2</sub>.

To eliminate the effect of non-specific recognition, sera were also tested on wells to which no gangliosides had been added. The absorbance at each serum dilution obtained on these wells was subtracted from that of the ganglioside-coated wells. Serological titer was defined as the inverse of the highest dilution yielding a final absorbance value higher than 0.1.

Determination of IgG isotype profile was performed using secondary isotype-specific biotinylated rat anti-mouse IgG1, IgG2a, IgG2b and IgG3 antibodies (PharMingen, San Diego, CA). Optimal secondary antibody dilutions were established by ELISA with the MAbs 14F7 (IgG1), T3 (IgG2a), T4 (IgG2b) (Center of Molecular Immunology, Havana, Cuba) and R24 (IgG3) (kindly provided by Dr. Philip O.

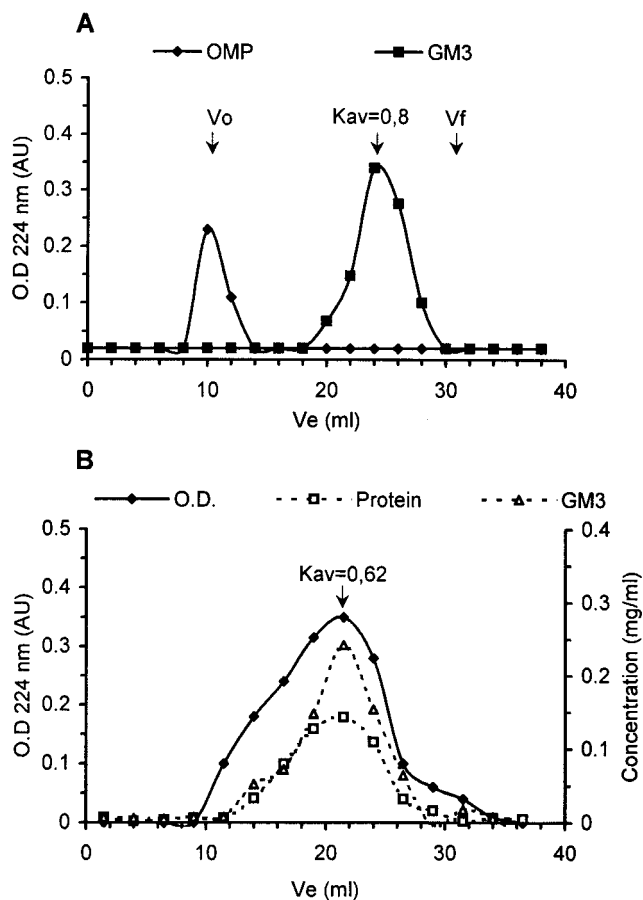


Fig. 1. Elution profile of vaccine lots GM3 and OMPC (A) and GM3/VSSP (B) on Sepharose CL-4B. Continuous lines represent the absorbance at 224 nm and broken lines the analytical results. Arrows signaled  $V_0$  and  $V_f$  indicate the void volume and the end of the run, respectively.

Livingston, Memorial Sloan Kettering Cancer Center, New York).

2.6. HPTLC and enzyme immunostaining

High-performance thin-layer chromatography (HPTLC) was carried out on Silica Gel 60 plates (E. Merck AG, Germany) and developed with chloroform–methanol–ammonia 2.5 M/KCl 0.25% (50:40:10, v/v). Ganglioside spots were visualized with resorcinol reagent (0.2% resorcinol plus 0.25 mM  $\text{CuSO}_4$  in 30% HCl).

Immunostaining on HPTLC plates was performed according to the method of Magnani et al. [24] with slight modifications. Briefly, gangliosides were spotted on plates eliminate this as described above. Plates were then dried in a warm air current, soaked in a 0.1% solution of polyisobutylmethacrylate (Aldrich Chemical Co., Ltd., Gillingham, Dorset) in hexane for

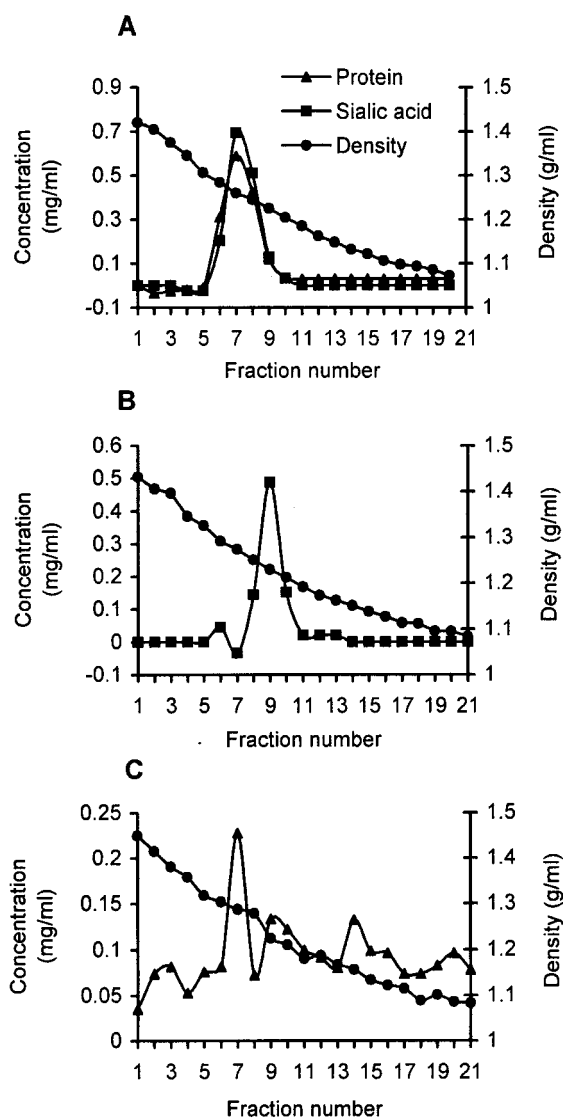


Fig. 2. Isopycnic CsCl density gradient centrifugation of vaccine lots GM3/VSSP (A), GM3 (B) and OMPC (C). Fractions were assayed for density by a gravimetric procedure (right axis) and for protein and sialic acid contents by spectrophotometric methods (left axis).

1 min and then allowed to air dry. To eliminate background, plates were blocked in PBS buffer containing 2% (w/v) skimmed milk at room temperature for 30 min. After overnight incubation with sera diluted 1:100 in blocking buffer, plates were extensively washed with PBS and incubated with biotinylated goat anti-mouse IgG antibodies. Following another washing step, Streptavidin-conjugated Alkaline Phosphatase (Jackson, WestGrove, PA) diluted in blocking buffer was added and incubated for 2 h at room temperature. Spots were visualized with a 0.1 mg/ml solution of 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M glycine buffer, pH 10.4. The reaction was stopped by washing with water.

### 3. Results

#### 3.1. Physicochemical characteristics of the conjugates

VSSP preparations are quite transparent solutions that can be easily filter-sterilized using a 0.2  $\mu\text{m}$  filter. Although, by electron microscopy, the OMPC displayed vesicular structure prior to its incorporation into VSSP (data not shown), no VSSP preparation did. Contrary to other proteosome-based preparations that completely precipitate, the VSSP insoluble fraction sedimenting by centrifugation for 1 h at 100,000g (step included in the production procedure) constituted less than 15% of the vaccine, indicating that VSSP possess increased solubility.

Chromatography of GM3/VSSP vaccine on Sepharose CL-4B (Fig. 1B) showed that both protein and ganglioside elute in a peak of  $K_{av}$  0.62 whose equivalent molecular weight is about 100–300 KDa when referred to globular proteins analyzed in the same conditions. Independent runs of the GM3 and lot OMPC (Fig. 1A) showed that GM3 alone eluted with a  $K_{av}$  of about 0.8, with OMPC eluting in the void volume. No significant deviations from this elution profile were observed among different ganglioside/VSSP preparations (data not shown).

More convincing data on the association between proteins and ganglioside was obtained by isopycnic gradient centrifugation of the vaccine in CsCl (see Fig. 2). Ganglioside alone (lot GM3) produced a single band at a density of 1.236 g/ml, whereas the ganglioside in the vaccine (lot GM3/VSSP) was found at a higher density (1.265 g/ml). Proteins in the vaccine were also found in a narrow band at 1.265 g/ml, whereas non-ganglioside-containing proteins (lot OMPC) were found dispersed throughout the gradient, with a peak at 1.282 g/ml. These results demonstrate an intimate association between ganglioside and the OMPC, probably hydrophobic in nature as indicated by the high resistance of the complex to very high concentrations of CsCl.

#### 3.2. Immunogenicity of NGcGM3/VSSP and GM3/VSSP in chickens

The very low-expressed NGcGM3 and the highly expressed GM3 gangliosides [25,26] were selected to

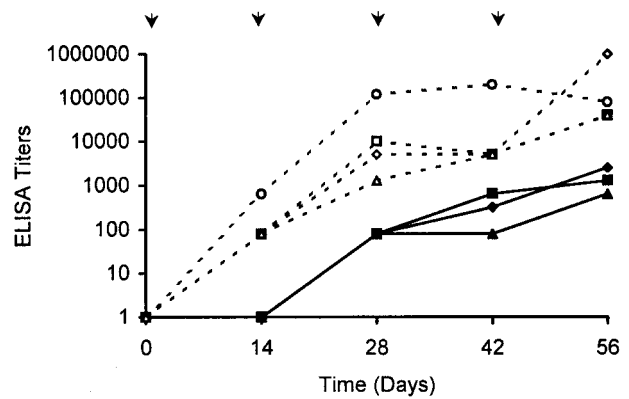


Fig. 3. IgG response against GM3 (closed symbols) and NGcGM3 (open symbols) in chicken immunized with GM3/VSSP and NGcGM3/VSSP, respectively, in CFA. Each symbol represent an individual chick; arrows, immunization days.

study the immunogenicity of VSSP in chicken. Four groups of four chickens were each immunized intramuscularly on days 0, 14, 28 and 42 with NGcGM3/VSSP or GM3/VSSP vaccines administered in either alum or emulsified complete Freund's adjuvant (CFA).

The serological study revealed that no animal developed antibodies against GM3 and only one of four against NGcGM3 (titer 40,000) when vaccines were administered in alum. On the contrary, when CFA was used (see Fig. 3) all chickens developed high titer IgG antibodies against both NGcGM3 and GM3.

#### 3.3. Immunogenicity of NGcGM3/VSSP, GM3/VSSP and GD3/VSSP in mice

Groups of ten mice each were immunized with NGcGM3/VSSP, GM3/VSSP and GD3/VSSP vaccines either alone or in Montanide ISA 51 adjuvant on days 0, 14, 28 and 42. Fourteen days after the last immunization (day 56), mice were bled and sera tested by ELISA against the specific ganglioside.

No animal developed antibodies against the studied gangliosides when vaccines were administered alone. On the contrary, immunization with the three vaccines in Montanide ISA 51 adjuvant was very effective (see Table 1). At least 80% of animals developed immunoglobulin titers equal to or higher than 80 (IgM and IgG). This response was not only observed in mice immunized with the more heterologous (very low-

Table 1  
Serological response induced by ganglioside/VSSP preparations in mice

Immunogen	Adjuvant	<i>n</i>	Anti-ganglioside IgG ELISA titers ( <i>n</i> )	Anti-ganglioside IgM ELISA titers ( <i>n</i> )
GM3/VSSP	Montanide ISA 51	10	10240 (3), 5120 (2), 640 (2), 320 (3)	640 (1), 320 (2), 160 (3), 80 (1), 0 (3)
NGcGM3/VSSP	Montanide ISA 51	9	1280 (2), 640 (5), 320 (2)	1280 (3), 640 (2), 160 (3), 80 (1)
GD3/VSSP	Montanide ISA 51	10	5120 (1), 1280 (1), 160 (2), 80 (4), 0 (2)	5120 (1), 1280 (1), 640 (4), 320 (3), 80 (1)

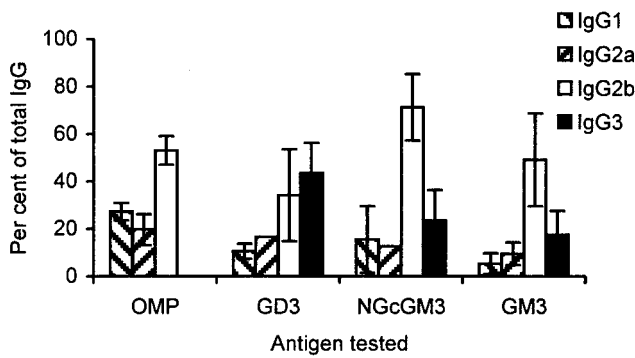


Fig. 4. IgG isotypes of serum antibodies. Groups of BALB/c mice were immunized four times with either NGcGM3/VSSP, GM3/VSSP or GD3/VSSP vaccines in Montanide ISA 51 adjuvant at 14 d intervals. Five day-56 sera of each immunized group were assayed by ELISA against the specific antigen. The geometric mean (n=5) of the ELISA titers were calculated for each particular immunoglobulin isotype. Data are plotted as the percent represented by each isotype ±SE within the total pool of IgG immunoglobulin.

expressed) GD3 but also with autologous GM3 and NGcGM3 gangliosides.

IgG antibodies induced by VSSP preparations against gangliosides included the whole panel of isotypes with slightly higher titers of IgG3 and IgG2b (Fig. 4) (cross-reactivity between isotype-specific secondary antibodies was insignificant). IgG antibodies against the OMPC, however, were restricted to the T-cell-dependent IgG isotypes IgG1, IgG2a and IgG2b. No IgG3 titers were detected in this case.

The specificity of IgG antibodies detected in mouse sera was studied by HPTLC immunostaining using as standard gangliosides GM1, GD1a, GT1b, GM3, GD3, NGcGM3 and NGcGM2. The results of a representative serum of each group are shown in Figs. 5 and 6. Only specific gangliosides were recognized by serum IgG. Similar results were obtained with sera of the other two animal models used in the study.

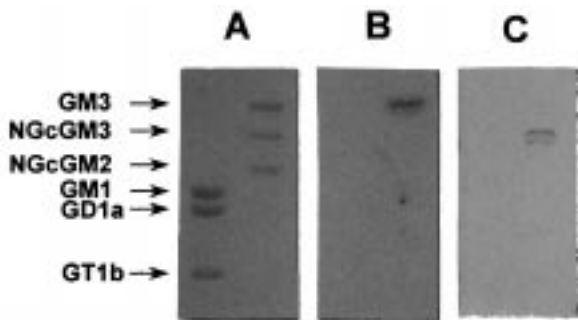


Fig. 5. HPTLC immune staining of sera of mice immunized with GM3/VSSP vaccine (B) and NGcGM3/VSSP vaccine (C) in Montanide ISA 51. Plate A was stained with resorcinol.

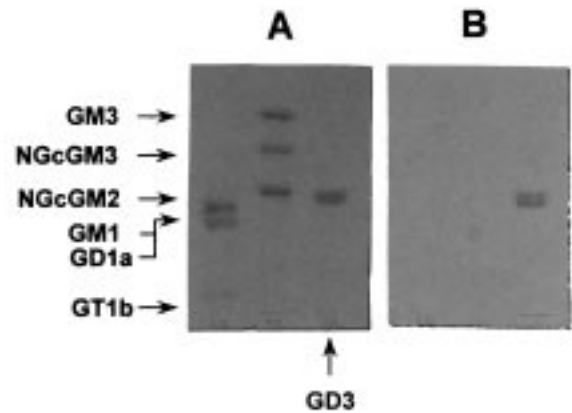


Fig. 6. HPTLC immune staining of sera of mice immunized with GD3/VSSP vaccine (B) in Montanide ISA 51. Plate A was stained with resorcinol.

### 3.4. Immunogenicity in monkeys

To evaluate the immunogenicity of autologous GM3/VSSP vaccine in an animal model closer to human beings, two monkeys were immunized intramuscularly with GM3/VSSP vaccine in Montanide ISA 51. Injections were given on days 0, 14, 28 and 42. Boosters were thereafter administered once a month and sera were collected before each immunization. Significant levels of both IgM and IgG antibodies were detectable as early as day 14 after the first immunization (see Fig. 7). IgM peaked on day 14 and IgG on days 42–56. After a short period of decline, levels of both immunoglobulins remained at a plateau for the next four months of the study.

### 4. Discussion

The term proteosome or proteoliposome was formerly used to designate liposome-like purified prep-

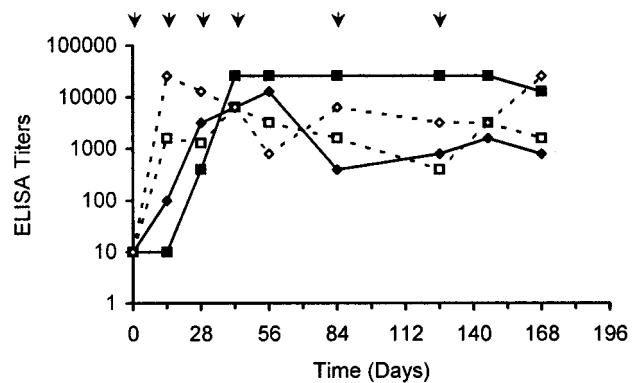


Fig. 7. Immunogenicity of vaccine GM3/VSSP/ Montanide ISA 51 in monkeys. Open symbols represent anti-GM3 IgM and closed symbols anti-GM3 IgG. each symbol represents an individual animal; arrows, immunization days.

arations of the outer membrane proteins of *N. meningitidis* [27,28]. The physicochemical properties of VSSP, however, differ from other proteosome-based preparations described before [29]. Ganglioside incorporation into the OMPC leads to a dramatic reduction in the size of the complex that, unlike other proteosome-based preparations, is not visible by electron microscopy. On the other hand, the relatively high density of VSSP (1.265 g/ml) reveals that VSSP are non-vesicular proteo-lipidic bodies from which most water has been excluded.

The basis for this compact structure might be explained if we consider the molecular properties of gangliosides. These glycosphingolipids have very large oligosaccharide moieties relative to their hydrocarbon portions. For reasons of molecular geometry, these structures are not thermodynamically compatible with the formation of bilayer vesicles but with micelles [30]. The presence of high concentrations of gangliosides in a solution of membrane proteins, highly dissociated by the presence of anionic detergents, leads to the formation of mixed micelles from which most water is excluded. After detergent removal, the system should reach a new equilibrium in which the ganglioside properties predominate.

Our study on the immunogenicity of VSSP confirms and extends a previous report [12], and shows that not only IgM but also high-level IgG anti-ganglioside antibodies can be induced in the mice. However, in addition to GD3 ganglioside, which is considered the most immunogenic in this animal model [13], highly expressed gangliosides such as GM3 and NGcGM3 were also highly immunogenic when used as VSSP. Similar results were obtained in the other two animal species studied. Unexpectedly, the anti-ganglioside IgG fraction in mice was not restricted to the typical T-independent isotype IgG3. High levels of the Th1-related IgG isotypes IgG1, IgG2a and particularly IgG2b were also found. These phenomena, however, were strongly dependent on the use of an oil-in-water adjuvant. VSSP overcame natural tolerance to ganglioside in an adjuvant-dependent fashion.

The mechanisms involved in VSSP immuno-enhancing properties are not yet understood. The observed effect is not inherent to the adjuvant itself since in previous experiences GM3/VLDL and NGcGM3/VLDL conjugates administered either in CFA or in Montanide ISA 51 were not immunogenic in mice. It is known that serotype proteins, which are the main components of the OMPC, induce proliferation and activation of lymphocytes and lead to secretion of IL-2 by a mechanism different to that of polyclonal activators such as LPS [31]. On the other hand, the adjuvant activity of oil-in-water emulsions has been suggested to be linked to their interaction with dendritic cells [32] which, at the same time, have been implicated in the

presentation of non-classical antigens that are very closely related to gangliosides [33]. The xenogenic environment created by the bacterial proteins and some residual lipids that, like LPS, are natural activators of the immune system, together with the very small size of the complexes, might facilitate ganglioside uptake by this subset of antigen-presenting cells that triggers the maturation of an incipient immune response.

No reactogenicity was observed even when VSSP of self-gangliosides such as GM3 were administered in Montanide ISA in any of the three animal species used. These gangliosides, as has been suggested before [34], should remain 'cryptic' in normal tissues, out of reach of circulating antibodies.

Based on these observations and on our recent finding that threshold inocula of syngeneic melanoma B16 tumor cells in mice immunized with GM3-containing VSSP leads to a very high tumor rejection rate and increased survival (submitted for publication), we have started two clinical trials, following international guidelines [35]. Patients suffering metastatic breast cancer have been immunized with GM3/VSSP and NGcGM3/VSSP vaccines in Montanide ISA 51 to evaluate the immunogenicity and the therapeutic potential of these preparations.

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