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Effects of Monophosphoryllipid-A on the Immunization of Mice with Keyhole Limpet Hemocyanin- and Muramyldipeptide-Ganglioside Gfpt1 Conjugates[1]

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Since it was considered that an active immunization against ganglioside Gfpt1 (IV^2Fuc-, II^3NeuAc-Gg_4Cer) expressed by human small cell lung cancer cells may be beneficial in the treatment of this neoplasm in humans, an optimal mode of vaccination in model mice was investigated. A novel Gfpt1-muramyldipeptide conjugate (Gfpt1-MDP) was synthesized. Its ganglioside carbohydrate-directed immunogenicity in mice as measured by serum antibody titers was comparable to that of the previously described Gfpt1-keyhole limpet hemocyanin conjugate (Gfpt1-KLH). Similar immunogenicity was displayed by free Gfpt1 in muramyldipeptide-phosphoethanolamine-containing phosphatidyl-choline, -serine (PC, PS) liposomes. Immunization with Gfpt1-vaccines in the presence of monophosphoryl-lipid A (MPL), in general, raised titers of anti-Gfpt1 antibodies effectively. Immunization with PC, PS-liposomes containing unconjugated Gfpt1 and MPL stimulated the highest titers observed, thereby effectively preventing tumor growth in Balbc nu/nu-mice challenged with human small cell lung cancer cells. However, there was a strong crossreaction of these and most other sera with the structurally related and widely distributed ganglioside Gtet1 (II^3NeuAc-Gg_4Cer). Only immunization with Gfpt1-KLH conjugate in the presence of MPL stimulated selectively high anti-Gfpt1 antibody titers showing comparably low crossreactivity to ganglioside Gtet1.

Key words: ganglioside IV^2Fuc-,II^3NeuAc-Gg_4Cer, keyhole limpet hemocyanin, monophosphoryllipid-A, muramyldipeptide, small cell lung cancer.

Gfpt1 (Fuc-GM1, IV^2Fuc-, II^3NeuAc-Gg_4Cer) was identified by Svennerholm and coworkers as a tumor-associated ganglioside of human small cell lung cancer (*2-4*). The characteristic expression of this sialo-fuco-glycosphingolipid on the surface of small lung cancer cells may, therefore, suggest its use as a marker for possible immunotherapeutic approaches by an active anti-tumor ganglioside vaccination. However, gangliosides are poor immunogens requiring the presence of strong adjuvants to elicit an effective humoral immune response (*5*). It had earlier been shown that chemical coupling of ganglioside to keyhole limpet hemocyanin (KLH) could considerably enhance their immunogenicity (*6*). In a further search for an optimally effective tumor-associated ganglioside vaccine that might be tested for its beneficial role in the treatment of human small cell lung cancer, the stimulation of the immunogenicity of KLH- as well as muramyldipeptide-(MDP)-Gfpt1 conjugates by monophosphoryllipid-A (MPL) was investigated.

MATERIALS AND METHODS

Antibodies—An anti-ganglioside Gfpt1 monoclonal antibody (F 12) was generously provided by Professor L. Svennerholm, Götheborg.

Cells—Human small cell lung cancer cells NCI H526 (*7*) were kindly grown for this study and provided by Dr. G. Jacques, Dept. Intern. Medicine, Universität Marburg.

Ganglioside Preparation—Gfpt1 (Fig. 3, lane 1) was isolated from bovine thyroid gland as described earlier (*8-10*).

Ganglioside-Aldehyde—A modified version of a procedure described earlier was used for the ozonolysis of glycosphingolipids (*6*): 10 mg of ganglioside Gfpt1 was

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Abbreviations: BSA, bovine serum albumin; G, gangliosides of the sialoganglio-series (Gg) are abbreviated as described before (*1*): an addendum to G designates the neutral carbohydrate portion, *e.g.*, Glac (lactose), Gtri (gangliotriaose, Gg_3), Gtet (gangliotetraose, Gg_4), Gfpt (gangliofucopentaose, IV^2Fuc-Gg_4). The number of sialic acid residues is given in Arabic numerals, sialic acid position isomers by a, b, c, *e.g.*, for gangliosides Glac2 [$II^3(NeuAc)_2$-LacCer], Gfpt1 (IV^2Fuc-,II^3-NeuAc-Gg_4Cer); KLH, keyhole limpet hemocyanin; MDP, muramyldipeptide; MDP-PE, muramyldipeptide-phosphatidylethanolamine conjugate; MPL, monophosphoryllipid-A; PBS, 10 mM phosphate-buffered saline, pH 7.2; PC, phosphatidycholine; PE, phosphatidylethanolamine; POD, horse raddish peroxidase; PS, phosphatidylserine.

dissolved in 5 ml of methanol under ultrasonification for 5 min. A very slow flow of ozone was passed through the solution with one to two gas bubbles per second from an ozone generator taking 40 liters of oxygen per h. After completion of the reaction, remaining ozone was removed by a stream of argon, 30 μl of dimethylsulfide was added and the reaction mixture kept for 4 h at room temperature. Subsequently the solvent was evaporated under a stream of nitrogen, the residue was suspended in 10 ml *n*-hexane by ultrasonification for 3 min and centrifuged at 4,000 rpm for 10 min. The supernatant was carefully removed and discarded, while the sediment containing the ganglioside-aldehyde was dried under vacuum in a desiccator (Fig. 3, lane 2). Further purification, in particular, separation from non-derivatized ganglioside, was performed by chromatography on a LiChroprep Si 60 (Merck, Darmstadt, Germany) column (v=25 ml, r=0.9 cm). The column was prewashed with 50 ml of *i*-propanol/*n*-hexane/water (55 : 37 : 8) and the reaction mixture applied in the same solvent, after its dissolution by repeated ultrasonification and centrifugation. Then the column was washed with 50 ml each of *i*-propanol/*n*-hexane/water (55 : 37 : 8 and 55 : 36 : 9). Non-derivatized ganglioside and ganglioside-aldehyde were then eluted with 250 ml each *i*-propanol/*n*-hexane/water (55 : 35 : 10 and 55 : 34 : 11) taking 25-ml fractions at a time. The yield of ganglioside-aldehyde was 90-95%, as determined after thin-layer chromatography and staining with resorcinol-HCl spray reagent by scanning with a Shimadzu CS-9000 TLC-scanner described earlier (*6*).

Coupling of Ganglioside-Aldehyde to KLH—Method 1: Gfpt1-aldehyde was coupled to KLH in PBS, pH 7.2, as described earlier (*6*). Method 2: 30 mg of KLH (free of endotoxin and pyrogen, Calbiochem, San Diego, CA, USA) was dissolved in 2 ml of water and dialyzed over night against 1 liter of 10 mM borate buffer, 0.15 M NaCl, pH 8.5 at 4°C, and the protein content of the final solution determined according to Lowry. From this solution an aliquot containing 20 mg of KLH was added to 1 mg of dry Gfpt1-aldehyde. After dissolution of the ganglioside derivative, 2 mg of sodium cyanoborohydride, freshly dissolved in 20 μl of water, was slowly added using a syringe under shaking. The reaction mixture was stirred for 16 h at room temperature under argon, and subsequently dialyzed for 24 h at 4°C against 5 changes each of 1 liter of PBS. The yield of KLH-coupled ganglioside was determined after thin-layer chromatography with a solvent system of chloroform/methanol/water (45 : 45 : 10) and staining with resorcinol-HCl reagent. The resulting color formation as measured at 580 nm of non-migrating resorcinol-HCl-positive Gfpt1-KLH conjugate band was compared with that of a migrating band of standard ganglioside II^{3}NeuAc-Gg$_4$Cer (Gtet1). KLH itself is not stained by the resorcinol-HCl reagent. Additionally, the ganglioside coupling yield to KLH was estimated according to Svennerholm (*11*), after non-reacted ganglioside-aldehyde had been removed by repeated extractions with chloroform/methanol/water (30 : 60 : 8) under ultrasonification.

Under the conditions of Method 2 and the application of 1 mg of ganglioside-aldehyde and 1 mg of KLH, 168±43 μg of Gfpt1 could be coupled to the KLH.

Preparation of Amino-Ganglioside Gfpt1—Gfpt1-aldehyde was reductaminated in the presence of ammonium ions and sodium cyanoborohydride as described before (*6*). An aliquot of 2 mg of reductaminated Gfpt1-aldehyde could be separated from non-reacted ganglioside-aldehyde by column chromatography on LiChroprep-Silicagel (column volume 2 ml, Merck, Darmstadt, Germany) prewashed with 5 ml of *i*-propanol/*n*-hexane/water (55 : 37 : 8). The reaction mixture of the reductamination dissolved in 5 ml of the same solvent was applied to the column. After a further application of the solvent, chromatography was performed with a stepwise gradient of 5 ml of *i*-propanol/*n*-hexane/water (55 : 36 : 9) and 20 ml (55 : 35 : 10). The stepwise eluates were collected and elution of compounds was monitored by thin-layer chromatography. Amino-Gfpt1 was finally eluted with 20 ml of *i*-propanol/*n*-hexane/water (60 : 25 : 20) (Fig. 3, lane 3).

MDP-N-Hydroxysuccinimidester—An equimolar mixture of 5 mg (9.7 μmol) of MDP (Fig. 3, lane 6), 2 mg of *N,N*-dicyclohexylcarbodiimide, and 1.1 mg of *N*-hydroxysuccinimid was dissolved in 100 μl of dimethylacetamide with the aid of ultrasonification and kept for 16 h under argon at room temperature. The yield of MDP-ester amounted to approximately 80-90% (Fig. 3, lane 7). Subsequently, the solution was diluted by addition of 400 μl of dimethylformamide.

Preparation of Gfpt1-MDP Conjugate—One milligram of amino-Gfpt1, dried *i.v.* over phosphorus pentoxide, was mixed under ultrasonification with 150 μl of MDP-*N*-hydroxysuccinimidester prepared as above, *i.e.*, an approximate 3- to 4-fold molar excess of the MDP-derivative. Subsequently, 5 μl of diisopropylethylamine in 100 μl of dimethylformamide was added dropwise, and the reaction mixture stirred over night at room temperature under an argon atmosphere. Completion of the reaction was monitored by thin-layer chromatography (Fig. 3, lane 4). Separation of the excess MDP-ester was achieved by column chromatography on 1 ml of DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) prewashed with chloroform/methanol (8 : 2). The reaction mixture was taken up with 3 ml of chloroform/methanol (8 : 2) and applied to the column, which was washed two times with the same solvent. After further washes with 3 ml each of the same solvent followed by chloroform/methanol (1 : 1) and 3 ml of methanol, the ganglioside-MDP conjugate was eluted with 3 ml of 0.2 M potassium acetate in methanol. Subsequently, the solvent was removed *i.v.*, and the salt separated by reversed-phase chromatography. To this end, 0.5-1 ml of Silicagel RP-18 (Millipore, Milford, MA, USA) suspended in methanol was packed into a glass pipette and washed with 2 ml of water. The sample was applied in 5 ml of water, and the salt removed by washing the pipette with 3 ml of water. The conjugate was eluted with 3 ml of methanol. The yield obtained amounted to 60-70% as estimated after thin-layer chromatography and color formation with resorcinol-HCl spray reagent by densitometry using a Shimadzu TLC-scanner at 580 nm.

Muramyldipeptide-Phosphatidylethanolamine (MDP-PE) Conjugate—Phosphatidylethanolamine was reacted with MDP-*N*-hydroxysuccinimidester in dimethylformamide according to Tarcsay *et al.* (*12*).

Preparation of Liposome Vaccines—One hundred micrograms each of phosphatidylcholine (PC) and phosphatidylserine (PS) (distearoyl-PC and -PS, Sigma, Munich, Germany) were dissolved in 20 μl chloroform/methanol (1 : 1). The antigen was added dissolved in 20 μl of

methanol, with or without MPL (8 μg of MPL from *Salmonella minnesota* Re 595, Sigma, Munich, Germany) dissolved in 8 μl of chloroform/methanol (1 : 1). The solvents were subsequently evaporated by rotary evaporation and the remaining lipid film dried for 2 h in a vacuum desiccator. After addition of 200 μl of PBS, the suspension was sonicated for 10 min at 37°C.

Immunizations—Each immunization was performed in parallel with 4 BALB/c mice, 8 weeks old, by four *i.p.* injections at weekly intervals of the antigens dissolved in 200 μl of PBS. The quantity of antigen used was calculated on the basis of Gfpt1-carbohydrate and amounted to 20 μg per mouse per injection. In the respective vaccines, 8 μg of MPL was added.

Enzyme-Linked Immunosorbent Assays—Two hundred nanograms of the respective ganglioside and 200 ng of ganglioside Glac2, as a negative control, were dissolved in 25 μl of methanol and placed into the wells of flat-bottom microtiter plates (Nunc, Polysorb F 16, Wiesbaden, Germany). After the solvent had evaporated, blocking was performed with 200 μl of 1% BSA in PBS for 30 min at room temperature. Sera (100 μl/well) were added in serial dilutions of 1:10 to 1:1,280, and incubated for 16 h at 4°C. Subsequently, the plates were washed four times with 200 μl/well of PBS. Further incubation was with 150 μl/well of horse raddish peroxidase (POD)-labeled monoclonal anti-mouse immunoglobulin (Dakopatts, Hamburg, Germany, 1:1,000, in 1% BSA in PBS) for 2 h at room temperature. After 4 washes with PBS, plates were developed with 150 μl/well of 0.15% *o*-phenylenediamine in citrate buffer, pH 5.0, 1 μl/ml of 30% H_2O_2. Color formation was stopped by addition of 50 μl of 0.25 M sulfuric acid, and optical density measured at 492 nm.

Anti-IgG and -IgM determinations with POD-labeled anti-mouse IgG or IgM antisera (Sigma, Munich, Germany, used at 1:1,000 dilution) were performed as described above, except for incubating with the immune sera at a dilution of 1:20 with 1% BSA in PBS.

For determination of IgG-subclasses, after blocking the wells with 200 μl of 1% BSA in PBS for 30 min, the first incubation was with 50 μl of the 1:20 diluted immune sera for 2 h at room temperature. The plates were washed four times each well with 200 μl of PBS, and then incubated with 75 μl of the 1:250 diluted biotinylated anti-IgG-1, -2a, -2b, and -3 in 1% BSA in PBS, for 2 h at room temperature (antibodies being a gift by courtesy of Dr. K. Boßlet, Behring Werke, Marburg, Germany). After a following washing step, the plates were finally incubated with 100 μl of 1:1,000 diluted streptavidin labeled peroxidase (Amersham, Braunsschweig, Germany), 2 h at room temperature. After 4 washes with PBS, color formation was carried out as described above.

Tumor Growth in Nude Mice—Three groups, each consisting of 4 nude mice (Balbc nu/nu), were challenged by subcutaneous injection of 10^6 NCI H526 cells (7) contained in 100 μl of PBS per animal. This human bronchial carcinoma cell line had previously been shown to express ganglioside Gfpt1 (*13*). Before challenge with tumor cells, group I of the nude mice had not been preimmunized, Group II had received four Gfpt1/MPL injections at weekly intervals, each consisting of 20 μg of Gfpt1, 8 μg of MPL, and 100 μg of PC/PS in 200 μl of PBS, well sonified before application, per animal. Group III, serving as a control, was immunized at weekly intervals four times with sonified 8 μg of MPL and 100 μg of PC/PS in 200 μl of PBS. After the challenge with tumor cells, immunization of groups II and III with Gfpt1/MPL- and MPL-liposomes, respectively, was continued with four weekly-interval injections, as before. After approximately four weeks, subcutaneous tumors in the control groups were well developed and all the animals were sacrificed. The sera of all animals were collected for anti-Gfpt1 titer determination. Tumors were carefully removed, wet-weighed and measured. The excised tumors were then lyophilized, the total lipids extracted with chloroform-methanol-water mixtures, and, after mild alkaline saponification and dialysis of the lipid fraction, the gangliosides isolated by ion-exchange chromatography (*14*). Ganglioside Gfpt1 was identified on thin-layer chromatograms by its migration velocity, resorcinol-HCl staining properties and immunereaction with monoclonal anti-Gfpt1 antibody as describerd before (*8-10*).

RESULTS

In an attempt to improve the ganglioside-related immunogenicity of the Gfpt1-KLH conjugate in mice as reported earlier, such conjugates were synthesized with a higher load of ganglioside relative to the KLH adjuvant. This was achieved by chemical coupling under more alkaline conditions than had been used previously, *i.e.*, at pH 8.5 instead of pH 7.2. However, it was observed that exposure of KLH to the more alkaline milieu decreased the ganglioside-related immunogenicity of the KLH-ganglioside adduct *per se*, irrespective of the load of ganglioside. Thus, immunization of mice with a Gfpt1-KLH pH 8.5 conjugate consisting of 50 μg of ganglioside and 15 μg per mg of KLH was in fact far less immunogenic than a comparable Gfpt1-KLH pH 7.2 preparation with a load of 15 μg of ganglioside per mg KLH (Fig. 1A).

There was no change in the ratio of IgG to IgM mouse serum antibodies with either the Gfpt1-KLH pH 8.5 or pH 7.2 vaccines (Fig. 2).

The lower adjuvant activity of the alkaline buffer-treated KLH may, therefore, be due to a change in molecular arrangement of this protein effected in a more alkaline milieu.

Muramyldipeptide was coupled to ganglioside Gfpt1 by reacting its *N*-hydroxysuccinimidester with amino-ganglioside prepared as reported earlier by ozonolysis followed by reductamination of Gfpt1 (*6*) (Figs. 3 and 4).

Using PC, PS-liposomes as carriers, a Gfpt1-MDP conjugate showed an increased ganglioside-related immunogenicity in mice when compared to a simple admixture of MDP and Gfpt1 (Fig. 1B). Gfpt1-MDP stimulated somewhat lower total anti-ganglioside Ig-titers than Gfpt1-KLH pH 7.2 conjugate showing a similar IgG to IgM distribution of the elicited serum antibodies (Fig. 2). However, even higher anti-Gfpt1 antibody titers could be raised by immunization with a PC, PS-liposome vaccine containing, besides native Gfpt1, MDP-PE as an immunoadjuvant (Fig. 1B, curve 3). In the case of immunization with liposomes containing either the Gfpt1-MDP conjugate or Gfpt1/MDP-PE, it was observed that individual mice showed unusually divergent anti-Gfpt1 serum antibody titers (see high maxima as compared to the mean values in Fig. 2).

Addition of MPL (8 μg/single immunization dose/

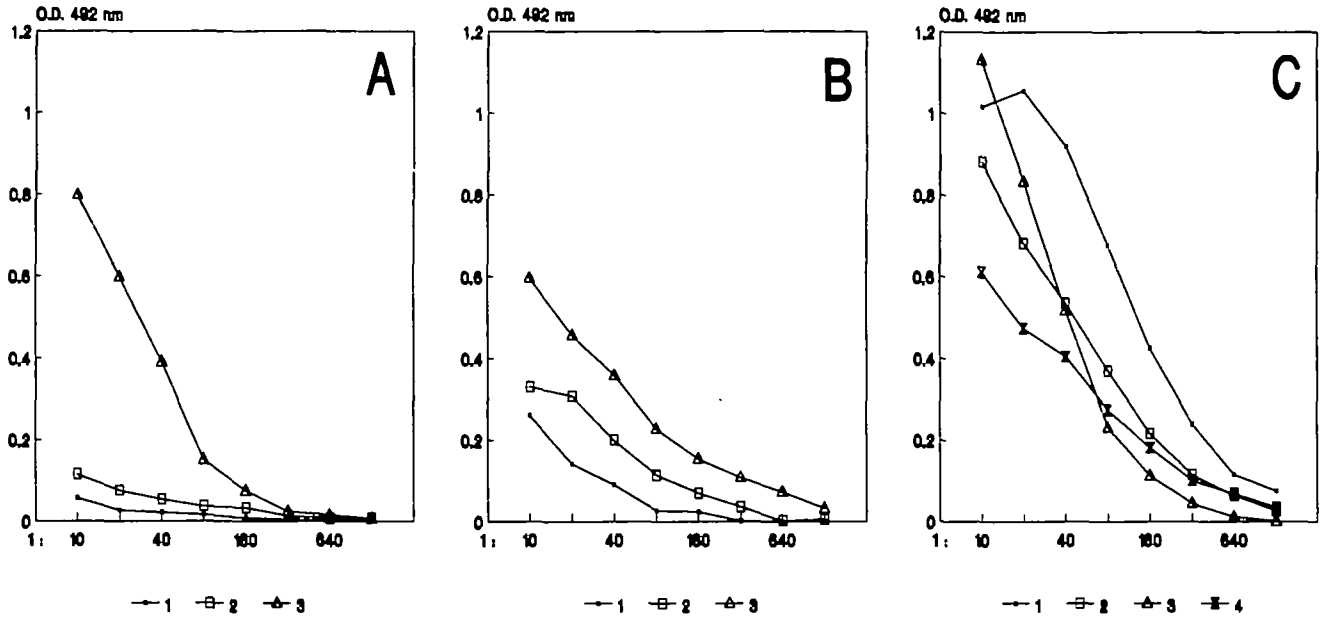

Fig. 1. **Total anti-Gfpt1-immunoglobulin response.** Mice were immunized with: A1, Gfpt1, PC,PS-liposomes; A2, Gfpt1-KLH pH 8.5 conjugate (50 μg G/mg KLH); A3, Gfpt1-KLH pH 7.2 conjugate (15 μg G/mg KLH); B1, Gfpt1, MDP (6.5 μg), PC, PS-liposomes; B2, Gfpt1-MDP, PC,PS-liposomes; B3, Gfpt1, MDP (6.5μg)-PE, PC,PS-liposomes; C1, Gfpt1+MPL (8 μg), PC,PS-liposomes; C2, Gfpt1-KLH pH 8.5 conjugate (50 μg G/mg KLH)+MPL (8 μg); C3, Gfpt1-KLH pH 7.2 conjugate (15 μg G/mg KLH)+MPL (8 μg); C4, Gfpt1-MDP+MPL (8 μg), PC,PS-liposomes. Measurement was by ELISA (for conditions, see "MATERIALS AND METHODS").

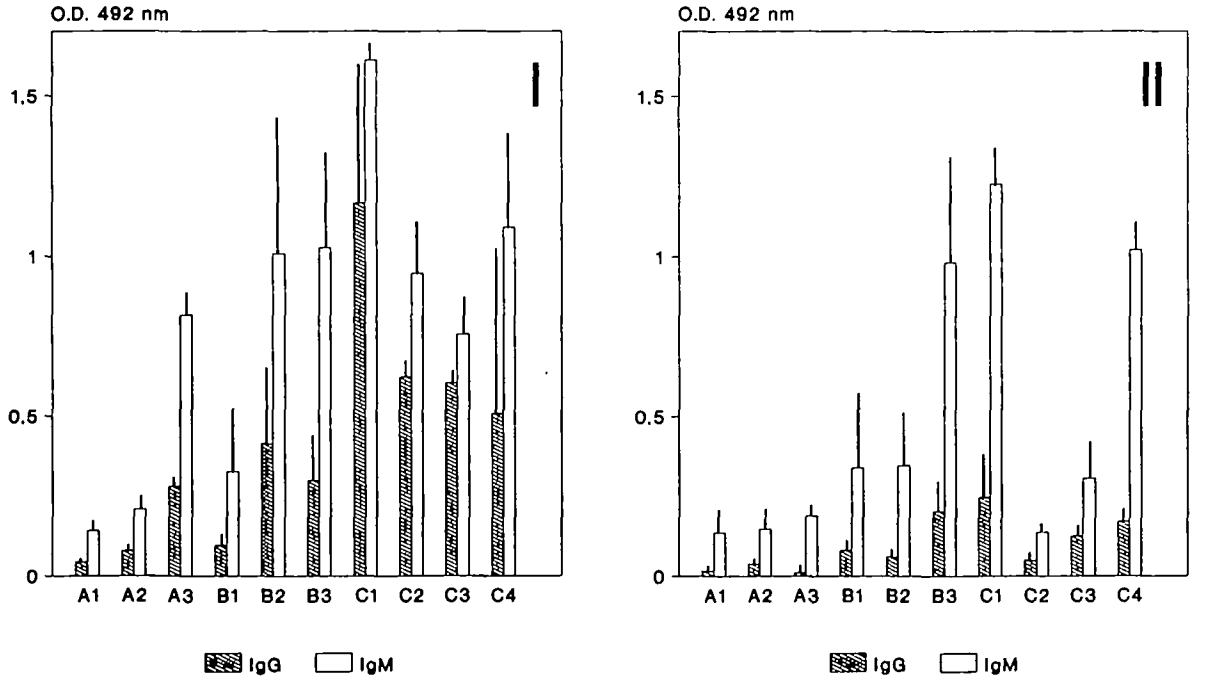

Fig. 2. **IgG/IgM anti-Gfpt1 (I) and anti-Gtet1 (II)-immunoglobulin response of the sera.** For designations, see Fig. 1 (for ELISA conditions, see "MATERIALS AND METHODS").

mouse) in the presence of Gfpt1 or its derivatives drastically elevated anti-Gfpt1 directed serum antibody titers in mice. In fact, the highest anti-Gfpt1 humoral antibody titers could be obtained in mice by immunization with the native free ganglioside in PC,PS-liposomes containing MPL, followed by the Gfpt1-KLH and Gfpt1-MDP conjugates (Fig. 1C). In addition to an enhancement of the total anti-Gfpt1 immunoglobulin, MPL also effected an increase in the ratio of anti-Gfpt1 serum IgG to IgM (Fig. 2). The MPL-induced increase in serum IgG correlated with an enhancement of all IgG-subtype titers, and in particular IgG3 and IgG2a (Fig. 5).

All sera obtained from mice that had been immunized with vaccines containing the Gfpt1-carbohydrate epitope also reacted with the Gfpt1-related ganglioside Gtet1 (GM1, II^{3}NeuAc-Gg$_4$Cer). However, of all mouse sera tested, crossreactivity between Gfpt1 and Gtet1 was lowest after immunization with Gfpt1-KLH conjugate (Fig. 2, C2).

Only in the case of immunization with Gfpt1-KLH did addition of MPL to the vaccine result in an improvement of the Gfpt1-selective recognition of the mouse serum immunoglobulins (Fig. 2). In no case of immunization of mice with any of the described vaccines could pathological phenomena be observed.

To see whether raised antibody titers against the human small cell lung cancer-associated ganglioside Gfpt1 would be effective in preventing tumor growth *in vivo*, athymic mice were preimmunized with the ganglioside in the presence of MPL. Control nude mice either were not immunized, or had in parallel received injections of MPL-PC/PS-liposomes. Only in those animals that had not been immunized with the Gfpt1/MPL-vaccine could tumor

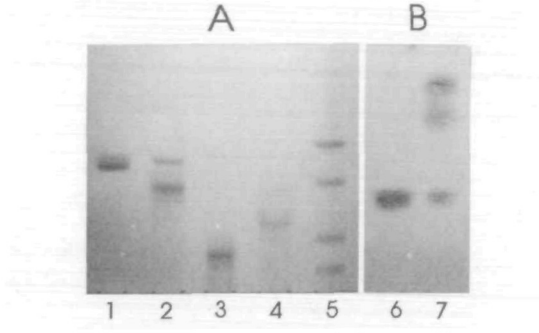

Fig. 3. **Thin-layer chromatogram of the coupling products of MDP to amino-ganglioside Gfpt1.** 1, Gfpt1; 2, Gfpt1-aldehyde; 3, amino-ganglioside Gfpt1, 4, coupling product of MDP-*N*-hydroxysuccinimidester and amino-ganglioside Gfpt1; 5, standard brain ganglioside (from top to bottom: Gtet-gangliosides 1, 2a, 2b, 3b); 6, MDP; 7, MDP-*N*-hydroxysuccinimidester. Visualization. A, orcinol-sulfuric acid spray reagent, B, chlorine-toluidine. Running solvent: chloroform/methanol/0.2% aqueous $CaCl_2$ (45 : 45 10).

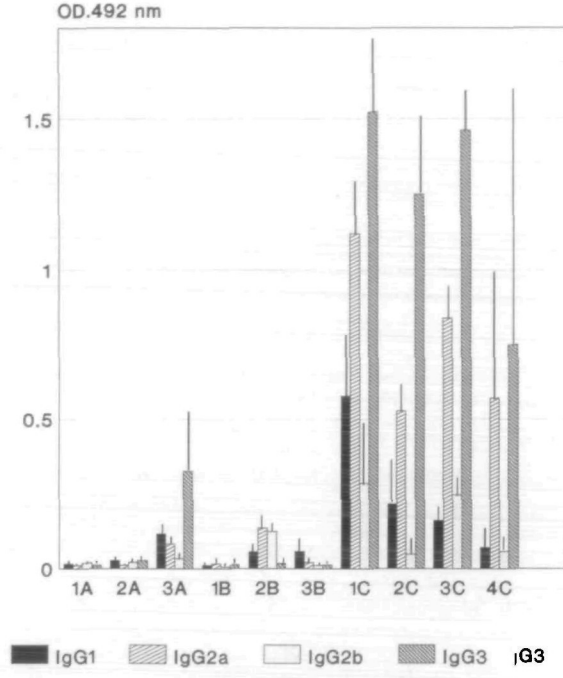

Fig. 5. **IgG-subclass determination.** For designations, see Fig 1 (for ELISA conditions, see "MATERIALS AND METHODS").

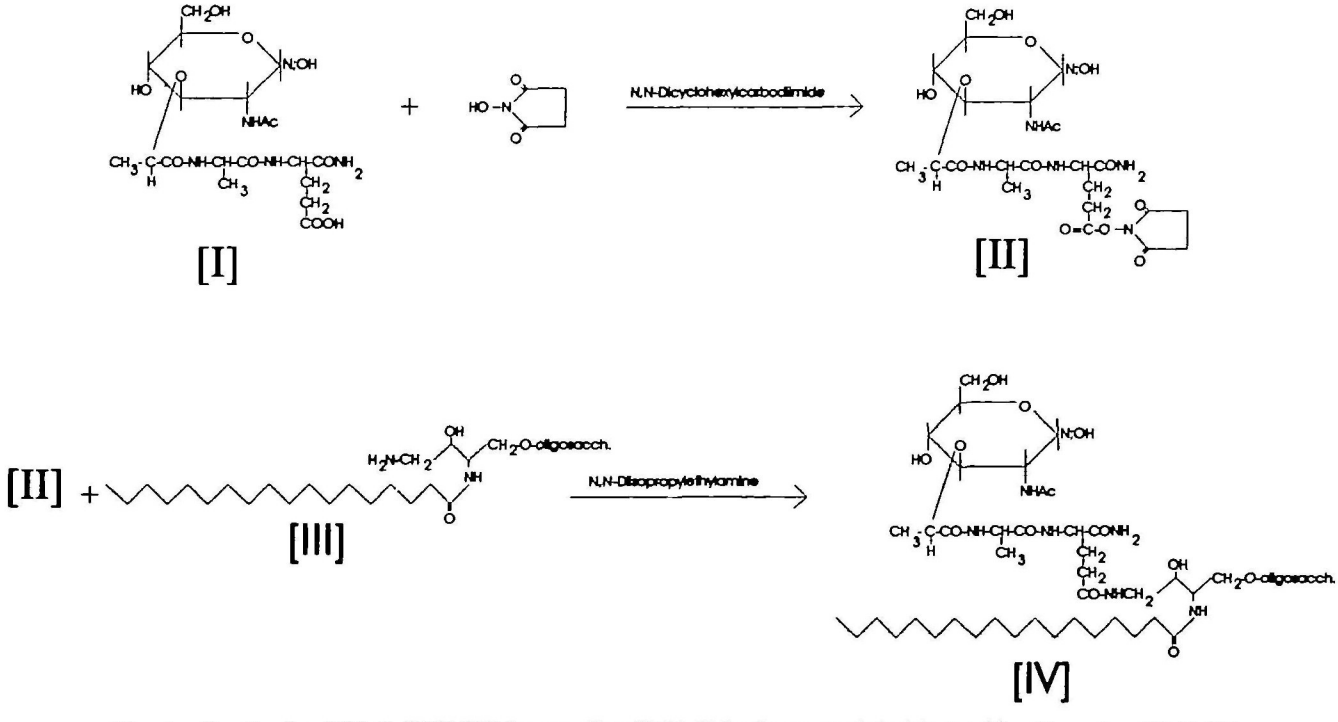

Fig. 4. **Synthesis of Gfpt1-MDP (III) by reacting MDP-*N*-hydroxysuccinimidester (I) with amino-Gfpt1 (II).**

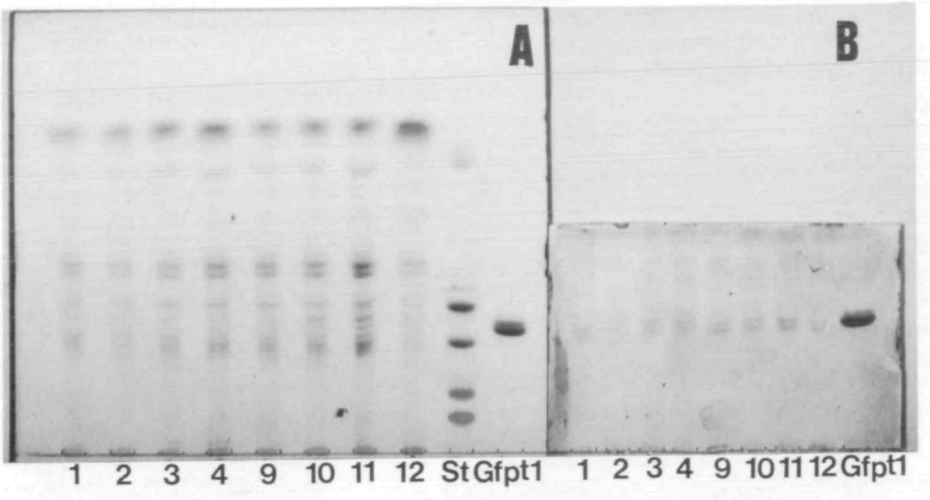

Fig 6 High performance thin-layer chromatogram of the ganglioside fraction of NCI H526-cell tumors grown in nude mice. 1-4, tumor gangliosides of animals not immunized, 9-12, tumor gangliosides of animals immunized with MPL-liposomes; ST, standard bovine brain gangliosides, Gfpt1, standard Gfpt1 Visualization: A, resorcinol-HCl spray reagent; B, immune overlay using anti-Gfpt1 monoclonal mouse antibody and POD-labeled anti-mouse-Ig immunoglobulin as described previously (*6*). Running solvent. chloroform/methanol/0.2% aqueous $CaCl_2$ (60 : 35 : 8).

TABLE I. **Tumor development in Balbc nu/nu athymic mice challenged with NCI H526-cells.**

No	Tumor weight (g)	Tumor vol (μl)	Serum anti-Gfpt1 titer OD at 1:80 serum dilution
Group I (non-immunized)			
1	0.30	986	0.001
2	1 49	1,884	0 066
3	0.12	208	0.006
4	0.55	613	0.032
Group II (Gfpt1/MPL-immunized)			
5	≤0 01	28	1.335
6	≤0 01	0.2	1 034
7	0.03	22.4	0.337
8	≤0.01	8.2	0.661
Group III (MPL-immunized)			
9	0 47	381	0 035
10	0 71	642	0 001
11	0.17	357	0.001
12	0.11	48	0 001

growth be observed (Table I). In parallel to the inhibition of tumor development, the Gfpt1/MPL-liposome-immunized animals showed serum antibody titers against ganglioside Gfpt1 (Table I). Furthermore, ganglioside Gfpt1 could be identified in all tumors that had developed in the nude mice (Fig. 6).

DISCUSSION

Human small cell lung cancer cells are characterized by the expression of ganglioside Gfpt1 (*2-4, 13*). This ganglioside was also found to be present in the serum of some of the patients carrying this tumor (*15*). Monoclonal antibodies that recognize Gfpt1 have been shown to lyse these tumor cells *in vitro* in the presence of complement and are able to inhibit a corresponding tumor growth in nude mice *in vivo* (*16, 17*). These observations may suggest that an anti-Gfpt1-directed immune response could have beneficial effects on the development of small cell lung cancer in human patients. Therefore, in an earlier study with the synthesis of a Gfpt1-KLH conjugate, the development of an effective anti-Gfpt1 immunogen was attempted which could possibly be used as a vaccine in human therapy (*6*). A similar immune conjugate using KLH and ganglioside Glac2 [$II^3(NeuAc)_2$-LacCer] (*18*) in the presence of a *Quillaja saponaria Molina* saponin adjuvant [fraction QS21 (*19*)] was more recently applied in a clinical phase-I trial for the treatment of human malignant melanoma (*20*). Another very effective general immunoadjuvant is monophosphoryllipid-A (MPL) (*21*). In contrast to diphosphoryllipid-A, the monophosphoryl-derivative was reported to be non-toxic in animals in small amounts. MPL has already been applied for the immune treatment of malaria in a clinical phase-I trial (*22*). In addition, Ravindranath and coworkers have shown that MPL considerably enhances the humoral immune response towards liposome-embedded ganglioside (*23*). Furthermore, these and other studies found that liposomes proved as the most efficient immunogen carriers, when they consisted of distearoylglycerol residues-carrying PC,PS mixtures in a molar ratio of 1:1 of these phospholipids (*24-26*).

The present study investigated the effect of MPL as a coadjuvant in the mouse model for the immunization with Gfpt1-derivatives, and the use of PC,PS-liposomes as immunogen carriers for the immunization with lipophilic Gfpt1-conjugates such as the novel chemical adduct of MDP and Gfpt1.

MPL very effectively stimulated anti-Gfpt1-directed serum antibody titers with PC,PS-liposomes solely containing Gfpt1. In addition, MPL raised anti-ganglioside serum antibody titers also with the chemical ganglioside conjugates, Gfpt1-KLH and Gfpt1-MDP. Concomitantly, however, antibody titers crossreactive with ganglioside Gtet1 were also elevated by the addition of MPL. Except for the case of Gfpt1-KLH, a further elevation of these MPL-stimulated titers was not linked to an increase in crossreactivity with Gtet1. Chemical linkage of the ganglioside-carbohydrate epitope to KLH, therefore, appears to have modified the immunoexpression of this glycoconjugate in such a way as to stimulate the polyclonal immunoglobulin formation more specifically site-directed with less cross-reactive overlap. In fact, the Gtet1-crossreactivity of these anti-Gfpt1 conjugate sera was even further reduced by the presence of MPL in the immunization mixtures.

In a preliminary experiment performed to observe a protective effect of an anti-Gfpt1 preimmunization on the growth of human small cell lung cancers, athymic mice were treated with the free ganglioside/MPL in PC/PS-liposomes. In this case, immunization with the unconjugat-

ed ganglioside was chosen, because of the considerably higher antibody titers achieved as compared to the KLH- and MDP-conjugates in presence of MPL. As a model for the possible curbing of growth and metastases of the tumor cells, preimmunization was performed. Thereby, we hoped to see whether preexisting antibodies in the animals could inhibit growth of the tumor. The results observed in the present study by mouse vaccination with Gfpt1/MPL showed an effective prevention of *in vivo* NCI H526-cell tumor growth in athymic mice. Nevertheless, among different Gfpt1-related immunogens, the application of Gfpt1-KLH conjugate vaccine together with MPL proved to be superior for a comparatively ganglioside-carbohydrate epitope selective immunization.

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