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Antibodies can be Spontaneously Loaded onto Monosialoganglioside Micelles Containing Oncological Drugs

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Abstract

Recently, we demonstrated that GM1 micelles transport paclitaxel and doxorubicin with high efficiency. When this GM1-drugs complex is incubated with whole serum, albumin was the only one protein that binds spontaneously to form GM1-drug-albumin complex. Here, we show that, under specific physicochemical conditions, these micelles interact with antibodies forming GM1-IgG complexes. The load of IgG in GM1 reaches a maximum at ratios of 1/4 (w / w) incubating to 4.5 and preheating the micelles of GM1 at 55-60°C. The IgG of the GM1-IgG complex obtained under these experimental conditions retains the biological activity against the soluble and cellular antigens and is not displaced from the micelles in the presence of albumin, the main competitive binding protein.

Treatment of GM1-IgG with pepsin, does not show the breakage of the IgG like control of free IgG, suggesting that IgG is deeply bound into GM1, probably via Fc. Moreover, the presence of 1 M NaCl does not prevent neither dissociate the complex, suggesting the hydrophobic nature of the interaction. The DLS and TEM results shows that GM1-IgG complexes have sizes significantly higher than those of GM1 micelles; this is directly related to the amount of IgG loaded. On the other hand GM1-IgG complex also retain the ability to encapsulate oncological drugs, but, an adequate sequence must be followed during the preparation, in order to obtain efficient GM1-drug-IgG ternary complexes. Moreover, the presence of IgG into GM1-oncological drugs complex do not affect the release or the citotoxic activity of the encapsulated molecules such as Ptx or Doxo.

Keywords: Immunomicelles; Monosialoganglioside (GM1); Targeted drug delivery; Nanomedicine; Cancer

Introduction

The use of monoclonal antibodies targeting to cell surface antigens on malignant cells, that are generally more numerous on the surface of cancer cells than healthy cells, has positively recognized itself as an effective approach for therapy of solid and hematological tumors. They are designed to function in different ways as flagging cancer cells, triggering cell-membrane destruction, blocking cell growth, preventing blood vessel growth, blocking immune system inhibitors, directly attacking cancer cells, delivering radiation treatment and delivering chemotherapy. Even, one strategy that has received much attention in recent times is the chemistry of attaching antibodies to nanocarriers for targeted cancer therapy to achieve a high degree of selectivity and improve drug absorption or to be used as a dual system. In this sense, numerous procedures have been described for attaching antibodies to carriers and even to drugs for active targeting, including covalent and non-covalent approaches [1-7]. In all cases, the main goal is to bind the antibody without losing its functionality after attachment. One of the most commonly used binding methods is through chemical conjugation using cross-linking reagents. This crosslinking leads to a more stable binding, which could improve the physicochemical stability and half-life of the antibodies and prevent competitive displacement by the blood components. Simultaneously, the incorporation of antibodies into the vehicles carrying the drugs offers clear advantages over the direct conjugation of the antibody into the drug itself [8,9], such as higher drug loading capacity and protection of the drug reaction conjugation. However, these methods often alter the biological functions of antibodies as well as drug transport structures.

Regarding the non-covalent binding of antibodies to the transporters, although methodologically it may be simpler and easier to carry out, there are a number of factors to consider since non-specific binding has less stability, which can determine the desorption or displacement from antibody by serum proteins which compete for adsorption sites [1,10,11]. Similarly, the correct orientation of antibody molecules is a key factor that can reduce or even eliminate the effectiveness of target binding. Therefore, depending on the chosen procedures, it is important to optimize the conditions and characterize the complexes obtained in terms of their physical properties and biological activity.

In previous studies, we describe a self-assembled sialo-glycosphingolipid (GM1) micellar system capable of loading and releasing hydrophobic (paclitaxel) and hydrophilic drugs (doxorubicin), or both, with high efficiency [12,13]. We also found that GM1 micelles loaded with drugs interact spontaneously with albumin (Human Serum Albumin-HSA) to give GM1-drug-HSA complexes. Here, we demonstrate that subtle specific changes in the conditions that affect both components GM1 and IgG it is possible to obtain the complex of GM1-IgG. We analyze influence of various physical factors in the non-covalent interaction, specially the temperature that modify substantially the properties of GM1 micelles [14] and the pH [15,16] to optimize amount of mAb bound to the micelles, its stability, biological activity and the capability to load oncological drugs.

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Materials and Methods

Materials

GM1 was supplied by TRB Pharma S.A. Total human IgG antibodies were provided by Laboratory of Hemoderivados, National University of Córdoba (Córdoba, Argentina). Rubella IgG antibodies were measured using Immulite / Immulite 1000 analyzer and anti-HBs were analyzed using Roche Elecsys immunoassay analyzer. Monoclonal antibodies CD4-FITC / CD8-PE / CD3-PC5 (OptiClone) were from Immunotech, Beckman, France. Anti-HER-2/neu (4B5) rabbit monoclonal primary antibody and anti-Ki-67 primary rabbit monoclonal antibody (IgG) directed against C-terminal portion of Ki-67 antigen were from Ventana Medical Systems, Inc., USA. Anti-FR IgG primary antibodies (folate receptor-targeted antibody), anti-LDLR IgG (anti-LDL receptor targeted antibody) and anti-CD44 IgG (antibody targeted against the receptor for hyaluronic acid) were purchased from Sigma Aldrich. Paclitaxel (Ptx) was acquired from Yunnan Smandbet Co. Ltd. (Kumming, China). The stock solutions of Ptx were prepared by dissolving the drug in dimethylsulfoxide (DMSO) at a final concentration of 50 mg mL-1. Doxorubicin (Doxo) was obtained from Sigma. Stock solutions of Doxo were prepared in physiologic solution at 6 mg mL^{-1}. Sephadex G-25 and G200 columns were purchased from GE Healthcare (Buckinghamshire, UK). All other reagents were of analytical grade, used as received from Merck (Darmstadt, Germany).

Methods

Cell lines cultures: The following continuous cell lines were used: human cervical epithelioma cells (HeLa), non-small cell human lung carcinoma cells (H1299) and highly metastatic mouse melanoma cells (B16).

For cell cultures, MEM for HeLa and DMEM-F12 for H1299 and B16 cells, were supplemented with irradiated fetal calf serum (NATOCOR, Córdoba, Argentina) at 1 or 10% (v/v) according to maintenance or growth conditions, respectively.

Preparation of IgG antibodies coupled to GM1 micelles: Stock solution of 250 $mg.ml^{-1}$ GM1 was prepared in bidistilled water 24 h prior to use. The solutions were maintained at 4-8°C for 24 h and centrifuged at 50,000 g for 15 min. The supernatant was finally filtered through 0.22 µm. The micelles (GM1) were then incubated with human IgG under different conditions of pHs (3, 4.5 and 7.4), temperatures (4, 25, 37, 45 and 55°C) and times (from 2 to 24 h).

The results of these assays were analyzed by chromatographic analysis.

Size exclusion chromatography (SEC): The GM1-IgG complexes were isolated and quantified by size exclusion chromatography. Samples and controls were run on an Åkta Explorer 100 system (GE Healthcare) fitted with a Superdex 200 column, previously equilibrated with 50 mM phosphate buffer (pH 7.4) and 150 mM NaCl at a rate of 0.4 mL min^{-1}. The elution profile was followed using a UV-visible detector at 200 and 280 nm for GM1 micelles and protein respectively [17].

Native SDS-PAGE analysis: The identification of antibodies after incorporation into the micelles and eluted by size exclusion chromatography was detected by SDS-PAGE and compared with the native antibody control. The gels were run under native conditions (without 2-mercaptoethanol) using a BioRad Mini-Protean II kit at a constant voltage of 200 V in Tris/glycine/SDS buffer. The gels were stained with Coomassie blue.

Determination of IgG concentration: IgG concentration was determined using a Lowry assay [18] or by direct absorbance at 280 nm.

Digestion of IgG incorporated in GM1 micelles with pepsin: 20 µl (0, 4 mg) of pepsin under acid conditions was added to 1 ml of GM1-IgG complex (5/1 w/w) previously prepared. The mixture was incubated at 37°C in buffer pH 4.5 in a shaking water bath and 20 µl aliquots were taken at 30 min for IgG control and at 15, 30, 60 and 90 min for GM1-IgG for analysis by gel electrophoresis.

Particle size and size distribution: Average particle size and size distribution of the nanoparticles were measured by laser light scattering (LLS, Delsa TM Nano, Beckman Coulter Instruments). The samples were diluted with PBS buffer pH 7.4 and filtered before measurement. Data were analyzed using Delsa ™ Nano Beckman Coulter software (version 2.2). All measurements were done in triplicate, for each of three micelle-independent batches.

Surface charge: The zeta potential of the GM1-IgG complexes was determined using a Delsa TM Nano zeta potential analyzer (Beckman Coulter Instruments) at room temperature. The samples were prepared by diluting the micelle solutions with PBS buffer pH 7.4. Particle sizes of the micelles were measured to assure that micellar structure was intact. Samples were diluted with PBS buffer (145 mM NaCl) pH 7.4 and filtered prior to measurement. The zeta potential was determined in triplicate and results were calculated automatically by the analyzer.

Transmission electron microscopy (TEM): A JEOL JEM-1200 EX II transmission electron microscope was used at 300000 X to establish the morphology of the modified micelles. 50 µl of each sample was loaded onto a carbon grid, incubated for 5 min and the grid was then dried. The gratings were then incubated with 50 µl of a 10% uranyl acetate solution for 1 min, dried and taken under a microscope for observation.

Drug encapsulation and loading efficiency: To assess whether IgG binding to GM1 micelles affects the loading capacity of the micelle, we evaluated the load of increasing amounts of Ptx or Doxo in GM1-IgG micelles of 10 $mg.mL^{-1}$ to reach micelle/drug molar ratios from 50/1 to1/1. Drug loading was performed at 20°C for 30 min before incubating the samples at 4°C for 24 h. After incubation time, the samples were dialyzed against PBS buffer pH 7.4 for 24 h at 4°C to remove the free drugs. In addition, the influence of the loading order of the drug and IgG was analyzed.

Determination of Ptx concentration: Ptx concentration was measured on a Curosil B C18 column (250×3.20 mm I.D., particle size 5 µm) and a Curosil BC18 guard column (30 × 4.60 mm I.D., particle size 5 µm) supplied by Phenomenex as we previously described [11]. The mobile phase was 60% (v/v) acetonitrile and 40% (v/v) bidistilled water. Flow rate was 0.7 mL min^{-1} and the eluent was monitored at 227 nm. Chromatography was performed at ambient temperature (20°C). Validation of the method was carried out according to FDA Guidance for Bioanalytical Method Validation.

Determination of doxo concentration: Doxo concentration was determined by absorbance at 490 nm using a calibration curve performed with a standard solution of Doxo in physiologic solution, as described by Abraham et al. [19].

Recognition properties of antibodies bound to GM1 micelles: The following methods were used to evaluate the functionality of the mAb on the micelle surface.

Flow cytometry: A combination of three anti-CD4-FITC / CD8-PE / CD3-PC5 (OptiClone) fluorescent monoclonal antibodies, previously absorbed on the micelles and eluted through a size exclusion column (Sephadex G- 200) was incubated with a whole blood sample. After incubation, the red blood cells were lysed using a non-washed procedure, the processed sample was then directly introduced into the flow cytometer for analysis. After excitation at 488 nm, each fluorescent marker emitted light at different wavelengths, allowing the simultaneous study of CD4, CD8 and CD3 antigens, respectively. The use of dispersion diagrams (representation of lateral dispersion vs. frontal dispersion) allows discrimination of lymphocytes waste, monocytes and polymorphonuclear cells.

As controls, free antibodies and uncoated micelles were analyzed.

Chemiluminescent immunoassay: A solid-phase, sequential chemiluminescent immunoassay IMMULITE/IMMULITE 1000 Rubella Quantitative IgG for *in vitro* diagnostics was performed to determine the functionality of the anti-Rub IgG antibodies absorbed on GM1 micelles. First, anti-Rub IgG antibodies were absorbed at the experimental conditions selected. Samples were then eluted by a size exclusion column to remove free antibodies. Finally, their biological activity was measured and compared with standard anti-Rub IgG antibodies. Likewise, anti-HBs IgG antibodies were absorbed into GM1 micelles and eluted through a size exclusion column to separate the free antibodies. Subsequently, their biological activity with standard anti-HBs IgG antibodies was measured and compared by a sandwich-type chemiluminescent immunoassay against hepatitis B surface antigen (HBsAg).

Immunohistochemistry: Anti-HER-2/neu and rabbit anti-Ki-67 IgG primary monoclonal antibodies were absorbed into GM1 micelles, under pre-established experimental conditions and then incubated with tumor tissue sections, which express the specific antigen, fixed in formalin for 16 to 32 min. Finally, the tissues with the samples were stained on a slide, with the detection kit provided by Ventana®.

The ability of antibodies to recognize their specific antigen after being absorbed into the micelles was compared to that of the same free antibodies using images obtained by optical microscopy of consecutive tissue sections.

***In vitro* interaction assays of GM1-antibody complexes with cells expressing specific receptors:** Different specific antibodies, anti-FR, anti-CD44, anti-HER-2/neu and anti-LDLR, were used to evaluate the surface expression of specific receptors in the different cell lines mentioned above. In parallel, cells were incubated with the same antibodies adsorbed into GM1 micelles to evaluate the specific interaction of GM1-anti receptor IgG with the cells. Briefly, confluent monolayers were incubated with the different anti-receptor antibodies, at concentrations between 1 and 20 $\mu g.ml^{-1}$ and 4 to 24 h at 37°C. After incubation, cells were washed extensively with PBS to remove unreacted material and a Peroxidase enzyme-labeled anti-IgG secondary antibody was added at different dilutions for 2 h at 37°C. Finally, the monolayers were washed again to remove the unreacted labeled anti-antibodies and the bound material was analyzed by colorimetric spectrophotometry. The final colorimetric evaluation was performed after the addition of a specific substrate (chromogenic), the ortophenylenediamine (OPD), on which the peroxidase enzyme acts and gives an observable and quantifiable colored reaction product. Absorbance measurements were performed on an automated plate reader (BioRad., CA, USA) at $\lambda = 490$ nm. The reaction blank for the absorbance of each sample was discounted. The untreated monolayers to which only the labeled secondary antibody and the chromogen were added for the reaction were taken as reaction blank. The results represent mean values of three measurements and their respective deviations.

***In vitro* cytotoxicity of Paclitaxel or doxorubicin loaded in GM1-drug-antibody complexes:** Cytotoxicity assays were performed using the cell lines which exhibited the expression of specific receptors (+), such as the non-small cell (H1299) human lung carcinoma cells, CD44 receptor (+) highly metastatic mouse melanoma cells (B16), FR and LDLR (+) and human cervical epithelial cells (HeLa), CD44 receptor (+).

Confluent monolayers grown in 96 well plates were incubated with GM1-Doxo or GM1- Doxo- IgG micelles with different Doxo final concentrations (10, 25, 50 $\mu g.ml^{-1}$) at different times (30 min to 8 h). After each time, the medium with the sample was replaced by fresh medium and incubated 24 h at 37°C and 5% CO_2. After 24 h the cells were washed three times with PBS, fixed with 10% formaldehyde and then stained with a solution of 0.4% crystal violet in methanol, widely used to evaluate the cytotoxic activity of drugs or biomaterials. Subsequently a solution of 5% acetic acid was added to the stained cells to dissolve the color after 10-15 min absorbance was determined.

Quantitative analysis (colorimetric assay of the fixed cells) was performed by absorbance measurements on an automated plate reader (Bio-Rad., CA, USA) at 620 nm. Cell viability was calculated and compared between samples. Untreated cells incubated with culture medium only were taken as negative control, 100% cell survival. The results represent mean values of three measurements and their respective deviations.

The assays with Ptx were performed in a similar way to those of Doxo; cells monolayers were incubated with Ptx, GM1-Ptx or GM1-Ptx- anti receptor IgG for 24 h and results were quantitatively analyzed by crystal violet assay.

Results

Characterization of the interaction of IgG antibodies with GM1 micelles

Previously, we described that GM1 micelles interact spontaneously with albumin, but not with immunoglobulins. In this work, we evaluated if modifying properties of GM1 or IgG under different experimental conditions allow achieving the interaction between micelles and antibodies. To do this, we begin evaluating whether the modification of the structure of IgG by changes in pH, in a range of 4.5 to 7.5, leads to interaction. The interaction was evaluated by size exclusion chromatography. Considering that pure GM1 micelles (~375 kDa) eluted with the V_0 (unretained fraction) of the column (data not shown) and IgG fraction (150 kDa) eluted in the fraction corresponding to a volume about 6 ml. When GM1 micelles (20 $mg.ml^{-1}$) were incubated with IgG (5 $mg.ml^{-1}$) at 25°C at pH7 and eluted at pH 7.4 each component kept its original profile with only less that 5 % of the added immunoglobulin interacts with micelles confirming that no association occurs under this pH condition (Figure 1A). However, when GM1 micelles (20 $mg.ml^{-1}$) were incubated with IgG (5 $mg.ml^{-1}$) and eluted at pH 4.5, a condition that produces changes in the Fc by exposure of hydrophobic components but it does not affect IgG activity nor GM1 structure, the elution profile showed a shift to a unique high molecular weight specie (≥ 375 kDa) where practically all the IgG elutes with the GM1 micelles, demonstrating a physical association between these two fractions. On the other hand, when the incubation is carried out at pH 4.5 and then eluted at pH 7, the appearance of a second peak of 150 kDa, corresponding to the IgG, indicate a partial dissociation of the complex.

Considering the reversible conformational denaturation of the IgG at acidic pH, with the consequent exposure of hydrophobic residues, mainly in the Fc region [15,16], our results about the binding of IgG to GM1 micelles at pH 4.5 suggest an interaction of hydrophobic nature. To better understand the nature of the GM1-IgG interaction at pH 4.5, we added 1 M NaCl to dissociate a possible electrostatic interaction of the complex, which did not happen, reinforcing the concept of the hydrophobic nature of the interaction.

To evaluate the effect of temperature on GM1-IgG interaction, we incubate the complex in two different experimental conditions and then molecular exclusion chromatography was also used. First one, increasing the incubation temperature in a range from 4°C until to 45°C, the maximum temperature that not affect the biological activity of IgG. Figure 1B show that the binding of the immunoglobulins to the GM1 micelles increase around from 4-5 times from 4°C to 45°C.

The second one is an assay where the temperature affects only the GM1 micelles. It has been reported that temperatures above 55°C induce the release of water molecules bound to the oligosaccharide chain of gangliosides, resulting in a significant reduction of the hydrophilic portion, accompanied by a slight extension of the hydrophobic region of the micelles [20,21]. When GM1 micelles were preheated at 55°C for 30 min at pH 4.5 and then stabilized at room temperature, the binding of IgG to this GM1 was higher than that for GM1-IgG complex heated together at 45°C described above (Figure 1C). These results, demonstrate that temperature and especially those that affect the GM1 play a key role in the interaction.

A common disadvantage of the physical adsorption of antibodies to nanocarriers is the competitive displacement by blood components [1,10,11]. Considering the spontaneous association of albumin (HSA) with GM1, demonstrated in previous reports of our own and other authors [12,17,22,23], we analyzed whether the presence of albumin affects the interaction of immunoglobulins with GM1 micelles.

To evaluate this, GM1 micelles (20 mg.ml^{-1}) at pH 4.5 were incubated during 30 min at 45°C with a solution containing both IgG (5 mg.ml^{-1}) with increasing concentrations HSA from 0 to 8.8 mg/ml and then the elution profile of proteins was analyzed. Figure 2A shows that the presence of increasing amounts of HSA (from 0 to 8.8 mg.ml^{-1}) induced the appearance of a new peak which correlates with the elution pattern of IgG (150 kDa) that increases with albumin concentration. These results demonstrate that, under this experimental condition, there is a competitive binding site for both proteins in GM1 micelles. Figure 2B shows the SDS-PAGE analysis of fractions eluted from the size exclusion column. With 2.2 mg.ml^{-1} HSA (Figure 2B- SDS-PAGE a), both proteins HSA and IgG appear to be bound to GM1 micelles and elute together in the column as can be seen in the same fractions of SDS-PAGE, whereas in presence of HSA 4.4 and 8.8 mg.ml^{-1} (2B- SDS-PAGE b) and (2B- SDS-PAGE c), only HSA can be seen in the initial fractions of SDS-PAGE while IgG appear in retarded fraction corresponding with MW 150 kDa, confirming the results described above.

Interestingly, when the complex of GM1-IgG is obtained at pH 4.5 and then adjusted to pH 7.4, the addition of HSA at 4, 4 and 8, 8

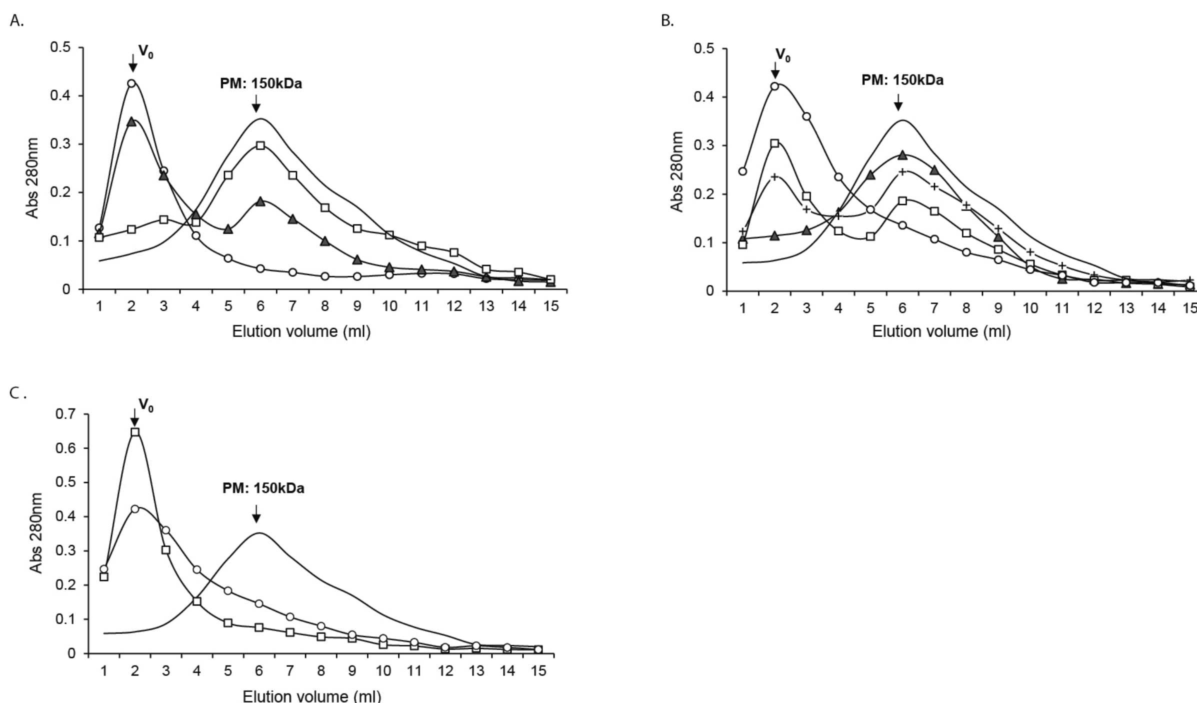

Figure 1: A- Pattern of chromatographic elution on Superdex 200® of IgG binding to GM1 at different pH: IgG control at pH 7.4 (▬), GM1 and IgG incubated and eluted pH 4.5 (○); GM1 and IgG incubated pH 4.5 and eluted pH 7.4 (▲) and GM1 and IgG incubated and eluted at pH 7.4 (γ). **B-** Pattern of chromatographic elution on Superdex 200® of IgG binding to GM1 at different temperatures. Samples of GM1 and IgG at pH 4.5 were incubated at temperatures indicated below for 30 min and then the elution was done at 25°C. IgG control (▬), GM1 and IgG at: 4°C (▲); 25°C (+); 37°C (γ) and 45°C (⊄). **C-** Pattern of chromatographic elution on Superdex 200® of IgG binding to pre-heated GM1at 55°C and then allowed to reach 25°C and incubated with IgG (γ) ; IgG and GM1 incubated at 45°C and eluted 25°C (⊄) and IgG control (▬). All samples were run at pH 4.5.

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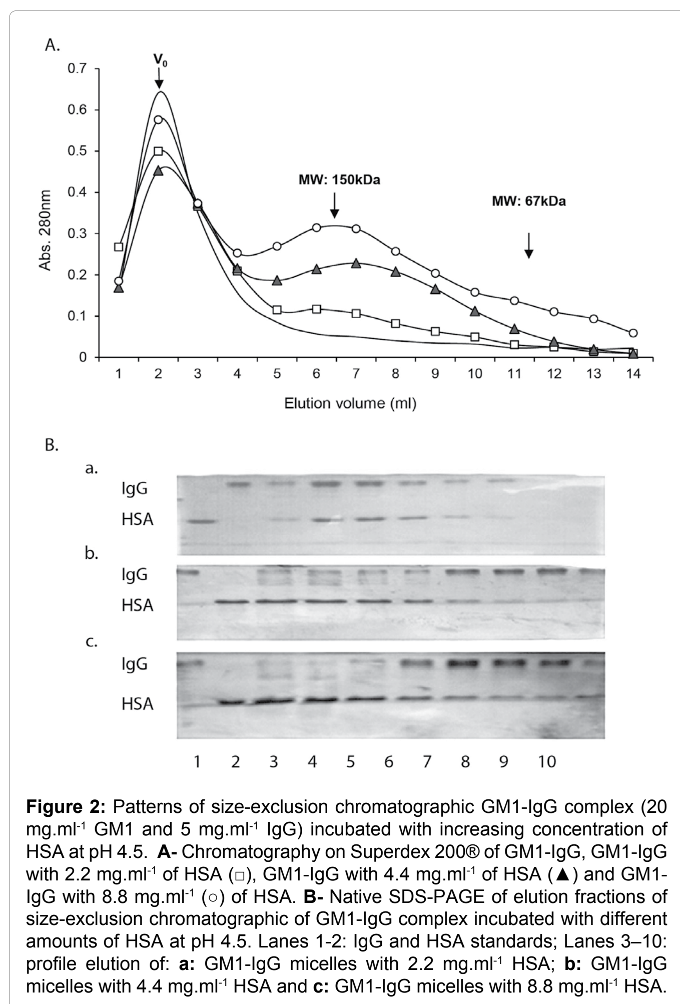

Figure 2: Patterns of size-exclusion chromatographic GM1-IgG complex (20 mg.ml^{-1} GM1 and 5 mg.ml^{-1} IgG) incubated with increasing concentration of HSA at pH 4.5. **A-** Chromatography on Superdex 200® of GM1-IgG, GM1-IgG with 2.2 mg.ml^{-1} of HSA (□), GM1-IgG with 4.4 mg.ml^{-1} of HSA (▲) and GM1-IgG with 8.8 mg.ml^{-1} (○) of HSA. **B-** Native SDS-PAGE of elution fractions of size-exclusion chromatographic of GM1-IgG complex incubated with different amounts of HSA at pH 4.5. Lanes 1-2: IgG and HSA standards; Lanes 3–10: profile elution of: **a:** GM1-IgG micelles with 2.2 mg.ml^{-1} HSA; **b:** GM1-IgG micelles with 4.4 mg.ml^{-1} HSA and **c:** GM1-IgG micelles with 8.8 mg.ml^{-1} HSA.

mg.ml^{-1} was not able to displaced the IgG such as describe above when both proteins were co incubated at same time since the complex GM1-IgG eluted in V_0, suggesting that under physiological conditions IgG would remain attached to the micelles.

As mentioned above, the binding of IgG to micelles is optimal after preincubating GM1 micelles at 55°C and allowed to reach room temperature before incubating with IgG at pH 4.5. Using this experimental condition, we evaluated the maximum binding capacity of IgG to a fixed concentration of GM1 micelles. The result revealed that GM1 reaches its maximum capability to bind IgG at GM1/IgG ratio 4/1 w/w (data not shown). Considering the apparent MW of the GM1 micelles which, when heated to 55°C, is 320 kDa [20,21] and the MW of the IgG, of 150 kDa, the complex would be formed by two antibody units per micelle of GM1.

Physicochemical characterization of GM1-IgG micelles

Here we characterize the physicochemical properties of these mixed micelles. Studies about particle diameter, polydispersity index, surface morphology and z potential were assessed. Table 1 shows the results of these studies of GM1-IgG in comparison with free GM1 micelles. The GM1-IgG micelles had a mean diameter in the range of 25.0 nm, while GM1 micelles shows only 11.9 nm, which shows that the presence of IgG on micellar surface increased the size about 100%. The polydispersity of different composition of GM1-IgG micelles (10/1 and 4/1 GM1/IgG weight /weight ratio) were very similar but with relatively high values of about 0.28 ± 0.09, however this result does not appear to be related to the amount of IgG on the surface. On the other hands, using transmission electron microscopy we also can visualize this size changes. The images of Figure 3A shows the particle size of GM1 micelles and in Figure 3B the changes observed in the GM1/IgG 4/1 weight /weight ratio that also show a longer dispersion interval.

Next we evaluate whether the presence of IgG in the surface of GM1 micelles induce changes on surface charge of final structure. Table 1 shows that the zeta potential values obtained for GM1-IgG in proportions of 10/1 and 4/1 w/w, were -10.6 ± 0.3 and -11.8 ± 1.2 respectively and -8.1 ± 1.0 for the micelles of GM1 alone. These results seems to indicate no substantial changes in zeta potential after the insertion of a high molecular weight protein like IgG into micelles, however, taking into account that GM1-IgG micelles were prepared from GM1 micelles preheated to 55 °C, that show a zeta potential of -23.6 ± 1.9, the zeta potential values of the complex would indicate a substantial molecular reorganization according to these results.

Topology of the IgG in the micellar structure

After demonstration of GM1-IgG interaction and the characterization of the physicochemical properties, we inquired about the final orientation of immunoglobulin in the micelle structure. To do this, we performed a proteolityc assay with pepsin, an enzyme which specifically cuts IgG separating Fab region from fragments of low molecular weight of Fc.

The results shows that pepsin on free IgG it produces the release of Fab fragment, however, under similar condition very little or none IgG was cleaved by pepsin from GM1-IgG complex, which suggest that the immunoglobulin would be bound to the micelle by the Fc region of the molecule, keeping the specific recognition of IgG (Figure 4).

Biological activity of antibodies associated with micelle surface

Here we evaluate whether the IgG incorporated in the GM1 micelles retain the biological activity by measurement the capability to

Table 1: Physicochemical characteristics of GM1 micelles and GM1-IgG modified micelles.

Parameters	Samples				
	IgG	GM_1	GM_1 (heated 55°C)	1-Oct GM_1/IgG w/w ratio	1-Apr GM_1/IgG w/w ratio
MD (nm)	6.4 ± 2	14.2 ± 3	11.9 ± 2	20.8 ± 2	25.1 ± 2
PI	0.16 ± 0.06	0.17 ± 0.08	0.11 ± 0.03	0.28 ± 0.09	0.28 ± 0.08
z (mV)	-7.8 ± 1.1	-8.1 ± 1.0	-23.6 ± 1.9	-10.6 ± 0.3	-11.8 ± 1.2

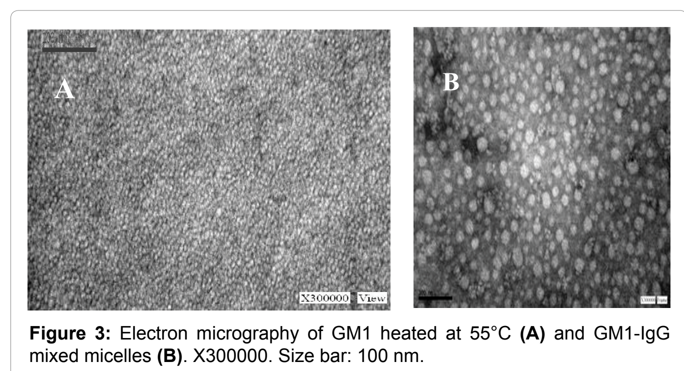

Figure 3: Electron micrography of GM1 heated at 55°C **(A)** and GM1-IgG mixed micelles **(B)**. X300000. Size bar: 100 nm.

recognize three different antigen species such as 1- soluble antigens, 2-cell surface antigens and 3- antigen expressed in sections of human tumor tissues.

For the first example, to analyze the recognition of soluble antigen, we used an anti-Rub IgG from a commercial kit of rubella virus adsorbed to the GM1 micelle. The biological activity of the antibody was determined by a chemiluminescence enzyme immunoassay. Table 2 shows that the purified complex of GM1-anti-Rubella virus antibodies retains their specific recognition ability against soluble antigens with a similar activity than control of anti-Rubella virus antibodies. Moreover, it was also observed a direct quantitative relationship between the biological activity and the amount of antibody adsorbed on the micelle. Nonspecific IgG antibodies bound to GM1 micelles were used as controls to rule out non-specific binding.

To evaluate the activity of GM1-antibodies against antigens located in the cellular surface we used a combination of three fluorescent monoclonal antibodies, anti-CD4-FITC /CD8-PE /CD3-PC5 (OptiClone), which were loaded onto GM1 and purified as GM1-IgG as mentioned before. The mixture was incubated with whole blood samples, after which red blood cells were lysed using a no-wash assay and finally the samples were analyzed by flow cytometry. The results indicate that the loaded of the immunoglobulins to the micelles does not affect their specific recognition property against antigens expressed in cells in suspension (Table 3).

On the hand the interaction of the GM1-antibodies with another class of cell surface antigen such as receptor were also evaluated. The specific receptors expressed on the cell surface was evaluated using anti-FR IgG, anti-CD44 IgG and anti-LDLR antibodies, which were absorbed to GM1 micelles and analyzed by colorimetric spectrophotometry. The results reveal no significant differences between the antibodies loaded to the micelles compared to the controls (Table 4). GM1 micelles alone and loaded with total human IgG were used as controls to confirm absence of nonspecific binding. All these results seem to prove that the incorporation of the antibodies into the micellar structure does not affect their functionality.

Finally we also studied the activity of anti-Her2 or anti-KI67 antibodies loaded in GM1 micelles by immune-histochemistry (IHC) to evaluate recognition of specific antigen expressed in sections of human tumor tissues. Immuno histochemical visualization of the antigen-antibody complex was performed by staining with a fluorochrome or enzyme, depending on the labeling of the antibody. Figure 5 shows that the GM1-antibodies are able to recognize their specific antigens on tissues, in this case the epidermal growth factor and the nuclear protein KI-67 in similar extension that control of free antibodies.

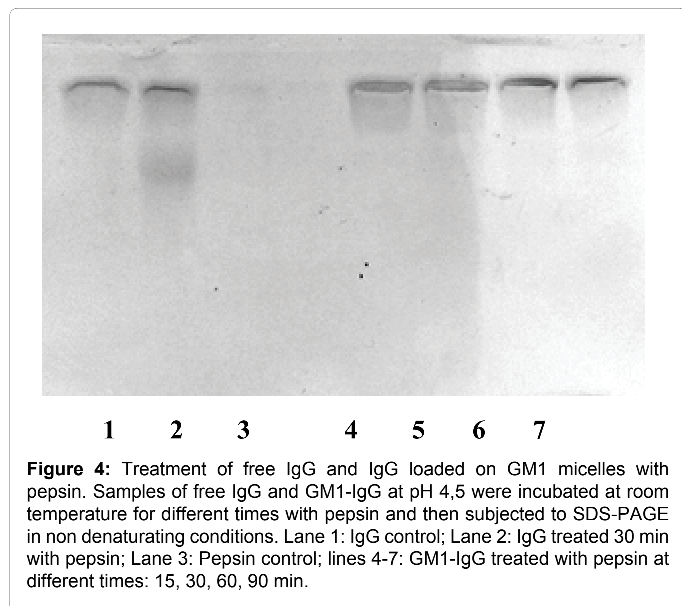

Figure 4: Treatment of free IgG and IgG loaded on GM1 micelles with pepsin. Samples of free IgG and GM1-IgG at pH 4,5 were incubated at room temperature for different times with pepsin and then subjected to SDS-PAGE in non denaturating conditions. Lane 1: IgG control; Lane 2: IgG treated 30 min with pepsin; Lane 3: Pepsin control; lines 4-7: GM1-IgG treated with pepsin at different times: 15, 30, 60, 90 min.

Effect of IgG in GM1 micelles on drugs encapsulation, release and cytotoxic activity of drugs

In previous reports we demonstrate that GM1 micelles were able to load hydrophobic or hydrophilic drugs such Ptx, Dtx, Doxo and AmB [12-13,17]. Here we evaluate capability of this GM1-IgG complex to discern whether this modification affects their drug loading capacity, drug release or the pharmacological activity.

We found that increasing the amount of IgG in the GM1-IgG complex produce a decreased in the amount of hydrophobic drug Ptx loaded in GM1-IgG micelles, Table 5 However, when the incorporation of the Ptx on GM1 occurs before the binding to IgG, the efficiency of Ptx encapsulation remain similar to that described above for pure GM1 micelles [12]. These results suggest that IgG could be located in external domains of GM1 micelles, which prevents subsequent entry of Ptx in the hydrophobic region of the micelles. By other hands, suggest that Ptx could be located deeply in the GM1 micelles as was proposed in previous report [12] a condition that not affect the binding of de IgG to GM1 micelles to form finally the GM1-Ptx-IgG complex.

The loaded of water soluble drug Doxorubicine into GM1-IgG micelles is substantially affected by the presence of IgG. At the GM1/IgG (4/1 w/w ratio) the saturating condition, the amount of Doxo that can be incorporated decreases four times with respect to its optimum capability to be loaded in free GM1 micelles (at molar ratio 5/1 GM1/Doxo). In this case, unlike what happens with Ptx, the load of increasing concentrations of Doxo into free GM1 micelles before adding IgG, produce a significantly decrease in the amount of IgG that binds to GM1-Doxo micelle. When GM1-Doxo complex is obtained at molar ratio of 5/1 GM1-Doxo, the maximun capability

Table 2: Test IMMULITE 1000. Quantitative measurement of IgG antibodies to rubella virus. Solid-phase, sequential chemiluminescent immunoassay for *in vitro* diagnostic use.

Sample	Biological Activity mUI/ml
10 µl anti-Rub antibody in PBS buffer	11.3 +/- 4.0
4 mg GM1 micelles + 10 µl anti-Rub antibody in PBS buffer	11.1 +/- 4.2
4 mg GM1 micelles + 30 µl anti-Rub antibody in PBS buffer	33.3 +/- 6.1

Table 3: Flow cytometry analysis of GM1 micelles pre-incubated with a three-color monoclonal antibody combination (OptiClone).

Monoclonal antibody	CD^{4+}	CD^{4+}	CD^{8+}	CD^{8+}	CD^{3+}	CD^{3+}
Sample	Counts/ mm^3	%	Counts/ mm^3	%	Counts/ mm^3	%
Control (+): 5 ul of Three-color Antibodies standards in Vf: 50 ul PBS buffer	2326	56	1039	25	3698	89
GM_1 micelles (1 mg) + 5ul of Three-color Antibodies standards in Vf: 50 ul PBS buffer	2410	58	977	23.5	3694	89
Control (-): GM1 micelles (1 mg) + 5 ul (Anti-Rabbit-FITC) Antibody in Vf: 50 ul PBS buffer	2	0	3	0	1	0

Table 4: Semi-quantitative colorimetric determination by absorbance at 490 nm of antibodies attached to the micelles against surface receptors expressed on *in vitro* cell lines HeLa, H1299, Hep-2 and B16. Anti-FR IgG: anti-folate receptor antibody; anti-CD44 IgG: anti-hyaluronic acid receptor antibody and anti-LDLR IgG: low density lipoprotein receptor antibody.

Cell Line	Hep-2				B16				H1299			
IgG (μg.ml^{-1})	5	10	15	20	5	10	15	20	5	10	15	20
Sample												
Anti-FR IgG	0.1	0.08	0.13	0.12	0.34	0.53	0.51	0.54	0.3	0.09	0.14	0.11
GM1/Anti-FR IgG	0.06	0.09	0.1	0.11	0.31	0.55	0.56	0.52	0.11	0.07	0.12	0.09
Anti-LDLR IgG	0.36	0.59	0.67	0.71	0.19	0.26	0.36	0.37	0.08	0.12	0.11	0.16
GM1/Anti-LDLR IgG	0.33	0.6	0.7	0.74	0.16	0.25	0.39	0.41	0.08	0.09	0.1	0.12
Anti-CD44 IgG	0.09	0.11	0.13	0.1	0.13	0.17	0.14	0.17	0.33	0.46	0.49	0.51
GM1/Anti-CD44 IgG	0.05	0.08	0.11	0.11	0.09	0.15	0.15	0.18	0.3	0.44	0.51	0.54
Total human IgG antibodies		0.13		0.11		0.09		0.13		0.09		0.11
GM1/Total human IgG antibodies		0.09		0.09		0.1		0.11		0.06		0.09

Table 5: Loading of Ptx into GM1-IgG micelles at different GM1-IgG weight ratios or into free GM1 micelles and then incubated with IgG at different GM1/IgG weight ratios. The loading of Ptx into GM1 and GM1-IgG were done at 20°C for 30 min, and the loading of IgG into GM1 or GM1-Ptx were done at 20°C for 1 h at pH 4.5.

Weight ratios GM1/IgG (w/w)	Ptx Soluble (%)	
	GM1-IgG-Ptx	GM1-Ptx-IgG
15/1	48	98
1-Oct	42	98
1-Aug	16	96
1-Apr	6	99
1-Feb	3	97

of GM1 to load Doxo [13], the percentage of IgG binding falls almost completely (data not shown). These results suggest a competitive relationship between Doxo and IgG by common sites within the micellar structure.

Studies of *in vitro* release and cytotoxicity activity of paclitaxel and doxorubicine loaded in GM1-IgG-drug complexes

Finally, we evaluate the cytotoxic effect of Ptx or Doxo present in this GM1-drug-IgG complexes and compare with previously report of GM1-Ptx [13], to determine if the presence of the immunoglobulin in the micelles produces any change in the biological activity *in vitro* of the loaded drug.

Paclitaxel was incorporated in two samples of GM1 at GM1-Ptx molar ratio 10:1, the maximun binding capability. Then, one sample was incubated with IgG (anti-CD44 IgG) in a ratio 4:1 to saturate GM1-Ptx to obtain GM1-Ptx-IgG complexes, and the citotxic effect of Ptx was evaluated on the H1299 cell line using GM1- Ptx -IgG $_{\text{inespecific,}}$ and GM1-Ptx as controls. The results showed that the cytotoxic activity of Ptx were similar in all cases (data not shown). Control of vehicle, GM1-IgG (anti-CD44), did not show toxic effects on the cellular monolayers (data not shown). Similar results were obtained when assays were performed with other specific antibodies in their respective susceptible cell lines (see materials and methods).

In the same way, assays performed with Doxo loaded on the different GM1-Doxo- IgGspecific formulations did not reveal significant differences in cell viability with respect to GM1- Doxo-$IgG_{\text{inespecific}}$ and GM1-Doxo. These results suggest that the presence of the antibody in the micellar structure does not modify the release and uptake of the drugs.

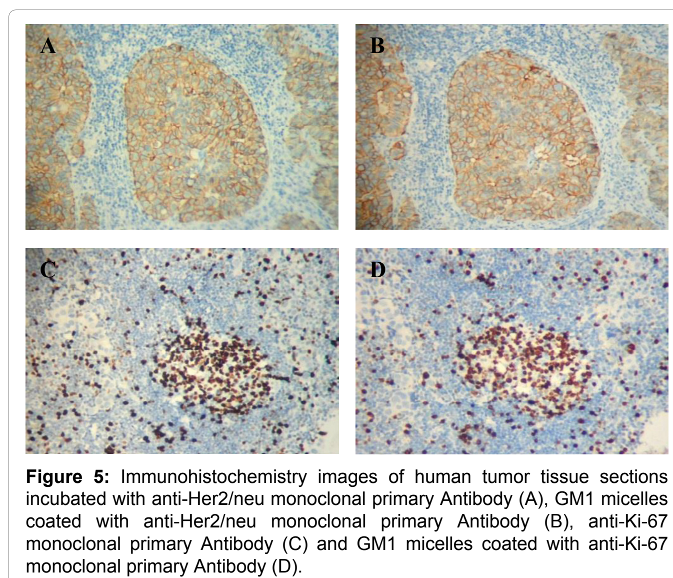

Figure 5: Immunohistochemistry images of human tumor tissue sections incubated with anti-Her2/neu monoclonal primary Antibody (A), GM1 micelles coated with anti-Her2/neu monoclonal primary Antibody (B), anti-Ki-67 monoclonal primary Antibody (C) and GM1 micelles coated with anti-Ki-67 monoclonal primary Antibody (D).

Discussion

The use of antibodies against selectively expressed antigens on the surface of cancer cells is a therapeutic strategy widely studied not only as directed immunotherapy but also for its potential to selectively target drugs or drug delivery systems [24,25].

In a previous report [12,13] we demonstrate that GM1 is able to load and release hydrophobic and hydrophilic oncological drugs in GM1-Drug complex. When this complex is incubated in presence of whole serum, there is only one protein, albumin, that spontaneously

bind in a non-covalent form to GM1-Drug-Albumin complex. In this report we describe a new procedure to load IgG into GM1 micelles to form GM1-IgG complex that under specific conditions also retain the capability of load drugs to generate the GM1-Drug-IgG.

This complex could be considered a delivery system for an immune-chemotherapeutic capable of combining the properties of drugs transported in the micelles [26,27] with the specific recognition capacity of the antibodies.

We demonstrate that the load of IgG into GM1 micelles was controlled by two principal factors such as the pH and temperature, conditions that affect IgG and GM1 structures to allow a non-covalent interaction,.

Changes in the pH from pH 7,4 to pH 4.5 produce significative increase in the interaction between GM1 micelles with IgG. This favorable interaction at pH 4.5 seems not related to changes in GM1 micelles since no modification were reported by GM1 but related to the conformational state that suffer IgG known as "reversible conformational denaturation", and especially for Fc fragment [15,16]. In this condition, there is a partial exposure of their hydrophobic residues of Fc involved in the interaction with micelles. On the other hand, the fact that addition of high salt concentrations in the incubation media, does not prevent interaction between micelles and antibodies reinforce the idea of the hydrophobic nature of the interaction.

Instead, the temperature was a critical factor for both molecules. When IgG and GM1 micelles were co-incubated from 4 to 45^{0}C, and moreover when free GM1 micelles were pre-heated at 55-60^{0}C, there is a clear increase in the capability of GM1 to load IgG.

The temperature shows two phases, until 45^{0}C both IgG and GM1 micelles seems to be modified to improve the interaction, but this temperature was the limit of IgG before an irreversible denaturation. However the thermal treatment at 55-60°C of free GM1 micelles before to incubation with antibodies produces the maximun GM1-IgG complex. In this case the effect is only for the conformational changes in the surface of GM1 micelles due to the dehydration of the polar groups of the ganglioside molecule that produce an increase of the hydrophobicity that undergoes the micellar structure at that temperature [20,21]. Under this experimental condition permit to avoids exposure of immunoglobulins to high temperatures, which compromise their functionality. After select the optimal interaction condition to obtain the more stable GM1-IgG complexes, pH 4,5 and heating GM1 micelles 55-60°C, the maximun ratio GM1-IgG was reached to 4/1 (w/w). Moreover under this condition the spontaneously binding of albumin to GM1 micelles mentioned above, does not occurs.

The characterization of the size and morphology of this GM1-IgG complexes by DLS and TEM showed a size considerably higher than that of free GM1 micelles, 25.1 nm and 11.9 nm respectively but with a similar rounded morphology, suggesting that IgG do not affect the geometry of complex. It is interesting to remark the results obtained with pepsin, a well-known protease that cleaves immunoglobulins in Fc and Fab fragments, the enzyme causes the release of Fab fragment in contact with free IgG, however, when exposed under similar conditions to the IgG bound to GM1-IgG complex, pepsin shows little activity on the immunoglobulin (Figure 4). Although it cannot be ruled out that this behavior respond to steric impediments that prevent the enzyme from accessing its target site, this fact may also be a consequence of the spatial location of the immunoglobulin in the micelle, with its Fc region protected inside.

On the other hand, we also evaluate whether antibodies retain their antigenic recognition ability after its physical association to GM1 micelles. The assays demonstrate that different specific monoclonal antibodies loaded onto GM1 micelles were able to recognize fragments of soluble antigens, or located in the cell surface such receptors or intracellular antigen used for histochemical. This study suggest that this GM1-IgG complex do not show any sterical impediment of IgG to react with different antigen. Both results are in agreement with the possibility that under this specific condition the insertion of IgG into GM1 micelles occurs through Fc leaving the biological active Fab fragment.

The results clearly demonstrate that GM1-IgG complexes are able to overcome two of the main drawbacks of using a non-covalent binding method, one referring to the displacement of antibodies by plasma proteins and the other to the loss of functionality due to a bad orientation of antibodies during binding to the micelle.

It is well known that antibodies are therapeutically useful molecules by themselves, capable of producing direct pharmacological effects, such as blocking the interaction of a receptor with a ligand, interfering with a multimerization process or activating receptor internalization or apoptosis of target cells with numerous intermediate cell modulation options [9,28]. The incorporation of antibodies into micellar structure could be considered as a strategy for improvement in the pharmacokinetic and biodistribution properties of the antibodies, which often do not achieve the effectiveness sought by low plasma half-life or because they do not reach the tumor tissues; variables in which GM1 micelles could offer substantial advantages such as crossing the blood-brain barrier and entering the brain [29]. This encapsulation of IgG into micelles of GM1 also could be used for improving the biodistribution by facilitating selective tumor accumulation through enhanced permeability and retention (EPR) effect [28-30].

On the other hand previously reports by owner and others shows that GM1 micelles were able to load and release oncological drugs such as Paclitaxel and Doxorubicine and moreover that the complex were able to cross brain blood barrier [12,13,29]. In these experimental conditions we also shown that GM1-IgG complex retain the capability of load drugs such as Ptx or Doxo, although it is necessary to follow a specific sequence in order to obtain stable GM1-IgG -drug complexes. For Paclitaxel, it must be incorporated into micelles before incubation with the antibodies, because the presence of IgG in the micellar structure prevents its incorporation. Following the order mentioned, Ptx can be loaded up to a GM1/Ptx molar ratio of 5/1 that represent is the maximum Ptx loaded capability in GM1 micelles alone, then IgG also can be loaded also at maximun weight/weight ratio capability GM1-IgG 4:1. These results suggest that antibodies occupy more external domains in the micelles structure which are critical for Ptx insertion.

With Doxo, its incorporation depends on the amount of IgG bound to the micelle and vice versa. At GM1-IgG 4/1 ratios (w/w) complex the amount of Doxo loaded is lower than 25% as was observed for GM1 alones, and when the amount of Doxo previously loaded onto GM1 micelles increase, the encapsulation IgG decrease until to be not detectable. This result suggesting that both molecules, IgG and Doxo, share common binding domains in the micelles.

The results of *in vitro* cytotoxicity assays revealed that drugs into GM1-$_{\text{Specific}}$ IgG micelles show a similar biological effect to drugs loaded into GM1-$_{\text{Non-specific}}$ IgG-drug and into GM1 micelles alone. This result demonstrates first, that the presence of the immunoglobulin in the

micellar structure does not affect the release of the drugs contained therein. On the other hand, was unexpected the similar toxicity observed for GM1-Drug loaded with specific or unspecific IgG. Although some kind of difference could be expected, mainly in the kinetics of the activity so, there are many basic points between the *in vitro* and the *in vivo* system that can be considered in the analysis. First, the use of antibodies bound either a nanocarrier or one drug itself is based on the potential selectivity of the immunoglobulin against the different cell types in the organism, which is a very different for the *in vitro* condition, where the assays are performed with pure cellular monolayers of a single cell type in each case. Likewise, there is a great variety of dynamic and structural factors that are not reflected in the *in vitro* model. All this, added to the high efficiency of the micellar system itself for the *in vitro* transport and drug delivery. The rapid fusion or interaction of GM1 micelles with cells may be masking antibody participation, together with the low internalization capacity of immunoglobulins.

Most monoclonal antibodies were developed to fulfill effector immune functions and not to internalize. In an active targeting system, the antibodies are mainly used as "hooks" to facilitate the binding of the nanotransporter to the cell expressing the ligands, increasing retention at the site of interest.

This report opens the possibility to incorporation of antibodies with different targets of action in a same nanocarrier that contain oncological drugs as a new alternative for different therapeutic purposes.

Conclusions

The results demonstrate the ability of GM1 micelles to interact and incorporate IgG into their structure, maintaining their ability to charge drugs with different physicochemical characteristics. In summary, the results described show that:

- IgG antibodies can be incorporated into GM1 micelles in a stable, simple and efficient manner.
- Incorporated antibody maintains its biological activity of recognition of its specific antigen.
- GM1 micelles modified with IgG maintain their capacity to carry drugs like Ptx and Doxo, but their preparation requires following an appropriate order to obtain the maximum load capacity.
- The GM1-IgG -drug model can be proposed as a dual or multifunctional system, in which the action of several antibodies is combined with the transport of drugs with the potential to be used for different therapeutic purposes.

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