3′-Sialyllactose neoglycoconjugates are not able to provide an immunological response against N-acetyl GM_3 ganglioside in mice and chickens

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INVESTIGACIÓN

ABSTRACT

Five different neoglycoconjugates containing 3'-sialyllactose or related structures linked covalently to Human Serum Albumin (HSA) were prepared as immunogens in order to elicit an immune response crossreacting with GM_3. ELISA, HPTLC-ELISA and inhibition experiments were used to test the sera of immunised animals against GM_3 and to a series of 3'-sialyllactose derivatives coupled to casein or polyacrylamide. In general, the neoglycoconjugates induced a moderate to strong immune response against the oligosaccharide, however the sera of immunised animals did not recognise the ganglioside GM_3.

Keywords: GM_3 ganglioside, 3´-sialyllactose, neoglycoconjugates, antibodies, ELISA

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RESUMEN

Neoglicoconjugados de la 3´-sialillactosa no son capaces de provocar una respuesta inmune contra el ganglió sido N-acetilGM_3 en ratones y pollos. Con el objetivo de preparar inmunógenos capaces de provocar una respuesta inmune contra el ganglió sido GM_3, se prepararon cinco neoglicoconjugados que contenían el trisacárido 3´sialillactosa o trisacáridos de estructura similar enlazados covalentemente a la albúmina sérica humana (HSA). Para verificar lo anterior, los sueros de los animales inmunizados con los neoglicoconjugados fueron analizados contra GM_3 y una serie de derivados de 3´sialillactosa acoplados a caseina o poliacrilamida, utilizando diferentes técnicas inmunológicas: ELISA, HPTLC-ELISA y experimentos de inhibición. De manera general, los neoglicoconjugados provocaron una respuesta inmune de moderada a fuerte contra el oligosacarido 3´sialillactosa, sin embargo el suero de los animales inmunizados no fue capaz de reconocer el ganglió sido GM_3.

Palabras claves: ganglió sido GM_3, 3´-sialillactosa, neoglicoconjugados, anticuerpos, ELISA

*I*ntroduction

Gangliosides are natural components of the plasma membranes. The ganglioside hydrophilic oligosaccharide chain is oriented towards the extracellular environment and has long been assumed to be involved in cell-cell recognition and cell-cell adhesion as well as in the interaction with hormones, lectins, viruses, interferon, bacterial toxins and the modulation of certain growth factors [1, 6]. Its abnormal over expression in some malignant tissues as compared to the normal ones has been considered by several groups of researchers as the basis for active specific immunotherapy approaches to destroy these tumors [7, 10].

GM_3 is by far the most abundant ganglioside in the majority of human tumor tissues and therefore can be considered as an attractive option for active specific immunotherapy of tumors.

Gangliosides -as most carbohydrates- are T-independent antigens and poor immunogens. Hence, the need to develop strategies for the covalent coupling of gangliosides to carrier proteins having T-helper cell epitopes able to promote an efficient, T-dependent, IgG isotype, booste immunological response. One of these approaches could be the covalent binding of 3'-sialyllactose (the oligosaccharide component of GM_3 ganglioside) to immunogenic proteins.

Ganglioside antibodies can be produced in several ways e.g. injection of its micelles and methylated bovine serum albumin [11] or by injection of gangliosides incorporated to hydrophobic proteins [12]. However, there is no evidence in the literature on the feasibility of obtaining antiganglioside antibodies by using neoglycoconjugates containing the oligosaccharide moiety of gangliosides as immunogens. This approach could provide an easy and relatively unexpensive way to obtain antibodies against the less abundant gangliosides based on the fact that the synthesis of oligosaccharides is less complex and cheaper than the complete ganglioside synthesis. This argument is also valid for the production of large amounts of immunogens used as anticancer vaccines in active specific immunotherapy approaches against tumor-containing gangliosides.

Several attempts to couple 3'-sialyllactose to proteins using different spacer arms have been made [13, 15], and the neoglycoconjugates have bean used in immunization experiments. In all cases, strong humoral immune responses were obtained against oligosaccharide moiety but the reactivity of the sera against the native ganglioside was not measured [13, 14] or was weakly positive in only one case [15].

1. Hakomori S. Glycosphingolipids in cellular interaction, differentiation and oncogenesis. Ann Rev Biochem 1981; 50: 733-64.

2. Ledeen RW, Yu RK. Gangliosides: structure, isolation and analysis. Methods Enzymol 1982; 83:139-91.

3. Stultz CLM, Sweeley C, Macher BA. Glycosphingolipids: structure, biological source, and properties. Methods Enzymol 1989;179:167-214.

4. Karlsson KA. Animal glycosphingolipids as membrane attachment sites for bacteria (1989). Annu Rev Biochem. 1989; 58: 309-50.

5. Paulson JC. Interactions of animal viruses with cell surface receptors. In: Conn M editor. The Receptors Vol. II , Academic Press, New York, 1985: p.131-219

6. Bremer EG, Schlessinger J, Hakomori SI. Ganglioside-mediated modulation of cell growth. Specific effect of GM_3 on tyrosine phosphorylation of the epidermal growth factor. J. Biol. Chem. 1986; 261: 2434-440.

7. Hakomori SI. Possible functions of tumor associated antigens. Current Opinion in Immunology. 1991; 3:646-53.

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This paper describes the preparation of five different neoglycoconjugates based on the coupling of natural or synthetic 3'-sialyllactose derivatives to Human Serum Albumin (HSA) and their use as immunogens in mice and chickens for trying to obtain specific antiGM_3 immunological response.

Material and methods

Chemicals

Silicagel-precoated thin layer chromatographic plates were obtained from Merck (Darmstadt, Germany). Biogel P-2 gel (fine, 45-90 μM) was purchased from Bio-Rad (Richmond, CA, USA). Sephadex G-50 and G-25 were supplied by Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade and were supplied by Sigma (St. Louis, USA).

Analytical methods

Sialic acid was measured with the HCl-resorcinol assay [16, 17] using pure NeuAc as the standard. Bio-Rad protein assay was used for determining protein content with human serum albumin (HSA) as the reference. Total amino groups were assayed with 2, 4, 6,-trinitrobenzenesulphonic acid (TNBS) [18] reagent. HPTLC was performed on Silicagel-precoated thin layer chromatographic plates (Merck, Darmstadt, Germany). The following solvent systems were used: ethanol-25% ammonia solution 80:20 (by volume, solvent 1) and pyridine-ethyl acetate-acetic acid-water 6:3:1:2.5 (by volume, solvent 2). The spots were visualized with ninhydrin, orcinol and basic potassium permanganate reagents.

Size-exclusion chromatography was performed by connecting the columns to a system composed of a P1 peristaltic pump (Pharmacia-LKB, Sweden), a Knauer differential refractomer (type 198.00, Germany) or a Pharmacia-LKB Uvicord SD fitted with a 206 nm filter, a Pharmacia-LKB Helifrac fraction collector and a REC 102 Pharmacia-LKB recorder.

Oligosaccharides

3'-sialyllactose **1** and disialyllactose were obtained from fresh harvested cow colostrum [19]. GM_3 ganglioside was obtained from dog erythrocytes using a modification of Folch´s method [20] which consisted of the substitution of the partition step by a mild base treatment followed by a solvent extraction with n-hexane. The 3'-sialyllactose derivative with azido-type spacer arm was obtained following a modification of the published procedures [21].

Coupling of 3'-sialyllactose derivatives to Human Serum Albumin (HSA)

The glycoconjugates **2**,**3**,**6**,**8** and **11** containing 3'-sialyllactose and HSA designed to be used as immunogens were prepared from 3'-sialyllactose or its derivatives using coupling procedures that will be described for each compound. The coupling degree of the conjugates was determined by the quantification of protein (BioRad test) and sialic acid (HCl-resorcinol reagent) and is expressed as moles of oligosaccharide per mole of protein.

Synthesis of glycoconjugate **2**. Reductive amination of 3'-sialyllactose **1** to HSA

The procedure used was that of Gray [22]. Briefly, compound 1 (16.5 mg) was dissolved in borate buffer pH 8.7 and added to a 1 mL (10 mg/mL) solution of the protein. Sodium cyanoborohydride (15 mg) and several drops of toluene were then added and the solution was allowed to react for 24 hours at 4 °C. After neutralization with 0.1M AcOH, the solution was desalted on a Sephadex G-50 column and the fractions containing the glycoconjugate 2 positive for both, the protein (absorption at 280 nm) and the oligosaccharide (resorcinol test) were pooled and freeze-dried.

Synthesis of glycoconjugate **3**. Carbodiimide mediated amidation of the carboxyl group of sialic acid

Several glycoconjugates were obtained by direct amidation of the carboxyl group of sialic acid varying the sialyloligosaccharide/HSA molar ratio and reaction temperature. In a typical reaction, 22 mg of compound **1** were dissolved in 1 mL of 0.2 M NaCl solution (pH 4.75) and 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC) was added until a 0.2 M concentration was reached. The pH was brought to 4.75 by adding 0.1 M HCl, then 10 mg of HSA were added. The solution was stirred for 24 hours at room temperature. Salts and the excess reagents were removed by Sephadex G-50 gel filtration giving the glycoconjugate with structure **3**.

Synthesis of glycoconjugate **6**. Carbodiimide mediated amidation with a spacer arm

A partially modified procedure of Stoll et al was used [23]. Briefly, in a reaction vial sealed with a teflon-lined cap, 3´-sialyllactose **1** (13 mg, 14 μmol) was dissolved in 4.8 mL of MeOH. The ACA (18.4 mg, 140 μmol) was dissolved in 0.2 mL of distilled water and both solutions were mixed when stirring at 50 °C for 2 hours. $NaBH_3CN$ (7 mg) in MeOH (500 μL) was then added and the mixture was again heated at 50 °C. Samples (5 μL) were withdrawn at intervals (8 h) and tested on HPTLC plates. Plates were developed with solvent 2 and developed with orcinol, ninhydrin and basic $KMnO_4$ reagents. The complete reaction was obtained after 40 h. The reaction mixture was dried under argon and an excess of $NaBH_3CN$ was decomposed with acetic acid. Desalting the modified oligosaccharide was done on a Biogel P-2 column (1.5 x 100 cm) using water as the eluent and the fractions containing the modified oligosaccharide **4**, pooled and freeze-dried.

Lactonization of compound **4** was performed according to the procedure of Yu et al [24]. Briefly, 20 mg of **4** exhaustively dried under heating and vacuum, were dissolved in glacial AcOH (10 mL) and the reaction was allowed to proceed for 4 days at room temperature. The extent of lactonization was followed by HPTLC in solvent 2. When more than 90% of the lactonization was achieved, the mixture was freeze-dried and used directly for coupling to HSA and casein.

In a typical reaction, HSA (10 mg) was dissolved in 0.2 M NaCl (pH 4.75) and 18.3 mg of lactone **5** (3 fold molar excess for free aminolysyl group in HSA) were added. Then, 1-ethyl-3-(3-dimethyl-aminopropyl)-

8. Portoukalian J, Carrel S, Dore JF, Rumke P. Humoral immune response in disease-free advanced melanoma patients after vaccination with melanoma associated gangliosides. EORTC Cooperative Melanoma Group. Int. J. Cancer 1991; 49: 893-99.

9. Livingston PO, Ragupathi G. Carbohydrates vaccines that induce antibodies against cancer. 1 Rationale. Cancer Immunol. Immunother: 1997; 45 :1-9.

10. Livingston PO, Zhang S, Lloyd KO. Carbohydrates vaccines that induce antibodies against cancer. 2 Previous experience and future plans. Cancer Immunol. Immunother. 1997; 45: 10-19.

11. Nagai Y, Osawa T. Production of high titer antisera against sialoglycosphingolipids and their characterization using sensitized liposome. Jpn. J. Exp. Med. 1974; 44: 451-64.

12. Estevez F, Carr A, Solorzano L, Valiente O, Mesa C, Barroso O, Sierra GV, Fernandez LE. Enhancement of the immune response to poorly immunogenic gangliosides after incorporation into very small size proteoliposomes (VSSP). Vaccine 2000; 18: 190-97.

13. Smith DF, Gingsburg V. Antibodies against sialyloligosaccharides coupled to protein. J. Biol. Chem.1980; 255: 55-9.

14. Zou W, Abraham M, Gilbert M, Akarchuk WW, Jennings H J. Allylmalonamide as a bivalent linker: Synthesis of biantennary GM_3-saccharide-Keyhole limpet hemocyanin glycoconjugate and the immune response in mice. Glycoconj. J. 1999; 16: 507-15.

15. Ding K, Ekberg T, Zeuthen J, Teneberg S, Karlsson KA, Rosen A. Monoclonal antibody against a lactose epitope of glycosphingolipids binds to melanoma tumor cells. Glycoconj. J. 1993; 10: 395-405.

16. Svennerholm L. Quantitative estimation of sialic acids. II A colorimetric resorcinol-hydrochloric acid method. Biochem. Biophys. Acta ,1957; 4: 604-11.

17. Miettinen J, Takki-Luukkainen. Use of butyl acetate in determination of sialic acid. Acta Chem. Scand. 1959; 13: 856-8.

18. Fields R. The measurement of amino groups in proteins and peptides. Biochem. J. 1971; 124: 581-90.

19. Laferriere CA, Roy R. Isolation, Modification and Conjugation of sialyl a(2®3)-Lactose. Methods Enzymol. 1994; 242: 102-08.

20. Folch PJ, Arsove S, Meath JA. Isolation of brain strandin, a new type of large molecular tissue component. J. Biol. Chem.1951; 191: 819-31.

21. Castro Palomino J C, Schmidt R, unpublished results.

22. Gray GR. Antibodies to carbohydrates: Preparation of antigens by coupling carbohydrates to proteins by reductive amination with cyanoborohydride. Methods Enzymol.1978; 50: 155-60.

23. Stoll MS, Mizouchi T, Childs R A, Feizi T. Improved method for the construction of neoglycolipids having antigenic and lectin-binding activities, from reducing oligosaccaharides. Biochem. J. 1988; 256: 661-4.

carbodiimide hydrochloride (EDC) was added until a 0.2 M concentration was reached. The pH was brought to 4.75 by adding 0.1M HCl and the mixture was stirred for 24 hours at room temperature or 55 °C. The solution was desalted in Sephadex G-50 and the fractions containing conjugate **6**, were pooled and freeze-dried. The coupling degree was determined as described before for other conjugates.

Synthesis of glycoconjugate **8**. Conjugation through the disulfide bond with the heterobifunctional linker N-succinimidyl 3-(2 pyridyldithio) propionate (SPDP)

3´-sialyllactose **1** was reductively aminated according to the procedure of Wiegandt and Ziegler [25]. The sialyllactose glycosylamine derivative was then coupled to the SPDP reagent as described by Carlsson et al [26] using 3 fold molar excess of the SPDP reagent (40 mm in EtOH) in 0.1 m Na_3PO_4, 0.1 m NaCl, pH 7.5, for 4 hours. Compound **7** was purified in a Biogel P2 column and freeze-dried.

The protein was also coupled to SPDP under the same conditions as described above and the new disulphur bridges were reduced with 25 mM dithiothreitol (DTT). The reduced protein was combined immediately with **7** (3 fold molar excess) and allowed to react at room temperature for 24 hours. The neoglycoconjugate **8** was purified through a Sephadex G-25 column.

Synthesis of glycoconjugate **11**. Coupling of synthetic 3´-sialyllactose derivative **10** to HSA

A solution of N-hydroxysuccinimidyl dithiopropionate (3.62 mg, 9 µmol) in DMF (0.1 mL) was added to a solution of HSA (20 mg, 0.3 µmol) in PBS (pH 8 with EDTA 5 mM, 4 mL). After 2 h, dithiotreitol (15.4 mg, 2.5 mM) was added under an N_2 atmosphere and the mixture was stirred at 4 °C for 1 h. The resulting solution was dyafiltered at pH 7.4 using N_2 as a pressure source (polysulfone membrane, 10 000 Da cutoff). The protein content was quantified as above and SH content was estimated by Ellman´s method [27]. Usually 18-23 moles of -SH per mole of HSA were obtained.

The oligosaccharide derivative **10** was obtained from **9** [21] according to our previously described procedure [28]. Compound **10** (3.1 mg, 3.5 µmol) in PBS (pH 7.4, 0.5 mL) was added under an N_2 atmosphere, to a solution of HSA-$(SH)_{18}$ (14.5 mg) in PBS (pH 7.4 with EDTA 5 mM, 3.5 mL). The solution was stirred at 4 °C for 24 h and dyafiltered over a polysulfone membrane (10 000 Da cutoff) against PBS (pH 7.4).The glycoconjugate **11** was analysed as above. Overall yields over 50% based on the oligosaccharide **10** are usually obtained by this procedure.

Synthesis of reagents for the study of the immune response

Casein (sodium salt, 200 mg) and EDC (500 mg) were dissolved in 0.2 M NaCl, pH 4.75 solution and ethylendiamine (EDA, 0.2 mL) was then added while stirring and the pH was adjusted to 4.75 by adding 6 M HCl, 200 mg of casein were dissolved in 2 mL of 0.2 M NaCl (pH 4.75 solution) and added to the previous solution. The solution was gently stirred overnight at room temperature, quenched by the addition of 0.5 M NaAcO and purified using a Sephadex G-25 column. Total amino groups were assayed with 2,4,6,-trinitrobenzenesulphonic acid (TNBS).

Lactose, 3´-sialyllactose, disialyllactose and glucose (3 fold molar excess per mol of amino group in the casein-EDA protein) were then coupled to casein-EDA by a reductive amination reaction with $NaBH_3CN$. Briefly, 10 mg of casein-EDA conjugate (20 µmol amine groups) were dissolved in 2 mL of borate buffer solution (pH 8.7) and 38 mg of 3'-sialyllactose (60 µmol) and few drops of toluene were added. The solution was heated with magnetic stirring at 55 °C and 10 mg of $NaBH_3CN$ were added. After 2 hours, 10 mg of $NaBH_3CN$ were added again and the solution was allowed to react overnight. Casein-EDA-3´-sialyllactose **14**, casein-EDA-disialyllactose **15**, casein-EDA-lactitol **12** and casein-EDA-sorbitol **13** were purified by a Sephadex G-25 column using water as the eluent. Two mL fractions were collected and each fraction was assayed in a HPTLC experiment using solvent 1 as eluent. The spots were visualised with nynhydrin and basic $KMnO_4$ reagents. The fractions containing the glycoconjugate were pooled and freeze-dried. The coupling degree was determined indirectly by calculating the difference of total amine groups before and after conjugation (the average of three independent determinations was used) and expressed as nmoles of hapten per mg of casein.

The p-nitrophenyl polyacrylate glycopolymers of 3´-sialyllactose **17**, lactose **16** and N-acetylneuraminic acid **18** were synthesised following a published procedure [29] starting from p-nitrophenyl polyacrylate and the corresponding oligosaccharide derivatives with a spacer containing an amino function at the terminal position [21].

ELISA experiments

Antibodies were measured by enzyme-linked immunosorbent assay (ELISA), in 96-well high-binding immunoplates (Costar, Cambridge, USA). Wells were coated with haptens-casein glycoconjugates **12-15** (0.16 nmol hapten/well) or with polyacrylate **16-18** at 5µg/mL in 50 mM carbonate-bicarbonate coating buffer pH 9.6 and incubated overnight at 4 °C. The plates were blocked with 1% gelatin (for chicken sera) or 3% skimmed milk (for mice sera) in tris-HCl buffer (50 mM, pH 7.8) for 2 hours at room temperature. Appropriate dilutions of serum samples were added to the plates and incubated at 37 °C for two hours. After incubation, plates were extensively washed with phosphate buffer (pH 7.3) containing 0.05% Tween-20. The second antibody consisting of alkaline phosphatase conjugated antichicken IgG (Sigma, St Louis, USA) diluted 1/1000 or biotin conjugated goat antimouse IgG+IgM (Jackson, West Grove, Pa., USA) diluted 1/5000 was added to the plates and incubated for one hour at 37 °C. After incubation, plates were washed again, and for mice assays, incubation with alkaline phosphatase conjugated streptavidin (1/1000) at 37 °C for 90 min was performed. The plates were then washed and visualisation of spots was achieved by adding p-nitrophenylphosphate substrate (1 mg/mL) dissolved in 1 M diethanolamine buffer, pH 9.8, plus 1 mM $MgCl_2$. Absorbance was read at 405 nm using an automatic microplate reader.

Reactivity of sera samples to GM_3 ganglioside was measured by ELISA experiments as previously reported [30]. GM_3 (0.16 nmol/well) in 50 µl of methanol was

24. Yu RK, Koerner AW, Ando S, Yohe AC, Prestegard JH. High-resolution proton NMR study of gangliosides. III Elucidation of the structure of ganglioside GM3 lactone. J. Biochem (Tokyo) 1985; 98:1367-73.

25. Wiegandt H, Ziegler W. The use of reductaminated sugars for the preparation of oligosaccharide conjugates. I Synthetic glycolipids containing glycosphingolipid-derived oligosaccharides. Hoppe-Seylers Z. Physiol. Chem.1974; 355: 11-18.

26. Carlsson J, Drevin H, Axen R. Protein thiolation and reversible protein-protein conjugation. N-succinimidyl 3-(2-pyridyldithio) propionate, a new heterobifunctional reagent. Biochem. J. 1978; 173: 723-37.

27. Ellman GL.Tissue sulfhydryl groups. Arch. Biochem. Biophys.1959; 82: 70-7.

28. Fernandez-Santana V, Gonzalez Lio R, Sarracent-Perez J, Verez-Bencomo V. Conjugation of 5-azido-3-oxapentyl glycosides with thiolated proteins through the use of thiophilic derivatives. Conjugation of 5-azido-3-oxapentyl glycosides with thiolated proteins through the use of thiophilic derivatives. Glycoconjugate J. 1998; 15: 549-53.

29. Bovin NV, Korchagina TV, Zemlyanukhina S, Byramova NE, Galanina OE, Zemlyakov AE, Ivanov AE, Zubov VP, Mochalova LV. Synthesis of polymeric neoglycoconjugates based on N-substituted polyacrylamides. Glycoconjugate J. 1993; 10: 142-151.

30. Karlsson KA. Animal glycosphingolipids as membrane attachment sites for bacteria. Annu Rev Biochem.1989; 58: 309-50.

dried in 96-well Polysorp immunoplates (Nunc, Roskilde, Denmark) for one hour at 37 °C. The subsequent steps were performed as described above.

Inhibition assays

The specificity of serum binding was evaluated in ELISA experiments. ELISA plates (Costar) were coated with GM_3, **14** (0,16 nmol) or **17** as described above. Serum samples (200 µL) at different dilutions were incubated with the inhibitor hapten (200 µL, concentrations 10^{-8} M-$4x10^{-5}$ M) overnight at 4 °C. The subsequent steps of the ELISA procedure were performed as described above.

High performance thin-layer chromatography and enzyme immunostaining HPTLC-ELISA

HPTLC-ELISA experiments were conducted on glass and alumina backed plates obtained from Merck (Darmstad, Germany). GM_3 ganglioside (2 µg) was developed in HPTLC plates using chloroform/methanol/aqueous 0.2% $CaCl_2$ (50:40:10). Chemical staining was achieved by using the orcinol reagent. Immunostaining on HPTLC plates was performed according to the method of Magnani [31]. Briefly, after spotting the gangliosides, HPTLC plates were dipped into a solution of 0.5% (w/v) polyisobutylmethacrylate in hexane/chloroform (1:3) for 1 min. After drying, the plates were soaked in 3% skimmed milk in TBS. The plates were then covered with a solution of the mice and chickens sera (1/80 dilution) and incubated overnight at 4 °C. After washing several times with PBS, the plates were incubated with alkaline phosphatase conjugated goat antimouse IgG+IgM in 3% skimmed milk in TBS for 3 hours at room temperature and after washing with PBS, 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) was added and the plates were incubated at 37 °C in an oven.

Immunization

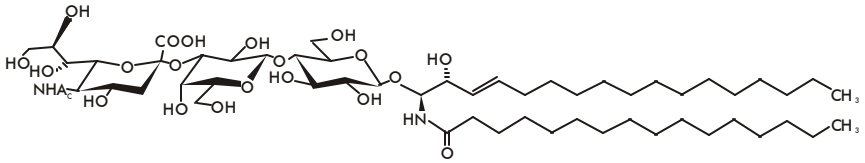
BALB/C, 7-8 week-old female mice (10 animals per group) and 10-12 week-old outbred chickens, line B4 (3 animals per group) were immunised intramuscularly. Each dose contained 10 µg of hapten in a final volume of 50 µL of saline solution. Adjuvant Montanide ISA 51 (50 µL per dose) was mixed vigorously with the immunogen solution immediately before the immunisation. Animals were boosted on days 14, 28 and 42, bled on the same days with a final bleeding on day 56. Sera were stored at –20 °C.

Statistical Analysis

Microsoft Excel and Microsoft Statistica were used for the analysis of variances and multiple ranges of Duncan´s test of the variables with significance assessed at the $P<0.05$ level.

Results and Discussion

Ganglioside GM_3 (Scheme 1) seems to be a good target for an active specific immunotherapy approach based on the evidence raised experimentally that several anti-GM_3 vaccine candidates are immunogenic and induce a specific, potent antitumor response against experimental tumor-bearing mice in which GM_3 ganglioside is expressed [32, 34, 12]. However, the use of glycolipids such as gangliosides as targets for active immunotherapy may have certain drawbacks. It is known that gangliosides (mainly GM_3) may exert immunosuppresive effects when shed from tumor cells [35, 38] or can down-regulate the response of T-cells to IL-2 in human melanomas [39].



Scheme 1

In our search for a vaccine candidate for active specific immunotherapy of GM_3 expressing tumors, we explore the ability of several conjugates formed by the covalent coupling of the GM_3 oligosaccharide to the immunogenic protein HSA, in promoting an antibody immunological response to GM_3. Several coupling methods in which the structure of the whole oligosaccharide was slightly modified and others in which it was preserved were used trying to understand the influence of the antigen structure in the capability to elicit specific immune responses. A second type of glycoconjugates **12-18** designed to be used for the evaluation of the immune response contained either casein or polyacrylamide.

Scheme 1

Synthesis of HSA-glycoconjugates. The synthesis of HSA-glycoconjugates is outlined in Scheme 2.

Glycoconjugates **2** and **3** were obtained by a direct coupling of 3'-sialyllactose **1** to HSA using the two available active functional groups; the terminal glucose free hemiacetal and the sialic acid carboxilic function.

In glycoconjugates **6**, **8** and **11**, a spacer arm was first introduced. This type of approach is usually more complex and specifically in **11** it allows us to preserve the terminal oligosaccharide structure intact rendering it more available for biological recognition.

Direct coupling of the trisaccharide **1** to HSA

Direct reductive amination of compound **1** to HSA afforded glycoconjugates **2** containing 5, 14 and 25 moles of hapten per mole of protein depending on the reaction time and temperature (Table 1). A higher coupling degree was obtained conducting the reaction at 55 °C for 24 hours as suggested by Laferriere and Roy [19] and using a 3-fold molar excess of hapten/amine group in the protein.

In this method, the aminated glucitol residues in the glycoconjugate function as a hydrophilic spacer arm, diminishing the rigidity of the hapten on the protein surface. However, the pyranosidic structure in the glucose moiety was destroyed as a result of the procedure.

The trisaccharide was also directly coupled to HSA by the carbodiimide amidation of sialic acid carboxyl groups. Considering a low reactivity of this carboxyl group, a four-fold molar excess of sialyloligosaccharides per mole of HSA aminolysyl groups was used in the reaction at several temperatures.

As observed (Table 1), even in the most extreme conditions a low coupling degree was obtained for 3´-

31. Magnani JF, Smith DF, Gingsburg V. Detection of gangliosides that bind cholera toxin: direct binding of ^{125}I-labeled toxin to thin-layer chromatograms. Anal. Biochem. 1980; 109: 399-402.

32. Jinnohara T, Tsujisaki M, Sasaki S, Hinosa Y, Taniguchi M, Imai K. Anti-tumor effect of internal image bearing anti-idiotypic monoclonal antibody in relation to GM3 ganglioside. Int. J. Cancer 1998; 76: 345-53.

33. Alonso DF, Gabri MR, Guthman MD, Fainboim L, Gomez DE. A novel hydrophobized GM3 ganglioside/Neisseria meningitidis outer-membrane-protein complex vaccine induces tumor protection in B16 murine melanoma. Int J Oncol. 1999; 5: 59-66.

34. Carr A, Mazorra Z, Alonso DF, Mesa C, Valiente O, Gomez DE, Perez R, Fernandez LE. (2001) A purified GM3 ganglioside conjugated vaccine induces specific, adjuvant-dependent and non-transient antitumor activity against B 16 mouse melanoma in vitro and in vivo. Melanoma Res. 2001;11: 219-27.

35. Grayson G, Ladisch S. Immunosuppression by human gangliosides. II. Carbohydrate structure and inhibition of human NK activity. Cell Immunol 1992; 139: 18-29.

36. Ladisch S, Becker H, Ulsh L. Immunosuppression by human gangliosides: I. Relationship of carbohydrate structure to the inhibition of T cell responses. Biochim. Biophys. Acta 1992; 1125: 180-88.

37. Ravindranath MH, Brazeau SM, Morton DL. Efficacy of tumor cell vaccine after incorporating monophosphoryl lipid A (MPL) in tumor cell membranes containing tumor-associated ganglioside. Experientia 1994; 50: 648-53.

38. Olshefski R, Taylor B, Heitger A, Hasegawa A, Ladisch S. Induction of programmed cell death and immunosuppression by exogenous sphingolipids are separate processes. Eur. J. Biochem.1996; 241: 47-55.

39. Hoon DS, Irie RF, Cochran AJ. Gangliosides from human melanoma immunomodulate response of T cells to interleukin-2. Cell. Immunol.1988; 11: 410-19.

Table 1 Characteristics of the HSA-neoglycoconjugates designed as immunogens in the present study.

3'Sialyllactose derivative (mg)	HSA (mg)	Conjugation procedure	neoglycoconjugate	Molar ratio*	Reaction time (h)	Temparature (°C)	Mole CHO/ Mole protein
1, (16.5)	(10)	Reductive amination	**2**	3:1	8	4	4-6
1, (16.5)	(10)	Reductive amination	**2**	3:1	24	4	13-15
1, (16.5)	(10)	Reductive amination	**2**	3:1	24	55	23-25
1, (22)	(10)	Amidation	**3**	4:1	24	4	1-3
1, (22)	(10)	Amidation	**3**	4:1	24	22	4
1, (22)	(10)	Amidation	**3**	4:1	24	55	9-10
4, (22)	(10)	Amidation	**6**	3:1	24	22	25
7, (20)	(10)	Disulfide bond formation	**8**	3:1	24	22	13-15
10, (3)	(18)	Thioether bond formation	**11**	1:1	24	4	10

* Oligosaccharide/active functional group in the protein

sialyllactose (9-10 aminolysyl groups out of 58 in HSA were coupled) and in the milder conditions only 1-2 moles of haptens were incorporated to HSA. These results indicate, as expected, that the carboxyl group of sialic acid displays a low reactivity probably due to steric hindrance.

Coupling of 3´-sialyllactose to HSA through a spacer molecule

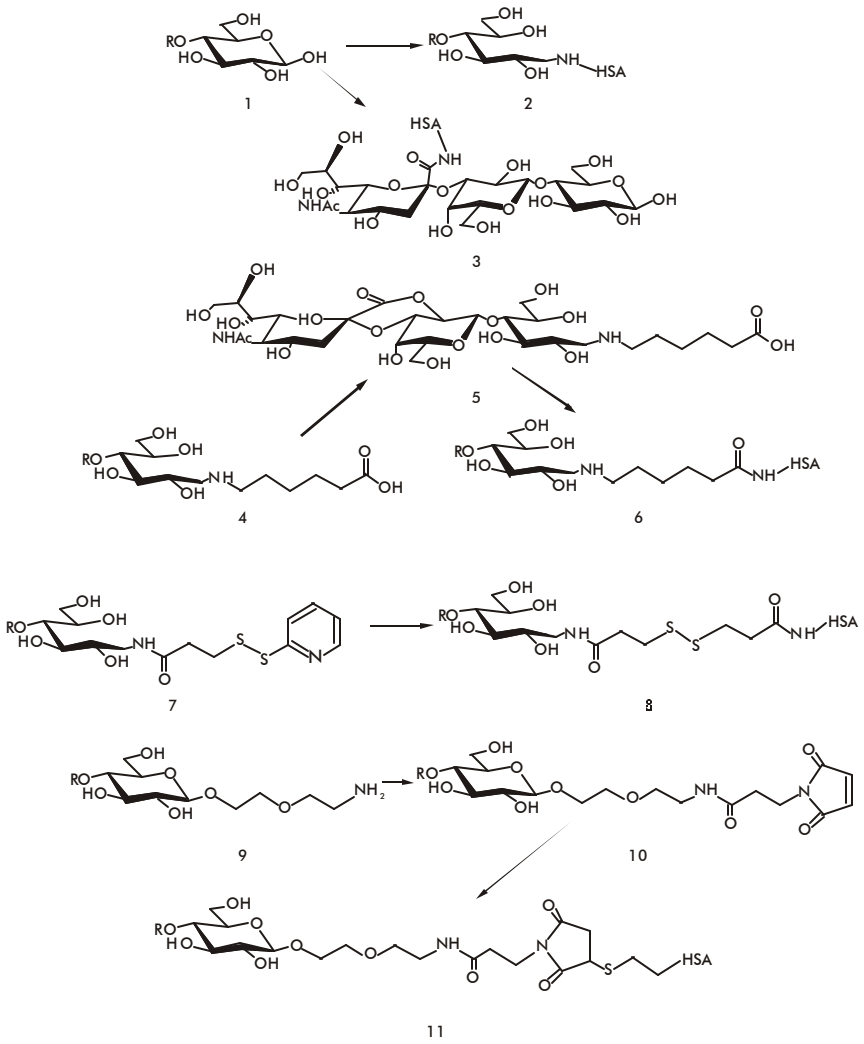
As the small size of the oligosaccharides used for immunisation may constitute a drawback for inducing a potent immunological response due to steric hindrance provoked by the proximity effects of the soluble carrier, a spacer arm was linked to the oligosaccharides prior to the conjugation reaction.

Glycoconjugates **6** were prepared by reductive amination of **1** with aminocaproic acid providing a spacer with an optimal length of 8 atoms [40] and a second more active carboxyl function in their terminus.

Initially, it could be thought that the reactivity of the new carboxyl group might be interfered by reactivity of the carboxyl group of sialic acid even when it was demonstrated that under certain conditions its reactivity is very low (see Table 1). To overcome this possible drawback, the carboxyl group of sialic acid was protected prior the conjugation to the protein. It is known that 3´-sialyllactose is prone to lactonization [19, 24] in an acidic medium. The inner ester is formed between the hydroxyl group of a carboxyl function of sialic acid and the hydroxyl group located at the carbon 2 position in the galactose ring although a second possible inner ester can also be formed with the carbon 4 hydroxyl group [41]. In fact, when aminocaproic acid was coupled to 3´-sialyllactose under reductamination conditions, a fast migrating compound in HPTLC experiments was formed which reacted with the orcinol reagent and was not stained with the ninhydrin reagent (not shown). This new compound was purified in BioGel P-2 with water as the eluent and freeze-dried. When this compound was treated with glacial AcOH, a fast migrating compound was observed in HPTLC plates which reverted to the starting material on standing in water solutions at room temperature. Alkaline treatment of this compound also produced a compound with similar RF to **4**, that proved that the lactone was formed.

The lactone **5** was then coupled to HSA using EDC under different conditions in order to obtain glycoconjugates with several coupling degrees of the haptens (see Table 1). The reaction was conducted at a pH under 5 allowing to conserve the structure of the lactone intact for more than 24 hours. The resti-

40. Lemieux RU, Bundle DR, Baker D. The properties of a ¨synthetic¨ antigen related to the human blood-group Lewis a. J. Am. Chem. Soc. 1975; 97: 4076-83.



Scheme 2

tution of the original structure of the saccharide **4** was attained after desalting in Sephadex G-50 using water as the eluent.

Glycoconjugate **8** containing SPDP as the spacer arm were also synthesised using a method already described [26]

In all these methods with the exception of the direct amidation of the carboxyl group of sialic acid of 3´-sialyllactose, the glucose residue was reductively opened with the subsequent loss of the original structure and of part of the information contained in the saccharide. This structural modification can be considered a potential neoantigen [42, 43] that is able to modify the immunological properties of the neoglycoconjugates.

Neoglycoconjugate **11** was prepared from the synthetic trisaccharide **9** [21] with an amino-type spacer arm containing the intact structure of the trisaccharide **1** α-NeuAc-(2-3)-β-D-Gal-(1-4)-β-D-Glc. The reaction of a trisaccharide derivative **9** with N-hydroxysuccinimidyl maleimidopropionate in DMF gave the active trisaccharide derivative **10** [28]. For the synthesis of the neoglycoconjugate **11**, HSA was thiolated by the reaction with N-hydroxysuccinimidyl dithiopropionate followed by a reduction with dithiothreitol. The coupling reaction between **10** and HSA-$(SH)_{18}$ proceeded smoothly giving glycoconjugate **11** containing 10 trisaccharide units in a 54% yield based on 10. As can be seen from formula **11**, this neoglycoconjugate maintains the intact pyranosidic structure of the glucose moiety.

*S*cheme 2

Synthesis of reagents for the ELISA evaluation of the serum

In order to evaluate the immune response induced by neoglycoconjugates **2**, **3**, **6**, **8** and **11** against the parent trisaccharide **1** and also against the di- and monosaccharide fragments, a series of derivatives were obtained. Additional amino groups were first introduced into casein by coupling with ethylendiamine. Then, lactose, 3´-sialyllactose, disialyllactose and glucose were coupled to casein by reductive amination. The structure of the neoglycoconjugates **12**, **13**, **14** and **15** obtained are represented in Scheme 3.

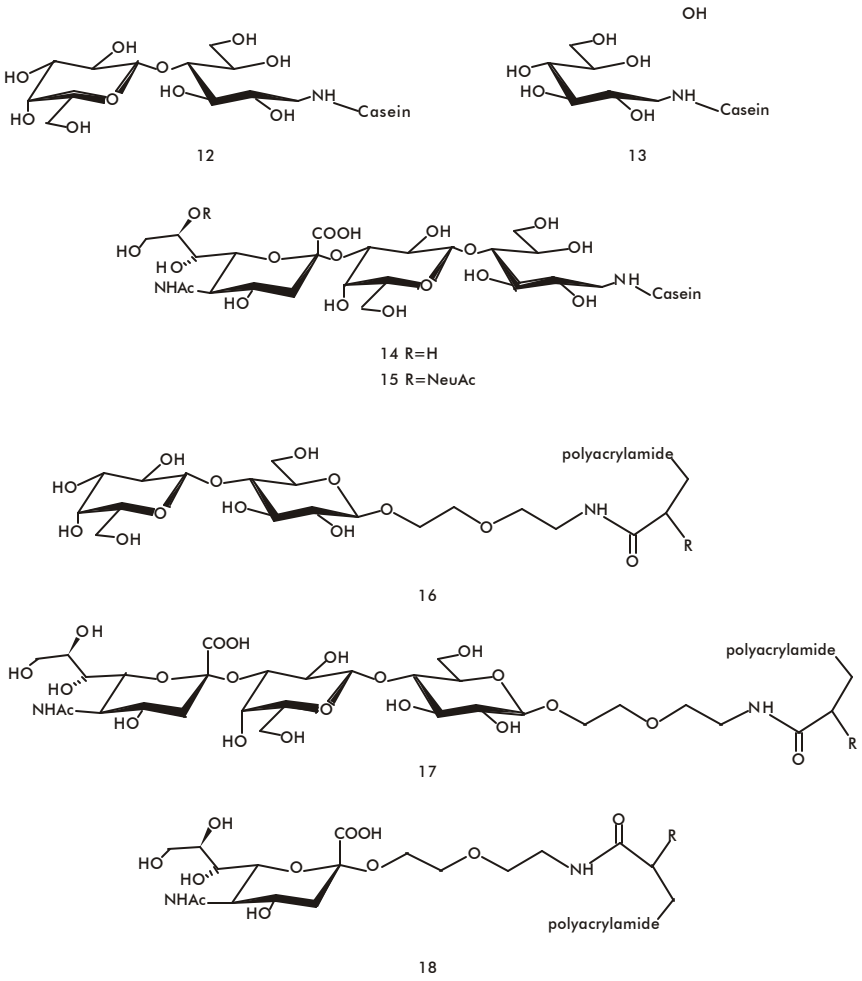
A second set of derivatives were obtained from lactose, 3´-sialyllactose and sialic acid with a spacer-arm with a terminal amino group and p-nitrophenyl polyacrylate. The glycopolymers **16**, **17** and **18** used for coating ELISA plates contained the structures represented in Scheme 3.

*S*cheme 3

Immune response

Two distinct animal species were immunised with the glycoconjugates trying to surpass a possible species-dependant immunological unresponsiveness and also to detect whether there are any differences in the specificity and crossreactivity to GM_3.

In the first experiment, chickens were immunised with the glycoconjugates **2**, **3**, **6** and **8**. The immune response was evaluated against the casein conjugate **14**. In all cases, a moderate to strong immune response was elicited against the trisaccharide moiety of conjugate **14**.



Scheme 3

The results obtained for all the glycoconjugates were also similar concerning the crossreactivity with other oligosaccharide-casein conjugates as well as for GM_3. The sera of animals immunised with conjugate **2** were chosen in order to illustrate the general pattern of reactivity.

As shown in Figure 1, the antibodies failed to recognise GM_3 at all. After the second dose, a moderate antibody response was induced that became stronger after the third and specially the fourth immunisation. The antibodies recognised to the same extent all four casein conjugates **12**, **13**, **14** and **15** but a specific recognition of sialyl-lactose casein conjugate **14** became more important after the last immunisation. There was practically, no recognition of sorbitol derivative **13** wich seems to show that no neoepitope was formed during the coupling reaction.

The inhibition of serum binding of chickens immunised with neoglyconjugate **2** by the oligosaccharide-casein conjugates **12**, **13**, **14** and **15** and GM_3 is shown in Figure 2. As expected, the best inhibitor is the homologous compound **14** with an IC50 value of 1.84 x 10^{-8} M, while lactitol-EDA-casein **12** and disialyllactose-

41. Nakamura T, Rubb WA, Saito T, Arai I, Urashima T. An NMR study of the lactonization of alpha-Nacetylneuraminyl-(2®3) lactose. Carbohydr. Res.2000; 329: 471-6.

42. Stowell CP, Lee YC. Neoglycoproteins. The preparation and application of synthetic glycoproteins. Adv. Carbohydr. Chem. Biochem.1980; 37: 225-81.

43. Zopf DA, Tsai CM, Gingsburg V. Carbohydrate antigens: coupling fo oligosaccharide-phenethylamine derivatives to edestin by diazotization and characterization of antibody specificity by radioimmunoassay. (1978) Methods Enzymol 1978; 50: 163-69.

EDA-casein **15** showed IC50 values of 1.96 x 10^{-7} M and 8.36 x 10^{-6} M, that is 11 and 454 times higher respectively. Casein-conjugate 13 and GM_3 were not able to inhibit the binding at the concentrations tested. These findings confirmed the results obtained previously.

In order to support the results obtained in chickens, all 4 neoglycoconjugates were also used for the immunization of mice. A similar immune response was elicited against the sialyllactose-casein **14**. The absence of antibody response of the sera against GM_3 ganglioside was again confirmed using ELISA, HPTLC-ELISA and specific inhibition experiments (Figure 5).

The degree of substitution in glycoconjugates is an important parameter that has a strong influence in the oligosaccharide presentation and the specificity of the response. Moreover, the optimal degree of substitution of the protein carrier ensures the preservation of its T-helper epitopes. In the case of conjugate **2**, products with different degrees of substitution (Table 1) were assayed in immunisation experiments and the results are shown in Figures 3 and 4.

As can be seen in Figure 3, the response against glycoconjugate **2** with 5 oligosaccharides per mole of protein is weaker while there were no significant differences in the response against glycoconjugates with 14 or 25 moles of oligosaccharides/mole protein. With a low degree of substitution, protein epitopes in the conjugate were probably preponderant.

The serum of animals immunized with glycoconjugate 2 with a D.S.25 of protein was further studied. As observed in Figure 4, with a dilution 1:80 a strong reaction was observed with casein conjugates **12-15** similar to those obtained with chickens. No reaction was observed with ethylendiamine-modified casein. However, with a more diluted serum (1:500) a specific binding of sialyl-lactose conjugate **14** was observed (Figure 5).

The inhibition of the binding of anti-3´-sialyllactose serum by casein-conjugates **12-15** and GM_3 ganglioside was also studied and is shown in Figure 5. At the hapten concentrations tested, only 14 (IC_{50} 7.42 x 10^{-8} M) and **15** in a lesser extent (IC_{50} 1.94 x 10^{-5} M) were able to inhibit the binding of mice anti-3´-sialyllactose serum to **14**. GM_3 ganglioside failed again to inhibit the binding, which proved that the antibodies that were raised during the immunization of mice with glycoconjugate **2** and reacting with **14** recognize a surface epitope that was not expressed or was not accessible in GM_3 .

The absence of sera reactivity of chickens and mice immunized with glycoconjugates **2**, **3**, **6** and **8** tested against native GM_3 ganglioside could be explained by the lost of the ring structure of glucose after the conjugation to a protein.

In order to verify this possibility, a new glycoconjugate **11** containing the intact structure of the oligosaccharide part of GM_3 and therefore with a greater resemblance to it, was included in this experiment.

Following two immunizations of mice with **11**, the immunogen elicited moderate levels of IgG antibodies, detected with conjugate **17**, which increased after the third immunization, as shown in Figure 6. The antibodies recognized only compound **17**, and neither sialic acid conjugate **18**, nor lactose-conjugate **16**, showing a strict specificity for the trisac-

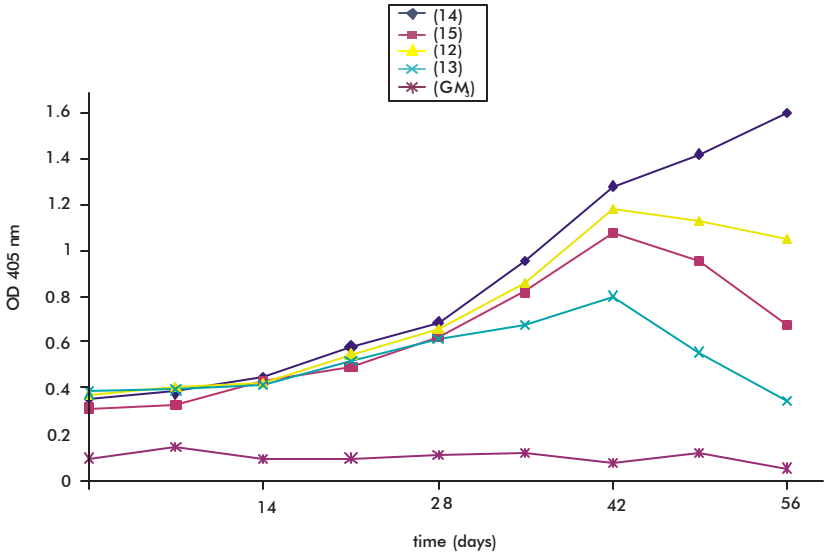


Figure 1. Time course experiment of the specific antibody response induced in chickens immunized with conjugate 2.

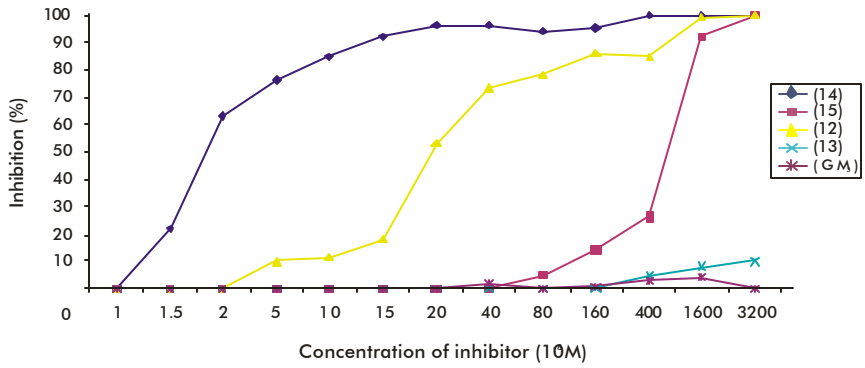


Figure 2. Inhibition by saccharides-casein glycoconjugates and GM_3 of 3´-sialyllactose binding to chicken anti-3´-sialiyllactose serum

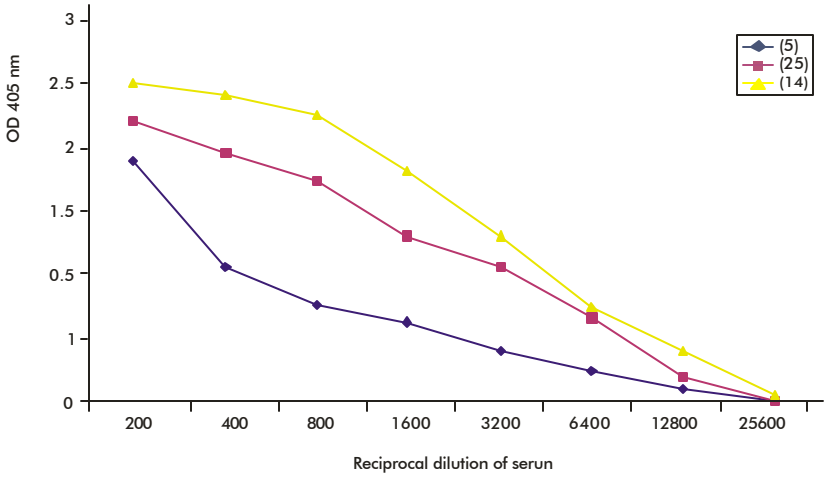


Figure 3. Influence of degree of subtitution of 3´-SL in 3´-SL-HSA conjugates on mice serum reactivity aginst 3´SL-casein conjugate

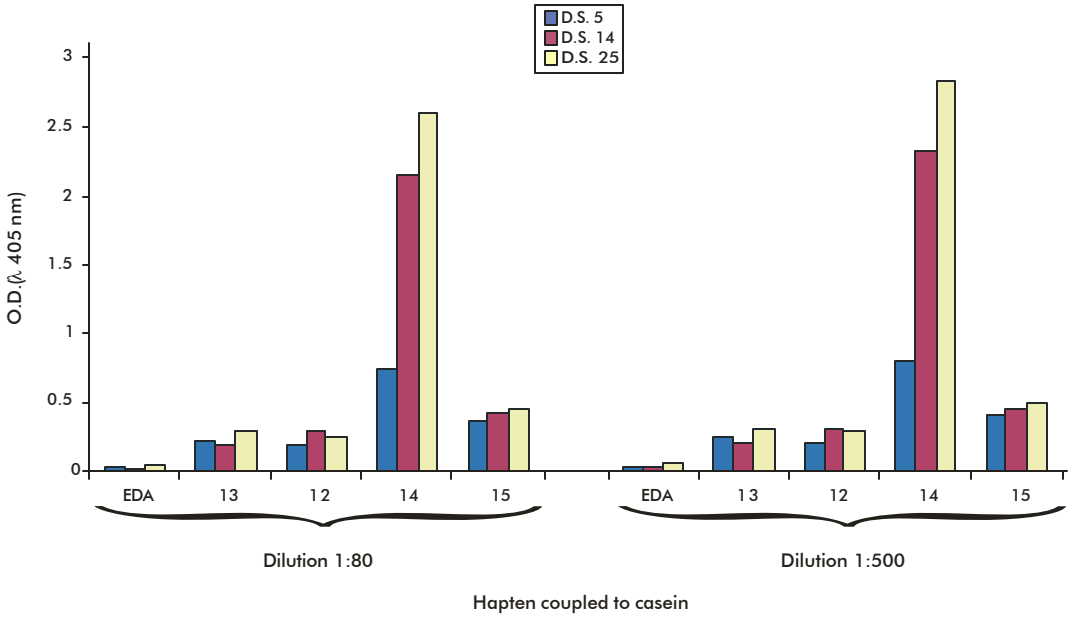


Figure 4. Antibody reactivity of mice sera. Influence of the degree of substitution of haptens and sera dilution.

charide were recognized. Surprisingly, the serum also failed to recognize GM_3.

An inhibition ELISA was performed to confirm the previous results. The binding between anti-**11** glycoconjugate serum and **17** was completely inhibited by synthetic trisaccharide **9**, showing an IC_{50} value of 10^{-8}M. The serum was only partially inhibited by sialic acid at high concentrations. Furthermore, lactose and GM_3 showed no inhibition. This finding suggests that the epitope recognized by the serum may include the whole trisaccharide.

Inhibition assays as well as direct ELISA`s clearly indicate that the reactivity of the serum was restricted to a whole trisaccharide molecule. Apparently the epitope recognized by this serum was larger than the epitope recognized by the serum obtained from the immunization with the previous glycoconjugates. Nevertheless, in any case there was no recognition of GM_3 ganglioside.

In another experiment (data not shown), a monkey serum raised against natural GM_3 ganglioside [12] was shown to react only with the parent ganglioside and not with any of the oligosaccharide-casein conjugates **12-15** or any of the polyacrylamide conjugates **16-18** confirming the data obtained for chickens and mice. This could be due to the fact that oligosaccharide is not exposed in GM_3 ganglioside in the same way as in neoglyconjugates. This result could also suggest an important role of lipid moiety in the immunogenicity of GM_3.

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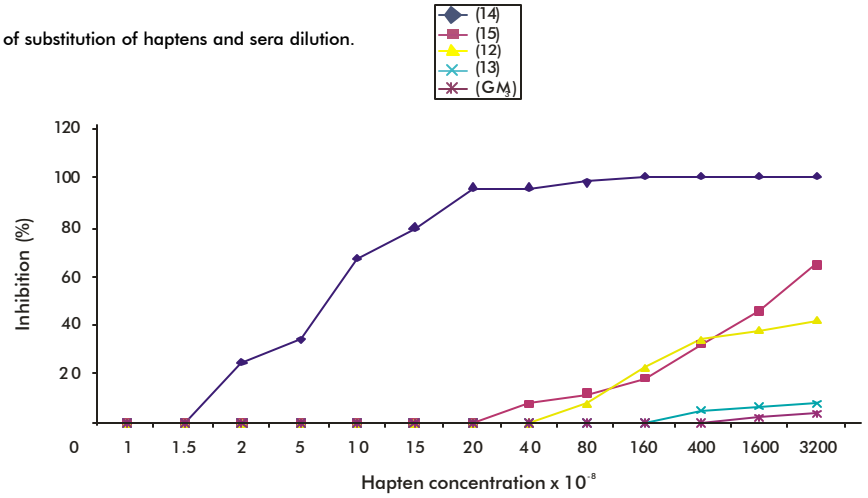

Figure 5. Sera inhibition of mice immunized with 3¨-SL-HSA conjugate (reductive amination, D.S.25). Sera dilution 1:500

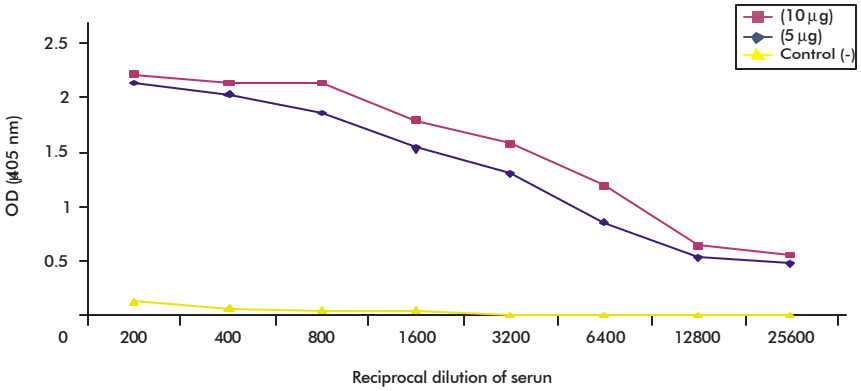

Figure 6. Antibody titer of mice immunized with neoglycoconjugate 11, measure using conjugate 17 as coating antigen.