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Affinity of mouse immunoglobulin G subclasses for sialic acid derivatives immobilized on dextran-coated supports

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Abstract

High-performance liquid affinity chromatography is a powerful method for the purification of biological compounds owing to its specificity, rapidity and high resolution. In our laboratory, we develop chromatographic supports based on porous silica beads. However, in order to minimize non-specific interactions between the inorganic surface and proteins in aqueous solution, the silica beads are coated with modified dextran. As previously reported, many affinity ligands can be covalently grafted onto dextran-coated silica. In this study, N-acetylneuramic acid, which belongs to the sialic acid family and is present in immunoglobulin G (IgG) epitopes, is used as an active ligand. The interactions of this affinity support and IgG subclasses are analyzed. This immobilized ligand enables purification of IgG3 antibodies.

Keywords: Immunoglobulins; Sialic acid

1. Introduction

The purification of monoclonal antibodies is essential, especially for immunotherapeutic applications. Of the numerous purification methods, affinity chromatography is becoming increasingly important and some affinity supports, such as phases grafted by protein A or protein G, are now commercially available.

Recently, new high-performance chromatographic supports have been developed. They are based on porous silica beads which are commonly used as high-performance chromatographic stationary phases because of their excellent mechanical properties [1]. However, it is necessary to modify the surface in order to decrease the non-specific interactions which occur between the deprotonated silanol groups on the silica surface, in aqueous solution, and proteins. To avoid this non-specific adsorption, as previously reported [2], the porous silica beads are coated with dextran bearing a calculated amount of positively charged diethylaminoethyl groups (DEAE), which neutralize the deprotonated silanols. Then, in order to get a better adsorption of the polymer layer at the silica surface, the dextran macromolecular chains are cross-linked using a diepoxide reagent. These dextran-coated silica supports, named SID, have the mechanical properties of the initial silica. The passivation of silica supports are verified by high-performance size exclusion chromatography of standard proteins at low ionic strength [2,3]. Moreover, these supports ally the performance of high pressure phases and the characteristics of low pressure gels, such as polysaccharidic phases (sephadex), due to the

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presence of the dextran layer. The hydroxyl groups of dextran polymers around the silica particles can be easily activated and functionalized by active ligands such as protein A, concanavalin A, heparin [4], benzamidine [5] and thiophilic ligand [6].

In this study, a glycosidic residue, N-acetylneuraminic acid (Neu 5 Ac) from the sialic acid family, is immobilized on the SID supports and used as the ligand in affinity chromatography. Interest in these compounds has recently increased because of their role in the phenomena of intercellular recognition and host parasite interactions [7]. Sialic acids are characterized by a carboxylic group at C1, a carbonyl group at C2 and a free or protected amino group in the C5 position. Neu 5 Ac, the prevalent sialic acid, is stable and presents an acetyl group on the amino group at C5, as shown in Fig. 1 [8]. The sialic acids are either free or bound to animal and bacterial glycoproteins, glycolipids and polysaccharides and are located at the end of the saccharidic terminal chains of these compounds. In particular, they are present in membrane receptors, named gangliosides [9], in normal cells such as nervous system cells [10], tablets [11] and endothelial cells [12]. Several studies have demonstrated that some antibodies decrease tumoral cell growth by a recognition mechanism of the gangliosides, whose density is abnormally high in tumoral cell membranes [13,14]. More precisely, the role of the ganglioside end terminal saccharidic chains and, notably, sialic acids, seems to be important in the recognition by these antibodies. Saito et al. [9] demonstrated the specificity of IgM and IgG3 for the GD2 ganglioside on human neuroblastoma cells. These authors suggest that sialic acid residues and N-acetylgalactosamine are involved in the epitope recognized by these antibodies. The end terminal location of the sialic acids and the flexibility of saccharidic chains may also participate in the recognition phenomena:

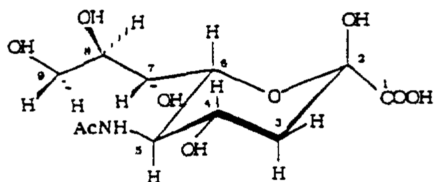

Fig. 1. Structure of N-acetylneuraminic acid.

Bechtel et al. [15] demonstrated, by conformational analysis, the specificity of the monoclonal antibody CA 19-9 for a carbohydrate antigen 19-9 whose structure is similar to that of gangliosides. Besides conformational aspects, the intrinsic structure of the sialic acid plays a significant role. Indeed, the replacement of an acetyl residue in Neu 5 Ac by a glycolyl residue cancels the specificity of IgG3 monoclonal antibodies on the lactone gangliosides GM3 [16].

It seems interesting to use sialic acid and notably, Neu 5 Ac, as an affinity ligand in order to observe the specificity of IgG for the gangliosides. Sialic acids are already used as affinity ligands in the purification of other biological compounds, revealing the important biological functions of these molecules. In previous studies, immobilized Neu 5 Ac enabled the purification of sialidases [17,18] and the affinity of lectin was demonstrated for an O-acetyl sialic acid grafted onto Sepharose 4B gel [19].

Mouse IgG contains four subclasses, named IgG1, IgG2a, IgG2b and IgG3. IgG3 differs from the others by the presence of a longer hinge region conferring a sensitivity to enzymatic digestion and also a higher molecular mass on these antibodies.

In this work, the interactions between Neu 5 Ac, immobilized on a dextran-coated silica support, and mouse monoclonal IgG subclasses are studied by high-performance affinity chromatography (HPAC). The influence of some support characteristics on the affinity of IgG subclasses is also analyzed.

2. Experimental

2.1. Synthesis of DEAE-substituted dextran

The average molecular mass of dextran (Pharmacia, Bois d'Arcy, France) was 70 000 g mol^{-1}. A solution containing 40 g of dextran and 79 g of sodium hydroxide in 120 ml of doubly distilled water was cooled and mixed at 4°C. The mixture was gently stirred for 20 min and 42.7 g of the hydrochloride form of 2-chloro-N,N-diethylaminoethane (Janssen, Pantin, France) were added. Then the temperature was raised to 55°C for 30 min of reaction time. After the reaction was complete, the

mixture was rapidly cooled and the pH was adjusted to 9 using concentrated hydrochloric acid. The DEAE–dextran polymer was then precipitated with methanol, filtered, washed with ethanol and dried under vacuum at 40°C overnight. The grafting rate of DEAE functions on the polymers was determined by elemental analysis and by acidimetry. The optimal conditions for good passivation of silica beads were already determined.

2.2. Coating of silica by DEAE–dextran (SID)

The porous silica beads were generously provided by Biosepra (Villeneuve-la-Garenne, France). The pore size of the silica particles was about 1250 Å and the bead sizes were 15–25 μm. The specific surface of these beads was 25 $m^2 g^{-1}$.

A 10-g quantity of DEAE–dextran was added to 100 ml of doubly distilled water and the pH was adjusted to 11.5, by the addition of 1 *M* sodium hydroxide. A 50-g sample of silica, degassed under vacuum for 1 h, was slowly added to this solution. After 30 min of impregnation, the packing was dried for 15 h at 80°C under vacuum. Next, the dextran layer was cross-linked. Then, the coated silica was added to 100 ml of diethyl ether containing 0.3% of 1,4-butanediol diglycidyl ether (Sigma, La Verpillière, France), and this mixture was stirred for 1 h at 40°C. After evaporation of the solvent, the silica was dried for 15 h at 80°C under vacuum.

2.3. Immobilization of Neu 5 Ac

Neu 5 Ac, extracted from birds' nests, was generously provided by Le Gallic [8]. We also used Neu 5 Ac purchased from Sigma or Neu 5 Ac freshly extracted from birds' nests in our laboratory, using a procedure previously described [20]. The control of sialic acid extracted from birds nests was observed by IR spectroscopy on a 1600 FTIR Perkin-Elmer (Saint-Quentin-en-Yvelines, France).

The ligand was grafted onto the dextran-coated support using two different coupling reagents, carbonyldiimidazole (CDI) and butanediol diglycidyl ether (BDGE). The activation of the basic support by CDI was achieved according to the following protocol: A 1-g quantitiy of CDI (Sigma) was dissolved in 10 ml of dried 1,4-dioxane. A 2-g quantity of dextran-coated silica was added to the solution and the suspension was gently stirred for 2 h at room temperature. The activated support was then collected by filtration and quickly washed with 200 ml of 1,4-dioxane and 200 ml of 0.1 *M* carbonate buffer, pH 9.

Activation by BDGE was achieved by adding 1 g of dextran-coated silica to 10 ml of diethyl ether containing 4 μl of BDGE. The suspension was gently stirred for 30 min at room temperature. The solvent was then evaporated at 40°C using a rotating evaporator and the activated support was dried under vacuum for 30 min.

Neu 5 Ac was coupled with these activated supports using either CDI or BDGE in a similar protocol: A 5-g quantity of activated support were suspended in 25 ml of 0.1 *M* carbonate buffer, pH 8.7, containing 100 mg of Neu 5 Ac. The suspension was gently stirred for 48 h at room temperature. The supernatant was kept for a further determination of the non-coupled ligand. The support was then filtered and washed with 200 ml of 0.1 *M* Tris–HCl buffer, pH 8.7. The excess reagent groups were deactivated in 25 ml of the latter buffer, under agitation for 3 h at room temperature. The support was successively washed with solutions of 0.05 *M* Tris–HCl, pH 7.4, 1 *M* NaCl and with 200 ml of the same buffer containing 0.05 *M* NaCl. The functionalized support was conditioned in this buffer at 4°C.

The quantity of Neu 5 Ac grafted onto the dextran-coated silica was indirectly calculated by determining the quantity of the non-coupled Neu 5 Ac by the periodate–resorcinol colorimetric method according to the method of Jourdian et al. [21]. Neu 5 Ac reacts with a chromogen substrate (resorcinol reagent) to give a colored complex. The absorbance at 635 nm is proportional to the sialic acid concentration present in the medium. A 400-μl sample of the solution was mixed with 100 μl of a 0.04 *M* solution of periodic acid. The mixture was cooled in ice for 20 min. A 1.25-ml volume of resorcinol reagent was then added and the solution was left in an ice bath for 5 min. Next, the mixture was heated at 100°C for 15 min. After cooling, 1.25 ml of terbutylic alcohol was added and the solution was agitated and incubated at 37°C for 3 min. The absorbance was then measured

at 635 nm. A calibration curve was drawn using solutions containing known concentrations of Neu 5 Ac, enabling determination of Neu 5 Ac present in the supernatant and the quantity of ligand grafted onto the supports.

2.4. Immunoglobulin G and immunotest

The monoclonal antibodies were extracted from mouse ascitic fluids provided by CisBioindustries (LAPAM, Bagnols sur Cèze, France). The anti-goat antibody mouse monoclonal IgG3 was purchased from Serotec (Paris, France) and the IgG2b (from a cell culture medium) was provided by Biosepra. The quantity of mouse monoclonal antibody was determined by an enzyme linked immunoassay (ELISA) from Boehringer Mannheim (Meylan, France). The ascitic fluids were diluted in the initial chromatographic buffer (50 m*M* Tris–HCl buffer, pH 7.4, containing 50 m*M* NaCl) and filtered through a 0.2-μm filter before injection.

2.5. HPLC apparatus and elution conditions

The HPLC apparatus consisted of a Merck-Hitachi 655 A-12 gradient system from Labs Merck-Clevenot (Nogent-sur-Marne, France) with a Rheodyne 7125 injection valve, connected to an LMC variable-wavelength monitor and to a D-2000 integrator.

The ascitic fluids were injected onto stainless columns (12.5 $\times$ 0.4 cm I.D.). The affinity supports were suspended in the initial chromatographic buffer (50 m*M* Tris–HCl buffer, pH 7.4, containing 50 m*M* NaCl). The column was filled with the suspension under mechanical vibration in order to obtain homogeneous packing.

After equilibration of the column, the samples were injected at a flow-rate of 1.0 ml/min in a 50 m*M* Tris–HCl buffer, pH 7.4, containing 50 m*M* NaCl. The desorption of antibodies was obtained by increasing the NaCl concentration to 1 *M* for 15 min in the same buffer. Then the column was thoroughly washed at this ionic strength and equilibrated again in the initial buffer.

3. Results and discussion

3.1. Synthesis of the supports

3.1.1. Extraction of Neu 5 Ac

Neu 5 Ac from different origins, one commercial and the others from birds' nests, was used. Of the latter type, one was extracted at our laboratory, the result of which is shown in Table 1. The IR spectrum of extract 3, shown in Fig. 2, reveals the same bands as for commercial Neu 5 Ac.

3.1.2. Immobilization of Neu 5 Ac on SID

The basic support used consisted of 40 mg of DEAE–dextran per gram of silica, with a 4.9% substitution of dextran by DEAE groups as determined by elemental analysis of carbon and nitrogen, corresponding to the optimal passivation of silica [4].

The Neu 5 Ac residue was grafted onto a dextran-coated silica support using conventional coupling methods. Two different reagents were used, CDI and BDGE. The former reacts with the hydroxyl groups of the dextran chains forming carbonyl–imidazole groups which then react with a hydroxyl group of Neu 5 Ac. The latter reacts with the hydroxyl groups of the polymer and generally with a hydroxyl group of the ligand forming an ether bond. As suggested by Suzuki et al. [18], the hydroxyl in position 1 of Neu 5 Ac is involved in the binding. This reagent creates a spacer arm between the support and the ligand and can probably increase the mobility of the active site.

The results of coupling the different Neu 5 Ac on SID using the two different coupling reagents are presented in Table 2. The amount of Neu 5 Ac immobilized on the solid support was indirectly determined by measuring the quantity of unbound

Table 1
Result of sialic acid extraction from a bird's nest

Weight of the nest (g)	Extraction yield (%)	Extract no.	Sialic acid quantity (mg)	Purity (%)
9.13	7.7	1	5	45
		2	30	60
		3	430	85
		4	240	60

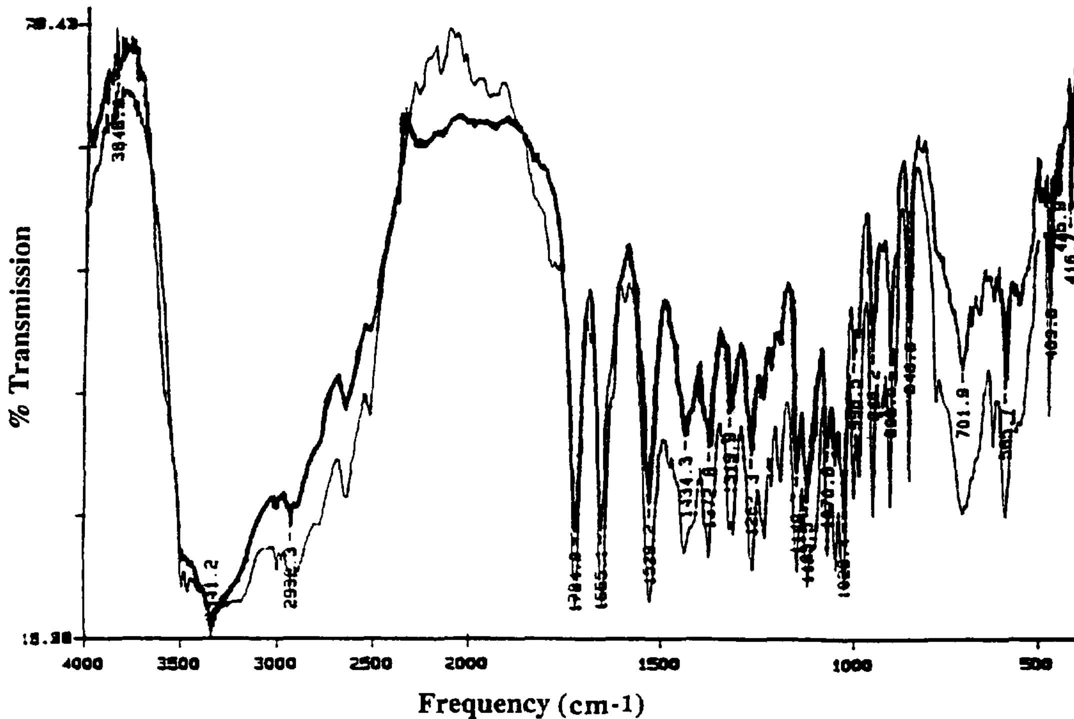

Fig. 2. FTIR spectra of commercial (—) N-acetylneuraminic acid and extract 3 (—).

sialic acid derivative, using the periodate–resorcinol method.

It seems that the origin of Neu 5 Ac does not affect the quantity of grafted ligand on the coated silica support. In contrast, the nature of the coupling reagent seems to be an important parameter. BDGE caused a higher grafted quantity of ligand to be coupled to the solid phase. This can be due to the higher chemical reactivity of the epoxide compound and the higher accessibility of the long arm of the reagent for the ligand.

3.2. *IgG elutions*

The affinity of the four IgG subclasses, IgG1, IgG2a, IgG2b and IgG3, extracted from mouse ascitic fluid was estimated by HPAC on the different supports grafted by Neu 5 Ac. In all cases, ascitic fluids were eluted from the column under the same conditions. The adsorption occurred at neutral pH in Tris–HCl buffer and the desorption of the antibodies was obtained by increasing the ionic strength of the adsorption eluent.

Table 2
Characteristics of the supports grafted by N-acetylneuraminic acid

Support	Coupling reagent	Neu 5 Ac origin	Initial quantity of Neu 5 Ac (mg/g support)	Grafted quantity of Neu 5 Ac (mg/g support)	Yield of coupling (%)
SID-NA1	CDI	JLG	20	6.0	30
SID-NA2	CDI	Sigma	20	6.0	30
SID-NA3	CDI	extract 3	20	5.4	27
SIDBNA	BDGE	JLG	25	10.0	40

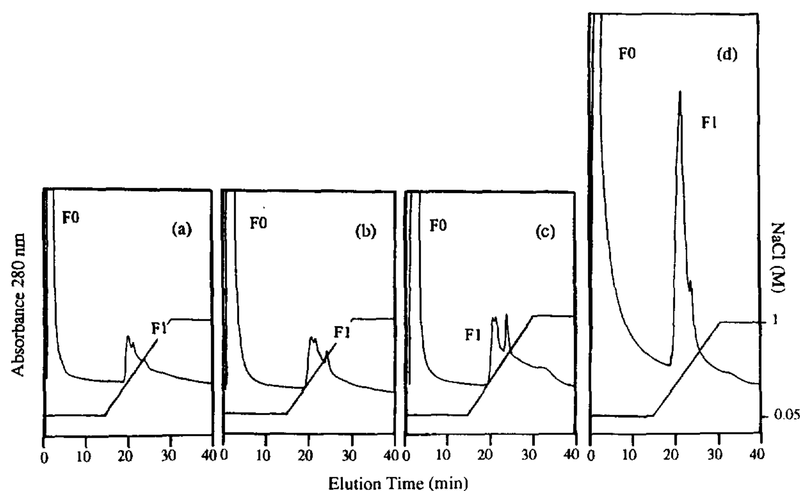

Fig. 3. Elution profiles of ascitic fluids containing IgG1 (a), IgG2a (b), IgG2b (c) and IgG3 (d) from a SID-NA1 support. Injected volume was 200 μl. F0 = non retained fraction; F1 = retained fraction.

3.2.1. IgG elutions on SID–NA1

The chromatograms of IgG subclasses eluted on the support SID–NA1 (where NA1 is the N-acetylneuraminic acid fraction used as ligand) grafted by Neu 5 Ac (JLG) provided by Le Gallic are presented in Fig. 3. IgG3 elution differs from the other subclasses and presents a sharper and higher desorption peak. The injected and collected quantities of antibodies were determined by ELISA (Table 3). These results were obtained by overloading the column during the injection period, so that a sufficient quantity of ascitic fluid to saturate all the functional sites on the column, is injected. The quantity of adsorbed and then eluted IgG3 is five times higher than the quantity of the other subclasses. This probably reflects a higher specificity of the affinity support for IgG3.

In order to measure the purification yield, the smallest quantity of ascitic fluid, below the overloading conditions of the column, was injected. As described in Section 2.4, the quantities of eluted IgG were determined by ELISA. A purification yield of 40% was obtained for the IgG3 subclass. For the three remaining subclasses, IgG1, IgG2a and IgG2b, under similar elution conditions, the amount of antibodies in the retained fraction (F1) was smaller and these IgGs were mainly eluted in the washing fractions.

The purity of the antibodies in the F1 was confirmed by SDS–PAGE and immunoblot of the eluted fractions [22].

The Neu 5 Ac dextran-coated silica support seems to develop a higher affinity for the IgG3 subclass rather than for the other subclasses. The better

Table 3
Antibody quantities injected on SID–NA1 and SIDBNA supports and collected in the eluted fractions

Antibodies	Injected quantity (μg)	Collected quantity in F0 (μg)		Collected quantity in F1 (μg)	
		SID–NA1	SIDBNA	SID–NA1	SIDBNA
IgG1	2680	310	314	26	27
IgG2a	3840	215	516	36	80
IgG2b	5060	426	665	33	47
IgG3	1240	379	136	144	127

These results were obtained using overloading conditions.

resolution observed in the elution profile of IgG3 demonstrates that the interactions between IgG3 antibodies and the immobilized Neu 5 Ac occur through better defined mechanisms.

In order to check and to improve the previous results, the influence of two parameters on IgG elution were then studied; the origin of Neu 5 Ac and the presence of a spacer arm between the support and the ligand.

3.2.2. *Effect of the origin of Neu 5 Ac*

The different ascitic fluids were eluted from the SID–NA2 support grafted by the commercial Neu 5 Ac compound (Sigma) using the same elution conditions as used for SID–NA1. Similar chromatograms were obtained. Moreover, the quantity of antibodies eluted in F1 are approximately identical to these determined for SID–NA1 and the IgG desorption occurs at the same ionic strength. These results indicate that the IgG subclasses have an equivalent affinity for SID–NA1 and SID–NA2.

The affinity of the four different IgG subclasses was then studied on the SID–NA3 phase grafted by Neu 5 Ac, freshly purified from extract 3 (Table 1). The elution profiles of IgG1 (a) and IgG3 (b) are presented in Fig. 4. The chromatograms of IgG2a and IgG2b on this affinity phase are similar to the elution profile of IgG1. In contrast to the previous results, the peak shapes of IgG1, IgG2a and IgG2b are better defined. However, IgG3 is eluted from this support with a longer retention time and at a higher ionic strength. The analysis of F1 by SDS–PAGE revealed the presence of many contaminant proteins. This support seems to be non-specific for IgG but exhibits, as with the previous supports, a stronger affinity for IgG3 antibodies compared with the other

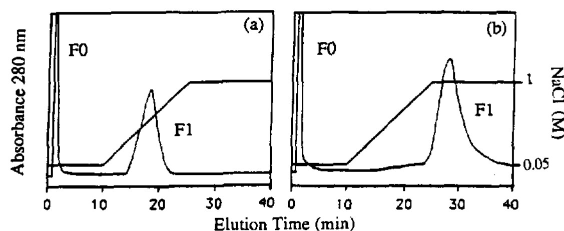

Fig. 4. Elution profiles of ascitic fluids containing IgG1 (a) and IgG3 (b) on a SID-NA3 support. Injected volume was 100 μl. Abbreviations as in Fig. 3.

IgG subclasses. This weaker specificity is perhaps related to insufficient purity of the Neu 5 Ac used as the affinity ligand.

3.2.3. *Effect of the spacer arm*

In this case, the pure ligand, Neu 5 Ac (JLG), was grafted onto the dextran-coated silica support using BDGE as a coupling reagent. The injected and collected quantities of antibodies are presented in Table 3. The results on this support, SIDBNA, in particular the elution profiles, are similar to those obtained on SID–NA1 support.

Consequently, the presence of a spacer arm between the ligand and the support does not modify, under our experimental conditions, the chromatographic performances of these affinity supports for the IgG separation. The presence of the polymeric layer probably creates, by itself, a mobility of the active site and the presence of the spacer arm does not improve the accessibility of the ligand.

4. Conclusion

The affinity of mouse monoclonal IgG subclasses for dextran-coated silica supports grafted by Neu 5 Ac has been studied. These new affinity supports allow a one-step separation of the IgG3 subclass from mouse ascitic fluids by HPAC. The adsorption of the other mouse IgG subclasses, IgG1, IgG2a and IgG2b, are weaker under our experimental conditions.

The desorption of IgG3, by increasing the ionic strength, demonstrates that ionic interactions take place, at least partially, between IgG3 and immobilized Neu 5 Ac. Further study of these interactions would be needed (effect of ionic strength, determination of affinity constants) in order to optimize the chromatographic performances of the supports. This might provide a better understanding of the recognition mechanism between IgG and gangliosides in vivo.

The physico-chemical characteristics of the supports are also important parameters. The coupling of a pure Neu 5 Ac is essential, showing that defined mechanisms exist between the ligand and the antibody. Moreover, dextran-coated silica supports are very interesting models. The presence of the dextran

layer on the silica surface allows one to examine the affinity mechanisms, by decreasing the non-specific interactions between the native silica and the proteins in solution. Additionally, the mobility of dextran macromolecular chains, studied in previous work [23,24], can facilitate a better accessibility of the ligand for the eluted compounds.

In order to check if the specificity of the IgG3 subclass for these supports depends on the origin of the monoclonal antibodies, complementary experiments were carried out; goat antibody mouse monoclonal IgG3 was injected and eluted using the chromatographic conditions described in Section 2.5. Similar results were obtained, as these antibodies were also adsorbed on this support and desorbed by a salt gradient. IgG2b antibodies, from a cell culture medium, were eluted on a Neu 5 Ac support; no adsorption occurred as already observed in the experiments presented here. These results confirm that the mouse IgG3 antibodies have a stronger affinity for the new support than the other subclasses.

By improving the chromatographic performance, particularly the capacity, of dextran-coated silica grafted by Neu 5 Ac, the use of these new affinity phases could lead to the development of a new purification process for IgG3 antibodies using mild and non-denaturing conditions.

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