

Anion exchange chromatographic distribution of human monoclonal immunoglobulin G is determined by heavy chain subclass and level of sialic acid expression

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Abstract

Anion exchange chromatography is a widely accepted method for purification of immunoglobulins. In this work, we used human monoclonal immunoglobulin G (IgG) with structure and solubility of normal human IgG as a model for studying chromatographic behavior of particular molecular forms of IgG. Human sera with monoclonal IgG were fractionated on a strong anion exchanger, Q Sepharose Fast Flow. With 20 mM Tris pH 7.5 as a start buffer, 42% of human monoclonal IgG passed through column, and 58% of them remained adsorbed. Bound monoclonal IgG were eluted from the exchanger by linear increasing of concentration of NaCl from 0 to 0.5 M. The chromatographic distribution of human monoclonal IgG correlated with their electrophoretic mobilities in agarose gels, and it was dependent on γ heavy chain isotype. Light chain type, as well as serum concentration of monoclonal IgG did not influence their chromatographic behavior. The level of heavy chain sialic acid expression, but not of galactose and *N*-acetylglucosamine, significantly determined chromatographic distribution of serum monoclonal IgG. In addition to the information on the chromatographic behavior of human monoclonal IgG, we believe that the presented data could provide useful information about the possible use of Q Sepharose Fast Flow matrix for the isolation of specific molecular forms of human IgG.

Keywords: human monoclonal IgG, anion-exchange chromatography, IgG subclasses, sialic acid.

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Human immunoglobulins G (IgG) are a heterogeneous group of molecules consisting of four subclasses (isotypes) and about 20 allotypes [1]. *N*-linked oligosaccharides in CH2 domain of heavy chains of all IgG molecules and oligosaccharides in variable domains of 15–20% IgG molecules also increase complexity of IgG structure [1]. There is a strong association of IgG isotypes and allotypes with antibody effector functions and with specific antibody response to different type of antigens [2,3]. Although IgG contains only 3% of carbohydrates, the oligosaccharide moieties, especially three outer arm sugars, sialic acid, galactose, and *N*-acetylglucosamine, influence physicochemical properties, antigen-binding capacity, and the effector functions of IgG molecules [1,4].

Nowadays, about ten different human or “humanized” monoclonal IgG for human immunotherapy exist on the market [5,6]. In addition to their therapeutic use, human monoclonal IgG are still a very important tool for studying the structure and the functional characteristics of antibodies. Independently of their uses or origin, a purification step of IgG molecules is

necessary. Due to extreme diversity in molecular characteristics of human IgG there is no unique method which can be applied for purification of all their molecular forms. Because of extreme selectivity towards antibodies, the most recommended method for purification of IgG is affinity chromatography on Protein A/G matrices [7,8]. However, strong interaction between the antibody and the ligand requires unfavorable elution conditions, sometimes causing damage to the molecules. In addition, contamination of antibody with ligand may occur during the affinity isolation. However, the greatest obstacle to the use of affinity chromatography is the high cost of Protein A/G matrixes. Ion exchange chromatography is a widely used alternative method for purification of IgG [8]. The ion exchangers are, compared to affinity matrices, considerably cheaper. They are more robust, have simpler cleaning and sanitization steps [7,8] and most importantly, good separation can be achieved under the mild condition of adsorption and elution where good recovery of antibody activity can be expected [7,9,10]. All this makes them very useful for laboratory-scale as well as large-scale purification of antibodies.

In this study, sera of patients with monoclonal IgG with structure and solubility of normal IgG were fractionated on a Q Sepharose Fast Flow anion exchanger. We analyzed whether serum concentration, heavy

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chain isotype, light chain type and N-acetylglucosamine, galactose, and sialic acid expressed on heavy and light chains influence chromatographic behavior of human monoclonal IgG. The results have shown that anion exchange chromatographic distribution of human IgG was strongly determined by their heavy chain isotype and the level of heavy chain sialic acid expression.

EXPERIMENTAL

Sample

The serum samples were obtained after the spontaneous coagulation of the venous blood, and centrifugation for 10 min, at 1250×*g*. Concentration of serum proteins was determined by tannin turbidimetric method [11]. Monoclonal immunoglobulins in patients' sera were detected by agarose gel electrophoresis [12] as homogenous fractions in γ or β zone. They were quantified by densitometry using ImageMaster TotalLab v1.11 software (Amersham Pharmacia Biotech, UK). Monoclonal immunoglobulins were immunoelectrochemically defined as IgG κ or IgG λ by serum immunoelectrophoresis [13] using specific antisera (Nordic, Tilburg, the Netherlands). Subclasses of monoclonal immunoglobulins were defined by Western blot [14] with mouse monoclonal antibodies specific for γ 1, γ 2, γ 3 and γ 4 heavy chains (Nordic, Tilburg, the Netherlands). Allotypic (Gm) markers of monoclonal IgG were determined by inhibition-ELISA [15] using murine monoclonal anti-G1m(f), anti-G1m(a), anti-G1m(z), anti-G3m(b1/u), anti-G3m(g1), and rabbit polyclonal anti-G2m(n) antibodies (CLB, Amsterdam, the Netherlands).

Ion exchange chromatography [7,9]

Sera (1.5 ml) were dialyzed against starting buffer, 20 mM Tris pH 7.5. Precipitated material was removed by centrifugation for 10 min, at 2500×*g*, at room temperature. The volume of dialyzed supernatants was adjusted to 5 ml by adding of starting buffer. Protein concentration in diluted serum samples was 29±4 mg/ml (range 19–39 mg/ml). The IgG concentration in diluted samples was within the range of 2 to 26 (9±4) g/l, and made 8 to 62% (30±10%) of total serum proteins. Serum samples were applied to a 1.6 cm×10 cm column packed with a strong anion exchanger Q Sepharose Fast Flow (Amersham Pharmacia Biotech, UK) previously equilibrated in starting buffer. According to the manufacturer, in 50 mM Tris buffer at pH 8.0, the exchanger binds 120 mg/ml of human serum albumin per ml. Applied sera did not contain more than 100 mg of albumin, whereby column overloading was avoided. The flow rate was 1.5 ml/min and the operating temperature was 22–25 °C. Chromatography was monitored by measuring UV absorbance (OD_{280}) of eluted proteins in UV cord, a flow spectrophotometer (LKB, Uppsala,

Sweden), and transmitting of optical signal in a graphic record (chromatogram). The eluted material was collected by a 17000 Minirac Fraction collector (LKB, Uppsala, Sweden), in 5 min intervals. The proteins that did not interact with anion exchanger had passed through the column with the first buffer volume, and the adsorbed proteins were eluted by linearly increasing concentration of NaCl from 0 to 500 mM in 20 mM Tris buffer. The linear gradient of NaCl concentration was performed using a gradient mixer GM-1 (Pharmacia, Uppsala, Sweden) in total volume of 500 ml. After each chromatography step, the anion exchanger was cleaned by passing 2 M NaCl and 1 M NaOH, which was followed by rinsing with sterile starting buffer. Additional washing with 70% ethanol was also performed. Cleaned column was stored in sterile 20 mM Tris buffer, pH 7.5, at 4 °C until its next use.

Protein fractions eluted in a form of one chromatographic peak were pooled and concentrated to 1–5 mg/ml by ultrafiltration in 200 ml Amicon stirred cells and PL-10 membrane (10 kDa cut-off, diameter of 63.5 mm) (Milipore, Billerica, MA). The buffer exchange (50mM Tris, pH 7.5/100 mM NaCl) was performed in the same device. The protein contents of each of chromatographic peaks were determined by agarose gel electrophoresis [15]. Western blot [14] with horse radish peroxidase conjugated mouse monoclonal antibody specific for human IgG (γ heavy chain) (CRB, Amsterdam, The Netherlands) and goat polyclonal antibodies specific for human IgA and IgM (α and μ heavy chain) were used to check purity of polyclonal IgG isolated from normal human sera (Sigma). The purity of isolated IgG was also verified by non-reducing and reducing SDS-PAGE [16] in 7.5% and 10% gels, respectively. Monoclonal IgG, which were partially purified by the anion exchange chromatography, were additionally purified by Protein G Sepharose Fast Flow affinity chromatography (Amersham Pharmacia Biotech, UK) according manufacturer instruction using 50mM Tris, pH 7.5/100 mM NaCl as starting buffer, and 100 mM glycine, pH 2.5 as elution buffer. Neutralization of eluted proteins was done with 1 M Tris-HCl, pH 7.5.

Lectin blotting

Localization and quantification of galactose, N-acetylglucosamine, and sialic acid were determined by lectin blotting assay [17]. The assay was performed by combined procedure of reducing SDS-PAGE and lectin binding to heavy and light chains of IgG. In brief: After SDS-PAGE of chromatographic fractions containing monoclonal immunoglobulins (5 μ g of proteins), the proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia, Biotech). After blocking of non-specific binding by 3% BSA in TBS-0.05% Tween buffer, membranes were incubated with one of biotinylated lectins from *Ricinus communis* (RCA I), *Grif-*

fonia (Bandeiraea) simplicifolia (GS II) and *Sambucus nigra* (SNA) (Vector laboratories, Burlingame, CA, USA) which detect terminal galactose, N-acetylglucosamine, and sialic acid, respectively. Biotinylated lectins were diluted in 3% BSA in TBS-0.05% Tween buffer according to the manufacturer's instructions. Having been labeled with avidin-peroxidase (Vector laboratories, Burlingame, CA, USA), the enzyme reactivity was visualized by using 4-chloro-1-naphthol (Serva, Heidelberg, Germany) as the peroxidase substrate. The terminal sugar expression was quantified by densitometry using ImageMaster TotalLab v1.11 software (Amersham Pharmacia Biotech, UK).

Statistical analysis

The results were presented as mean \pm standard deviation. Statistical significance of differences between two groups was determined by Student's *t*-test. Correlation between analyzed parameters was tested by linear regression. Differences with *P*-value of <0.05 were considered significant. All statistical analyses were carried out in OriginPro 8 program. An interactive calculation for chi-square tests was made by using software on <http://www.quantpsy.org/chisq/chisq.htm> webpage.

RESULTS AND DISCUSSION

Anion exchange chromatography is broadly accepted method for purification of IgG. However, the obtained results, regarding the purity and composition of molecular forms, are strongly influenced by the type of anion exchanger [18]. Both weak and strong anion exchanger can be equally used for isolation of IgG [19], but which of the numerous ion exchangers is suitable for isolation of specific molecular form of IgG can be known only after having been tested for specific application. In this study, we used a strong Q Sepharose Fast Flow anion exchanger, with quaternary amine as the ion exchange group, for fractionation of human serum with polyclonal and monoclonal IgG.

Anion exchange chromatographic behavior of normal human serum IgG

Using the aforementioned protocol, normal serum proteins were separated into four chromatographic fractions (peaks) (Figure 1a) with electrophoretically slower proteins eluted earlier than rapidly moving proteins. The first peak contained negatively charged proteins that passed through the column without binding to the exchanger. The remaining proteins were adsorbed to the anion exchanger, and eluted by linearly increasing concentration of NaCl. In accordance with previously published data [9], IgG molecules had broad chromatographic distribution and that they were found throughout the chromatogram (Figure 1b). Immunoblot analysis (Figure 1b) showed that approximately 70% of

total isolated IgG, were found in first two peaks. No IgA and IgM were detected in these fractions (Figure 1c). In the peaks III and IV, it was found 30% of total IgG. They were significantly contaminated with other serum proteins.

Anion exchange chromatographic distribution of human monoclonal IgG

Sera of 92 patients with monoclonal IgG were fractionated by anion exchange chromatography on Q Sepharose Fast Flow exchanger. The monoclonal fraction level was within the range of 6 to 82 g/l, and made 8 to 62% of total serum proteins. From 92 monoclonal IgG, 66 belonged to IgG1 (59 with G1m(f), and 7 with G1m(az) allotype), 9 to IgG2 (all but one with G2m(n) allotype), 12 to IgG3 (all with Gmb allotype), and 5 to IgG4 subclasses. The κ/λ ratio for total human monoclonal IgG was 1.6. The κ/λ ratio for monoclonal IgG1, IgG2, IgG3 and IgG4 was 1.5, 8.0, 1.4, and 0.7, respectively. Any of monoclonal IgG did not precipitate at temperature lower than 37 °C and did not precipitate in low ionic strength buffer, *i.e.*, they had no properties of either cryoglobulins or euglobulins.

Q Sepharose Fast Flow elution profiles of three representative human sera containing monoclonal IgG, as well as agarose gel electrophoresis of separated protein fractions are shown in Figure 2.

By SDS-PAGE it was confirmed that both normal serum polyclonal IgG and monoclonal IgG were isolated in form of complete IgG molecules containing both heavy and light chains (Figure 3b) and had molecular weight of ~150 kDa (Figure 3a). In preparation of isolated monoclonal IgG, fractions of lower molecular weight were found. They can occur because of IgG degradation during purification process. Additionally, it is known that normal synthesis of IgG is performed through the synthesis of different intermediates of lower molecular weight HL, HL₂, H₂ and H₂L [20]. The assembling of IgG molecules in normal B lymphocytes is precisely controlled. However, in malignant B cells, heavy and light chains' synthesis can be unsynchronized and synthetic intermediates can be secreted in plasma. After reducing SDS-PAGE, beside heavy and light chains, heavy chain dimmers were detected (Figure 3b). Other authors also detected them [21], and believed that the altered structure of IgG secreted by malignant plasma cells is responsible for their formation.

Monoclonal IgG had wide chromatographic distribution (Table 1, Figure 4). Among the monoclonal IgG analyzed, 42% were not adsorbed by the Q Sepharose Fast Flow anion exchanger, and passed through with first buffer volume, and 48% were weakly bound to anion exchanger and their maximal elution were achieved with 0.09 to 0.19 M buffer. The remaining of 10% monoclonal IgG were strongly bound, and their maximal elution were achieved with 0.20–0.43 M buf-

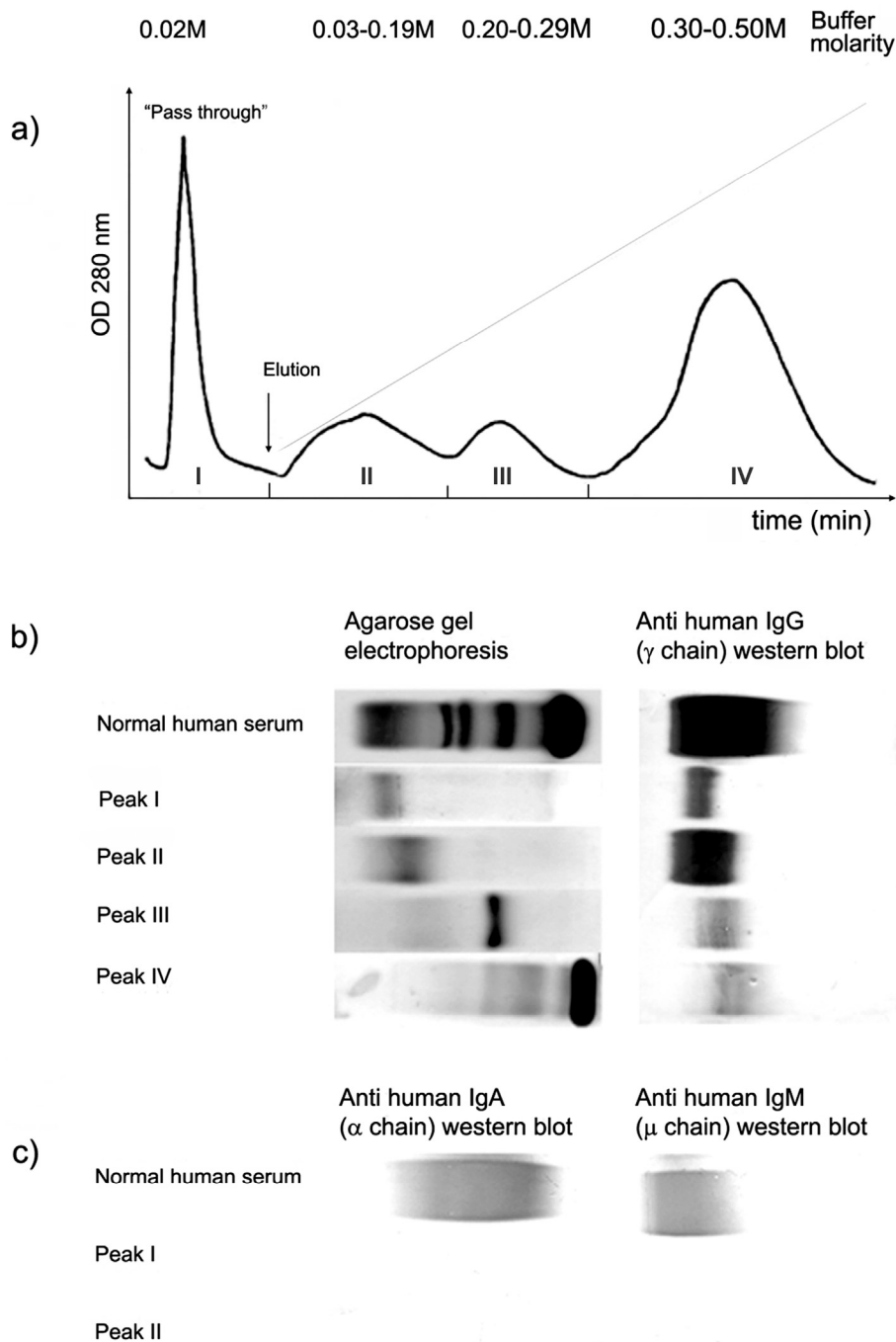


Figure 1. Q Sepharose Fast Flow anion exchange chromatography of normal human serum. a) Chromatogram. Sample: diluted normal human serum; concentration of total protein – 20 mg/ml; concentration of IgG – 3 g/l. Sample volume: 5.0 ml. Column 1.6 cm×10 cm. Starting buffer: 20 mM Tris-HCl, pH 7.5. Elution: 333 min linear gradient to 500 mM NaCl. Flow rate: 1.5 ml/min. Detection: OD 280 nm. The grey line follows linear increasing of concentration of NaCl in the elution buffer. b) Agarose gel electrophoresis and anti human IgG western blot of chromatographic fractions I and II. c) Anti-human IgA and IgM western blots of chromatographic fractions I and II.

fer. Only monoclonal IgG which passed through the column without binding to the exchanger, were not contaminated with other serum proteins (Figure 2). Monoclonal IgG recovery, after anion exchange chromatography and ultrafiltration, was $64 \pm 13\%$.

The chromatographic distribution of human monoclonal IgG was not influenced by their serum concentrations ($R = -0.009$). Although the κ/λ ratio decreased with the increase of molarities of elution buffer, the change was not statistically significant ($\chi^2 = 1.109$, $df = 2$, $p = 0.58$), i.e., chromatographic distribution was

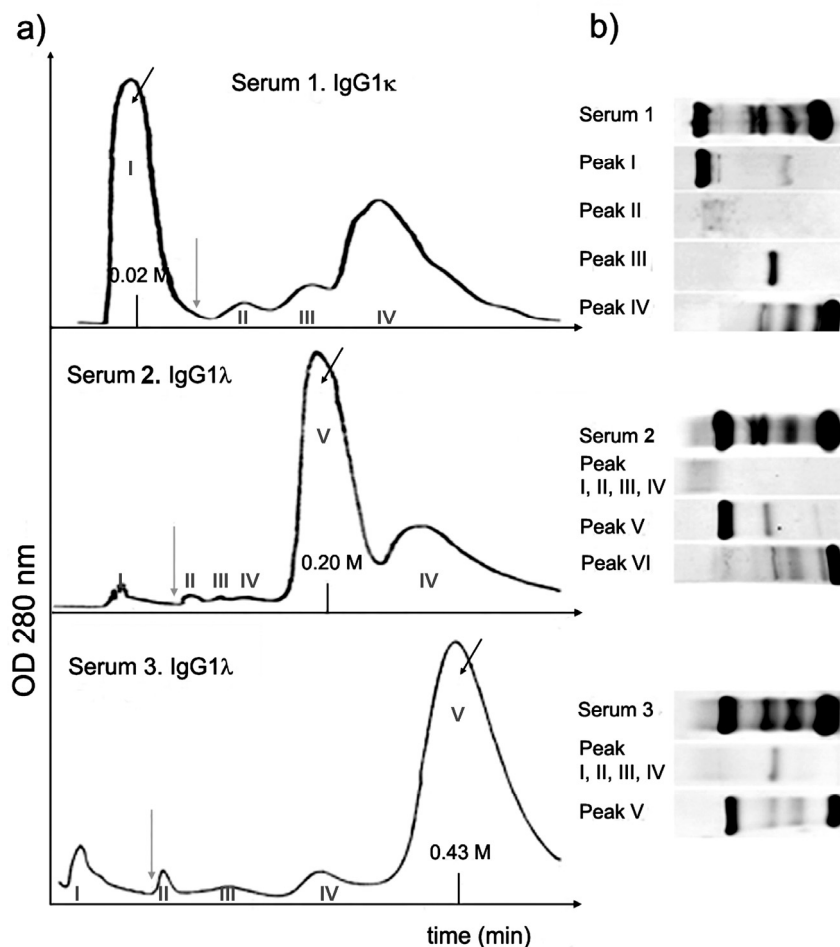


Figure 2. Q Sepharose Fast Flow anion exchange chromatography of human sera with monoclonal IgG. a) Chromatograms. Samples: diluted human serum with monoclonal IgG1. Serum 1 – total protein 29 g/l, mIgG 7 g/l; serum 2 – total protein 27 g/l, mIgG 10 g/l; serum 3 – total protein 30 g/l, mIgG 8 g/l. Sample volume: 5.0 ml; column 1.6 cm×10 cm; starting buffer: 20 mM Tris-HCl, pH 7.5; elution: 333 min linear gradient to 500 mM NaCl; flow rate: 1,5 ml/min; detection: OD 280 nm. The grey arrows indicate start point of linear gradient, the black arrows show peak which contain monoclonal IgG, and the vertical bars indicate buffer molarity at which maximum of elution of monoclonal IgG was obtained. b) Agarose gel electrophoresis of chromatographic fractions.

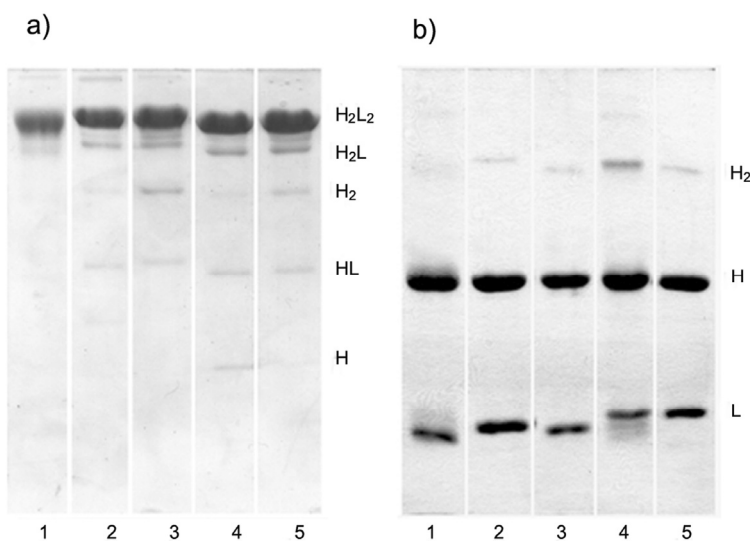


Figure 3. Non-reducing (a) and reducing (b) SDS-PAGE of human polyclonal IgG (1) and monoclonal IgG (2-5) isolated on Q Sepharose Fast Flow anion exchanger with first buffer volume. H – heavy chain, L – light chain.

Table 1. Chromatographic distribution of serum human monoclonal IgG on Q Sepharose Fast Flow anion exchanger

Parameter	Molarity of Tris-HCl/NaCl buffer pH 7.5, at which maximum elution of monoclonal IgG was obtained					
	0.02		0.03–0.19		0.20–0.50	
	No.	%	No.	%	No.	%
IgG	39	42	44	48	9	10
IgG1	36	55	22	33	8	12
IgG2	3	33	6	67	0	0
IgG3	0	0	12	100	0	0
IgG4	0	0	4	80	1	20
IgG κ	23		27	45	7	
IgG λ	16		17	44	2	
κ/λ	1.44		1.59		3.50	

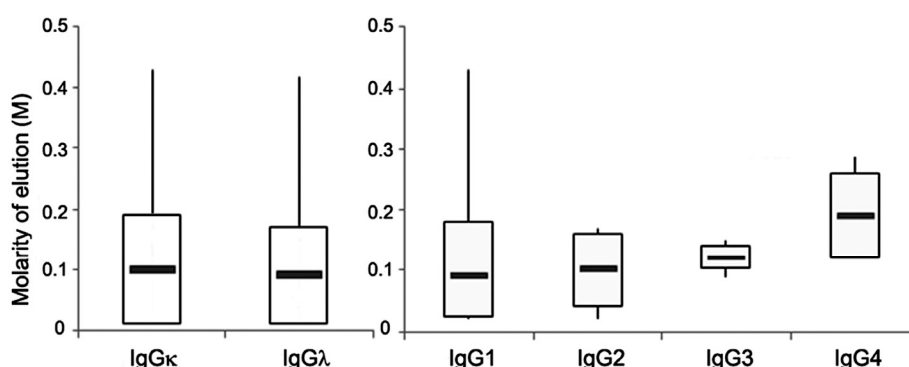


Figure 4. Chromatographic distribution of human serum monoclonal IgG isolated on Q Sepharose Fast Flow anion exchanger. Molarity of elution represents molarity of Tris-HCl/NaCl buffer at which maximum of elution of monoclonal IgG was obtained. The horizontal bars represent means values. The box plots display mean \pm SD. Vertical bars indicate minimum and maximum values.

not dependent on light chain type. The chromatographic distribution of monoclonal IgG significantly correlated with their electrophoretic mobility in 1% agarose gel ($R = 0.58$, $p < 0.001$). In addition, isotype of monoclonal IgG strongly determined their chromatographic distribution. Monoclonal IgG1 had the widest chromatography distribution. Of the monoclonal IgG1 analyzed, 55% passed through the column with first buffer volume. The remaining 45% of monoclonal IgG1 were bound to the exchanger, and eluted with broad range of concentrations of Tris-HCl/NaCl buffer (0.10–0.43 M). This broad chromatographic distribution was not a consequence of differences in chromatographic properties of two IgG1 antithetic allotypes (G1m(f) and G1m(z)) ($\chi^2 = 0.887$, $df = 2$, $p = 0.64$). One third of IgG2 passed through column, whereas two thirds bound weakly and were eluted with 0.10–0.17 M buffer. All monoclonal IgG3 were weakly bound to the exchanger and eluted within very narrow concentration range (0.09–0.15 M). Just like the monoclonal IgG3, all monoclonal IgG4 bound to the anion exchanger. However, the binding was stronger and monoclonal IgG4 were eluted with 0.14–0.32 M buffer.

The chromatographic elution pattern of monoclonal IgG of different subclasses obtained in this work corre-

ponded to the pattern of their mobility in cellulose acetate electrophoresis [22] and was in correlation with isoelectric focusing electrophoretic pattern of monoclonal [23] and normal human IgG subclasses [22].

Influence of N-acetylglucosamine, galactose, and sialic acid expression level on anion exchange chromatographic distribution of human monoclonal IgG

Studies in which the influences of oligosaccharide moieties on chromatography characteristic of human polyclonal and monoclonal IgG are being estimated are extremely rare [24–26]. As rare as they are, they show that the type of chromatography determines glycosylation profile of isolated IgG. Thus, the human polyclonal IgG isolated with first buffer volume by DEAE anion exchange chromatography expressed more sialic acid, galactose, and N-acetylglucosamine than IgG isolated by protein G affinity chromatography [24]. Also, it was shown that particular glycoforms of single IgG molecules could be differentiated by cation exchange chromatography [25] or protein G versus protein A affinity chromatography [26]. According to our knowledge, the studies of the effect of the expression of N-acetylglucosamine, galactose, and sialic acid on human

monoclonal IgG to their anion exchange chromatography behavior do not exist.

The expression of three terminal sugars *N*-acetylglucosamine, galactose, Gal, and sialic acid was observed in a group of 34 monoclonal IgG (28 of IgG1, 3 of IgG2, 2 of IgG3, and 1 of IgG4 subclasses, 20 with κ and 14 with λ light chains) that was selected from the group of 92 molecules previously analyzed for their chromatographic characteristics (see above). The distribution of IgG subclasses, as well as κ/λ ratio in the group of 34 monoclonal IgG, did not significantly differ from total IgG molecules ($\chi^2 = 1.896$, $df = 3$, $p = 0.60$; and $\chi^2 = 0.103$, $df = 1$, $p = 0.75$ respectively). For that reason the studied group, based on the aforementioned properties, could be regarded as a representative group of total IgG population analyzed.

Sialic acid, galactose, and *N*-acetylglucosamine expression was estimated by reactivity of their heavy and light chains IgG with SNA, GS II and RCA I lectins, using the lectin blotting technique (Figure 5).

The obtained results showed that all γ heavy chains, 17 κ , and 6 λ light chains were reactive with at least

one of three lectins. The number of human heavy and light chains reactive with RCA I, GS II and SNA lectins as well as the mean intensity of their reactivity, was shown in Table 2. In the analyzed group of monoclonal IgG, the level of expression of reactivity with these three lectins was not dependant on heavy chain subclasses and light chain type (Table 2).

Of the 34 human monoclonal IgG, 18 (16 of IgG1 and 2 of IgG2) passed through the column, and the other 15 remained bound to the anion exchanger. Of all the attached IgG, 10 (6 of IgG1, 1 of IgG2, 2 of IgG3 and 1 of IgG4) were eluted with 0.03–0.19 M buffer, and 6 (all of IgG1 subclass) were eluted with 2.0–5.0 M Tris-HCl/NaCl buffer. The chromatographic distribution of this group did not significantly differ from the chromatographic distribution of all 92 analyzed monoclonal IgG ($\chi^2 = 3.865$, $df = 2$, $p = 0.15$), and the studied group could be, based on its chromatographic distribution, regarded as a representative group of total IgG analyzed.

The chromatographic properties of analyzed human monoclonal IgG were not dependent on level of expression and localization of galactose, and *N*-acetylgluco-

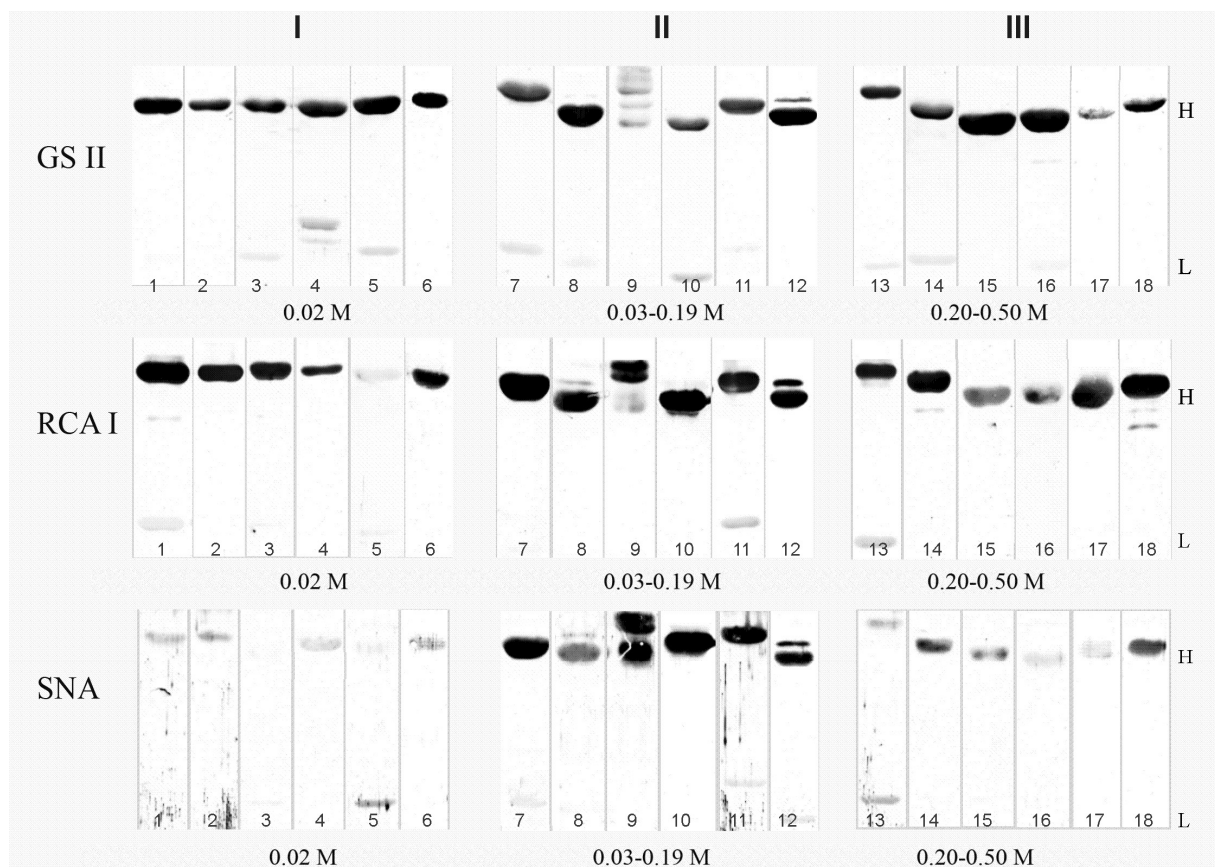


Figure 5. Reactivity of human monoclonal IgG with RCA I, GS II and SNA lectins. Groups of IgG molecules were formed based on their chromatographic distribution. Group I) monoclonal IgG that did not bind to Q Sepharose Fast Flow anion exchanger (lines 1–6): 1 – IgG1 κ , 2 – IgG1 λ , 3 – IgG1 κ , 4 – IgG1 λ , 5 – IgG1 λ , 6 – IgG1 κ . Group II) monoclonal IgG weakly bound to Q Sepharose Fast Flow and eluted with 0.03–0.19 M Tris-HCl pH 7.5/NaCl buffer (lines 7–12): 7 – IgG1 κ , 8 – IgG4 λ , 9 – IgG3 κ , 10 – IgG1 κ , 11 – IgG1 λ , 12 – IgG1 κ . Group III) monoclonal IgG strongly bound to Q Sepharose Fast Flow and eluted with 0.20–0.50 M Tris-HCl pH 7.5/NaCl buffer (lines 13–18): 13 – IgG1 κ , 14 – IgG1 κ , 15 – IgG1 κ , 16 – IgG1 κ , 17 – IgG1 λ , 18 – IgG1 λ .

Table 2. Reactivity of human monoclonal IgG heavy and light chains with RCA I, GS II and SNA lectins

Light chain	Total	RCA I reactive		GS II reactive		SNA reactive	
	No.	No.	Intensity ^a	No.	Intensity	No.	Intensity
γ	34	34	4310±2990	32	2750±1590	33	1750±1780
γ1	29	29	4520±3620	27	2820±1750	28	1450±1390
γ2	3	3	1680±820	3	2160±210	3	1500±1350
γ3	2	2	5390±20	2	2530±70	2	5970±3050
γ4	1	1	4210	1	3140	1	2160
κ	20	11	910±950	12	320±210	11	700±1220
λ	14	2	1210±490	6	270±90	2	70±10

^aExpressed as number of pixel (mean ± SD) obtained by densitometric quantification of lectin reactive bands

samine, but they were significantly dependent on the level of expression of sialic acid on γ heavy chains. Monoclonal IgG with a statistically significant higher level of sialic acid expression on the heavy chains were weakly bound to the exchanger and eluted with 0.03–1.9 M elution buffer (Figure 5 and Table 3). Within this range of molarity of buffer, all IgG subclasses were eluted. With an aim to eliminate the possible influence of different IgG subclasses on these results, we analyzed the influence of sialic acid expression on chromatographic properties of monoclonal IgG1 only. The results showed that the chromatographic properties of monoclonal IgG1 were, identical to the total monoclonal IgG analyzed, dependent on heavy chain sialic acid expression (Table 3). Whether the sialic acid was located on V or CH2 domain of γ heavy chain of analyzed monoclonal IgG it is not known, and it could be determined only after fragmentation of IgG to Fab and

Fc regions.

Similarly to our results, Bond and coworkers (1993) showed that polyclonal human IgG, which did not bind to DEAE cellulose anion exchanger, contained more sialic acid, than adsorbed IgG [24]. These data can be important knowing that a higher level of sialylation of IgG Fc regions results in lower affinity to Fcγ receptors, and a reduction in ADCC activity [27,28]. In addition, it was shown that anti-inflammatory activity of therapeutic human IgG is mediated by a small fraction that expresses sialic acid [27]. The isolation of molecules enriched or impoverished in sialic acid can result in an enhancement of effector functions of therapeutic IgG.

CONCLUSION

Preparative low-pressure Q Sepharose Fast Flow anion-exchange chromatography was used for fractionation of normal human sera or human sera with

Table 3. Intensity of reactivity of human monoclonal IgG heavy (γ) chains, grouped according to their chromatographic distribution, with RCA I, GS II and SNA lectins. Molarity of elution represents Tris/NaCl buffer molarity at which maximum of elution of monoclonal IgG was obtained. The intensity of reactivity in lectin blot was expressed as number of pixel (mean ± SD) obtained by densitometric quantification of lectin reactive bands

Parameter	IgG			IgG1		
	Molarity of elution					
	0.02 (n = 18)	0.03–0.19 (n = 10)	0.20–0.50 (n = 6)	0.02 (n = 16)	0.03–0.19 (n = 6)	0.20–0.50 (n = 6)
	GS II					
Mean ± SD	3112±1790	2354±1243	2311±1364	3223±1884	2199 ± 1679	2311 ± 1364
Min	574	202	746	574	202	746
Max	7253	4809	4217	7253	4809	4217
	RCA I					
Mean ± SD	4510±3480	4270±2433	3978±2978	4788±3584	4540 ± 2832	3471 ± 2337
Min	641	745	2012	641	2302	2012
Max	11596	9448	8369	11596	9448	8369
	SNA					
Mean ± SD	1036±983***	3461±2092	919±891*	981±9073***	3461 ± 2092	919 ± 891*
Min	72	1326	216	72	1326	216
Max	2777	8120	2373	2558	4822	2373
p-Value	***0.0004		*0.02	***0.0007		*0.02

monoclonal IgG. It was showed that, using 20 mM Tris-HCl, pH 7.5 as a start buffer, and elution by linearly increasing concentration of NaCl from 0.0 to 0.2 M, the major part (70%) of polyclonal IgG from the normal sera can be isolated in pure form. Human monoclonal IgG, with the structure and solubility of normal human IgG, were used as a model in the study of chromatographic behavior of particular molecular form of human IgG. The results have shown that only monoclonal IgG1 and IgG2 pass through column without interaction with the exchanger, and can be isolated in pure form. All IgG subclasses were found among IgG weakly bound to Q Sepharose Fast Flow (eluted with 0.03–0.19 M NaCl/20 mM Tris-HCl, pH 7.5 buffer). Strongly bound monoclonal IgG were of IgG1 and IgG4 subclasses. The chromatographic distribution of analyzed human monoclonal IgG was not dependent on the level of expression galactose, and *N*-acetylglucosamine, but it was significantly dependent on the level of expression of sialic acid on γ heavy chains. Monoclonal IgG with a statistically significant higher level of sialic acid expression on the heavy chains were weakly bound to the exchanger and eluted with 0.03–1.9 M elution buffer. We estimate that obtained pattern of chromatographic distribution might be used for the prediction of chromatographic behavior of the specific form of monoclonal IgG. We also believe that this data provide information about the possibility of using Q Sepharose Fast Flow matrix for the isolation of specific molecular forms of human IgG.

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ИЗВОД

ПОДКЛАСА ТЕШКИХ ЛАНАЦА И НИВО ЕКСПРЕСИЈЕ СИЈАЛИНСКЕ КИСЕЛИНЕ НА ЊИМА ОДРЕЂУЈУ ХРОМАТОГРАФСКУ РАСПОДЕЛУ ХУМАНИХ МОНОКЛОНСКИХ ИМУНОГЛОБУЛИНА Г

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Због велике молекулске хетерогености хуманих имуноглобулина Г (IgG) (4 изотипа, 20 алотипова и преко 600 могућих гликоформи) не постоји јединствен протокол за изоловање свих молекулских форми IgG. Јоноизмењивачка хроматографија је широко прихваћен метод за изоловање и пречишћавање хуманих IgG, пре свега због чињенице да се сепарација одвија у благим условима, што поред ефикасног пречишћавања омогућава да структура и функција IgG као антитела остане очувана. За изоловање хуманих IgG могу се користити и слаби и јаки анјонски измењивачи. Међутим, који ће од бројних матрикса који данас постоје на тржишту бити одговарајући за изоловање специфичних молекулских форми хуманих IgG, може се знати само након тестирања. У овом раду, хумани моноклонски IgG из серума болесника са моноклонским гамапатијама су изоловани препаративном хроматографијом на *Q Sepharose Fast Flow* анјонском измењивачком матриксу. Када је као стартни пуфер коришћен 20 mM Tris-HCl, pH 7,5, 42% анализираних моноклонских IgG није се везивало за матрикс и било је могуће изоловати их у чистој форми. Преосталих 58% моноклонских IgG, различитим интензитетом везивали су се за матрикс, и са њега су елуирани 0,09–0,43 M Tris, pH 7,5/NaCl. Хроматографска расподела моноклонских IgG је била у корелацији са њиховом електрофоретском покретљивости у гелу агарозе и подкласом у тешког ланца, а није била одређена типом лаког ланца и серумском концентрацијом моноклонског IgG. Ниво експресије сијалинске киселине на тешким ланцима је значајно утицао на хроматографску расподелу хуманих моноклонских IgG, док је ниво експресије друга два терминална шећера, галактозе и N-ацетилглюкозамина, био без утицаја. Сматрамо да приказани резултати могу бити од користити у креирању протокола за изоловање хуманих моноклонских IgG јер поред тога што дају информације о њиховом хроматографском понашању дају и информацију о могућности коришћења *Q Sepharose Fast Flow* анјонског матрикса за изоловање појединих молекулских форми хуманих IgG.

Кључне речи: Хумани моноклонски IgG
• Анјонска измењивачка хроматографија
• Подкласе IgG
• Сијалинска киселина