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Galactosylation of IgG from rheumatoid arthritis (RA) patients – changes during therapy

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Abstract It is well documented that serum IgG from rheumatoid arthritis (RA) patients exhibits decreased galactosylation of its conservative N-glycans (Asn-297) in CH2 domains of the heavy chains; it has been shown that this agalactosylation is proportional to disease severity. In the present investigation we analyzed galactosylation of IgG derived from the patients using a modified ELISA-plate test, biosensor BIAcore and total sugar analysis (GC-MS). For ELISA and BIAcore the binding of IgG preparations, purified from the patients' sera, to two lectins: *Ricinus communis* (RCA-I) and *Griffonia simplicifolia* (GSL-II) was applied. Based on ELISA-plate test an *agalactosylation factor* (AF, a relative ratio of GSL-II/RCA-I binding) was calculated, which was proportional to actual disease severity. Repeated testing of several patients before and after treatment with methotrexate (MTX) alone or in combination with Remicade (a chimeric antibody anti-TNF-α) supplied results indicating an increase of IgG galactosylation during the treatment. This introductory observation suggests that IgG galactosylation may be an additional indicator of the RA patients' improvement.

Keywords IgG · Rheumatoid arthritis · Surface plasmon resonance · *Ricinus communis* lectin · *Griffonia simplicifolia* lectin

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Abbreviations

IgG	immunoglobulin G
RA	rheumatoid arthritis
RCA	lectin from *Ricinus communis*
PVL	lectin from *Psathyrella velutina*
GSL	lectin from *Griffonia simplicifolia*
ESR	erythrocyte sedimentation rate
CRP	C-reactive protein
GC-MS	gas chromatography-mass spectrometry
MTX	methotrexate
NC	nitrocellulose
DTT	dithiotreitol
AF	agalactosylation factor

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune, inflammatory disease that affects mainly the diarthrodial joints [1]. The disease affects about 1% of white population and may occur at any age, but the patients are most commonly between 40–70 years old. Rheumatoid arthritis is still a subject for biochemical investigations directed, among others, towards defining the best biochemical marker for its diagnosis and towards establishing the correlation between modes of treatment and improvement of the clinical symptoms of the patients.

Human IgG is a glycoprotein with approx. 2.4 oligosaccharides per molecule [2]. Most of the carbohydrates are present in the Fc fragment (2 oligosaccharides in one Fc fragment) at the conserved N-glycosylation sites (Asn-297) of both heavy chains; the remaining sugars are present in the Fab fragment of IgG and their position is random. Conserved N-glycans in IgG are poorly sialylated (only *ca* 25% of the available positions are occupied by sialic acid) and it has

been observed that in rheumatoid arthritis these carbohydrate chains lack some of the terminal galactose residues; percentage of the N-glycans completely lacking galactoses (fraction G[0]) is closely correlated with a severity of the disease.

The routine procedure in clinical practice to follow the disease is to determine in RA patients' serum the anti-IgG antibodies, which is called a *rheumatoid factor*. Although the rheumatoid factor is a popular immunologic hallmark of RA, it has a modest disease specificity (up to 66% of the cases). As far as the published literature is concerned there is no data available on the correlation between effectiveness in RA treatment and IgG galactosylation improvement. To approach this problem we decided to compare various methods of determination of IgG galactosylation to follow the galactosylation status of IgG from RA patients under clinical treatment. Our introductory results obtained for several patients treated with methotrexate, a traditional therapeutic agent used in RA or with a combination of methotrexate and Remicade (a monoclonal anti-TNF-α antibody, also called infliximab) indicated the improvement of IgG galactosylation during therapy of RA patients.

Materials and methods

Chemicals

BIAcore sensor chips CM5, amine coupling kit containing N-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(3-dimetylaminopropyl) carbodiimide (EDC) and ethanolamine hydrochloride were obtained from Biacore International, AB (Uppsala, Sweden). The working buffer HBS (10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA, BIAcore P20 surfactant 0.05% (v/v), pH 7.4) was prepared according to the manufacturer's prescription. Trifluoroacetic acid (TFA), sodium borohydride, BCIP and NBT were from Fluka (Switzerland), pyridine and xylose were from Merck (Germany). Protein A-Sepharose, 2-mercaptoethanol, bovine serum albumin (BSA), acetic anhydride, ExtrAvidin–alkaline phosphatase conjugate, anti-human IgG (γ-chain specific) alkaline phosphatase conjugate (product no. A 3187), *p*-nitrophenylphosphate and *Ricinus communis* agglutinin I (RCA-I) were purchased from Sigma (USA). *Psathyrella velutina* lectin (PVL) was obtained from WAKO (Germany) and *Griffonia simplicifolia* II (GSL-II) was from Vector Laboratories Inc. (USA); the lectins were biotinylated as described [3]. Maxisorp microtiter ELISA-plates were from Dako (Denmark). β-Galactosidase from *Charonia lampas* was purchased from Seikagaku Kogyo Co. Ltd (Japan).

Specimens

Serum samples of RA patients were obtained from Rheumatology Department under the permission of the Commission of Bioethics (Medical University, Wroclaw, Poland); the patients between 25–79 years old (mean 52) were diagnosed by the criteria recommended by American College of Rheumatology [4]. They were treated, during present investigation, with methotrexate alone or with methotrexate/Remicade.

Isolation of IgG

Immunoglobulin G was isolated from the serum samples by affinity chromatography on Protein A-Sepharose column [5]. Each sample was diluted 1:1 with 50 mM TBS, pH 8.0 and centrifuged 15 min. at 1400 $\times$ g. The solution was applied on the column (5 ml) and washed using TBS. IgG, retained on the column, was eluted with 0.1 M glycine-HCl, pH 3.4 and was immediately neutralized with 1 M TRIS; to estimate the elution profile the content of IgG was monitored by measuring the absorbance at 280 nm. The selected fractions were analysed for the presence of IgG using SDS-PAGE [6] and staining, according to Schaffner *et al*. [7].

Reduction of IgG disulfide bonds

Reduction was performed according to a previously described procedure [8]. Purified IgG fraction was diluted with 50 mM Tris-HCl, pH 8.0 to a concentration 1 $\div$ 2 mg/ml. Nine volumes of IgG solution were mixed with one volume of 1 M 2-mercaptoethanol in 50 mM Tris-HCl, pH 8.0 and incubated at 37°C for 2 h. Generated sulfhydryl groups were blocked with iodoacetamide, added directly to the solution to a final concentration 0.1 M; incubation was performed overnight at room temperature. Reduced IgG was dialyzed to the relevant solution.

Gas chromatography–mass spectrometry (GC-MS)

The monosaccharide composition of the oligosaccharides was estimated by GC-MS. Samples containing 300 μg IgG (as determined for protein by BCA method) were hydrolysed in 4 M trifluoroacetic acid (TFA) at 100°C for 4 h, cooled and supplemented with xylose as an internal standard, evaporated to dryness and neutralized by Dowex AG3-X4A(OH^-) resin. The generated monosaccharides were converted into corresponding alditol acetates [9] and analysed in a Hewlett-Packard 5890 gas chromatograph, equipped with the HP-1 fused-silica capillary column (0.2 mm $\times$ 12.5 m) and a mass detector 5971A. A temperature program 150–230°C, 8°C/min, was used and quantification of sugars was based on the total ion current (TIC). The sugar-containing peaks were identified by comparing with the mass spectra of the standards.

Western blotting

Purified IgG (5 μg) was dissolved in a sample buffer (2% SDS, 10% glycerol, 5% 2-ME, 0.06 M Tris and 0.025% bromophenol blue), heated at 100°C for 5 min. and subjected to SDS-PAGE [6] using 12.5% acrylamide gel under reducing conditions. Then the proteins were electrophoretically transferred onto nitrocellulose (NC) using a semi-dry blotting system [10]. NC membranes with blotted proteins were treated at 4°C overnight with 5% BSA. For the specific staining the membranes were incubated in the solutions (TBS-T, pH 7.4, containing 1% HSA and 1 mM ions: Ca^{2+}, Mg^{2+}, Mn^{2+}) with the following biotinylated lectins: RCA-I (*Ricinus communis*, 0.5 μg/ml), GSL-II (*Griffonia simplicifolia*, 1.0 μg/ml) and PVL (*Psathyrella velutina*, 2.0 μg/ml). The membranes were washed three times with TBS-T as above, ExtrAvidin-AP conjugate was added and incubated for 1 h. The membranes were then washed four times in TBS-T and the bound lectins were visualized by the addition of AP substrate solution (Sigma 104 phosphatase substrate tablets), containing 1 mM $MgCl_2$.

Preparation of IgG G(0) fraction

A sample of IgG (1 mg), from a healthy individual, in 1 ml of 0.1 M acetate buffer, pH 5.0, was digested with 200 mU neuraminidase at 37°C for 24 h under toluene vapour. After completing the incubation, environment was changed (0.1 M citric acid, pH 4.0, 0.5 M NaCl) and 100 μl of β-galactosidase (specific activity 0.1 U/2.5 ml) was added; incubation proceeded at 37°C for three days. Then the sample was dialyzed against 0.1 M phosphate buffer, pH 7.0; asialo-agalacto IgG was purified on the affinity column with protein A, as described above.

ELISA-plate test

Serum IgG galactosylation was measured using an ELISA-plate test, separately with two lectins: *Ricinus communis* (RCA-I) and *Griffonia simplicifolia* (GSL-II). All reagents were added in a volume of 50 μl/well; washing of the plates was done with TBS-T (10 mM Tris-HCl, containing 1% Tween 20) of pH 7.4 and all the steps, if not stated otherwise, were performed at room temperature. The IgG sample purified from the pooled serum samples of 6 healthy persons served as a control. Triplicate wells for one sample on a microplate were coated, each with 0.5 μg of IgG purified from sera of RA patients or healthy persons, in PBS, pH 7.4 at 4°C overnight. The plate was washed 3× with TBS (10 mM Tris-HCl, pH 7.4) and IgG was reduced directly on the plate by incubation with dithiotreitol (2 mg/ml) in 0.1 M Tris-HCl, pH 8.0 at 37°C for 70 min. Then the plate was overlayed with the biotinylated lectin: RCA-I (1 μg/ml in TBS-T, pH 7.4) or GSL-II (5 μg/ml in TBS-T, pH 7.8) for 90 min; both lectins were used in the presence of 1 mM ions Ca^{2+}, Mg^{2+}, Mn^{2+} and 1% BSA. After washing, ExtrAvidin-AP solution diluted with TBS-T was added to the wells for 1 h. Finally, a substrate for AP dissolved in 0.05 M carbonate buffer, pH 9.6, containing 1 mM $MgCl_2$, was added and an absorbance was read after 40 min. at 405 nm. The relative binding of GSL-II and RCA-I was expressed as the absorbance given by the tested sample, divided by the absorbance of the control sample; the ratio of relative GSL-II/RCA-I binding was termed as *agalactosylation factor* (AF). The amount of IgG samples, coated in individual wells, was monitored simultaneously in a separate ELISA test, using an anti-human IgG antibody, conjugated with AP and was found to be almost identical in all wells.

SPR analysis

Analyses in a biosensor BIAcore-1000 (Biacore AB, Uppsala, Sweden) were performed at 25°C. Immobilization of RCA-I (0.01 M acetate buffer, pH 4.0) and GSL-II (0.01 M acetate buffer, pH 4.5) to the dextran matrix on CM5 sensor chip was performed using the general procedure for amine groups, as described [11], at a flow rate 5 μl/min. Immobilization levels for RCA-I and GSL-II were 5800 and 5300 resonance units (RU), respectively. TBS containing 1 mM Ca^{2+}, Mg^{2+} and Mn^{2+} cations was used as a running buffer for RCA-I (pH 7.4) and GSL-II (pH 7.8). For binding experiments the reduced IgG samples were dissolved in 1 M NaCl containing divalent cations as above at 200 μg/ml (for RCA-I) and 100 μg/ml (for GSL-II), the binding was measured for 5 min. at a flow rate 60 μl/min. The working channel with GSL-II was regenerated by 1-min. injection of 2.5 M guanidine hydrochloride at 60 μl/min; the RCA-I surface was regenerated using 0.01 M HCl at 20 μl/min for 2 min.

Results

Binding of three lectins to IgG samples on the blot

IgG samples, isolated from the sera of RA patients, were run in SDS-PAGE under reducing conditions, then were transferred onto nitrocellulose and overlayed with three biotinylated lectins: *Ricinus communis* (RCA-I, specific for the terminal Gal residues [12]); *Griffonia simplicifolia* (GSL-II) [13] and *Psathyrella velutina* (PVL) [14]–both lectins recognize the terminal GlcNAc, but PVL is less specific and reacts also with sialic acid residues; the results obtained for six randomly chosen IgG samples are presented (Figure 1). As can be seen, either lectin reacted only with heavy chains of the analysed IgG samples. The reaction with RCA-I lectin was reciprocal to the reaction with two other lectins:

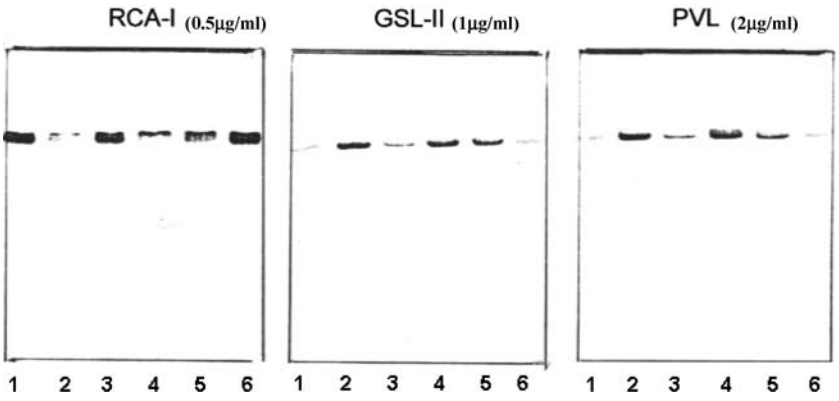

Fig. 1 Electroblotting of six example IgG samples, derived from the RA patients' sera; SDS-PAGE was performed under reducing conditions. The nitrocellulose sheets were overlayed with three biotinylated lectins, as indicated.

GSL-II and PVL, indicating that decreased galactosylation was accompanied by the increased exposure of terminal GlcNAc residues. This experiment clearly showed that the used lectins are suitable to determine terminal Gal (RCA-I) and terminal GlcNAc (GSL-II) residues, therefore, they were used in ELISA-plate test.

ELISA-plate test of IgG samples from RA patients

IgG samples were coated on the ELISA microplates, reduced using dithiotreitol (DTT) and overlayed separately with RCA-I and GSL-II lectins (Figure 2). The dots representing binding of both lectins to IgG samples from RA patients are moved to the left upper corner of the diagram, as compared with the samples of IgG from the healthy individuals; this is an indication of the decreased galactosylation of IgG from RA patients. Four IgG samples, presented in the frame, exhibited low reaction with RCA-I and the highest reaction with GSL-II, even higher than IgG(G0) sample, which corresponds to the highest degree of agalactosylation among the studied samples.

To facilitate a comparison of agalactosylation status of individual IgG samples, the reaction of both lectins with each IgG sample was recalculated as a ratio of relative binding GSL-II/RCA-I and was named *agalactosylation factor* (AF). Accordingly, the AFs were calculated for 42 IgG samples from RA patients and 8 samples from healthy people (Figure 3). The AF of the control sample (pooled sera from six healthy persons) was set to 1.0; the AFs of IgG samples from healthy individuals were close to 1. For IgG samples from the RA patients the AF values were distributed between the range of more than 6 and a little less than 1; a half of the tested IgG samples from RA patients showed the AF values ≥ 1.5.

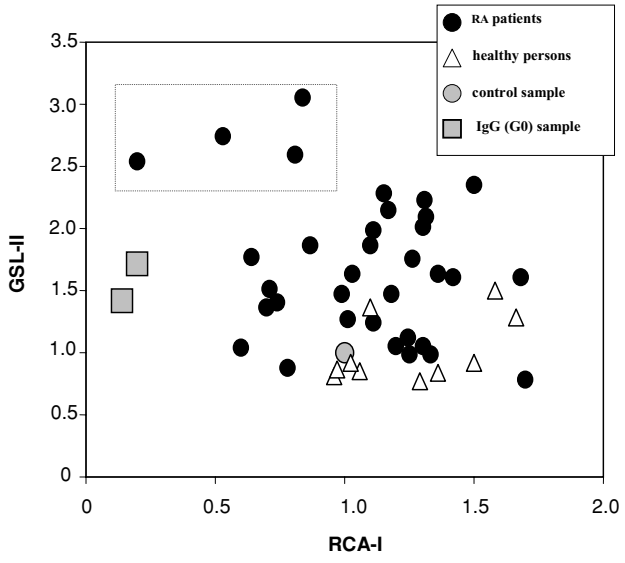

Fig. 2 ELISA-plate test; IgG samples reacted with two lectins: RCA-I and GSL-II. Four samples, included in the frame, exhibit the highest agalactosylation status.

ELISA-plate test of IgG from RA patients during therapy

In the separate set of experiments with ELISA-plate test, using the same two lectins as above, the IgG samples from RA patients during therapy were analysed, Table 1. The patients were treated with MTX alone or with MTX/Remicade. The blood samples were collected from the same patient at the beginning of treatment and after time intervals ranging from 1.5 to 10 months. The determined AF values were compared with the typical clinical parameters, such as erythrocyte sedimentation rate (ESR) and serum concentration of C-reactive protein (CRP), determined in the same samples (Table 1). The AF values decreased during therapy in 7 out of 11 patients and it correlated with a concomitant decrease of initially elevated clinical parameters and with general improvement of patients' health. The AF values of the remaining 4 patients were relatively less elevated at the beginning of treatment and then slightly increased which was accompanied by an increase or lack of improvement of both or at least one of the

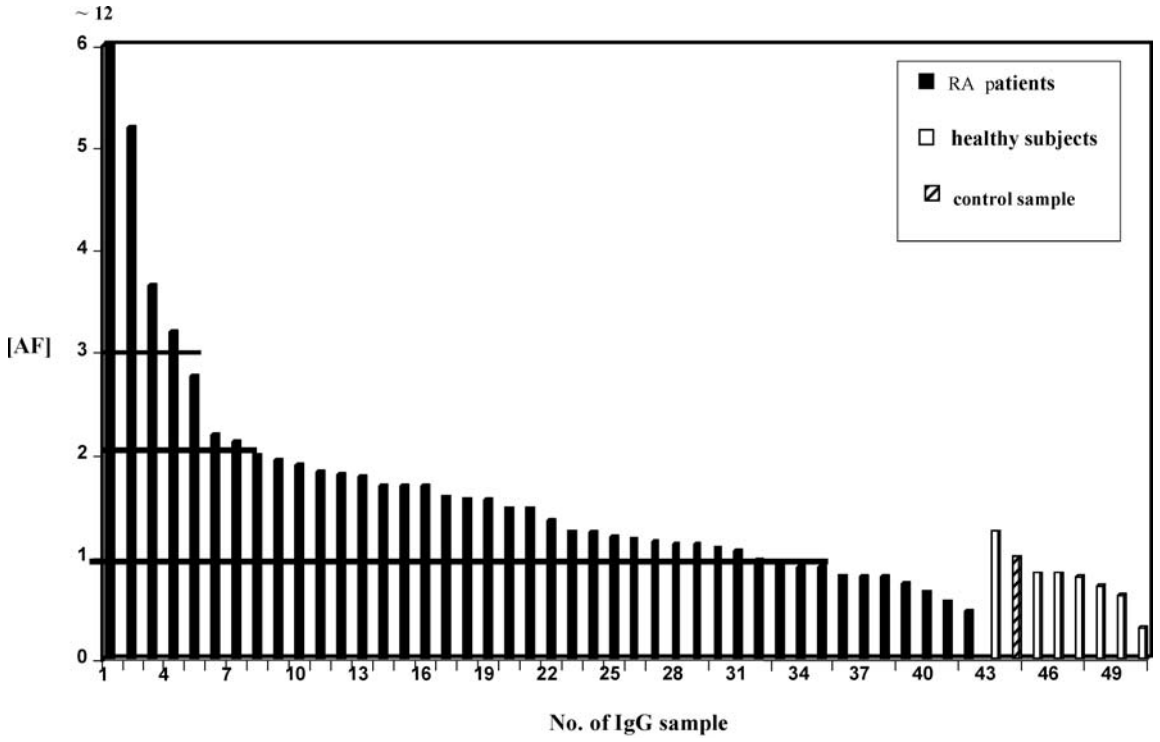

Fig. 3 ELISA-plate test; the *agalactosylation factor* (AF) calculated for the IgG samples from RA patients (see the main text for a calculation methodology of this factor). AF for the control IgG sample was set to 1.0.

clinical parameters. These results indicate that 1° IgG galactosylation status is generally lower in RA patients (higher AF values) and is accompanied by the significantly elevated ESR and CRP concentration and 2° it is improved during successful treatment (lower AF values).

Interaction of IgG with lectins, measured by SPR technique

To confirm the results of ELISA method and to get insight into a possibility to measure the interactions of IgG and lectins by an advanced instrumental method, a biosensor BIAcore-1000 was used, equipped with a surface plasmon resonance (SPR) detector. In the course of experiments two separate working channels with two ligands were used: one with RCA-I lectin and the other with GSL-II lectin, respectively. In Figure 4 an example of binding of IgG samples from the patient BD (from Table 1), is shown. Three consecutive IgG samples from this patient, undergoing combined therapy with methotrexate/Remicade, showed an increasing reaction with RCA-I lectin (Figure 4 part **A**) with concomitant decreasing reaction with GSL-II (Figure 4 part **B**). Both interactions with two lectins are in accordance with increasing IgG galactosylation during applied therapy. Similar changes were observed for IgG samples from the other patients. Interactions of IgG samples from four randomly chosen patients together with two healthy individuals are presented in Figure 5.

Correlation of biosensor data and galactose content

Nineteen IgG samples were assayed with GSL-II lectin in biosensor BIAcore and concomitant sugar analyses, using GC-MS method, were performed. The N-glycans on IgG molecules are the complex-type biantennary structures, containing three mannose residues in oligosaccharide core. It is known that mannose content in IgG is not affected by RA status [15]; therefore, we calculated the number of galactose residues per three mannoses in IgG samples and created a correlation between these two types of analyses. The results in Figure 6 show that a higher content of galactose in IgG correlated with a weaker reaction with GSL-II lectin, which fits well to the described above results regarding ELISA-plate test and biosensor BIAcore.

Discussion

It is obvious that the best method to determine IgG galactosylation would be quantitation of individual oligosaccharides and their regular structural analysis [16–18]. This method, however, is not applicable to determine IgG

Table 1 Comparison of clinical parameters: erythrocyte sedimentation rate (ESR) and concentration in serum of C-reactive protein (CRP) with IgG agalactosylation factor (AF) for the patients who were tested at least two times in the course of treatment.

Patient	Age [years]	Days[a]	ESR	CRP[b] [mg/dl]	AF
MZ[c]	26	0	60	1.2	9.3
		89	14↓	1.2	4.1↓
DB	49	0	110	9.0	15.9
		100	82 ↓		5.9 ↓
		173	71 ↓	5.9 ↓	2.6 ↓
GS	45	0	51		4.0
		191	37 ↓	1.9	2.4 ↓
		303	27 ↓	0.2 ↓	0.9 ↓
HW	49	0	90	12.3	3.9
		98	20 ↓	0.3 ↓	2.0 ↓
DJ	27	0	41	8.5	1.8
		78	11 ↓	1.5 ↓	1.5 ↓
HB	61	0	80	5.4	3.4
		71	31 ↓	0.5 ↓	1.5 ↓
ED	45	0	22	0.5	1.5
		58	14 ↓	1.0	1.0 ↓
DB	41	0	10	1.2	1.5
		275	34 ↑	3.7 ↑	1.7 ↑
EM	65	0	100	17.5	1.8
		139	100	10.5 ↓	3.3 ↑
HL-V	40	0	20	1.4	1.2
		60	22	1.9 ↑	1.3
TK	53	0	30	2.8	1.2
		47	41 ↑	1.6 ↓	1.5 ↑

[a] Time intervals between the first (0) and next measurements.
[b] Physiological range 1–5 mg/dl.
[c] Patient treated with MTX alone, other patients were treated with MTX/Remicade. Arrows indicate a decrease (↓) or increase (↑) of the studied parameters.

galactosylation in multiple serum samples of RA patients. Therefore, the most frequently method used is an analysis of interaction of IgG samples with the lectins recognizing terminal Gal residues (RCA-I) and GlcNAc residues (GSL-II, PVL). Various methods of determination of this interaction were reported, including fractionation of IgG-derived oligosaccharides on immobilized lectin columns [19], dot-blotting [20,21], SDS-PAGE combined with lectinoblotting [22], microtiter plate lectin-binding assay [23] or surface plasmon resonance [11]. In the present investigation we decided to use ELISA-plate test and, at the beginning, we focused on the selection of the optimal experimental conditions. The following modifications were introduced to increase the sensitivity of the method and reproducibility of the results: 1° the untreated plates were coated directly with the purified IgG samples, instead of IgG binding from diluted sera onto Protein A or Protein G-coated plates; 2° bound IgG was reduced, with dithiotreitol, directly in the wells; this procedure was less time-consuming and gave a higher lectin binding as compared with reduction in a solution before coating; 3° two biotinylated lectins were used: RCA-I, specific for terminal galactose and GSL-II, specific for terminal *N*-acetylglucosamine; the results were expressed as a relative *agalactosylation factor* (AF). The GSL-II lectin was chosen for ELISA-plate test to detect terminal GlcNAc residues, because PVL, which reacted with IgG on the blots similarly to GSL-II (Figure 1), gave a weaker and less reproducible binding to the coated plates.

The results shown in Figure 2 demonstrated that using two lectins in ELISA-plate test, instead of one lectin, is more informative, because some samples showed an increased both GSL-II and RCA-I binding, and *vice versa*. Moreover, two enzymatically desialylated and degalactosylated IgG samples (G0) showed the lowest RCA-I binding, but not the highest GSL-II binding. One of the possible reasons for these discrepancies may be a microheterogeneity of IgG N-glycans and the second–a possible presence of additional N-glycans in the Fab fragments. Concerning the relatively low GSL-II binding to IgG (G0) samples it cannot be ruled out that one of the commercial glycosidases used was contaminated with β-*N*-acetylhexosaminidase, which gave a partial release of GlcNAc residues.

The selected IgG samples were also tested by other methods, such as binding to lectins in a biosensor BIAcore and determination of total carbohydrate composition by GC-MS. Finally, the modified ELISA-plate test was shown to be useful for monitoring multiple serum samples from RA patients and a correlation was observed between the results regarding galactosylation status of IgG, obtained by two different methods.

In this paper we confirmed that IgG galactosylation was significantly decreased in most RA patients studied, but extent of this phenomenon was greatly differentiated (Figures. 1 and 2), similarly to other studies [16–18]. It has been reported that degree of IgG galactosylation in RA patients depends on the stage of disease development, and may serve as a prognostic factor [22,24]. A general question arises how useful may be an analysis of IgG galactosylation for monitoring the RA patients during therapy. We attempted to approach this problem by testing IgG from several RA patients under treatment with MTX and with a combined treatment MTX/Remicade [25]. After therapy (the period of 1.5–10 months) a decreased AF was found for IgG from seven patients, which correlated with improvement of the clinical parameters (ESR, CRP concentration); this effect was particularly pronounced in the patients with a high initial AF value (Table 1). Therefore, we have shown for the first time that successful, from the clinical point of view, treatment of the RA patients concomitantly improves the galactosylation status of serum IgG, i.e. it influences the biochemical marker of this disease. The results obtained in the present investigation seem to be promising; they indicate that analysis

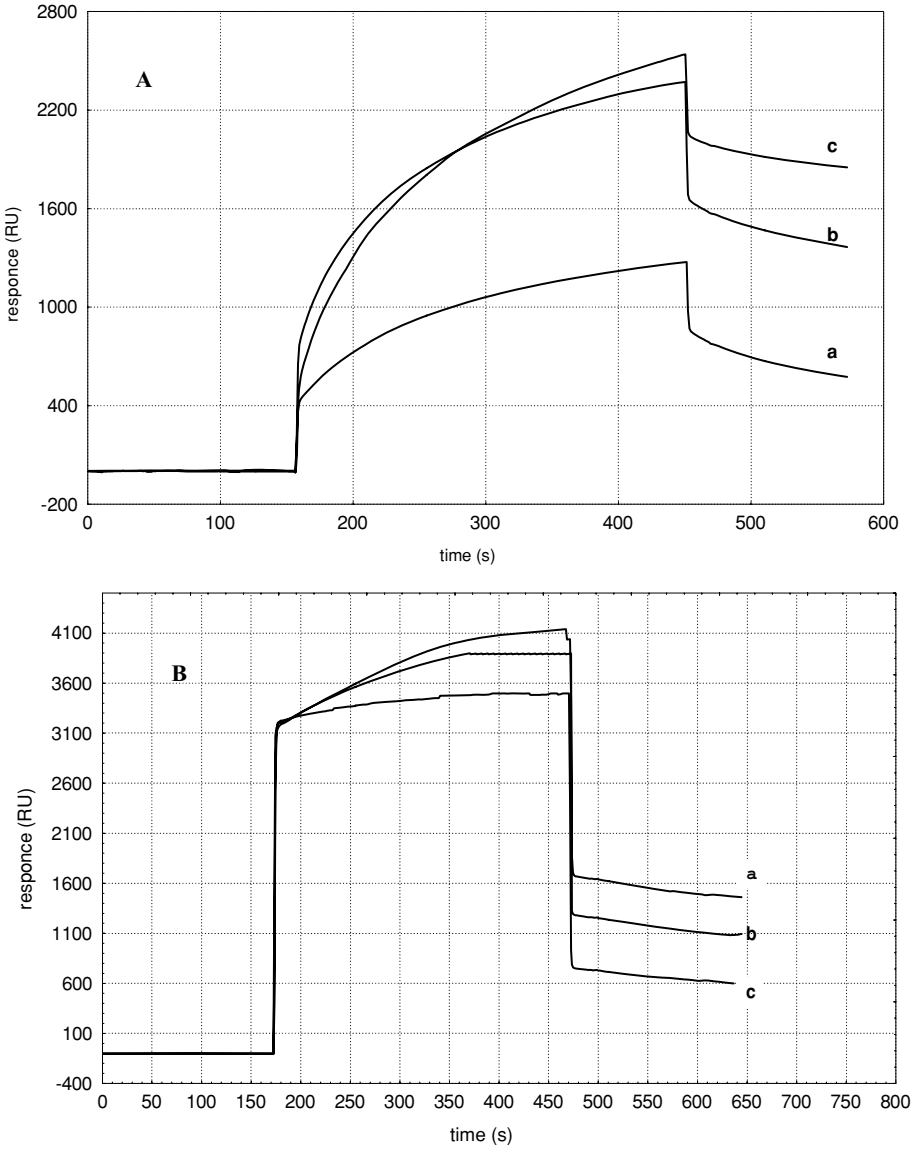

Fig. 4 Biosensor BIAcore; interaction of IgG samples from the patient no. 2 (depicted in Figure 4, treated with MTX/Remicade), with RCA-I (part **A**) and GSL-II (part **B**). a—patient's serum before treatment; b—patient's serum after 3 months treatment; c—patient's serum after 6 months treatment.

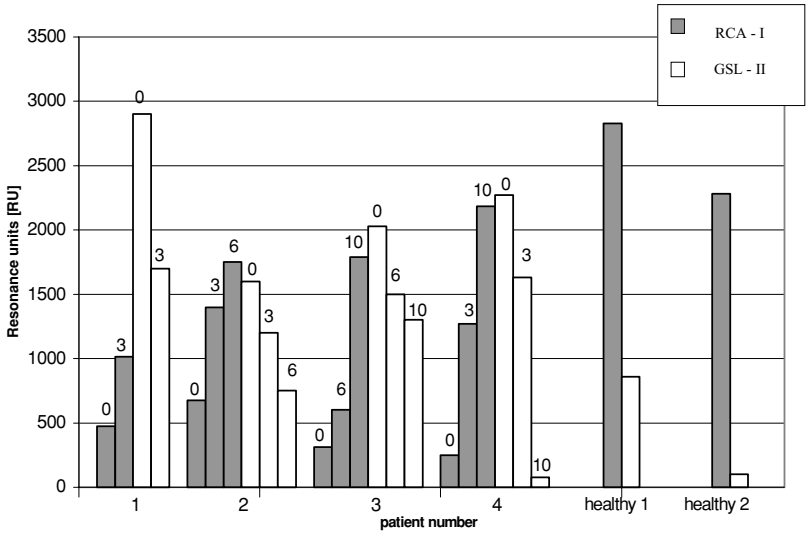

Fig. 5 Biosensor BIAcore; interaction of IgG samples, derived from four RA patients treated with MTX/Remicade, with two lectins as indicated.

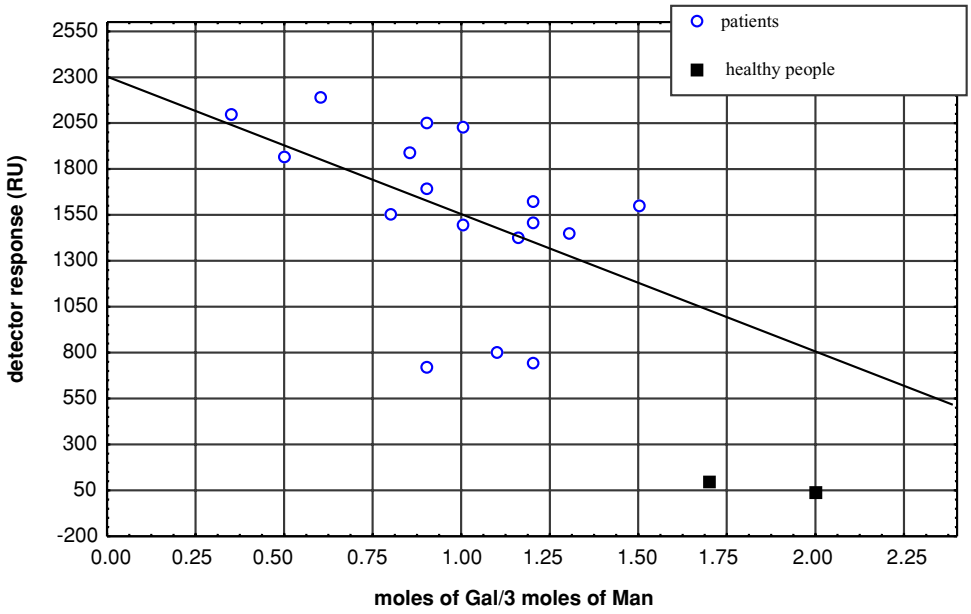

Fig. 6 Correlation between galactose content in IgG samples from RA patients (determined by GC-MS) and IgG reaction with GSL-II, measured in biosensor BIAcore.

of IgG galactosylation in RA patients may have a clinical relevance.

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