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Analysis of agalacto-IgG in rheumatoid arthritis using surface plasmon resonance

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It is well established that IgG from rheumatoid arthritis (RA) patients are less galactosylated than IgG from normal individuals. Determination of agalacto-IgG may therefore aid in diagnosis and treatment of RA. The decrease in galactosylation of IgG leads to an increase in terminal N-acetylglucosamine residues, which can be detected using a specific lectin from *Psathyrella velutina*. In the present study IgG from RA and control serum was purified using affinity chromatography. The samples were then, after reduction, analyzed on a BIOCORE® 2000 system with immobilized *Psathyrella velutina* lectin. Using this technique it was possible to discriminate between IgG from RA patients and IgG from control individuals with respect to its content of IgG with terminal N-acetylglucosamine. The affinity biosensor technique makes it possible to detect binding without labeling or using secondary antibodies.

Keywords: **rheumatoid arthritis, agalacto-IgG, glycosylation, surface plasmon resonance, affinity biosensors, *Psathyrella velutina* lectin**

Introduction

The glycosylation of proteins is important in several biological phenomena. Examples are cell–cell adhesion, protein turnover, and protein transport [1].

Furthermore, protein glycosylation has been shown to change in response to several inflammatory conditions and malignancies [2,3]. Thus, there is an extensive need to explore to what extent the oligosaccharide composition of various glycoproteins can give more detailed information in a clinical situation. Complete structural elucidation of glycoprotein oligosaccharides requires a combination of chemical, enzymatic and chromatographic techniques combined with mass spectrometry and NMR analyses. These procedures are time- and labor consuming and are not generally applicable for routine analysis of clinical samples. Changes in glycosylation associated with disease often occur as shifts in the distribution of specific oligosaccharide structures. These shifts can be monitored using lectins or antibodies directed against specific oligosaccharide structures. Methods such as affinity chromatography, crossed affino-immunoelectrophoresis, capillary electrophoresis, dot-blotting, and ELISA have previously been used to detect glycosylation changes associated with disease [2,4].

Affinity biosensing based on surface plasmon resonance (SPR) is another technique that utilizes interactions between biomolecules for detection [5,6]. The technique detects mass changes in a dextran matrix by measuring changes in refractive index [7]. Such changes occur when an analyte binds to, or dissociates from, an immobilized ligand. The obtained sensorgram (a response over time curve) can be used to determine both the kinetics and the affinity of the analyte-ligand interaction [8,9], and to quantify the concentration of analyte in the sample [9,10]. Due to its real-time response it is a rapid technique suitable for studying weak affinity interactions such as lectin-carbohydrate interactions.

Studies of lectins interacting with carbohydrates on glycosylated peptides [11,12], and proteins [13,14] have been made with SPR based affinity biosensors. These studies have indicated the potential to use SPR in analysis of glycoproteins expressing specific oligosaccharides in a heterogeneous glycoprotein population in clinical samples. To our knowledge such studies has not yet been performed.

One example of changes in glycosylation associated with disease is the altered glycosylation of IgG in patients with rheumatoid arthritis (RA). Human IgG is predominantly glycosylated with biantennary N-linked oligosaccharides. Due to microheterogeneity in glycosylation it expresses

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more than 30 different oligosaccharide structures [15]. Several studies have shown that serum IgG from patients with RA contains an increased portion of oligosaccharides lacking galactose compared with IgG from healthy individuals [15,16]. This results in an accumulation of IgG carrying oligosaccharides with terminal N-acetylglucosamine (GlcNAc). These IgG glycoforms are commonly referred to as agalacto-IgG. Lectins specific for the involved terminal oligosaccharide structures have been used for analysis of agalacto-IgG in clinical samples in methods such as ELISA [17] and dot-blotting [18–20]. Lectins that have been used in these assays include *Psathyrella velutina* lectin (PVL) and *Bandeiraea simplicifolia* II (BS II) that bind to terminal GlcNAc, *Ricinus communis* agglutinin 120 (RCA_{120}) which binds terminal galactose. Of the GlcNAc binding lectins PVL has the highest specificity to terminal GlcNAc in agalacto-IgG [21,22]. The present study describes an SPR based affinity biosensor assay using immobilized PVL for the analysis of agalacto-IgG from clinical samples.

Materials and Methods

Materials

The BIACORE 2000 system, sensor chip CM5, HBS-EP buffer (10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA, 0.005% surfactant P-20, pH 7.4), and amine coupling kit containing N-hydroxy-succinimide (NHS), N-ethyl-N′-(3-dimethyl-aminopropyl) carbodiimide (EDC) and ethanolamine hydrochloride were obtained from Biacore International AB (Uppsala, Sweden). HiTrap 1 ml protein G columns were from Amersham Pharmacia Biotech AB (Uppsala, Sweden). PVL was from Wako Chemicals (Neuss, Germany). Protein A, bovine serum albumin (BSA), trizma-base and iodoacetamide were purchased from Sigma (St. Louis, USA), 2-mercaptoethanol was from Bio-Rad Laboratories (Hercules, USA), and trifluoroacetic acid (TFA), sodium borohydride, pyridine, and acetic anhydride from Fluka (Steinheim, Switzerland). Gas chromatography was performed on a Hewlett-Packard 5890A gas chromatograph using a WCOT fused silica column (25 m × 0.25 mm). A Hewlett-Packard 5970 mass sensitive detector was used as detector. Serum samples were obtained from 9 RA patients (Table 1). All patients had suffered from RA for several years. Serum from 10 blood donors was used as control samples.

Methods

IgG purification

For each sample, 0.5 ml serum was diluted with 0.5 ml PBS (10 mM KH_2PO_4/Na_2HPO_4, 0.15 M NaCl, pH 7.4) and centrifuged for 15 min at 15 000 *g*. IgG was purified by applying the diluted serum to a HiTrap protein G column. After washing with 3 ml PBS and 3 ml citrate buffer (10 mM, pH 4.5) IgG was eluted with 1.5 ml of 10 mM citric acid.

Table 1. Clinical data on the RA patients included in the study.

Patient	*Sex*	*Age*	*Disease activity*[1]
1	Male	62	Mild
2	Female	75	Medium
3	Male	74	Medium
4	Female	77	Mild
5	Male	66	Mild
6	Female	53	Medium
7	Male	67	Severe
8	Female	60	Mild
9	Female	63	Mild

[1]As defined by acute phase protein analysis by electroimmunoassay according to Laurell [31].

Eluted IgG was immediately neutralized with 150 µl of Tris-HCl (0.5 M, pH 8.0).

Reduction of disulfide bonds

Purified IgG was diluted with 50 mM Tris-HCl (pH 8.0) to a concentration of 70 ± 3.5 µg/ml, as determined by absorbance at 280 nm, using $\varepsilon = 1.43$ [23]. Nine volumes of the diluted IgG sample was mixed with one volume of 2-mercaptoethanol (1 M in 50 mM Tris-HCl buffer, pH 8.0) and incubated for 2 h at 37 °C. To block thiol-groups one volume of 0.2 M iodoacetamide was added to the reduced sample (final concentration 0.1 M). Incubation was performed over night at room temperature.

Protein immobilization

Immobilization of BSA, Protein A, and PVL to the dextran-matrix on CM5 sensor chips was performed using the general procedure for amine coupling recommended by the manufacturer [24]. PVL was immobilized using a concentration of 50 µg/ml in 10 mM sodium acetate buffer (pH 5.4). For immobilization of BSA and Protein A 10 mM sodium acetate buffer (pH 3.8) was used. Protein concentrations were 20 µg/ml and 100 µg/ml, respectively. Immobilization was performed at a flow rate of 5 µl/min, and 35 µl of the protein solutions were injected. Immobilization levels for PVL, BSA, and Protein A were 11 000, 11 000, and 3300 response units (RU) respectively.

SPR analysis

Analyses were performed at 25 °C. HBS-EP was used as running buffer. Flow rate was 70 µl/min if not otherwise stated. Prior to injection samples were automatically diluted 50 times in running buffer, yielding an IgG concentration of 0.63 ± 0.03 µg/ml (unless otherwise stated). A sample injection time of 3 min (210 µl) was used. As a measure of binding the difference in RU before and 3 min and 30 s after the start of the sample injection phase was used. PVL and BSA surfaces were regenerated using 0.5 M GlcNAc, and the Protein A

surface was regenerated with 0.1 M citric acid. The configuration of the flow system in BIACORE 2000 allows samples to be analyzed in up to four differently modified flow cells. Immobolized BSA, Protein A, and PVL was used in three serial flow cells, respectively, to simultaneously study the interaction between the IgG sample and these proteins.

Monosaccharide analysis

Analysis of the galactose content on IgG was performed using monosaccharide analysis as previously described [25]. Briefly, purified IgG was dialyzed against water. Samples containing 70 µg IgG (as determined by absorbance at 280 nm) were then hydrolyzed over night in 4 M trifluoroacetic acid at 100 °C, and the generated monosaccharides were converted into their corresponding alditol acetates. Samples were analyzed by gas-chromatography mass-spectrometry. Retention times for peaks corresponding to galactose and mannose were verified using a standard mixture. Galactose and mannose were quantified from an ion trace chromatogram of $m/z = 115$. Oligosaccharide chains on IgG consist of complex type diantennary structures all containing three mannose residues. Further, the mannose content in IgG is not affected by RA statues [26]. Thereby, dividing the galactose peak area with a third of the mannose peak area gives the content of galactose as an average number of galactose residues in each oligosaccharide chain.

Results and Discussion

Purification of IgG

IgG was purified using protein G affinity chromatography. This is known to accurately purify all human IgG isoforms without contamination from other immunoglobulins. It has been reported that purification of IgG using protein G affinity chromatography and ion-exchange chromatography generates differently glycosylated populations of IgG, and that ion-exchange chromatography leads to an enhanced discrimination between RA and normal IgG [27]. However, protein G purification has previously been used successfully in agalacto-IgG assays [17], and was chosen due to its simplicity.

Reduction of IgG is necessary for binding to the PVL surface

Reduced and non-reduced IgG were injected over both PVL and Protein A sensor surfaces, with a BSA surface as a negative control, using an IgG concentration of 1.2 µg/ml. When non-reduced IgG was injected over Protein A, which binds to the Fc region of the IgG molecule, binding was 480 ± 3 RU. There was almost no binding of non-reduced IgG to immobilized PVL (12.6 ± 0.4 RU). On the contrary, reduced IgG bound to the PVL surface with 167 ± 15 RU, while binding to Protein A decreased to 20.7 ± 1.5 RU. When Protein G was used instead of Protein A the same effect was observed (data not shown). Both reduced and non-reduced IgG bound less than 5 RU to the surface with immobilized BSA, thus the observed binding responses over PVL was not due to nonspecific protein-protein interactions or interactions between IgG and the dextran-matrix. The reduction of disulfide bonds opens up the tertiary and quaternary protein structure of IgG and reduces the binding to Protein A and Protein G. However the opened tertiary and quaternary protein structure enable lectins to bind to oligosaccharides attached in the Fc part of IgG [19]. These oligosaccharides are normally hidden within the folded structure of IgG [28]. The same effect was reported when PVL was used to detect agalacto-IgG by ELISA [17]. However, in that study binding of IgG to protein G remained after reduction. The biosensor system makes it easy to directly follow both binding to protein A and PVL to optimize the conditions for reduction to obtain maximal sensitivity for PVL binding. The protein A sensor surface was used in parallel with the PVL surface in all subsequent analyses to assure complete reduction of IgG.

The IgG-PVL interaction is blocked by free GlcNAc

The specificity of PVL has been thoroughly investigated. It is known from hemagglutination- and ELISA-inhibition assays [21], and from affinity chromatography studies [22], that PVL binds to oligosaccharide chains that have a terminal GlcNAc. To further test the specificity of the interaction between IgG and PVL observed in the biosensor, the IgG sample was mixed with different amounts of free GlcNAc before injection. There was a dose dependent inhibition of binding using a concentration range of GlcNAc between 0.05–1 mM, where 1 mM showed complete inhibition, whereas 10 mM galactose had no effect on the binding (data not shown).

Binding responses is flow rate dependent

In some cases binding of analyte to immobilized protein is faster than the diffusion of new analyte into the binding matrix. This will cause the concentration of unbound analyte near the sensor surface to be lower than the bulk concentration. The effect is termed mass transport limitation, and results in lower binding responses. When increasing the flow rate, transport of molecules in to the binding matrix will increase giving smaller differences in concentrations between the bulk and near the surface. The resulting increase in binding response makes it possible to measure at lower concentrations. The effect of flow rate on binding of reduced IgG to PVL was tested by injecting reduced IgG for 3 min at increasing flow rates. The concentration of reduced IgG was 6 µg/ml. An increased response was observed up to a flow rate of 70 µl/min. When the flow rate was further increased to 100 µl/min a slight decrease in response was observed compared to when a flow rate of 70 µl/min was used (Fig. 1). To obtain maximum sensitivity for the PVL-biosensor assay of agalacto-IgG all subsequent analyses were performed at a flow rate of 70 µl/min.

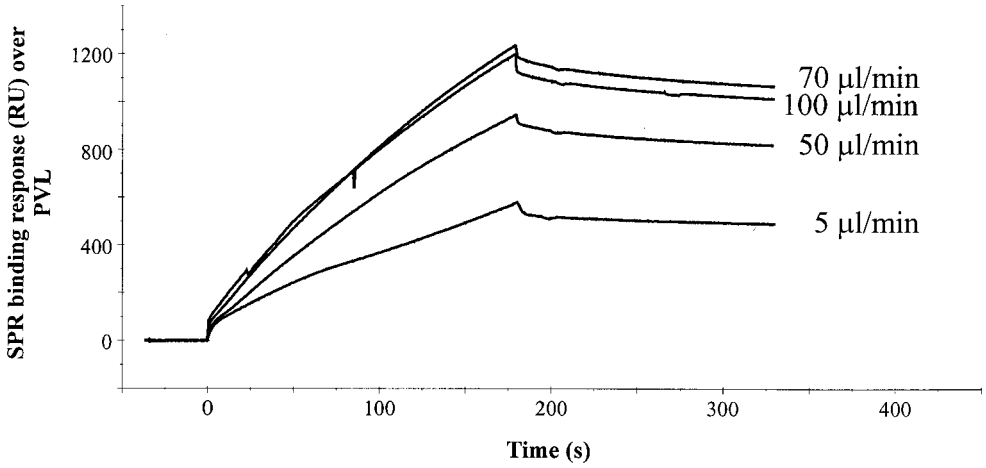

Figure 1. Sensorgrams showing reduced IgG injected over PVL for 3 min with different flow rates.

Determination of a suitable IgG concentration

In order to obtain a suitable concentration of total IgG to be used in the PVL-biosensor assay, binding of reduced IgG to immobilized PVL was analyzed using different IgG concentrations. A purified IgG sample from one RA patient was diluted before reduction to yield final concentrations between 0.3 and 1.2 µg/ml. Two samples were prepared at each concentration, and analyzed in duplicate runs. In this concentration range binding of agalacto-IgG to PVL was proportional to the total IgG concentration and did not reach surface saturation (data not shown). A total IgG concentration of 0.6 µg/ml gave a clearly detectable response (100 RU) compared with BSA background (5 RU) without a remaining signal after surface regeneration (Fig. 2). Therefore, a total IgG concentration of 0.6 µg/ml was used for all subsequent analyses. Thus, for each analysis 0.2 µg IgG is used. The amounts used, and the sensitivity of the assay is in the same range as previously used lectin assays, such as ELISA [17] and dot-blot techniques [19].

Reproducibility

The overall reproducibility for the PVL-biosensor assay was tested by separately purifying and reducing eight IgG samples from the same serum sample. After reduction the eight samples were injected at a protein concentration of 0.6 µg/ml. The coefficient of variation (CV) were 9% (n = 7). Injecting the same preparation 8 times gave a CV for the biosensor detection of 4%.

The PVL-biosensor assay can discriminate between IgG from control individuals and RA patients, respectively

Samples from 10 control individuals and 9 RA patients were analyzed in duplicates using the PVL-biosensor assay. The

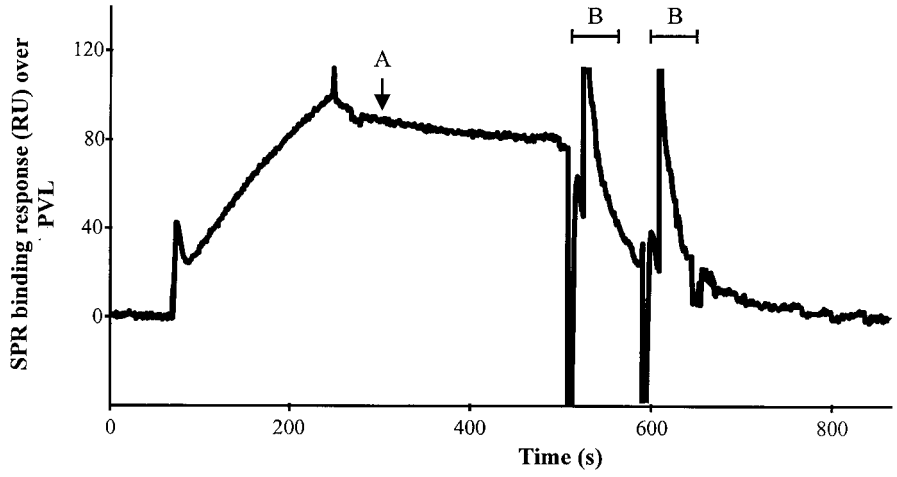

Figure 2. Sensorgram showing reduced IgG injected over a surface with immobilized PVL after subtraction of the response from a surface with immobilized BSA. The concentration of IgG was 0.6 µg/ml. (A) The arrow indicates the time for determination of bound IgG. (B) Bars indicate surface regeneration using two pulses of 0.5 M GlcNAc.

mean RU values ranged between 18.3–60.8 and 37.2–86.3 with medians at 41.1 and 72.3 for the control individuals and RA patients respectively (Fig. 3). A statistical significant difference was obtained between control individuals and RA patients using Mann-Whitney U-test ($p < 0.005$). An increase of agalacto IgG associated with rheumatic disease has been observed in a number of reports [15,16]. Furthermore, the degree of IgG hypogalactosylation in RA patients is correlated with the degree of disease activity [29]. However, IgG glycosylation has also been shown to be age-dependent [30], therefore it can not be excluded that some of the differences observed between the RA patients and the control group in this study can be explained by a somewhat higher mean age of the RA patients compared to the control group.

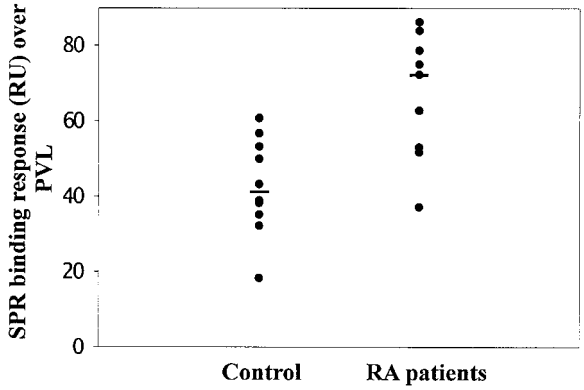

Figure 3. Binding (RU) of reduced IgG from 10 control individuals and 9 RA patients, respectively, to PVL. The vertical line marks the median value in each group.

Validation of the PVL-biosensor assay

Two samples, one with low and one with high galactose/oligosaccharide ratio (0.72 and 1.56 respectively, as obtained by monosaccharide analysis) were mixed in different proportions. Total IgG concentrations were in each case 0.6 μg/ml. Each sample was injected in duplicate, and the average binding response was plotted against galactose/oligosaccharide ratio (Fig. 4). The observed inverse proportionality ($r = -0.99$ using linear regression) between binding response and galactose/oligosaccharide ratio shows that binding to PVL changed solely as a consequence of changes in galactosylation. This shows that the PVL-biosensor assay can be used to measure the relative content of agalacto-IgG in purified IgG samples. Extrapolation of the obtained regression line to 0 RU in SPR binding gives a galactose/oligosaccharide ratio of 2.01. The expected galactose/oligosaccharide ratio for fully galactosylated IgG that does not bind to PVL is 2.

Galactose/oligosaccharide ratio of the control individuals and RA patients were obtained in duplicates using monosaccharide analysis. The results from the PVL-biosensor assay were plotted against the galactose/oligosaccharide ratio (Fig. 5). The correlation coefficient, r, between the two methods was −0.80. This is comparable with other studies using lectins to detect agalacto-IgG by ELISA [17] and dot-blot techniques [19]. These studies reported r-values of 0.90 and 0.82, respectively, when correlated to chemical methods. The monosaccharide analyses were performed using the whole IgG molecule. As undergalactosylation occurs only on oligosaccharides in the Fc region of IgG, the performed monosaccharide analysis may overestimate the galactose/oligosaccharide ratio of oligosaccharides from the Fc region. However this does not affect the correlation between the monosaccharide analysis

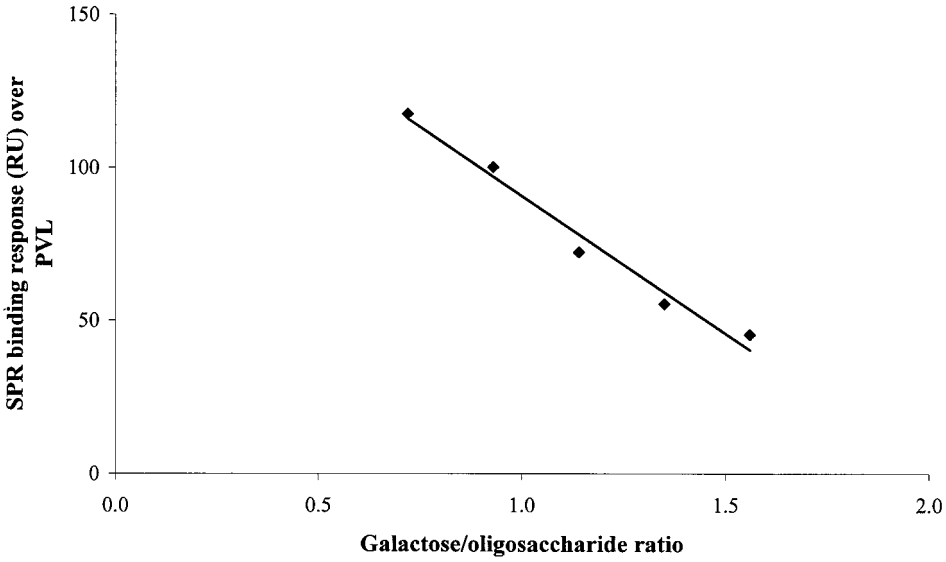

Figure 4. Two IgG samples containing different amounts of agalacto-IgG obtained by monosaccharide analysis were mixed in different proportions. Binding responses over PVL in RU were plotted against galactose/oligosaccharide ratio calculated from the used mixing proportions ($r = -0.99$).

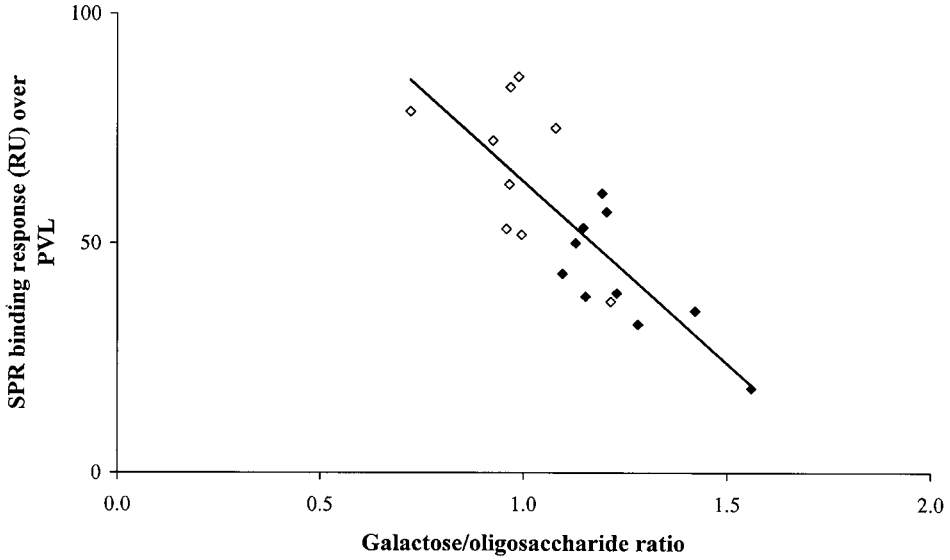

Figure 5. Agalacto-IgG binding response in RU plotted against galactose/oligosaccharide ratio for the 10 control individuals (closed diamonds) and 9 RA patients (open diamonds). The correlation coefficient between the two methods was −0.80.

and the PVL-biosensor assay.

In conclusion, this work shows the potential of SPR based affinity biosensors to measure the relative content of glycoproteins with specific oligosaccharide structures in a heterogeneous glycoprotein population. This can be utilized to detect and to follow changes in glycosylation during disease and treatment. The affinity biosensor technique makes it possible to detect binding without using labeling or secondary antibodies. The direct detection feature also enables measurements at non-equilibrium. This speeds up the analytical procedure and can be used to broaden the dynamic range of a method [7], which might be of use in the development of rapid analytical systems for clinical applications.

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