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B lymphocyte galactosyltransferase protein levels in normal individuals and in patients with rheumatoid arthritis

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We have quantified the level of β4-galactosyltransferase protein in human B lymphocytes using an ELISA-based assay. Between 1-10ng of β4-galactosyltransferase was detected per mg total cellular protein, indicating that this enzyme constitutes <0.001% of B lymphocyte cellular protein. Akin to previous studies, individuals with rheumatoid arthritis exhibited reduced lymphocytic galactosyltransferase enzyme activity compared with normal controls when using ovalbumin as the acceptor substrate. The levels of enzyme protein present in B lymphocytes from patients with rheumatoid arthritis was, however, not reduced suggesting that the B lymphocyte galactosyltransferase catalytic activity may be regulated post-translationally.

Keywords: **Galactosyltransferase; B lymphocyte; Antibody; Rheumatoid arthritis; Agalactosyl IgG.**

Abbreviations: β4GalT, β1,4-galactosyltransferase; BSA, bovine serum albumin; GlcNAc, *N*-acetylglucosamine; IgG G0, agalactosyl IgG; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; pITC, phenylisothiocyanate; RA, rheumatoid arthritis

Introduction

Little is known regarding the regulation of glycosyltransferase activity. Potential control points include transcription, mRNA stability, translation, and post-translational events. The consequences of aberrant control of glycosylation on the properties of a glycoprotein may be numerous, including altered functional activity and/or immunogenicity, increased proteolysis and altered clearance from the circulation [1]. In the case of IgG it is known that a lack of galactose can affect the effector functions of the antibody [2]. When both arms of the N-linked biantennary oligosaccharide on the IgG Cγ2 domain lack galactose the molecule is referred to as agalactosyl IgG, or IgG G0. In RA there is an increased proportion of IgG G0 [3–5], a glycoform that is pathogenic in an animal model of RA [6] and which correlates with a relatively poor prognosis in patients with RA [7]. Galactose is added to terminal *N*-acetylglucosamine (GlcNAc) on complex-type N-linked oligosaccharides by β4-galactosyltransferase (β4GalT). Reduced GalT activity has been detected in the B lymphocytes of patients with rheumatoid arthritis (RA) [8–10] and therefore B cells isolated from these patients provide a model system in which the regulation of GalT can be explored.

In addition to the classical β4GalT (β4GalT-I), five more human β4GalT enzymes have recently been cloned (β4GalT-II to β4GalT-VI) [11–13]. However, our recent data using an antisense approach in human B cells clearly demonstrate that if β4GalT-I expression is sufficiently decreased, the levels of galactose on the IgG are also decreased [14]. One explanation for reduced B cell β4GalT activities in RA could therefore be a simple quantitative difference, i.e., there is less enzyme present in the B cells. Alternatively, there may be normal enzyme levels but the enzyme activity is impaired. In order to distinguish between these two possibilities we have developed a monoclonal antibody (mAb) based assay for quantifying β4GalT protein and compared the levels of protein with the activity of the enzyme.

Materials and methods

Subjects

Peripheral blood was obtained from a total of 12 patients with RA (11 females, mean age 60.7, age range 49–73; one 64-year-old man) who fulfilled the revised criteria of the

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American Rheumatism Association [15] and from 12 healthy volunteers and non-RA patients (six females, mean age 60, age range 50–74; six males, mean age 64.7, age range 58–77). Two individuals in the control group had lumbar spondylosis, four had osteoarthritis and the remainder were normal healthy individuals with no arthritis. It has previously been shown that the percentage of IgG G0 structures in lumbar spondylosis and osteoarthritis do not deviate from age-matched controls [3,4].

B lymphocytes

B cells were isolated using anti-CD19 Dynabeads (Dynal, UK) as previously described [16]. Viable cells attached to the anti-CD19 Dynabeads were regarded as purified B cells and constituted >98% of the cells used in these experiments.

Preparation of cell extracts

Approximately 10^6 cells per 100 µl of ice-cold lysis buffer (0.2% Triton X-100 in 0.15 M phosphate-buffered saline pH 7.4 [PBS]) were lysed for 30 min at 4 °C. Following centrifugation at 15 000 g in an IEC Centra-3RS microfuge for 1 h at 4 °C, aliquots of the supernatants were collected and stored at −70 °C. Prior to assaying for GalT activity, the lysates were centrifuged at 126 000 g for 5 min at room temperature in polyallomer tubes (5 × 20 mm, Beckman, UK) using an A-100/30° fixed angle rotor in a Beckman CLS ultracentrifuge.

Measurement of GalT activity

A radiochemical assay which detects the transfer of [^{3}H]Galactose from UDP-[^{3}H]Galactose onto the acceptor was used as previously described [17]. The samples were quantified using a bovine milk β4GalT standard curve run each time and the activity expressed in nmol/mg of protein/h.

Quantification of β4GalT protein levels

A novel assay to measure β4GalT protein levels was developed in order to undertake the present study. Fifty µl/well of streptavidin (Sigma S-4762) at 5 µg/ml was coated overnight at 4 °C in 96-well Maxisorp immunoplates (Nunc, Denmark), the plates then washed four times with PBS-0.05% Tween 20 (PBS-T), and blocked with 100 µl/well of PBS-1% bovine serum albumin (BSA) for 2 h at 37 °C. After washing, one half of the plate was incubated with 50 µl/well of biotinylated isotype-matched control MOPC-21 IgG1κ mAb (Sigma M-9269) at 5 µg/ml in PBS, with the other half of the plate incubated with 50 µl/well of a mixture of biotinylated anti-human β4GalT mAbs, UCLgt1B6 and UCLgt1H11, each at 2.5 µg/ml in PBS. These two mAbs recognise spatially distinct protein epitopes on the enzyme to which they bind synergistically [18]. Plates were incubated for 2 h at 37 °C and then overnight at 4 °C. Following washing, the plates were incubated with 50 µl/well human milk β4GalT standard (purified as previously described [19]) at doubling dilutions in PBS-T from 60 ng/ml, or with 50 µl/well of the B cell lysates. Samples were assayed in duplicates on both sides of the plates. Plates were incubated for 2 h at 37 °C, then washed and incubated with 50 µl/well affinity-purified rabbit anti-human β4GalT [20] at 5 µg/ml in PBS-T-1% BSA for 1 h at 37 °C. After washing, the plates were incubated with 50 µl/well of goat $F(ab')_2$ anti-rabbit IgG-horseradish peroxidase (Jackson ImmunoResearch Laboratories, USA) at 1 : 2000 in PBS-T-1% BSA for 1 h at 37 °C. Following the final washing steps, the plates were developed using 100 µl/well of 1 mg/ml *o*-phenylenediamine in 0.1 M citrate phosphate buffer, pH 5.0 with 0.03% H_2O_2. The reaction was stopped with 50 µl/well 3N H_2SO_4 and absorbances read at 490 nm using a Dynatech MR5000 ELISA plate reader. The amount of enzyme protein was quantified using the human β4GalT standard curve constructed with each experiment. Total cellular protein concentration in the cell lysates were determined using the Bio-Rad protein micro-assay (Hemel Hempstead, UK), with BSA as a standard [21].

IgG G0 measurement

The IgG G0 was measured using a previously described enzyme-linked lectin assay [22]. Samples containing known amounts of IgG G0 determined by the Department of Biochemistry, University of Oxford using the hydrazinolysis method were used to produce a standard curve of the absorbance ratios of *Bandeiraea simplicifolia* II and *Ricinus communis* agglutinin against the known amounts of IgG G0 and the test sample absorbances interpolated. The IgG G0 results were scored in relation to previously defined age-population curves determined for normal serum IgG [22] and expressed as standard deviation units about the normal mean.

Results

Levels of β4GalT protein

An assay based upon a pair of mAbs which synergistically bind β4GalT was developed for the quantification of β4GalT protein. The bound enzyme was detected using a polyclonal anti-β4GalT which recognizes additional epitopes on the enzyme. This ELISA-based method proved to be highly reproducible and specifically detected the enzyme protein down to a sensitivity of 1 ng/ml (50 pg per well) (Figure 1). The interplate variation was between 1.3–6.1% cv using β4GalT protein concentrations between 0–60 ng/ml, with an average cv of 3.11% ± 1.81.

The concentration of β4GalT in B cell lysates was assayed using this method. The line of best-fit was deter-

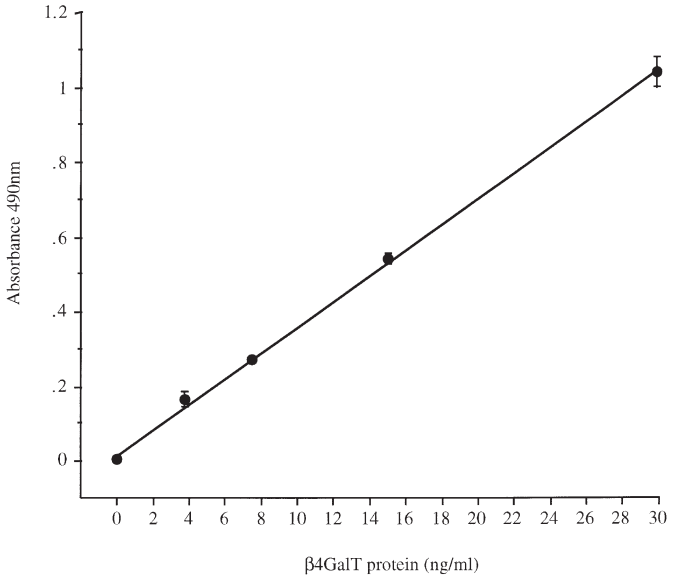

Figure 1. Standard curve of human milk β4GalT measured using the newly developed β4GalT protein quantification assay. Biotinylated anti-β4GalT mAbs UCLgt1B6 and UCLgt1H11 (each at 2.5 μg/ml) were bound to streptavidin-coated ELISA plates and the captured β4GalT detected using an affinity-purified rabbit anti-β4GalT antibody. Error bars indicate one standard error of the mean. r = 0.99.

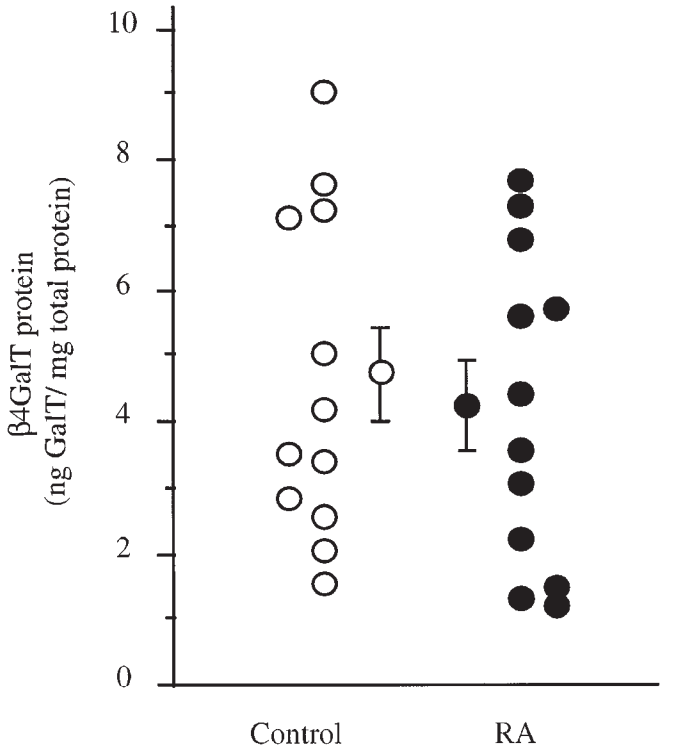

Figure 2. Quantification of β4GalT protein in B cell lysates of RA patients (n = 12, black circles) and controls (n = 12, white circles). Error bars indicate one standard error of the mean.

mined for the standard curve using linear regression and the regression equation used to calculate β4GalT protein in the samples (InStat, GraphPad Software, USA). No significant difference was observed between the amounts of B cell β4GalT protein from the RA patients and controls (Figure 2).

GalT activity

The B lymphocytic GalT enzyme activity was measured using two different acceptor substrates. With the GlcNAc-pITC-BSA acceptor substrate, there was no significant difference between the GalT activities in B cells from patients with RA and controls (p = 0.1279 unpaired Student's t test, Figure 3a). However, when ovalbumin was used as the acceptor the GalT activities detected in the RA patients' B lymphocytes were significantly lower than those seen in the control group (p = 0.0176, Figure 3b). B cell GalT activities from the RA patients showed a mean value of 324 pmol/mg protein/h, compared to the control group value of 770 pmol/mg protein/h.

IgG glycosylation

In the cohort of RA patients used in the present study there was no statistically significant increase in the levels of IgG G0 compared to the control group (p = 0.352) (Figure 4).

Discussion

Using a sensitive and reproducible ELISA-based assay we have quantified β4GalT protein from cell lysates. In this assay the mAbs UCLgt1B6 and UCLgt1H11 jointly capture the enzyme prior to quantification with affinity-purified rabbit anti-human β4GalT. The six β4GalT enzymes cloned so far (β4GalT-I - β4GalT-VI) [11–13] share 33–55% sequence homology and it has yet to be established if, in addition to β4GalT-1, the protein quantification assay detects any of these other enzymes. The mAb UCLgt1B6 binds to the poorly conserved stem region of β4GalT-1 [18] and is therefore unlikely to bind the other enzymes. In contrast, the mAb UCLgt1H11 may recognise the more highly conserved catalytic domain on the other enzymes [18]. Given that UCLgt1B6 and UCLgt1H11 bind β4GalT in a synergistic manner it is, however, likely that the assay at least preferentially detects β4GalT-1. Using this assay we have shown that the reduction in GalT activity observed in RA B cells is not due to a reduced amount of β4GalT protein in these cells. This accords with our previous observations that there was no reduction in the level of β4GalT-1 mRNA expression in B cells either in patients with RA or in the arthritis-prone MRL-*lpr/lpr* mouse [16].

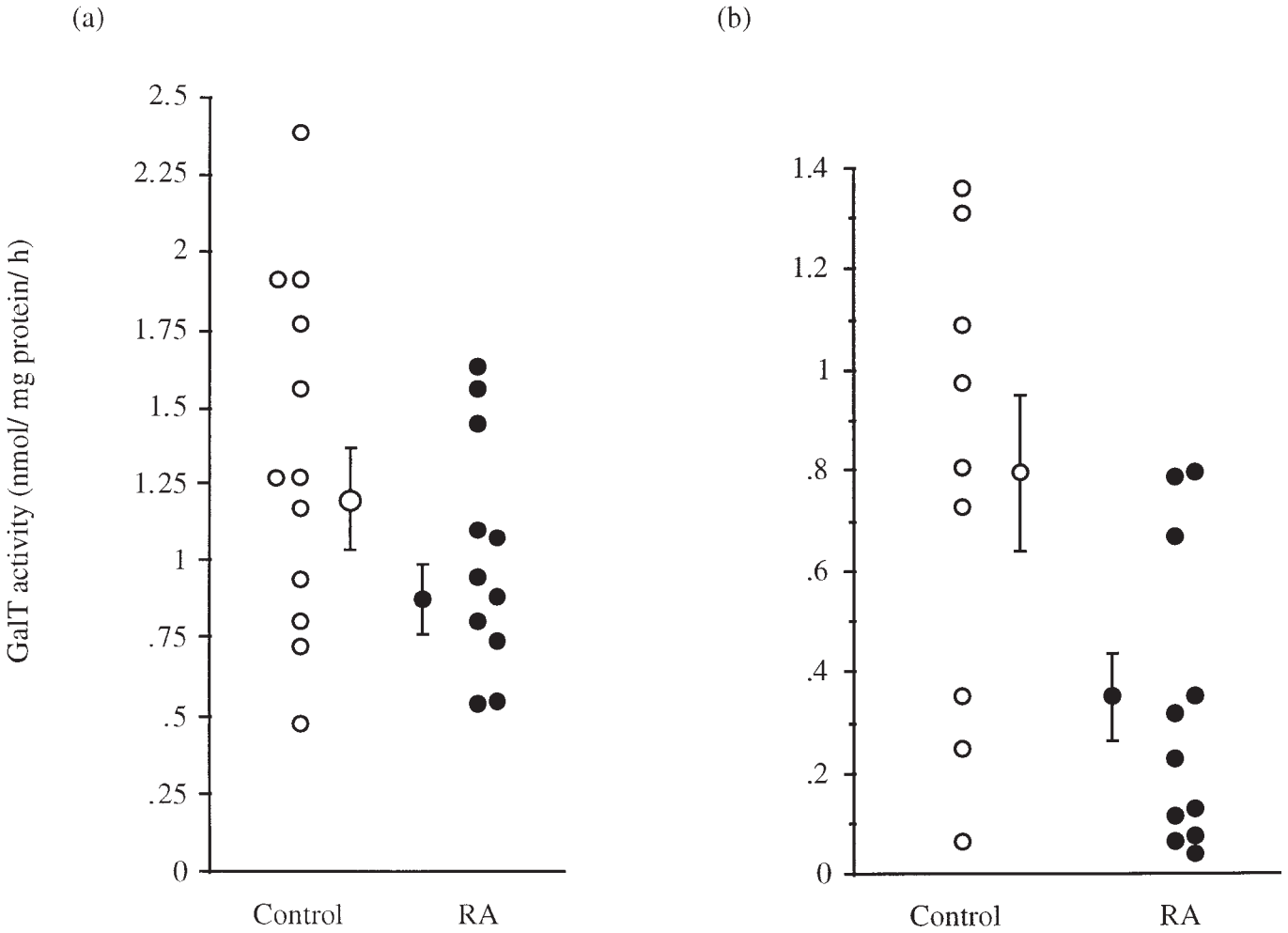

Figure 3. GalT activity in B lymphocytes from patients with RA (●) and controls (○) using (a) GlcNAc-pITC-BSA and (b) ovalbumin as an acceptor. Error bars indicate one standard error of the mean.

Although the amount of enzyme was quantitatively similar between the two groups, there was decreased activity of the enzyme in the patient group when using ovalbumin as the acceptor. Decreased levels of GalT activity in RA B cells have previously been reported using this acceptor [8,10]. However, together with our previous study on the enzyme activity for the GlcNAc-pITC-BSA acceptor [17], the present results suggest that the enzyme 'defect' in these patients exhibits a degree of substrate specificity. In a study by Furukawa and colleagues, the reduction was only observed when using asialoagalacto-IgG (AsAg-IgG) as the acceptor molecule [9]. The precise status of assays measuring GalT activity has been clouded somewhat by the recent discovery of other β4GalT family members some of which appear to differ in their fine substrate preferences and in their optimal assay conditions.

Despite the fact that, rather atypically, only two of the patients available to us for the present study had G0 values above the normal range, there was an overall statistically significant decrease in lymphocytic GalT activity for the entire group of RA patients compared to the control group. Therefore, there does not appear to be a direct correlation between the enzyme activity level and the IgG galactosylation. Indeed, the most straightforward explaination that the reduced GalT activity is responsible for the increase in G0 has been questioned [5] and the present data would certainly argue against a simple relationship between enzyme activity and IgG glycosylation.

Potential mechanisms for post-translational regulation of β4GalT clearly exist, such as serine phosphorylation [23]. In patients with RA the affinity of lymphocytic GalT for UDP-Gal is reduced [9], suggesting a qualitative defect in the enzyme. Decreased galactosylation of glycoproteins could also occur due to mislocalization of β4GalT following a stressful insult to the cell. Segregation of β4GalT and α2,6-sialyltransferase, which normally co-localize in the *trans Golgi/trans* Golgi network, can be induced by monensin treatment of cells [24] and there is evidence in autoim-

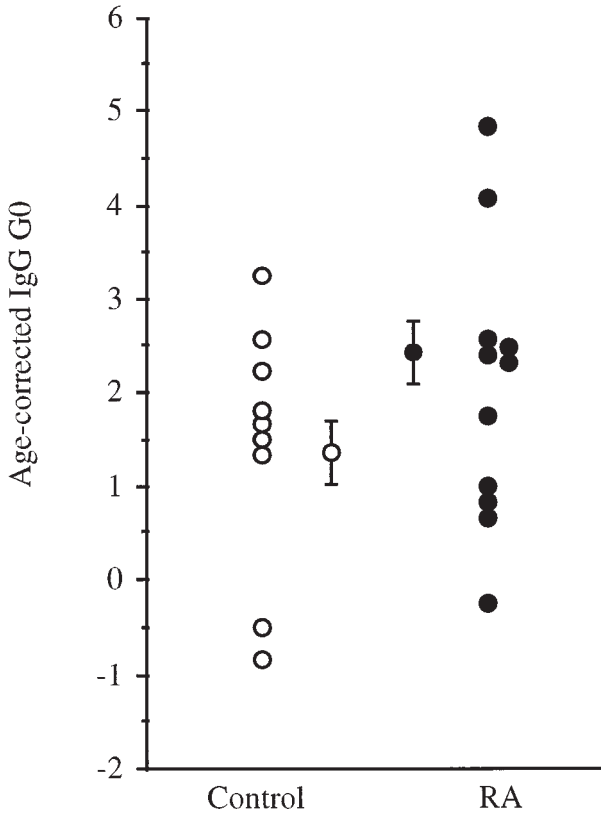

Figure 4. IgG from the peripheral blood of the RA (●) and control (○) individuals presented in figure 3 was assayed for IgG G0. Values were age-matched and expressed as standard deviations of the age-related population mean [22]. Error bars indicate one standard error of the mean.

mune diseases such as RA that heat-shock and other stress proteins are elevated [25]. Any stress-induced perturbation to the Golgi might influence the correct targeting of glycosyltransferases, thereby altering protein glycosylation.

Acknowledgments

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