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Serum Galactosyltransferase Isoform Changes in Rheumatoid Arthritis

AZITA ALAVI, JOHN S. AXFORD, and ANDREW J. POOL

ABSTRACT. Objective. To investigate glycosylation changes associated with rheumatoid arthritis (RA) by determining whether there are β1,4-galactosyltransferase (GTase) isoforms specific to or altered in the serum of patients with RA.

Methods. Serum GTase isoform profiles were determined using isoelectric focusing (IEF) in patients with active RA (n = 9), disease controls (DC; n = 9), and healthy individuals (HI; n = 10).

Results. There was a highly significant difference (p < 0.0001) between the IEF profiles. The RA IEF profile was significantly (p < 0.0001) different from that of the DC or the HI group. There was, however, no significant difference between the DC and HI profiles. Serum GTase samples from 8/9 RA, 9/9 DC, and 9/10 HI resolved into 2 distinct peaks of activity. The RA isoform profile was associated with an acidic shift. There were no significant differences in the pH value of the first peak; the second peak was found to be significantly more acidic in the RA group (mean pH 5.02) compared to the DC and HI group (mean pH 5.20; p < 0.05). The RA associated isoform constituted a significantly greater proportion of total enzymatic activity in the RA sera (16.1%) compared to DC and HI (13.5%; p < 0.05 and 12.6%; p < 0.01, respectively). RA and HI serum GTase desialylation resulted in an alkaline shift of the isoforms into similar pH bands: 5.25–5.50, 5.70–5.85, and 6.20–6.40. GTase was found to be on average 75% more active in its desialylated form than in its sialylated state.

Conclusion. RA is associated with a differential expression of GTase isoforms. This may be due to increased hypersialylation, which has the potential to adversely affect the catalytic activity of the enzyme, thus providing a possible mechanism for posttranslational regulation of GTase activity in RA. (J Rheumatol 2004;31:1513–20)

Key Indexing Terms: RHEUMATOID ARTHRITIS ISOFORM GALACTOSYLTRANSFERASE IMMUNOGLOBULIN G

The link between rheumatoid arthritis (RA), reduced β1,4-galactosyltransferase (GTase) enzyme activity1–7, and immunoglobulin G (IgG) hypogalactosylation is a well-documented phenomenon that may be linked to the pathology of RA8–11.

β1,4-galactosyltransferase is a subfamily of the glycosyltransferase superfamily, which comprises at least 7 members that use different types of glycoproteins and glycolipids as substrate12–15. The most widely distributed, principal member of this family, and the most extensively studied in terms of both RA and the control of galactosylation, is the classical GTase-I13,15. The other more recent additions (GTase-II to GTase-VII) identified by primary sequence similarities15 are differentially expressed, often present at comparatively low concentrations, and are still under investigation for their biological significance, if any, in terms of the control of galactosylation14,16–18.

GTase has a wide range of biological functions, and has been extensively studied in relation to RA and its role in the galactosylation of the terminal N-acetylgalcosamine on the complex N-linked biantennary oligosaccharides located in the CH2 domain of IgG1,4–6,12,19. These sugars are an integral feature of IgG and are known to affect various Fc-mediated effector functions20.

Reduced B cell GTase activity and the corresponding decreased IgG galactosylation (IgG-G0) in patients with RA and various animal models5,7,8,10 appear to be directly linked to the pathogenic features associated with RA9,11. For example, agalactosylation of IgG can trigger the inappropriate activation of complement21, is an important component of rheumatoid factor-IgG complexes in RA22,23, and has been shown to be pathogenic in animal models of this disease6,11. In addition, IgG-G0 has been found to be a significant diagnostic and prognostic feature of RA that, together with rheumatoid factor (RF) status, predicts a more severe disease24–26.
GTase, in particular GTase-I, has been shown to be the principal regulator of IgG galactosylation, as demonstrated by the specific alteration of β1-4GTase-I expression in a human IgG-secreting cell line, via transfection with sense/antisense human β1-4GTase-I cDNA. GTase galactosylation of IgG is a presecretory event that occurs principally within the trans-Golgi apparatus. In addition to its biosynthetic function within the cell, GTase is also involved in a wide variety of other complex biological processes, including cell-cell and cell-matrix interactions, and is therefore widely distributed and important in cell migration, matrix formation, and signal transduction. GTase is, therefore, present in the Golgi as well as on the cell surface and in a soluble form in various body fluids, e.g., serum.

The metabolism of GTase has been extensively studied. Structurally, GTase consists of a short, positively charged region attached to a tightly folded globular (catalytic) domain that is attached to the membrane by a heavily glycosylated stem region. The enzyme is synthesized in the rough endoplasmic reticulum and has a half-life of roughly 20 hours, as a membrane-bound glycoprotein in the Golgi apparatus, after which it is released (via proteolytic cleavage of the stem region) in a catalytically active soluble form.

Regulation of GTase activity is complex and is controlled in part by transcription, translation, and posttranslational modifications, e.g., the degree of phosphorylation.

However, to date, no evidence has been found to explain the reduction in GTase activity in RA. Studies of transcriptional and translational control have found no evidence of reduced GTase mRNA expression or reduced amount of GTase protein in RA B cells. Further, there is also no evidence of unique B cell polymorphisms of the GTase gene or the gene controlling phosphorylation in patients with RA, or any evidence for an intracellular inhibitor of GTase in RA.

In a study of 9 patients with RA (7 women, mean age 53.5 yrs, range 32–76), a disease control (DC) group (n = 9; one woman, mean age 50.5 yrs, range 21–80), and a group of healthy individuals (HI; n = 10; 5 women, mean age 39.0 yrs, range 25–59), the possibility that RA may be associated with the differential expression of GTase isoforms was suggested by pilot studies in which we observed (1) the differential incorporation of galactose onto a variety of different acceptor molecules, and (2) significant changes in the gross isoelectric focusing (IEF) (pH 3–10) profiles of soluble serum and peripheral B cells (CD19). GTase derived from a group of patients with RA and healthy individuals.

Our aim was to extend our preliminary IEF investigations and to determine whether there are GTase isoforms specific to or elevated in patients with RA, and to investigate whether these changes could be attributed to possible sialylation differences.

### MATERIALS AND METHODS

#### Identification of Serum GTase Isoforms

**Study subjects.** Serum was obtained from blood taken from patients with RA (n = 9; 7 women, mean age 53.5 yrs, range 32–76), a disease control (DC) group (n = 9; one woman, mean age 50.5 yrs, range 21–80), and a group of healthy individuals (HI; n = 10; 5 women, mean age 39.0 yrs, range 25–59).

The RA and DC groups were selected from the rheumatology clinic at St. George’s Hospital and the HI included volunteer hospital workers. The RA patients satisfied the American College of Rheumatology criteria for disease as defined by serological and clinical measures. The patients were taking nonsteroidal antiinflammatory drugs (NSAID) plus weekly methotrexate (MTX).

The DC group comprised patients with peripheral arthritis: 2 with ankylosing spondylitis and 7 with psoriatic arthritis, and had clinically active disease as defined by serological and clinical measures.

**IEF.** Serum GTase was separated by charge using solution-phase IEF (Rotofor IEF; Biorad, UK). A 2% ampholyte solution, with pH specificity of 4–6, was added to a 60 ml Rotofor chamber and focused for 1 h using a constant power of 12 W. The temperature of the solution was maintained between 1° and 4°C by infusing cold water at a rate of 50 ml/min through the cooling finger of the Rotofor.

On completion of prefocusing, 2 ml serum were added to the chamber. Focusing was continued until a voltage plateau was achieved. The final voltage ranged from 1050 to 1150 V and this was achieved in 4–5 h; 20 fractions were harvested and the pH of each sample was determined.

**GTase activity assay.** The samples were prepared for the assay by neutralizing each fraction to pH 6.8, using 4 M Tris-HCl. A solution of 20 mM MnCl₂, 0.5 mM ATP, 0.1 mM UDP-galactose, 1 μCi uridine diphospho-[6-³H]galactose: activity = 1 μCi ml⁻¹ (Amersham, UK), 1 mg of ovalbumin (the acceptor molecule), and 50 μl of each fraction was made up to a total volume of 100 μl using 0.1 M Tris-HCl, pH 6.8. The mixture was incubated 2 h in a shaking water bath at 37°C. The reaction was stopped by the addition of 1.8 ml of 1% phosphotungstic acid in 0.5 M HCl. The resulting precipitate was left to stand for 15 min and then centrifuged for 10 min at 2000 g. This was repeated twice to remove any unbound radioactive material. The remaining pellet was dissolved in 0.5 ml NCS tissue solubilizer (Amersham, UK). The amount of radioactivity (cpm × 10³) was then determined using a liquid scintillation counter. To account for endogenous serum acceptors (endogenous activity was negligible and accounted for only 1–10% of total counts), background activities were determined of the observed charge heterogeneity of GTase isoforms, e.g., in cancer, and although its function is unknown, it may influence the enzymatic activity of GTase, which has been shown to be influenced by its pI value.

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determined and subtracted from the total GTase activity. The yield of activity after IEF was at least 90%. To standardize the data from each assay, the enzyme activity of each sample was expressed as a percentage of the sum of the activity of all the fractions (percentage total GTase activity).

Galactosyltransferase Desialylation

Subjects. Two patients with clinically active RA (2 women ages 35 and 48 yrs), previously found to have abnormal GTase isofrom profiles, were selected and compared to a patient with inactive RA (woman aged 55, not previously investigated) and 2 HI (one woman, one man, ages 36 and 53). The 2 patients with active RA had clinically active disease (see above) and were IgM RF positive. These patients were taking NSAID plus weekly MTX. The patient with inactive disease was taking NSAID only.

Desialylation of serum. Serum samples (2 ml) were incubated with 2.5 units of Clostridium perfringens sialidase (Oxford GlycoSystems, Abingdon, UK) in the presence of sodium acetate buffer, pH 5.0, for 18 h at 37°C. The sialidase-treated serum was added to the IEF chamber and focused as described above, using ampholytes with a pH range of 5–7. The resulting samples were neutralized and assayed by the method described above.

Desialylation of purified GTase. We compared activity of the enzyme in both its sialylated state and after treatment with sialidase. In the absence of sources of nonrecombinant human GTase, purified bovine GTase, a well characterized and readily available source of the enzyme, was used. It shares > 90% cDNA sequence identity with human GTase-15. Purified commercial bovine GTase (Sigma, Poole, UK) was desialylated using the method described above.

Standard dilution curves (0–50 µU; mean of duplicate assays) of GTase were determined before and after treatment with sialidase. The assay was also repeated in the presence of sialidase to determine its effect on the assay.

Statistics. Analysis of variance (ANOVA) for repeated measures was used to test the null hypothesis that the shape of the IEF profile of serum GTase differs between the 3 populations. Comparative analyses of the pH and height of the first and second peaks of GTase activity were made using Student’s t-test.

RESULTS

IEF profiles. Statistical comparison of the results for 26 subjects (RA, n = 8; DC, n = 9; HI, n = 9) examined at 20 pH points (4.00–4.99 to 5.00–5.99) revealed a highly significant (p < 0.0001) difference between the shapes of the IEF profiles among the 3 groups examined.

ANOVA applied to each pair of groups showed that the difference in the shape of the IEF profile was highly significant for RA compared to DC or HI (p < 0.0001; Figure 1).

There was no significant difference between the IEF profiles of the DC and HI groups.

Identification of GTase isoforms. In all 3 groups examined, over 90% of GTase activity was seen between pH 4.00 and 6.00, with maximum activity in the pH band 4.30–5.35. In 8/9 RA patients, 9/9 DC, and 9/10 HI, 2 distinct peaks of activity were seen, as follows (Figure 2).

Peak 1 analysis. The mean pH values of the first peak were 4.49 (range 4.30–4.65), 4.63 (range 4.38–4.86), and 4.60 (range 4.40–5.00) for the RA, DC, and HI, respectively. There were no significant differences in the mean pH value of this peak among the 3 groups examined, although the peak in the RA group was more acidic (Figure 3).

There were no significant differences in the height of these peaks, which constituted 19.7% (range 17.2–26.0), 16.9% (range 12.1–22.8), and 17.7% (range 9.3–27.3) of the total GTase activity in the RA, DC, and HI groups, respectively.

Peak 2 analysis. The mean pH values of the second peak were 5.02 (range 4.83–5.11), 5.20 (4.99–5.39), and 5.20 (range 5.00–5.35) for the RA, DC, and HI, respectively.

The peak in the RA group was less heterogeneous and occurred over a narrower pH range.

There were significant differences in the pH values of the second peak among the 3 groups. The RA peak was significantly more acidic compared to the peak in the DC (p < 0.05) and the HI group (p < 0.01; Figure 4).

The RA second peak constituted a significantly greater proportion of the total GTase activity [16.1% (range 13.3–18.8)], compared to the second peak in the DC [13.5% (range 10.2–18.0; p < 0.05)] and HI [12.6% (range 8.9–17.7); p < 0.01] groups.

GTase desialylation

Predesialylation GTase IEF analysis. Both the patients with active RA had typical RA GTase isoform profiles, with a marked acidic shift of the second peak (pH 4.83 and 4.99). However, the patient with inactive RA had a profile similar to that of the HI group, with an isoform second peak in the pH range 5.17–5.21 (Figure 5A).

Postdesialylation GTase IEF analysis. Desialylation resulted in an overall alkaline shift (4.30–5.20 to 5.25–6.40). The postdesialylation serum samples resolved into a total of 3 pH bands: pH 5.25–5.50, 5.70–5.85, and 6.20–6.40 (Figure 5B). Of the 3 RA patients, one active and one inactive patient showed 3 peaks of activity, similar to those in the HI group. Interestingly, the RA patient with the most acidic GTase isoform profile (for both peaks 1 and 2) resolved into a single peak of activity with a pI of 6.26. All patients and HI showed a common peak of activity occurring in the pH range 6.2–6.4.

Effect of desialylation on the activity of purified GTase. This experiment was carried out to determine the effect of desialylation on the enzymatic activity of bovine GTase. GTase activity increased by an average of 75% after desialylation with C. perfringens sialidase (Table 1). The presence of sialidase in the assay mixture did not interfere with or have a significant effect on the final counts.

DISCUSSION

GTase in RA B cells has reduced activity1,3-6 and abnormal kinetics2 that are not due to any known genetic abnormality or the aberrant expression of the GTase gene34, or due to reduced expression of GTase in RA6,19. These findings raise the possibility that the reduced lymphocytic GTase activity reported in RA may be due to a postsynthetic modification of the enzyme.

Several postsynthetically modified GTase isoforms have
been identified in both healthy persons and persons with malignancy, where a large degree of the observed charge heterogeneity has been attributed to changes in the degree of sialylation\textsuperscript{35,36,38}. In keeping with the findings of these previous studies, possible variations in RA lymphocytic GTase isoforms should be reflected in the soluble form of the enzyme in serum. We investigated whether there were RA-associated serum changes in the GTase isoform composition, and whether these changes could be attributed to possible sialylation differences. Whole serum, which represents secreted GTase from a number of different cell types, including lymphocytes, was used in preference to purified GTase, as the purification procedure may have selectively removed certain GTase isoforms in preference to others\textsuperscript{37}.

Solution-phase IEF revealed highly significant differences between the groups studied. The RA IEF profile over the entire pH gradient of 4–6 was found to be significantly different from those of the DC and HI groups. These differences were in agreement with the results of a pilot study\textsuperscript{43} in which we observed gross changes (pH gradient 3–10) in the IEF profile of RA serum and peripheral B cell (CD19) GTase (using solution-phase and agarose-gel IEF, respectively).

In the present more detailed IEF study, we observed that the IEF differences are predominantly the result of changes in the pI of 2 distinct, albeit broad, peaks of GTase activity. The RA isoform profile revealed an acidic shift compared to that of the DC and HI. There were no significant differences in the pH values of the first peak in the 3 groups examined. However, the pH of the second peak was significantly more acidic in the RA group, compared with the relatively less acidic and heterogeneous second peak in both the DC and HI groups. Further, this acidic RA GTase isoform constituted a significantly greater proportion of the total GTase activity in the RA sera. These results would indicate that the RA patients are synthesizing larger quantities of the relatively more acidic GTase isoforms.

These results are in general accord with previous IEF studies, and indicate that all GTase isoforms occur within the pI range of 4–6\textsuperscript{37}. Solution-phase IEF did not provide sufficiently high resolution to obtain the more detailed profiles previously described\textsuperscript{37}. The peaks of activity we describe are therefore likely to be a combination of 2 or more different GTase isoforms with similar pI values, the pH of each peak being determined by the relative quantities and pI values of the GTase isoforms present. These may, possibly, be further resolved into separate smaller peaks with higher resolution IEF. However, although interesting from an analytical point of view, further resolution of these 2 major peaks of GTase activity is unlikely to affect or alter our findings in any significant manner.

It is unlikely that the acidic isoforms seen in RA are...
unique to this disease, as a small percentage of the DC and HI show a second peak in a similar position. This is not surprising, since a study examining GTase isoforms in HI and those with malignancy has shown that HI express most isoforms of the enzyme. The difference between disease states, e.g., RA and cancer patients compared to HI, lies in the relative quantities of each individual isoform, as indicated by our results.

Comparing the IEF profile of RA in relation to the DC group, it is apparent that the association between the acidic GTase isoform and RA is especially strong. This signifies that the changes in the GTase isoform profile are specific to RA and not due to inflammation per se as all the DC patients investigated had active disease at the time of sampling.
Comparing serum RA GTase profiles with those found in malignant disease, there appear to be a number of similarities. Solution-phase IEF of sera from patients with malignancy showed the presence of a large and prominent peak at pH 4.75 and the absence of a peak normally found at pH 5.1045, suggesting that isoforms with greater overall negative charge predominate in cancer patients. This has been confirmed by similar studies using solid-phase IEF30.

Sialidase treatment of sera from both RA patients and HI with markedly differing GTase isoform profiles revealed 3 peaks of activity with comparable pH values, which would suggest that the acid shift in the RA profile is due in part to the presence of isoforms with higher sialic acid content.

In both RA and malignant disease30,36, the differences in GTase isoform profiles appear to be primarily due to the presence of increased quantities of hypersialylated GTase, suggesting that there may be a common mechanism by which this occurs. The most likely explanation for these observations is the high level of sialyltransferase found in both RA and malignancy46,47. Although the sialic acid content appears to be the principal determinant of the observed charge heterogeneity of serum GTase isoforms, other possible postsynthetic modifications cannot be ruled out. These include the degree of relative deamidation of negatively and positively charged amino acids and possible changes in phosphorylation, acetylation, and sulfation. Hyperphosphorylation of GTase is unlikely in RA, for not only is there normal expression of p58GTA gene, but increased phosphorylation is likely to enhance GTase activity34.

The finding that GTase activity can be influenced by its degree of sialylation supports earlier observations that the enzymatic activity of GTase may be influenced in part by its pl value, isoforms with low pl values having decreased and those with higher pl values having increased enzyme activity36.

The differences we describe are only significant across a small band of GTase isoform(s), i.e., peak 2. Whether the isoform(s) in this pl band are representative of the isoform(s) responsible for IgG galactosylation remains to be tested. To prove a direct cause and effect between increased serum GTase sialylation and IgG hypogalactosylation would require the solid-phase IEF of Golgi GTase extracted from large numbers of B cells. This would be quantitatively difficult, as GTase constitutes less than 0.001% of B lymphocyte cellular protein6 and would require B cells from pooled peripheral blood obtained from groups of patients and controls rather than individuals.

Finally, our data raise the question, is the altered RA serum GTase IEF profile a reflection of changes in the expression of one or more of the other members of the GTase (GTase II to GTase VII) gene family? Although we cannot completely rule out this possibility, we believe that

| GTase Activity Increase in Activity, % |
|----------------|----------------|----------------|
| Sialylated, cpm | Desialylated, cpm |
| µ Units | Activity | Activity |
| 3.10 | 7.94 | 14.38 | 81 |
| 6.25 | 16.12 | 25.79 | 60 |
| 12.50 | 33.97 | 63.24 | 86 |
| 25.00 | 79.15 | 138.46 | 75 |
| 50.00 | 190.61 | 326.30 | 71 |

Figure 5. The effect of sialidase treatment on the IEF profile of serum GTase in 3 patients with RA: inactive (○), active RA1 (■), and active RA2 (▲) and 2 HI (□, △). (A) Predesialylation and (B) postdesialylation (bracket bars represent the range of activity for each peak). Sialidase treatment resulted in similar IEF profiles of GTase in both RA and HI.
the evidence to date strongly favours GTase-I as the principal enzyme responsible for the galactosylation of IgG and other major glycoproteins. Indeed, recent GTase-I ablation studies have shown that the absence of GTase-I results in a dramatic switch from B1-4 to B1-3 galactosylation in both N- and O-glycans. The results of these knockout experiments clearly indicate either that the recently discovered members of the B1-4 GTase gene family (GTase-II to VII) may be biologically irrelevant or that they play only a minor role in the overall control of galactosylation.

We observed quantitative and qualitative changes in the RA serum GTase isoform profile. This is likely due to a greater proportion of hypersialylated isoforms, which have the potential to adversely affect the catalytic activity of the enzyme, thus providing a possible mechanism for posttranslational regulation of GTase activity in RA. It also provides further evidence that RA glycosylation changes may be more general than previously indicated and encompass proteins other than IgG.

REFERENCES


