Elevated Thymic and Serum Galactosyltransferase with Low-Molecular-Weight Acceptor Activity in Murine Leukemia

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ABSTRACT

Galactosyltransferase activity (UDP-galactose: N-acetyl-D-glucosamine-D-galactosyltransferase) could be measured in thymus and sera from different strains of mice. Total thymic homogenates or thymocyte preparations obtained from thymoma carrying AKR/J mice exhibited higher enzyme activity compared to nonleukemic control mice. A similar difference was also noted in Swiss mouse thymus which develop thymic leukemia upon a single injection of 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboximide. Galactosidase, which was 25 times less active than galactosyltransferase, was not responsible for this difference. These observations were extended to an evaluation of the serum level of the enzyme as a potential tumor biomarker. A 3- to 4-fold increase in the activity of galactosyltransferase was detected in serum samples obtained from leukemic mice models (AKR/J and Swiss) compared to the controls, whereas the sera from P388 tumor-bearing DBA/2 mice showed a statistically nonsignificant increase of only 20%. The data indicate that serum galactosyltransferase (that accepts the low-molecular-weight acceptor, N-acetyl-D-glucosamine) levels are elevated in the presence of thymic leukemia, and suggest the possibility of shedding of this enzyme from the tumor cells to the systemic circulation of the host. The implications, including the potential diagnostic significance of the results, are discussed.

INTRODUCTION

Enzymes known generically as GTases participate in the transfer of sugar molecules to suitable substrates such as low-molecular-weight acceptors (free monosaccharides) or high-molecular-weight glycoconjugates, including glycoproteins and glycolipids. Of these transferases, GTases (which are usually associated with membrane portions such as Golgi apparatus and plasma membrane of cells) have been tested for changes in tissue or serum activity under a variety of malignant situations, by using high-molecular-weight acceptors, usually a glycoprotein (1-9). In general, no consistent elevations in serum GTase activity could be demonstrated (2), unless in such tumor bearers there was a concomitant hepatic involvement (4). However, liver was not believed to be the source of serum sialyltransferase activity in rats bearing mammary tumors (10). The presence of an electrophoretically distinct isoenzyme of GTase was detected in the sera of patients with certain cancers, usually of alimentary tract origin (7, 9, 11). However, the substrate used was a high-molecular-weight acceptor protein, and the methodology involved a lengthy procedure of discontinuous polyacrylamide electrophoretic separation of serum samples (9). Also, entirely different data might emerge from the use of high- or low-molecular-weight acceptors, e.g., serum α-2-L-fucosyltransferase activity was found to be low in acute adult myeloid leukemic patients when low-molecular-weight acceptor (phenyl-β-D-galactoside) was used (5); in contrast, use of a high-molecular-weight acceptor (desialylated fetuin) showed elevated activity in such patients and in those with non-Hodgkin's lymphomas (12, 13). There has been no study dealing with the quantitation of GTase that accepts low-molecular-weight acceptor (e.g., GlcNAC) in experimental or human leukemia.

Earlier work from this laboratory has revealed an elevated thymus and serum level of acidic glycosphingolipids in AKR/J mice bearing thymic lymphoma (14, 15). We have also reported an altered pattern of glycolipid: sialyltransferases concomitant with the biosynthetic profile of gangliosides in such mice (16). Although GlcNAc-containing gangliosides have been reported in tumor cell lines, as well as in normal cell types, including human lymphocytes (17-20), glycolipid-accepting GTase activity of human lungs did not show difference between control and cancer patients (8). Basu et al. (21) could detect the activity of a GTase in thymic lymphocytic tumor cell lines, wherein galactose bound to UDP was transferred to a GlcNAc-containing glycolipid. Also, the presence in serum and tissues of a GTase with GlcNAc-acceptor activity has been described (22-25). In order to monitor if any change in the GTase with a low-molecular-weight-acceptor activity is associated with leukemogenesis, thymic UDP-galactose:GlcNAc (β1-4)-GTase (EC 2.4.1.22) activity in normal and tumor-bearing mice was measured. This enzyme catalyzes the formation of galactosyl-GlcNAc, i.e., N-acetyllactosamine, and is one of the well-characterized GTases. In this communication an elevation in the activity of the above enzyme in thymus and sera from mice bearing thymic lymphomas is reported. The oncomavirus-induced spontaneous thymoma of the AKR/J mice and the thymoma of the Swiss mice induced by a single injection of DTIC were used in these experiments. In contrast, no such dramatic increase in GTase activity was noted in the sera from DBA/2 mice carrying P388 tumor cells. The biological implications and potential diagnostic significance of these results are discussed.

MATERIALS AND METHODS

Mice

Three different animal models of malignancy were used. Eighty to 90% of AKR/J mice develop thymic lymphomas at about 6 to 9 months of age. Male AKR/J mice of this well-defined model were divided into 2 groups. (a) nonleukemic group of mice 4 to 7 months old with no evidence of leukemia; and (b) leukemic group of mice with thymic lymphomas and frequently enlarged spleens. A complete description of this model has been published by this laboratory (27).
Starting at 10 weeks, following a single i.p. injection of DTIC into 4-week-old outbred Swiss mice, thymic lymphomas start to appear, and more than 90% will have had tumors within 18 weeks from injection (28). The control (vehicle injected) or leukemic (DTIC injected) group were about 18 to 24 weeks of age at the time of sacrifice.

The third model involves 3-month-old DBA/2 mice given injections i.p. of 1 x 10^6 transplantable P388 tumor cells. The mice carried the fast-growing-malignant cells in the peritoneal cavity for 1 week, at which time they were sacrificed. Sera from such tumor-carrying or control mice were kindly provided by Dr. M. Stern (Department of Microbiology, The Chicago Medical School).

**Sera**

Blood collected from a cut in the jugular blood vessels was allowed to clot and centrifuged at 12,000 x g for 1 to 2 min, and clear sera were stored at -10°C until used. In some experiments fresh sera were also used.

**Chemicals**

UDP-[U-14C]galactose (specific activity 274 mCi/mmol) was obtained from New England Nuclear, Boston, MA. HEPES, bovine serum albumin, mannitol, and dithiobitric acid standards, unlabeled UDP-galactose, GlcNac, and 5'-AMP were from Sigma Chemical Co., St. Louis, MO. Bio-Rad AG 1 x 8 (200 to 400 mesh) resin was purchased from Bio-Rad Laboratories, Richmond, CA.

**Preparation of Enzyme**

Thymocytes were prepared as described elsewhere (29) and were suspended in phosphate-buffered saline (in g/liter: NaCl, 8; KCl, 0.2; NaHPO4, 1.15; KH2PO4, 0.2, pH 7.4), and were centrifuged at 350 x g for 10 min at 4°C. The pellet was homogenized in 5 volumes of 0.2 M caccodylate-HCl buffer, pH 6.4, containing 0.1% -mercaptoethanol and Triton X-100 (0.3 mg/ml) in an all-glass homogenizer. A 350 x g (10 min) supernatant of this homogenate was used as the enzyme. Whole thymus homogenates were prepared in 5 volumes of 0.2 M HEPES buffer, pH 7.4, containing Triton X-100 (0.3 to 0.75 mg/ml), centrifuged at 350 x g for 10 min at 4°C, and the supernatant was used as the source of enzyme. Serum was diluted in 0.32 mM sucrose.1 mM EDTA:0.1% -mercaptoethanol solution before assay.

**GTase Assay**

The enzyme activity was measured as described before (21, 24) with slight modifications, using the low-molecular-weight sugar acceptor, GlcNac. The reaction mixture contained in a final volume of 55 μl: HEPES buffer, pH 7.4 (10 μmol); MnCl2 (0.25 μmol); Triton X-100 (75 μg); 5'-AMP (25 nmol); GlcNac (1 μmol); the donor, UDP-[U-14C]galactose (25,000 to 50,000 cpm/10 nmol), and 10 μl of thymus homogenate (60 to 80 μg protein), or 1 to 5 μl of the serum suitably diluted in sucrose (0.32 M):EDTA (1 mM):-mercaptoethanol (0.1%) solution. After incubation for 1 to 2 h at 37°C, the reaction was stopped with 10 μl of 50% ethanol containing 1.25 μmol of EDTA. Separation of the product was achieved either by high-voltage electrophoresis (for serum samples), or by a Bio-Rad AG 1 x 8 column method (for tissue homogenates).

**High-Voltage Electrophoresis**

The contents were applied on Whatman 3 MM paper and subjected to high-voltage electrophoresis in 1% borate solution, pH 9.1, for 1 h at 2,000 volts (24). Under the conditions of electrophoresis, UDP-galactose and most of free galactose were removed from the column, allowing quantitative recovery of the product. The activity of the GTase from serum was assayed by electrophoresis (for serum samples), or by a Bio-Rad AG 1 x 8 column method (for tissue homogenates).

**GTase Activity in Normal and Leukemic Mice.** Using the low-molecular-weight acceptor, GlcNac, the amount of product formed was found to be proportional to the concentration of the enzyme used (Chart 1). The enzyme activity from AKR/J leukemia thymocyte homogenate was also linear, but showed higher activity at all concentrations tested. Also, homogenate preparations from both normal and leukemic thymuses showed linearity with the time of incubation (Chart 1). In contrast to the progressive increase in the (exogenous) enzyme activity with time, the endogenous value remained low with increasing time, e.g., at 30- and 150-min points, the endogenous values were 0.22 and 0.39 nmol/mg protein for nonleukemic AKR, and 0.15 and 0.24 for leukemic AKR thymic homogenates, respectively, thereby suggesting a low level of nonenzymatic breakdown.

**Comparison of GTase Activity from Virally or Chemically Induced Leukemic Thymuses.** Table 1 presents the data on changes in the GlcNac acceptor activity of the enzyme during 20 ml of distilled water before applying the sample (23). The product N-acetyl-14C]glucosamine was eluted with 4 ml of distilled water, collected in scintillation vials, and radioactivity was determined after adding 15 ml of Budget-Solve (Research Products, Inc., Mt. Prospect, IL). Unreacted UDP-galactose and free galactose (which forms an anionic-borate complex) are retained by the column, allowing quantitative recovery of the product (23). Necessary quenching corrections were made and the enzyme activity was expressed as nmol of product formed/2 h/mg protein, or nmol/hr/μl of serum.

For each serum sample and tissue homogenate preparation the product formed in absence of exogenous acceptor (GlcNac) was determined, and this value was subtracted from the total enzyme activity. The endogenous activity did not show consistent proportionality with the time of incubation, or source of enzyme (see "Results"; Chart 1; Tables 1 and 2). Optimal conditions for the assay of tissue enzyme were met with regard to substrate (UDP-galactose) and acceptor (GlcNac) concentrations; the enzyme exhibited saturability and had an apparent Kₘ of 7.6 x 10⁻⁴ M and 4 x 10⁻⁴ M, respectively (data not presented).
GlcNac:GTase IN LEUKEMIA

Chart 1. GTase activity of thymocytes from leukemic and nonleukemic AKR/J mice as a function of protein concentration and time. The reaction mixture contained the following in a final volume of 55 μl: HEPES buffer, pH 7.4 (10 mmol); MnCl₂ (0.25 μmol); Triton X-100 (75 μg); 5'-AMP (25 nmol); GlcNac (1 μmol); UDP-galactose (U-14C) (25,000 to 50,000 cpm/10 nmol), and thymocyte homogenate at varying concentrations. Left, after incubation at 37°C for 1 h, reaction was terminated and the product was separated by column chromatography, as described in “Materials and Methods.” All values were corrected for the usually low endogenous activity (e.g., 0.028 and 0.055 nmol/h at the highest nonleukemic and leukemic enzyme protein values, respectively). Right, for time kinetics experiment, the total thymus homogenate protein concentration was 60 to 80 μg/assay. All values (nmol N-acetylactosamine/mg protein) were corrected for endogenous values (range: 0.35 to 0.59 nmol/mg protein) run in absence of the acceptor, GlcNac. The zero-time value (the contribution of UDP-galactose to the column eluates) was equivalent to 0.02 nmol. All values are average of 2 experiments. ●, nonleukemic; ○, leukemic.

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzyme source</th>
<th>Endogenous</th>
<th>Exogenous</th>
<th>Leukemia:nonleukemia ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKR/J</td>
<td>Thymocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nonleukemic</td>
<td>0.81</td>
<td>42.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leukemic</td>
<td>1.38</td>
<td>110.52</td>
<td>2.61</td>
</tr>
<tr>
<td></td>
<td>Total homogenate</td>
<td>2.34 ± 0.94</td>
<td>11.91 ± 0.99</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>Nonleukemic</td>
<td>2.33 ± 0.67</td>
<td>24.22 ± 2.80</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>Leukemic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swiss</td>
<td>Nonleukemic</td>
<td>2.40 ± 1.41</td>
<td>21.45 ± 3.96</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>Leukemic</td>
<td>3.84 ± 2.60</td>
<td>37.92 ± 3.23</td>
<td>(3)</td>
</tr>
</tbody>
</table>

- Homogenates prepared from nonleukemic (average age, 18 weeks for AKR/J and Swiss) or leukemic (average age, 36 and 20 weeks for AKR/J and Swiss) mice were assayed over 2-h incubation periods. Values are mean ± SE, with the number of experiments indicated within parentheses.
- Determined in the absence of exogenous acceptor (GlcNac).
- Pooled cells from 6 mice in each group.
- Significantly different from nonleukemic (P < 0.01, > 0.001).
- Significantly different from nonleukemic control (P < 0.01).

leukemogenesis in 2 models of murine thymic leukemia. Under optimal conditions, the average activity of the enzyme from AKR/J leukemic thymocyte homogenates and leukemic thymus total homogenates were 2.61- and 2.03-fold higher than the respective normal cell and tissue enzyme preparations. Also, the increase in thymus tissue enzyme activity in leukemia is mostly due to a higher enzyme activity in the lymphoid cells of the tissue. Thymus homogenates from Swiss mice carrying chemically induced thymic lymphoma showed comparable results, the magnitude of increase due to leukemia being 1.78-fold.

Galactosidase Activity. Hydrolytic enzymes such as galactosidases may degrade the product of the GTase reaction, i.e., N-acetylactosamine, and it is possible that the differences observed in Table 1 or Chart 1 are partly due to low galactosidase activities in malignant thymocytes. Although specific assays were not performed for this purpose, the appearance of free, radioactive galactose in the GTase reaction mixture at the end of the 2-h assay (as determined by the high-voltage electrophoretic separation procedure in which free galactose moves towards the anode) may be taken as a rough indicator of the activity of enzymes that split galactose from galactose-containing compounds. Thus, in a typical experiment, the galactosidase activity was about 25 times less than the GTase activity (0.36 versus 8.5 nmol/h/mg protein, respectively). Furthermore, the galactosidase activity was about 60% less in nonleukemic AKR thymic homogenates, a result which would further enhance the real difference in GTase activity between the normal and tumor tissues. Our results are in agreement with those reported on GTase that accepts high-molecular-weight acceptors, wherein the influence of hydrolases was excluded because the β-galactosidase activity was undetectable and the UDP-galactose pyrophosphatase activity was low and similar in the sera from both normal donors and cancer patients (7). Similar conclusions were reached by others, based on the use of inhibitors of these hydrolases (1, 6). It may be mentioned here that our standard assay includes the inhibitor, 5'-AMP.

Serum GTase Activity. Shedding of macromolecules by neoplastic cells is well known (1, 2, 13, 33), and these may play a role in the pathophysiology of cancer. In particular, thymus has actively proliferating T-lymphocytes, and thymocytes are known to "shed" some of their membrane components into the extracellular environment (34, 35). Accordingly, it was predicted that sera from leukemic mice might exhibit higher GTase activity compared to sera from mice not suffering from thymic leukemia. Chart 2 depicts the activity of GlcNac-accepting GTase as a function of the volume of normal murine sera. The enzyme activity could be detected in the sera from 3 different strains of mice, the rate of product formation being proportional to the quantity of the enzyme protein used, and the method is sensitive enough to detect the enzyme activity in as little as 1 μl of serum. In a separate series of experiments, activities of serum GTase obtained from normal and tumor-bearing mice representing the 3 models of malignancy were compared (Table 2). Nonleukemic AKR/J mice showed the lowest activity, while DBA/2 mice had the highest activity. Under the same conditions, pooled sera from

![Graph](https://via.placeholder.com/150)

Chart 2. Serum GTase activity of different strains of normal mice. Experimental conditions were the same as in Chart 1, except that sera were used at indicated volumes, and the product was separated by high-voltage electrophoresis, as described in "Materials and Methods."
normal C57 mice gave a value of 0.6 nmol/5 μl serum/2 h. Interestingly, the sera from AKR/J and Swiss mice bearing thymic lymphoma exhibited 3- to 4-fold higher GTase activity (P < 0.01) than did their respective controls.

These changes are most probably due to the presence of the tumor rather than to age differences. Within the limited number of tissue samples examined, there was no apparent age-dependent variation in the enzyme activity. Normal thymuses from AKR/J mice 13, 18, and 26 weeks old had similar GTase activities: 0.51, ±0.16 and 0.54 nmol/2 h/5 μl serum at 24, 30, and 36 weeks respectively (±8% SE). Also, thymuses from 13-, 18-, and 28-week-old Swiss mice exhibited overlapping enzyme activities (16.9, 21.45, and 16.81 with a ±18% SE). Furthermore, sera from normal AKR/J mice had similar enzyme activities: 0.51, ±0.16 and 0.54 nmol/2 h/5 μl serum at 24, 30, and 36 weeks old. Finally, our previous experience with elevated serum lipid-bound sialic acid level (which positively correlates with leukemogenesis in AKR/J mice) suggests that these changes are most likely to be tumor dependent rather than age dependent (14).

Due to the limited number of samples within each tumor weight group, especially for AKR/J thymic tumors, which ranged from 0.2 to 1.55 g in wet weight, any correlation between tumor weight and the serum enzyme activity could not be attempted. Eighty-eight % of individual serum samples from AKR/J mice bearing thymic tumors showed enzyme values higher than the mean control serum enzyme activity. In contrast, only a statistically insignificant 20% increase in the enzyme activity was observed in the sera of DBA/2 mice bearing i.p. injected P388 tumor cells. This raised the question if the P388 cells had any "shedding" of the enzyme from the tumor cells, or there was no shedding at all. Nonetheless, the pattern of change in all of the 3 malignant models was the same whether the enzyme activity was expressed as specific activity (per mg serum protein) or per unit volume of serum. Thus, there appeared to be an apparent association of elevated GTase activity with the 2 models of leukemia, whether induced chemically or virally.

**DISCUSSION**

Several investigators have tested sera from patients with malignant diseases and animals bearing tumors for changes in the activities of glycosyltransferases. A number of high-molecular-weight acceptors including mucin, fetuin, agalactofetuin, asialomucin, and ovalbumin have been used by other investigators previously (1-9). Availability of such an extensive data on GTases that accept high-molecular-weight acceptors, combined with the demonstration of a distinct serum GTase that accepts a low-molecular-weight acceptor (22, 25) and the narrow focus of our study on the latter enzyme activity excluded the question of the dynamics of the former type of enzyme activity in the present investigation. The report about higher "endogenous galactosyltransferase" activity in serum and peritoneal fluid of BALB/c mice transplanted with Yc8 lymphoma is of interest, although undefined acceptor(s) was used (serum itself was the source of enzyme and acceptor) and the model was different (36). In the current study, presence of a GTase (EC 2.4.1.22) which catalyzed the formation of A/-acetyllactosamine by utilizing the low-molecular-weight acceptor, GlcNac, has been detected in the thymus and sera from different strains of mice (Tables 1 and 2). The enzyme activity can be measured in one million cells and it appears to be stable. Sera and thymuses frozen at -10°C for up to 2 years had activities not far different from freshly obtained samples, although the magnitude of difference in activities between normal and leukemic enzymes was slightly less (results not shown). Data presented in Table 1 suggest that the enzyme activity is probably associated with the thymocytes; the difference in enzyme activity between normal and leukemic AKR/J thymus is slightly greater if cells were the source of enzyme rather than the total homogenates. Approximately 85% of wet weight of thymus (leukemic or nonleukemic) is due to the thymocytes.6

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**Table 2**

Comparison of GTase activities in sera from normal and tumor-bearing mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serum Enzyme Activity</th>
<th>Tumor: Normal Serum Ratio</th>
<th>Tumor: Normal Serum Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKR/J Nonleukemic</td>
<td>0.51 ± 0.16 (20)</td>
<td>0.99 ± 0.31</td>
<td>2.81</td>
</tr>
<tr>
<td>Leukemic (viral)</td>
<td>1.92 ± 0.43 (14)</td>
<td>2.84 ± 0.47</td>
<td>2.54</td>
</tr>
<tr>
<td>Swiss Normal</td>
<td>1.15 ± 0.39 (25)</td>
<td>2.10 ± 0.70</td>
<td>2.54</td>
</tr>
<tr>
<td>Leukemic (chemical)</td>
<td>3.63 ± 1.33 (8)</td>
<td>5.34 ± 1.95</td>
<td>2.54</td>
</tr>
<tr>
<td>DBA/2 Normal</td>
<td>3.34 ± 0.45 (6)</td>
<td>6.85 ± 0.88</td>
<td>2.10</td>
</tr>
<tr>
<td>Tumor-bearing (P388 cells)</td>
<td>4.01 ± 0.30 (12)</td>
<td>8.09 ± 0.6</td>
<td>2.13</td>
</tr>
</tbody>
</table>

*Enzyme activities are given as mean ± SE, with number of mice used in parentheses. The overall endogenous activity registered in the absence of GlcNac was 18.7 ± 4.8% (±SE) of the total enzyme activity measured in presence of the exogenous acceptor.

#Unpublished observations.
The reason(s) for increased activity of the enzyme in leukemic thymus is not clear. It may be due to high turnover of the enzyme in rapidly proliferating leukemic thymocytes, or due to a shift in the specificity of enzyme toward the substrate. Based on mixing experiments, appearance of an activator or loss of an inhibitor of the AKR/J thymic tissue enzyme as a result of leukemogenesis has been ruled out as the cause of the difference in activity (results not presented). An increased rate of shedding or release of the enzyme from leukemic thymocytes into the circulation might explain a higher enzyme activity in leukemic sera. A similar suggestion was offered for the presence of 5'-nucleotidase in the sera of breast cancer patients (2), fucosyltransferases in non-Hodgkin's lymphoma patients (13), and glycoprotein (high-molecular-weight) accepting GTase in mammary carcinoma of rats (1). Involvement of a nonthymic tissue in the release or as the sole source of GTase remains as a possible but less likely cause.

Finally, the method used avoids chemical-enzymatic preparation of high-molecular-weight acceptors (e.g., fetuin, orosomucoid) which measure more than one enzyme activity. The simplicity and sensitivity of the method (requiring as little as 1 μl of serum) should be useful in monitoring the presence of thymic lymphoma in mice. Interestingly, women with primary or metastatic breast carcinoma (25), and rats bearing prostate and mammary adenocarcinomas (24), were reported to have higher serum or tissue GlcNac-accepting GTase activity than did normal or disease control patients and animals, respectively. Since the AKR/J mouse model is believed to represent human malignant situations (e.g., acute leukemia, malignant lymphoma, and myeloma), extension of these observations to human leukemic patients may be useful.

ACKNOWLEDGMENTS

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