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CO-PURIFICATION OF SOLUBLE HUMAN GALACTOSYLTRANSFERASE AND IMMUNOGLOBULINS

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SUMMARY: Preparations of human malignant effusion galactosyltransferase activity purified according to previously published techniques using enzyme-specific affinity chromatography consistently produced antibodies directed toward immunoglobulins with no detectable antigalactosyltransferase. Double immunodiffusion analysis of the antigen showed the presence of both IgG and IgA. Affinity chromatography with anti-human IgG-Sepharose and anti-human serum-Sepharose resulted in a 48,000-fold purification of galactosyltransferase activity with no detectable IgG by radioimmunoassay. Immunization of rabbits with this preparation produced antibodies directed against galactosyltransferase activity and minimal anti-Ig. The persistence of immunoglobulins during the purification of soluble galactosyltransferase activity through two enzyme-specific affinity chromatographic steps suggests an association of immunoglobulins with galactosyltransferase activity.

INTRODUCTION

Although galactosyltransferase:SGF-fetuin (GT) activity is primarily localized to cell membranes (1,2) it has also been detected in various extracellular fluids including milk (4), serum (5), and malignant effusions (6). Purification of galactosyltransferase(s) has been described from several sources, including bovine (7) and human milk (8), human serum (4,5), and malignant effusion (6,9).

Elevations of total serum GT activity have been reported in patients with cancer (10,11) but these elevations were neither consistent nor specific

Abbreviations: SGF-fetuin = Sialyl-, galactosyl-free fetuin; GT = galactosyltransferase:SGF-fetuin activity; GlcNAc = N-acetylglucosamine; EDTA = ethylenediaminetetraacetic acid; NHS = Normal human serum.

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for cancer. An isoenzyme of serum GT (GT-II) has been described as more consistently associated with cancer (12,13). Two recent articles have suggested the clinical usefulness of GT-II for detection of potentially curable colon cancers (12) and for diagnosis of pancreatic carcinoma (14). The purification of the cancer-associated and normal isoenzymes were reported by Podolsky and Weiser (6) using DEAE-cellulose and two enzyme substrate affinity chromatographic steps. Attempts to use this previously published protocol resulted in a similar 5000-fold purification but the preparations, by immunologic criteria, contained immunoglobulins. Our present data indicate that these immunoglobulins can only be removed by anti-immunoglobulin chromatography. The data suggest that the earlier report (6) on the structural characterization of GT-I and II requires re-evaluation and that there may be a special relationship between immunoglobulins and soluble GT.

MATERIALS AND METHODS

Materials. Radioactive UDP[^{3}H]galactose (Amersham) and non-radioactive UDP-galactose (Sigma) were mixed to 54.8 dpm/pmole. Filter sheets (934-AH) used for the GT assays were purchased from Whatman. Asialo, agalactosylfetuin was prepared as described (15). α-Lactalbumin was purchased from Sigma. Sepharose 4B was purchased from Pharmacia. Antibodies specific for normal human serum proteins (Miles-Yeda), bovine IgG (Miles-Yeda), human IgG (Cappel), human IgA (Miles-Yeda) and rabbit Ig (Cappel) were used for immunological procedures. Goat anti-human Ig used in enzyme purification was a generous gift of Dr. N. Tanigaki, Roswell Park Memorial Institute, Buffalo, New York. Purified IgG (Sigma) and IgA (Miles-Yeda) were used to characterize antisera. Purified bovine milk GT (Lot #120F-8085) was purchased from Sigma. All other chemicals were of reagent grade or better.

Assay of GT activity. Assay conditions were measured as previously described (6). Product was measured by spotting onto glass fiber sheets wetted with 5% phosphotungstic acid in 1N HCl. The sheets, with up to 30 assay spots, were washed to eliminate unbound radioactivity. The five washes consisted of a 10 min wash in 5% phosphotungstic acid in 1N HCl, two washes of 10 min in 0.1 M HCl, and two washes of 5 min in 95% ethanol, all at room temperature. The filter sheets were blotted dry between washes. The washed filters were dried and individual assay spots cut out and counted in OCS (Amersham) at an efficiency of 27%.

GT purification: Human malignant effusions, known to contain both GT-I and II by polyacrylamide gel electrophoretic separation (16), were pooled. Effusions were filtered through cheese cloth and the 20-50% ammonium sulfate fraction isolated, resuspended in 0.1 M NaCl to one-fourth the original volume and dialyzed against changes of CK buffer (25 mM Na cacodylate + 40 mM KCl, pH 7.2). The dialyzed 20-50% ammonium sulfate fraction was adjusted to 5mM GlcNAc and applied to a column of α-lactalbumin-Sepharose (5 x 40 cm) which bound essentially all GT activity. The column was washed with application buffer (CK buffer + 5 mM GlcNAc) until absorbance at 280nm was reduced to less than 0.20. GT activity was recovered as a single peak by eluting with CK buffer The pooled GT in CK buffer was made to 0.5 μM UDP and 25 mM $MnCl_2$ and applied to a GlcNAc-Sepharose column (1.5 x 30 cm). The column was washed with application

buffer until absorbance at 280nm approached zero. GT was then eluted from the column with CK buffer containing 5 mM GlcNAc and 25 mM EDTA. Fractions of effluent containing GT activity were pooled and made to 0.005% Triton X-100 to stabilize GT activity (17), concentrated by ultrafiltration (Amicon PM-30 filter) and dialyzed against CKT buffer (CK buffer + 0.005% Triton X-100) Partially purified GT was applied in 5 ml aliquots to columns (0.7 x 9 cm) of anti-normal human serum- or anti-human IgG-Sepharose and 1 ml fractions collected at 22°. The columns were washed with CKT buffer until all GT activity had eluted. Washes containing enzyme activity were pooled and recycled through affinity columns until protein concentration had stabilized. The GT-containing washes were concentrated to approximately 0.075 mg protein/ml.

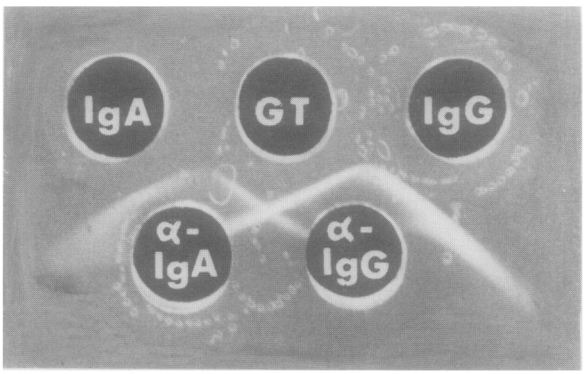
Production of antibodies. A solution of purified GT (750 µg in 1 ml) was emulsified with an equal volume of Freund's complete adjuvant. Rabbits were immunized with half of this mixture intradermally at 8-10 sites along the back and the other half was used for intramuscular and footpad injection. This protocol was repeated 10 days later. At 2 and 4 weeks after the first immunization purified GT (20 µg in .2 ml) in CKT buffer was injected into the ear vein. Animals were bled and sera tested for GT antibodies approximately 3 weeks after primary immunization and at 2-3 week intervals. Sera were heated to 56° for 45 min and denatured protein and lipid removed by centrifugation at 40,000 x g for 1 hr.

Preparation of affinity adsorbents. α-Lactalbumin-Sepharose was prepared as outlined by Trayer, et al. (18). N-Acetylglucosamine-Sepharose was prepared according to the method of Bloch and Burger (19) using p-aminophenyl-GlcNAc, prepared by reduction of p-nitrophenyl-GlcNAc with 0.1 M $Na_2S_2O_4$ (19). Other proteins (e.g. antibodies or antigens) were coupled to cyanogen bromide activated Sepharose 4B according to Porath (20). Gluteraldehyde cross-linked α-lactalbumin-Sepharose effluent was prepared as outlined by Avrameas and Ternynck (21).

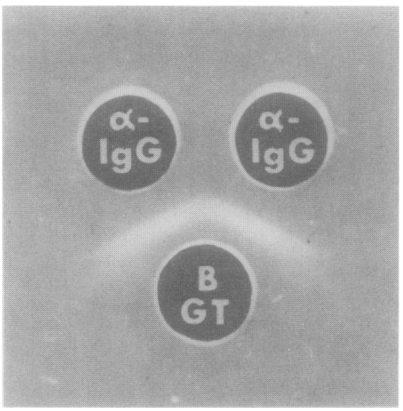
RESULTS AND DISCUSSION

Using a previously published method (6) GT-I and GT-II were purified from human malignant effusions and attempts were made to induce antibodies against these proteins in goats and rabbits. The antibodies produced were directed toward serum proteins other than GT. Both anti-IgG and anti-IgA showed immunoprecipitin lines with components in the purified GT preparation, suggesting the presence of immunoglobulins (Fig. 1). Immunodiffusion analysis of a commercial preparation of purified bovine milk GT also demonstrated bovine IgG (Fig. 2) However, anti-human IgG did not precipitate or inactivate GT activity of human serum and malignant effusions.

A purification procedure for human GT was subsequently developed which eliminated non-GT antigens using immuno-affinity chromatographic steps (Table I). Immunoadsorbant chromatography of GT with anti-IgG-Sepharose markedly increased galactosyltransferase specific activity and reduced IgG content to levels below the sensitivity of RIA determination (<5µg/ml).



(1)



(2)

Fig. 1. Double immunodiffusion of "purified" GT prepared according to the method of Podolsky and Weiser (6) vs. antibodies of human IgG and IgA. Human IgG and IgA are included for comparison. It is apparent that the preparation contains IgG and IgA.

Fig. 2. Commercial preparation of bovine milk GT contains immunoglobulin. Double immunodiffusion of 10 mg/ml bovine milk GT (lower well) and goat anti-bovine IgG (upper wells).

Production of antibodies in rabbits by immunization with the product from the final purification step of Table I is shown in Fig. 3. Double antibody immunoprecipitation of GT using these antisera demonstrated that rabbit anti-GT was present (Fig. 3). The amount of GT in unfractionated serum, about 150 ng/ml (5), is too low to be detected by double immunodiffusions. Thus any precipitin lines which appear between NHS and anti-GT antisera are probably due to the presence of non-GT antibodies. Evaluation of the anti-GT antisera by double immunodiffusion demonstrated the presence of antibodies to human serum components including IgG and IgA. Removal of all but one non-GT antibody was accomplished by affinity chromatography of rabbit antisera with Sepharose-bound human effusion antigens (minus GT) isolated from an α-lactalbumin-Sepharose purification step effluent. Repeated absorption with a gluteraldehyde cross-linked α-lactalbumin-Sepharose effluent (minus GT) was required to eliminate any detectable reaction to human serum by double immunodiffusion. Absorbed antiserum retained the ability to inhibit GT activity. Berger et al. (22) found it necessary to use a galactosyltransferase-agarose affinity column for isolation of anti-GT antibodies produced in rabbits by immunization with purified human milk GT. This

TABLE I. PURIFICATION OF HUMAN GALACTOSYLTRANSFERASE FROM HUMAN MALIGNANT EFFUSION

Step	Specific Activity	Purification	Yield	IgG Content	Protein Content
	Units*/mg x 10^3	fold	%	mg/ml[+]	mg/ml
Crude Effusion (2634 ml)	.01	1.0	100	6.2	36.0
20-50% $(NH_4)_2SO_4$ ppt	.032	3.1	44.9	12.0	40.0
α-Lactalbumin-Sepharose	.875	85.0	48.5	0.4	1.0
GlcNAc-Sepharose	102	9,911	158.2	0.25	0.49
Anti-NHS-Sepharose	95	9,199	163.7		0.39
Anti-IgG-Sepharose (67.2ml)	486	47,143	134.2	<0.005	0.078

*one unit = 1 mMole of galactose incorporated/min

[+]Radioimmunoassay of human IgG was performed by a competitive binding solid phase system. Plastic tubes were coated with rabbit anti-human IgG. ^{125}I-labeled human IgG was incubated in the presence of standard and test samples containing IgG. The ^{125}I bound to the plastic tube was measured and standard curve over the range 2.5 to 67ng was used to determine IgG.

would substantially improve the specificity of anti-GT and remove non-GT antibodies.

The co-purification of immunoglobulin and other serum components with GT during affinity chromatographic purification is not understood at present. Other cases of immunoglobulin associations with serum enzymes such as creatine kinase (23), lactate dehydrogenase (24) and amylase (25) are well documented. In addition, a high-molecular-mass alkaline phosphatase complex has been identified in sera of patients with liver disease (26) which appears to be due to an enzyme interaction with lipoprotein (27). More recently, Sharma et al. (28) have detected macromolecular albumin-IgG complexes as normal constituents of human serum. The co-purification of galactosyltransferase activity with several serum proteins, particularly immunoglobulins, may stem from the need for this Golgi membrane-associated enzyme to seek a hydrophobic anchor in extracellular fluids. The use of Triton X-100 to preserve enzymatic activity after affinity chromatography on α-lactalbumin- and GlcNAc-Sepharose may have altered the association of GT with serum proteins and permitted the removal of most of the accompanying immunoglobulins. It is unlikely that the association of GT with immunoglobulins in malignant effusions represents interaction with

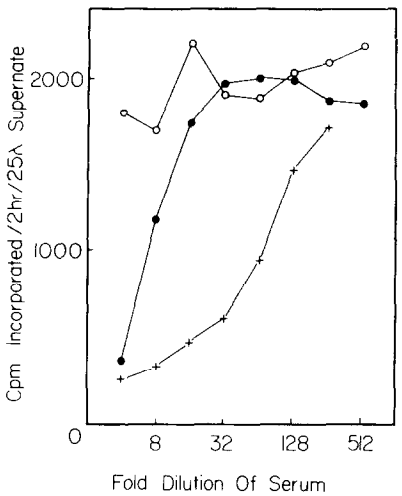


Fig. 3. Test for production of rabbit antibodies to human GT purified as outlined in Table I. Normal human serum (20µl) was incubated with an equal volume of either preimmune or GT-immunized sera (20µl diluted 1:1 with CK buffer) for 3 hr at room temp. A second antiserum (20µl) directed against goat or rabbit IgG, was then added and the mixture incubated overnight at 4°C. The mixtures were then centrifuged for 30 min at 2000 rpm and 25µl of the supernatant assayed for GT activity. Serum dilutions: preimmune (O—O), first bleed at 3 weeks (●—●) and second bleed at 6 weeks (+—+) after immunization are shown. All anti-sera were diluted with preimmune serum.

anti GT antibody since 1) no loss of GT activity was observed when immunoglobulins were removed by affinity chromatography (Table I), and 2) precipitation of effusion and serum immunoglobulins by anti-Ig did not remove GT activity.

In view of our observations showing that immunoglobulin accompanies GT preparations purified by the method of Podolsky and Weiser (6), structural characterization of the normal human enzyme (GT-I) and the cancer associated enzyme (GT-II), reported in that earlier paper, must be re-evaluated. Neither the amino acid nor the carbohydrate composition in the report by Podolsky and Weiser (6) were comparable to values reported by Fujita-Yamaguchi and Yoshida for normal human serum GT (5) or by Trayer and Hill for bovine milk GT (7). Contamination of GT by immunoglobulins, known to contain a variety of oligosaccharides (29), could be responsible for the differences observed.

In conclusion, we have found that GT purified from human malignant effusion requires affinity chromatographic steps directed not only at binding GT but also at removing specific contaminants including immunoglobulins. Anti-

body to GT can be produced using antigens purified by our method. These antibodies should prove useful in studies of GT serum levels in malignancy.

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REFERENCES

1. Schachter, H. and Roden, L. (1973) in Metabolic Conjugation and Metabolic Hydrolysis, ed Fishman, W.H. (Academic Press, New York, pp. 1-149).

2. Whur, P., Herscovics, A. and Leblond, C.P. (1969). J. Cell Biol. 43, 289-300.

3. Hill, R.L. and Brew, K. (1975) Adv. Enzymol. 43, 411-590.

4. Bella, A., Whitehead, S.S. and Kim, Y.S. (1977) Biochem. J. 167, 621-628.

5. Fujita-Yamaguchi, Y. and Yoshida, A. (1981) J. Biol. Chem. 256, 2701-2706.

6. Podolsky, D.K. and Weiser, M.M. (1979) J. Biol. Chem. 254, 3983-3990.

7. Trayer, I.P. and Hill, R.L. (1971) J. Biol. Chem. 246, 6666-6675.

8. Gerber, A.C., Kozdrowski, I., Wyss, S.R. and Berger, E.G. (1979) Eur. J. Biochem. 93, 453-460.

9. Rittenhouse, H., Schenck, J., Tomito, J., Hirata, A., Weiser, M.M., Albini, B., Wilson, J.R., and Berger, E., 1981. Fed. Proc. (FASEB) 40, 1675.

10. Chatterjee, S.K., Bhattacharya, M., and Barlow, J.J. (1979) Cancer Res. 39, 1943-1951.

11. Paone, J.F., Waalkes, T.P., Baker, R.R. and Shaper, J.H. (1980) J. Surg. Oncol. 15, 59-66.

12. Podolsky, D.K., Weiser, M.M., Isselbacher, K.J. and Cohen, A.M. (1978) N. Engl. J. Med. 299, 703-705.

13. Weiser, M.M., Podolsky, D.K., and Isselbacher, K.J. (1976) Proc. Natl. Acad. Sci. USA 73, 1319-1322.

14. Podolsky, D.K., McPhee, M.S., Alpert, E., Warshaw, A.L. and Isselbacher, K.J. (1981) N. Engl. J. Med. 304, 1313-1318.

15. Kim, Y.S., Perdomo, J., and Nordberg, J. (1971) J. Biol. Chem. 246, 5466-5476.

16. Podolsky, D.K. and Weiser, M.M. (1975) Biochem. Biophys. Res. Commun. 65, 545-551.

17. Fraser, I.H. and Mookerjea, S. (1976) Biochem. J. 156, 347-355.

18. Trayer, I.P., Barker, R., and Hill, R.L. (1974) in Methods in Enzymology, eds. Jakoby, W.B. and Wilcheck, M. (Academic, New York) Vol. 34, pp. 359-360.

19. Bloch, R. and Burger, M.M. (1974) FEBS Letters 44, 286-289.

20. Porath, J. (1974) in Methods in Enzymology, eds. Jakoby, W.B., and Wilchek, M. (Academic, New York) Vol. 34, pp. 13-30.

21. Avrameas, S., and Ternynck, T. (1969) Immunochemistry 6, 53-66.

22. Berger, E.G., Mandel, T. and Schilt, U. (1981) J. Histochem. Cytochem. 29, 364-370.

23. Prabhakaran, V., Nealon, D.A. and Henderson, A.R. (1979) Clin. Chem. 25, 112-116.

24. Ganrot, P.W. (1967) Experientia 23, 593.

25. Berk, J.E., Kizu, H., Wilding, P. and Searcy, R.L. (1967) N. Engl. J. Med. 277, 941-946.

26. Crofton, P.M. and Smith, A.F. (1981) Clin. Chem. 27, 867-874.

27. Crofton, P.M. and Smith, A.F. (1981) Clin. Chem. 27, 860-866.

28. Sharma, N.C., Mohammad, S.F., Chuang, H.Y.K. and Mason, R.G. (1981) Proc. Natl. Acad. Sci. USA 78, 7750-7753.

29. Baenziger, J., Kornfeld, S. (1974) J. Biol. Chem. 249, 7270-7281.