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Serum α-6-L-Fucosyltransferase is Released from Platelets during Clotting of Blood

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Activity of serum α-6-L-fucosyltransferase is strictly correlated with the original concentration of blood platelets. The plasma enzyme activity is confined to intact platelets and appears in serum during the course of coagulation of blood. Activity of serum α-3-L-fucosyltransferase does not depend on platelets.

Glycosyltransferases occur in the body as membrane-bound enzymes in cells and as soluble enzymes in blood serum and secretions (see [1-3]). Human serum contains a number of different glycosyltransferases including α-2-L-fucosyltransferase or *H* enzyme (EC 2.4.1.69) [4, 5], α-3-L-fucosyltransferase (EC 2.4.1.52) [4] and α-6-L-fucosyltransferase (EC 2.4.1.68). The latter enzyme was probably the one described in human serum by Munro and Schachter [5] which was later characterised as an α-6-L-fucosyltransferase [6].

The origin and mechanism of entry of glycosyltransferases into serum are unknown. Genetic evidence broadly implies a bone marrow origin of serum α-2-L-fucosyltransferase [7] whereas glycosyltransferases specified by blood group *A* and *B* genes are only partly derived from the hemopoietic tissue [8, 9].

Here we report that α-6-L-fucosyltransferase is released into serum during coagulation of blood. The enzyme activity in plasma is confined to intact platelets. Some aspects of this study were communicated at the 621st Biochemical Society Meeting, London, December 1986 [10].

Abbreviations: AML, acute myelogenous leukemia; CGL, chronic granulocytic leukemia; CLL, chronic lymphocytic leukemia; ALL, acute lymphocytic leukemia, IV^3NeuAc-nLcOse$_4$Cer, sialosylparagloboside, NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcCer; EGTA, ethyleneglycolbis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid

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Materials and Methods

Blood Samples

Samples of blood from patients with various forms of leukemia were obtained from the Clinic of Internal Diseases and the Outpatient Department of the Institute of Hematology, Warsaw. None of the patients was treated with cytostatic drugs. Normal blood was supplied by volunteers. Samples of serum or plasma were kept frozen at -20°C and were thawed only once just before transferase assays were performed. "Bombay" serum was kindly supplied by Prof. H. Seyfried of this Institute.

Radioactive Nucleotide Sugars and Enzyme Acceptors

Labeled GDP-[^{14}C]fucose (200 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, England. Phenyl-β-D-galactoside was from Sigma Chemical Co., St Louis, MO, USA. Sialosylparagloboside (IV^{3}NeuAc-nLcOse$_4$Cer) was isolated from human erythrocyte stroma by the method outlined in [11]. The glycolipid was purified to homogeneity by HPLC with a Knauer HPLC apparatus (pumps, model 64; programmer, model 50) on a column (0.8 × 150 cm) packed with unmodified silica Lichrosorb Si 60, 10 μm diameter (Merck, Darmstadt, W. Germany) in chloroform. The column was eluted with chloroform/methanol/water, 10/80/10 by vol, and chloroform/methanol/water, 90/10/0.5 by vol, programmed so that the concentration of the first solvent mixture increased from 20-60% final concentration.

IgG glycopeptide was isolated from pronase digests of human IgG (16 g), at the Institute of Hematology, Warsaw, Poland, by fractionation on Sephadex G-25, Sephadex G-75 (Pharmacia, Uppsala, Sweden) and Dowex 50W-X2 (200-400 mesh; Fluka, Buchs, Switzerland) columns exactly as described in [12]. The main carbohydrate peak, containing ~85 mg hexose and ~10 mg protein, eluted from Dowex 50W-X2 was desalted on a Bio-Gel P-10 column (1.5 × 35 cm) and then subjected to acid hydrolysis with 0.8 M HCl at 80°C for 1 h to remove sialic acid, fucose and bisecting *N*-acetylglucosamine residues [13]. The material was again desalted on Bio-Gel P-4 (1.5 × 35 cm) and then exhaustively digested with jack bean β-galactosidase (Sigma) using the method of Li and Li [14]. Briefly, the partially hydrolysed glycopeptide (80 mg) was treated with 0.4 ml of 0.2 M sodium citrate buffer pH 4.0 and 2 ml (4 Units) of the enzyme. The mixture was incubated for 8 h at 37°C. Subsequently, more enzyme (1 ml) was added and the incubation was continued for 40 h under toluene. The mixture was then filtered successively through columns of Bio-Gel P-4 (2 × 30 cm) and Bio-Gel P-10 (2 × 30 cm) and the appropriate hexose-containing peaks were collected. The final eluate was evaporated under reduced pressure.

The material was subjected to methylation analysis employing GLC and GLC-MS techniques (for methods and apparatus see [15]). The sugar composition was as follows: 2-*O*-substituted mannose (2 mol); 3,6-di-*O*-substituted mannose, 0.7 mol; 3,4,6-tri-*O*-substituted mannose, 0.3 mol; terminal *N*-acetylglucosamine and 4-*O*-substituted *N*-acetylglucosamine were present but not quantified; terminal galactose was not detected but some terminal fucose was still present. Thus it was concluded that the preparation contained a bi-antennary glycopeptide [13] terminated with *N*-acetylglucosamine residues. This glycopeptide preparation will be further referred to as stripped IgG glycopeptide.

Fucosyltransferase Assays

α-2-L-Fucosyltransferase activity was determined by the method of Chester *et al.* [16] with phenyl-β-D-galactoside as substrate. The incubation mixture contained in 100 μl; phenyl-β-D-galactoside, 4 μmol; GDP-[^{14}C]fucose, 0.6 nmol; Tris-HCl buffer, pH 7.2, 5 μmol; neutralised ATP, 0.5 μmol; NaN_3, 0.8 μmol; serum, 50 μl. After incubation for 16 h at 37 °C, the radioactive reaction product was isolated by method A [16] and counted in a liquid scintillation counter.

α-3-L-Fucosyltransferase activity was measured under similar conditions to those described in [17]. The incubation mixture contained in 100 μl; IV^{3}NeuAc-nLcOse$_4$Cer, 50 nmol; GDP-[^{14}C]fucose 0.5 nmol; Tris-HCl buffer, pH 7.0, 2.5 μmol; neutralised ATP, 1 μmol; $MgCl_2$, 0.75 μmol; NaN_3, 0.75 μmol; Triton X-100, 100 μg; serum, 75 μl. After incubation for 24 h at 37°C the reaction was terminated by sequential additions of 25 μl 0.2 M EDTA, pH 7.2 and 1 ml chloroform/methanol, 1/1 by vol. Samples were mixed, left overnight at room temperature and centrifuged at 500 × *g* for 15 min. Supernatants were collected and the residues washed once with 0.25 ml portions of chloroform/methanol, 1/1 by vol. Combined supernatants were dried under a stream of nitrogen, and redissolved in 0.25 ml portions of chloroform/methanol/0.05 M NH_4OH, 5/5/1 by vol. The solubilized materials were spotted on Whatman 3MM papers and chromatographed overnight with 1% sodium tetraborate, pH 9.1. Radioactivity remaining at the origin and that of two adjacent 2 × 2 cm strips were determined by scintillation counting.

α-6-L-Fucosyltransferase. Incubation mixtures contained in 120 μl: stripped IgG glycopeptide, 100 μg; GDP-[^{14}C]fucose 0.37 nmol; sodium cacodylate buffer, pH 6.0, 4 μmol; EGTA, 0.8 μmol; $MgCl_2$, 0.8 μmol; NaN_3, 1 μmol; serum 25μl. After 16 h at 37°C, reactions were terminated by the addition of 80 μl water and 200 μl of absolute ethanol. Samples were mixed and clarified by centrifugation at 500 × *g* for 15 min. Supernatants were withdrawn and tubes washed once with 200 μl of 50% ethanol. Combined supernatants were passed through Dowex 1 (acetate, 200-400 mesh) columns (0.5 × 1.5 cm). The columns were washed successively with five 0.5 ml portions of 50% ethanol. Combined eluates were dried under a stream of nitrogen, redissolved in 200 μl of 50% ethanol and chromatographed overnight with absolute ethanol/0.01 M EDTA, 8/2 by vol. Radioactivity remaining at the origin and that of two adjacent 2 × 2 cm strips was determined by liquid scintillation counting.

Isolation of Platelets

Blood (50 ml) was collected into 8 ml of acid-citrate-dextrose anticoagulant in a polycarbonate tube, mixed and centrifuged at 300 × *g* for 10 min at room temperature. The supernatant was withdrawn with a plastic pipette, mixed, and recentrifuged at 200 × *g* for 10 min. Platelet-rich plasma prepared in this way (19 ml) contained approximately 60% of the platelets originally present in whole blood but was completely free of erythrocytes and leukocytes. Platelet concentrate was prepared from the platelet-rich plasma by centrifugation at 1500 × *g* for 30 min. Sedimented platelets were resuspended in 1.5 ml Eagles's Minimum Essential medium (Biomed, Lublin, Poland) containing 0.1% Triton X-100 and homogenized in a glass-teflon Potter homogenizer. The homogenate was used immediately for α-6-L-fucosyltransferase assays.

Table 1. α-3-L- and α-6-L-Fucosyltransferases in sera of leukemic patients with different white cell and platelet counts.

Patient	Blood group	Diagnosis	White blood cell count x 10^3	Blasts x 10^3	Platelet count x 10^3	L-Fucosyltransferase activity	
						α-3[a]	α-6[b]
MG	O	CGL at blast crisis	48.3	26.0	30.0	8.0	8.3
AP	A	AML	114.0	37.0	79.0	55.0	14.7
SF	A	CLL	49.0	—	165.0	21.0	24.3
MD	A	CGL	282.0	5.6	358.0	9.6	40.7
JK	O	Thrombo-cytosis	17.9	—	1869.0	12.9	180.7

[a,b] pmol [^{14}C]fucose incorporated into a) IV^{3}NeuAc-nLcOse$_4$Cer/100μl of serum/24 h, or b) stripped IgG glycopeptide/100 μl of serum/16 h. Both reactions were linear within the time limits of the experiments. For compositions of the reaction mixtures see the Materials and Methods section.

Results

Activities of α-2-L-, α-3-L- and α-6-L-fucosyltransferases were determined with phenyl-β-D-galactoside, sialosylparagloboside and stripped IgG glycopeptide as acceptor substrates, respectively. All three substrates employed should be unambigous and measure only single enzyme activities (see the Discussion section).

While studying enzyme activities in patients with various forms of leukemias [10] we noticed a correlation between platelet counts and serum α-6-L-fucosyltransferase but not α-3-L-fucosyltransferase (Table 1). The enzyme activities did not correlate with high concentrations of other morphotic elements of blood. Determination of serum α-6-L-fucosyltransferase activity in more patients as well as normal volunteers established a strict correlation between α-6-L-fucosyltransferase and the concentration of platelets (Fig. 1). In keeping with the results presented in Table 1 and Fig. 1 the activity of α-6-L-fucosyltransferase is reduced by ~ 90% in platelet-free plasma as compared to serum (Table 2). On the other hand, the activity of α-2-L-fucosyltransferase is reduced by only 30-50% and that of α-3-L-fucosyltransferase is the same in serum and platelet-free plasma.

In spite of the absence of terminal galactose in stripped IgG glycopeptide we measured the activity of α-6-L-fucosyltransferase in a "Bombay" serum sample. The activity was normal, i.e. it corresponded to a platelet concentration of 300 000/μl, although activity of α-2-L-fucosyltransferase was totally absent.

Finally, we measured the activity of α-6-L-fucosyltransferase in isolated platelets. As shown in Table 3 the activity of the enzyme in plasma is largely confined to intact platelets and is recovered in the platelet concentrate. In these experiments α-6-L-fucosyltransferase was measured in the presence of 0.1% Triton X-100. Presence of the detergent had no effect upon serum enzyme activity (data not shown).

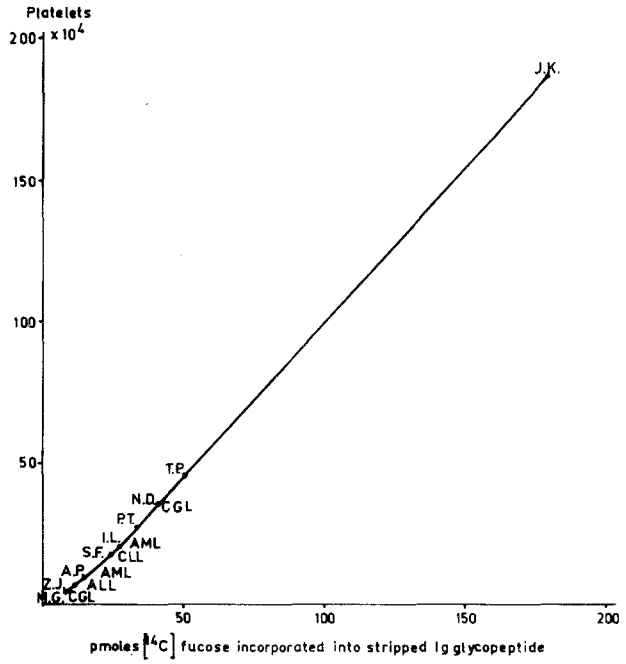

Figure 1. Concentration of platelets expressed as number per μl blood versus serum activity of α-6-L-fucosyltransferase. Initials of patients or volunteers are shown above the line. Diagnosis is indicated below the line. Patient JK with the highest concentration of platelets probably suffers from myelofibrosis.

Table 2. Activities of α-2-L-, α-3-L- and α-6-L-fucosyltransferase in serum and platelet-free plasma of two healthy individuals.

		L-Fucosyltransferase activity		
Material	Donor	α-2-[a]	α-3-[b]	α-6-[b]
Serum		212.0	46.0	63.4
Plasma, heparin[c]	AP	162.5	46.0	4.4
Plasma, EGTA		135.0	44.1	10.0
Serum		292.5	39.0	59.9
Plasma, heparin	TP	160.0	39.0	6.8
Plasma, EGTA		155.0	44.0	14.0

[a] pmol [^{14}C]fucose incorporated into phenyl-β-D-galactoside/100 μl of plasma or serum.
[b] Activities of α-3-L- and α-6-L-fucosyltransferase are expressed as in Table 1.
[c] For plasma collection, blood samples were drawn into heparin or EGTA (10 mM final concentration). Samples were then centrifuged at 1600 $\times$ g for 30 min.

Table 3. α-6-L-Fucosyltransferase in isolated platelets.

Enzyme source	α-6-L-Fucosyltransferase		
	Activity/100 μl plasma or platelet concentrate[a]	Total activity[b]	Recovery %
Leukocyte-free, platelet-free plasma	6.8	—	—
Leukocyte-free, platelet-rich plasma[c]	23.0	4.4	(100%)
Platelet concentrate	226.4	3.4	76

[a] pmol [^{14}C]fucose incorporated/16 h.
[b] nmol [^{14}C]fucose incorporated/total volume/16 h. Total volumes for leukocyte-free, platelet-rich plasma and platelet concentrate were 19.0 ml and 1.5 ml, respectively.
[c] The platelet concentration was 256 600/μl; when leukocyte-free, platelet-rich plasma was centrifuged in order to prepare platelet concentrate (see the Materials and Methods section) some platelets (19 000/μl) remained in the supernatant. Substrate: stripped IgG glycopeptide.

Discussion

α-6-L-Fucosyltransferase transfers fucose to position 6 of asparagine-linked *N*-acetylglucosamine residues of *N*-glycans [13]. The enzyme requires acceptor substrates containing a terminal β(1-2)-linked *N*-acetylglucosamine residue on the Manα1-3 arm of the core. Bi-antennary glycopeptides with two terminal β(1-2)-linked *N*-acetylglucosamine residues are also excellent substrates. A bisecting *N*-acetylglucosamine residue linked to the mannose core prevents attachment of fucose to asparagine-linked *N*-acetylglucosamine. The stripped IgG glycopeptide which we used as substrate in α-6-L-fucosyltransferase assays contained a typical mannose core and terminal (1-2)-linked *N*-acetylglucosamine residues as suggested by the results of methylation analysis (see the Materials and Methods section). The amount of material with a bisecting *N*-acetylglucosamine residue, as calculated from 3,4,6-tri-*O*-substituted mannose, did not exceed 30% of the total glycopeptide, and terminal galactose was absent. Thus we may assume that fucose incorporated into this substrate was due to the action of α-6-L-fucosyltransferase and not α-2-L- or α-3-L-fucosyltransferases, which are also present in human serum [4, 5]. The acceptor substrate for α-2-L-fucosyltransferase requires a terminal galactose residue [1] whereas that for α-3-L-fucosyltransferase needs a Galβ1-4GlcNAc structure which may be also terminated with α(1-2)-linked fucose or α(2-3)-linked sialic acid [18, 19]. Recent evidence suggests that the α-3-L-fucosyltransferase which transfers fucose to Galβ1-4GlcNAc and Fucα1-2Galβ1-4GlcNAc is different from that which conveys fucose to NeuAcα2-3Galβ1-4GlcNAc (P.H. Johnson and W.M. Watkins, personal communication). Thus, using sialosylparagloboside as substrate for α-3-L-fucosyltransferase we may have measured only one form of enzyme

activity. Of three α-L-fucosyltransferases of serum studied, only one, i.e. α-6-L-fucosyltransferase, is strictly correlated with platelet concentration. This fact, as well as the presence of α-6-L-fucosyltransferase in isolated platelets and its absence from platelet-free plasma, indicates that the enzyme is released from platelets during clotting of blood. Another enzyme which is but partly released during blood clotting is α-2-L-fucosyltransferase. Here we confirm the results of P. Skacel and W.M. Watkins (personal communication) who found α-2-L-fucosyltransferase in platelets.

The finding of a platelet-dependent release of α-6-L-fucosyltransferase during blood clotting is of major interest. It raises the question about a possible function of this enzyme, and glycosyltransferases in general, in blood coagulation.

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