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Shape Change and the Percentage of Sialic Acid Removed by Neuraminidase from Human Platelets[1] (40282)

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N-acetylneuraminic acid is the only sialic acid found in human platelets (1) and 7–15 μg is found per mg of platelet protein (2). Bacterial neuraminidases from *Clostridium perfringens* and *Vibrio cholera*, which cleave the α-ketoside linkage between sialic acid and the penultimate galactose or galactosamine, liberate 40–60% of sialic acid from human platelets (1–5). This is thought to be derived from surface membrane glycoproteins.

In a recent study, Motamed *et al.* (6) conclude that platelet surface sialic acid increases after shape change. This report is based on data obtained from platelets fixed in plasma. A nonspecific attachment of plasma proteins to the platelet surface is observed after fixation (7). As many of these plasma proteins contain sialic acid, and the platelet surface area increases after shape change (6), it is difficult to establish on the basis of these studies whether the increase in the amount of sialic acid removed by neuraminidase represents increased platelet surface sialic acid, or merely an increase in the amount of plasma proteins fixed onto the platelet membrane.

Ku and Wu (4, 5), using washed platelets, reported that thrombin-, collagen- and ADP-induced platelet activation increased the amount of neuraminidase-removable sialic acid. The increases observed after thrombin and collagen treatment were inhibited by aspirin, suggesting that material containing sialic acid is released during platelet activation, and that the increase in sialic acid observed after activation does not represent surface sialic acid.

This study was designed to determine whether ADP-induced shape change alters the amount of sialic acid on the surface of unfixed, aspirin-treated, gel-filtered platelets.

Materials and methods. C. perfringens neuraminidase (Type VI, Sigma) was purified according to the method of Hatton and Regoeczi (8) and shown to be free of proteolytic activity against radioactive tosylarginine methyl ester (TAMe) (9) and fibrinogen (10). The activity of the purified enzyme was determined according to the method of Warren (11), and one unit is defined as the amount of enzyme required to liberate 1 μmole of *N*-acetylneuraminic acid from bovine submaxillary mucin (Sigma) per min at 37°, pH 5.0.

Nine ml of blood from normal volunteers was collected into 1.5 ml of acid–citrate–dextrose (ACD) solution (12) and 0.5 ml of 1 m*M* acetylsalicylic acid (Merck) to inhibit the ADP-induced release reaction (13). After 15-min incubation at 37°, platelet-rich plasma (PRP) was prepared by centrifugation at 280*g* for 8 min at room temperature. PRP was removed and centrifuged at 2100*g* for 20 min. The resulting platelet button was resuspended in ½ the PRP volume with Tyrode's solution containing no added calcium (14), but with 0.2% bovine serum albumin (Sigma), and 0.1 mg/ml potato apyrase (Sigma purified Grade 1). The buffer was adjusted to pH 7.4 and the platelet concentrate gel-filtered (15) through Sepharose 2B (Pharmacia) equilibrated with Tyrode's solution containing albumin but no added glucose, calcium or apyrase. The platelets were eluted with the same buffer and their ability to aggregate with 5 μ*M* ADP was established on a small aliquot after adding 0.5 mg/ml fibrinogen (Kabi).

The suspension, containing 0.5–1.0 × 10^6 platelets/μl, was divided into four 0.9 ml aliquots and brought to 37°. Two samples were treated with 0.1 ml 50 μ*M* ADP, and two with 0.1 ml 0.15 *M* NaCl. The tubes were gently inverted twice, and a small aliquot of each was fixed in 1% formalin for examination under the phase contrast microscope.

Approximately 1 min after the addition of ADP, the pH of the samples was adjusted to

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6.5 with 0.1 *N* HCl. One ADP-treated and one saline control sample than received 40 μl of neuraminidase (0.21 U/ml). The other ADP-treated and saline control sample received 40 μl of ammonium acetate buffer. All samples were incubated for 15 min at 37°. After incubation, phase microscope observation showed that the saline control platelets had retained their discoid shape and the suspension exhibited the characteristic "swirl" when agitated. In contrast, the ADP-treated samples were spiny spheres and failed to swirl when shaken. A portion of the platelet suspensions not treated with neuraminidase was subjected to mild acid hydrolysis (0.1 N H_2SO_4, 1 hr, 80°) and total sialic acid was determined. Platelets were counted in another portion with a Coulter Counter. The remainder of the control samples as well as the neuraminidase-treated samples were then centrifuged in a Serofuge (Clay Adams) for 20 min. The supernatants were removed and sialic acid measured without hydrolysis (11).

In order to determine whether sialic acid is released from platelets after treatment with thrombin and connective tissue as reported by Ku and Wu (4), 9 ml of blood was collected into 1.5 ml of ACD containing 10 μl ^{14}C serotonin (Amersham, 8 μCi/ml) (16) and processed as previously described. Fifty μl of the suspension of ^{14}C-serotonin-labeled gel-filtered platelets was placed in glass counting vials containing 10 ml Aquasol (New England Nuclear) and counted in a Packard Liquid Scintillation Counter. Another aliquot, 190 μl, was used to determine total sialic acid after mild acid hydrolysis. The remaining suspension was divided into three equal aliquots and treated with 1 U/ml (final concentration) of highly purified human thrombin (kindly given to us by Dr. John W. Fenton II, New York State Department of Health, Albany), a suspension of ground human subcutaneous connective tissue (given to us by Dr. H. Lackner, New York University Medical Center), or isotonic saline. After gentle mixing, the suspensions were incubated at 37° for 10 min. The samples were then chilled and centrifuged at 4°C at 2100 g for 20 min to pellet the platelets. The sialic acid was measured on 190 μl of each supernatant after mild acid hydrolysis (11) and 50 μl of each supernatant was used to measure ^{14}C.

Results. The total sialic acid content of the ADP-treated and saline control samples did not differ. Hence, both values were included in the average, which was 60 nmoles/10^9 platelets (Table I). Neuraminidase removed 47% of sialic acid from both control and ADP-treated platelets. There was no sialic acid in the supernatants of samples treated with buffer instead of enzyme, even after hydrolysis.

Two experiments were carried out in which platelets were incubated with thrombin or connective tissue for 10 min without shaking. The platelets lost their "swirl" but no aggregates were seen on gross inspection. ^{14}C-serotonin release in the saline control platelets was 13.6% and 4.2% (Table II). This material is presumably released when platelets come into contact with Sepharose beads during gel filtration (17). Platelets treated with thrombin released 89.1% and 81.2% of their ^{14}C-serotonin, and 30% and 43% of their total sialic acid, respectively. Platelets treated with collagen released 57.6% and 33.2% of their ^{14}C serotonin, and 30% and 31% of their total sialic acid.

Discussion. ADP-induced shape change does not cause the release of material containing sialic acid from aspirin-treated platelets, as none was detected in the supernatants even after hydrolysis. Thus the sialic acid measured in the supernatant of suspensions treated with neuraminidase presumably represents sialic acid cleaved from membrane glycoproteins.

The amount of sialic acid removed by neuraminidase is not altered by ADP-induced shape change. This finding agrees with the results of Bunting and Zucker (3) who demonstrated that the same amount of tritium was incorporated into the sialic acid of discoid platelets and platelets which had undergone shape change after exposure to ADP.

Like others (2, 18), we found that collagen and thrombin release material containing sialic acid from human platelets. As neuraminidase will cleave sialic acid from both the platelet membrane and the released material, it is essential to prevent the release reaction in order to quantitate changes in membrane sialic acid during platelet stimulation.

Since we find no difference in the amount of sialic acid removed by neuraminidase from

TABLE I. THE EFFECT OF SHAPE CHANGE ON THE AMOUNT OF SIALIC ACID REMOVED BY NEURAMINIDASE FROM HUMAN PLATELETS.

Expt. No.	Total sialic acid (nmoles/10^9 platelets) NaCl	ADP	Avg.	Sialic acid removed by neuraminidase (%) NaCl	ADP	Change in sialic acid removed
1	74	74	74	40	39	−1
2	90	91	90	40	40	0
3	58	58	58	42	42	0
4	50	46	48	64	66	+2
5	40	44	42	52	52	0
6	80	82	81	33	30	−3
7	45	50	47	59	63	+4
8	42	44	43	49	44	−5
Mean			60	47	47	−0.375[a]
SE			0.6	0.8	0.8	0.98

[a] t = 0.382, not statistically significant.

TABLE II. PERCENT RELEASE OF ^{14}C SEROTONIN AND SIALIC ACID BY STIMULATED PLATELETS.

Expt. No.	Agent added	^{14}C Release (% of Total)	Sialic acid in supernatant (% of Total)
1	NaCl	13.6	0
	Thrombin	89.1	30
	C.T.	57.6	30
2	NaCl	4.2	0
	Thrombin	81.2	43
	C.T.	33.2	31

discoid platelets and spiny spheres, we conclude that ADP-induced shape change does not alter platelet surface sialic acid. In contrast, sialic acid appears to be lost from platelets which have been aggregated with ADP and disaggregated (3).

Summary. The sialic acid of human gel-filtered platelets was studied before and after ADP-induced shape change. Neuraminidase cleaved 47% of the total sialic acid from both discoid control platelets and platelets that had become spiny spheres after treatment with 5 μM ADP.

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