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Increase in Sialic Acids Removable by Neuraminidase during the Shape Change of Platelets

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Human or rabbit platelets were activated by ADP or 5-hydroxytryptamine and rapidly fixed with glutaraldehyde. The shape change associated with activation gave rise to an increase in sialic acids removable by neuraminidase. This increase, like the shape change, was prevented by adenosine or methysergide added before ADP or 5-hydroxytryptamine respectively. The results indicate the exposure of additional glycoprotein(s) on the platelet surface.

ADP or 5-hydroxytryptamine causes platelets of several species, including man and rabbit, to transform rapidly from discs with almost smooth surfaces to spheroids with variously shaped protrusions (Macmillan & Oliver, 1965; Born, 1970). This morphological change, which precedes aggregation, is associated with a decrease in light-transmission through and an increase in light-scattering by the suspension (Michal & Born, 1971). During the morphological change the average platelet volume remains constant (Born, 1970), but the surface-to-volume ratio increases considerably (Born *et al.*, 1972; G. V. R. Born, R. Dearnley & J. Foulks, unpublished work). The origin and nature of the newly exposed surface are unknown. The outer membrane of platelets contains glycoproteins, and the removal of sialic acids from them by neuraminidase alters platelet aggregability (Hovig, 1965; Greenberg *et al.*, 1975). The present paper shows that the increase in surface area of platelets during the shape change is associated with an increase in enzymically labile sialic acids.

Experimental

Human or rabbit platelet-rich plasma was separated from citrated blood by centrifugation at 300*g* for 15min. Samples were warmed to 37°C and 20μl of 0.4M-ethanedioxybis(ethylamine)tetra-acetic acid (EGTA) (Koch–Light Laboratories, Colnbrook, Bucks., U.K.) was added/ml of plasma to prevent aggregation of the platelets. Then 10s later 20μl of either 0.1mM-ADP [Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.], 0.5mM-5-hydroxytryptamine (May and Baker, Dagenham, Essex, U.K.) or saline (0.9% NaCl) was added to 1ml of rapidly stirred plasma, and 5s later 3vol. of glutaraldehyde fixative was added to 1vol. of platelet-rich plasma. To prepare the fixative glutaraldehyde (TAAB Laboratories, Reading, Berks., U.K.) was diluted to 0.41% and the osmolality adjusted to 310mosM with NaCl; the pH was adjusted to 7.4. The plasma plus fixative was left at room temperature for 1h. The plasma was centrifuged and the fixed platelets were washed three times in 0.154M-NaCl and finally suspended in saline at pH5.4 at a concentration of 10^9 platelets/ml. Samples of the suspension mixed with 10μl of 0.13M-$CaCl_2$/ml and with 15 units (Schramm & Mohr, 1959) of neuraminidase (*Vibrio cholerae*; Behringwerke AG, Marburg, Germany) were incubated at 37°C for 60min. After centrifugation at 9000*g* for 4min, 0.2ml samples of the supernatant were used for sialic acid assay. For total sialic acids, 0.2ml samples of the platelet suspensions without neuraminidase were sealed in glass ampoules with 0.2ml of 1M-HCl and heated at 110°C for 16–24h. Sialic acid was assayed by fluorophore formation (Hess & Rolde, 1964) with purified 3,5-diaminobenzoic acid (Aldrich Chemical Co., Gillingham, Dorset, U.K.), and the fluorescence was measured in a Perkin–Elmer MP4 ratio recording spectrophotometer. Lack of proteolytic activity was confirmed by assay of sialic acid-containing glycoprotein [human fraction VI from Schwarz–Mann Division of Becton Dickinson (U.K.) Ltd., Wembley, Middx., U.K.]; the enzyme yielded only sialic acids, and in the correct quantity. Reproducibility of the assay with standard *N*-acetylneuraminic acid solutions was within 3%.

Light-microscopy (Leitz phase-interference contrast) and electron microscopy (Philips 300) confirmed that our glutaraldehyde fixation preserved control platelets in their discoid form and was capable of arresting very rapidly the morphological reactions to ADP or 5-hydroxytryptamine. Moreover, fixed platelets retained their shapes throughout subsequent washings.

Results and Discussion

Fixation did not interfere with the release of sialic acids by neuraminidase. Neuraminidase removed 40.3 and 39.7% of the total sialic acid from fixed control human or rabbit platelets respectively (Table 1). Our estimates of total and neuraminidase-labile sialic acids are similar to values obtained with washed platelets (Gröttum & Solum, 1969; Kuroyanagi & Saito, 1974; Greenberg *et al.*, 1975).

Rabbit platelets that had been changed in shape by either ADP or 5-hydroxytryptamine released more sialic acids with neuraminidase than did control platelets (Table 2); the increases were 22.2% for ADP and 14.6% for 5-hydroxytryptamine. With human platelets there were similar increases after the shape change. When the shape change was inhibited by adenosine (20 μM) or methysergide {*N*-[1-(hydroxymethyl)propyl]-1-methyl-(+)-lysergamide} (0.25 μM) added before ADP or 5-hydroxytryptamine respectively, the amount of sialic acid released was similar to that released from control platelets. Adenosine by itself had no effect on sialic acids removable from control platelets; and methysergide by itself may actually have caused a small diminution (Table 2).

The results show that neuraminidase removed more sialic acids from platelets that had undergone the characteristic shape change produced by ADP or by 5-hydroxytryptamine than from platelets in their normal discoid form. This suggests that activation of platelets is associated with an increase in the glycoproteins exposed on the cell surface. The possibility has not been excluded that this increase may represent adsorption of plasma glycoproteins, but it seems unlikely. The newly exposed glycoproteins may be involved in aggregation if they should turn out to be receptors for fibrinogen, which is an essential cofactor for aggregation (Born & Cross, 1964; Cross, 1964; Born, 1968).

Quantitative electron microscopy has established that the total surface area exposed by platelets to the environment during the shape change increases 2–3-fold (G. V. R. Born, R. Dearnley & J. Foulks, unpublished work). The increase in enzymically labile sialic acids is much smaller. Therefore most of the

Table 1. *Comparison of the amount of sialic acid released from rabbit and human platelets by hydrolysis or neuraminidase*

Platelets were fixed in glutaraldehyde, washed and resuspended in saline. Hydrolysis and neuraminidase treatment are described in the text. The results are expressed as means ± S.E.M. for 18 observations with rabbit and 21 with human platelets.

Source of platelets	Sialic acid released (nmol/10^9 platelets)		Enzymically liberated sialic acid (% of total)
	By acid hydrolysis	By neuraminidase	
Rabbit	55.3 ± 0.33	22.0 ± 0.45	39.7
Human	67.2 ± 0.63	27.1 ± 0.37	40.3

Table 2. *Effect of shape change of rabbit platelets by ADP or 5-hydroxytryptamine and its inhibition on the amount of sialic acid releasable by neuraminidase*

Adenosine or methysergide were incubated in platelet-rich plasma for 10 min before addition of ADP or 5-hydroxytryptamine. Fixation and neuraminidase treatment of platelets are described in the text. Four assay values were obtained for each experiment.

Agent(s) added	No. of experiments	Sialic acid liberated (nmol/10^9 platelets)	Increase (+) or decrease (−) in sialic acid (%)
Control		22.1	
ADP (2 μM)	12	27.0	+22.2 ± 1.2*
Control		21.5	
5-Hydroxytryptamine (10 μM)	8	24.6	+14.6 ± 1.8*
Control		22.7	
Control + adenosine (25 μM)		22.0	−3.1
ADP (2 μM)	2	28.4	+25.1
ADP + adenosine (25 μM)		22.8	+0.4
Control		20.2	
Control + methysergide (0.25 μM)		18.8	−6.9
5-Hydroxytryptamine (10 μM)	2	23.4	+15.8
5-Hydroxytryptamine + methysergide (0.25 μM)		18.7	−7.4

* Means ± S.E.M. of paired samples; *P* value of the differences < 0.005.

newly exposed surface is either free of sialic acid-containing glycoproteins, or, if present, they are not accessible to added neuraminidase. When the platelet membrane is enriched in the sialic acid *N*-acetylneuraminic acid, aggregation by ADP is diminished, whereas aggregation by 5-hydroxytryptamine is accelerated (Mester *et al.*, 1972); at the same time the uptake of 5-hydroxytryptamine and the associated K^+ exchange are accelerated (Michal *et al.*, 1972; Szabados *et al.*, 1975). These observations and others (Glynn, 1973) suggest that a sialic acid-containing glycoprotein is a component of the receptor for 5-hydroxytryptamine on the platelet membrane. So far there is no indication whether the glycoproteins exposed during the shape change have functions in relation to 5-hydroxytryptamine.

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