Acanthocytosis with normolipoproteinaemia: biophysical aspects

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Summary

The electrokinetic properties of normal and pathological red cells and platelets are compared.

The electrophoretic mobilities and isoelectric points of normal and pathological erythrocytes do not differ significantly, adsorption of calcium and uranyl ions occur to the same extent and treatment with neuraminidase induces almost identical mobility decreases.

The pathological platelet-plasma system is more sensitive to adenosine diphosphate (ADP) than normal systems; platelet aggregation and electrophoretic mobility increases are induced by much lower concentrations of ADP than those normally required. The pathological platelet system shows a greater tendency to aggregate spontaneously. The abnormal sensitivity to ADP of the pathological platelet-plasma system is considered in terms of plasma components.

Introduction

The unique morphology of the acanthocytic red cell has been attributed to abnormal lipid structures in the red cell membrane (van Buchem *et al.*, 1966) and there is electronmicrographic evidence of membrane irregularities (Mars *et al.*, 1969). Since the electron micrographs of the red cells in this particular case of acanthocytosis with normolipoproteinaemia, revealed no membrane defects (Critchley *et al.*, 1970) it is proposed to examine the membrane surface in more detail using cell electrophoresis. The technique has been used to assess surface abnormalities in pathological states and when used in conjunction with reagents specific to particular chemical groupings, it enables unusual membrane structures to be detected (Ambrose, 1965). The electrokinetic properties of blood platelets are influenced by adsorbed plasma components (Grötum, 1969) and Hampton & Mitchell (1966a) observed that abnormal plasma components markedly affect the rise in platelet mobility which may be induced by adenosine diphosphate (ADP). In the acanthocytic

patient under examination any unusual platelet response to ADP may indicate an abnormal plasma condition and provide a guide for further biochemical investigations.

Materials and methods

Blood was drawn from the antecubital vein into a plastic syringe and slowly discharged into plastic tubes containing 3.8% sodium citrate to yield a final concentration of 4 mg/ml collected blood. It was noted that the characteristic morphology of the acanthocyte disappeared upon washing, therefore unwashed red cells were used in all electrophoresis experiments. Whole blood was carefully diluted 1/1000 with 0.145 M NaCl, pH 7, solution and introduced into the electrophoretic cell. Platelet aggregation was followed turbidimetrically using the 'EEL Titrator' (Evans Electroselenium). Platelet mobilities were determined by diluting platelet-rich plasma (PRP) with 9 volumes of either 0.145 M NaCl pH 7 solution or platelet-poor plasma (PPP) prior to introduction into the electrophoresis cell. Micro-electrophoretic measurements were made at 25° C in a cylindrical chamber assembly with platinum electrodes and following the technique described by Bangham *et al.* (1958a). Adenosine diphosphate (ADP) and neuraminidase specimens were obtained from Sigma Chemical Co. Neuraminidase treatment of red cells was effected by adding 1 ml of neuraminidase solution (10 mg neuraminidase dissolved in 10 ml of 0.145 M NaCl pH 7 solution) to 100 ml of the red cell/saline suspension and after adjusting the pH to 5.0, the mixture was incubated at 37° C, for specified times. Electrophoretic mobility readings on the treated red cells were made after the pH had been adjusted to 7.0.

Preliminary experiments

Whole blood diluted with 0.145 M NaCl pH 7 solution contained 40-50% of the red cell population

as acanthocytes but after twice washing with 0.145 M NaCl pH 7, no acanthocytes could be detected. Resuspension of the washed red cells in the patient's own plasma, or in foreign plasma of the same blood group, or in Ringer's solution resulted in the re-appearance of about 20% acanthocytes in the population.

Electrophoretic mobility of red cells

The distribution of electrophoretic mobilities of the red cells showed no marked irregularity or skew. Fig. 1 compares the distribution of 700 readings on pathological cells with sixty readings on normal erythrocytes from a volunteer.

The spread of mobilities of the pathological specimen was greater than that found in normal specimens. Table 1 sets out the standard deviations of

sixty readings taken on each of four specimens from the patient against the standard deviations of the same number of readings on each of the specimens taken from three healthy volunteers. The standard deviation of the readings on the pathological specimen were significantly greater than those taken from the controls ($P < 0.05$) in seven of the twelve possible combinations. There was no significance in the difference between the two extreme standard deviations of specimens from the controls.

Comparison of normal and acanthocytic red cell membrane characteristics

At pH 7 (Fig. 2) the mean electrophoretic mobility of normal unwashed erythrocytes in saline ($1.44 \mu\text{m s}^{-1}/\text{V cm}^{-1}$) was close to that recorded by Seaman, Kok & Heard (1962) for unwashed erythrocytes in

FIG. 1. Distribution of electrophoretic mobilities in erythrocyte population of patient (—) (L.H. scale) and healthy volunteer (---) (R.H. scale).

TABLE 1. Comparison of standard deviations of erythrocyte mobilities at pH 7. The four samples from the patient are compared with each of the samples from three volunteers and the significance of the difference assessed using the *F* test.

Samples		Patient SD	1 0.114	2 0.096	3 0.083	4 0.101
Volunteers	1	0.077	$P < 0.01$	$P < 0.05$	$P > 0.1$	$P < 0.025$
	2	0.087	$P < 0.025$	$P > 0.1$	—	$P > 0.05$
	3	0.075	$P < 0.01$	$P < 0.05$	$P > 0.1$	$P < 0.025$

FIG. 2. Variation, with pH, of the electrophoretic mobility of human erythrocytes. Whole blood (citrate) was diluted 1:1000 with 0.145 M NaCl and pH adjusted using 0.1 M HCl. ● = Patient; × = mean of three volunteers. Vertical bars to left of data points, SD patient, to the right of data points, SD volunteers.

serum but corrected for viscosity difference ($1.47 \mu\text{m s}^{-1}/\text{V cm}^{-1}$). The isoelectric point is higher than that determined by Bangham, Pethica & Seaman (1958b) ($\text{pH} < 2$) for unwashed erythrocytes: the difference may be attributed to adsorbed plasma components.

The behaviour of the pathological red cells did not differ significantly from that of normal red cells. The mobility at pH 7 was within the normal expected variation and the isoelectric points of the two series were almost identical.

Fig. 3 compares normal and acanthocytic red cell mobilities in various concentrations of uranyl ion and calcium ion. The data indicate that normal and acanthocytic red cell mobilities are influenced to the same extent by adsorption of the two divalent ions.

FIG. 3. Effect of uranyl ion (pH 3.8) and calcium ion (pH 7.0) on the electrophoretic mobility of human erythrocytes. Uranyl ion, ● = patient, × = control. Calcium ion, × = patient, ● = control: vertical bars to left of data points, SD patient, to right of data points, SD control.

Neuraminidase has been shown to release sialomucopolymers from washed red cells and to reduce the electrophoretic mobility by 80% (Cook, Heard & Seaman, 1961). Comparisons were made between pathological red cells and normal red cells. The results (Fig. 4) show that only 65% of the surface-charge of unwashed erythrocytes may be attributed to sialomucopolymers. The charge is removed at approximately equal rates and the charge reduction is almost identical in the two cases. The difference in charge reduction between washed and unwashed cells implies that adsorbed plasma components affect the nature of the charged groups in the outer regions of the membrane and reduce the fraction of the charge attributable to sialomucopolymers.

Comparison of normal and pathological platelet-plasma reactions

In Fig. 5, the electrophoretic mobility changes induced by ADP treatment of platelets from the patient are compared with those induced in platelets from healthy volunteers. The maximum increase in mobility requires a concentration of $5 \times 10^{-2} \mu\text{g/ml}$ ADP (Hampton & Mitchell, 1966b) but to induce the maximum increase in mobility in the pathological platelets a concentration of only $5 \times 10^{-5} \mu\text{g/ml}$ ADP was required. The increased platelet sensitivity to ADP of the pathological specimen is manifested in 1:10 PRP:PPP and in 1:10 PRP:0.145 M NaCl. The sensitivity seemed consistent since 2 months elapsed between the two series of experiments.

The increased sensitivity of the patient's platelets to ADP was also extended to platelet aggregation. Mitchell & Sharpe (1964) in a survey of twenty-five normal samples of human platelet-rich plasma noted that the lowest concentration of ADP which would still produce detectable visible clumping of platelets

FIG. 4. Variation of electrophoretic mobility of human erythrocytes at pH 7 with neuraminidase incubation times. O = Patient; X = volunteer; whole blood was diluted 1:1000 with 0.145 M NaCl pH 7.

was 4×10^{-2} $\mu\text{g/ml}$ ($\text{SEM} \pm 0.3 \times 10^{-2}$) ADP. Fig. 6 shows that at 5×10^{-2} $\mu\text{g/ml}$ ADP the pathological platelets undergo significant reversible aggregation whereas normal platelets are only slightly affected. Furthermore, after keeping PRP for 1 hr

FIG. 5. Effect of ADP on the electrophoretic mobility of human platelets. Upper graph: PRP diluted 1:10 with 0.145 M NaCl pH 7, X = normal (mean of three) O = patient. Lower graph: PRP diluted 1:10 with PPP. ● = normal (Hampton & Mitchell, 1966b), Δ = patient (mean of two specimens).

at 4°C, merely stirring induces reversible platelet aggregation in the acanthocytic specimen but the normal specimen is unaffected.

Discussion

The data show that the ionization characteristics of red cells in normolipoproteinaemic acanthocytosis do not differ significantly from those of normal erythrocytes. The isoelectric points of the normal and abnormal red cells are almost identical which suggests similar ionogenic groupings in the plane of shear. Negative carboxylate and phosphate groupings bind calcium ions (Manery, 1966) and uranyl ions (Anderson & Pethica, 1955). The presence of unusual structures or groupings in the outer region of the membrane could result in abnormal divalent ion adsorption. The results presented imply that the outer membrane structures of the pathological and normal red cells do not differ significantly. Enzymatic treatment of erythrocytes show that sialomucopolymers are not only located at the surface of electrokinetic shear but also in structures deeper into the membrane (Cook *et al.*, 1961). The enzymatic attack on these structures by neuraminidase thus disrupts the surface in depth as well as in the plane of the charges. The data presented in this paper show that the mobility-time curves of normal and acanthocytic red cells during neuraminidase treatment are similar and suggest that the two types of cell have indistinguishable structures immediately below the plane of shear as well as in the plane of shear. Thus the biophysical comparison of normal with acanthocytic red cells did not reveal any evidence to suggest structural differences in the outer region of the membrane.

A comparison of the reaction to ADP of the platelet-plasma system in the acanthocytic patient

FIG. 6. Optical density changes during platelet aggregation. Upper graph: effect of $0.05 \mu\text{g/ml}$ ADP in PRP from patient (mean of three) = $\times$ and control = $\bullet$. Lower graph: effect of stirring upon PRP kept at 4°C for 60 min, patient = Δ , control = $\blacktriangle$.

with that of normal healthy volunteers showed that the pathological system was surprisingly sensitive to ADP. Recent reports have characterised ADP-induced mobility changes and have specified factors which influence both these changes and ADP-induced aggregation. The electrophoretic mobility changes induced by ADP are biphasic in character (Betts, Betts & Nicholson, 1968; Hampton & Mitchell, 1966b); low concentrations of ADP ($\sim 0.05 \mu\text{g/ml}$) increase platelet mobility and high concentrations ($> 0.5 \mu\text{g/ml}$) decrease mobility. Hampton & Mitchell (1966a) reported that in acute pathological states, e.g. myocardial infarction, platelets show increased sensitivity to ADP and concentrations much less than $0.05 \mu\text{g/ml}$ induce mobility increases. Bolton, Hampton & Mitchell (1967) showed that abnormal plasma phospholipid levels could sensitize platelets to ADP and Betts *et al.* (1968) showed that ADP-induced mobility increases are influenced by the presence of fibrinogen.

There is evidence to suggest that platelet aggregation is also affected by abnormal lipid or fibrinogen levels. Farbiszewski, Skrzydlewski & Worowski (1969) refer to reports of increased platelet adhesiveness in atherosclerosis and hyperlipaemia: they

present evidence to show that high *beta*-lipoprotein concentrations accelerate platelet aggregation. Haslam (1964) showed that free fatty acids induce platelet aggregation. On the other hand Deykin, Pritzker & Scolnick (1965) demonstrated that fibrinogen was a necessary component for platelet aggregation and Brinkhaus, Read and Mason (1965) noted that afibrinogenaemic plasma was devoid of a platelet-aggregation factor. The latter authors conclude that ADP and fibrinogen act synergistically in inducing platelet aggregation.

It seems reasonable to assume that the increased sensitivity of the platelets of the acanthocytic patient to both ADP-induced mobility changes and ADP-induced aggregation manifests a common underlying cause. Our current knowledge indicates that the cause may be either abnormal lipid levels or abnormal fibrinogen levels. The clinical biochemical data on this patient (Critchley *et al.*, 1970) show that the $S_f > 400$ (chylomicrons) and the $S_f 0-20$ (*beta*-) lipoprotein levels were normal but the $S_f 20-400$ (pre-*beta*-) lipoprotein level was high though not in the range of significant abnormality. The fibrinogen level, on the other hand, was abnormally high (813 mg/100 ml). Accepting that ADP and fibrinogen act syner-

gistically (Brinkhaus *et al.*, 1965) it would be anticipated that in the presence of high concentration of fibrinogen, the electrophoretic mobility and aggregation response of platelets would be elicited by unusually low concentrations of ADP. It is interesting to note that Mars *et al.* (1969) in their report on a case of familial hypo- β -lipoproteinaemia recorded unusual clumping of the platelets. It is thus possible that high fibrinogen levels and increased sensitivity of platelets to ADP is a common feature in the neurological syndromes involving acanthocytosis

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