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Self-Digestion of Human Erythrocyte Membranes

ROLE OF ADENOSINE TRIPHOSPHATE AND GLUTATHIONE

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Intact human erythrocytes incubated at 37°C, pH 7.4, release a sialoglycopeptide similar in its chemical composition, immunological and aggregation properties to the glycopeptide released by isolated 'ghost' membranes. The presence of ATP or reduced glutathione at physiological concentrations in the incubation medium of 'ghost' membranes inhibits this self-digestion process.

Sialic acid residues are responsible for the negative charge at the cell surface in human erythrocytes (Eylar *et al.*, 1962), and are involved in M- and N-group specificity (Ebert & Jurgen, 1972). The sialic acid content decreases as erythrocytes age (Balduini *et al.*, 1974; Baxter & Beeley, 1975), and its role as a determinant of the erythrocyte life-span is described in several reports (Jancik & Schauer, 1974; Durocher *et al.*, 1975; Gattegno *et al.*, 1975). Human erythrocyte 'ghost' membranes can digest their own sialoglycoproteins and release into the incubation medium a glycopeptide containing sialic acid, glucosamine, galactosamine, galactose and mainly polar amino acids (Brovelli *et al.*, 1976). If glycoprotein breakdown has a physiological role in erythrocyte aging, it must also take place in intact cells; therefore we have tested intact human erythrocytes for the ability to digest *in vitro* their membrane glycoproteins. Moreover, it seems reasonable that a mechanism must exist by which this process is inhibited in the young cell. On the basis of metabolic considerations, the ability of ATP and glutathione to modulate membrane self-digestion was hypothesized, and the effect of these metabolites on sialoglycopeptide release was studied, by evaluating linked sialic acid released by human erythrocyte 'ghost' membranes in the presence of ATP or glutathione. Membrane glycoprotein patterns obtained on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after incubation of 'ghost' membranes with ATP or glutathione at physiological concentrations were also investigated.

Experimental

Experiments on self-digestion of intact erythrocytes

Fresh human blood (40–60 ml) of the O–A–B–AB Rhesus-positive group was collected for each experiment from the veins of donors, with the use of 3.8% (w/v) sodium citrate as anti-coagulant. Plasma and buffy coat (leucocytes) were carefully removed after centrifugation at 4°C at 1000*g* for 15 min. For each sample 4–6 ml of cells was suspended in 1 vol. of 0.9% NaCl or Krebs–Ringer phosphate buffer [100 vol. of 0.9% NaCl, 4 vol. of 1.15% (w/v) KCl, 3 vol. of 1.22% (w/v) $CaCl_2$, 1 vol. of 3.82% (w/v) $MgSO_4,7H_2O$, 20 vol. of 0.1 M-sodium phosphate buffer, pH 7.4], put into a dialysis bag and immersed in 200 vol. of the same solution. The samples were incubated for increasing periods at 37°C, with shaking. Control experiments were performed by incubating cells at 4°C in the same buffer for the same period. All the experiments were carried out with sterile material. The addition of ampicillin (50 μg/ml of Krebs–Ringer solution) to some samples resulted in the absence of bacterial contamination at the end of 30 h incubation. No difference in the autolytic process was observed in the presence of antibiotic. After incubation, the cell suspension was centrifuged at 1000*g* for 5 min; the supernatant was then filtered on Dowex 2 (X8; acetate form) for the isolation of the sialic acid-containing material, which was then purified by gel filtration on Bio-Gel P-30 or P-150 as previously described (Brovelli *et al.*, 1976). Sedimented erythrocytes were lysed and washed as described by Marchesi & Palade (1967): 'ghost' membranes were used for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Experiments on self-digestion of isolated membranes

'Ghost' membranes, prepared as previously described (Brovelli *et al.*, 1976), were suspended in 0.5 vol. of 0.05 M-Tris/HCl buffer, pH 7.4, and incubated for 4 h at 37°C, with shaking. In some experiments, ATP, ADP, AMP, NAD^+, NADH, $NADP^+$, NADPH, GSH† or GSSG was added, at

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† Abbreviations: GSH and GSSG, reduced and oxidized glutathione.

physiological concentrations (Pennell, 1974). The effect of GSH concentrations two and four times the physiological ones was also investigated. After incubation, the samples were centrifuged at 25000*g* for 15min; the supernatant was tested for linked sialic acid as described by Svennerholm (1958), after the sialic acid-containing material had been isolated on Dowex 2 (X8). The sedimented membranes were used for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This was carried out on sodium dodecyl sulphate-solubilized 'ghost' membranes as described by Fairbanks *et al.* (1971), with the previously described modifications (Brovelli *et al.*, 1976). The gels were stained with periodic acid/Schiff reagent (PAS) (Fairbanks *et al.*, 1971). The electrophoretograms were recorded with a Saitron 803 densitometer (Florence, Italy) by using the yellow–green filter.

Analytical methods

Hexosamines and amino acids were determined with the Hitachi–Perkin–Elmer liquid chromatograph by the method of Moore & Stein (1951). Total sialic acid content of 'ghost' membranes was determined after hydrolysis of fresh 'ghosts' for 1h at 80°C in 0.05M-H_2SO_4 and isolation of the sialic acid by chromatography on a column (1.5cm×2cm) of Dowex 2 (X8; acetate form) under the conditions of Brovelli *et al.* (1976). The sialic acid content of the glycopeptide released in the self-digestion experiments was determined by the same procedure, excluding the H_2SO_4 hydrolysis step. Adenosine triphosphatase was assayed by the method of Kielley (1955). Phosphate was determined by the molybdate/vanadate method of Zilversmith & Davis (1950). Protein content of 'ghost' membranes was determined by the method of Lowry *et al.* (1951), with serum albumin as standard. GSH was assayed as described by Beutler *et al.* (1963). Inhibition of M–N-group haemagglutination was tested as described by Lisowska & Duk (1975). Glycophorin was prepared by the procedure of Marchesi & Andrews (1971).

Results

Self-digestion of intact erythrocytes

Sialic acid release from intact cells begins after 9h of incubation and reaches its highest value (50–70% of the total membrane sialic acid) after 25–30h of incubation. The product of this self-digestion process was isolated on a column (1.5cm×4cm) of Dowex 2 (X8) and purified by gel filtration on a column (1.5cm×85cm) of Bio-Gel P-30 or P-150 under the conditions previously described (Brovelli *et al.*, 1976). The sialoglycopeptide was eluted from both the columns in the void volume; this gel-filtration pattern supports the hypothesis that the sialoglycopeptide is obtained in an aggregated form and therefore behaves like the fragment released by 'ghost' membranes (Brovelli *et al.*, 1976). Its chemical composition is reported in Table 1; some components are present in the same amount as in the sialoglycopeptide released by 'ghost' membranes (sialic acid, aspartic acid, serine, proline, alanine, valine, isoleucine), but others are significantly different (glucosamine, galactosamine, threonine, glutamic acid, methionine, leucine). Both the sialoglycopeptides released from 'ghosts' and from intact erythrocytes inhibit M–N-group haemagglutination. Electrophoretograms of 'ghost' membranes prepared from incubated cells show that after 30h of incubation a modification of the ratio of bands PAS 1/PAS 2 appears (Fig. 1). It is noteworthy that sialoglycopeptide release appears to be correlated with GSH content of the erythrocyte; at the point during incubation when the cellular content of GSH decreases, the self-digestion process begins. The results of six experiments indicate that, while GSH concentration is maintained at about 60–80% (or above) of the concentration at zero time, no sialic acid release takes place, but when a 30–50% decrease in the GSH concentration occurs (9–12h of incubation) sialoglycopeptide release becomes evident (3–10% of the total membrane sialic acid). It was also shown that when 0.3mM-adenine is present in the

Table 1. *Chemical composition of the sialic acid-containing material isolated by gel filtration on Bio-Gel P-30 or P-150*

Gel filtration was carried out after incubation of intact erythrocytes at pH7.4 and isolation of sialic acid-containing material on Dowex 2 (X8); sialoglycopeptide was eluted from both the Bio-Gels in the void volume. Basic amino acids are present in very small amounts and are not reported in the Table. Values are means±S.D. of three experiments.

	Content (μmol/100μmol)
Sialic acid	32.19±1.66
Glucosamine	4.79±0.21
Galactosamine	11.82±0.82
Aspartic acid	5.09±1.07
Threonine	10.60±1.02
Serine	10.30±2.08
Glutamic acid	6.96±1.03
Proline	Traces
Glycine	4.16±0.99
Alanine	4.74±0.08
Valine	4.21±0.72
Methionine	Traces
Isoleucine	2.82±0.86
Leucine	2.33±0.62

incubation medium a 20–40% inhibition of the self-digestion process occurs after 30h of incubation. These results seem to indicate that energy and redox metabolism affect the membrane self-digestion process.

Regulation of the membrane self-digestion process

To investigate the correlation between membrane autolysis and the redox and energetic situation of the cell, the effect of different metabolites on this process was studied by using 'ghost'-membrane preparations. The effects of the following compounds at physiological concentrations (Pennell, 1974) were tested (three to four experiments for each substance): ATP, ADP, AMP, NAD^+, NADH, $NADP^+$, NADPH, GSH and GSSG. Since in 'ghost'-membrane preparations a residual adenosine triphosphatase activity is present, 4mM-ATP was added to the incubation medium to obtain a physiological concentration (0.6mM) of this nucleotide after 4h of incubation. In such conditions the sialoglycopeptide release is completely inhibited. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of these membranes shows the same periodic acid/Schiff-stain pattern as that of unincubated membranes (Fig. 1). AMP and ADP have no effect. When GSH concentrations (3.25mM) similar to those present in fresh erythrocytes were used, a 50% inhibition of the self-digestion process was achieved; for higher concentrations (6.5mM and 13mM), total inhibition was observed; the glutathione concentration remains constant during the incubation. In these conditions, the periodic acid/Schiff-stain electrophoretic pattern is superimposable on that of unincubated 'ghost' membranes (Fig. 1). GSSG, NAD^+, NADH, $NADP^+$ and NADPH at physiological concentrations have no effect on membrane autolysis. Inhibition is restored if NADPH and glutathione reductase are added to 3mM-GSSG in the incubation medium. Other thiol-group-containing compounds (5mM-dithiothreitol) do not influence the autolytic process.

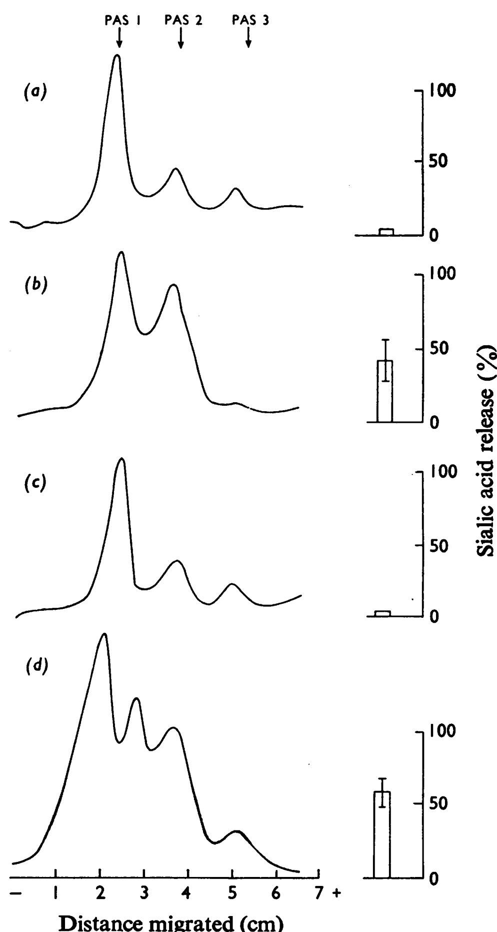

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of sodium dodecyl sulphate-solubilized 'ghost' membranes*

(*a*) Unincubated 'ghost' membranes; (*b*) membranes incubated for 4h at 37°C, pH7.4; (*c*) membranes incubated for 4h at 37°C, pH7.4, in the presence of 0.6mM-ATP or 13mM-GSH; (*d*) membranes from cells incubated for 30h at 37°C in 0.9% NaCl. The electrophoresis was carried out as described by Fairbanks *et al.* (1971); the current used was 3–3.5mA/tube. Acrylamide concentration was 5.6% (w/v). Gels were stained with periodic acid/Schiff reagent (PAS).

Discussion

The mechanisms involved in erythrocyte aging are only partially known (Brewer, 1974), and, in particular, no information is available about the factors determining the removal of sialic acid from old erythrocyte membrane (Balduini *et al.*, 1974; Jancik & Schauer, 1974; Baxter & Beeley, 1975; Durocher *et al.*, 1975; Gattegno *et al.*, 1975). It has been shown in our laboratory that human 'ghost' membranes are able to release, by an autolytic process, a glycopeptide in which 30–60% of total membrane sialic acid is present (Brovelli *et al.*, 1976). Also intact human cells incubated at physiological pH digest their own membrane glycoproteins and release a sialoglycopeptide which is similar with respect to its chemical composition to that obtained after the incubation of 'ghost' membranes. Some substantial differences appear in the molar ratio of some constituents; this can be ascribed to modifications of the supramolecular arrangement occurring during 'ghost'-membrane preparation, which could also be responsible for the different periodic acid/Schiff-stain electrophoretograms of residual membranes.

However, immunological properties and aggregation in solution indicate that both sialoglycopeptides behave like glycophorin (Janado, 1974; Lisowska & Duk, 1975). Moreover, the regulatory mechanisms of the autolytic process in 'ghost' membranes and intact cells show some similarities. The presence of physiological concentrations of GSH or ATP in the incubation medium inhibits the sialoglycopeptide release from 'ghost' membranes. The similarity in periodic acid/Schiff-stain electrophoretograms obtained from unincubated 'ghost' membranes and those incubated with GSH and ATP confirms the protective action of these two metabolites. The GSH effect seems to be specific, since other thiol-group-containing reagents, such as dithiothreitol, are quite ineffective. Moreover, when GSSG, NADPH and glutathione reductase are present in the incubation medium, the autolytic process is similarly inhibited; this must be a consequence of GSH production, since it has been shown that GSSG and reduced nicotinamide nucleotides alone do not produce such an effect. These results are in accordance with the observations that sialoglycopeptide release is significantly lower when intact cells are incubated in the presence of adenine, and that the autolytic process only starts when a decrease in cellular GSH concentration occurs. Our results suggest that membrane glycoprotein structure is related to cellular redox metabolism and ATP synthesis. These processes are known to decrease in the old erythrocyte, since glucose 6-phosphate dehydrogenase activity and energy charge are significantly decreased (Brewer, 1974). Therefore it is suggested that, when the erythrocyte loses the ability to maintain its GSH and ATP concentrations, the membrane proteolytic mechanism becomes active, causing sialoglycopeptide release and surface modifications that enable haemocatheretic organs to remove the old cells from the circulation. A similar mechanism could also take place in the haematological disorders characterized by a decrease in redox or energy metabolism.

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