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An Examination of Complement Proteins on Membranes of Paroxysmal Nocturnal Haemoglobinuria (PNH) and PNH-like Red Cells

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SUMMARY. Proteins obtained from membranes of PNH erythrocytes and of red cells treated with 2-aminoethylisothiouronium bromide (AET) following lysis in acidified serum (Ham's test) were compared by sodium dodecylsulphate-polyacrylamide gel electrophoresis. The peptide patterns were identical, providing support that AET-treated red cells afford a suitable experimental substitute for PNH red cells in studies involving complement fixation.

Paroxysmal nocturnal haemoglobinuria (PHN) is a disease characterized by intravascular haemolysis of red cells due to their remarkable sensitivity to complement, activated both by the classical and alternate pathways (Hinz, 1976). Although a defect in the erythrocyte membrane proteins has been suspected (Canellos *et al*, 1970) the actual biochemical basis has remained an enigma during the past 40 years since Ham's original observations on the lysis of PNH red cells in acidified serum (Ham, 1939; Ham & Dingle, 1939).

One approach to the problem has been to generate PNH-like red cells by treating normal cells with a variety of agents. These include proteolytic enzymes, sodium periodate (Yachnin *et al*, 1961), various sulphydryl containing compounds (De Sandre *et al*, 1968; Kann *et al*, 1968; Sirchia *et al*, 1965), oxidizing and reducing agents (Goldstein, 1974), and even tea (Benjamin *et al*, 1977). Treatment of normal cells with 2-aminoethylisothiouronium bromide (AET) increases their sensitivity to complement lysis in a manner similar to that of PNH erythrocytes (Sirchia & Ferrone, 1972). In this study we have compared complement proteins from membranes of PNH and AET-treated red cells following lysis in acidified serum (Ham's test) using the technique of sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Blood. Studies were performed on erythrocytes from five known cases of PNH and from healthy blood donors. Fresh erythrocytes, collected in acid–citrate–dextrose solution, were washed three times in isotonic saline and the buffy coat removed.

Serum. Fresh human serum was stored at −70°C for not more than 3 months.

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AET-treated cells. Normal erythrocytes were reacted with AET according to Sirchia *et al* (1965).

Ham's test. 0.8 ml of a 50% suspension of erythrocytes in isotonic saline were incubated at 37°C for 1 h in 8 ml of compatible normal human serum acidified to pH 6.5 with 0.8 ml of 0.2 M HCl. The lysed erythrocyte membranes were separated and washed according to Fairbanks *et al* (1971).

Complement-lysis of normal red cells. 5 ml of a 2% suspension of erythrocytes in isotonic saline were incubated at 37°C for 1 h with 10 ml of diluted (1:2 with isotonic saline) noncompatible human serum. The lysed erythrocyte membranes were isolated as described above.

One-dimensional gel electrophoresis. Membrane preparations were solubilized in 1% solution of SDS containing 160 mM dithiotreitol and subjected to electrophoresis in 5% polyacrylamide gel also containing 1% SDS (Fairbanks *et al*, 1971). In certain runs, dithiotreitol was omitted from the solubilized membrane solutions. Gels were stained with Coomassie Brilliant Blue dye and absorbance monitored at 550 nm using a Gilford spectrophotometer equipped with a linear transport. Calibration of molecular weight was determined from the mobility of standard protein markers: human spectrin (250 000), *E. coli* β-galactosidase (130 000), bovine serum albumin (68 000), human IgG heavy chain (50 000), pepsinogen (42 000), human IgG light chain (25 000), trypsin (23 000), myoglobin (17 200) and α-chymotrypsin (13 000).

Two-dimensional gel electrophoresis. SDS-polyacrylamide gel electrophoreses in which disulphide cleavage took place between the first and second dimensional runs, were carried out according to the method of Wang & Richards (1974). The peptide spots were visualized by staining with Coomassie Brilliant Blue dye.

RESULTS

Fig 1A shows the densitometric profile of normal erythrocyte membrane proteins; the major peptides have been labelled according to the nomenclature of Fairbanks *et al* (1971). There was no change in the peptide pattern when dithiotreitol was added to the membrane preparation (Fig 1B). Membrane peptides from hypotonic lysed PNH erythrocytes were indistinguishable from those of normal red cells.

The densitometric profiles of complement-lysed normal human erythrocyte membrane peptides, electrophoresed in the absence and in the presence of dithiotreitol (Figs 1C and 1D respectively) were similar to those reported by Bhakdi *et al* (1974a). Minor differences in staining intensity were however observed, presumably due to species differences in the origin of the target membranes; in the previous studies of Bhakdi *et al* (1974a) the target cells were sheep erythrocytes. Based upon their relative mobilities the nonmembrane peptides have been identified and labelled according to the nomenclature of Bhakdi *et al* (1974a).

The peptide pattern of complement-lysed PNH erythrocyte membrane, solubilized in the absence of dithiotreitol, revealed a corresponding series of new peptides (arbitrarily labelled P (190 000 daltons), Q (167 000), R (130 000), S (67 000) and T (55 000)) superimposed upon the membrane protein profile; a typical densitometric scan is shown in Fig 1E. Upon cleavage of disulphide bonds, all the new peptides with molecular weight greater than 100 000 daltons disappeared and there was a concomitant increase of bands in the molecular weight range of 85 000, 82 000, 69 000, 58 000 and 27 000, arbitrarily labelled U, V, W, Y and Z respectively.

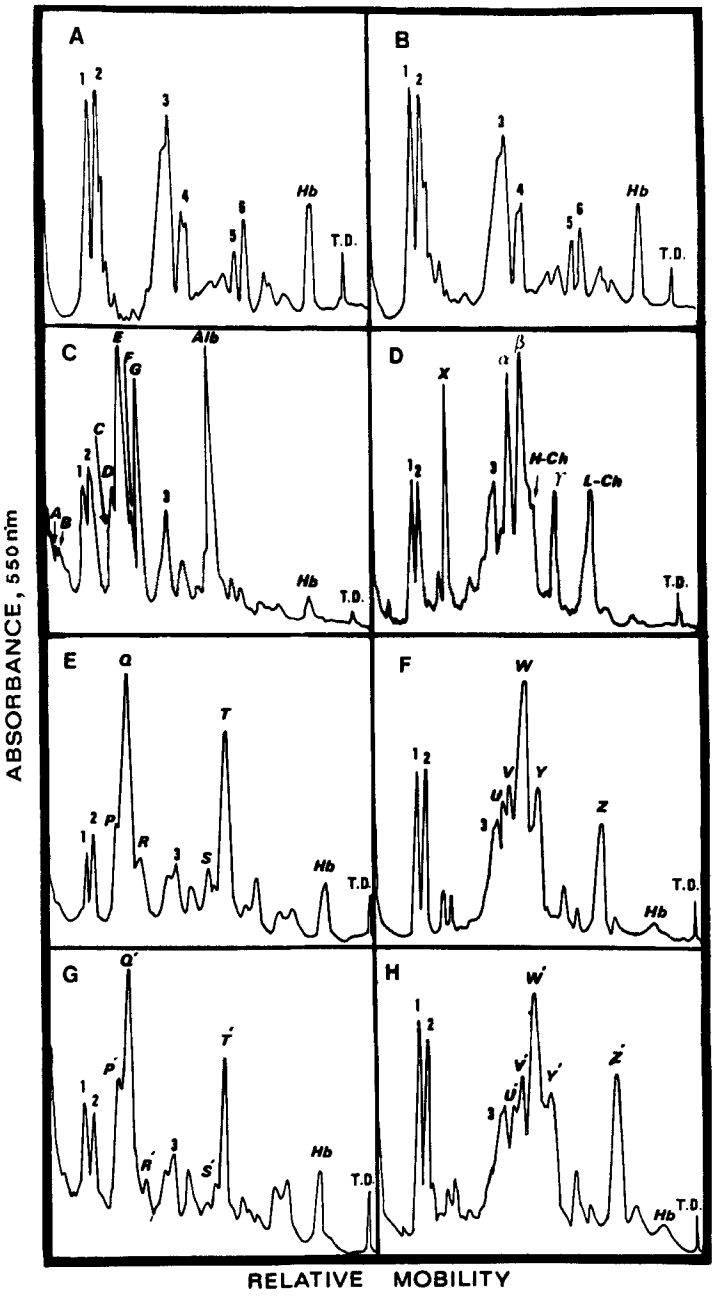

FIG 1. Sodium dodecylsulphate-polyacrylamide gel electrophoresis peptide patterns of erythrocyte membranes. (A) and (B), normal erythrocyte membranes, following hypotonic lysis. (C) and (D), normal erythrocyte membranes, following complement lysis. (E) and (F), PNH erythrocyte membranes, following complement-lysis. (G) and (H), AET-treated erythrocyte membranes, following complement-lysis. Dithiotreitol added to (B), (D), (F) and (H). The membrane peptides are labelled with arabic numerals and nonmembrane peptides are labelled with latin and greek letters. Hb, haemoglobin; Alb, albumin; H-Ch, heavy chain; L-Ch, light chain; T.D., tracking dye.

AET-treated red cell membrane, following complement lysis, produced densitometric peptide profiles that were essentially identical to those of PNH membranes (compare Fig 1E with 1G, and Fig 1F with 1H).

The changes in mobility of peptides following disulphide cleavage can be conveniently followed by two-dimensional gel electrophoresis, in which reduction occurs during the first and second dimensional runs. The peptide spots visualized were remarkably similar for complement-lysed PNH and AET-treated red cells (Fig 2). Furthermore, Fig 2 demonstrates that peptides PQR (or P′Q′R′) were split by dithiotretiol into peptides of lower molecular weight, namely UVWY (U′V′W′Y′) and Z (Z′), and that a contribution of the intensity of peptide W (W′) was due to the presence of peptide T (T′) whose mobility had been reduced as a result of dithiotreitol reduction (thereby appearing above the diagonal of unmodified peptide spots).

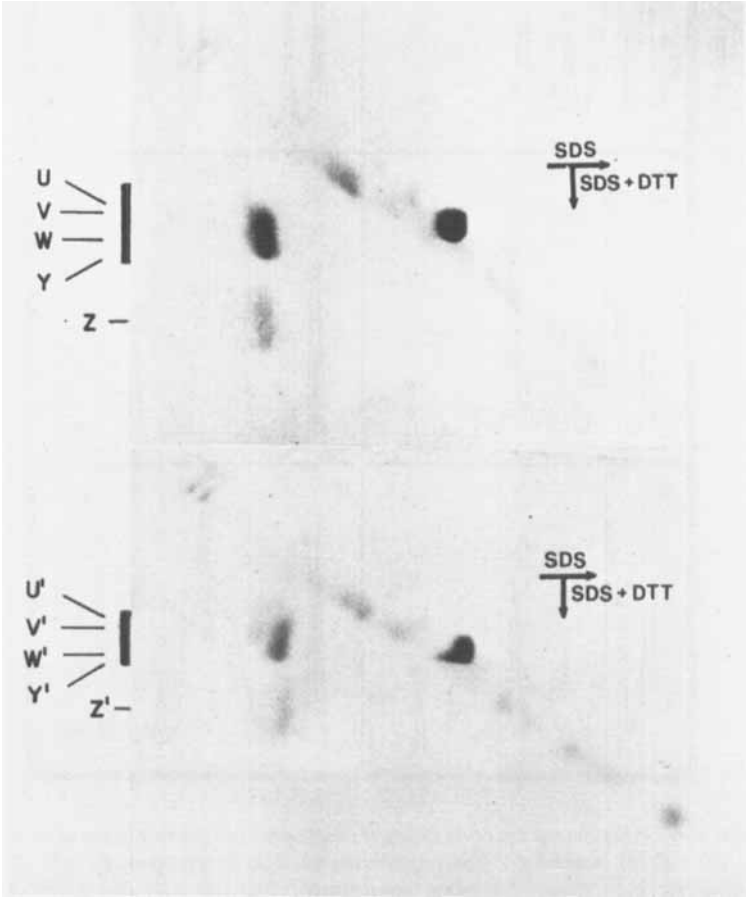

FIG 2. Two-dimensional sodium dodecylsulphate-polyacrylamide gel electrophoresis of erythrocyte membranes following complement lysis. Disulphide cleavage between first and second dimensional runs. Top, PNH erythrocyte; bottom; AET-treated erythrocyte. The peptides labelled U–Z and U′–Z′ are those similarly identified in Figs 1F and 1H respectively. SDS, sodium dodecylsulphate; DTT, dithiotreitol.

DISCUSSION

Serum proteins from complement-lysed (Ham's test) membranes of PNH and AET-treated red cells were identical when analysed by one- and two-dimensional SDS-polyacrylamide gel electrophoresis. This provides additional evidence to the contention that normal red cells treated with AET represent a good experimental substitute for PNH red cells (Sirchia & Ferrone, 1972).

Bhakdi *et al* (1974a, b) have examined membrane-bound complement proteins from sheep erythrocyte lysed by antibody and activated human complement using two-dimensional polyacrylamide gel electrophoresis. The main nonmembrane proteins visualized were early acting components of the complement system. Subcomponents of C3 corresponded to peptides of molecular weight in the range 160–180 000 daltons which generated smaller peptides of molecular weight 80 000, 45 000 and 30 000 upon reduction with dithiotreitol. A similar behaviour was observed in the corresponding nonmembrane peptides from complement-lysed normal human, PNH and PNH-like membrane (Fig 1). The presence of C4 on complement-lysed sheep erythrocyte membrane was characterized by the appearance of a peptide of molecular weight 170 000 following dithiotreitol treatment of the membrane preparation; this peptide was observed in the case of complement-lysed normal human membrane (band X in Fig 1D) but was not evident on PNH and PNH-like membranes. Serum albumin was found to be nonspecifically attached to the lysed PNH and PNH-like membranes (bands T and T′ in Figs 1E and 1G respectively) as was the case with lysed human (Fig 1C) and sheep erythrocyte membrane (Bhakdi *et al*, 1974a).

Binding of C3 to PNH red cells has been previously observed based on immunological techniques (Jenkins *et al*, 1970; Logue *et al*, 1973; Yachnin, 1965). The absence of C4 on PNH and AET-treated red cell membrane is consistent with the notion that lysis in the Ham's test takes place by activation of the complement system through the alternate pathway (for references, see Hinz, 1976).

The basic defect in PNH red cells has been connected to a change in membrane protein. Righetti *et al* (1973) and Dalmasso *et al* (1974) reported modification of a membrane glycoprotein. Changes in the peptide patterns obtained by electrophoresis in SDS-polyacrylamide (Atlas *et al*, 1973) and in a urea starch-gel system (Kotsifopoulos, 1976) have been observed. However, such membrane alterations were not substantiated in this study.

Recently it has been suggested that increased lysis of red cells in PNH is due at least in part to more efficient penetration of the PNH membrane by the terminal lytic sequence of complement (Rouault *et al*, 1978). Examination of complement components on target membranes provides one method for analysing details concerning complement assembly on cell surfaces. This approach should yield further insight into the unique sensitivity of PNH and PNH-like cells to complement fixation.

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REFERENCES

ATLAS, S.J., SHAPIRO, B. & GREEN, J.W. (1973) Surface properties of erythrocytes: normal, paroxysmal nocturnal hemoglobinuria and glutathione-treated cells. *Biochimica et Biophysica Acta*, **323**, 194–206.

BENJAMIN, L.J., GOLDSTEIN, B.D., DISTENFELD, A. & TROLL, W. (1977) Production of paroxysmal nocturnal hemoglobinuria-like red cells by tea. *American Journal of Hematology*, **2**, 245–249.

BHAKDI, S., KNÜFERMANN, H., SCHMIDT-ULLRICH, R., FISCHER, H. & WALLACH, D.F.H. (1974a) Interaction between erythrocyte membrane proteins and complement components. I. The role of –S–S linkages as revealed by two-dimensional sodium dodecylsulfate-polyacrylamide gel electrophoresis. *Biochimica et Biophysica Acta*, **363**, 39–53.

BHAKDI, S., KNÜFERMANN, H., FISCHER, H. & WALLACH, D.F.H. (1974b) Interaction between erythrocyte membrane proteins and complement components. II. Identification and peptide composition of complement components C3 and C4 desorbed from erythrocyte membranes. *Biochimica et Biophysica Acta*, **373**, 295–307.

CANELLOS, G.P., BRAIN, M.C. & DACIE, J.V. (1970) Effects of sulphydryl inhibition on the erythrocyte in paroxysmal nocturnal haemoglobinuria. *British Journal of Haematology*, **18**, 269–277.

DALMASSO, A.P., PIZZIMENTI, M.C., VACS, E. & DIAZ, A. (1974) Abnormal solubilization by triton X-100 of erythrocyte membranes from patients with paroxysmal nocturnal hemoglobinuria. *Proceedings of the Society for Experimental Biology and Medicine*, **147**, 273–279.

DESANDRE, G., VETTORE, L., CORROCHER, R., CORTESI, S. & PERONA, G. (1968) Ham-positive red cells induced *in vitro* by N-acetylcysteine or D-penicillanine. *British Journal of Haematology*, **15**, 437–441.

FAIRBANKS, G., STECK, T.L. & WALLACH, D.F.H. (1971) Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*, **10**, 2606–2617.

GOLDSTEIN, B.D. (1974) Production of paroxysmal nocturnal haemoglobinuria-like red cells by reducing and oxidizing agents. *British Journal of Haematology*, **26**, 49–58.

HAM, T.H. (1939) Studies on destruction of red blood cells. I. Chronic hemolytic anemia with paroxysmal nocturnal hemoglobinuria: an investigation of the mechanism of hemolysis, with observations on five cases. *Archives of Internal Medicine*, **64**, 1271–1305.

HAM, T.H. & DINGLE, J.H. (1939) Studies on destruction of red blood cells. II. Chronic hemolytic anemia with paroxysmal nocturnal hemoglobinuria: certain immunological aspects of the hemolytic mechanism with special reference to serum complement. *Journal of Clinical Investigation*, **18**, 657–672.

HINZ, C.F., JR (1976) Acid hemolysis revisited. *Seminars in Hematology*, **13**, 201–209.

JENKINS, D.E., JOHNSON, R.M. & HARTMANN, R.C. (1970) Uptake of anti-C4 and anti-C3 by erythrocytes from paroxysmal nocturnal hemoglobinuria hemolytic systems. *Journal of Clinical Investigation*, **49**, 49a.

KANN, H.E., JR, MENGEL, C.E., MERIWETHER, W.D. & EBBERT, L. (1968) Production of *in vitro* lytic characteristics of paroxysmal nocturnal hemoglobinuria erythrocytes in normal erythrocytes. *Blood*, **32**, 49–58.

KOTSIFOPOULOS, P.M. (1976) Red cell membrane proteins abnormality in paroxysmal nocturnal hemoglobinuria and in *in vitro* induced PNH-like erythrocytes. *Acta Haematologica*, **56**, 328–333.

LOGUE, G.L., ROSSE, W.F. & ADAMS, J.P. (1973) Mechanisms of immune lysis of red blood cells *in vitro*. I. Paroxysmal nocturnal hemoglobinuria cells. *Journal of Clinical Investigation*, **52**, 1129–1137.

RIGHETTI, P.G., PERRELLA, M., ZANELLA, A. & SIRCHIA, G. (1973) The membrane abnormality of the red cell in paroxysmal nocturnal haemoglobinuria. *Nature: New Biology*, **245**, 273–276.

ROUAULT, T.A., ROSSE, W.F., BELL, S. & SHELBURNE, J. (1978) Differences in the terminal steps of complement lysis of normal and paroxysmal nocturnal hemoglobinuria red cells. *Blood*, **51**, 325–330.

SIRCHIA, G. & FERRONE, S. (1972) The laboratory substitutes of the red cell of paroxysmal nocturnal haemoglobinuria (PNH): PNH-like red cells. *Series Haematologica*, Vol. V, No. 3, pp. 137–175. Munksgaard, Copenhagen.

SIRCHIA, G., FERRONE, S. & MERCURIACI, F. (1965) The action of two sulfhydryl compounds on normal human red cells. Relationship to red cells of paroxysmal nocturnal hemoglobinuria. *Blood*, **25**, 502–510.

WANG, K. & RICHARDS, F.M. (1974) Behavior of cleavable cross-linking reagents based on disulfide groups. *Israel Journal of Chemistry*, **12**, 375–389.

YACHNIN, S. (1965) The hemolysis of red cells from patients with paroxysmal nocturnal hemoglobinuria by partially purified subcomponents of the third complement component. *Journal of Clinical Investigation*, **44**, 1534–1546.

YACHNIN, S., LAFORET, M.T. & GARDNER, F.H. (1961) pH dependent hemolytic systems. I. Their relationship to paroxysmal nocturnal hemoglobinuria. *Blood*, **17**, 83–96.