ABNORMALITY OF PHOSPHOLIPIDS IN RED CELLS OF PATIENTS WITH PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA

BY

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Crosby (1953) concludes a detailed review of paroxysmal nocturnal haemoglobinuria (P.N.H.) by saying that it is an acquired disease in which abnormal red cells, white cells, and platelets are produced; that the lesion of the cells probably involves the stromal proteins so that they become susceptible to the proteolytic effect of a system of normal plasma enzymes; and that the severity of the disease depends upon the degree of susceptibility of the cells to the plasma system.

Whilst there have been a number of investigations into the plasma haemolytic factors in this disease, little attention has been paid to the red cell. Conflicting evidence of the electron microscopic abnormalities of the cells have been reported (Matthes *et al.*, 1951; Douglas and Eaton, 1955; Braunsteiner *et al.*, 1956), but otherwise there are few published data about abnormalities in these cells other than their well-known susceptibility to haemolysis at acid pH. The following investigation was designed to study the state of phospholipids in P.N.H. cells, since it is known that some of these are in a dynamic state (Altman, 1953; Prankerd, 1957) whilst the protein component of the cell is static (Muir *et al.*, 1952).

Methods

The basic method used in this work has been that described by Dawson (1954) in his analysis of phospholipid in liver cells. Cells were separated from whole blood by centrifuging and washed once with 0.9% saline. The cells were packed by centrifuging at 3,000 g. for 10 minutes and then haemolysed with an equal volume of distilled water. The haemolysate was extracted with boiling ethanol-ether (2:1) for 5-10 minutes and the mixture allowed to stand at room temperature for 15 minutes. The subsequent steps in the purification and alkaline hydrolysis of the extracted lipids were carried out as described by Dawson, and the final aqueous solution of phosphate esters was dried on Whatman No. 1 paper and chromatographed two-dimensionally in saturated phenol-water for eight hours and in trichloroacetic acid-*tert*-butanol for six hours. The individual spots were developed by spraying with ninhydrin and then later with a molybdate spray (Hanes and Isherwood, 1949). Each spot was finally cut out and the phosphorus in it estimated (Prankerd and Altman, 1954). Analyses for total lipid, by drying to constant weight; cholesterol, by method of Bloor (1916); lipid nitrogen, by method of Koch and McMeakin (1924); and phospholipid, by method of Youngburg and Youngburg (1930), were also made, after extraction by refluxing for two hours with the same solvent.

Results

Table I gives the results of analyses of total lipid and some of its fractions in 10 instances of six cases of P.N.H. and 24 control subjects. The significant differences would

appear to be the higher values for lipid nitrogen and cholesterol found in the P.N.H. cells with normal total lipids and lipid phosphorus. In three cases of P.N.H. the serum cholesterol was also estimated and found to be below the normal range.

Table II summarizes the amounts of phosphate ester derivatives separated chromatographically from the phospholipids in normal and P.N.H. subjects. The methods used in separation and hydrolysis of the phospholipids cannot be expected to give quantitatively comparable yields on each occasion, but since the same method is employed in each case it may be assumed that any loss is randomly distributed amongst the individual lipids, and the two ratios of different types of phospholipid to each other at the bottom of Table II would therefore be comparable.

The statistical differences between the normal and P.N.H. groups can be seen to lie in the highly significant (at 1% level) differences in the ratios of choline phosphate to serine phosphate found on the chromatograms. The individual

TABLE I

	Total Lipid (mg./ml. Cells)	Cholesterol (mg./ml. Cells)	Lipid N ₂ (mg.N ₂ /ml. Cells)	Lipid P (μg.P/ml. Cells)
Normals (24)	0.0125±0.0005	1.76±0.058	0.156±0.012	80.5±2.6
P.N.H. (10) (6 cases)	0.0123±0.0008	2.17±0.154	0.232±0.023	74.8±6.8
Difference of means	0.002	0.41	0.076	5.7
Test of significance:				
t	0.038	3.05	3.24	0.94
P	>0.10	<0.01	<0.01	>0.10

TABLE II.—Quantities of Phosphate Esters Separated Chromatographically. (Figures Represent μg. Phosphorus Derived from 1 ml. Cells)

Normals:	1	2	3	4	5	Mean Value	
Phosphoryl choline (C)	19.3	21.0	22.3	17.1	21.2		
Phosphoryl ethanolamine (E)	9.5	8.3	6.9	5.0	8.7		
Phosphoryl serine (S)	3.0	4.3	5.0	2.0	5.5		
C/E	1.9	2.5	3.2	3.7	2.4	2.7±0.31	
C/S	6.3	5.0	4.5	8.5	4.0	5.7±0.80	
P.N.H.:	1	2	3	4	5	6	Mean Value
Phosphoryl choline (C)	11.0	17.0	11.0	19.5	16.5	14.5	
Phosphoryl ethanolamine (E)	2.5	8.0	6.0	16.5	7.0	12.0	
Phosphoryl serine (S)	8.0	8.0	5.0	14.0	5.5	10.0	
C/E	4.3	2.1	1.8	1.2	2.4	1.2	2.7±0.43
C/S	1.4	2.1	2.2	1.4	3.0	1.4	1.9±0.26

Test of significance:

Difference of means C/E .. t=1.3, P>0.2.

" " " C/S .. t=4.85, P<0.001.

amounts of these esters suggest that the difference is due both to a deficiency in choline phosphate and to an increase in serine phosphate.

Discussion

At present we can only conclude that there is a fundamental difference between the phospholipids of the P.N.H. cell and the normal, such that there appears to be less phosphatidyl choline and more phosphatidyl serine per unit of packed cell, and that these cells contain greater amounts of lipid nitrogen and cholesterol than normal. It also appears that the serum cholesterol in P.N.H. is reduced; but similar reductions have been reported in other anaemias (Muller, 1930), so that this finding may not be specific. Several of the patients were studied at various periods after transfusion, but none had been transfused within two months of examination, and it is likely that the presence of normal cells in the analysed sample would weight the results towards normal, so that, taking this into consideration, the differences become even more significant.

A number of other types of anaemia have been investigated in this way and changes such as these have not been found, nor has the presence of reticulocytes been shown to

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affect the results. The results reported by no means cover all the lipids or even phospholipids in the red cell, and it may well be that other constituents are involved also. This, and the relationships these abnormalities have to the mechanism of the disease, form a problem under further investigation.

We thank Professor J. V. Dacie for kindly providing many of the samples of P.N.H. blood for this study.

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RED-CELL SURVIVAL IN PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA

EFFECT OF PHENINDIONE AND CORTISONE

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Paroxysmal nocturnal haemoglobinuria (P.N.H.) is a rare form of haemolytic anaemia in which the essential abnormality lies in the cells rather than in the plasma (Dacie and Firth, 1943; Dacie and Mollison, 1949; Crosby, 1953; Dacie, 1954). The only treatment of proved value in the condition is repeated blood transfusion, but it has been claimed that anticoagulant therapy may be beneficial. The basis for this form of treatment is the observation by Crosby and Dameshek (1950) that haemolysis is produced by a factor normally present in plasma which is activated by thrombin. A patient with P.N.H. was given dicoumarol and the degree of haemolysis diminished. Haemolytic crises, however, still occurred, and the authors conclude merely that "dicoumarol offers an intriguing possibility for treatment." They mention its additional value as a protection against the spontaneous thromboses to which these patients are so liable. In a later paper Crosby (1953) refers to three cases treated by dicoumarol, with apparent success in two.

Other cases subjected to anticoagulant therapy have been recorded. Kalant and Cyr (1952) noted that treatment with dicoumarol "coincided with a remission lasting five weeks." Perkins *et al.* (1955) report four cases, in two of which they thought the evidence was in favour of a diminished degree of haemolysis when the patient was receiving dicoumarol. Wang *et al.* (1956) describe a case in which both phenindione ("dindevan") and biscoumacetate ("tromexan") were thought to have extended the interval between transfusions given at a fixed haemoglobin level.

Both corticotrophin and cortisone have been used clinically in the treatment of P.N.H. Crosby (1953) states categorically that these substances are of no benefit. Fudenberg *et al.* (1954), however, describe a case in which the patient showed symptomatic improvement during six months of corticotrophin treatment. There was no evidence of intravascular thromboses over this period. Feichtmeir *et al.* (1955) discovered a patient suffering from P.N.H. who had present in his serum a haemolysin active against normal red cells and in whom cortisone produced a remission. Dicoumarol treatment had failed.

From the evidence in favour of these two forms of treatment it is clear that the chief difficulty in assessing their value lies in the tendency for spontaneous fluctuation in severity to occur in this disease. When conventional haematological criteria are used studies must necessarily be continued over weeks or months. A more precise index of improvement is the mean lifetime of red cells, and this can be measured by the use of red cells labelled with radioactive chromium (^{51}Cr). This method has the important advantage of providing a quick assessment of results. Accordingly, the effect of phenindione and of cortisone on the survival of ^{51}Cr -labelled cells was studied in a case of P.N.H. The survival time of the patient's cells transfused into a normal recipient and that of normal donor cells transfused into the patient were investigated at the same time.

Case Report

The patient, a 37-year-old man, was first seen by us in February, 1956. His presenting complaint was of dyspnoea on exertion. He had had no illness until March, 1951, when he had been investigated elsewhere for the same symptoms. He was then considered to be suffering from an iron-deficiency anaemia of undetermined aetiology, and thereafter received sporadic doses of oral iron, and two courses of intravenous iron therapy. He continued to feel moderately lethargic and short of breath, and these symptoms became worse after a bout of "influenza" in December, 1954. He then first noticed that his urine was at times dark in colour. He was admitted to the urological department, where routine urine tests, cystoscopy, and excretion pyelography revealed no abnormality. The haemoglobin was 9.5 g. per 100 ml. and the M.C.H.C. 29%. Occult blood was absent from the faeces, and barium meal and follow-through yielded negative results. Sternal marrow smears showed normoblastic hyperplasia. Free acid was present in the gastric juice. Nothing further was added on this occasion to the previous diagnosis of iron-deficiency anaemia of undetermined aetiology. The patient was discharged on oral iron therapy, and continued to feel unwell in a greater or less degree until his admission to the medical unit. His complaint was of breathlessness on exertion, tiredness, and weakness.

On examination there was obvious pallor of skin and conjunctivae. No enlargement of liver, spleen, or lymph nodes was noted. The results of laboratory investigations were: Hb, 5.9 g. per 100 ml.; P.C.V., 20.5%; M.C.H.C., 28.9%; W.B.C., 4,000 per c.mm.; reticulocytes, 8%; platelets, 80,000 per c.mm. No significant abnormality was detected in a stained film of peripheral blood. Occult blood was not present in the faeces. The urine contained urobilin, and tests for haemoglobin were strongly positive despite the absence of red cells on microscopical examination. The serum bilirubin was 0.6 mg. per 100 ml., and 24-hour faecal urobilinogen (mean of four-day period) was 280 mg. The Wassermann and Donath-Landsteiner reactions were negative. The direct Coombs test was negative on two occasions. Ham's acidified serum test (Ham, 1939) and Crosby's acid-serum-thrombin test (Crosby, 1950) were positive on each of three occasions. Spectroscopic examination of the