Erythrocyte Diphosphopyridine Nucleotidase (NADase) in Paroxysmal Nocturnal Hemoglobinuria[1] (36000)

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Deficiency of acetylcholinesterase (AChE) activity has been described in the erythrocytes of patients with paroxysmal nocturnal hemoglobinuria (PNH) (6, 9). The significance of this finding has been recently reviewed (5, 10). Those PNH cells with the more marked AChE deficiency appear more susceptible to hemolysis. By contrast, there is no evidence that AChE deficiency is directly responsible for the peculiar susceptibility of the PNH erythrocyte to hemolysis. Nevertheless, the enzyme defect remains the most consistent biochemical abnormality of the PNH red cell and provides an interesting marker.

There is likewise no evidence that AChE deficiency reflects a "general poverty" of enzymes in the defective PNH erythrocyte. Other red cell enzyme activities have been reported as normal or increased, the latter probably reflecting the presence of many young red cells (5, 8, 13, 14). However, the other enzymes studied were "soluble enzymes;" whereas AChE is a "stromal-bound" enzyme. According to Firkin and Wiley (7), of the more than 20 enzymes reported to be associated with erythrocyte stroma, it seems probable that AChE, diphosphopyridine nucleotidase [NAD glycohydrolase (EC3.2.2.5) NADase], and carboxylesterase are at least firmly bound if not a portion of the structural membrane. The activity of adenosine triphosphatase, which seems intimately related to the red cell membrane, has been reported elevated in PNH erythrocyte membranes (2). For this reason, it was considered of importance to determine one of the other "stromal-bound" enzymes, NADase, in PNH. Although the role of NADase in the red cell remains unknown (12), such a study could provide some information as to whether in PNH there exists any "general poverty" of stromal-bound enzymes.

Clinical material. PNH case 1 died prior to the present study. However, the original case numbers of the other patients were maintained since the clinical protocols of cases 2–8 have been previously documented and details are available in these publications (9–11). All patients were between the ages of 41 and 68 years at the time of this study. Case 2 is a black male who also has erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD) deficiency, demonstrated by family studies to be of the typical primaquine-sensitive red cell variety seen in American blacks (11). Case 6 was a black female; case 7, a white female; and the rest of the patients were white males. For control studies, 51 determinations of red cell NADase were carried out on 14 normal white males and females, ages 18 to 25.

Enzyme assays. Fifty percent suspensions of thrice-washed red cells obtained from freshly drawn and defibrinated blood were used as the enzyme source. The reaction mixture consisted of erythrocyte suspension, 1.0 ml; NAD[3] solution (1.0 mg/ml, 0.85% sodium chloride), 0.1 ml; phosphate buffer (0.1 *M*, pH 6.95), 0.1 ml; and 0.85% sodium chloride solution, 1.5 ml (1). During incubation at 37°, aliquots were removed at 0, 5, 10, and

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[3] NAD (Pabst, No. 300).

20 min, the reaction was stopped by the addition of 1.5 ml of 10% trichloroacetic acid, and the filtrate was analyzed for residual NAD by the method of Carpenter and Kodicek (3) as modified by Colowick and Kaplan (4), using a Coleman C12 or Turner fluorometer with thiamine filters and quinine standards. Standard curves for NAD concentration were obtained by plotting fluorometric values for solutions containing 0.125–2.0 μg of NAD/ml, and for aliquots taken at various times from a single reaction mixture containing a set amount of NAD without, however, added erythrocytes.

Since the red cell contains NAD as well as the NADase to be assayed, it was important to determine whether the enzyme assay procedure with added NAD would be influenced by any reaction between intraerythrocytic NAD and intraerythrocytic NADase. No change in fluorescence occurred when PNH and normal red cells were incubated in the reaction mixture in the absence of added, exogenous NAD. There was no difference in the erythrocytic total pyridine nucleotide concentration of PNH (11.84 μmoles/100 ml RBC) and of normal (11.2 μmoles/100 ml RBC) red cells.

Expression of Results. Although a volume measurement of erythrocyte suspension was used for the determinations, final expression of enzyme activity was in terms of dry weight. Duplicate 0.1 ml aliquots of red cell suspensions were dried in small tinfoil cups to a constant weight at 200°. Since the first 10 min of incubation of the reaction mixture at 37° showed the greatest changes in fluorescence, enzyme activity was measured as the slope of decrease of NAD in the first 10-min period. Results are expressed as micrograms of NAD hydrolyzed per milligram of dry weight per minute.

Results. The results of assays for NADase activity in the red cells of 14 normal subjects and 7 PNH patients are shown in Table I. The range of values obtained in the normal subjects was rather wide (0.023 to 0.101). Most of the values for the PNH patients were within these normal limits. The single low values in cases 3 and 6 could not be confirmed on retesting. There was no correlation between the values obtained in the PNH patients and the degree of anemia or reticulocytosis, presence or absence of gross hemoglobinuria, and therapy with androgen, iron, or transfusions. Likewise there was no correlation between enzyme values and leukocyte and platelet counts. This is of interest since leukopenia (as in patients 5, 7, and 8) and thrombocytopenia (as in patients 2–4, and 8) are common findings in PNH.

The fact that as a group the PNH patients were not very anemic could be attributed largely to improvement with androgen and iron therapy (10, 11). Case 8 had mild, untreated disease. Case 2 had previously experienced complete relief of anemia with iron therapy alone, but at the time of this study was in the recovery phase following a severe hemolytic episode due to Methimazole given for hyperthyroidism. It is of interest that in this patient, who also has erythrocyte G-6-PD deficiency, the red cell NAD and NADase were both normal.

NADase quantitations in our PNH population were repeated several years later. Five normal individuals had on 8 independent determinations an average activity of 0.030 μg of NAD destroyed/mg of dry wt/min, with a range of 0.013–0.042 while seven PNH patients on 14 independent determinations had an average activity of 0.029 μg of NAD destroyed/mg of dry wt/min, with a range of 0.010–0.047. The seven PNH patients included five previously studied (cases 2–5 and 8) and two new patients (cases 10 and 12, both white females). The slightly lower averages compared to the study of 1962 are readily explained by the use of a purer NAD[4] preparation, new fluorimetric equipment, and different individuals to perform the assays. However, there was again no difference between PNH patients and simultaneously determined controls.

Discussion. Both the clinical and laboratory findings in PNH may vary strikingly from patient to patient and in the same patient from time to time (5, 10, 11). For this reason repeated study of a sizable group of patients

[4] NAD (C.F. Boehringer and Sons, Mannheim, West Germany).

TABLE I. Erythrocyte NADase in PNH.

PNH (patient)	Hematocrit (%)	Reticulocytes (%)	Gross hemoglobinuria	NADase activity ($\Delta\mu$g/mg of dry wt/min)
14 normals (51 expts.)	—	—	—	0.062 (0.023–0.101)
2	31	5.9	+	0.038
	37	2.8	0	0.037
	42	1.2	0	0.055
	43	1.1	0	0.086
3	18	30.0	+	0.021
	26	21.4	+	0.039
4	42	3.9	0	0.054
5	46	7.5	0	0.086
6	39	4.3	0	0.015
	34	7.0	+	0.107
	25	13.0	0	0.040
7	32	7.0	0	0.041
8	38	4.0	0	0.040
	38	7.0	0	0.112

with this rare disorder is important in any thorough assessment of a possible PNH erythrocyte defect. Erythrocyte NADase was normal in all nine of our PNH patients studied despite a variety of clinical circumstances. Thus, even though erythrocyte AChE is depressed in PNH, the finding of normal NADase provides evidence against any "general poverty" of stromal-bound enzymes in the PNH erythrocyte.

Androgen and iron therapy has relieved the anemia of some of our PNH patients, and this could conceivably have restored depressed erythrocyte NADase activity to normal. However, patient 2, treated with iron alone; patient 8, untreated; and patients 3 and 7, with persistent anemia despite combined androgen-iron therapy, all had normal values. Furthermore, erythrocyte AChE activity has remained severely depressed even in those cases with complete relief of anemia on combined androgen-iron therapy (11).

Summary. Erythrocyte NADase activity was found to be normal in nine PNH patients, including with one with concomitant G-6-PD deficiency. Thus the erythrocyte AChE deficiency in PNH does not likely reflect any "general poverty" of stromal-bound enzymes in this disorder.

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