Biochimica et Biophysica Acta, 551 (1979) 452—458
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BBA Report

BBA 71374

ELECTRON SPIN RESONANCE, HEMATOLOGICAL, AND DEFORMABILITY STUDIES OF ERYTHROCYTES FROM PATIENTS WITH HUNTINGTON'S DISEASE

D. ALLAN BUTTERFIELD[a,*], MARK J. PURDY[a] and WILLIAM R. MARKESBERY[b]

[a] *Department of Chemistry, University of Kentucky, Lexington, KY 40506 and* [b] *Departments of Neurology and Pathology, University of Kentucky Medical Center, Lexington, KY 40506 (U.S.A.)*

(Received September 6th, 1978)

Key words: Huntington's disease; Deformability; Membrane defect; ESR; Fluidity; (Erythrocyte membranes)

Summary

Electron spin resonance, hematologic, and deformability studies of erythrocytes from patients with Huntington's disease have been performed. A decreased deformability of Huntington's disease erythrocytes compared to normal controls was demonstrated. No difference in erythrocyte hematologic indices, osmotic fragility, reticulocyte counts, or intracellular Na^+ concentration was found. Huntington's disease serum had no demonstrable effect on electron spin resonance parameters of a protein-specific spin label attached to membrane proteins in control erythrocytes compared to the effect of control serum. This finding suggests that under the conditions employed no serum component or circulating factor is responsible for the changes in the physical state of membrane proteins in Huntington's disease erythrocytes (Butterfield, D.A., Oeswein, J.Q. and Markesbery, W.R. (1977) Nature 267, 453—455). No alteration in lipid fluidity of Huntington's disease erythrocyte membranes could be discerned suggesting that the underlying molecular defect in Huntington's disease involves a membrane protein. The results of the present studies on erythrocytes strongly support the concept that Huntington's disease is associated with a generalized membrane abnormality.

Huntington's disease, inherited as an autosomal dominant trait, is characterized clinically by progressive choreiform movements and dementia [1], pathologically by neuronal loss in the neostriatum and cortex [2], and biochemically by decreased levels of γ-aminobutyric acid [3] and decreased activ-

*To whom correspondence should be addressed.

ity of glutamic acid decarboxylase and choline acetyltransferase in the basal ganglia [4]. The molecular basis for this disease remains unknown.

Although Huntington's disease has been considered a defect of the basal ganglia, recent biophysical and biochemical experiments in our laboratory suggest that this disease may have more widespread membrane involvement [5—8]. Electron spin resonance (ESR) studies of erythrocyte membranes have suggested alterations in the conformation and/or organization of membrane proteins in Huntington's disease [5]. Scanning electron microscopic investigation of unmanipulated erythrocytes have demonstrated an increased number of stomatocytes in Huntington's disease compared to controls [6]. The activity of the membrane-bound sodium plus potassium-stimulated adenosinetriphosphatase ($(Na^+ + K^+)$-ATPase) was observed to be increased in Huntington's disease erythrocyte membranes [7], consistent with an altered physical state of membrane proteins in Huntington's disease and supporting the ESR results. Further ESR studies have demonstrated a differential effect of γ-aminobutyric acid on Huntington's disease and control erythrocyte membranes [8]. Also, upon incubation of γ-aminobutyric acid with Huntington's disease erythrocyte membranes the relevant ESR parameters of spin-labeled membrane proteins were indistinguishable from those of untreated controls [8]. These studies on membranes outside the central nervous system formed the basis for our hypothesis that Huntington's disease is associated with a generalized cell membrane abnormality.

The present communication reports on additional ESR studies designed to compare the membrane fluidity in Huntington's disease to that in several other inherited neurological diseases [9] and to assess the extent to which changes in the physical state of membrane proteins in Huntington's disease erythrocytes may be caused by an altered serum component. To determine the degree to which other membrane characteristics were altered in this proposed generalized cell membrane disease, hematologic and deformability experiments were also performed.

The spin labels employed in these studies were 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl (5-nitroxide stearate or 5-NS) and 2,2,6,6-tetramethylpiperidin-1-oxyl-4-maleimide (MAL-6) and were obtained from Syva. Polycarbonate filters used in the deformability experiments were 25 mm in diameter, of 3 μm pore size, having a pore density of $2.4—3.0 \cdot 10^6$ pores/cm^2, and were purchased from Nucleopore Corp. All chemicals used were of the highest purity available.

Blood was obtained in heparinized syringes from each Huntington's disease subject and normal control. All Huntington's disease patients had signs of involuntary movements and/or dementia and a positive family history of Huntington's disease. In each set of experiments some patients were on no drugs and some were on phenothiazines or assorted medications. Most patients were ambulatory and active but a few were bedridden. The majority of patients were on a regular at-home diet, while others were on a regular hospital diet.

Intact erythrocytes and erythrocyte membranes (ghosts) were prepared and the spin labeling using MAL-6 and 5-NS was performed as previously described [5,9]. Magnetic resonance experiments were performed on a Varian

E-109 electron spin resonance spectrometer using an E-238 cavity and quartz aqueous sample cell. Membrane protein content was estimated according to Lowry et al. [10].

The serum incubation experiments were performed as follows: serum from four Huntington's disease patients and from four age, sex, and blood type matched controls was used. Control packed, intact cells were divided into two aliquots. Huntington's disease serum was added to one aliquot while control serum was added to the other to give a 50% hematocrit in both cases. The aliquots were incubated at room temperature for 24 h after which ghosts were prepared and the electron spin resonance experiments using MAL-6 performed.

Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), hematocrit and reticulocyte counts and osmotic fragility experiments on control and Huntington's disease erythrocytes were performed on cells from six Huntington's disease patients according to standard methods [11]. To determine intracellular Na^+ levels, packed cells were washed five times with three times the volume of isotonic choline chloride. One part of these washed, packed cells was mixed with 40 parts deionized, distilled water and intracellular Na^+ levels per cell were determined using an Instrumentation Laboratory, Inc., Model 153 atomic emission spectrophotometer. The actual number of cells in this volume were determined by a Coulter Counter using the same volume of packed cells in isotonic choline chloride.

The deformability experiments were performed as previously described [12]. Basically, the time required for 2 ml of a 2% hematocrit suspension of erythrocytes (actual cell counts were determined with a Coulter Counter) to go through a 3 μm polycarbonate filter under 5 cm H_2O pressure was determined. The nine separate experiments, each involving a different Huntington's disease patient and control, were performed in a blind fashion and the results for Huntington's disease were computed as a percentage of controls.

The fluidity of the lipid phase of erythrocyte membranes in Huntington's disease was examined by electron spin resonance. The spin label employed, 5-NS, is thought to orient in the membrane with its polar acid group near the polar head groups of the phospholipids and its alkyl chain parallel on the average to the alkyl chains of the lipids [13]. Rapid anisotropic rotation is thought to occur about the long axis of the probe whose nitroxide group reports on an environment near the membrane surface [14]. Spectra similar to that obtained from 5-NS in intact erythrocytes are given in previous publications from our laboratory [9,15]. The order parameter S is a measure of the fluidity of the local environment in which the paramagnetic center of the spin label is found. Further discussion of the order parameter, its calculation, interpretation, and use can be found in several review articles [15—17]. The mean values of the order parameter were compared by a two-way analysis of variance [18], a statistical test which minimizes possible fluctuations between experiments which often occur in biological samples. No significant differences in the value of S could be found in intact erythrocytes in Huntington's disease compared to normal controls (Table I).

Additional ESR studies using MAL-6 were performed. Intact cells from

TABLE I

COMPARISON OF THE ORDER PARAMETER S FOR 5-NS IN NORMAL AND HUNTINGTON'S DISEASE INTACT ERYTHROCYTES

S is calculated by $S = \frac{T'_{\parallel} - T'_{\perp}}{(T_{\parallel} - T_{\perp})_{XL}} \cdot \frac{a_{XL}}{a'}$ where the primed T-tensor values are obtained experimentally and $a' = (T'_{\parallel} + 2T'_{\perp})/3$. The unprimed crystal values were obtained from the results of Jost et al. [29] on doxyl-propane. Further discussion of the order parameter and membrame fluidity may be found in several references [15—17].

	Normal	Huntington's disease
Mean ± S.D.	0.67 ± 0.02	0.66 ± 0.02
n	8	8
	$P^* > 0.5$	

*P value is calculated by a two-way analysis of variance [18].

age, sex, and blood type matched controls were incubated separately with control and Huntington's disease serum for 24 h at room temperature. Ghosts were prepared and MAL-6 was used to spin label the membrane proteins. MAL-6 is covalently bonded to membrane protein sulfhydryl (SH) groups, although a very small amount (<5%) of amino group labeling may occur [19]. Spectra resulting from erythrocyte membranes labeled with MAL-6 and the interpretation of these spectra with respect to motion of labeled sulfhydryl groups have been discussed previously [5,15—17]. The ratio of the ESR spectral amplitude of the low-field line of MAL-6 attached to weakly immobilized SH groups (W) to that of MAL-6 attached to strongly immobilized SH groups (S) is a convenient and sensitive monitor of membrane protein conformational and/or organizational changes [5,15—17].

The W/S ratios obtained in these experiments involving 24 h incubation were larger than those obtained in studies in which ghosts were made immediately after serum removal, an observation noted previously [20]. No difference in the W/S ratio of MAL-6 attached to control erythrocyte membranes which had previously been incubated with control or Huntington's disease serum could be discerned. This observation suggests that under these conditions no circulating factor is present or essential component absent in Huntington's disease serum which could explain the alteration in the physical state of membrane proteins previously observed [5].

No definitive change in MCV, MCHC, MCH, hematocrit or osmotic fragility could be demonstrated in Huntington's disease erythrocytes. No change in Huntington's disease reticulocyte count was evident, suggesting that the red cell life span may not be altered in Huntington's disease. No definitive differences in internal Na^+ levels could be observed in Huntington's disease erythrocytes.

In order to investigate mechanical properties of Huntington's disease erythrocytes, deformability studies were performed. In seven of nine separate experiments a significantly reduced deformability of Huntington's disease erythrocytes was observed (Fig. 1). Deformability equal to controls was observed in two experiments involving patients in the late stages of the disease who were bedridden. Some patients in the nine experiments were on no drugs and some were on phenothiazines. Those on no drugs were among the patients whose erythrocytes were less deformable than controls. No clear effect of

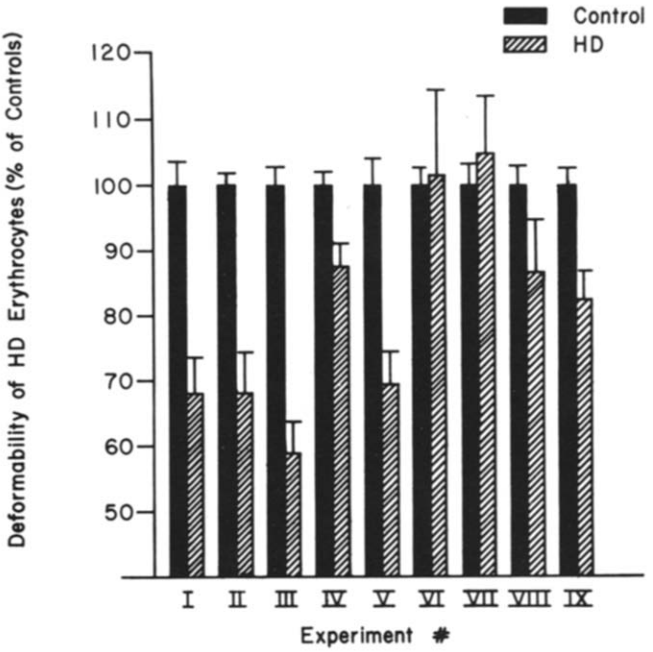

Fig. 1. Comparison of erythrocyte membrane deformability in Huntington's disease (HD) to that of normal controls. Nine separate experiments, each involving a different Huntington's disease patient and control, were performed in a blind fashion. The time required for a known number of erythrocytes (determined by a Coulter Counter) in 2 ml of a 2% hematocrit suspension to pass through a 3-μm polycarbonate filter under 5 cm H_2O pressure was determined. The results for Huntington's disease were computed as a percentage of control values which were set at 100%. The relative mean values of 4—6 trials for each control and Huntington's disease sample are represented by the bar graph. Standard deviations of these trials are also indicated.

phenothiazines on membrane deformability could be demonstrated in additional experiments with non-Huntington's disease patients who were on phenothiazines. These findings suggest that the decreased erythrocyte membrane deformability observed in Huntington's disease (Fig. 1) is not a result of medication.

The results of the present serum incubation studies suggested that under the conditions employed no factor in Huntington's disease serum caused an altered physical state of membrane proteins in Huntington's disease. This finding suggests that the changes in the physical state of membrane proteins in Huntington's disease previously observed [5] arise from an intrinsic membrane defect. With the possible exception of an altered α_2M-macroglobulin, no other significant alteration in Huntington's disease serum has been reported [21], a finding consistent with our results.

The absence of an alteration in the fluidity of the lipid phase of erythrocyte membranes in Huntington's disease (Table I) lends specificity to our previous ESR and biochemical studies in several other inherited neurological disorders. An increased lipid fluidity was observed in erythrocyte membranes in myotonic muscular dystrophy and congenital myotonia [9], suggesting that the molecular bases for these diseases are different than that in Huntington's disease. No difference in fluidity was observed in erythrocytes

from patients with Duchenne muscular dystrophy by first-harmonic in phase ESR [9], but an altered ability of 5-NS to undergo lateral diffusion in Duchenne muscular dystrophy erythrocytes has been reported by others using saturation-transfer ESR [22]. An altered physical state of membrane proteins has been observed in erythrocyte membranes in both Huntington's disease [5] and Duchenne muscular dystrophy [20]. However, the nature of these alterations is probably different. Membrane-bound protein kinase has increased activity in Duchenne muscular dystrophy erythrocytes [23], while this enzyme is unaltered in Huntington's disease [7]. In addition, it is reported that ouabain stimulates rather than inhibits the activity of the $(Na^+ + K^+)$-ATPase enzyme in Duchenne muscular dystrophy ghosts [24], while in Huntington's disease we have observed this enzyme to be of intrinsically higher activity compared to controls [7].

The absence of fluidity changes in the lipid phase of Huntington's disease erythrocytes, together with the previous ESR studies of membrane proteins in Huntington's disease [5] as well as the present serum incubation experiments, suggest that the primary defect in Huntington's disease membranes involves a membrane protein. Experiments designed to test this possibility are currently in progress in our laboratory.

A decreased deformability of Huntington's disease erythrocytes has been demonstrated in this study (Fig. 1). The mechanical properties of red cells are thought to be located in the network of proteins involving spectrin, actin, Band 3, glycophorin, and perhaps others [25]. If any of these proteins were altered in Huntington's disease, one might expect the deformability of erythrocytes to be affected. A decreased deformability of erythrocytes has been observed in Duchenne muscular dystrophy [26] where our previous ESR studies had shown an altered physical state of membrane proteins [20], and others have shown that the phosphorylation of spectrin is significantly increased [23]. The method of measuring deformability used in our experiments has been employed successfully to study hereditary spherocytosis and other disorders [12,27,28]. This method has been criticized because it studies a population of erythrocytes and not individual cells [27]. While this comment has merit, the method used in the current study does give relative differences in deformability of this population in an exact manner [12,27].

Routine hematological studies showed no change in erythrocyte indices. This result suggests that the increased stomatocyte formation of unmanipulated Huntington's disease erythrocytes previously reported [6] is not the result of an increased cell volume but results from the response to fixative by an abnormal membrane. The Na^+ level inside Huntington's disease erythrocytes was found to be normal. This finding suggests that the increased activity of the $(Na^+ + K^+)$-ATPase in Huntington's disease ghosts [7] is not the result of an increased stimulation by Na^+, but results from an altered enzyme or altered milieu in which the enzyme is located.

This study has shown an apparent lack of a causative factor in Huntington's disease serum that affects the physical state of proteins in erythrocyte membranes. While the interrelationships between this finding, decreased deformability, and the molecular basis for the alterations in Huntington's disease erythrocytes are unclear, the present results support our

concept that Huntington's disease is associated with a diffuse membrane defect.

We thank Dr. Jon Gockerman, Department of Medicine of the University of Kentucky Medical Center, for help and advice with the hematologic aspects of this study and Marcia Butterfield for technical assistance. This work was supported in part by the National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health (NS 13791-01A1) (D.A.B.) and NS-14221-01 (W.R.M.), the Muscular Dystrophy Association of America (D.A.B.), and the Graduate School of the University of Kentucky (D.A.B.).

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