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Biochimica et Biophysica Acta 1234 (1995) 184–190

BBA Biochimica et Biophysica Acta

Differential electrophoretic behavior in aqueous polymer solutions of red blood cells from Alzheimer patients and from normal individuals

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Received 28 July 1994; accepted 24 November 1994

Abstract

The recently reported phenomenon that red blood cells (RBC) from Alzheimer disease (AD) patients and normal individuals, which have identical electrophoretic mobilities (EPM) in phosphate-buffered saline (PBS), have different EPM in appropriately selected polymer solutions, has been further explored. Of a number of in vitro treatments to which AD and normal RBC were subjected prior to EPM measurements in bottom phase (from a dextran-poly(ethylene glycol) (PEG) aqueous phase system) only trypsin eliminated the difference. Thus, the differential polymer interaction between AD and normal RBC, thought to be the basis for their dissimilar EPM, can be abolished by appropriate proteolytic modification of the cell surfaces and suggests protein as a source of difference. Because young and old RBC from normal individuals, which have the same EPM in PBS, have different EPM in certain polymer solutions, and the RBC from AD patients have been reported to age abnormally, we also compared the young and old RBC subpopulations from these two sources. By the criterion of cell electrophoresis in polymer solutions the differences between AD and normal RBC and between young and old RBC are distinct. The EPM of AD and normal RBC differ in bottom phase or PEG but not in dextran solution; while the EPM of young and old RBC differ predominantly in dextran. We speculate that since the observed difference in EPM of RBC from AD patients and normals depends on protein(s) yet is anticoagulant-related (being obtained only when blood is collected in citrate or oxalate) it might be the result of an interaction (Ca^{2+}-mediated?) between the surfaces of these cells and protein component(s) of their respective, compositionally differing sera.

Keywords: Alzheimer's disease; Electrophoretic mobility; Red blood cell

1. Introduction

Extra-neuronal abnormalities in Alzheimer disease (AD), including altered red blood cell (RBC) membranes [1] and aging patterns [2,3], have been the subject of many reports (see [4] for a review). These findings prompted us initially to examine whether such changes are reflected at the surface of RBC from AD patients by comparing these cells' partitioning and electrophoretic behavior to those of RBC from subjects without memory impairment or dementia ('normals'). Neither cell partitioning in a charge-sensitive dextran-poly(ethylene glycol) (PEG) aqueous phase system (i.e., one with a Donnan potential between the phases, top phase positive [5,6]) nor cell electrophoresis in phosphate-buffered saline (PBS) revealed any difference between these cell populations.

Recently we related the fortuitous circumstances that led us to examine the electrophoretic mobilities (EPM) of RBC from AD patients and from normals in a number of polymer solutions. We discovered significant differences in EPM between these RBC populations in some polymer solutions (i.e., bottom phase from a dextran-PEG phase systems; PEG solution in PBS) but not in others (i.e., top phase; dextran solution in PBS) [7]. RBC from AD patients have a higher mobility in the indicated polymer solutions than do RBC from normals. The altered EPM associated with RBC from AD patients was not found with RBC from patients with Parkinson's disease and the alteration is, therefore, not a phenomenon shared by RBC in all neurologic diseases [7].

Young and old RBC from normal individuals, which have identical EPM in PBS [8], have also been found to

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SSDI 0005-2736(94)00302-5

have unequal EPM in polymer solutions, young RBC < old RBC [9,10]. Here EPM differences are readily apparent in dextran solution [9] and also, but on a percentage basis to a much smaller extent, in PEG solution, and in top and bottom phases [10]. These cells' behavior suggests thereby the involvement of surface properties other than or in addition to those permitting differentiation of RBC from AD patients and normals (see above).

Studies on RBC from different species have shown that partitioning in a charge-sensitive dextran-PEG aqueous phase system and electrophoresis in PBS or in selected polymer solutions (e.g., dextran-rich bottom phase; PEG-rich top phase) detect surface charge or charge-related properties [11]. At the same time each of these physical measures represents a distinct probe capable of reflecting different and cell population-specific surface properties, a phenomenon ascribed to differences in the nature of polymer-specific interaction with cell surfaces [11].

In the present work we have (a) determined that protein is responsible for the EPM differences between RBC from AD patients and normals, (b) studied the cell- and suspending medium-specific effects on the EPM differences between RBC from AD patients and normals and also between young and old RBC in each of these cell populations, and (c) examined the effect of a number of anticoagulants on the relative EPM of RBC from AD patients and normals.

2. Materials and methods

2.1. Reagents

Dextran T500 (lot No. 01 06905) was obtained from Pharmacia LKB (Piscataway, NJ). PEG 8000 (Carbowax 8000) was from Union Carbide (Long Beach, CA). Trypsin, chymotrypsin and paraformaldehyde were from Sigma (St. Louis, MO). All salts and organic solvents used were of analytical reagent grade.

2.2. Collection of blood from normal individuals and Alzheimer patients

Blood obtained by venipuncture from normal individuals and from AD patients was collected in citrate vacutainers. As previously described in detail [7] all patients met criteria for Dementia [12] and for Probable or Possible AD [13]. Consent was obtained from patients and respective responsible parties per institutional guidelines and approved procedures. Patients were selected after thorough evaluation including physical and mental status examination, blood studies, electrocardiography, electroencephalography (EEG), and brain imaging with X-ray CT scan or magnetic resonance imaging of the brain [7]. The majority also had functional imaging with quantitative EEG and/or Single Photon Emission Computed Tomography (SPECT). Subjects had been followed longitudinally for a minimum of 6 months (maximum 9 years) in a specialized program designed to establish progression of dementia and to clarify the role of any identified concomitant medical disorders. Blood in these experiments was not obtained during acute medical illness. Confirmation of clinical diagnoses of Probable AD post mortem from the clinical program (not including any members of the sample reported here) has been 100% to date (unpublished data).

Blood from AD patients and from normals, comprising some spouses of Alzheimer patients, some other elderly individuals and some younger donors who were employees of the VAMC and spanning the age range from 22 to 83 years, was collected. When young and old red blood cells were to be prepared, the blood was processed (see below) within 1 h of collection. RBC were used within one week in all other experiments. For comparative purposes, in one set of experiments (where indicated), blood was collected in oxalate, heparin or EDTA vacutainers or was defibrinated by gently swirling 10 ml of blood with 4 glass beads in a 50 ml Erlenmeyer flask for 10 min.

2.3. Preparation of two-polymer aqueous phase systems and other standard solutions used

Aqueous two-phase systems having the dextran and PEG concentrations and salt compositions and concentrations indicated in the tables or text were prepared as previously described [5,6]. PBS contained 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 6.8. Solutions containing 4, 8 or 12% (w/w) dextran in PBS or 1, 2, 3, 5, 6, 8% (w/w) PEG in PBS were prepared by weighing out the appropriate amounts of concentrated respective stock solutions [5,6] (i.e., of 20% (w/w) dextran, 40% (w/w) PEG, 0.44 M sodium phosphate buffer, pH 6.8, 0.60 M NaCl).

2.4. Preparation of young and old red blood cell populations from normal and Alzheimer patients' blood

Young and old RBC were separated by the centrifugation method of Murphy [14] using approx. 2 ml of packed RBC per 10.7 × 77 mm centrifuge tube. About 7% of the upper and lower centrifuged blood cell layers were collected to obtain RBC populations enriched with respect to young (top layer) or old (bottom layer) cells as previously established [8,15]. The cells were washed three times in PBS and used in electrophoresis experiments (see below).

2.5. In vitro treatments of red blood cells from normal individuals and Alzheimer patients

Chymotrypsin and trypsin treatment [10]

1 ml of packed RBC were washed three times with 10 volumes of PBS, pH 7.4. 0.1 ml of washed RBC diluted in 1 ml of PBS were incubated together with 50 μg of either

trypsin (1 mg/ml saline) or chymotrypsin at 37° C for 60 min. Controls were incubated in the absence of enzyme. The cells were washed three times with PBS and used in electrophoresis experiments.

Formaldehyde fixation of red blood cells [10]

RBC (from blood collected in citrate vacutainers) were washed three times with 10 volumes of PBS, pH 6.8. 1% paraformaldehyde in PBS was heated at about 80° C for 30 min to obtain dissolution and the solution was then allowed to cool. 2 ml of washed packed RBC were added with continuous swirling to 18 ml of the formaldehyde solution. The mixture was permitted to remain at room temperature overnight. The clear formaldehyde solution was poured off and replaced with an equal volume of fresh 1% formaldehyde solution. The cells were resuspended and stored in the cold (4–5° C) for a minimum of 1 week. Fixed cells were then removed and washed three times with 10 volumes of PBS before use in experiments.

Lipid extraction of formaldehyde-fixed red blood cells with chloroform / methanol [10]

Formaldehyde-fixed cells were washed three times in 10 volumes of PBS and an additional two times with 10 volumes of distilled water and one time with 10 volumes methanol. The cells were suspended in 7 volumes of methanol. 14 volumes of chloroform were added, the tube was capped and allowed to sit at 21–24° C for 1 h. The cells were then centrifuged and washed twice with 10 volumes of methanol, twice with 10 volumes of distilled water, and twice with PBS before use in experiments.

Lipid extraction of formaldehyde-fixed red blood cells with ethanol [10]

Formaldehyde-fixed cells were washed as in the previous section. The cells were suspended in 10 volumes of ethanol and kept at 21–24° C for 1 h. The cells were washed in PBS and prepared for analysis as in the previous section.

2.6. Viscosity determinations of suspending media used in electrophoretic mobility measurements

The viscosities of the various suspending media were estimated by means of an Ostwald viscometer immersed in a tank thermostated at 25 ± 0.2° C.

2.7. Electrophoretic mobility measurements on erythrocytes in various suspending media [10,11]

RBC (fresh, enzyme treated, fixed, fixed and lipid-extracted) were washed three times with PBS and a suitable cell aliquot was, finally, suspended in PBS.

Phase systems were mixed and permitted to settle in a separatory funnel overnight at 21–24° C. Top and bottom phases were then separated with the material at the interface being discarded. The PEG-rich top and the dextran-rich bottom phases were centrifuged at 12000 × g for 15 min to ensure that phase separation was complete. Top phase was removed leaving all remaining bottom phase and some top phase behind in the centrifuge tube. Bottom phase was pipetted out of the latter from the middle of the bottom phase being careful to keep residual top phase from entering the pipette.

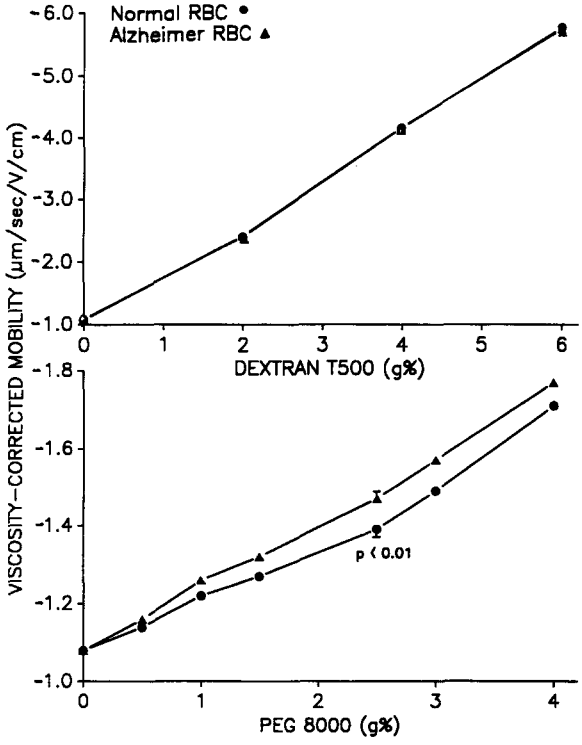

Fig. 1. Viscosity-corrected electrophoretic mobilities (μm/s per V per cm) of red blood cells (RBC) from normals and Alzheimer patients at different concentrations of dextran T500 (top) and poly(ethylene glycol) (PEG) 8000 (bottom). See text for discussion.

Aliquots of the cell suspensions in PBS (see above) were diluted 1:1 (by weight) with top or bottom phase (of the phase system indicated in the tables), 4, 8 or 12% (w/w) dextran in PBS or 1, 2, 3, 5, 6 or 8% (w/w) PEG in PBS for EPM measurements (as indicated in Fig. 1).

Cell microelectrophoresis was carried out in a cylindrical chamber (Rank Brothers, Cambridge, UK) at 25 ± 0.2° C with transillumination [16]. In each sample the rates of migration of ten individual RBC were obtained at the stationary level for the calculation of EPM in μm/s per V per cm [16]. The rates of migration were observed in alternate directions.

2.8. Presentation of data

The EPM of the RBC in the different media were corrected to the viscosity of water [16]. The EPM obtained in the different suspending media are presented, in each case, as the mean ± S.D. with the number of different

individuals in parentheses. Since the S.D. were small in experiments with AD and normal untreated blood samples, the selection of the smaller number of samples subjected to the various treatments (Table 1) was arbitrary. P values were obtained by one way analysis of variance (ANOVA).

3. Results and discussion

3.1. Effect of different suspending media on the relative electrophoretic mobilities of RBC from Alzheimer patients and normal individuals

RBC from AD patients and normal individuals have EPM that are identical in PBS but differ significantly in some polymer solutions (e.g., PEG, bottom phase (from a dextran-PEG aqueous phase system)) [7]. The relationship between the viscosity-corrected EPM of AD or normal RBC and PEG concentration is shown in Fig. 1.

Despite the differences in EPM between AD and normal RBC in PEG or dextran-rich bottom phase (diluted 1:1 with PBS) (Table 1 and [7]) it is intriguing that no mobility difference can be found in dextran solutions (Fig. 1) or in diluted PEG-rich top phase [7]. We have suggested [7] that this phenomenon may be a consequence of the contaminant(s) (probably originating in dextran) which is visible at the interface when dextran-PEG phase systems are made and permitted to phase separate. The interface is discarded when the PEG-rich top and dextran-rich bottom phases are collected and the bottom phase is thereby depleted of the contaminant(s). Particulates often partition between the interface and the PEG-rich top phase in dextran-PEG phase systems [5,6] and it is not unlikely that the contaminant(s) also partitions in this manner. This would explain the elimination in PEG-rich top phase of the difference in EPM between AD and normal RBC found in PEG solutions (Fig. 1).

To probe the above-described hypothesis a number of experiments were carried out (data not shown). (1) A solution composed of dextran, PEG and salts approximating the composition of bottom phase (as used in the tables) was made. This 'bottom phase' yielded no difference in EPM between AD and normal RBC indicating that phase separation is essential to obtain the indicated EPM difference. (2) Bottom phase, obtained by phase separation, collected either together with the interface or to which interfacial material (5–10% by volume) was added did not yield a difference in EPM between AD and normal RBC. (3) PEG solutions (5% (w/w) PEG) to which interfacial material (5–10% by volume) from a dextran-PEG phase system had been added also gave no difference in EPM between AD and normal RBC. (4) Bottom phase to which top phase (5 or 10% by volume) obtained after phase system separation had been added, gave no difference between AD and normal RBC; while bottom phase to which PEG solution (to a concentration of 0.25% or 0.5% (w/w) PEG in addition to that already present in bottom phase) was added continued to give a difference in EPM between AD and normal RBC. These latter experiments show that it is not the high concentration of PEG in (1:1 diluted) top phase that prevents detection of the indicated difference in EPM, but rather something present in the phase-separated PEG-rich top phase. All of these results reinforce the conclusion that the interface and the top phase contain material which eliminates the EPM difference between AD and normal RBC observed in bottom phase or PEG solutions.

3.2. Electrophoretic mobilities of native and chemically modified RBC from Alzheimer patients and normal individuals in phosphate-buffered saline and in bottom phase

The nature of the surface component(s) responsible for the EPM difference in bottom phase between AD and normal RBC was probed by treating these RBC in vitro in a number of different ways (Table 1) and determining the effect of each treatment on their relative EPM. In these studies bottom phase rather than PEG solution was chosen

Table 1
Viscosity-corrected electrophoretic mobilities [a] of fresh, enzymatically treated, formaldehyde-fixed, and formaldehyde-fixed, lipid extracted red blood cells (RBC) from Alzheimer patients and normal individuals in different suspending media

Treatment	PBS [b]			Bottom phase [c]		
	normal RBC	Alzheimer RBC		normal RBC	Alzheimer RBC	
None	-1.08 ± 0.01 (18)	-1.08 ± 0.01 (18)	N.S.	-4.56 ± 0.05 (18)	-4.87 ± 0.07 (18)	$P < 0.01$
Chymotrypsin	-0.91 ± 0.01 (8)	-0.92 ± 0.02 (8)	N.S.	-3.81 ± 0.12 (8)	-4.14 ± 0.09 (8)	$P < 0.01$
Trypsin	-0.81 ± 0.01 (7)	-0.81 ± 0.02 (7)	N.S.	-3.48 ± 0.14 (7)	-3.53 ± 0.16 (7)	N.S.
Formaldehyde fixed	-1.09 ± 0.01 (8)	-1.09 ± 0.01 (8)	N.S.	-4.61 ± 0.09 (8)	-4.88 ± 0.11 (8)	$P < 0.01$
Fixed, $CHCl_3$/MeOH extracted	-1.08 ± 0.01 (8)	-1.08 ± 0.01 (8)	N.S.	-3.97 ± 0.08 (8)	-4.17 ± 0.07 (8)	$P < 0.01$
Fixed, EtOH extracted	-0.96 ± 0.02 (8)	-0.95 ± 0.02 (8)	N.S.	-3.41 ± 0.07 (8)	-3.62 ± 0.11 (8)	$P < 0.01$

[a] Data present the mean electrophoretic mobilities, EPM, (μm/s per V per cm), $\pm$S.D. with the number of experiments in parentheses.
[b] Phosphate-buffered saline (PBS) was composed of 0.15 M NaCl + 0.01 M sodium phosphate buffer, pH 6.8 (NaPB).
[c] Bottom phase was from a system containing 5% (w/w) dextran (Dx) T500, 3.5% (w/w) poly(ethylene glycol) (PEG) 8000, 0.15 M NaCl and 0.01 M NaPB. Bottom phase is Dx-rich. Bottom phase was diluted 1:1 with the indicated RBC suspension in PBS followed by measuring cell EPM.

Table 2
Viscosity-corrected electrophoretic mobilities [a] of young and old red blood cells [b] (RBC) from Alzheimer patients and normal individuals in different suspending media

Suspending media	RBC					
	normal young [c]	normal old [c]		Alzheimer young	Alzheimer old	
PBS [d]	−1.08 ± 0.01 (9)	−1.08 ± 0.01 (9)	N.S.	−1.08 ± 0.01 (6)	−1.08 ± 0.01 (6)	N.S.
Dextran [e]	−3.99 ± 0.13 (9)	−4.33 ± 0.13 (9)	$P < 0.01$	−4.05 ± 0.10 (6)	−4.41 ± 0.13 (6)	$P < 0.01$
Bottom phase [f]	−4.54 ± 0.04 (6)	−4.61 ± 0.06 (6)	$P < 0.03$	−4.77 ± 0.07 (6)	−4.87 ± 0.10 (6)	N.S.
PEG [e]	−1.41 ± 0.01 (6)	−1.44 ± 0.03 (6)	$P < 0.04$	−1.45 ± 0.02 (6)	−1.48 ± 0.03 (6)	$P < 0.01$
Top phase [f]	−1.82 ± 0.03 (6)	−1.86 ± 0.03 (6)	$P < 0.04$	−1.83 ± 0.03 (6)	−1.86 ± 0.03 (6)	N.S.

[a] Data present the mean electrophoretic mobilities, EPM, (μm/s per V per cm), ±S.D. with the number of experiments in parentheses.
[b] Obtained by centrifugal fractionation method.
[c] Data of normal young and old red blood cells are taken from Walter and Widen, Biochim. Biophys. Acta. (1994) 1194, 131–137.
[d] Phosphate-buffered saline (PBS) was composed of 0.15 M NaCl + 0.01 M sodium phosphate buffer, pH 6.8 (NaPB).
[e] EPM were measured in a 4% dextran (Dx) T500 solution in PBS and a 2.5% poly(ethylene glycol) (PEG) 8000 solution in PBS.
[f] Top and bottom phases were from a system containing 5% (w/w) Dx T500, 3.5% (w/w) PEG 8000, 0.15 M NaCl and 0.01 M NaPB. Top phase is PEG-rich and bottom phase is Dx-rich. Top and bottom phases were diluted 1:1 with the indicated RBC suspension in PBS followed by measuring cell EPM.

because the EPM difference between AD and normal RBC is larger, on a percentage basis, in this suspending medium [7].

First note that (Table 1), measured in PBS, the EPM of both AD and normal RBC is the same (−1.08 μm/s per V per cm) [7]. When treated with chymotrypsin these cells' EPM is reduced by about 16% (−0.91 μm/s per V per cm) and with trypsin by 25% (−0.81 μm/s per V per cm). Formaldehyde-fixation or formaldehyde-fixation followed by membrane lipid extraction with chloroform/methanol causes no change in mobility while fixed cell extraction with ethanol results in a reduction in EPM of 12% (−0.96 μm/s per V per cm).

Measured in bottom phase AD RBC have a higher viscosity-corrected EPM (−4.87 μm/s per V per cm) than normal RBC (−4.56 μm/s per V per cm) [7]. Chymotrypsin- or trypsin-treated or formaldehyde-fixed normal RBC have EPM reduced, respectively, by 16, 24 and 1% in relation to the untreated normal RBC. These percentage reductions in EPM are comparable to those found for these cells in PBS (see above). Fixation of normal RBC with formaldehyde followed by chloroform/methanol or ethanol extraction gives EPM that are substantially reduced with respect to untreated cells (i.e., 13 and 25%, respectively) and thus differ from these cells' electrophoretic behavior in PBS. This result emphasizes (see also [11]) that electrophoretic gauging of cell surface charge in PBS and of surface properties manifested by interaction between cell surface and polymer in the suspending medium can differ. AD RBC have a percentage reduction in EPM following each treatment, with respect to untreated AD RBC, that mimics that of normal RBC with the exception of cells treated with trypsin. Trypsin causes a larger reduction in EPM of AD RBC (−4.87 to −3.53 μm/s per V per cm, 28%) than of normal RBC (−4.56 to −3.48 μm/s per V per cm, 24%) thereby eliminating the EPM difference between trypsin-treated AD and normal RBC (Table 1). Thus, a difference

Table 3
Viscosity-corrected electrophoretic mobilities [a] of young and old red blood cells [b] (RBC) from Alzheimer patients and normal individuals in different suspending media

Suspending media	RBC					
	normal young [c]	Alzheimer young		normal old [c]	Alzheimer old	
PBS [d]	−1.08 ± 0.01 (9)	−1.08 ± 0.01 (6)	N.S.	−1.08 ± 0.01 (9)	−1.08 ± 0.01 (6)	N.S.
Dextran [e]	−3.99 ± 0.13 (9)	−4.05 ± 0.10 (6)	N.S.	−4.33 ± 0.13 (9)	−4.41 ± 0.13 (6)	N.S.
Bottom phase [f]	−4.54 ± 0.04 (6)	−4.77 ± 0.07 (6)	$P < 0.01$	−4.61 ± 0.06 (6)	−4.87 ± 0.10 (6)	$P < 0.01$
PEG [e]	−1.41 ± 0.01 (6)	−1.45 ± 0.02 (6)	$P < 0.01$	−1.44 ± 0.03 (6)	−1.48 ± 0.03 (6)	$P < 0.03$
Top phase [f]	−1.82 ± 0.03 (6)	−1.83 ± 0.03 (6)	N.S.	−1.86 ± 0.03 (6)	−1.86 ± 0.03 (6)	N.S.

[a] Data present the mean electrophoretic mobilities, EPM, (μm/s per V per cm), ±S.D. with the number of experiments in parentheses.
[b] Obtained by centrifugal fractionation method.
[c] Data of normal young and old red blood cells are taken from Walter and Widen, Biochim. Biophys. Acta. (1994) 1194, 131–137.
[d] Phosphate-buffered saline (PBS) was composed of 0.15 M NaCl + 0.01 M sodium phosphate buffer, pH 6.8 (NaPB).
[e] EPM were measured in a 4% dextran (Dx) T500 solution in PBS and a 2.5% poly(ethylene glycol) (PEG) 8000 solution in PBS.
[f] Top and bottom phases were from a system containing 5% (w/w) Dx T500, 3.5% (w/w) PEG 8000, 0.15 M NaCl and 0.01 M NaPB. Top phase is PEG-rich and bottom phase is Dx-rich. Top and bottom phases were diluted 1:1 with the indicated RBC suspension in PBS followed by measuring cell EPM.

in EPM between AD and normal RBC is retained subsequent to every in vitro treatment to which these cells were subjected (Table 1) other than trypsin.

It appears that proteolytic modification of the RBC surfaces with an appropriate enzyme eliminates the EPM difference between AD and normal RBC in bottom phase.

3.3. Electrophoretic mobilities of young and old RBC from Alzheimer patients and normal individuals in a number of different suspending media

Human normal young and old RBC (obtained by a centrifugation method [14]) have, like unfractionated AD and normal RBC populations, identical EPM in PBS [8]. Unlike AD and normal RBC they display markedly different EPM in solutions of dextran [9]. Much smaller differences in EPM, on a percentage basis, are also found for both normal and AD patient young and old RBC in PEG solution and, for normal young and old RBC, in bottom and top phases (see [10] and Table 2).

The reported increase of IgG on the AD RBC surface [3], which mimics the behavior of normal old RBC, has led to the suggestion that AD RBC age abnormally [1–3]. We therefore compared the EPM of young and old RBC from AD patients to each other and to their normal counterparts. In Table 2, we show the EPM of AD young and old RBC in the five above-indicated suspending media. Again no difference in EPM is in evidence in PBS while a clear difference between AD young and old RBC is found in dextran and a smaller difference also in PEG. It is unlikely that the borderline differences, in terms of percentage, found between normal young and old RBC in top and bottom phases and their absence between AD young and old RBC are of importance. However, when normal young and AD young RBC or normal old and AD old RBC are compared (Table 3) the only differences in EPM observed are in bottom phase and in PEG solution, which thus duplicate the behavior of the respective unfractionated RBC [7].

By the criterion of cell electrophoresis in polymer solutions the differences between AD and normal RBC and between young and old RBC are distinct. The EPM of AD and normal RBC differ in bottom phase or PEG but not in dextran solution; while the EPM of young and old RBC differ predominantly in dextran.

3.4. Electrophoretic mobilities in phosphate-buffered saline and in bottom phase of RBC from Alzheimer patients and normal individuals from blood collected in different anticoagulants

Even though RBC are washed repeatedly with PBS prior to suspension in the medium selected for EPM measurements (see Materials and methods), the manner of blood collection (i.e., anticoagulant employed or defibrination) is found to markedly influence the results obtained with AD and normal RBC. (The difference in EPM between young and old RBC obtained in dextran solution is, however, irrespective of anticoagulant tested.)

We examined the effect of a few anticoagulants on the relative EPM of AD and normal RBC in bottom phase. Table 4 shows that, while the EPM in PBS of these RBC is the same irrespective of blood anticoagulant used, only collection in citrate or oxalate results in an EPM difference between AD and normal RBC in bottom phase. The EPM values of AD and of normal RBC, respectively, and the EPM differences between AD and normal RBC in these two anticoagulants are identical (Table 4).

RBC from normal and AD patient blood collected in heparin, EDTA or by defibrination cannot be differentiated in bottom phase. Of these only heparin gives rise to an EPM in bottom phase for normal RBC similar to that of these cells obtained using citrate or oxalate (Table 4). Hence, the higher mobility in bottom phase of AD RBC from citrated or oxalated blood would appear to be reduced to that of normal RBC with heparin. On the other hand, normal RBC cells have, with respect to RBC in blood collected in citrate, oxalate or heparin, elevated EPM when it is collected in EDTA or is defibrinated (Table 4). In any case, as judged by cell EPM in bottom

Table 4
Viscosity-corrected electrophoretic mobilities [a] of red blood cells (RBC) from Alzheimer or normal blood collected in different anticoagulants or defibrinated

Anticoagulation treatment	PBS [b]			Bottom phase [c]		
	normal RBC	Alzheimer RBC		normal RBC	Alzheimer RBC	
Citrate	-1.08 ± 0.01 (8)	-1.08 ± 0.01 (8)	N.S.	-4.50 ± 0.07 (8)	-4.85 ± 0.07 (8)	$P < 0.01$
Oxalate	-1.09 ± 0.01 (5)	-1.09 ± 0.01 (5)	N.S.	-4.49 ± 0.04 (5)	-4.83 ± 0.04 (5)	$P < 0.01$
Heparin	-1.08 ± 0.01 (5)	-1.08 ± 0.01 (5)	N.S.	-4.53 ± 0.06 (5)	-4.56 ± 0.08 (5)	N.S.
EDTA	-1.08 ± 0.00 (5)	-1.09 ± 0.01 (5)	N.S.	-4.73 ± 0.08 (5)	-4.75 ± 0.11 (5)	N.S.
Defibrination	-1.09 ± 0.01 (5)	-1.08 ± 0.01 (5)	N.S.	-4.72 ± 0.08 (5)	-4.75 ± 0.07 (5)	N.S.

[a] Data present the mean electrophoretic mobilities, EPM, (μm/s per V per cm), $\pm$S.D. with the number of experiments in parentheses.
[b] Phosphate-buffered saline (PBS) was composed of 0.15 M NaCl + 0.01 M sodium phosphate buffer, pH 6.8 (NaPB).
[c] Bottom phase was from a system containing 5% (w/w) dextran (Dx) T500, 3.5% (w/w) poly(ethylene glycol) (PEG) 8000, 0.15 M NaCl and 0.01 M NaPB. Bottom phase is Dx-rich. Bottom phase was diluted 1:1 with the indicated RBC suspension in PBS followed by measuring cell EPM.

phase, the surface of normal RBC from blood collected in EDTA (or defibrinated) differs from that of RBC collected in the other three anticoagulants.

Currently, one can only conjecture as to the basis for the differential anticoagulant effects on the relative EPM of RBC from AD patients and normals. Disturbances in calcium homeostasis in AD have been reported [17,18] as reflected in increased calcium levels and decreased levels of calcium-binding proteins in AD brain tissue [17]. Calcium abnormalities have also been found in blood plasma in AD [4]. Possibly citrate or oxalate which bind calcium, thus preventing blood from clotting, reflect aspects of these differences. In contrast, heparin inactivates thrombin in the presence of serum and thus acts in a part of the clotting cascade unrelated to calcium. Heparin also binds many proteins (e.g., β-amyloid protein [19]) and such binding may be related to the absence of the above-indicated relative difference in EPM. The action of EDTA, which prevents clotting by removing calcium, is more wide-ranging than that of either citrate or oxalate. Its reactivities include chelation of metals, some of which are reported to differ [20] or be involved in modulatory effects [21] in AD, and inhibition of metal-requiring proteinases. Defibrination involves the physical removal of the (predominantly) fibrin clot as it forms and thus also gives rise to a cell environment distinct from those obtained with the above anticoagulants.

Since the difference in EPM between AD and normal RBC appears to be protein-dependent and anticoagulant-related, it may involve an anticoagulant-associated interaction between the respective cells and their compositionally differing [4] plasma proteins. Such interaction would have to take place before or at the time blood is collected in anticoagulant because when AD and normal RBC (blood-group matched) are incubated in each other's plasma or serum the EPM of the cells and, hence, the EPM difference between them, is unaffected (data not shown).

Acknowledgements

We thank Dr. Stephen L. Read for the evaluation of patients for Alzheimer disease and Marilyn Tacio-Maravillas for making available blood samples of selected patients (John Douglas French Center for Alzheimer's Disease, Los Alamitos, CA); Dr. Geoffrey V.F. Seaman (Western Biochemical Research Institute, Eugene, OR) for instruction in the use of the microelectrophoresis apparatus. This work was supported by the Medical Research Service of the U.S. Department of Veterans Affairs.

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