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CELL ELECTROPHORESIS IN PERCOLL DENSITY GRADIENTS

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INTRODUCTION

The existence of surface charges on living cells was recognized very early by Jurgensen in 1860 when he reported a migration of erythrocytes toward the anode(1). Since then, numerous attempts at qualitatively or quantitatively describing these charges have been made, which are now generally accepted as arising from the carboxyls of sialic acid residues(2). But because of serious theoretical and practical limitations of previous designs, these surface charges are not often measured.

Four general methods for measuring cell surface potentials have been described in the literature. Microelectrophoresis, as described by Abramson, 1929 (3), is the most widely reported method. Microelectrophoresis involves placing a test cell in a glass capillary tube filled with an electrolyte solution. When an electric field is applied across the ends of the tube, negatively charged cells should be attracted to the anode. However, some difficulties arise since the glass tube is negatively

charged itself, which causes an electroosmotic movement in the opposite direction. Consequently, the electrophoretic movement of the cell toward the anode may be suppressed, indeed totally reversed by electroosmosis. Theoretical corrections of electroosmosis are possible, but practical limitations of visualizing cells in a curved tube and the constant settling of cells may result in inaccurate measurements with this arrangement (4), This electroosmotic backflow can be rendered nearly uniform by coating the inner wall and closing both ends with a gel layer of agarose (5). Cell separation and preparation is not possible with this method.

The STAFLO method of Mel (6) (also known as free-flow electrophoresis) involves a constant flow of fluid through a rectangular box stabilized by a flowing density gradient. The position of cells in the fluid is influenced by a vertical electric field much like an electron in a cathode ray tube. The problems of excessive heat production, construction and large quantities of sample cells are major and quite involved. Other continuous flow electrophoresis methods can be found in reference (7).

The vertical density gradient apparatus described by Boltz (8) minimizes some of the convection problems of elctroosmosis. A continuous density gradient of Ficoll and sucrose tends to confine fluid movement to a small layer. However, the Ficoll-sucrose density gradient is highly osmolar which may render cells nonviable. Additionally, the described procedure requires a large sample and much heat dissipation. The vertical ascending cell electrophoresis method described by van Oss, et al. (9) alleviates the problem of osmolarity with the use of a D_2O density gradient.

Gilbert (10) suggests titrating the negative surface charge by changing the intracellular fluid and measuring the transmembrane potential. The technique of transmembrane, intracellular potential recording is quite difficult and unsuitable for routine measurements.

The method proposed in this paper involves several modifications to the vertical density gradient method. These modifications allow for simplicity of operation, low cost, and use of isotonic solutions suitable for clinical or laboratory investigations of living cells.

METHODOLOGY

A Hoeffer 12-tube, disc-gel electrophoresis apparatus equipped with platinum wire electrodes servers as the elctrophoretic chamber. Each glass tube measures 15 cm long and has an inside diameter of 0.4 cm.

The problem of electroosmosis is minimized through the use of a methyl cellulose coating described by Catsimpoolas, et al. (8). The coating material is made by first dissolving 0.4 gm methyl cellulose into 30 ml boiling water. Next, 70 ml of Cold (4° C) water is added and stirred in a cold room until the solution becomes clear. 7.0 ml formic acid and 35 ml formaldehyde are added with additional stirring. This solution is clarified by filtration and can be stored for extended periods at 4°C. The glass electrophoresis tubes are rinsed thoroughly with a detergent solution followed by hot, cold, and deionized water and then dried. The cleaning procedure is repeated. The prepared methyl cellulose solution is then poured into Parafilm stoppered tubes for 5 min. The solution is slowly pipetted out with a 9 inch disposable pipet. The tubes are dried for 40 min at 120°C and the coating and drying procedure is repeated. Air bubbles on the tube walls must be carefully avoided and a vertical arrangement of the tubes for uniform coating should be maintained.

A 1 cm, 15% polyacrylamide plug is utilized to support the density gradient at the bottom of the tube, 4.7 ml water is combined with 5.45 ml buffer solution, 10.0 ml of a solution containing 30% Acrylamide and 0.8% Bis, and 10 μl TEMED supplied by Bio-Rad. This is degassed for 15 min in a 125 ml suction flask. 6 ml 2% ammonium persulfate solution is then added and pipetted into the bottom of the Parafilm stoppered, coated and dried tubes. The acrylamide polymerizes within one hour.

The density gradient consists of varying dilutions of Percoll (polyvinyl-pyrrolidone coated colloidal silica) supplied by Pharmacia Corp, as a 1.13 gm/cm^3 solution. This material offers the benefits of being non-toxic, and having low osmolarity, viscosity, and conductivity, making it eminently applicable for electrophoresis. Initially, the Percoll is diluted to a 90% stock solution using 10 ml of 10X concentrated HEPES (n-2-hydroxyethyl piperazine-n'-2-ethane sulfonic acid) buffer solution into 90 ml Percoll. A 70% solution is used for the heavy component of the density gradient by mixing 35 ml of 90% Percoll with 10 ml 1X HEPES buffer solution. Similarly, 20% and 10% are made using 10 ml and 5 ml of 90% Percoll made up to 45 ml with buffer solution. The 20% solution becomes the light component of the density gradient and the 10% solution is the suspension medium of the sample cells. This step gradient at the top of the column

provides a concentrating mechanism for the cells much like the step concentration procedure described by Ornstein for proteins. The range of 20% to 70% Percoll converts to a density range of 1.02 to 1.10 gm/cm^3. It should be noted that in this density range and pH of Percoll solution, the viscosity is approximately the same as water (12).

The density gradient is formed in the tubes by a micro-density gradient maker (MRA) joined to a piece of plastic tubing (Intramedic) which will fit freely into the glass tubes. The glass tubes will accept a 0.6 ml gradient. Thus 0.3 ml of light and dense solutions are used in each chamber of the gradient maker. During the formation of the gradient, the connecting tube is slowly withdrawn to allow the lighter solutions to layer on top of the gradient. The sample cells suspended in 10% Percoll are added by pipet to the top of the gradient. The top and bottom electrophoretic chambers are then filled with buffer solution.

The buffer used to dilute the Percoll and provide for the top and bottom electrolytes is a buffer of 25mM HEPES, 2.5 mM KCl, 0.81 mM $MgSO_4$, 0.95 mM NaH_2PO_4, 2.38 mM $NaHCO_3$, 1.84 mM $CaCl_2$, 5.55 mM d-glucose, NaOH to adjust the pH to 7.4, and NaCl to yield a 300 mOsm solution (approx. 143 mM). The osmolarity is verified with a Osmette osmometer to be 300 mOsm $\pm$20.

Approximately two million cells in 0.1 ml 10% Percoll are introduced at the top of each tube. The cells are electrophoresed for 2 hours at 4°C with sufficient current to equal 6.67 mamp per tube. This arrangement produces an electric field of 2.0 V/cm, using the formula $E = I/\gamma A$ where I is the current, γ is the conductivity (26.5 mmho/cm for 0.3M NaCl), and $A = 0.126$ cm^2 for the glass tubes in this experiment. Both the total time and current are variable and may be adjusted for different applications. Any power supply may be utilized for the application of current, but a current meter should be inserted into the circuit to insure constant current through the tubes.

After electrophoresis, the cells appear as bands in the density gradient. These bands can be photographed and measured for quantitative calculations of electrophoretic mobility.

Fractionation of the density gradient can be accomplished using a 1 ml syringe with a long needle, 0.2 ml fractions withdrawn from the bottom of

the gradient provide excellent separation. These fractions can be centrifuged and washed for further testing and checks on viability. Yields can be determined by counting the cells in each fraction with a haemocytometer. The density gradient can be checked by comparing the refractive index (R.I.) to calibrated curves of percent Percoll versus R.I. using a refractometer (Bausch-Lomb).

Chick embryo heart cells, human promyelocytic leukemia cells (HL-60), and human erythrocytes from patients with Huntington's disease were kindly supplied by Drs. Robert DeHaan (13), Joseph Kinkade (14), and John Madden.

The quantification of surface charge density from electrophoretic mobility is theoretically based and, therefore, subject to certain assumptions (15). The zeta-potential derived from electrophoretic mobility is given by:

$$\zeta = (4 \pi \eta / D)\ u$$

where η is viscosity (1.5 cP for 50% Percoll) and D is the dielectric constant (85.9 for 5°C).

RESULTS

The procedure described above was applied to several cell systems. Seven-day and fourteen-day-old embryonic chick heart cells were electrophoresed for 2 hours. Figure 1 depicts the total distance of migration of the cells. The seven day cells had a greater electrophoretic mobility than the fourteen day cells. This electrophoretic velocity difference was maintained even when the sample consisted of a mixture of seven and fourteen day cells which could be distinguished by their aggregative ability. Direct observation of the spontaneously beating heart cells confirmed the viability of the cells after electrophoresis.

Erythrocytes from patients with Huntington's disease were compared with normal erythrocytes for differences in surface charge density. Electrophoresis of these cells did not show any difference in the mean migration distance. However, the erythrocytes from patients with

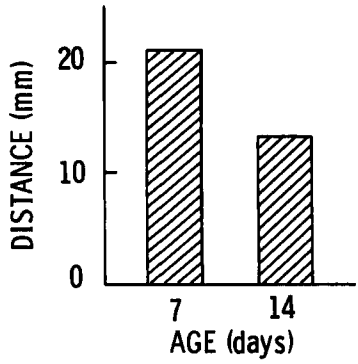

FIGURE 1
Total distance of migration for 7 and 14 day embryonic heart cells after electrophoresis for two hours in an electric field of 2 V/cm.

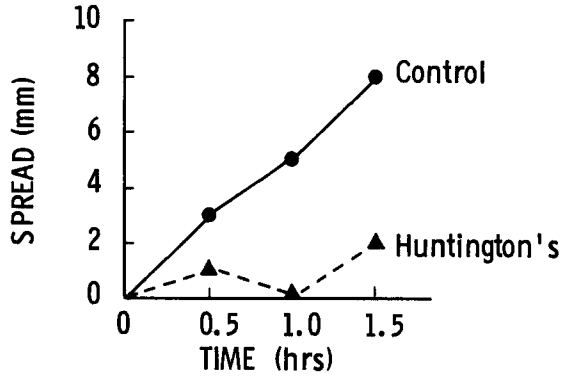

FIGURE 2
Width of cell band (spread) versus time of electrophoresis for erythrocytes from Huntington's diseased patients and normal humans in an electric field of 2 V/cm.

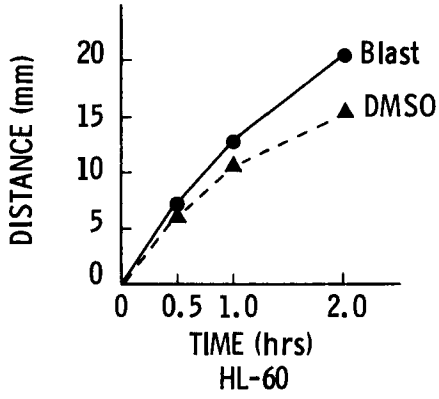

FIGURE 3
Distance versus time of electrophoresis for HL-60 cells in an electric field of 2 V/cm.

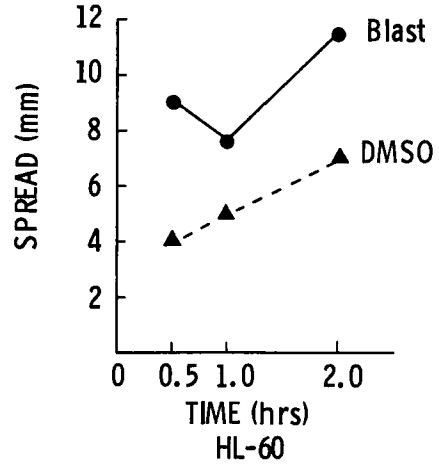

FIGURE 4
Width of cell band versus time of electrophoresis for HL-60 cells in an electric field of 2 V/cm.

TABLE I

Electophoretic Mobility and Surface Charge Density of Tested Cells.

Cell Type	v ($\frac{\mu m}{sec}$)	u ($\frac{\mu m/sec}{V/cm}$)	ζ (mV)
7 d Heart	-2.9	-1.45	-29
14 d Heart	-1.8	-0.90	-18
HL-60, Blast	-2.7±1.5	-1.4	-27±15
HL-60, DMSO	-2.1	-1.0	-21
normal RBC	-3.3±0.8	-1.6	-33±8

Huntington's disease maintained a sharp band throughout the experiment; whereas, the normal control erythrocytes moved in a wider band as shown in figure 2.

The human leukemia line of HL-60cells can be induced to differentiate by 1.25% dimethyl sulfoxide (DMSO). Comparing uninduced promyelocytic cells with seven day transformed cells, the DMSO cells showed less electrophoretic mobility. Figure 3 depicits the distance of migration versus time of elctrophoresis. The HL-60 cells treated with DMSO additionally show a smaller range of spread than uninduced cells as shown in figure 4.

The surface charge density can be calculated from the equations given in the methodology section. The results of these calculations is shown in Table I.

DISCUSSION

Small differences in the mean and range of surface charge density can be detected by vertical density gradient electophoresis through Percoll. The spread is proportional to the standard deviation of electrophoretic mobility of two million cells. This indicates that the control RBC had a greater variation of surface potential and that the Huntington's RBC tested were more homogeneous. The low amount of spread was true for samples from both symptomatic and asymptomatic patients.

The trend of decreasing surface charge density with development, as shown by decreases in electrophoretic mobility for embryonic chick heart cells and HL-60 cells, is paralleled by reports that aggregation increases over the same time frame in B-lymphocytes in mice (16) and HL-60 cells induced by DMSO (14). Embryonic chick heart cells show a similar pattern of aggregation with age. The two phenomena may be linked by the hypothesis that electrostatic repulsion due to high surface potentials decreases during development, allowing attractive forces such as hydrogen bonding to aggregate cells (17).

Quantitation of the surface charge density of various cells can provide a check of other biochemical determinations of membrane structure. Calculations from these experiments are similar in magnitude to previous studies of biological cells (18-22). However, this method can reveal slight differences in the heterogeneity of surface potential of a large population of cells which may be masked in a few observations of individual cells under microelectrophoresis. The amount of electrophoretic spread is proportional to the variation in surface potential on different cells. Thus, the electrophoretic spread seen in the uninduced HL-60 cells may account for the extreme variation in the timings of peripheral lymphocytes seen by Pritchard, et al. (23). Both age and cell differentiation influence the membrane composition suggesting that one should expect a variety of surface potentials in a sample of peripheral erythrocytes (4). Thus, it is quite remarkable that the RBC from the patients with Huntington's Disease exhibited such a uniform surface potential.

Selective perturbations in membrane sialic acids, e.g. caused by neuraminidase, could be detected by this method via quantitative or qualitative differences (24). Relative differences in electophoretic mobility can also distinguish subsets of cells for clinical diagnoses or further laboratory studies.

The described procedure for electrophoresis of cells resolves several limitations of previous methods through low cost and easy availability of the Hoeffer electrophoretic apparatus, low distortion from electroosmosis without large heat generation, and simplicity of operation suitable for routine clinical and basic science applications.

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