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ELECTROPHORETIC SEPARATION AND ANALYSIS OF LIVING CELLS FROM SOLID TISSUES BY SEVERAL METHODS

HUMAN EMBRYONIC KIDNEY CELL CULTURES AS A MODEL

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SUMMARY

Preparative electrophoresis of living cells has been considered for some time as a potential tool for isolating, from heterogeneous mixtures, subpopulations of cells according to function. Such a purification depends upon the retention of electrophoretic heterogeneity and the retention of function. Human embryonic kidney cells that had been in monolayer culture for 1–5 subcultivations were resuspended by treatment with trypsin and/or EDTA and suspended in a variety of electrophoresis buffers, ranging in ionic strength from 0.0015 to 0.15 *M*. Analytical electrophoresis with a Zeiss Cytopherometer or Pen Kem 3000 automated light-scattering electrophoretic analyzer indicated that electrophoretic heterogeneity was retained under the full range of conditions tested. Preparative electrophoresis by three methods —in a density gradient, with continuous flow, and in microgravity— indicated that electrophoretic heterogeneity coincided with functional heterogeneity; for example, some electrophoretically isolated subpopulations produced increased levels of urokinase while others produced increased level of tissue plasminogen activator.

INTRODUCTION

Preparative electrophoresis of living cells has been considered for some time as a potential tool for purifying, from heterogeneous mixtures, subpopulations of cells according to function. Such purification depends upon the retention of electrophoretic heterogeneity and the retention of function.

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Several examples of the electrophoretic purification of specific mammalian cell types from populations of cells that occur naturally in suspension or in suspension cultures have been described. Examples include graft-*vs.*-host cells of bone marrow[1], mouse T-lymphocytes[2–4], human peripheral blood leucocytes[5], and ascites tumor cell subpopulations[6,7]. Cells from solid tissues and monolayer culture have also been subjected to electrophoretic study and separation, including adult rat and rabbit kidney[8–10], virus infected cultured cells[11], canine pancreas[12], rat pituitary[13], and cultured human embryonic kidney cells[14], and cells from human and animal tumors[15,16].

Several methods suitable for cell purification by electrophoresis have been developed and tested. These include stable-flow free-boundary density gradient electrophoresis[17], continuous flow electrophoresis[18], density gradient electrophoresis[19–21], continuous flow electrophoresis (CFE)[22–24], and electrophoresis in a re-orienting density gradient[25,26]. Preparative isoelectric focusing methods have also been applied to living cells[7,11,16,27].

Electrophoretic isolation of subpopulations of cells from solid tissues or monolayer culture presents several challenges. These challenges must be met by conditions that fulfill the following criteria:

(1) The starting population must be electrophoretically heterogeneous; *i.e.*, the electrophoretic mobility (EPM) distribution of a typical population of cells from the particular source must have a standard deviation greater than 10% of the mean EPM. Distributions with multiple peaks are further indication of the possibility of functional heterogeneity related to electrophoretic heterogeneity.

(2) Electrophoretic heterogeneity should be consistent among cell populations; *i.e.*, similar cell suspensions from different animals or from different cultures should be similarly heterogeneous and have similar EPM distributions.

(3) The method of cell dispersal should produce single-cell suspensions and not adversely affect EPM or EPM distributions. While the method of producing single cells may modify absolute EPM values, such modifications must not compromise the electrophoretic and functional heterogeneity of the starting cell population.

(4) The electrophoresis buffer should not adversely affect EPM or EPM distributions. Different electrophoretic methods may require different electrophoresis buffers having a wide range of ionic strengths. While it is well known that decreasing ionic strength increases absolute EPM, and the purely physical relationship between ionic strength and EPM predicts that all electrophoretic subpopulations should increase their EPM proportionately, the physiological effects of decreased ionic strength may modify the cell surface, and the "scaling" of EPM distributions with ionic strength must be examined in each case.

(5) If the purification of a subpopulation with a particular function is desired, unrelated factors must not dominate the motion of the cell in an electric field. For example, in density-gradient and free-flow electrophoresis the cell sedimentation rate, which depends on size and density according to Stokes' relationships, affects electrophoretic migration distances of cells[12,13]. This means that other variables upon which cell size depends, such as cell cycle position, may affect the purification of subpopulations by these two methods.

(6) There should be a demonstrated relationship between cell function and cell EPM. This relationship need not be causal nor proven to be causal, if it can be

empirically demonstrated. The desired function simply needs to be found at a satisfactorily increased level in the purified subpopulation. In some instances this will mean that cells with specific unwanted functions are excluded and in others it will mean that the separated subpopulation of cells has a quantitatively increased capacity to produce a desired product, such as a hormone or an enzyme.

(7) The combination of methods must not comprise the function of the cells being purified. Purified subpopulations of cells must be tested for the retention of the desired function.

In the research described below these postulates were tested by using human embryonic kidney cell monolayer cultures as heterogeneous starting material and urokinase production as the desired function in experiments in which various methods of analytical and preparative cell electrophoresis were used. The study of this starting material was originally inspired by the discovery of electrophoretic subpopulations by the continuous electromagnetic electrophoresis method and by the potential commercial value of urokinase[40].

EXPERIMENTAL

Cells and culture

Frozen suspensions or cultured monolayers of freshly prepared human embryonic kidney cells were obtained from MA Bioproducts (Rockville, MD, U.S.A.). They were cultured in one of three types of medium: Medium 199[28], a mixture of equal portions of Dulbecco's modification of Eagle's minimal essential medium and Ham's nutrient mixture F-12[29–31], or a three-component medium, consisting of a mixture of three standard media with specific additives[32]. All media contained 10% fetal bovine serum. To test cultures for urokinase production, nutrient medium was removed when cells were 95% confluent, and a high-glycine, serum-free medium[33] was added in its place. This medium was sampled at intervals of a few days and frozen for later assay on the basis of fibrin clot lysis or artificial substrate hydrolysis[32]. Urokinase activity was determined in international CTA (Committee on Thrombolytic Assay) units per ml on the basis of a standard, prepared by the World Health Organization.

Analytical electrophoresis

Two methods of analytical cell electrophoresis were used. Microscopic electrophoresis was performed with the aid of a Zeiss cytopherometer with Cam-Apparatus electrodes and off-line computerized analysis of the electroosmotic flow parabola, which produced mean EPM, standard deviations, and EPM histograms, as previously described[11,13]. In principle, cells are timed with a manually operated stopwatch as they migrate between two markers, as shown in Fig. 1a. Laser light-scattering electrophoresis was performed with a Pen Kem 3000 Automated Electrokinetic Analyzer[34]. This fully automated device analyses the frequency of modulation of light scattered through a rotating grating from moving cells in comparison with the modulation frequency of light from a stationary source, as shown schematically in Fig. 1b. The resulting power spectrum, produced by a fast Fourier transform, is used to calculate an electrophoretic mobility histogram from corresponding absolute velocities and the automatically measured electric field.

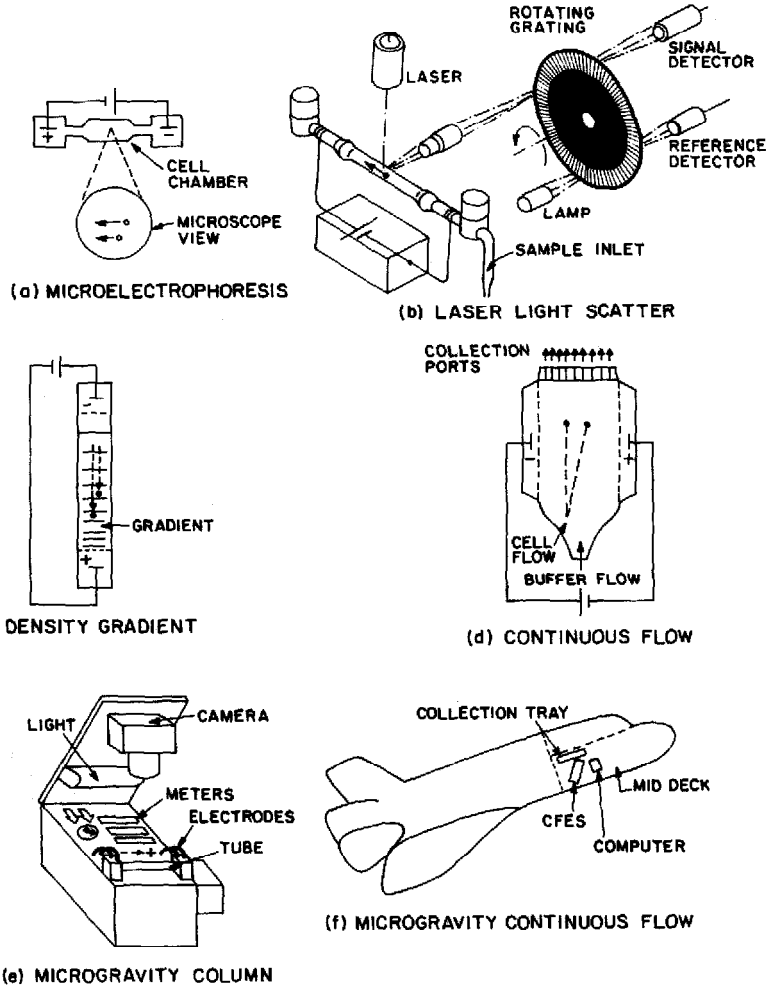

Fig. 1. Six methods of cell electrophoresis used in this study. For details see text.

Preparative electrophoresis

Four methods of preparative electrophoresis were used in the search for electrophoretic subpopulations of cultured human embryonic kidney cells that produce urokinase:

(1) In density-gradient electrophoresis[35], cells migrated upward in an electric field applied vertically through a gradient of Ficoll, balanced osmotically by sucrose. This is sketched in Fig. 1c, where it is also shown that downward migration can be used successfully.

(2) In continuous-flow electrophoresis (Fig. 1d), carrier buffer was pumped upward through a flat chamber, 1.5 mm thick, 6.0 cm wide, and 110 cm long, at the top of which buffer and sample exited through 197 outlets, each leading to a fraction-collecting vessel[12,32]. In the normal configuration, sample was admitted at the lower left of the chamber, and electrophoretic migration was from left to right, as in the figure.

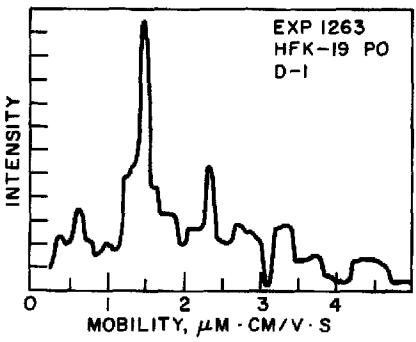

Fig. 2. Electrophoretic mobility histogram of human embryonic kidney cells of strain HFK-19 after resuspension by trypsin treatment of the first monolayer formed in culture (passing zero) in D-1 buffer, 0.015 *M*. At least three broadly distributed subpopulations are present. The laser light-scattering method was used.

(3) Static-column electrophoresis in microgravity was performed on the Apollo–Soyuz test project[36], with a semi-automated device, similar to that sketched in Fig. 1e. Cells were frozen in glycerol-containing buffer A-1 (see Table I) in a disk that was inserted at the origin of the electrophoresis column. Columns were interchanged through quick connections to the gas-recycling electrodes, and the current was switched on after the frozen disk of cells in buffer had thawed. At the end of electrophoresis, the columns were frozen in place, removed from the unit in their columns, stored in liquid nitrogen, and returned to Earth, where they were carefully extruded while still frozen and cut into sections for thawing and further analysis and for culturing, as previously described in detail[36].

(4) Microgravity continuous-flow electrophoretic separations of kidney cells were performed on Space Shuttle flight STS-8 (Fig. 1f). The same procedure was used as in continuous flow electrophoresis on the ground, but, owing to microgravity advantages, the chamber could be made bigger: 3.0 mm thick, 16 cm wide, and 110 cm long. Of the 197 fraction outlets at the "top" of the device, only 50 were used, and each collected 10 ml of effluent into 3 ml of calf serum. In the course of an experiment, cells were subjected to one day in suspension in microgravity at 5–9°C, 12 min in buffer in the electric field, and five days in buffer plus serum at 5–9°C before being returned to the culture medium[32].

Buffers

Compositions and characteristics of each buffer used with each of the methods are listed in Table I. All buffers could be used in the two analytical electrophoresis methods, while the preparative methods required low-conductivity buffers to avoid convective mixing due to Joule heating.

RESULTS AND DISCUSSION

Electrophoretic heterogeneity

Early-passage cultured human embryonic kidney cells are electrophoretically heterogeneous. The EPM distribution in Fig. 2, obtained by using the Pen Kem 3000

TABLE I

COMPOSITIONS OF BUFFERS USED IN ELECTROPHORESIS OF CELLS PREPARED FROM MONOLAYER CULTURES

Buffer name	*Concentrations (mM)*								*Ionic strength*	*K (mmho/cm)*
	$MgCl_2$	*NaCl*	*KCl*	*KH_2PO_4*	*Na_2HPO_4*	*Glucose*	*Sucrose*	*$NaHCO_3$*		
Standard saline	0	145.0	0	0	0	0	0	0.30	145	12.5
"0.03"	0.48	0	2.65	1.47	8.10	5.55	199	0	30	1.5
D-1*	0	6.42	0	0.367	1.76	222	0	0	15	0.9
A-1*	0	6.42	0	0.367	1.76	222	0	0	15	

* A-1 contains 5% glycerol; D-1 contains 5% dimethyl sulfoxide; both contain 0.336 m*M* EDTA.

TABLE II

ELECTROPHORETIC MOBILITIES OF HUMAN EMBRYONIC KIDNEY CELL STRAINS AT VARIOUS PASSAGES AND IN VARIOUS BUFFERS

The C.V. value is, in all cases, indicative of electrophoretic heterogeneity.

Experiment	*Cells*	*Passage number*	*Ionic strength (M)*	*EPM*	*S.D.*	*C.V. (%)*	*Conditions*
1429	8514	3	0.015	1.47	0.25	17	
1433	8514	5	0.015	1.39	0.27	19	EDTA
1433	8514	5	0.015	1.23	0.21	17	EDTA and trypsin
1520	8514	1	0.0015	1.47	0.27	18	Not stored
1520	8514	1	0.0015	1.28	0.26	20	Stored
1557	HFK40	1	0.015	1.62	0.20	12	
1559	HFK40	2	0.015	1.48	0.28	19	
1561	HFK40	3	0.015	1.38	0.22	16	
1563	HFK40	2	0.015	1.46	0.17	12	Aged
11/79	HFK5		0.030	1.43	0.26	18	

instrument, of cells at the first passage in culture is broad and shows several peaks. Consistent with this breadth is a coefficient of variation (C.V.) of over 10%, the maximum typically found in homogeneous cell suspensions. The magnitude of typical C.V. values of early-passage cells can be appreciated by reading Table II.

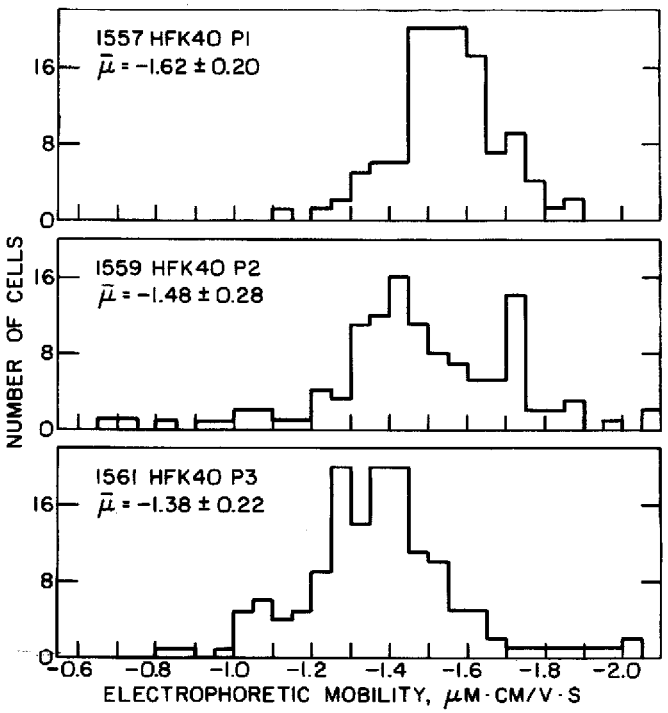

Fig. 3. Electrophoretic mobility histograms of cell strain HFK-40 in the first three passages in culture. Electrophoretic heterogeneity persists, although not all subpopulations of cells remain at a constant level. The microscopic method of electrophoresis was used.

Consistency of heterogeneity

Table II also reveals that, from condition to condition, the C.V. does not vary greatly, and as cells progress from generation to generation in culture, at least through the first five generations, electrophoretic heterogeneity is retained. This is seen in Fig. 3, which consists of EPM distributions of one cell strain (HFK-40) over its first three passages in culture.

Effect of cell dispersal method

It is normally necessary to use trypsin and EDTA mixtures to remove cells from their plastic culture vessels. With appropriate care, EDTA can be used without trypsin, thereby making it possible to evaluate the effect of proteolysis on the cell surface. The EPM distributions of suspensions of cells, prepared from identical cultures with and without trypsin, are compared in Fig. 4. Trypsin was found to raise or lower slightly the EPM of all populations tested, but electrophoretic heterogeneity was always retained.

Effect of buffer

As Table I shows a wide range of ionic strengths was used, partly to avoid the

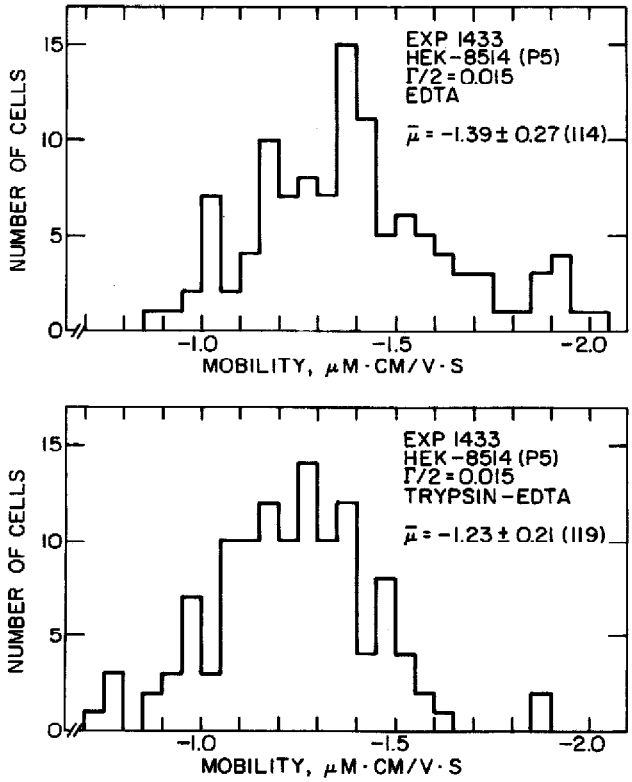

Fig. 4. Effect of trypsin on EPM of cultured human embryonic kidney cells, strain HEK-8514. Trypsin treatment resulted in a slight reduction in both EPM and electrophoretic heterogeneity. The microscopic method of electrophoresis was used.

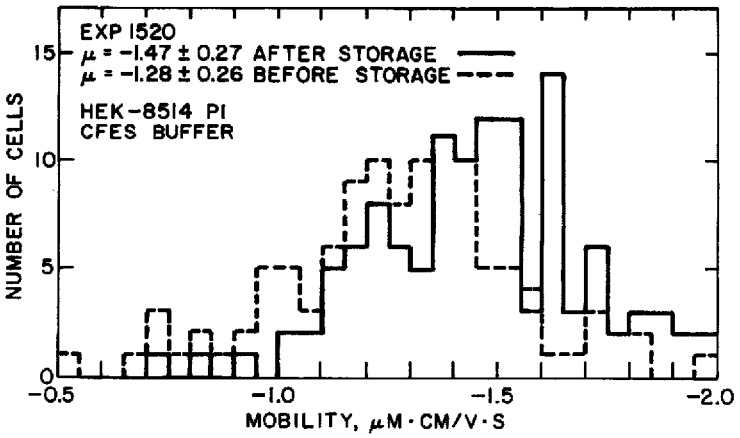

Fig. 5. Effect of storage for 1 day in low ionic strength (0.0015 *M*) buffer on EPM of cultured human embryonic kidney cells, strain HEK-8514. Storage resulted in a slight increase in EPM but no change in electrophoretic heterogeneity. The microscopic method of electrophoresis was used.

application of high currents in preparative separations. Table II shows the EPM and C.V. values determined in three different buffers. The dependence of the absolute EPM on ionic strength is not as strong as originally expected, and there appears to be no relationship between C.V. and ionic strength. For example, the EPM distribution of cell strain HEK-8514 in D-1 buffer (0.015 *M*) in the lower panel of Fig. 4 may be compared with that found in continuous flow electrophoretic separator (CFES) buffer (0.0015 *M*) and presented as the solid histogram in Fig. 5. It is concluded that electrophoretic heterogeneity is retained over a wide range of ionic strengths and that long exposures to these unusual buffers do not result in large changes in either EPM or C.V., as shown in Fig. 5.

Role of cell size

Isolated electrophoretic fractions, collected after density-gradient electrophoresis, were analyzed individually with a Coulter Electronics TA II particle-size distribution analyzer[37]. No consistent relationship between size and electrophoretic fraction was found, as indicated in Fig. 6. This finding implies that, despite the fact that gravity and electrokinetic vectors are antiparallel in density-gradient electrophoresis, size does not play a dominant role in determining the electrophoretic migration of cultured human embryonic kidney cells.

Role of the cell cycle

Unless other sources of heterogeneity exist, the cell volume can vary by as much as a factor of 2 on the basis of progression through the intermitotic cell cycle alone[37]. Fractions were collected after separation of cells by density-gradient electrophoresis. Then, cell suspensions were fixed and stained by the acriflavine Feulgen procedure, which results in cell fluorescence in direct proportion to DNA content[38]. The resulting DNA distributions, shown in Fig. 7, reveal no trends relating G1 and G2 peak heights, for example, to electrophoretic fraction. This finding implies that any changes in cell size or surface charge density that may be related to cell position

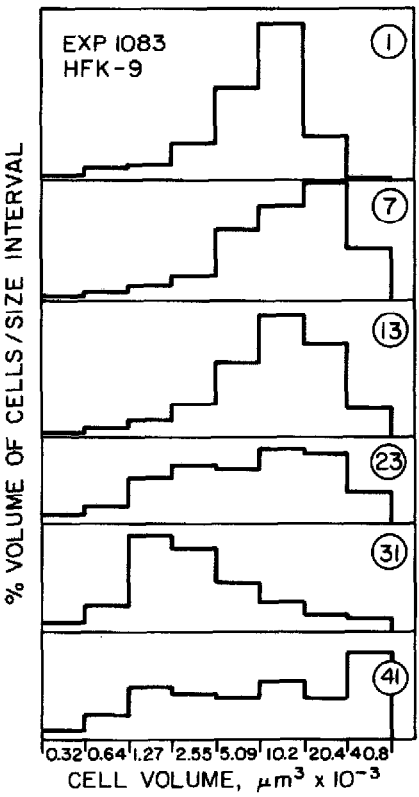

Fig. 6. Volume distributions of cells in electrophoretic fractions, separated by density-gradient electrophoresis. Cell volume was measured with a calibrated TA II volume distribution analyser (Coulter Electronics). Increasing fraction numbers (in circles) correspond to decreasing EPM. There was no systematic relationship between cell volume and EPM in human embryonic kidney cell strain HFK-9.

play very little role in determining the electrophoretic migration of these particular cells, although this may not be the case for other cell types[39].

Relationship between EPM and cell function

Subsequent to the original demonstration of electrokinetic heterogeneity[40], one of the earliest lines of evidence that urokinase-producing kidney cells might have a specific EPM appeared in the results of experiment MA-011 on the Apollo–Soyuz test project. The data of Allen and co-workers[36,41] are presented in Fig. 8, in which cell count per fraction sliced from the electrophoresis column is plotted against distance migrated. The vertical solid line marks the electrophoretic fraction from which cells with a nearly 5-fold increase in urokinase production were grown. The arrow indicates one additional fraction with higher than average urokinase production. The vertical, dashed lines mark the expected limits of cell migration in the electrophoresis column. Although it was not possible to grow and test an adequate number of cells from all fractions collected, the data are consistent with the existence of at least one electrophoretic subpopulation enriched with urokinase-producing cells.

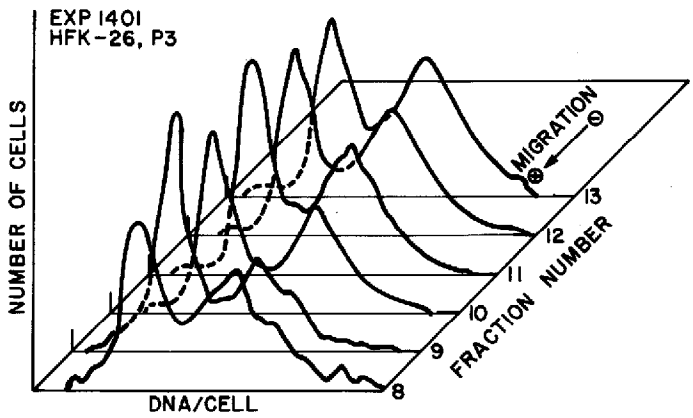

Fig. 7. DNA distributions of cells in electrophoretic fractions separated by density-gradient electrophoresis. DNA per cell was determined by acriflavine staining of formalin-fixed cells and measuring the fluorescence intensity of individual cells with a Cytofluorograf 4800A flow cytometer (Bio/Physics Systems). Increasing fraction numbers (on *z*-axis) correspond to decreasing EPM. There was no systematic relationship between DNA distribution (and hence cell cycle position) and EPM in human embryonic kidney cell strain HFK-26.

The subsequent application of three preparative electrophoresis methods revealed that cell subpopulations enriched with urokinase-producing cells exist and that they have the same approximate EPM as suggested by the data of Fig. 9. In Fig. 9 are presented electrophoretic distributions (cell count *vs.* fraction number) from experiments involving three cell strains and three methods. In all cases, the solid vertical line marks the fraction from which cells that produced the largest amount of urokinase were grown. Arrows indicate fractions that produced cultures with above-average urokinase production. The upper two panels describe experiments with strains HEK-17 and HEK-18 separated by means of density-gradient electro-

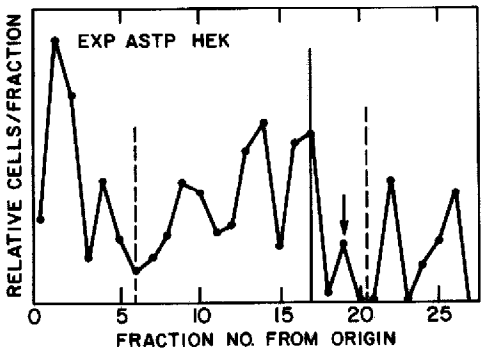

Fig. 8. Electrophoretic distribution of human embryonic kidney cells, separated by free-zone electrophoresis in a static column on Apollo–Soyuz test project. Data are from ref. 35, re-drawn to show (between vertical, dashed lines) electrophoretic heterogeneity and (vertical, solid line) the fraction of cells that produced the greatest amount of urokinase upon subcultivation. Arrow indicates one additional fraction that produced cells with above-average urokinase production. The occurrence of cells near the origin is unexplained[35,41].

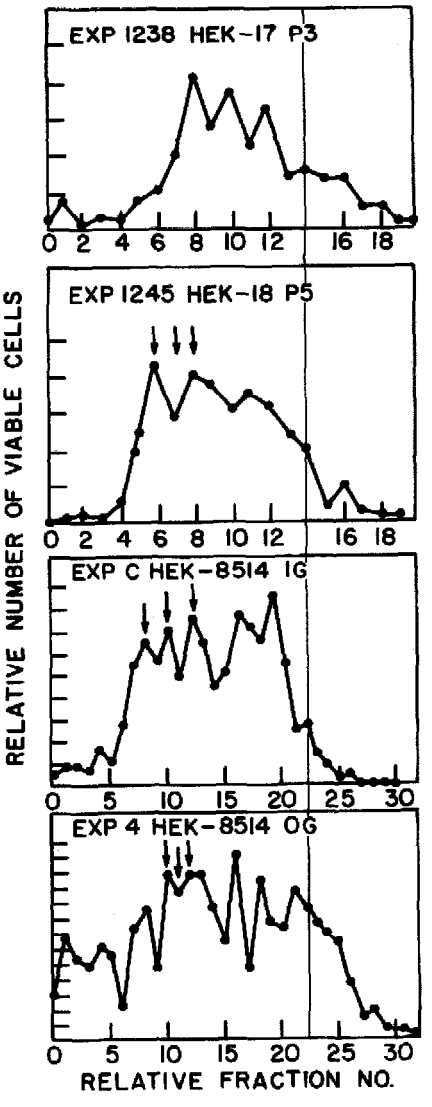

Fig. 9. Electrophoretic profiles of three cell strains, separated by three different methods of preparative cell electrophoresis for comparison. In each case, the relative number of viable cells is plotted against electrophoretic fraction, renumbered, and scaled so that direct comparisons are possible. The cell strain is indicated on each panel. The data in the top two panels were obtained from density-gradient electrophoresis experiments, and the lower two panels are data from continuous-flow electrophoresis experiments. Experiment 4, (in the bottom panel) was performed in the microgravity environment of Space Shuttle Flight STS-8[32]. The solid, vertical line marks the fraction which, in every case, gave rise to cells with above-average urokinase production.

phoresis, while the lower two panels describe experiments in which continuous-flow electrophoresis was used to separate cell strain HEK-8514. Additional detailed results of these experiments have been presented previously[32]. Migration in all cases was from left to right on the graphs. Fraction 0 indicates the shortest migration distance

at which cell counts were plotted, not the zero-field migration distance. Data from the two types of experiments have been plotted on comparable fraction scales.

In all cases, the largest amount of plasminogen activator production occurred in cultures from high (but not the highest)-mobility fractions. A broadly distributed low-mobility subpopulation of cells that gives rise to cultures with above-average plasminogen activator production appears to exist in nearly all separations (shown by arrows in Fig. 9). This subpopulation could be rich in cells that produce "tissue plasminogen activator". Evidence favoring this interpretation will be published elsewhere[12].

CONCLUSION

A rationale has been developed for the use of free-fluid electrophoresis methods for the separation of tissue cells with specific function. Experimental data indicate that cells suspended by trypsinization from cultures of human embryonic kidney are electrophoretically heterogeneous, tolerate a wide range of electrophoresis buffers and conditions without significant attenuation of function, do not separate electrophoretically on the basis of size or cell cycle position alone, and can be separated according to their ability to give rise to progeny that produce specific plasminogen activators.

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