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KIDNEY CELL ELECTROPHORESIS IN SPACE FLIGHT: RATIONALE, METHODS, RESULTS AND FLOW CYTOMETRY APPLICATIONS

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SUMMARY INTRODUCTION

The human kidney performs an incredible number of different functions. There are individual cells responsible for the production of renin, erythropoietin, enzymes of vitamin D metabolism, plasminogen activators of at least 3 types, granulocyte conditioning factor, the maintenance of electrolyte balance, and the clearance of soluble wastes from the blood, to mention a few. The sharing of these functions among cells will take decades to fully charactarize, and many of these functions, if they could be isolated, are also of commercial importance to biotechnology. For example, the kidney is perhaps the second most prolific tissue with respect to plasminogen activator production, while plasminogen activator is one of today's "hottest" biotechnology future market items (Kadouri and Bohak, 1985). The purpose of the research project was to develop the most effective means of purifying subpopulations of cultured human embryonic kidney (HEK) cells and produce living subpopulations for study. Originally primarily urokinase, a plasminogen activator found in urine, was the product goal. Since primary cultures of differentiating epithelioid cells are not immortal, adequate numbers of cells could not be purified by cloning or cell sorting. Historically, Barlow and Kolin showed some three major subpopulations by "continuous belt" electrophoresis in a magnetic field (Barlow et al., 1977). This finding was not pursued at the time of its discovery, but it was confirmed as an early goal in this project, and the original premise that electrophoretic subfractions would separate according to function was also confirmed. A series of ground-based electrophoretic and enzymologic studies led to an experiment on the Apollo-Soyuz mission in which evidence for urokinase-rich cell subpopulations was found. The first objective of the present project was to reproduce this result with adequate statistical data and superior technique but with the same apparatus. The "EEVT" experient on STS-3 included two columns of fixed erythrocytes, which were photographed in flight. The remaining 6 tubes were dedicated to living kidney cells that had to be frozen for several weeks before and immediately after electrophoresis in space. Liquid nitrogen loss in a ground refrigeration unit destroyed all samples from this flight, and only photographic data from the fixed erythrocytes was usable (Snyder et al., 1985; Morrison and Lewis, 1983; Sarnoff, et al., 1983). Owing to the development of a Joint Endeavor Agreement with McDonnell Douglas Astronautics Co. NASA made time available on the Continuous Flow Electrophoresis System (CFES) aboard the space shuttle orbiters. Without the necessity of freezing cells and without worrying about the influence of particle sedimentation or zone convection it became possible and logical to separate cells by CFES in microgravity. This undertaking was suggested by the clear finding of a gravity-dependent component of pituitary cell separation in free fluid electrophoresis methods (Plank et al, 1983). Prior to the Challenger tragedy it was only possible to separate two samples of kidney cells, both on STS-8. Extensive reporting has been accomplished on these cells, but there are still numerous analytical results to be completed and published. The following text (1) introduces the subject, (2) describes the space-based electrophoresis apparatus and its testing, (3) reviews results of kidney cell electrophoresis, and (4) presents results in the application of flow cytometry to kidney cell electrophoresis in low gravity.

WHY STUDY CELL ELECTROPHORESIS IN SPACE?

Any cell type with limited or no proliferative capabilities that is needed in pure form must be separated by a technique that provides adequate purity, adequate yield, adequate relationship to cell type, and adequate function after separation. Cell electrophoresis, high gradient magnetic filtration, two-phase partitioning, and affinity chromatography have been identified as such processes. All are in their infancy, and some processes may not yet have been discovered. The reasons for purifying suspended animal and plant cells are numerous, and the activity itself is amply justified by the demands for pure cell populations as objects of chemical research, living material for transplantation, and sources of uncontaminated bioproducts. These reasons include the anticipated needs of biochemists and biotechnologists in the 1990's.

1. Stokes heterogeneity of cells with similar charge and function

Cell parameters related to Stokes sedimentation, namely density and radius, are distributed values in any living cell population.These may or may not be related to cell function. For example, neutrophils and somatotrophs are extraordinarily dense owing to their densely packed granules of secretory proteins. Sedimentation helps separate such cells from others with which they are naturally mixed. Heterogeneity of sedimentation in a pure subpopulation interferes with purity and yield in most purification processes. Figure 1 is an indication of the heterogeneity of a population of human embryonic kidney cells with respect to diameter. Terminal sedimentation velocity increases with the square of the radius, so there could be an 8-fold difference in the sedimentation velocites of the most and least rapidly sedimenting cells. In populations with less size heretogeneity it has been shown that density differences also affect vertical electrophoretic migration (Plank et et al., 1983) and probably horizontal electrophoretic migration in continuous flow electrophoresis (Todd, 1985), as indicated schematically in Fig. 2.

2. Ambiguity of electrophoretic migration

Given the above, in continuous flow electrophoresis cell type X could sediment into the same electrophoretic pathway as cell type Y, or vice versa, as shown in Fig. 2. Thus, two electrophoretic subpopulations would be collected as one, or an electrophoretically homogeneous population could be detected as widely distributed.

3. Discovery of new cell types

Extensive electrophoretic studies on pituitary cells (Plank et al., 1983; Hymer et al., 1987) and kidney cells (Todd et al., 1986) demonstrate that different cells in a tissue perform different secretory, synthetic, and metabolic functions despite morphological similarity. While these findings may seem amusing to histochemists, who routinely make similar discoveries, histochemistry will not reveal the subtle differences that have been discovered by cell purification, such as immunologically similar molecules with different biological function produced by separable, morphologically similar, cells (Hymer et al., 1987). Histochemistry cannot measure the molecular weights or determine gene sequences in such cells; cell purification has made it possible to do these. Owing to electrophoretic purification, we now know, for example, that two pituitary cell types make two types of growth hormone and that two electrophoretic subpopulations of kidney cells make urokinase molecules having different molecular weights.

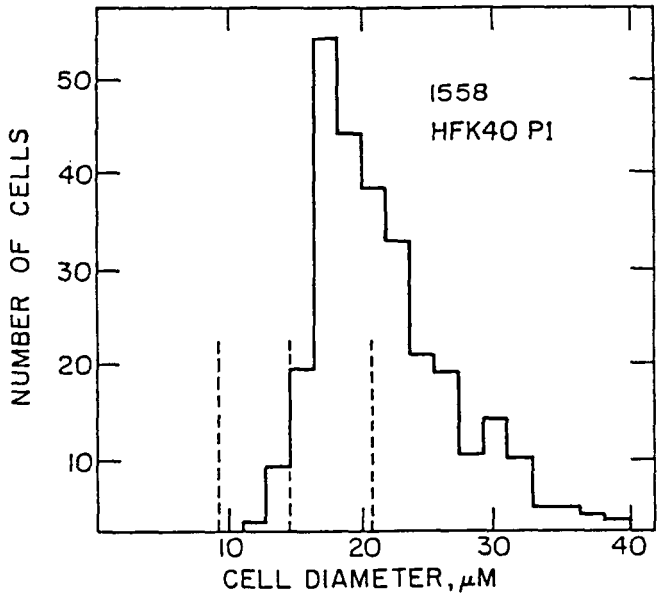

Figure 1. Histogram of microscopically determined sizes of passage-1 HEK cells. The three dashed lines indicate the calibration points based on the diameters of 9.5 μm, 14.5 μm, and 20.5 μm spheres also determined microscopically.

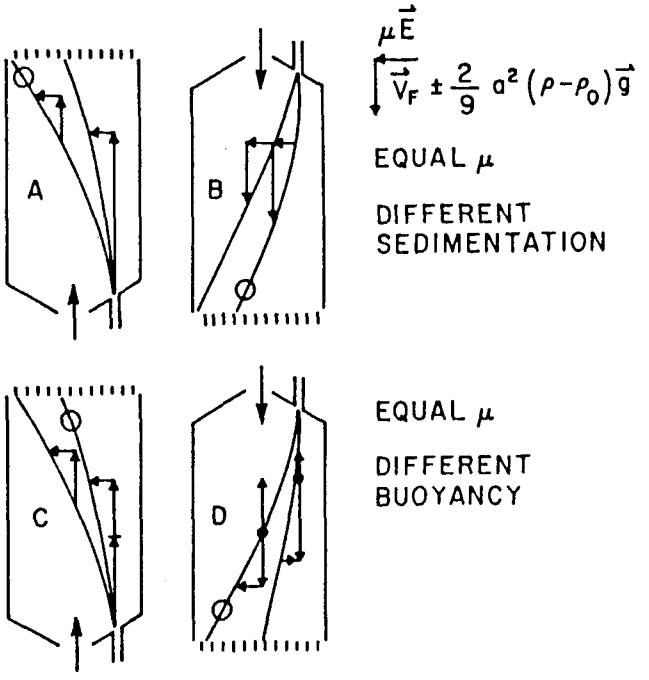

Figure 2. Effects of particle sedimentation on continuous flow electrophoresis of cells. In A and B the circles show the path of more rapidly sedimenting cells. In C and D the circles show the path of more buoyant cells. All cells are considered to have the same electrophoretic mobility.

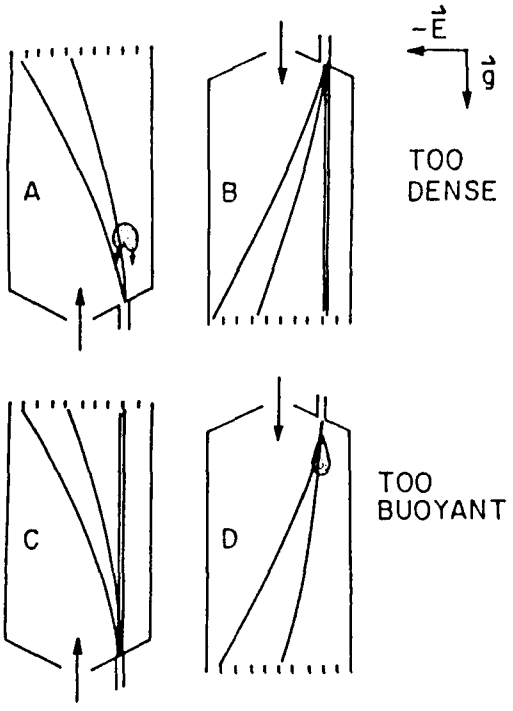

Figure 3. Sketch of the consequences of sample zones that are too dense or too buoyant in continuous flow electrophoresis. Sample flow is stopped or too fast, depending on relative densities of sample and carrier buffers.

4. Rare cell types

A popular activity in flow cytometry is the detection of rare cell types--one in a million or so. Detection alone does not produce workable amounts of material. To perform any analysis or post-separation study requires adequate cell numbers. Consider that a biochemical measurement requires 1 million cells -- a typical lower limit. Obviously the starting population must be 10^{12} cells. Passing these through a cell sorter at current maximum rates of 10,000/sec (depending on the complexity of sort logic) would yield the desired number of cells in 100 million seconds (or 3 years). A technique for accomplishing the same thing in a day would be useful, but ground based electrophoresis is not equal to this task owing to limited capacity and limited resolution. The erythropoietin gene is only an early example of a problem of this magnitude; its original isolation required a few years by other methods.

5. Cells for transplantation

The treatment of congenital or chronic diseases by a single injection or implantation of cells has been a goal of several disease-specific research programs and projects. Many believe that cell therapy is safer than gene therapy. It is necessary to devise methods that prevent the transplantation of unwanted cells, such as graft-vs-host cells in bone marrow, alpha cells in pancreatic islets, or mammotrophs in pituitary. These specific examples have been addressed in ground-based CFES, which has produced adequate quantities of the appropriate cell types for research (Hymer et al., 1987). Whether or not ground based electrophoresis is equal to the same task for medical transplantation needs to be determined.

6. The capacity problem

The previous two items point to the need for high capacity separation accompanied by adequate resolution. Cells in suspension have a very low diffusion coefficient, so they do not diffuse out of the zone in which they are injected into a free-fluid separator. Rapidly diffusing substances flow into the zone, and the zone sediments rapidly if the cell concentration is above a critical value (Mason, 1976). The effect of this phenomenon on CFES is described in Fig. 3. For most nucleated cells the critical concentration is below 10 million cells per ml (Boltz and Todd, 1978). It has been mentioned that for certain research purposes the capacity of ground-based CFES is adequate because the availability of cells is limiting (Hymer et al., 1987). However,

there will be cases in which it is not. Biochemical assays on single fractions without reculturing, in vivo implantation of single fractions, and mechanistic studies on isolated rare cell populations will require the separation, within a few hours, of larger pure cell populations have been heretofore available.

Thus, the ability to process more than 30 million cells per hour (current practice) will eventually be needed. In microgravity zone sedimentation is absent, and such capacity is feasible.

7. The resolution problem

In CFES samples are injected in a fine stream into a flowing buffer stream. The small distance between the walls causes laminar flow in a steep parabolic profile from front wall to back wall. Cells near the walls move more slowly and spend more time in the field and hence are swept anodally a greater distance than are their counterparts at the center of the stream. The ideal sample stream is one whose diameter is negligible compared to the chamber thickness; hence a thick chamber is desirable, but chamber thickness is fixed by the need to reject heat uniformly to the cooling jacket at the walls. The quantitative nature of this problem can be modeled by computer and studied in low gravity where buoyancy driven convective flow is absent and thicker chambers can be used. Stable operating ranges of CFES in microgravity have been explored by computer simulations, and 4 g/hr processing rate can be achieved in a 10.0 mm thick chamber (McCreight, 1977). A typical commercial chamber is 0.3 - 0.5 mm thick. The McDonnell Douglas Astronautics Co. chamber is 1.5 mm thick, and the space flight CFES has a 3.0 mm thick chamber. The study of thicker chambers is desirable, but the thermal stability of the sample also must be considered. Cells could withstand 10 min. at 41^{o}C, for example, so a chamber thickness that maintains this temperature at useful currents could be studied in space. To date, modifications of sample stream diameter have been studied in space and on earth, and a 4-fold enhancement of resolution has been possible within the working constraints of the studies.

8. Current carrying capacity of the separands

Highly concentrated cells in very low conductivity fluid will themselves carry a substantial amount of current, thereby increasing the current passed through the sample zone relative to the carrier buffer if the buffer is of very low conductivity. In the case of cells, the influence of this phenomenon on resolution is not known, because adequate

concentrations must be achieved to study it. These concentrations can be achieved in low gravity space experiments.

The above several types of experiments and processes require heroic measures or long projects to perform. In most of these, low gravity offers a short-cut to early answers or unavailable products. Nevertheless, low gravity research exellence in this limited field depends on maintaining a relevent research capability that supports the progress of comparable ground-based technologies.

METHODS AND EVALUATION OF CONTINUOUS FLOW CELL ELECTROPHORESIS IN LOW GRAVITY

The paragraphs that follow are adapted from the major paper by Hymer et al., (1987). They describe, as concisely as possible, the physical methods used on Space Shuttle flights and the results of specific tests, including kidney cell separation experiments using the McDonnell Douglas CFES.

1. The McDonnell Douglas Continous Flow Electrophoresis Systems

In continuous flow electrophoresis (CFE), cells or molecules are separated from one another on the basis of their surface charge density. The instrument used to conduct the cell separations differs from other designs (Hannig et al., 1975) in thickness and width of the separation chamber, and in the method of cooling. It consists of a rectangular separation chamber sandwiched between two electrode chambers which are also cooling jackets (Snyder et al., 1985 Plank et al., 1983; Hymer et al., 1987; Rose and Richman, 1979) and are separated from the chamber at its edges by semipermeable membranes (Fig. 4A,C). A stable electric field is maintained by ion flow through the membranes. Carrier buffer (low conductivity triethanolamine-potassium acetate buffer, pH 7.25, 300 mOsm per liter for cells or barbital buffer for proteins) flows upward through the separation chamber at a rate of 20 ml/min. The electrode and separation chambers are differentially pumped. Computerized flow balancing tends to eliminate transverse flow and pressure gradients in the sample chamber, enhancing cell stream stability and reproducibility of results. Dimensions of both ground and space instruments are described in Fig. 4. Cells ($\sim 10^7$/ml) previously washed and suspended in carrier buffer are injected into the bottom of the chamber using an infusion pump (4 ml/hr). Cells are exposed to the electric field for 4 minutes before they exit the top of the flow chamber through 197 ports and are collected as "fractions". The separands migrate across the

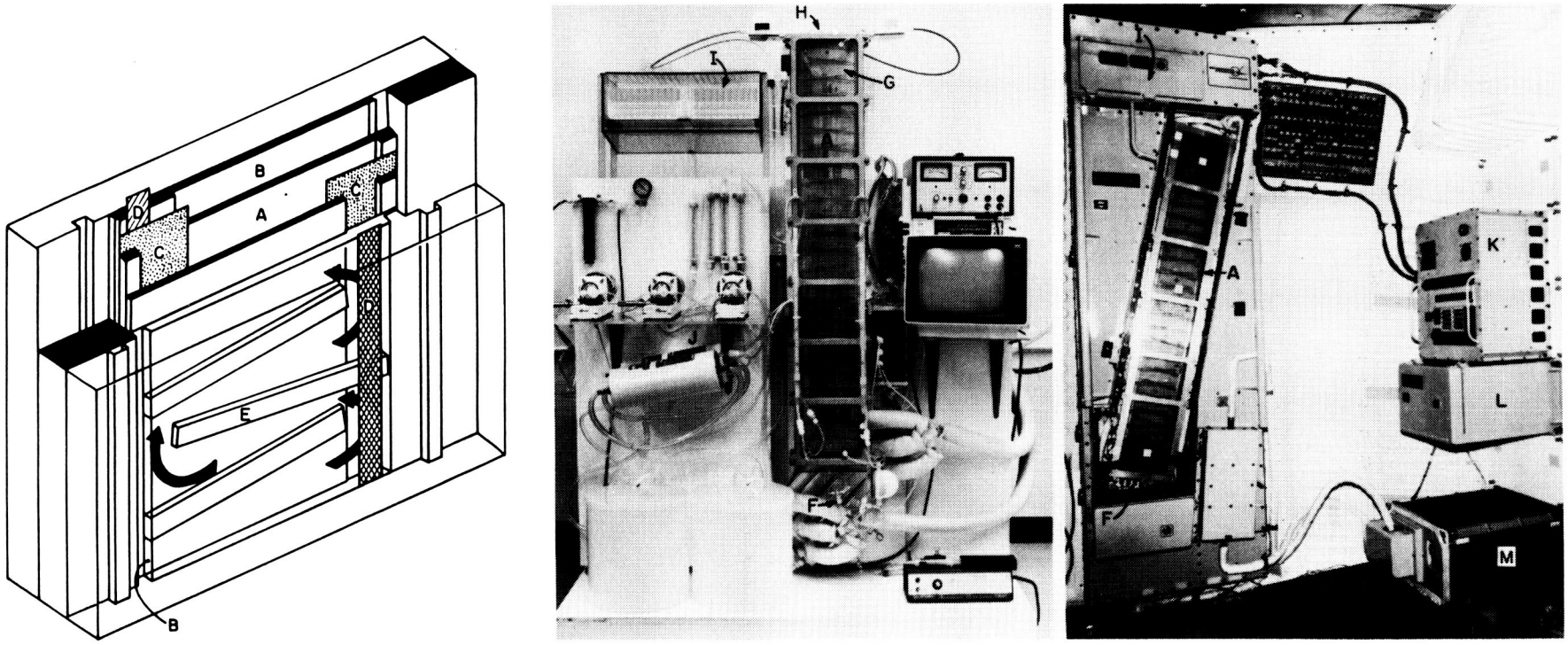

Figure 4. Continuous flow electrophoretic separators used in the laboratory and in space flight. Left: Diagram indicating how the separation chamber is surrounded by the coolant/electrode flow chambers. Center: Photograph of laboratory model. Right: Photograph of space flight unit installed on Shuttle Orbiter. The following components are labeled: (A) separation chamber, which is 0.15 cm thick and 6 cm wide in the laboratory model and 0.30 cm thick and 16 cm wide in the space flight unit and 120 cm long in both units; (B) front and back coolant/electrode chambers, through which electrode buffer (which also serves as coolant) is pumped in a serpentine path under computerized pressure control; (C) two semipermeable membranes which separate coolant/electrode chambers from sample chamber fluid while permitting free passage of ions for the maintenance of a stable electric field across the chamber; (D) a platinum ribbon electrode placed parallel to the semipermeable membrane in each of the two coolant/electrode chambers; (E) baffles which force serpentine flow of coolant/buffer fluid between the electrodes and membranes; (F) sample inlet; (G) flared region of the chamber of the laboratory model, to accommodate outlet tubes; (H) 197 sample outlets that permit fractions to exit through Tygon tubes at the top of the chamber; (I) thermostated fraction collection and storage area containing tubes (on the ground) or plastic bags (in space); (J) buffer pumping and cooling unit. In addition the space flight system consists of a separate computer module (K) for controlling separation commands, a sample storage module (L) for cooling up to six 20-ml samples and 6 trays having either fifty 15-ml fractions for cell separation or 197 fractions, 1.4 ml each, for protein collections; and a thermoelectric cooler (M).

separation chamber at different velocities depending on their net surface charge and viscous drag cross section and therefore leave the chamber through different outlet tubes. Lateral migration distance thus depends on the time, t, spent by the cells in the chamber, the applied field, E, and the cell surface charge density. The product is Et, expressed in volt-min-cm^{-1}.

The CFE unit for space flight (Fig. 4) has been used for separations of various materials, including test proteins on Shuttle flight STS-4 and STS-6, particles on STS-7, and mammalian cells on Shuttle flight STS-8. The collection system was reduced to 40 tubes to accommodate a fraction volume of 13 ml required when living cells were collected. During operation in space the astronaut first removes a sample collection tray and syringe from a thermostated cabinet and places them into the fraction collection drawer and sample injection device, respectively. The electrophoresis process is initiated by a command at the computer keyboard. Sample enters the separation chamber, and fractions exit through tygon collection tubes ending with 20-gauge needles. At collection command, each of these needles penetrates a rubber septum covering the collection trays. The astronaut hears a signal to remove the sample collection tray and syringe, and the process can be repeated several times in one flight.

In space, temperature during storage prior to separation was 4°C; during separation temperatures were 6°C at chamber inlet, 12°C at outlet tubes and 16°C during collection. After collection, samples were maintained at 4°C. Samples processed in the ground chamber were collected into tubes at 4°C at the top of the unit. Temperatures within the chamber ranged from 6 to 8°C.

2. Test Protein Separations

Droplet sedimentation and convection currents caused by uneven Joule heating distort free-fluid electrophoretic separations. Since these gravity-dependent effects would theoretically play no role in the electrophoretic separations performed in microgravity, it should be possible to process samples containing greater concentrations of protein in space. This possibility was demonstrated by comparing laboratory and space flight separations.

A sample containing 0.1% rat serum albumin and 0.1% ovalbumin was electrophoresed at Et=110. A 4 tube peak-to-peak separation, with a 5 tube overlap between the two proteins, was routinely achieved. Whenever

the protein concentration was increased, the higher sample density resulted in decreased resolution. On Shuttle flight STS-4 a sample containing 12.5% rat serum albumin and 12.5% ovalbumin (25% protein w/v) was separated to the same 4-tube peak separation that was achieved on earth with 0.2% protein solution.

The diameter of the sample stream in the space-flight chamber was twice that of the laboratory chamber (see Fig. 4), so a four-fold increase in sample volume/unit time was realized. This plus the increased sample concentration (permitted by lack of zone sedimentation) resulted in 500-fold increased "throughput" achieved by performing the separation in microgravity.

3. Test particle experiments

The ability of CFE to separate particles on the basis of charge was confirmed in experiments designed by Dr. R. S. Snyder and co-workers (1986). Mixtures of test particles having known electrophoretic mobilities and different sizes were used. Polystyrene latex spheres having diameters (in μm) of 0.56 (red), 0.30 (white) and 0.80 (blue) were synthesized by Interfacial Dynamics Co., Portland, OR (white particles), and Particle Technology, Inc., Bethlehem, PA (red and blue particles). These were separated from each other on Space Space Shuttle flight STS-7, prior to the use of living cells on STS-8, to test for separability, absence of sedimentation, particle band width, and effects of conductance discontinuities. Mobilities of the particles were 3.5± 0.2 (red), 2.4± 0.2 (white) and 1.6 ± 0.1 (blue) $\mu m\text{-}cm\text{-}V^{-1}\text{-}s^{-1}$ (Snyder et al., 1986).

KIDNEY CELL ELECTROPHORESIS

1. Historical Introduction

Barlow's experiment (Barlow et al., 1977; Allen et al., 1977) on Apollo Soyuz in 1975 using a free-fluid column electrophoresis device showed that human kidney cells could be separated into 7 or 8 subfractions which are capable of producing high levels of plasminogen activators. Many difficulties were presented by freezing cells before flight and on orbit (after separation), then harvesting the cells postflight from the frozen ice column. Cell viability was low, and the small sample size and cell recovery methods precluded extensive study of the most interesting fractions. The corresponding technique on the ground, vertical density gradient electrophoresis, separates cells with satisfactory resolution (Platsoucas, 1983; Todd et al., 1981), but the

number of cells per fraction is not adequate to allow extensive studies of the highest producing subpopulations. The availability of the continuous flow electrophoresis system (CFES) in the middeck of the Space Shuttle offered much greater throughput at adequate resolution and eliminated the need for the freeze-thaw procedures used in the static column device. Ground-based separation of human embryonic kidney (HEK) cells using the CFES began in 1981 by a NASA/ university/ industry principal investigator team. Early results showed that: 1) extensive qualification of the candidate cell lots was mandatory (Morrison and Lewis, 1983); 2) standard assays for target cell secretory function were often inadequate to distinguish among multiple subfractions (Lewis et al., 1984); and 3) HEK cells could be separated by CFES into more than 30 different fractions, all of which produced some level of plasminogen activators (Lewis et al., 1982). Prior to the first CFES separations of live cells on the Space Shuttle, research was performed to determine the effects of cell lot, harvesting and handling procedures, post-separation storage, and culture conditions on electrophoretic mobility (EPM) distribution and plasminogen activator production. Three different methods of analytical electrophoresis were used to characterize the EPM distribution of cultured HEK cells under a variety of conditions (Todd et al., 1986), and comparisons were made between density gradient electrophoresis (DGE) and CFES which confirmed the results of the first separation of HEK cells in space (Barlow et al., 1977; Allen et al., 1977). The following sections describe the most significant results of the first CFES separation of HEK cells under microgravity conditions on STS-8, the major compromises which occurred, and the subsequent research which has been done or is planned to prepare for future CFES flight experiments. Only the basic relationship between the EPM distribution and initial production of urokinase (u-PA) is discussed here. The details of the secretion of different PA's produced by the subpopulations following electrophoresis is described in the report by Barlow et al (1987) in this series.

2. Materials and Methods

Frozen suspensions of human kidney cells were obtained from MA Bioproducts (Rockville, MD, U.S.A.) and cultured in a serum supplemented medium according to previously published methods (Lewis et al., 1984). More than 35 cell lots were screened for viability, growth, karyotype, PA production, and morphology (Morrison and Lewis, 1983). For plasminogen production cells were plated into complete growth medium, allowed to multiply to confluence and changed to a serum-free PA production medium (UKPM) developed by Barlow et al., (1977a). The medium was harvested at

4 or 6 day intervals for 28 days, and the conditioned medium samples were frozen and analysed later for PA secretion rates. PA activity was determined by fibrin clot lysis, artificial substrate hydrolysis, or Enzyme Linked Immunosorbent (ELISA) assays according to methods previously published (Morrison and Lewis, 1983; Lewis et al., 1984; Morrison et al., 1984, 1984a). A detailed description of the culture and assay methods is found in the accompanying paper by Barlow et al (1987). For the Shuttle control and flight experiments the HEK cells (lot #8514) were grown on flasks, trypsinized and recultured on microcarrier beads. At 46 hours before separation (20 hours before STS-8 launch) the cells were trypsinized, harvested and suspended in a low ionic strength triethanolamine buffer containing 10% dialysed horse serum and antibiotics. Cells were stored in this buffer at 4^{0}C until injection into the CFES, at which time they were mechanically redispersed and inserted as described in detail elsewhere (Morrison et al., 1984). Separations on STS-8 were performed at a field strength of 26 V/cm and a sample residence time of 12 minutes. Cells were injected at two different concentrations: 2.6×10^{6} cells/ml and 8.0×10^{6} cells/ml, whereas ground control experiments using a similar value of Et were conducted at concentrations of 2.5 and 7.2×10^{6} cells/ml. Each of 50 separated cell fractions was collected in 13 ml bags containing serum and stored at 4^{0}C until return to Earth. Postflight the cells were recovered from the CFES collection tray, grown to 95% confluence in growth medium, then maintained on serum-free UKPM for up to 28 days. Supernatant medium was removed at 4-day intervals and frozen for PA analysis.

3. Results of STS-8 experiment and related research

The results confirmed that HEK cells are suitable test cells for microgravity separations. They are heterogeneous in EPM and function, predictable in growth and maintenance culture, stable in karyotype, and hardy enough to withstand the combined rigors of pre- and post-flight handling, the electrophoresis processing, and extended storage in the CFES buffer.

By using three methods of cell electrophoresis, the EPM distributions of early passage cultures of human embryonic kidney cells were determined in a variety of low ionic strength buffers of different chemical composition (Morrison and Lewis, 1983; Todd et al., 1986). HEK cells were found to be quite heterogeneous, having a broad range of EPM's and often distributions with multiple peaks. Electrophoretic heterogeneity, as measured by the coefficient of variation, was retained throughout several subcultures and in a variety

of buffers with various ionic strengths. Trypsin and EDTA, normally used to remove cells from the culture surface, had only a minor effect on the EPM distribution. In the CFES buffer (triethanolamine, ionic strength=0.0015 M) the mean EPM was found to be -1.47 $\pm$ 0.27 um-cm/V-sec; however, after storage the mobility decreased to -1.28 $\pm$ 0.26 um-cm/V-sec. Thus storage in these low ionic strength buffers does not cause major changes in EPM or its CV. The role of cell cycle and size was also studied using density gradient electrophoresis (DGE), and no systematic relationship between either of these parameters and EPM was found (Todd et al., 1986).

Cell separation experiments using DGE (Todd et al., 1981 , 1986) demonstrated that HEK cells could be separated into some 20 fractions with several major groups of cells producing high levels of u-PA. The highest secreting cells were found to have a characteristic EPM that was approximately 15 percent lower than that of the most mobile cells. In comparison, CFES separations showed a bandspread of 35 electrophoretic fractions, containing five or six major subpopulations, which produced high levels of u-PA (Morrison and Lewis, 1983; Lewis et al., 1982). In these studies the highest producing subpopulation typically had an EPM approximately 30 percent less than the that of the most mobile cells. The relationship of these combined findings to those of microgravity experiments is detailed below (see Figure 8).

Replicated quantitative assays of the slowest and fastest electrophoretic fractions are seldom feasible, owing to limited cell numbers per fraction. Attempts to increase sample input concentration greater than 2.5×10^6 cells/ml in CFES ground-based experiments resulted in a reduction in the mean migration distance and in the bandspread of the distribution. This could be due to cell-to-cell interactions which have been shown to affect the EPM of cultured mammalian cells, but not erythrocytes (Todd and Hjertén, 1985).

In cell separations in microgravity, separated HEK cells were collected in 45 fractions from CFES experiments on STS-8, however, low cell numbers and viability in the most mobile cells resulted in only 36 cultured subpopulations. Figures 5a and 5b show the electrophoretic distributions of cells separated in the flight and control experiments. The mean migration distance of cells in the flight experiment was approximately 30 percent greater than that of the ground controls (see Figure 5a). The breadth and heterogeneity of the EPM distribution in the space experiments was also greater.

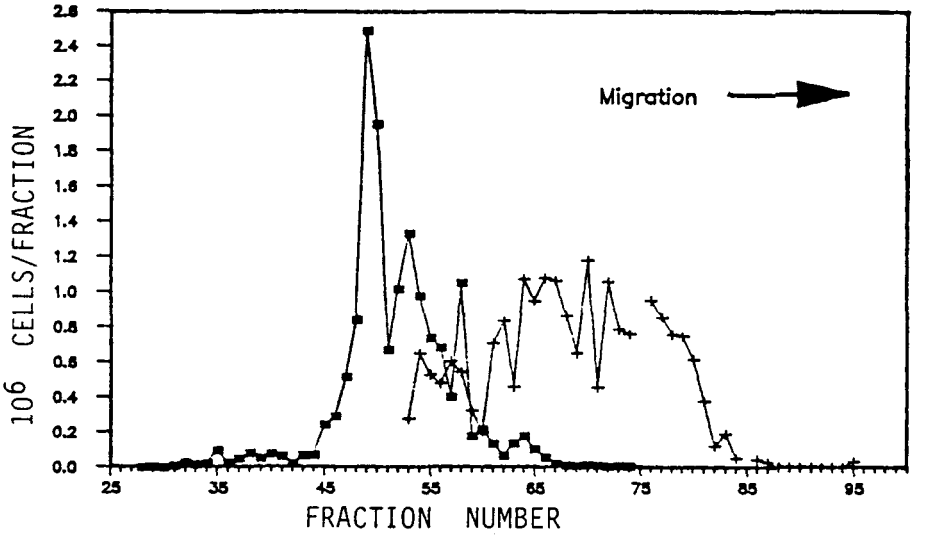

Figure 5a. Electrophoretic distribution of HEK cells separated by CFES in 1-g (squares) and micro-g (+). In micro-g the cells migrated farther, although the separation potential was similar. The mean migration in both flight experiments was 14 fractions closer to the anode than that of the ground controls. Input sample concentration was 7.2 x 10^6 cells/ml for ground control Run 5c and 8.0 x 10^6 cells/ml for Run 4, in flight Migration is shown from left to right and fraction number is relative to sample inlet tube.

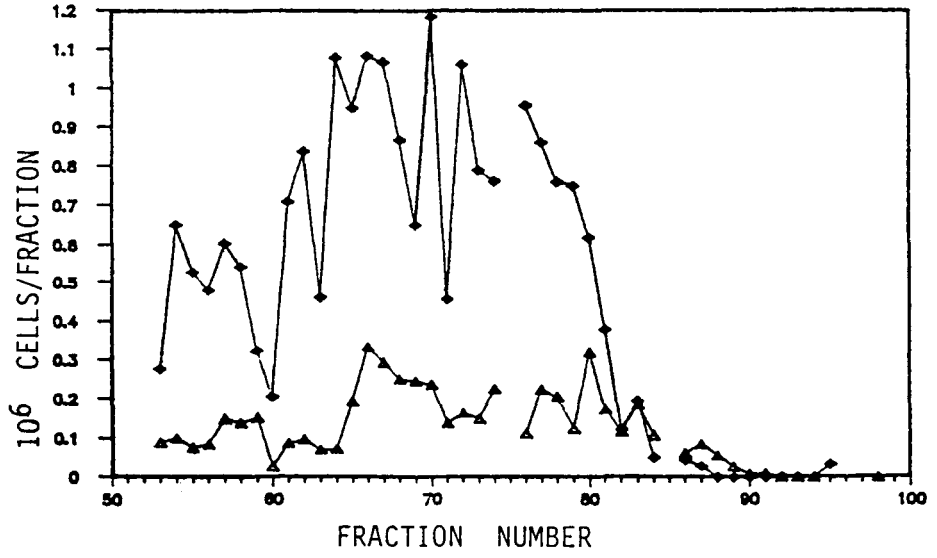

Figure 5b. Distributions of HEK cells from both CFES runs conducted in micro-g. Input cell concentration was 2.6 x 10^6 cells/ml for Run 3 (triangles) and 8.0 x 10^6 cells/ml for Run 4 (diamonds). Narrowing of the EPM distribution did not occur at cell concentration > 2.6 x 10^6 cells/ml as had been observed in ground-based CFES experiments.

Calculations indicated that six to eight of the lowest mobility fractions might not have been collected in the flight experiments due to preflight selection of the 50 sample outlets that were connected to the 50 bags in the sample trays. Figure 5b shows that the EPM patterns of the two flight experiments were similar. No reduction in mean migration distance or bandspread was noted at the higher input cell concentration (Run 4). Figure 6a shows the urokinase (u-PA) production rate of the ground control fractions as measured by the S-2444 chromogenic substrate assay. Most of the fractions in the upper 15 percent and lower 50 percent of the distribution produced low levels of u-PA. Several fractions in the upper 50 to 80 percent of the distribution produced high levels of u-PA. Figure 6b shows that the u-PA secretion from flight cells was considerably greater in the lowest 30 percent, and some productive fractions were found in the uppermost 20 percent of the electrophoretic distribution. In both the ground and flight distributions the highest producing subpopulation was found at approximately the same point in the distribution (86% of maximum migration distance). Secretion of other PA's and PA inhibitors also was found and is described in detail by Barlow et al., (1987, this volume).

Postflight analysis of the EPM distribution of individual fractions, collected in space and then subcultured on Earth, indicated that the progeny cells studied had mobilities that were related to the fractions from which they were collected. However, Figure 7 shows that the fraction studied with the lowest original mobility (Exp 1530) produced differentiated progeny cells which had a greater EPM (Morrison et al., 1984a). Summarized comparisons of the results of different methods of preparative electrophoresis, both in 1-g and in micro-g, show that the subpopulations of HEK cells that produce PA's at the greatest or higher than average rates have the same relative EPM's (Todd et al., 1986) (see Figure 8). Microscopic studies of cells cultured after separation showed a unique distribution of four morphological types which are believed to have different physiological functions (see next section: Figures 9 and 10).

4. Discussion of procedures and results

Minor in-flight problems occurred during redispersion of the cells prior to injection into the CFES and with the automatic syringe drive mechanism, and fewer cells were processed in micro-g than planned. An apparent preflight microbial contamination of the CFES device could not be eliminated due to Shuttle turnaround operations that did not allow the CFES to be removed from the Challenger between STS-7 and STS-8. The contamination caused significant changes in the postflight procedures.

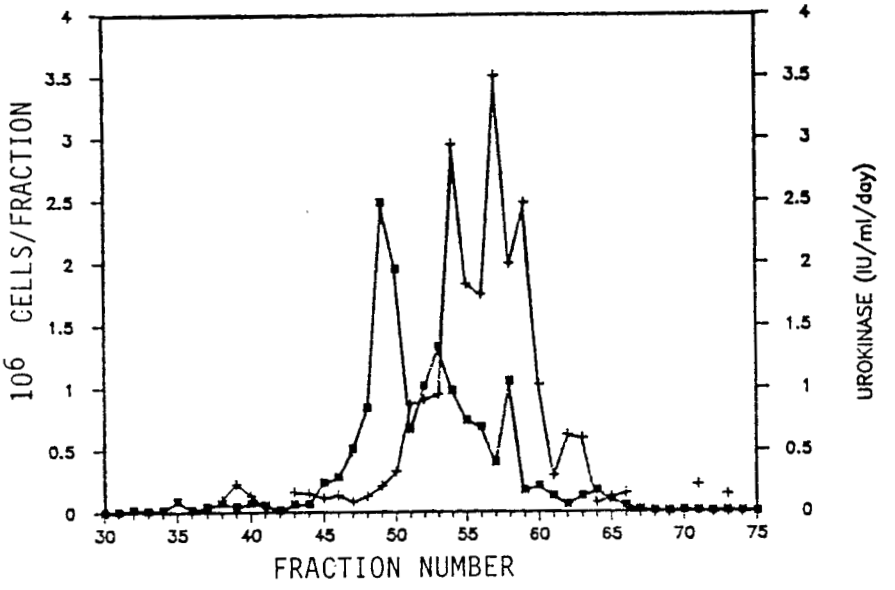

Figure 6a. Electrophoretic distributions of cell number (squares) and urokinase production asmeasured bu S-2444 chromogenic substrate assay (+) following separation of HEK cells by CFES at 1-g, then cultured in 8-10 replicated cultures per fraction seeded at 3000 cells/cm^2, grown to 95% confluence and changed to u-PA production medium. Most fractions in the lower half of the EPM distribution (Fractions 30-50) produced minimal levels of urokinase. Fraction number is relative to sample inlet tube. (Ground experiment 5c).

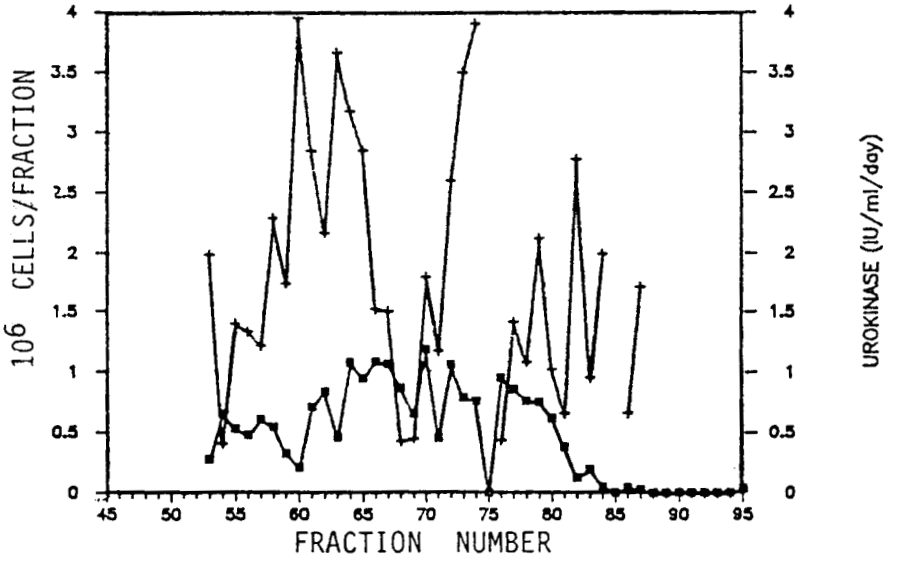

Figure 6b. Comparison of cell number (squares) and u-PA production (+) by HEK cells separated by CFES in microgravity. EPM distribution was broader than that of 1-g experiment (Figure 6a). Most fractions in lower mobility range (fractions 53-66) were high producers of u-PA as compared with ground control experiment (Figure 6a). (Flight experiment4).

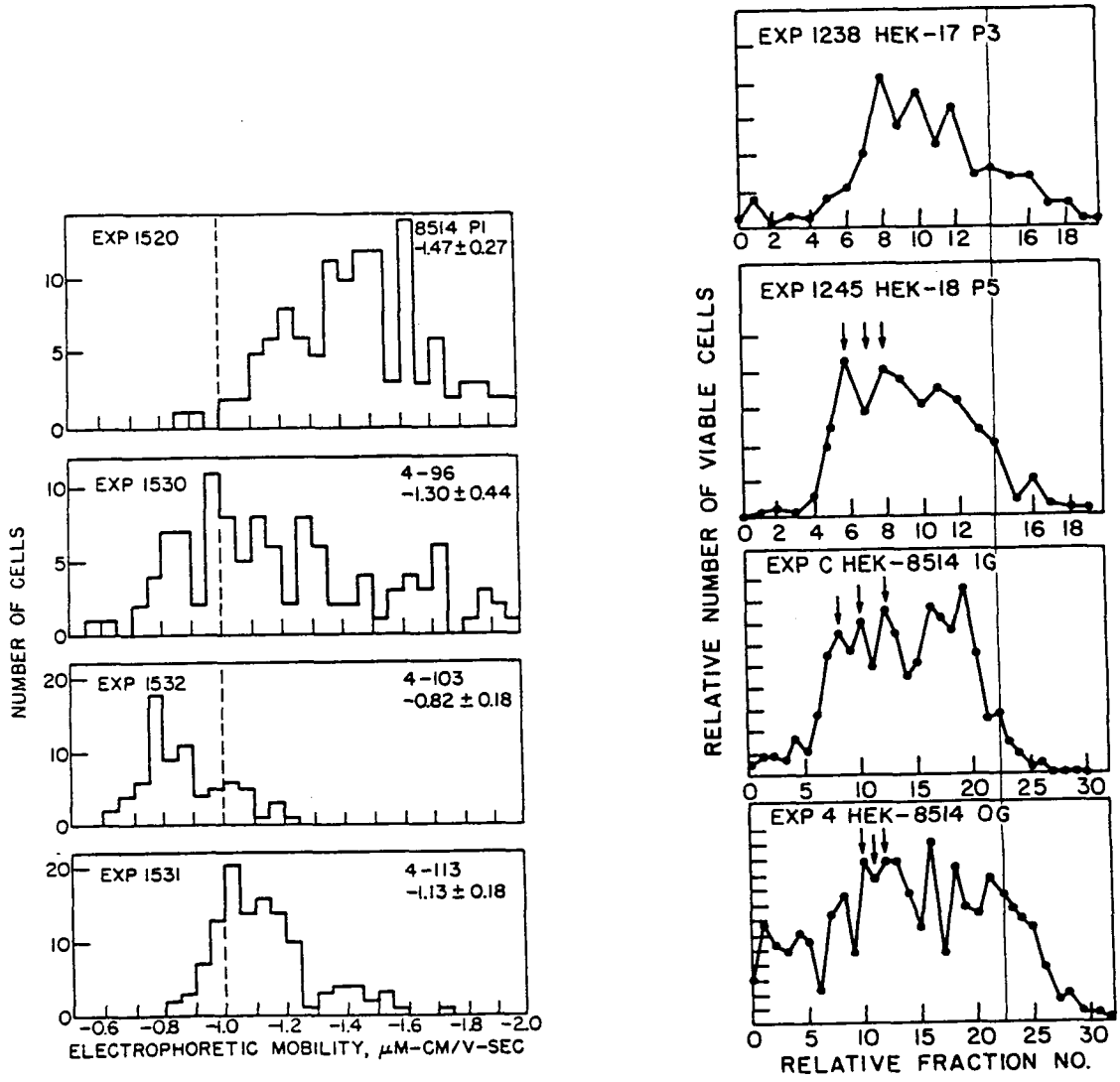

Figure 7. (Left) Electrophoretic mobility (EPM) distributions of starting mixture (top) and 3 fractions of human embryonic kidney cells (strain HEK-8514) separated by Continuous Flow Electrophoresis in microgravity and subsequently grown in cell culture. Original EPM is linearly related to fraction number (96, 103, and 113 in this case). The low EPM fraction (number 96) yielded higher-EPM progeny cells (from Morrison et al., 1984a).

Figure 8. (Right) Electrophoresis profiles of three kidney cell strains, separated by three different methods of preparative cell electrophoresis. The relative number of viable cells is plotted against electrophoretic fraction, renumbered, and scaled so that direct comparisons are possible. The cell strain number 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 on Space Shuttle Flight STS-8. The solid, vertical line marks the fraction which, in every case, gave rise to cells with the highest level of plasminogen activator production, while downward arrows indicate fractions with above-average production (from Todd et al., 1986).

The bacteria were resistant to antibiotics which HEK cells can tolerate, and this contamination eventually resulted in the loss of 25 percent of the HEK flight samples.

FLOW CYTOMETRY APPLICATIONS IN THE ANALYSIS OF CFES FRACTIONS

1. Cell subpopulations in human embryonic kidney cultures

Flow cytometry was applied to the analysis of fractions of electrophoretic cells that produce PA's. As there are five morphological subpopulations identifiable in cultures of HEK cells (domed, fenestrated, large and small epithelioid, and fibroblastoid cells), shown as sketched in Figure 9, different types of cells should exhibit different light scatter properties. Unseparated and electrophoretically separated kidney cells were therefore analysed on the EPICS V cell sorter (Coulter Electronics, Hialeah, FL) to attempt to characterize the light scattering signatures of the various morphological types and to attempt to relate morphology to enzyme production.

2. Materials and Methods

HEK cells were separated by microgravity electrophoresis on space shuttle flight STS-8 (Morrison et al., 1984, 1984a). Counts of viable cells were made on each sample. The cells were then plated for propagation in culture. After allowing time for attachment and flattening, the medium was replaced with urokinase production medium (UKPM), and after several days the amount of urokinase per sample was determined. Several fractions were further propagated, and the per cent of each morphological cell type was determined by phase contrast microscopy by counting at least 200 cells in each fraction.

HEK cells obtained from MA Bioproducts were put into a single cell suspension using 0.05% trypsin and 0.37% EDTA in saline A and were analysed on the EPICS V cell sorter. Two-parameter 488 nm light scatter distributions were obtained (forward angle light scatter and 90^{o} integrated light scatter pulse) as well as 90^{o} pulse-width "time-of-flight" for cell sizing (Leary, et al., 1978). Two-parameter distributions combining these measurements were obtained of HEK cell subpopulations that had been purified by microgravity electrophoresis and subsequently propagated in culture.

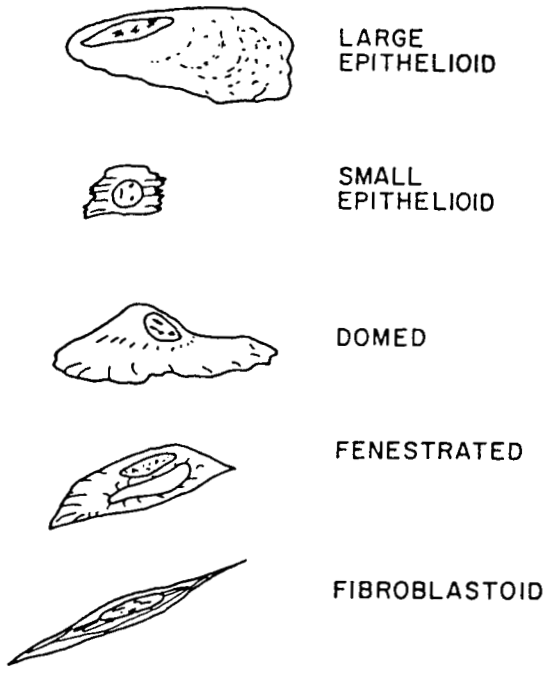

Figure 9. Sketches of the 5 major identifiable cell types found in early-passage human embryonic kidney cell cultures.

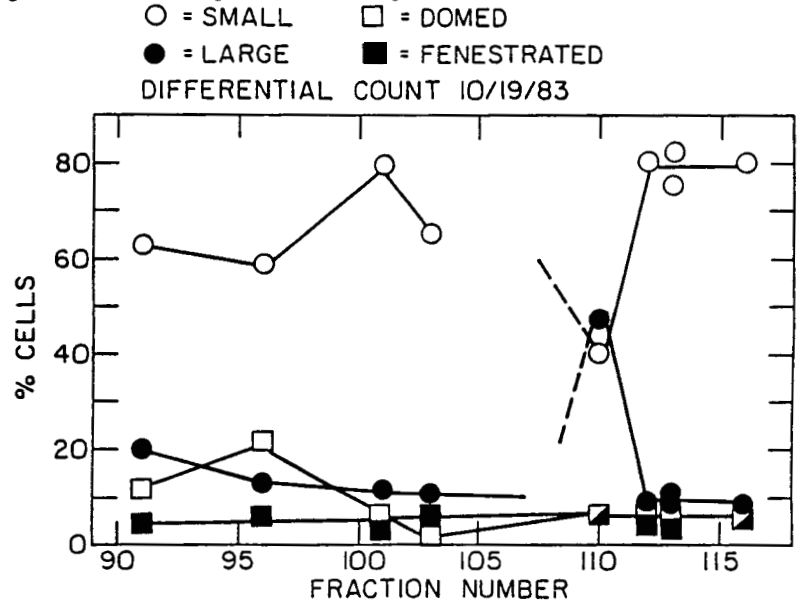

Figure 10. Electrophoretic distribution of the 4 morphological types of cultured HEK cells retrieved from STS-8 separation experiment. Cultures consisted of progeny of cells one passage after retrieval.

Flow cytometry can be used to identify plasminogen activator containing cells by using a fluorescent staining method for cells that contain plasminogen activators, especially urokinase. The general approach to this problem was similar to that used by Dolbeare and Vanderlaan (1979). First a simple staining technique was tested in which a fluorogenic substrate was mixed with cells and the cells were analyzed by fluorescence flow cytometry. The kidney cell strain which was designated "1593", and which was found to be high in cells producing PA's, was tested. Cells were maintained either on complete culture medium or urokinase production medium (UKPM)(Barlow et al., 1977a). Cells were suspended by trypsinization and exposed briefly to the fluorogenic substrate CBZ-gly-gly-arg-MNA. Cleavage of the basic arginyl amide bond releases 4-methoxy-2-naphthylamine, which can be stimulated to fluoresce by the 488 nm light from the argon ion laser of the EPICS V cell sorter. Fluorescent light was detected through a 590 nm band pass filter.

3. Results

The first passage progeny of cells obtained from fractions 91, 96, 101, 103, 110, 112, 113, and 116 of flight experiment 4 were counted according to morphological type at various times after retrieval. The distributions of the four principal morphological types among these fractions is shown in Figure 10.

The size measuring capability of the EPICS V sorter, which is a pulse-height-independent measure of pulse width using the unintegrated 90^{o} light scatter signal ("time of flight") was calibrated with different sizes of microspheres, and a resolution of 0.45 um/channel was obtained, as shown in Figure 11. Microscopic measurements of HEK cells were also performed using 9.5, 14.5 and 20.5 um microspheres as standards. The size distribution is shown in Figure 1, where it is seen that the majority of these passage-1 HEK cells are between 15 and 25 um in diameter. Size was used as a correlated parameter with light scatter intensity in Figures 12-14.

The integrated 90^{o} light scatter signal intensity reflects the internal structure of the cell, including granulation (Goolsby, 1985), nuclear size (Brunsting and Mullaney, 1972), and shape (Latimer et al., 1978). Granular cells should scatter more light at 90^{o} than non-granular cells. Forward angle light scatter (2.5-19 degrees) is a measure of size and refractive index in live cells (Leary, Notter and Todd, 1978; Goolsby, 1984). Cells with a high refractive index have reduced forward angle light intensity.

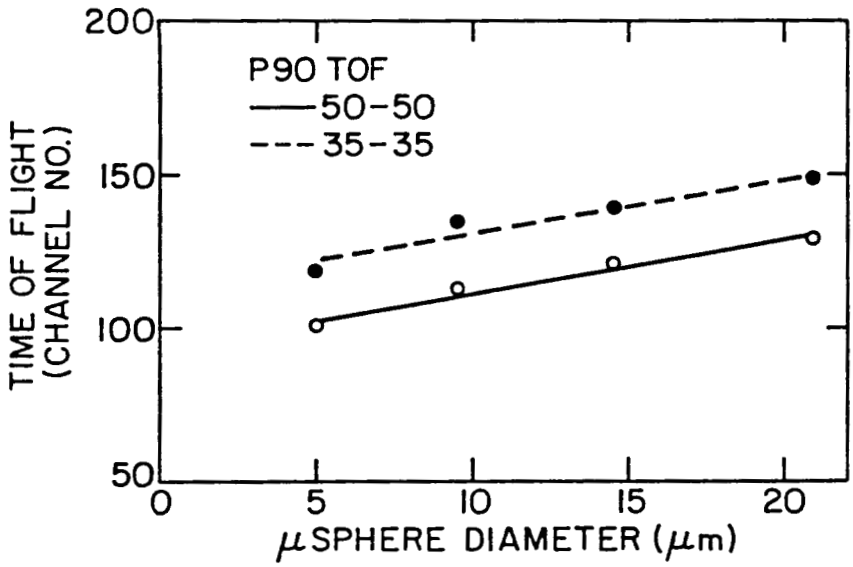

Figure 11. Calibration curve for pulse-height-independent time-of-flight sizing using 5, 10, 15, and 20 um spheres. Pulse width measurements were made at 50% (solid line) and 35% (dashed Line) of the peak height of the raw (unintegrated) 90^{0} ($70\text{-}110^{0}$) light scatter signal.

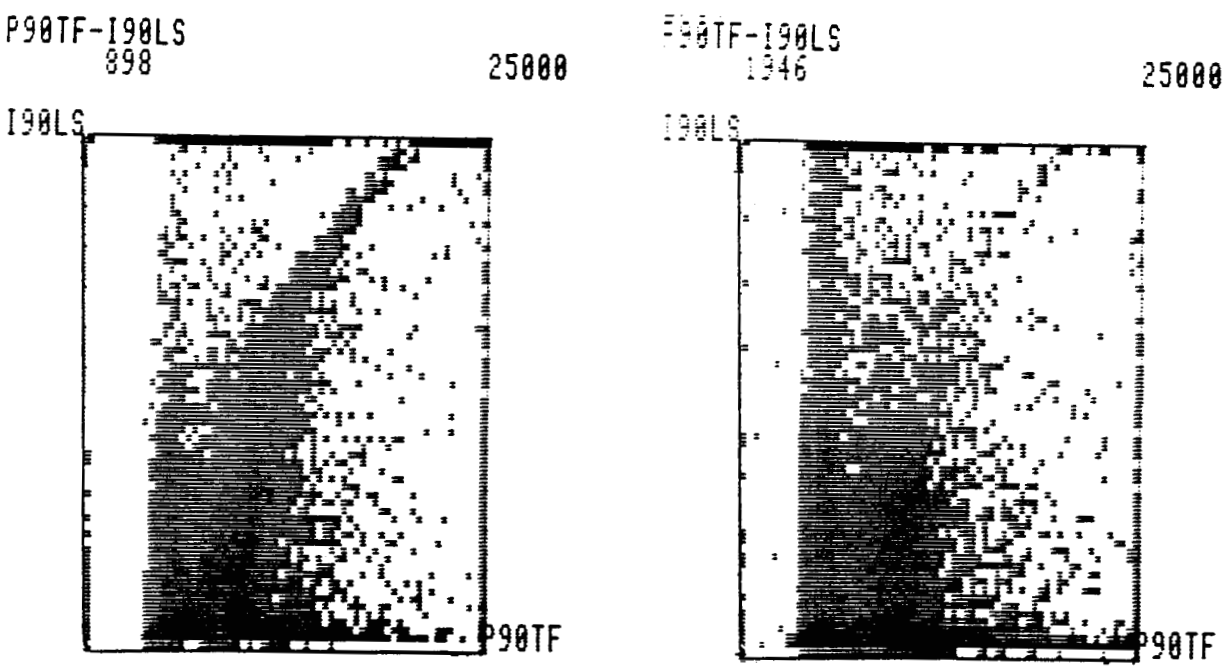

Figure 12. Two examples of raw histograms from the EPICS V cell sorter. Four two-parameter light scatter distributions were obtained on each fraction available after the Shuttle flight. These are scattergrams of integrated 90^{0} light scatter signals (ordinate) vs of time-of-flight cell diameter (abscissa) for two of the separated fractions from the STS-8 experiment. Darker areas indicate higher cell counts.

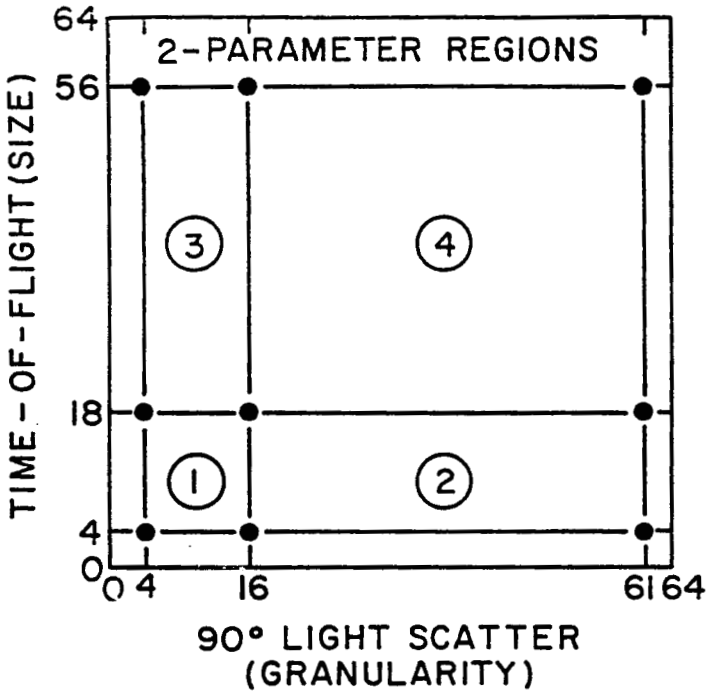

Figure 13. A diagram of the method of analysis of the histograms of the type shown in Figure 12. Each two-parameter histogram was divided into four regions, and the percentages of cells falling into each region was determined. Note inversion of axes relative to Figure 12 (region 3 cells lie along the abscissa in Figure 12).

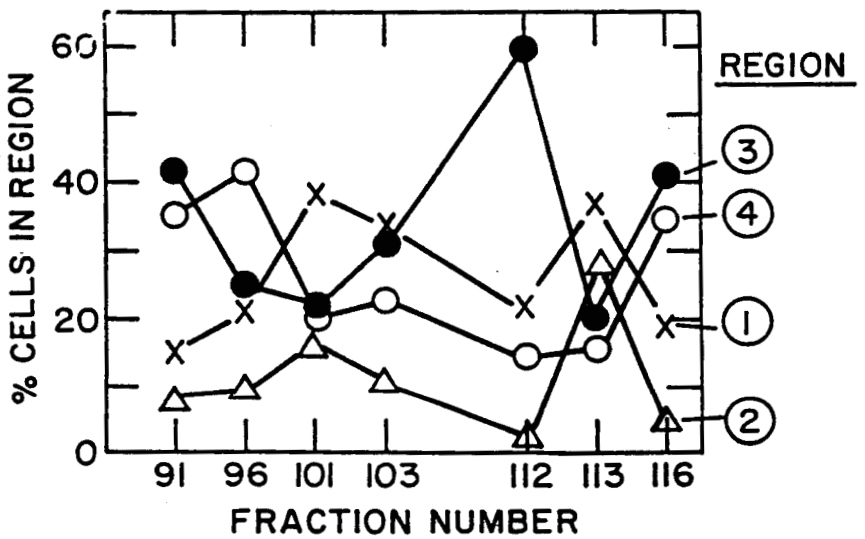

Figure 14. Percentages of cells from each electrophoretic fraction falling into each of the 2-parameter regions defined in Figure 13. Region 1 contained debris and was therefore eliminated from further analysis.

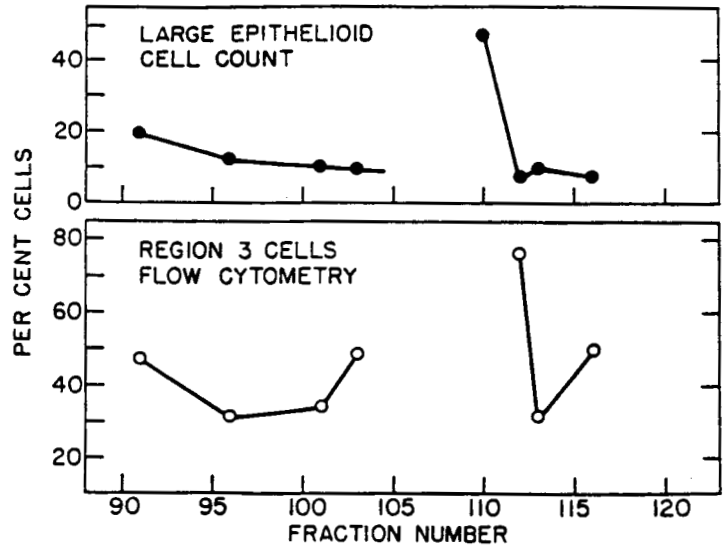

Figure 15. Comparison of morphological and flow cytometric light scatter analyses of cells separated on Space Shuttle flight STS-8. Percentage of large epithelioid cells as determined microscopically from cells in culture is given on the upper graph, and the percentage of cells in Region 3 (see Figures 13 and 14) determined by flow cytometry is given on the lower graph. Large size and low granularity are consistent with appearance of large epithelioid cells. Highest plasminogen activator activity was found in fraction 110, which corresponds to fraction 73 of Figure 6b.

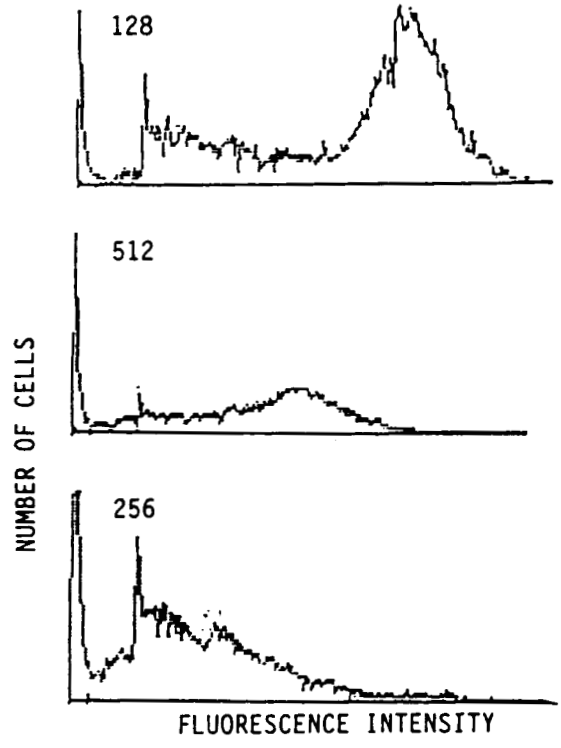

Figure 16. Fluorescence intensity distributions of cultured human embryonic kidney cells "1593" at the 5th passage stained with CBZ-gly-gly-arg-4-methoxy-2-naphthylamide after cultivation in microcarrier medium "MM1" plus 10% fetal bovine serum (TOP distribution) or 3 days after transfer to serum-free production medium "UKPM" (MIDDLE distribution) compared to unstained cells (BOTTOM distribution).

Single cell suspensions of the above-mentioned electrophoretics fractions were analyzed on the EPICS V cell sorter. Two-parameter distributions of integrated 90^0 light scatter vs time-of-flight, integrated 90^0 light scatter vs forward angle light scatter, and integrated 90^0 light scatter vs peak 90^0 light scatter were acquired. Two examples of these distributions are shown in Figure 12. Each distribution was divided into four regions (Figure 13), and the percentage of cells in each region was determined for each fraction (Figure 14).

A selected portion of these results, is compared with and the morphological observations are shown in the 2 graphs of Figure 15. Large size and low granularity correlated with a high frequency of large epithelioid cells, as expected, and a high frequency of epithelioid cells correlates with high total plasminogen activator production (Figures 6 and 8).

When cells were stained for plasminogen activator content by the fluorogenic substrate method the resulting fluorescence distributions revealed subpopulations of stained and unstained cells. Incubation in UKPM actually decreased cell fluorescence (possibly due to such cells having already secretred their PA), and unstained cells had the same fluorescence distribution (roughly) as the low intensity population in stained cells. These findings are illustrated by the three fluorescence distributions given in Figure 16.

4. Conclusions

Results of flow cytometric light scattering experiments with prepurified cultured human kidney cells indicate that subpopulations of living cells that were high in plasminogen activator also contained the highest percentage of cells with large size and low 90^0 light scatter intensity and that these cells corresponded to the large epithelioid population.

By staining cells with a fluorescent substrate, it has been shown, in preliminary experiments, that plasminogen activator is synthesized and retained by cells multiplying in growth medium and released by cells transferred to serum-free production medium.

CONCLUSIONS AND FUTURE RESEARCH

Cultures of human embryonic kidney cells consistently contain an electrophoretically separable subpopulation of cells that produce high levels of urokinase and have an EPM about 85% as high as that of the most

mobile HEK cells. This subpopulation is rich in large epithelioid cells that have relatively little internal structure. When resolution and throughput are adequate free-fluid electrophoresis can be used to isolate a broad band of low mobility cells which also produces high levels of PA's.

In the course of performing this research we have discovered that all electrophoretic subpopulations of cultured human embryonic kidney cells produce some plasminogen activators and that separate subpopulations produce high quantities of different types of PA's. This information and the development of sensitive assays for this project have provided new insights into cell secretion mechanisms related to fibrinolysis. These advances would probably not have been made without the NASA program to explore fundamental questions of free-fluid electrophoresis in space and McDonnell Douglas Astronautics Co.'s Joint Endeavor Agreement with NASA.

The results of STS-8 demonstrated some advantages of cell separations in microgravity; however, the experiments must be repeated under optimized conditions and with the use of refined assay techniques to determine the full potential of CFES for live cell separations. Enough viable cells in each subpopulation must be returned for the functional assays to be able to identify the important fractions, which then can be subcultured for continued studies. Postflight research will determine the factors that affect the relationship between cell surface charge in an electrophoresis buffer and subsequent secretory function in a complex medium following growth and maintenance under various culture conditions.

The current and planned research is focused on preparations for the next CFES flight opportunity and on improvements in micro-g experiment conditions and sample storage. Ground control studies include quality control of flight cell lots, optimization of CFES buffers, improvements in pre- and post-separation storage conditions, testing effects of chemical disinfectants used in the CFES, and cell tolerance to antibiotics required for microbial contamination control. Post-separation growth and function studies are used to determine cell viability ranges and the typical retention of target cell function following storage and successive subcultures. This work is being conducted in collaboration with the Biochemical Assays of Kidney Cells and Bioseparations (CFES) research project at the Bioprocessing Research Center at Houston (Barlow et al, 1987, this volume) and at Johnson Space Center.

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