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ELECTROPHORETIC SEPARATION OF CELLS

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CONTENTS

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1. INTRODUCTION

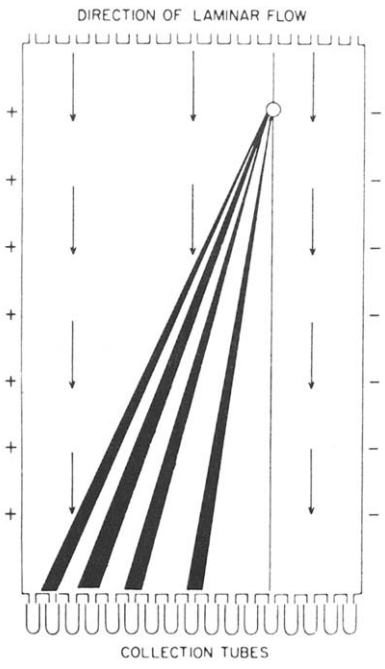
Isolated cell populations are required in studies of the biological characteristics of single cell populations and in attempting to understand the role that a cell population plays within the total organism. Furthermore, in medical practice it is important to determine the biological features of isolated cell populations of the human body, because many pathological abnormalities are due to dysfunctions of distinct cell populations. For these reasons considerable efforts have been made during the past few decades to develop methods for purification of cellular subpopulations.

One of these methods, cell electrophoresis [1], can be used to separate cells according to their negative surface charge density, which is a natural physical parameter of cells. Many cellular subpopulations of complicated organisms with different negative surface charge densities have different biological functions and activities.

Cell electrophoresis belongs to the class of physical cell separation methods. Other physical cell separation methods are density gradient sedimentation [2, 3] and counter-current centrifugal elutriation [4]. During density gradient centrifugation, cells are separated according to the specific density. Various cell populations have different specific densities because their content of proteins, granules and other particles is different. A list of the specific densities of a lot of cellular subpopulations is shown in [5]. Cell separation according to volume can be achieved by counter-current centrifugal elutriation. The volume of human cellular subpopulations varies from a few μm^3 (platelets have 8 μm^3) up to thousands of μm^3 (macrophages).

In addition to physical separation methods, further possibilities for purifying cell populations have been found. For example, lymphocytes have been cloned [6]. This method was successfully applied to prepare homogeneous populations of cells, which produce distinct substrates such as antibodies or interferon. Cells with common surface antigens were labelled by antibodies, and labelled and unlabelled cells were then separated by cell sorting [7], by panning [8] or by complement lysis [9]. Cells were isolated by adherence to ligands, such as plastic surfaces or nylon wool [10–12]. The cell populations obtained with the help of these methods could be used for a lot of very successful studies.

Despite the many methods that are available for cell purification, cell electrophoresis is still an important tool for obtaining homogeneous cellular subpopulations for further investigations. In this review I shall describe problems of cell purification that can be solved by cell electrophoresis. The application of cell electrophoresis is now more worthwhile because all kinds of cell electrophoresis instruments, especially the free-flow electrophoresis apparatus, have reached a very high standard of separation accuracy.



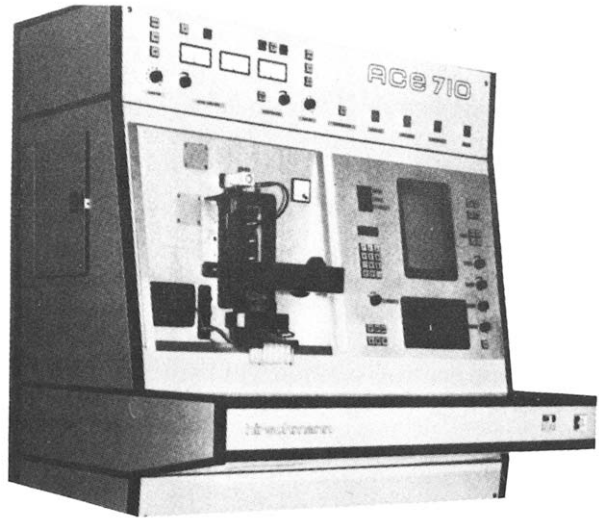


Fig. 1. Basic principle of free-flow electrophoresis.

Fig. 2. Front view of the free-flow electrophoresis apparatus ACE 710.

2. METHODS OF CELL ELECTROPHORESIS

There are several techniques that can be used to separate cells depending on their negative surface charge densities. Several methods of cell electrophoresis have been successfully applied either to characterize or to isolate various cellular subpopulations from both animals and humans (for references, see refs. 13 and 14).

2.1. Free-flow electrophoresis

2.1.1. Basic principle

One kind of cell electrophoresis is the free-flow electrophoresis developed by Hannig [1]. The basic principle of free-flow electrophoresis is shown in Fig. 1. A laminar buffer stream flows vertically from the top to the bottom. An electric field is applied perpendicular to the buffer flow. Near the top cells are introduced into the vertically flowing electrophoresis buffer. Cells exposed to the electric field migrate laterally towards the positively charged electrode in the vertically flowing buffer. The migration velocity depends on the negative surface charge density. Thus cells with different negative surface charge densities migrate at different speeds, arrive at different points along the bottom line and can be collected for preparative isolation.

2.1.2. Apparatus design

During the past few decades several types of machines were developed [13]. Earlier types were the VAP machines (Bender & Hobein, Munich, F.R.G.). Recently the size of the separation chamber was reduced in order to further improve the separation accuracy of the free-flow electrophoresis apparatus. At present the ACE 710 (Hirschmann Gerätebau, Unterhaching, F.R.G.) appears to be very suitable for performing accurate cell electrophoresis (Fig. 2) [13,15]. The heart of this machine is the separation chamber, which is composed of two parallel glass plates 0.3 mm apart. It is 18 cm high and 4 cm wide. The size seems to be an optimal compromise between separation capacity and accuracy of electrophoresis. The chamber is large enough to separate $3 \cdot 10^7$–$10 \cdot 10^7$cells per hour. Fewer cells must be electrophoresed when the cells have a high tendency to stick together. At the same time the chamber is so small that any curvature of the two parallel glass plates can be totally excluded. Since the buffer film is only 0.3 mm thick, the cooling system is able to remove the heat produced by the electric current so quickly that no disturbance of the laminar stream occurs and the variability of the temperature is kept below 0.1 °C. Thus variations in the voltage are negligible. The small size of the chamber makes it possible to electrophorese cells so fast that they remain within the electric field for only 30 s.

Several features of the apparatus are very helpful for performing exact cell electrophoresis: (i) an efficient electronically controlled cooling system; (ii) an implement for preparative isolation of cells that does not disturb the laminar flow of the buffer film; (iii) a videosystem, which supervises continuously the separation profiles: any shift of a distribution curve caused by air bubbles or clotting cells can immediately be detected and corrected; (iv) at the point of injection the cells are focused to a sample stream diameter of 0.2 mm, so contact of cells with the chamber walls is avoided; (v) on both sides of the buffer film there are membranes above which the electrophoresis buffer is in electrical contact with the electrode buffer: electrophoresis and electrode buffers are separated to prevent damage of cells by electrolyte products.

2.1.3. Buffer system

A further critical point in cell electrophoresis is the buffer system. A buffer of low ionic strength is required if a high voltage and low heat production are to be realized. Furthermore a lot of ions are changed by the electrical current. In particular, chloride is readily converted into hypochlorite, which is a strong poison for cells. Zeiller et al. [16] spent a lot of time finding low-ionic-strength buffers that do not damage cells. We now use a pH 7.2 electrophoresis buffer consisting of 16.5 m*M* triethanolamine, 4 m*M* potassium acetate, 1 m*M* glucose, 216 m*M* glycine and 26.4 m*M* saccharose. The pH is adjusted by acetic acid. The cells are suspended in this buffer for ca. 1 h. They are then resuspended in RPMI 1640 (Gibco, Karlsruhe, F.R.G.) medium supplemented by 10% of any kind of serum. A 75 m*M* triethanolamine–4 m*M* potassium acetate solution (pH 7.2) flows along the electrodes.

2.1.4. Personal experiences in handling the ACE 710

The free-flow electrophoresis instrument ACE 710 can be handled easily. The chamber construction makes it possible to get very sharp bands. Nevertheless, the right-hand slope of an erythrocyte electrophoretic mobility (EM) distribution curve is sometimes found to be less steep than the left-hand one. This indicates that a proportion of the cells interacts with the walls of the separation chamber. The interaction can be avoided if the glass walls of the separation chamber are coated with bovine serum albumin (BSA). Thus the chamber should be filled with a 3% BSA solution a few hours before each separation experiment. The sharpness of the bands also depends on correct placement of the needle that is used to introduce the cells into the chamber. It should also be ensured that the cells are pumped in exactly the same direction that the separation buffer is flowing, and not against the walls of the chamber.

In many experiments the fractionated cells have to be incubated for a long time. The machine is not designed for sterile operation. However, if the system is cleaned well, all tubing is rinsed with 0.1 *M* sodium hydroxide, and sterile buffers are used, bacterial contamination of the electrophoresed cell fractions is very low. Thus the purified cells can be incubated for longer periods in media containing suitable antibiotics.

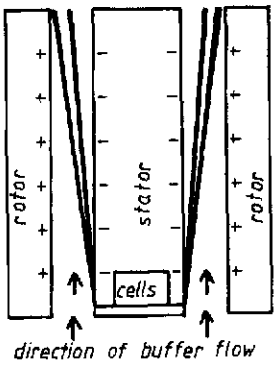
2.2. Scaled-up free-flow electrophoresis

2.2.1. Basic principle

There is a limit to the number of cells that can be separated by a free-flow electrophoresis apparatus such as the ACE 710. Sometimes it is a problem to get enough cells to enable investigation of slowest and fastest mobility fractions. Therefore efforts have been made to overcome the difficulties of scaling up. If the gap between the walls becomes broader, the heat produced by the electrical current cannot be adequately removed. The consequence is that convection occurs [13,17]. If the cell concentration is increased, aggregation and sedimentation of cells prevents accurate cell electrophoresis. Sedimentation is of great importance in large-scale cell electrophoresis because the residence time of the cells within the chamber is ca. 10 min. Therefore a large-scale electrophoresis instrument was constructed which allowed vertical flow of buffer from the bottom to the top of the chamber. Under these conditions, cells with higher sedimentation rates are exposed to the electrical current for longer and therefore migrate farther towards the positively charged electrode on their way through the chamber. The second problem is thermoconvection. It has been overcome in two ways. Mattock et al. [18] counter-balanced the thermoconvection by rotating one of the walls of the chamber, and a group of American investigators [17] tried to perform cell electrophoresis in space where no thermoconvection occurs because gravity is zero.

2.2.2. Apparatus design

The machine that was used to perform cell electrophoresis in space was built by McDonnell Douglas. There are only a few published data on this machine. The separation chamber consists, as do other free-flow electrophoreses, of two parallel



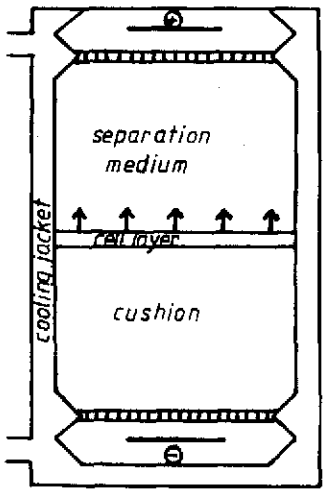


Fig. 3. Basic principle of the scaled-up free-flow electrophoresis described by Mattock et al. [18].

Fig. 4. Basic principle of column electrophoresis [21-26].

glass plates, which are up to 3 mm apart. It is 1.2 m high and ca. 30 cm wide and 3 ml of a sample can be injected per hour. The highest possible concentration of human erythrocytes is 10^9 cells per ml. A voltage of 40 V/cm at 14 °C is usually applied. The separated cells can be collected at 197 outlets. Electrophoresis and electrode buffers are separated. The sample residence time in the electrophoresis chamber is ca. 12 min. The throughput of this machine seems to be ten-fold higher than the throughput of the Hannig free-flow electrophoresis [17,19,20].

The machine constructed by Mattock et al. [18] consists of two concentric cylinders (Fig. 3). The inner cylinder is held stationary, the outer rotates. Electrophoresis takes place in the 3 mm wide annulus between the cylinders. The electrophoresis buffer flows upwards through the annulus. An electric field is applied perpendicular to the buffer flow. Stable flow of the carrier solution is maintained because the relative movement of the cylinders prevents convection turbulence caused by resistive heat. Cells are introduced into the carrier solution near the base of the stationary cylinder. On their way upwards the cells migrate to the positively charged electrode, which is placed in the outer cylinder. The migration velocity depends on the surface charge density. This means that different cell populations arrive at the top of the chamber in different concentric zones and can be collected in different fractions. (Further information about this machine may be obtained from CJB Developments, Portsmouth, U.K.) A characteristic feature is the tremendous throughput. Up to 3 l of sample can be separated per hour. This opens up the possibility of electrophoresing total samples of blood or bone marrow transplants.

2.2.3. Buffer system

In large-scale electrophoresis, low-ionic-strength buffers are used similar to those used for the Hannig free-flow electrophoresis. However, they have been

modified. For example, in space electrophoresis a buffer is used which consists of 6.42 m*M* sodium chloride, 0.367 m*M* potassium dihydrogenphosphate, 1.76 m*M* disodium hydrogenphosphate, 222 m*M* glucose, 0.336 m*M* Na_2EDTA, and 5% (v/v) dimethyl sulphoxide. The pH is 7.2 [19,20].

2.3. Column electrophoresis

2.3.1. Basic principle

An alternative electrophoresis method is column electrophoresis. Several similar variants have been developed by different investigators [21–26]. One of them is the so-called "preparative ascending electrophoresis" developed by Van Oss [21]. Its principle is shown in Fig. 4. A cylindrical column is in part filled by a starting cushion. This starting cushion is an isotonic buffer with a specific density higher than the specific density of the cells that are to be separated. Cells are layered on the cushion, and the column is filled to the top with an isotonic medium. An electrical field is applied between the top and the bottom of the column. Cells exposed to the electrical current start to migrate against gravity towards the positively charged electrode. The migration rate depends on the negative charge density. During an electrophoresis experiment, which usually takes ca. 1 h, distinct zones of cells are formed. After electrophoresis the isotonic buffer above the cushion is fractionated. Cells from the different zones are collected in different fractions.

2.3.2. Apparatus design

At least to my knowledge, no kind of apparatus for performing column electrophoresis is commercially available. The laboratory machines, described in the literature, have a similar construction [21,22]. The cylindrical columns have cooling jackets through which cooling water circulates. The electrode wires are separated from the chamber where the cells are electrophoresed. Electrical contact between the separation and the electrode chambers is established either above filter papers or above agarose gels. Further equipment is used for filling the chambers and collecting the separated cells. The separation capacity of column electrophoresis depends on the apparatus design. The machine of Van Oss separates $2 \cdot 10^6$ cells per run [21]. The machine described by Petrov et al. [26] can be loaded with $2 \cdot 10^8$ cells suspended in 10 ml. The yield is ca. 80%.

2.3.3. Buffer system

Electrophoresis and electrode media are buffered by phosphate to pH 7.1. Isotonicity is established either by neutral compounds (low-ionic strength buffer) or by sodium chloride. The specific density of the cushion is increased by Ficoll–Hypaque medium, by sucrose or by deuterium oxide [21,26].

3. EXTENSION OF APPLICATIONS

3.1. Problem

The highly developed electrophoresis machines that are now available can be used to separate cells differing in their EM by only 5%. However, many cell pop-

TABLE 1

PHYSICAL PARAMETERS OF CELL POPULATIONS OF THE HUMAN PERIPHERAL BLOOD

Population	EM (10^{-4} cm^2 V^{-1} s^{-1})	Volume (μm^3)	Specific density (g/cm^3)	Relative frequency
Granulocytes	0.95	400	> 1.077	100
Erythrocytes	1.1	90	> 1.077	65000
Platelets	0.9	8	< 1.077	–
T-lymphocytes	1.1	175	< 1.077	24
B-lymphocytes	0.9	165	< 1.077	7
Monocytes	0.95	400	< 1.077	8
Dendritic cells	0.9	180	< 1.077	0.4

ulations have almost identical EM and cannot be separated by cell electrophoresis. Table 1 shows the EM of several subpopulations in peripheral blood. There are many important cell populations with identical EM. Furthermore, the frequencies of the different kinds of cells are not equal: 99.8% of all human blood cells are erythrocytes. The total distribution of the EM of erythrocytes overlaps the EM range of other populations, although the mean EM of some populations is different from the mean EM of erythrocytes. Even when the population of the mononuclear leukocytes (MNLs) is isolated by Ficoll–Hypaque gradient centrifugation [2], cell electrophoresis cannot be applied to gain really pure subpopulations. The reason for this failure is that although the MNLs consist of at least four major subpopulations, the total population shows a unimodal distribution curve with only a slight shoulder at the left side (Fig. 5).

3.2. Ways to overcome the problem

In Hannig's department, purification of cellular subpopulations of human MNLs was used as a model for finding possibilities for successful application of cell electrophoresis in cases where several cellular subpopulations are contained within a narrow EM distribution curve. The protocols developed for purification

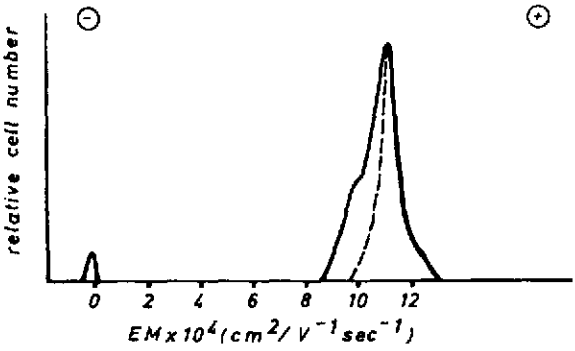


Fig. 5. EM distribution curves of human erythrocytes (– – – –) and human MNLs (———).

of subpopulations of MNLs can be applied to other cell purification experiments.

3.2.1. Antigen-specific electrophoretic cell separation (ASECS)

Many cellular subpopulations from the human body are very well characterized by monoclonal antibodies. Antibodies are positively charged, and if they are bound to particular cell populations the negative surface charge density of the target cells is reduced [27]. In many cases the reducing effect of the first antibody is too small to enable separation, but the effect can be intensified by attaching a second antibody, raised against the first, to the original antibody (sandwich method). Chemically cationized antibodies have even higher slowing effects [28]. If the decrease in negative surface charge density of labelled cells is made use of, labelled cells can be separated from unlabelled cells on a preparative scale. This method usually leads to very homogeneous cell populations. However, there is the danger that cell labelling by antibodies may change the prevailing in vivo status of the cells. Thus in each case it is necessary to test whether the functional state of the cells is altered during an ASECS experiment [29]. The ASECS technique can be very helpful for removing distinct cellular subpopulations from a total cell sample [30]. Usually target cells that have bound a particular antibody are removed from a total fraction by complement lysis. During complement lysis, however, many enzymic reactions occur, which have strong influences on many unlabelled cells [9]. These influences can be avoided if the labelled cells are removed by cell electrophoresis according to the ASECS technique. Van Oss has proposed using large-scale electrophoresis machines to remove antibody-labelled killer cells from bone marrow transplants (personal communication).

3.2.2. Preseparation by counter-current centrifugal elutriation (CCE)

Another way of extending the application possibilities of cell electrophoresis is to combine the method with other physical methods that do not have an influence on the prevailing in vivo status of cells. Such methods are density-gradient centrifugation and (CCE) [2–5,31]. In this way it is possible to preseparate cell populations with identical EM or to reduce the frequency of an abundant cell population. Abundant cell populations very often cover minor cell populations with their total distribution, even if the latter have a different EM.

Fig. 6 shows how subpopulations of MNL can be purified from human blood using natural physical parameters alone. The cell isolation protocol is compared with the protocol for isolation of the complement component C1s, to show the parallels between enzyme and cell enrichment according to physical parameters. C1s is precipitated together with the other euglobulins from human serum. Then it is further enriched according to its surface charge and its Stokes radius [32]. The MNLs and platelets are separated from the rest of the cells in human peripheral blood by density-gradient centrifugation, because they have a specific density below 1.077 g/cm^3 [5]. These cells are fractionated according to their volume into platelets, lymphocytes and monocytes. Then the CCE fractions are further purified by free-flow electrophoresis. This method was used to obtain monocyte populations with purification degrees higher than 95%, and T-lymphocytes were isolated free from all antigen-presenting cells. The method of combining several

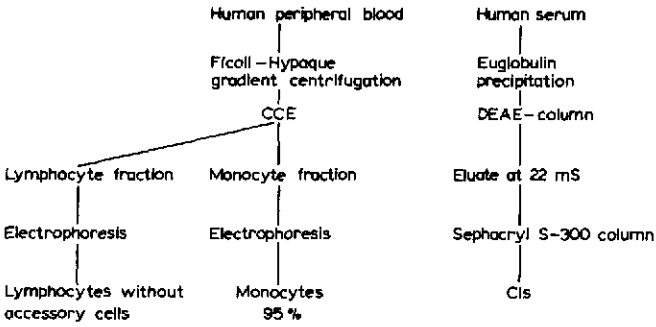


Fig. 6. Protocols for isolation of MNL subpopulations and of the complement component C1s.

physical methods has the advantage that desired cell subpopulations can be highly enriched while their prevailing in vivo status remains virtually unchanged.

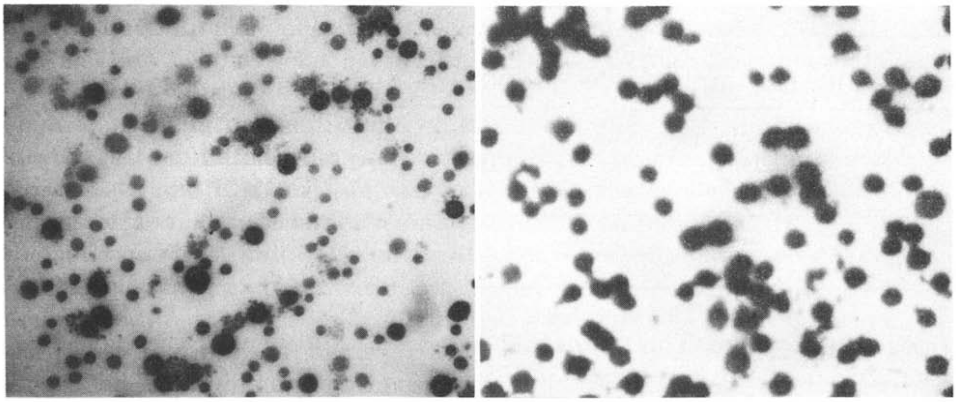
The commercially available elutriator (Beckman Instruments, Palo Alto, CA, U.S.A.) has the drawback that the percentage of cell loss becomes very high when the machine is loaded with fewer than $2 \cdot 10^8$ cells. For clinical practice this means that ca. 200 ml of blood are required from a patient in order to perform good cell separation experiments. An elutriator is being developed in Hannig's department, which is intended to operate well when loaded with ca. $5 \cdot 10^7$ cells. The combination of the free-flow electrophoresis instrument ACE 710 with the new elutriator might well be more suitable for the separation of cells in clinical practice.

4. APPLICATION FOR THE ISOLATION OF CELLULAR SUBPOPULATIONS

4.1. Purification of cells used in biological assays

One purpose of cell separation is to obtain homogeneous cell populations that can be used to perform biological assays. Homogeneous cell populations are required if the biological activity determined in an assay is to be attributed to a distinct cell population. Tests of biological cell activities are useful not only for basic research but also for clinical diagnosis. Many kinds of sickness result from dysfunctions of distinct cell populations.

Cells are able to switch their activities on and off. In basic research, and especially in medical practice, it is of interest to determine the level of activity that cells have while they are still in the human body. For such determinations cells are required that have retained their in vivo status during the isolation procedure. Thus methods that provide activation signals can hardly be applied to isolate cells for biological assays. If antibodies are attached to the surface of a target cell the activation status of the cell may be changed. It is known that antibodies such as anti T-3 [33] or anti immunoglobulin (Ig) M [34] trigger target cell proliferation. Plastic adherence activates monocytes and T-lymphocyte-rosetting challenges the T-lymphocytes [35,36]. Cloned cells are transformed cells and are thus not identical with cells from healthy human beings. Thus physical methods seem to be the most suitable for purification of cellular subpopulations, if the



a b

Fig. 7. Smears of an MNL fraction (a) and of purified monocytes (b), which were stained according to the esterase staining technique [82].

prevailing in vivo status of cells is to be determined. The advantages of physical methods are that (i) the isolation procedure takes only a few hours and (ii) the cells are kept in suspension throughout the isolation procedure, making it unlikely that the cells will receive any activation signals during the process.

In this chapter I shall describe the separation of homogeneous subpopulations of cells from human peripheral blood by free-flow electrophoresis. When cells are isolated from healthy human beings they show resting states of activation, which are presumably close to the state of activation that they have in the human body.

4.1.1. Monocytes

The monocytes are a small cell population in human peripheral blood [4,10]. Despite their low frequency in peripheral blood (Table 1), monocytes have important functions. Tests of some of these functions are already used in clinical diagnosis. We have developed an isolation procedure that gives very homogeneous monocyte populations (Fig. 7) [37]. Three important features of the isolated monocytes were tested.

One of the first functions turned on when monocytes come into contact with foreign particles, such as bacteria, is the oxygen-reduction mechanism. With the help of an NADPH-dependent enzyme, monocytes reduce oxygen molecules to the very reactive O_2^- molecular ions, which destroy foreign particles [38]. It is very important for human beings that this monocyte function works well. Severe inherent immunodeficiencies result from low oxygen-reduction activity in phagocytosing cells [39]. In some places oxygen-reduction tests are performed in monocytes for clinical diagnosis. Table 2 shows the oxygen-reduction capability of monocytes isolated from healthy human beings by physical methods. The reduction activity was tested according to the chemiluminescence method described in ref. 40. It can be seen that the monocytes isolated by our method have a low background. After addition of opsonized zymosan or human IgG the activity was increased sixty-fold. In comparison, if preactivated monocytes were used, a two-fold increase in the baselines and an almost three-fold increase in the maximum

TABLE 2

OXYGEN-REDUCTION ACTIVITY OF MONOCYTES

	Resting monocytes (cpm)	Activated monocytes (cpm)	Resting monocytes plus lymphocytes (cpm)
Baseline	2000	5000	1200
Stimulated	115000	310000	305000

activities were observed. This shows that preactivation of the monocytes changes the results.

Another possibility for preparing resting monocytes for an oxygen-reduction test might be to adjust the number of monocytes to a standard number and leave the lymphocytes within the sample. Lymphocytes do not reduce oxygen, but they have a strong influence on the activity of the monocytes [41]. Table 2 shows that $5 \cdot 10^5$ monocytes reduced more oxygen molecules when 10^6 lymphocytes were present. From our studies it seems likely that monocytes isolated by CCE and free-flow electrophoresis may give more reliable results in oxygen-reduction tests.

A second physiological feature of monocytes is their ability to migrate from the peripheral blood into the tissue and differentiate to macrophages [42]. Tissue macrophages play a very important role in eliminating cancer cells. The process of differentiation was studied in vitro. These studies revealed that the differentiation is not a preprogrammed time-dependent process, but depends on the surrounding environment, on the activation state of the monocytes and on the influence of lymphocytes. It was also found that non-activated monocytes increase their negative surface charge density during differentiation [43]. In contrast, activated monocytes showed no change in EM within six days of culturing [44]. The results suggested that it is important to distinguish between resting and activated monocytes if their differentiation behaviour is to be investigated and furthermore, that human monocytes are able to change their EM. This may be of interest in connection with the macrophage electrophoretic mobility (MEM) tests described by others [45,46].

A third function of monocytes is antigen presentation [47]. A pathway of T-lymphocyte activation has been established on the basis of a greater number of studies [48]. According to this pathway, T-lymphocytes are only stimulated when a stimulus is presented by accessory cells such as monocytes. Thus the first step in an immune response is the incorporation of an antigen or a mitogen into the membranes of accessory cells, mainly monocytes. Then the accessory cells bearing the antigen or mitogen interact with T-lymphocytes, triggering them to start proliferation. Attempts to separate lymphocytes and accessory cells have been made for several reasons. In basic research it is of interest to understand the mechanism of cellular immune response. In clinical practice it may be important to determine whether a low immune response is due to a decrease in antigen presentation activity or to a low proliferation capability of lymphocytes [49]. Furthermore the influence of drugs on both kinds of cells can be determined [29].

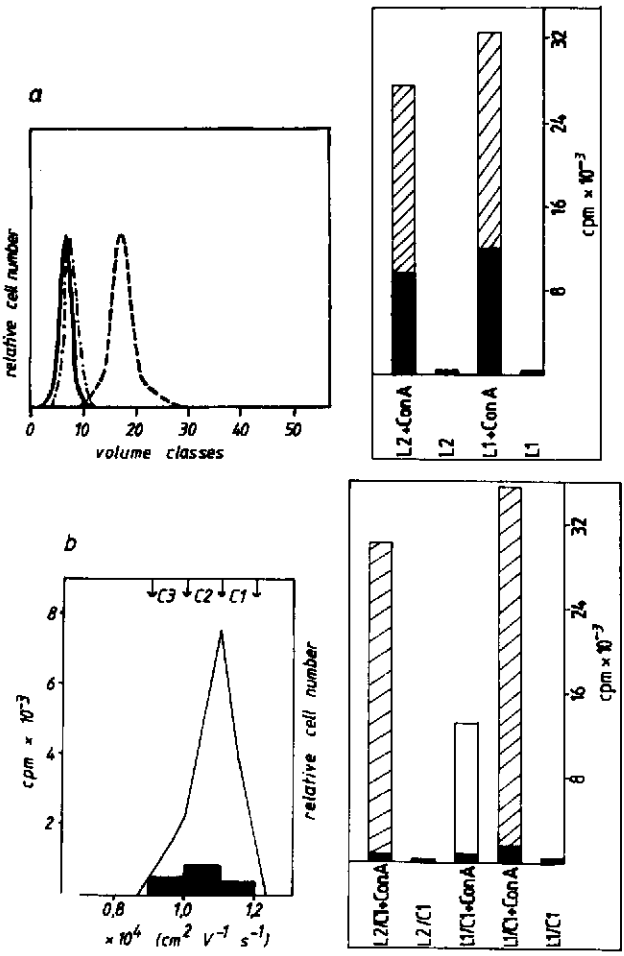


Fig. 8. (Left) Volume (a, solid line) and EM (b) distribution curves of the lymphocyte fraction L1 obtained by CCE. (Right) Con A-stimulated [^{14}C]thymidine incorporation activity of lymphocyte fraction L1 (a, black bars) and of the subfraction L1/C1, further purified after preseparation by free-flow electrophoresis (b, black bars). The hatched bars show the incorporation activity in the presence of monocytes.

We used physical methods to purify monocytes and responder lymphocytes (Fig. 6)[44]. Fig. 8b shows that, with the help of free-flow electrophoresis, a cellular subfraction L1/C1 was obtained, which did not respond to concanavalin A (Con A) in the absence of monocytes (black bars). When monocytes were re-added (hatched bars), the [^{14}C]thymidine incorporation was increased fifty-fold.

Monocytes induced a fifty-fold enhancement of [^{14}C]thymidine incorporation activity when the cells were suspended in a culture medium supplemented with 10% (v/v) fetal calf serum (FCS), but not when the culture medium was supplemented with human plasma (HP). Monocytes purified by physical methods to a degree of 98% had significantly less stimulation activity in HP media. Mono-

cytes preactivated by lipopolysaccharide before the stimulation experiment, however, had equal stimulation capacity in both kinds of media. Further investigations revealed that Con A is able to interact directly with T-lymphocytes, rendering them responsive to Interleukin 2 (IL-2) (first step in lymphocyte activation, see ref. 48) and that human plasma contains a protein that prevents direct interaction of Con A with the T-cell receptor [50]. This plasma protein, which prevents an immune response outside the control of activated monocytes, could only be detected because, during our isolation procedure, monocytes from healthy human beings retained their resting status and lymphocytes were not challenged to produce factors that could activate the monocytes.

All experiments on monocytes have shown that this cell population, which is very susceptible to stimulating signals, can be purified to homogeneity by physical cell separation methods without their resting status being changed. This resting state seems to be near to the in vivo status of monocytes in healthy people. Thus monocytes isolated by physical methods are very suitable for studying the first steps in an immune response and for determining the prevailing in vivo status of cells for diagnostic purposes.

4.1.2. Lymphocytes

Purified responder T-lymphocytes did not proliferate in the absence of accessory cells, but they were triggered by Con A or phytohaemagglutinin (PHA) to express IL-2 receptors [44,48]. T-lymphocytes bearing IL-2 receptors can be used to determine the concentration of IL-2 in plasma or other body fluids. Determination of IL-2 is already carried out in medical practice. The usual test cells are cloned T-lymphocytes. T-responder cells isolated from peripheral blood by physical methods could offer an alternative test system.

Fig. 8 shows that free-flow electrophoresis is required to purify responder T-lymphocytes. The lymphocyte fraction still showed significant response to Con A after the CCE purification step (Fig. 8a). The responsiveness appeared to be due to dendritic cells [51], which have a similar volume to T-lymphocytes. Since these cells have a different EM, they can be separated from the responder T-lymphocytes by free-flow electrophoresis. Little is known about human dendritic cells because their frequency in peripheral blood is low (Table 1) and it is very difficult to purify them. Studies on animal dendritic cells, however, revealed that these cells play a very important role in the immune system. Free-flow electrophoresis could become a help in investigations of dendritic cells.

4.1.3. Platelets

There is another cell population in peripheral blood, in addition to monocytes, which is very susceptible to activation signals. These are the platelets. They have the function of aggregating around injuries and stopping bleeding [52,53]. When platelets are activated a lot of intracellular functions are turned on. Platelet dysfunction can have a severe effect on the health of humans, so several clinical tests of platelet function are performed. Usually platelets are isolated by several steps of centrifugation but, during centrifugation, they often become aggregated. This itself may induce activation signals. Platelets have an EM of $0.9 \cdot 10^{-4}$ cm^2 V^{-1}

s^{-1}, whereas erythrocytes and some leukocyte populations have an EM of ca. $1.1 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}. Cell electrophoresis has been used to isolate platelets from whole blood samples in the scaled-up free-flow electrophoresis apparatus manufactured by CJB Developments. One of the cell fractions obtained after a short time had a high frequency of platelets (personal communication). The electrophoresis of whole blood samples might be a way to enrich platelets without centrifugation.

4.1.4. Erythrocytes

Erythrocytes from healthy humans show a very homogeneous EM distribution curve with a peak at $1.1 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}. Abnormal erythrocytes can be recognized by a change in their EM distribution curve. For example, hyperagglutinating red blood cells were electrophoretically separated from normal cells [18]. Free-flow electrophoresis has also been applied in malaria research. In investigations of this disease, *Plasmodium falciparum* is cultured in the presence of human erythrocytes. When the *Plasmodia* are harvested for further investigations, the human erythrocytes have to be lysed and the *Plasmodia* released from the erythrocytes separated from the erythrocyte ghosts. Heidrich et al. [54] succeeded in separating erythrocyte ghosts and *P. falciparum* by free-flow electrophoresis.

4.2. Preseparation of cells for immunological characterization

The width of the EM distribution curve of a homogeneous cellular subpopulation is narrower than the width of the EM distribution curve of a mixed cell fraction, such as the MNLs or bone marrow cells [55,56]. Rychly et al. [55] analysed the EM distribution curve of the total MNLs and showed that it is composed of several EM distribution curves belonging to the subpopulations. The EM distribution curves of the subpopulations overlap so that purification of a distinct subpopulation is often impossible. Even so enrichment can often be achieved.

In clinical diagnosis it is useful to determine the type and amount of antigen markers expressed on the surface of distinct cell populations. Many monoclonal antibodies are offered commercially for this purpose. Usually the binding of antibodies to the cell surface is measured by fluorescence microscopy and flow cytometry [57,58], but there are cases when the frequency of the target cells is so low that reproducible measurements cannot be made. Especially in early stages of diseases or malignancies it is difficult to get reliable results. Presorting of cells by free-flow electrophoresis has proved useful for enhancing the efficiency of these methods [59].

4.2.1. Human B-lymphocytes

An example of pre-enrichment of cells is the enrichment of human B-lymphocytes [60]. The frequency of human B-lymphocytes in the MNL fraction is ca. 15%. Immunological determination of this cell population is not easy because human monocytes are able to bind antibodies unspecifically via their Fc receptors. This may explain why the EM of human B-lymphocytes was a matter of

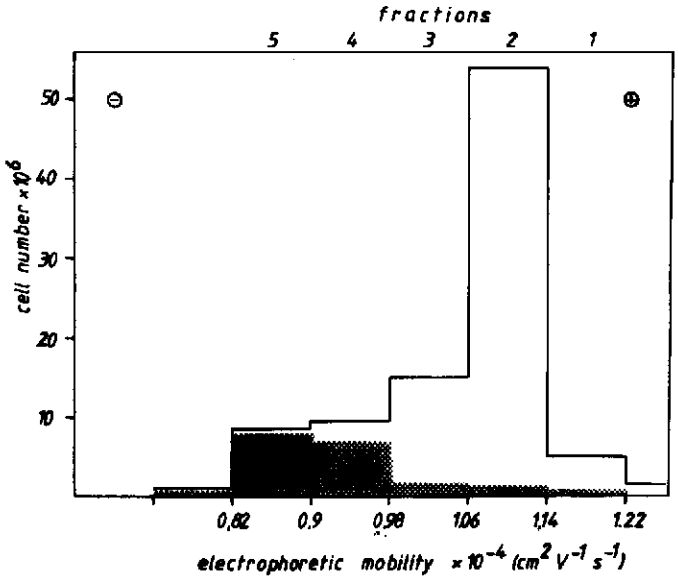


Fig. 9. EM distribution curve of a group of lymphocytes with volume of ca. 165 μm^3 (solid line). The dotted area shows the distribution of Ig^+ cells (B-lymphocytes).

controversy for a long time [14,61,62]. We reinvestigated the EM of resting B-lymphocytes from peripheral blood. For this purpose the MNL were preseparated by CCE before the Ig^+ B-lymphocytes were investigated. A group of lymphocytes was obtained by CCE at a counter flow-rate of 16 ml/min, which contained 25% Ig^+ cells (lymphocytes bearing immunoglobulins) but no monocytes [63]. The mean volume (165 μm^3) of these cells was smaller than that of unseparated lymphocytes. These cells were further separated by free-flow electrophoresis, and the proportion of Ig^+ cells in each electrophoresis fraction was determined. Fig. 9 shows that 70–80% of the lymphocytes in the electrophoresis fractions 4 and 5 ($EM = 0.82–0.98 \cdot 10^{-4}\ cm^2\ V^{-1}\ s^{-1}$) were Ig^+ cells (dotted area), whereas fractions 1 and 2 contained less than 5% Ig^+ cells. A cell fraction containing 70% of B-lymphocytes can be better characterized by flow cytometry than the unseparated fraction (Fig. 10).

4.2.2. Leukemic cells

The exact diagnosis and classification of leukemias are decisive prerequisites for the selection of optimum therapies, and in this context immunological analysis of surface markers has become an important diagnostic tool. With the aid of monoclonal antibodies it has become possible to identify a variety of membrane antigens and to gain information regarding the degree of differentiation and origin of cells. Leukemic cells have also been studied extensively by analytical cell electrophoresis [55,64–66]. Both leukemic cells of lymphoid malignancies and the corresponding hematopoetic cell lines retain the EM of the original cell, so that leukemic cells of different origins can be distinguished on the basis of their EM. The EMs of different kinds of leukemic cells are shown in ref. 55. Leukemic

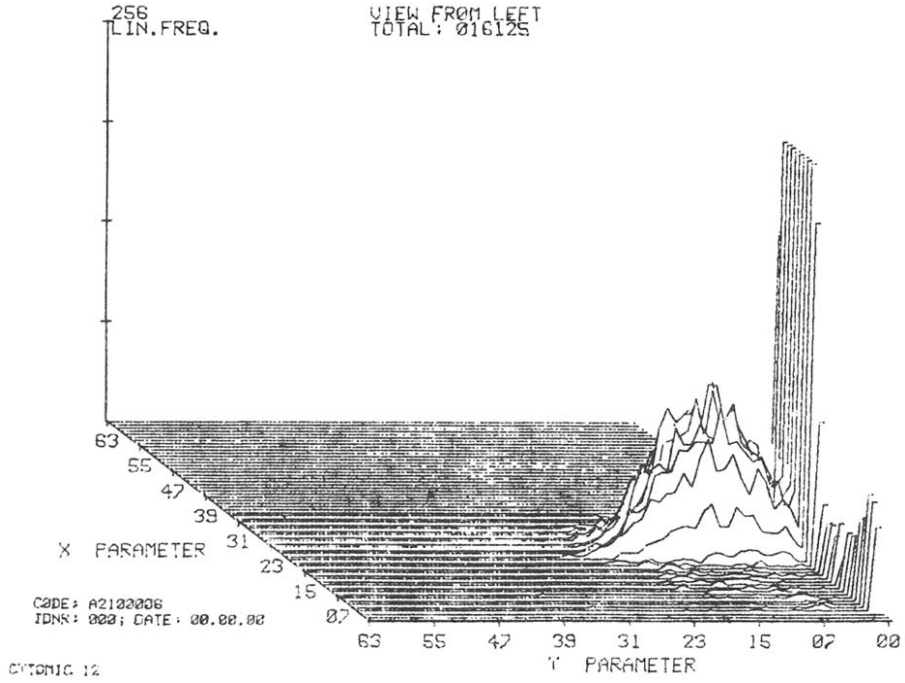


Fig. 10. Flow cytometry measurement of the cells of the fractions 4 and 5 shown in Fig. 9. The cells were stained by fluorescein isothiocyanate-labelled anti-Ig antibodies and measured in a Fluvo-Metricell [83]. The volume is shown by the x parameter, the fluorescence by the y parameter.

cells have a narrow unimodal distribution curve like that of homogeneous cellular subpopulations. This means that the width of the distribution curve of leukemic cells is narrower than the width of the distribution curve of the total mononuclear cell fraction. Thus cell electrophoresis may be useful in the diagnosis of leukemias in two ways: (i) the EM of leukemic cells may be used as an additional marker in a multicentric analysis of leukemic cells; (ii) in early stages of leukemia the frequency of the leukemic cells in the cell sample can be increased by separating a total MNL or lymphnode fraction by preparative cell electrophoresis. As mentioned above, better analysis of cells can be performed by flow cytometry when the frequency of the target cells is higher.

4.2.3. Cancer cells

It seems to be a general rule that cells retain their EM during proliferation. This has been shown for leukemic cells and for cells stimulated by Con A or by pokeweed mitogen (PWM) [55,60,64–67]. In animal models it was also found that the width of the EM distribution curve of cancer cells is as narrow as the width of homogeneous cell populations. Miller et al. [68] isolated specific types of cells that emerged during chemical hepatocarcinogenesis in livers of carcinogen treated rats, and Hannig and Wrba [69] were able to separate malignant and normal cells from ascites fluid from rats. In this case the isolated cells were reimplanted into other rats, and the cell fraction containing the malignant cells determined. On the basis of these experiments it seems that it might also be possible

to enrich human cancer cells from different organs. Especially in the early stages of malignancy such an enrichment of cancer cells might be of help in further analyses by monoclonal antibodies.

4.3. Enrichment of cells that secrete products for use in medical therapy

Cell products useful for medical therapy are obtained in two ways. Either they are isolated from serum or other body fluids, or they are obtained from cells that produce the substances under culture conditions. For several reasons the latter method is becoming increasingly important. For in vitro production of distinct substances the cells that secrete the desired product have to be immortalized and cloned. Immortalization can be achieved by transformation of the cells or by hybridization of the cells with suitable cancer cells [6,70]. The chance of performing successful immortalization and obtaining clones is better if cell fractions with a high frequency of the desired cells are available. Thus it might be helpful to enrich the cells of interest, before performing further experiments.

4.3.1. Kidney cells

Earlier studies showed that rabbit kidney cells have a very broad EM distribution curve. Within this distribution curve only the cells in the fast mobility fractions produce renin [71]. Recently an American group of investigators, who have developed a scaled-up free-flow electrophoresis system, investigated human embryonic kidney cells [17,20,72]. They cultured the cells under conditions in which they produced urokinase and other plasminogen activators (PAs). When the number of the cells in the primary cultures was enough, the cells were harvested and electrophoresed. During electrophoresis they were separated into as many as 31 fractions that produced PAs. Of these fractions, five or seven produced high levels of PA and some fractions appeared to produce predominantly the high- or low-molecular-mass forms of urokinase. The separation experiments were performed both on the ground and in the space shuttle. The aim of the space shuttle experiments was to increase the throughput of the cell electrophoresis. The free-flow electrophoresis had greater separation capabilities in the space shuttle. The distribution of the separated cell subpopulations appeared to be broader compared with on the ground, although the separating conditions were essentially the same. The throughput was ten times higher than on the ground. The viability was significantly lower than in the ground experiments. After separation, long-term cultures were set up, and 75% of the 400 separated kidney cell cultures survived.

It is important for medical practice to get high numbers of kidney cells that produce large amounts of PA. However, a ten-fold enhancement in throughput is unlikely to be of such importance that it would justify the expense of using the space shuttle in routine experiments. A sufficient enrichment of PA-producing kidney cells can probably be achieved on the ground using other cell electrophoresis machines.

4.3.2. Human antibody-secreting cells

We have attempted enriching human antibody-secreting cells using the free-flow electrophoresis apparatus ACE 710 [60]. Antibodies, particularly monoclonal antibodies, are important tools in medical diagnosis and therapy. Production of mouse monoclonal antibodies is a well established technique [6]. Mouse monoclonal antibodies, however, cannot be used for therapy, because they cause an immune response in the host. Thus efforts were made to obtain human monoclonal antibodies.

Antibodies are produced by B-lymphocytes. Investigation of B-lymphocytes is of interest in basic research [73]. B-Lymphocyte activation is also of medical interest, because B-lymphocytes may produce antibodies useful in patient therapy. In order to obtain large amounts of human antibodies against defined antigens it is necessary to stimulate resting B-lymphocytes and to clone the activated B-lymphocytes that produce the desired antibody. Cloning of human antibody-secreting cells is still very difficult. The major problem is that the frequency of cells that produce a desired antibody is often too low for successful immortalization.

The main reason why human antibody-secreting cells are so infrequent is that only lymphocytes from human peripheral blood can be stimulated in vitro. In vitro stimulation of B-lymphocytes by distinct antigens is possible [74], but only a few antigens, such as sheep red blood cells (SRBCs), trigger antibody production. Other antibodies are obtained in vitro when B-lymphocytes are stimulated by mitogens such as PWM. During such stimulation experiments a great number of cells is obtained, secreting many kinds of antibodies. Amongst the great number of antibody-secreting cells obtained after PWM stimulation, there are sometimes cells that produce antibodies against defined antigens. Antibodies against SRBCs have been found in many experiments, but antibodies against hepatitis B surface antigens or *P. falciparum* have also been detected [75,76].

An important step toward the goal of obtaining human monoclonal antibodies would be to increase the frequency of cells secreting a desired antibody. High numbers of antibody-secreting cells can be obtained when either the B-lymphocytes bearing the genetic information for production of the desired antibody are enriched before stimulation, or the antibody-secreting cells are enriched after stimulation. We have tried both methods.

First, we enriched antibody-secreting cells after stimulation. To obtain antibody-secreting cells the small B-lymphocytes (165 μm^3) were cultured in the presence of T-lymphocytes, monocytes and PWM [60,77,81]. After seven days of incubation the stimulated lymphocyte–monocyte mixtures released antibodies into their supernatants.

In order to determine the EM of the cells that released human antibodies into the supernatants, the viable cells were isolated after seven days of incubation and separated by free-flow electrophoresis. The cells of each fraction were then counted (Fig. 11a), resuspended in 1 ml of culture medium, and incubated for another two days. The supernatants from the different cell fractions were screened for human antibodies. During this additional period of incubation only cells in fractions 4 and 5 produced human antibodies (Fig. 11b, solid lines). The cells in

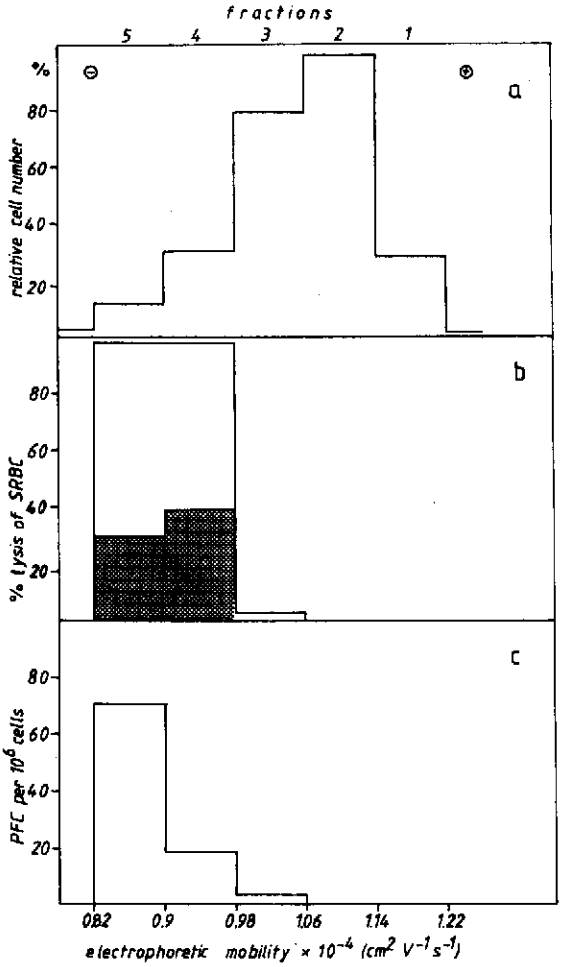


Fig. 11. EM distribution curves of the total cell population isolated from PWM cultures (a), of antibody-secreting cells (b) and of plaque-forming cells (c). The solid line of panel b shows the antibody-dependent lysis of protein A-coated SRBCs, and the dotted area shows the lysis of uncoated SRBCs. The antibodies were produced either by $2 \cdot 10^5$ cells (fraction 5) or by 10^6 cells (fractions 1-4).

fractions 1 and 2 never released antibodies into their supernatants, even when more than $5 \cdot 10^6$ cells were incubated. Thus we concluded that antibody-secreting cells had an EM of ca. $0.9 \cdot 10^{-4}\ cm^2\ V^{-1}\ s^{-1}$. The greater part of the non-secreting cells from PWM cultures had a higher EM. This suggested that the fractions of low EM may be enriched in antibody-secreting cells.

In order to determine the factor of enrichment, cell cultures were selected whose supernatants contained anti-SRBC antibodies. When these cells were electrophoresed and the cells of the different fractions further incubated, anti-SRBC antibodies were only produced by the cells in fractions 4 and 5 (Fig. 11b, dotted area). This indicated that cells that secreted distinct antibodies had a narrow distribution within the distribution profile of the total cell population. The fre-

quency of the cells that secreted anti-SRBC antibodies was determined by performing plaque assays. An aliquot of the cultured cells was assayed immediately after harvesting. The rest of the cells were separated by free-flow electrophoresis and the cells in each fraction investigated. Only fractions 4 and 5 contained plaque-forming cells (PFCs) (Fig. 11c). The frequency of the PFCs in fraction 5 was nine times higher than in the corresponding unfractionated population. Thus free-flow electrophoresis can be used to enrich cells producing defined antibodies. Although the enrichment factor of 9 may seem to be low it can, for example, mean that only 1000 culture supernatants have to be screened instead of 9000. This would seem to be a considerable advantage.

The identity of the EM of resting B-lymphocytes and antibody-secreting cells (Figs. 9 and 11) indicates that human B-lymphocytes, like mouse B-lymphocytes [79], maintain their EM during development to antibody-secreting cells. The EM of antibody-secreting cells that we found during this study rendered an earlier consideration by Hannig and Kruesmann [80] very interesting. They claimed that the electrophoretic slower moving fractions from freshly isolated human lymphocytes might contain antibody-secreting cells.

In order to test this speculation we searched for MNL fractions that produced antibodies when cultured for a short time in the absence of a mitogen. From the literature [81] it was known that human peripheral blood contains small and large B-lymphocytes, and that only the large B-lymphocytes bear surface markers specific for activated B-lymphocytes. Thus the MNLs were fractionated according to their volume by CCE. The small B-lymphocytes with a volume of 165 μm^3 and an EM of $0.9 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1} left the elutriator at a counter flow-rate of 16 ml/min. The large B-lymphocytes were eluted from the CCE together with monocytes at a counter flow-rate of 25 ml/min. The large B-lymphocytes, but not the small ones, had a high tendency to secrete antibodies when cultured for a few days in the absence of a mitogen. The CCE fraction containing the large B-lymphocytes was further subdivided into fast-moving and slow-moving subfractions by free flow-electrophoresis. The cell fractions were suspended in culture medium and incubated. After seven days of incubation the supernatants were assayed for IgG and IgM. The results revealed that the fast-moving large B-lymphocytes produce mainly IgG molecules, whereas the slow-moving large B-lymphocytes produce IgM (Fig. 12, manuscript in preparation). IgG-producing cells are memory cells. This means that they have already been stimulated in vivo by a distinct antigen. Thus it might be speculated that B-lymphocytes from survivors of complex diseases can be enriched with the help of CCE and free-flow electrophoresis.

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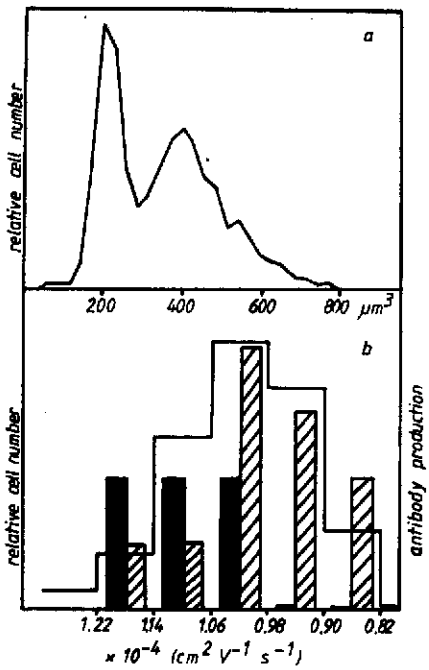

Fig. 12. Volume (a) and EM (b) distribution curves of a CCE fraction obtained at a counter flow-rate of 25 ml/min. The bars in panel b show the IgG (black bars) and IgM (hatched bars) production of $6 \cdot 10^5$ cells of the respective fractions.

6. SUMMARY

There are several kinds of cell electrophoresis. The most important are free-flow electrophoresis, scaled-up free-flow electrophoresis and column electrophoresis. All kinds of cell electrophoresis, especially free-flow electrophoresis, have been improved to a very high standard of separation accuracy, and their application possibilities are extended when antigen-specific electrophoretic cell separation is performed, or when cell electrophoresis is combined with other physical methods for cell separation. Cell electrophoresis and other physical cell separation methods have the advantage that the functional state of the cells remains virtually unchanged during the isolation procedure. With the help of cell electrophoresis monocytes, T-lymphocytes, platelets and other cells from human peripheral blood could be purified. Other human cells were enriched for immunological characterization. Furthermore, cells that secreted human plasminogen activators or human antibodies were electrophoresed to give cell fractions with increased frequencies of the cells of interest.

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