〔教育講演Ⅱ〕

Biomedical and clinical applications of automated
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SUMMARY

The use of automated devices in the last ten years have made biomedical applications of cell electrophoresis more attractive. We are using routinely the automated single cell electrophoresis microscope PARMOQUANT. Using the PARMOQUANT we applied this method to discriminate lymphocytes and study the interaction of substances with cells and synthetic particles. Electrophoretic histograms allowed the determination of changes in the proportion of lymphocyte populations after kidney transplantation, during dialysis treatment, open heart surgery and during pregnancy. Discrimination of leukemic cells on the basis of electrophoresis was used as an additional parameter in diagnosis. In a mouse tumor model histograms determination enabled evaluation of the *in vivo* effect of tumor necrosis factor on immune cells. Cell electrophoresis was shown to be suitable to detect the influence of antibodies, lectins and bacteria on the cell surface. Protein adsorption was studied on synthetic particle using cell electrophoresis. This method was applied in investigating the phenomena of blood interaction with biomaterials for use in artificial organs and to determine differences in the protein composition in serum or other body fluids connected with diseases. On the basis of this principle a test to detect heterozygotes in cystic fibrosis is now in progress.

INTRODUCTION

Cell electrophoresis is a simple, classical biophysical measuring method to determine the net surface charge density of small particles suspended in an electrolyte solution and likewise cells suspended in a physiological medium. The application of this method in many different fields is extensively described in literature (for review see Ref. 1-3). In principle, on the basis of differences of surface charge density, it is possible to characterize various cell types and their subpopulations and furthermore to determine the relative proportion of subpopula-

tions present. Cell electrophoresis may also be used to detect the influence of substances on cells or particles. Under certain circumstances it is possible to relate this physical parameter to biological functions and/or pathological changes of cells. In the seventies many applications in different clinical fields and especially the so-called Magrophage-Electrophoresis-Mobility-Test for detection of cancer⁴⁾ stimulated the development of automated measuring techniques with high accuracy and productivity. On the basis of this technical progress many groups tried to extend the field of application of cell electrophoresis for biomedical,

自動化細胞電気泳動法の生物医学的および臨床的応用

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clinical and basic research. Results obtained by our interdisciplinary group at the Department of Internal Medicine will be described in summarized form in this contribution.

TECHNIQUES

The measurement of the electrophoretic mobility (EPM) of biological cells has been traditionally carried out with analytical electrophoretic equipments that require visual and manual timing of migrating cells in a microscope. This technique is to slow, of limited accuracy, and extremely wearing on those making the measurements. Investigating cells with small changes of mobility, in comparison to controls, is limited by the subjective selection of cells.

Several working groups tried to automate the process of cell electrophoresis by applying different physical principles and updated versions of devices in order to expand the suitability of this biophysical method. The different measuring principles applied can be distinguished into two groups:

a) The electrophoretic movement of cells stabilized in zones by different procedures can be followed macroscopically. These techniques can be used for preparative purposes. Automated tracking of the cell zones are possible:

- Free Flow Electrophoresis
- Stabile Flow Free Boundary Electrophoresis
- Endless Fluid Belt Electrophoresis
- Free Zone Electrophoresis
- Density Gradient Electrophoresis

b) Single cells moving only within microscopic distances are measured in a short time by direct and indirect methods:

- Laser Doppler Electrophoresis
- Image Transduction
- Video Signal Analysis
- Image Processing

The most frequently used techniques capable of measuring electrophoretic mobilities of many cells in a short time are based on the Laser-Doppler principle introduced by Ware⁵⁾ and on Free Flow Electrophoresis as developed by Hannig⁶⁾ with a

slit scanning photometric technique used to detect the cell path optically. Goetz⁷⁾ has invented a technique for transducing the focussed microscopic image to a modulated signal of scattered light intensity as a function of migration velocity. He combined the measuring system with a sample preparation system in order to measure the electrophoretic mobility of particles in dependence of pH value and conductivity automatically (fingerprints).⁸⁾ All these principles are suitable for measuring submicroscopic particles suspended in solutions with low ionic strength. Since the intensity of light scattered at a given angle is influenced by the size, shape, orientation and refractive index of the cell, the interpretation of frequency distributions from an unknown population may be inaccurate. These are disadvantages with negative consequences for the study of cells with different sizes in one suspension. Finally no absolute mobility values from individual cells can be obtained. Therefore attempts have been made to compensate for these disadvantages by projecting the cells moving in classical electrophoretic chambers on a TV screen and using automated electronic timing devices. The automated analytical electrophoretic microscope developed under NASA contract by Bartels, Bier, Brooks and Seaman⁹⁾ permits rapid multiple velocity measurements per cell to be taken resulting in reliable determinations of electrophoretic mobilities. In our PARMOQUANT, developed together with Carl Zeiss Jena (licensed to Kureha Chem. Ltd., Tokyo), individual cells which are sharply focussed in dark field illumination are tracked automatically using a computer-controlled video-system. Cells within a depth range of $\pm 5-8\mu\text{m}$ around the stationary layer are selected objectively. In order to estimate the migration speed, the position and area of all sharply imaged cells with a constant area will be recorded simultaneously every $1/3$ of a second. The absolute electrophoretic mobility of many cells can be measured quickly and accurately.¹⁰⁾ Repeated measurements on single cells shows an accuracy of $\pm 1\%$ during a migration time of 2-3 s. The lowest relative standard

deviation obtained repeatedly for erythrocytes from a certain donor was below $\pm 2\%$. The measuring time necessary for obtaining a frequency distribution for 500 mobility values is some 10-12 min.

BIOLOGICAL APPLICATIONS

1. Investigation of cells in basic research

Under constant conditions different cell types and the same cell type of various species show mostly separated electrophoretic mobilities (EPM) thus enabling the detection of different cell types using cell electrophoresis. Lymphoid cells in mice reveal a distribution with electrophoretically separated peaks which were identified as being lymphocyte subpopulations. Electrophoretic measurements of density gradient separated human mononuclear cells reveal a histogram of heterogeneously distributed cells regarding the EPM. In our laboratory a dissection of such electrophoretic histograms into immunologically defined lymphocyte subpopulations was obtained using monoclonal antibodies against surface markers in a complement dependent lysis assay combined with electrophoretic measurements.¹¹⁾ In this way a correlation between the EPM and different lymphocyte subpopulations as well as monocytes were obtained. Interestingly, CD 4- and CD 8-lymphocytes also differed in their EPM. Thus, alterations of the percentage of lymphocyte subpopulations in patients can be simply detected by the electrophoretic method.

2. Analysis of mononuclear cells

For the determination of changes in the percentages of subpopulations in diseases and different altered physiological conditions we set borderlines between high and low mobility cells (T cells and B cells plus monocytes respectively) and between fast and slow T cells (CD 4 and CD 8 cells respectively) in the electrophoretic histograms. In this way we detected changes in the percentage of T cells after kidney transplantation, after open heart surgery, during haemodialysis, during pregnancy, and in cord blood.^{12,13)} Changes were also found in the ratio of fast and slow T-cells which we regarded as an alteration of the immunoregulatory

Fig. 1. Electrophoretic histograms before (above) and after (below) open heart surgery and the relationship to the immunological phenotyping.

quotient CD 4/CD 8. Figure 1 demonstrates the correlation between histogram and immunophenotyping after open heart surgery.

Although it is not possible to determine the exact percentage of each cell subpopulation the method allows detection of the degree of change in the composition of different lymphocyte populations by one single measurement and is mainly suitable in follow up studies.

As with human mononuclear cells electrophoretic histograms of spleen cells, lymph node cells and thymocytes from mice enable the percentage of different lymphocyte subpopulations to be evaluated. We used this possibility in a comprehensive study to evaluate the effect of tumor necrosis factor (TNF) in a mouse tumor model. Histogram measurements revealed an increased cell population with intermediate EPM in the spleen of tumor mice which was reduced by treatment with TNF.¹⁴⁾ Changes were also found in the cell composition of thymus and lymph node by TNF *in vivo* which

Table 1. Range of electrophoretic mobilities of leukemic blasts in acute leukemias.

ALL-subtypes		AML-subtypes	
T-ALL	1.07-1.20	M 2	1.15-1.20
C-ALL	0.97-1.01	M 4	1.09-1.15
pre-B-ALL	0.90-0.92	M 5	0.88-1.02
B-ALL	below 0.88		

enable us to draw conclusions regarding the influence of TNF on the immune cells.¹⁵⁾

3. Characterization of cells in leukemias

We evaluated the suitability of the EPM as a parameter in leukemic diagnosis.¹³⁾ In acute lymphocytic leukemias a subtyping which correlates with immunological phenotypes is possible. In acute myeloid leukemias the FAB (French American British) type M 2 can be discriminated from the M 4 type and also the M 5 type has a distinct EPM (Table 1). In these leukemias the EPM can be introduced as an objective parameter to support the more subjective diagnosis according to the FAB classification.

In chronic leukemias a discrimination can be made between T and B leukemias and it seems possible to discriminate the hairy cell leukemia from the B-CLL.¹⁶⁾ From our experiments we conclude that the EPM is a suitable parameter in a multiparameter diagnostic protocol for leukemias.

4. Electrophoretic investigation of interactions with the cell membrane

The second general application of cell electrophoresis is the detection of interaction of cells with substances *in vitro* such as antibodies, antigens, and lectins.

The interaction of the cell membrane with specific antibodies leads to changes in the surface charge density and thus in the EPM. In this way we obtained information about binding sites for antibodies, kinetic of antibody binding and the mechanism underlying changes of the surface charge after antibody interaction.¹³⁾ Moreover, the specific action of antibodies on the surface charge of cells corresponds to immunological markers and can be used for detecting the antibody specificity during

antisera production.¹⁷⁾ The detection of monoclonal antibodies by electrophoresis is more difficult and requires indirect procedures.

The electrophoretic method can also be applied to the antigen/antibody detection on synthetic microspheres. In contrast to usual methods for the antigen/antibody detection the cell electrophoretic approach requires no labelling of the substances involved, whereas its sensitivity is lower than those of other methods.

The simple cell electrophoretic detection of antigen-cell interaction can be used for the characterization of sensitized lymphocytes. The EPM of sensitized lymphocytes changes after *in vitro* contact with the specific antigen.¹³⁾ Considering the disadvantages of immunological methods for detecting lymphocyte sensitization this simple electrophoretic approach is worth consideration.

Electrophoretic investigations of the interaction of lectins with the cell surface were mainly performed with mitogens for lymphocytes, like Con A and PHA in connection with their stimulatory effects on cells.¹⁸⁾ In our electrophoresis studies we used peanut lectins (PNA) and wheat germ agglutinin (WGA) to identify and discriminate cell populations. PNA reduced the EPM of immature cortical thymocytes whereas of mature T and B cells only after treatment with neuraminidase.¹⁹⁾ WGA had a dramatic effect at very low concentration on the electrophoretic mobility of various human cells tested. Quantitative differences in this parameter between cell types we discussed in connection with differences in the structure or amount of surface carbohydrates which is related to the biological behaviour of cells such as homing or invasion (Rychly *et al.*, submitted).

5. Assessment of parameters of biocompatibility

A further field for application of cell electrophoresis is that concerned with investigations of blood compatibility above all at the level of blood-material interaction.

There are several means by which cell electrophoresis can be applied to contribute to the assess-

ment of selected parameters of blood compatibility.

It is possible :

- to determine changes in subpopulations of lymphoid cells after *in vitro* and *in vivo* bio-material-blood contact,
- to detect the presence of released cytokines following *in vitro* mononuclear cell (MNC) —biomaterial contact,
- to investigate protein adsorption behaviour on latex particles of different surface structure.

Characterization of lymphoid cells

Using cell electrophoresis it is possible to determine the relative proportions of immunologically defined subpopulations of human peripheral blood lymphocytes (PBL) and mouse spleen cells in a frequency distribution because of their different EPM as described above. Above all the electrophoretically low mobility cells are the adherent cells and therefore *in vitro* investigations of lymphoid cells enable assertions with regard to the adherence behaviour of lymphocyte subpopulations as well as in *in vitro* and during extracorporeal circulation.^{20, 21)} Our investigations in this direction concern 1. *in vitro* investigations of the adherence behaviour of isolated mouse spleen cells following incubation of these cells with different biomaterials²⁰⁾ and 2. *ex vivo* investigations of the behaviour of PBL of uremic patients before, during and after hemodialysis treatment in dependence on the type of dialyzer used.²¹⁾ The PBL-histogram of a renal disease treated (RDT) patient during hemodialysis using a Cuprophane dialyzer and the influence of different kinds of dialysis membranes on the relative proportion of low mobility cells in the PBL-histogram during the first 15 min are summarized in Fig. 2. These results correlate with the increase of the C3a desArg plasma concentration and the decrease of total count of neutrophils.²²⁾

These studies indicate that the application of cell electrophoresis is a useful method for the characterization of lymphoid cells during extracorporeal circulation which may be an additional parameter of blood compatibility.

Fig. 2. Electrophoretic histograms of peripheral blood lymphocytes of a dialysis patient in dependence of time of dialysis using a Cuprophane dialyzer (above) and relative proportion of low mobility cells in histograms of peripheral blood lymphocytes of uremic patients during hemodialysis using different types of dialyzers (related to initial value).

Detection of cytokines

Cell electrophoresis is a simple method for the non-specific detection of cytokines/cell products released/generated from mononuclear cells following incubation with a variety of stimulators. This so-called electrophoretic mobility (EM) test can be used as an *in vitro* assay for cellular immunity.⁴⁾ The EM-test consists of two independent steps. 1. Induction of the possible release of cell products from cells by *in vitro* incubation of cells with stimulators (in our case small strips of membranes). 2. The subsequent detection of those cytokines in the cell free supernatant by means of cell electrophoresis using indicator particles (e. g. tanned sheep

red blood cells, latex particles or macrophages). There is a stronger decrease in the EPM of indicator particles in the presence of released cytokines in the supernatant than in the corresponding controls (incubation without stimulators). To answer the question does the contact of MNC with a biomaterial lead to the release of cytokines, the EM-test principle was used with mouse spleen cells as a source of MNC. Investigations with different dialysis membranes are shown in Fig. 3 and demonstrate clearly the different behaviour of dialysis membranes with regard to the measurable release of cytokines from mononuclear cells in dependence on both the surface area and the chemical structure of the membrane. Results shown were obtained independently using different types of indicator particles. It has furthermore been shown that the charge changing cytokines are closely connected to the interleukins (Thomaneck *et al.* submitted for publication).²³⁾

We can conclude that the EM-test is a relatively simple but non-specific *in vitro* test system for the measurement of released charge changing cytokines. The advantage of this method lies in its ability to measure the combined effect of all released cell products on target cells.

6. Investigation of synthetic particles and protein adsorption

The electrical surface charge density is an important parameter which influences the interaction of synthetic particles with substances. This includes action of particles in biological experiments (protein adsorption) and clinical practice (latex agglutination test, phagocytosis). A considerable progress for medical use is the procedure of electrokinetic fingerprinting, which is a new *in situ* analytical technique for characterizing the surface of polymer microspheres.⁸⁾ This technique requires the measurement of the EPM of particles over all pertinent electrochemical variables and allows one to produce representations of EPM as a function of both potential determining ion (pdl) concentration and overall ionic transport properties (measured to a first approximation by the specific conductivity). Analysis of

the data produces a mobility-pdl concentration profile from which it is possible to correctly differentiate surface functional groups and ion adsorption. We have applied this technique successfully to evaluation of latex microspheres which were used for body fluid tests.

The understanding and control of interaction of proteins with solid surfaces is important in a number of fields such as pharmacy, protein processing, biotechnology and biomaterial research. In contrast to other methods such as radioisotope labelling the very simple method of particle electrophoresis allows rapid, direct measurements without modification of molecules involved. The adsorption of proteins on synthetic particles (e.g. latex particles, glass particles) is manifest by changes in the electrical surface charge density of the particles compared to bare particles and thus altered EPM. Most protein-surface pairs exhibit a limiting surface concentration corresponding presumably to the capacity of the surface to accommodate protein. Thus adsorption isotherms showing EPMs of protein coated particles as a function of the solution concentration of protein, approach a plateau (Fig. 4).²⁴⁾ It was shown that important plasma proteins have different electrophoretic plateaus and thus electrophoretic measurements can detect adsorption of competing proteins on solid particles. In addition, particle

Fig. 3. Cytokine release as a function of membrane area following *in vitro* incubation of mouse spleen cells with different dialysis membranes.

Fig. 4. Adsorption of plasma proteins on polystyrene latex particles (○) and glass particles (●).
Electrophoretic mobility versus protein concentration.

electrophoresis was used to study the dynamic behaviour of protein adsorption layers and the specific interaction of antibodies with adsorbed proteins.¹³⁾ Regarding the structure and orientation of polymer bound proteins a model for monolayer binding to identical, non-interacting sites was developed which corresponds well to experimental electrophoretic data.²⁵⁾

7. Detection of cystic fibrosis gene carriers

Based on adsorption processes mentioned above a simple method for detecting pathological changes in body fluids has been developed.¹³⁾ The principle of this method is based on the observation that pathological changes of body fluids cause modified adsorption layers on microspheres and consequently changes of the surface electrical charge/electrophoretic mobility. The procedure is simple, rapid and requires small amounts of body fluids. Briefly, defined amounts of body fluids (such as serum) are added to a suspension of synthetic particles which

Fig. 5. Electrophoretic mobility of polystyrene latex particles as a function of immunoglobulin G (IgG) concentration in phosphate buffered saline: IgG prepared ● from serum of healthy subjects, IgG prepared from serum of cystic fibrosis ▲₁ patients, IgG prepared from serum of cystic fibrosis heterozygotes ▲₂

will be measured after 3 min incubation time. We have mainly focused on cystic fibrosis (CF), the most common inherited disease in the Caucasian population. Particle electrophoresis is capable of detecting differences in the EPM of microspheres incubated with serum of CF homozygotes and heterozygotes on the one hand and serum from control subjects including various diseases on the other hand.

A summary of results from our clinical studies including 49 CF patients, 61 CF heterozygotes and 250 control subjects reveals that compared to DNA analysis an exact diagnosis was made in 85–90% of all cases. The screening of CF heterozygotes which is an important problem is not possible at present and in the near future. For that reason the simple electrophoretic approach for diagnosing CF heterozygotes may become valuable in clinical practice.²⁶⁾

Regarding the mechanisms underlying the test we have clearly demonstrated that important plasma proteins such as IgG prepared from serum of CF gene carriers show another adsorption behaviour than IgG prepared from control subjects (Fig. 5).

Using electrophoretic fingerprinting we have found that only certain types of indicator particles respond in the test.⁸⁾ The technique may help to predict the suitability of particles for clinical tests. Attempts to find exactly the localization for the changed adsorption properties of plasma proteins from CF gene carriers and to standardize the test procedure are now under continuing study.

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