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Efficacy of Local Anesthetics in Detachment of Normal 3T3 Mouse Fibroblasts and Prostate Cancer AT-2 Cells from Substrata, in Maintenance of Viable Cells in a Non-Adherent State, and in Preservation of Cell Surface Markers Detected with FlowSight Image Cytometry*

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The local anesthetics procaine, lidocaine and tetracaine permit the reversible detachment of viable cells and their passaging or preservation in a non-adherent state in the absence of proteolytic enzymes. The effects of these anesthetics, dissolved in various media, on cell viability, cell detachment from substrata and preservation of cells in a non-adherent state, were compared using the AT-2 line of rat prostate carcinoma cells of moderate malignancy and the 3T3 mouse fibroblast cell line. It was found that all three local anesthetics can induce cell rounding followed by detachment of over 95% of viable cells in both lines in Ca^{2+}/Mg^{2+}-free PBS. Tetracaine in 1 mM concentration was the most effective in induction of fast cell detachment. However, procaine and lidocaine in 16 mM concentrations were found to be optimal for preservation of cells in a non-adherent state and for the maintenance of cell viability for at least 2 h. The tested anesthetics also cause cell rounding and detachment when present in various cell culture media but these processes occurred much more slowly and less efficiently than in Ca^{2+}/Mg^{2+}-free PBS. Normal 3T3 mouse fibroblasts after detachment and passaging undertake growth reaching the same saturation density in cultures after detachment with procaine or lidocaine as after passaging using trypsin solution. The results suggest that the application of local anesthetics can be a very simple and effective technique for cell passaging in tissue cultures. This technique might decrease side-effects and cell injury caused by trypsinization or cell scraping. The preservation of cells in suspension in a non-adherent state may facilitate analysis of cell surface properties and fractionation of cell mixtures. Avoiding the use of trypsin allows for the preservation of cell surface proteins ICAM, CXCR4, and HCAM analyzed with FlowSight® image flow cytometry.

Key words: Local anesthetics, cell detachment, cell passaging, cell surface markers.

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Methods commonly used for harvesting cells growing when attached to solid substrata are based on the application of proteolytic enzymes. One often applied method is cell trypsinization causing cell rounding and detachment from substrata and from neighboring cells, practically without changes to cell viability. The concentration of trypsin and time required for effective cell detachment have to be adjusted for each cell type and culture conditions and the action of the enzyme must be terminated by trypsin inhibitors or serum. Trypsin treatment modifies epitopes of cell surface markers studied with cytometry (GRAY *et al.* 2001), causes changes in cell membrane physical proper-

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ties and shows mitogenic activity (SEFTON & RUBIN 1970; BURGER 1970,1971). Modification of the cell surface with enzymes should be considered in research concerning cell surface properties. Pronase or trypsin treatment of cells causes cell membrane modifications which persist for more than 24 hours. In addition, even gentle proteolytic enzyme treatment acts as a mitogenic agent inducing stationary cells from confluent cultures into the S phase of the cell cycle (SEFTON & RUBIN 1970,1971; BURGER 1970). Treatment of anchorage dependent cells with proteinases to obtain cell suspensions causes modification of cell surface receptors, adhesion molecules and other cell surface markers which are often later analyzed in assays of cell adhesion (SEFTON & RUBIN 1971; DELHOTAL *et al.* 1983; AGGELER 1990; BEREITER-HAHN *et al.* 1990).

Because of the side-effects of cell trypsinization, attempts have been made to develop alternative methods of viable cell detachment (RABINOVITCH & DESTEFANO 1975, 1976; SIT & WONG 1991). Cell detachment in the presence of calcium chelators was found to be useful for weakly attached embryo or cancer cells and some blood cells. It prevents modification of cell surface markers studied with cytometry (GRAY *et al.* 2001), but not all cell types can be detached with chelators. Cell scraping with a silicone policeman is often used for flow cytometry research of cell surface markers. This method modifies the cell membrane to a lesser extent but causes injury to many cells (DELHOTAL *et al.* 1983). Among the most successful alternatives to trypsinization are the methods based on cell detachment in the presence of local anesthetics. RABINOVITCH and DESTEFANO (1976) described reversible cell rounding and detachment in the presence of cationic local anesthetics. Their results were published before cell surface markers became extensively studied and are now almost forgotten. The authors concentrated on qualitative observations and aimed at developing a suitable method for cell passaging. For this purpose the detachment of the cell fraction is sufficient. A quantitative comparison of the efficacy and the optimization of conditions for the application of local anesthetics for detachment of cells from solid substrata and maintenance of cells in a non-adherent state for a few hours can be useful in cell biology research. Cell rounding can be effectively used for cell detachment since it resembles natural cell rounding and detachment during mitosis (ABERCROMBIE & ABERCROMBIE 1962). This phenomenon was used for cell synchronization by harvesting mitotic cells, as described by TERASIMA and TERASIMA (1963) and studied and described in numerous papers (cf. SANGER & SANGER 1980; MITTAL & BEREITER-HAHN 1985; PASTERNAK 1986; CRESPO *et al.* 1987; AGGELER 1990; SIT *et al.* 1990; SIT & WONG 1991; KOROHODA & WÓJCIAK 1992; MADDOX & BURRIDGE 2003).

The experiments described in this communication were carried out with relatively weakly attached rat prostate tumor cells of moderate malignancy (AT-2 line) and strongly attached mouse fibroblasts 3T3, often used as normal anchorage-dependent cells. We also tested the effects of cell detachment with local anesthetics on cell growth after passaging and on some cell surface markers studied with image flow cytometry of single cells.

Material and Methods

Cell culture

Experiments used the well-characterized rat prostate AT-2 adenocarcinoma cell line of the Dunning R-3327 model and NIH 3T3 mouse fibroblasts. AT-2 prostate cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and gentamycin (1 μg/ml). Mouse 3T3 fibroblasts were grown in DMEM supplemented with 10% calf serum and gentamycin. Cells were cultured in 25 cm^2 Falcon flasks in a humidified atmosphere with 5% CO_2 at 37°C. Initial cell density was 10 x 10^4 cells/cm^2.

Cell detachment experiments and preparation of cell suspensions

For experiments the cells were trypsinized and transferred to 6-well plates (Corning) at 10 x 10^4 cells/cm^2 density in a proper culture medium. The cell detachment experiments were carried out after 3 days. Cells were detached by washing with PBS and i/ incubation with trypsin (0.25%) with EDTA (0.02%) solution in PBS Ca^{2+}/Mg^{2+}-free, ii/ washing and incubation with procaine, lidocaine or tetracaine hydrochloride monohydrate dissolved in Ca^{2+}/Mg^{2+}-free PBS in concentration and time shown in Results. Undetached cells in the presence of anesthetics dissolved in culture media or PBS were trypsinized and separately counted. A total of 250 to 400 cells were counted in a Bürker haemocytometer for determination of percentages of detached and attached cells in each sample. Data are expressed as means ±SD and analyzed with two-sample-independent student's *t*-test; $P<0.01$. All results are representative of three independent experiments.

Cell viability determination

Cell viability was tested using the fluorescein diacetate (FDA) and ethydium biomide (Eth Br)

tests and a fluorescence microscope (Jenavert, Carl Zeiss Jena) as earlier described (GRYS *et al.* 2014). In some experiments cell viability was additionally determined with the often used trypan blue exclusion test. Each determination was based on counting at least 250 cells in one sample. Both methods yielded consistent results. Data are expressed as means ±SD and analyzed with two-sample-independent student's *t*-test; *P<0.01. All results are representative of three independent experiments.

Effects of cell detachment and incubation in the presence of anesthetics on cell growth

The effects of the method of cell detachment followed by incubation of cells in the presence of anesthetics for 60 minutes (procaine and lidocaine) or 30 minutes (tetracaine) was examined by seeding cells in suspension ($15x10^3$ cells) to 6 well plates. Growth curves of 3T3 mouse fibroblasts after passaging in the presence of trypsin (control), or after cell detachment and pre-incubation in the presence of procaine (60 minutes, 18 mM), lidocaine (60 minutes, 16 mM) or tetracaine (30 minutes, 1 mM) were plotted. For determination of cell numbers in the following days of cell culture, the cells were detached with trypsin and counted in a Bürker haemocytometer. At least 250 cells were counted in each sample. Data are expressed as means ±SD. All results are representative of three independent experiments.

Image Stream cytometry

Image Stream cytometry was applied to examine the effect of the method of cell detachment from substratum upon chosen cell surface markers. AT-2 cells, immunolabeled with Alexa 488 (ICAM), APC (CXCR4) phycoerythrin (HCAM) were analyzed with a FlowSight® imaging cytometer (Amnis Corp. Seatle WA) equipped with krypton-argon lasers (excitation 488 nm (50mW) and 658 nm (60mW). Brightfield and light scatter images were collected in channels 1, 9 (white) and 6, while Alexa 488, PE, 7AAD and APC fluorescence were collected in channels 2 (green), 3 (orange) and 12 (cyan), respectively. Post-Acquisition spectral compensation was performed using the IDEAS® image analysis software package (Amnis Corp.) on unstained cells, cells stained with a single antibody/fluorophore combination, and multi-stained cells, respectively. Compensation for inter-channel spectral crosstalk was performed by plotting best-fit lines of signal intensities from each fluorochrome in the reference and spectrally adjacent channels. The values in the compensation matrix were then applied to each pixel of the image. After compensation, a similar analysis was done on in-focus single cell images. In-focus single cells were classified based on their small bright field area, high bright field aspect ratio (width to high ratio) and high nuclear contrast (measured by the gradient max feature). At least 100 000 images were collected for data analysis. Data are expressed as means ±SD and are representative of three independent experiments.

Results and Discussion

In the first series of experiments we examined which concentrations of the tested local anesthetics can be applied for cell detachment from the substratum without significant reduction of cell viability. Detached cells as a cell suspension are used commonly not only for cell passaging, but also for further research. In this case often cell fractionation and preparation of possibly pure cell subpopulations is required. Since fractionation of mixed cell suspensions usually requires about one hour, we compared survival of cells after treatment for 120 minutes with the tested anesthetics.

The AT-2 rat prostate cancer cells and 3T3 mouse fibroblasts were incubated in the presence of anesthetics in the range of concentrations suggested by RABINOVITCH and DESTEFANO (1975, 1976) in growth media (RPMI-1640 supplemented with 10% FCS and DMEM with 10% FCS, respectively). Cell viability dependent upon time was tested. In the presence of 12 mM and 14 mM procaine for 120 minutes, 89% and 86% of AT-2 cells survived (Fig. 1A), in the presence of 16 mM

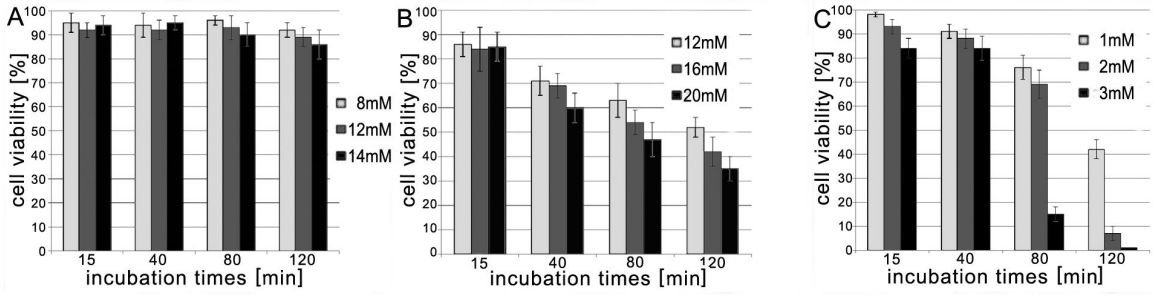

Fig. 1. Viability of 3T3 cells after 120 min incubation with (A) procaine (B) lidocaine (C) tetracaine. All anesthetics were dissolved in RPMI culture medium supplemented with 10% FCS. Data are expressed as means ±SD and analyzed with two-sample-independent student's *t*-test; *P<0.01.

and 20 mM lidocaine, 42% and 35% of cells survived (Fig. 1B), and in the presence 1 mM and 2 mM tetracaine, 42% and 7% survived, respectively (Fig. 1C). Similar results were obtained for 3T3 fibroblasts (data not shown). More than 85% of cells of both types survived 120 minutes of treatment only in the presence of 12 mM and 14 mM procaine. Under these conditions cells survive and remain non-adhesive to glass and plastic substrata. This shows that the best survival of cells after two hours of cell incubation in the presence of the tested local anesthetics is preserved in the presence of procaine. This observation corresponds to the report of STURROCK *et al.* (1979) who observed low toxicity of 7 mM procaine when continuously present in the cell culture medium.

Further experiments concerned quantitative analysis of cell detachment, survival, and the rate of cell proliferation after removal of anesthetics. For experiments on cell detachment, anesthetics dissolved in various media were applied because RABINOVITCH and DESTEFANO (1975, 1976) noticed that the type of medium influences the effectiveness of cell detachment in the presence of anesthetics. The results are shown in Fig 2. The percentage of cells detached after 60 min did not exceed 60% when the tested anesthetics were applied in cell culture media. When we tested the same local anesthetics applied in PBS, PBS free of Ca^{2+} and Mg^{2+} cations and in solutions supplemented or not-supplemented with FCS, the effects of serum and Ca^{2+}/Mg^{2+} ions on cell detachment became apparent (Fig. 3). The most efficient and very fast (within 3-5 minutes) cell detachment was observed when the anesthetics were applied in Ca^{2+}/Mg^{2+} free PBS. In these conditions almost 100% of AT-2 and 3T3 cells were rounded and detached and almost all (over 98%) detached cells remained alive. The results are shown in Fig. 3.

After removal of the anesthetics by washing in culture medium and re-seeding the cells, they attached to substratum, spread, and commenced

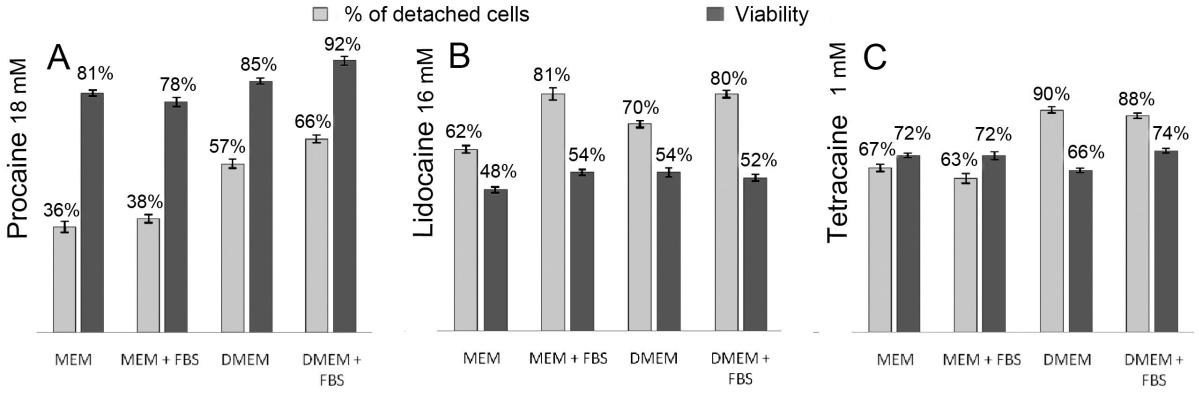

Fig. 2. Efficiency of cell detachment and viability of detached 3T3 cells as dependent on the presence cell growth media: MEM, MEM+ FBS, DMEM, and DMEM + FBS and the anesthetic used. Data are expressed as means ±SD.

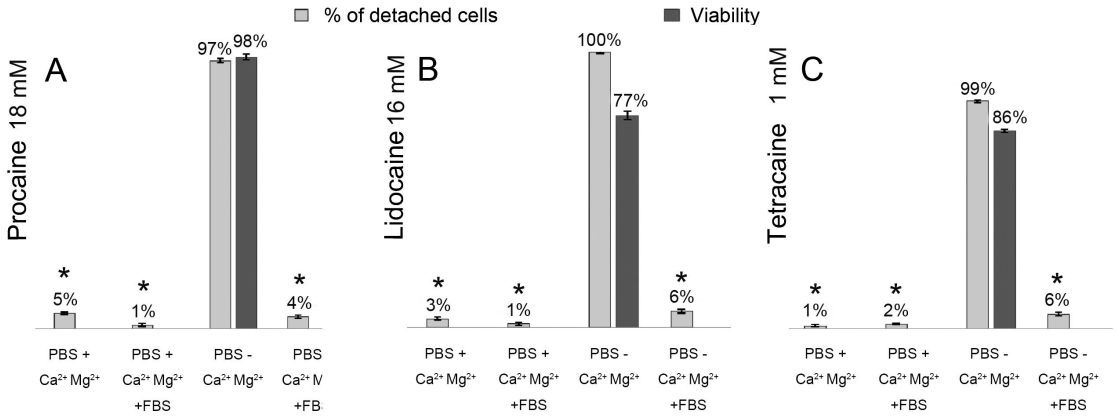

Fig. 3. Efficiency of detachment and viability of 3T3 cells after incubation in the presence of the tested anesthetics in PBS with and without divalent cations and serum. Data are expressed as means ±SD and analyzed with two-sample-independent student's *t*-test; *P<0.01.

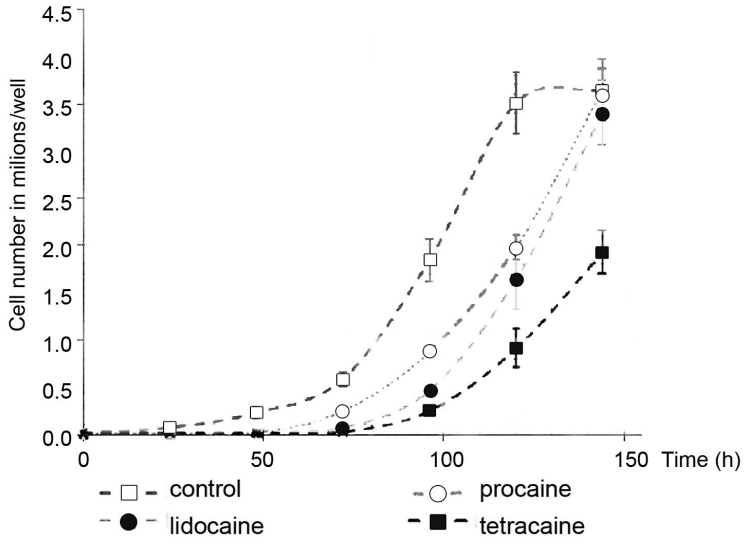

Fig. 4. Growth curves of 3T3 mouse fibroblasts after passaging in the presence of trypsin (control), or after cell detachment and incubation in the presence of procaine (60 minutes, 18 mM), lidocaine (60 minutes, 16 mM) or tetracaine (30 minutes, 1 mM). Data are expressed as means ±SD.

growth. The growth curves (Fig. 4) show that mouse 3T3 fibroblasts pre-incubated in suspension for one hour in the presence of procaine or lidocaine and 30 minutes in tetracaine after removal of anesthetics commenced growth. They grew with some delay in comparison to trypsinized cells but after 6 days of culture the 3T3 cells detached in the presence of procaine and lidocaine reached the same saturation density as the cells detached with trypsin. The difference with the initial growth of trypsinized cells and procaine-treated cells can be due to the mitogenic action of trypsine treatment (SEFTON & RUBIN 1970; BURGER 1970, 1971).The detachment, passaging and maintenance of living detached cells in non-adherent state was found to be the best when the cell cultures were treated with PBS free of Ca^{2+}/Mg^{2+} ions supplemented with 12 mM procaine. This method can be used for cell passaging without a need for applying proteinases and their inactivators.

On the grounds of the results presented in this communication and our experience, we advise the following procedure for preparation of suspensions of anchorage dependent cells:

i) wash the cell culture twice with Ca^{2+}/Mg^{2+}-free PBS warmed to 37°C, ii) add warm PBS supplemented with 14 mM procaine or 16 mM lidocaine to cell cultures for 3 to 5 minutes, iii) gently shake the cultures and check whether all cells have already detached. If some cells remained attached incubation with an anesthetic can be prolonged to 10-15 minutes, iv) transfer the cell suspension to test tubes, v) condense the cells by centrifugation and re-suspend in the culture medium, vi) count the cells and prepare aliquots of cell suspensions for passaging or further research. Cell suspensions prepared in this way can be used not only for cell culture but also for cell fractionation or flow cytometry or other research methods of cell surfaces.

To assess the effects of cell detachment with trypsin, EDTA and local anesthetics on ICAM, CXCR4 and HCAM epitopes present on the cell surface, we compared the exposition of these markers with FlowSight cytometry. The results show (Fig. 5) that best preservation is observed after detachment of cells with EDTA or with lidocaine and procaine. Much worse preservation was found for cells detached with trypsin and tetracaine. This observation confirms results by GRAY *et al.* (2001) showing that application of trypsin for cell detachment and harvesting for cytometric studies can strongly modify expression of cell surface marker proteins. Cell adhesion molecules (such as ICAM and HCAM) which extend over 10 nm into the cell medium from the surface of lipids are particularly exposed to the action of trypsin.

Cell pretreatment and contact with dextran T500 often used for cell isolation causes coating of proteins near the external surface of the lipid bilayer (SROKA *et al.* 2009), whereas cell trypsinization eliminates the parts of proteins extending more than 5 nm from this surface. Cell rounding induced by mechanical stimulation lasts less than a few seconds and re-spreading is very fast, much faster than after trypsinization (BEREITER-HAHN *et al.* 1990; SIT *et al.* 1990; KOROHODA *et al.* 1992; KOROHODA *et al.* 1993). Fast cell rounding and detachment from substratum are usually associ-

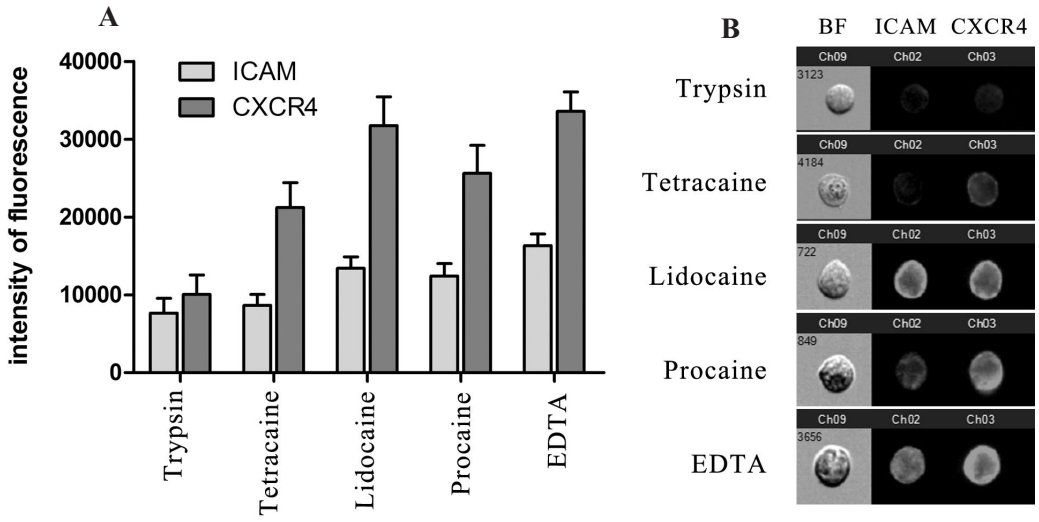

Fig. 5. Preservation of cell membrane marker proteins ICAM and CXCR4 as dependent upon the method of cell detachment.
A. Relative fluorescence measured as described in Materials and Methods.
B. Images of single cells detached in the presence of trypsin, tetracaine (1 mM), lidocaine (16 mM), procaine (18 mM), and EDTA as observed with SigthFlow image flow cytometry. Ch1 – BF (braight field), Ch2 – ICAM, Ch3 – CXCR4.

ated with disassembly of stress fibers and disorganization of the cytoskeleton due to isotonic contraction (KOROHODA & WOHLFARTH-BOTTERMANN 1976; NICOLSON *et al.* 1976; KOROHODA & KAJSTURA 1982; KOROHODA & WÓJCIAK 1992; PIERZCHALSKA *et al.* 1998). These changes in cytoskeleton architecture are reversible on cell attachment to substratum, formation of focal contacts and cell re-spreading.

Procaine and lidocaine also induce a variety of other effects upon cells besides changes in cell shape associated with changes in cytoskeleton organization. Changes in pinocytotic activity, in electric properties of cell surface as studied with cell electrophoresis, in sensitivity to electric fields used for cell electroporation and in cell movement have been described (NICOLSON *et al.* 1976; STURROCK *et al.* 1979; DICKSTEIN *et al.* 1984; JOHNSON & DOWSE 1986; PIERZCHALSKA *et al.* 1998; JOHNSON *et al.* 2002; MICHALIK *et al.* 2003; GRYS *et al.* 2014). All these effects were, however, reversible and cells return faster to the original state than after treatment with trypsin.

Perhaps some cell modification during preparation procedures (such as cell detachment, isolation or fractionation) of cells used for further research are unavailable, but nevertheless should be considered, carefully analyzed, and minimized as far as possible. In each case an analysis of cell preparation procedure should be performed before proper study of cell properties and activities.

In summary, the improvement of the method of RABINOVITCH and DESTEFANO (1975) for detachment from substratum of anchorage-dependent cells presented in this communication increases its efficiency and speeds up the preparation of cell suspensions. The use of procaine or lidocaine in Ca^{2+}/Mg^{2+}-free PBS rather than in media for cell culture causes an increase of cell detachment efficiency from 40-60 percent (depending on the type of cells and medium) to 95-100%. Moreover, the time required to detach the cells is reduced to 3-5 minutes (exceptionally to 10 minutes). 3T3 fibroblasts prepared by this method grow to the same density as after passaging with the use of proteolytic enzymes. Omission of the need for trypsin avoids modification of cell surface molecules used as markers for flow-cytometry (GRAY *et al.* 2001). It also eliminates the side effects of proteolytic enzyme application, including the mitogenic activity of enzymes associated with induction of DNA synthesis, transition of G1 to S phase of the cell cycle (SEFTON & RUBIN 1970, 1971; BURGER 1970, 1971), and changes in the transport of substances across cell membranes (SEFTON & RUBIN 1971). Treatment of cells with serum or proteinase inhibitors is unnecessary, which is important if the cells are later cultured in defined, serum-free media. The method described here causes fewer changes to the cell surface than proteinases, similarly as the use of chelators (EDTA, EGTA) and faster cell rounding and detachment than EDTA (cf. RABINOVITCH & DESTEFANO 1976) but it is not limited to embryonic and cancer cells weakly attached to the substratum. Maintaining suspended cells in solution in non-adherent state for several hours makes it possible, as will be shown in separate contributions (in preparation), to improve methods of cell

cloning and several methods of fractionation of cell mixtures.

We hope the presented modification of the method originally proposed by RABINOVITCH and DESTEFANO (1975) for cell passaging due its straightforward application and the above mentioned advantages will prove suitable in research in cell biology and biotechnology.

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