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Effect of serum on in vitro electrically mediated gene delivery and expression in mammalian cells

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Abstract

In many cell systems, electric pulses can efficiently mediate gene transfer with a high level of expression in vitro. In vivo results have been reported where decrease in efficiency was obtained. The mechanisms involved in the process are unknown. Since, in vivo, the efficiency of non-viral methods of gene transfer is generally limited by the presence of serum, we report here the effect of serum on in vitro electrically mediated chinese hamster ovary cell membrane permeabilization, viability, gene transfer and expression. The results indicate that permeabilization and gene transfer are not inhibited by serum. By acting as a protector of cell viability, serum indeed increases gene transfer and expression.

Keywords: Electropermeabilization; Electroporation; Gene transfer; Serum; Chinese hamster ovary cell

1. Introduction

The use of in vivo gene delivery methods makes it possible to deliver a gene in the living host. Despite high transfection efficiencies, viruses present different drawbacks such as toxic–inflammatory and induce host–immune responses, cytopathicity or scaling-up difficulties that limit their therapeutic applications [1]. Optimization of in vivo non-viral delivery methods of DNA therefore represents a challenge. Synthetic cationic lipid complexes with DNA have been developed. However, the presence of serum prevents the use of such complexes [1,2] even though new conditions could maintain the transfection efficiency when up to 8% serum is present [3]. Particle bombardment is an efficient method but it suffers from its limited penetration [4].

An alternative method for gene delivery is the use of electric fields. Since the first experiments of Neumann and collaborators in 1982, electropulsation of cells in the presence of plasmid DNA has been reported to be an efficient method to induce in vitro gene transfer and expression [5]. In vivo applications of electric fields have been developed, primarily to introduce anti-tumor drugs into cancer cells on patients, a method called electrochemotherapy [6], and more recently to get gene expression. Electro-mediated gene transfer experiments have been performed in skin [7], in rat liver [8], murine melanoma [9], as well as in muscle and cardiac tissues [10–12]. This method is of great interest since it allows the targeting of gene transfer in tissues. Electric pulses are

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delivered directly at the site of DNA injection by a direct contact between the tissue and the electrodes, but the molecular processes involved in such phenomena remain poorly understood. While the level of safety is very high, the in vivo use appears restricted by their relative low efficiency in comparison with viral vectors. The efficiency of gene transfer and expression indeed decreases by at least one order of magnitude from in vitro to in vivo studies [7–9]. This decrease in transfection efficiency can have different origins. In particular, the presence of serum has been shown to strongly affect cationic lipid-mediated gene transfer [1]. Serum could either prevent the free exchange of macromolecules across the permeabilized membrane, affect cell viability or, by a direct interaction with DNA, affect gene transfer as has been shown with chemical vectors.

To go further into the mechanism, we examined in the present study the influence of serum on in vitro cell membrane permeabilization, cell viability and transfection.

2. Materials and methods

2.1. Cell culture and plasmid

Chinese hamster ovary (CHO) cells were used. The WTT clone which is not strictly anchorage dependent was selected. It grows at 37°C in monolayers on Petri dishes (35 mm in diameter, Nunc, Denmark), but has been adapted for suspension culture under gentle agitation (100 rpm) in Eagle's minimum essential medium (MEM0111, Eurobio, France) supplemented with glucose (3.5 g/l), tryptose phosphate (2.95 g/l), sodium bicarbonate (3.5 g/l), vitamins and 8% newborn calf serum. Antibiotics, penicillin (100 U/l) and streptomycin (100 μg/ml), and glutamine (0.585 ng/ml) are added extemporally. Cells are maintained in the exponential growth phase (4×10^5–1.2×10^6 cells/ml) by daily dilution of the suspension. Cells in suspension have a mean radius equal to 6.5 μm. Cells grown in suspension can be replated readily on Petri dishes and kept at 37°C in a 5% CO_2 incubator (Jouan, France). The ability of cells in suspension to replate is evidence of their viability.

Plasmid pUT531 used to transfect cells is a pBR322 shuttle vector carrying the β-galactosidase gene under the control of the SV40 early promoter. It is extracted from *Escherichia coli* and purified by standard procedures (Qiagen plasmid kit).

2.2. Cell electropulsation

The cell electropulsation protocol has been described elsewhere [13]. Briefly, cells were centrifuged for 5 min at $350\times g$ (1000 rpm, Jouan C500 centrifuge, France) and resuspended in the 'pulsing buffer' (10 mM phosphate, 250 mM sucrose, 1 mM $MgCl_2$, pH 7.2, conductivity of 1400 μS) at a concentration of 10^7 cells per ml. The low ionic content saline buffer allows the delivery of pulses of long duration (ms) at high voltages (up to 1000 V). One-hundred μl of the cell suspension was put at 21°C between two flat and parallel electrodes seated on the bottom of a culture dish that formed the pulsation chamber. The electrodes were connected to a voltage generator which gave square-wave electric pulses (CNRS cell electropulser, Jouan, France). The voltage pulses were monitored with an oscilloscope. The experiments were performed under a laminar flow hood. Ten pulses lasting 5 ms were applied with different field strengths at a 1 Hz frequency. These long pulse duration, mild electric field strength conditions lead to the transfer of macromolecules into mammalian cells while preserving their viability [13].

In the case of experiments performed with serum, new-born calf serum was used (Gibco BRL, France). It was added to the cell suspension either before pulsation or immediately after it at concentrations ranging from 5 to 50% (v/v). To keep constant the final volumes of cell suspension, precise amounts of pulsing buffer were added. Under these conditions, the osmolarity did not change (0.28 Osm/kg without serum, 0.30 Osm/kg with 50% serum).

2.3. Electropermeabilization of cells

Electropermeabilization was quantified by penetration of dyes to which the membrane was normally impermeable. Propidium iodide, PI (100 μM in pulsing buffer, P4170 Sigma, USA), was used to monitor permeabilization. Cells were pulsed, incubated 10 min at room temperature and then tested for permeabilization. Pulsed cells were analyzed by flow cytometry (Becton Dickinson, FACScan, USA). Flow cytomet-

ry can be used to measure both the percentage of fluorescent cells and the amount of associated fluorescence. Laser excitation was 488 nm, fluorescence was detected above 600 nm.

2.4. Determination of electropulsed cell viability

Cells were pulsed, kept 5 min at room temperature and then grown (37°C, 5% CO_2) in Petri dishes after adding 2 ml of culture medium. Viability was measured by observing the growth of cells over 24 h by the crystal violet coloration method [13].

2.5. Electrotransfection procedure

0.8×10^6 cells were incubated on ice in 100 μl pulsing buffer containing the plasmid (25 μg/ml) 10 min before electropulsation. They were loaded between electrodes and pulsed as described above (10 times, 5 ms, 1 Hz, 0.6 kV/cm) at room temperature. They were immediately incubated for 10 min at 37°C. Two ml of culture medium was added and the cells were cultured on dishes for 24 h. As only viable cells are prone to plating, plating efficiency gives an easy assay of cell viability. Plated cells were then tested for the expression of the β-galactosidase activity as described in [13]. Cells expressing β-galactosidase appeared deep blue. Transfection frequency is defined as the ratio of blue cells over the total number of surviving cells. 300–500 cells were observed under an inverted digitized video microscope (Leica, Germany). Expression of β-galactosidase was used to evaluate the occurrence of DNA delivery and expression at the single cell level. Detection of the activity is the evidence that at least one copy of plasmids introduced into the cell has been expressed.

2.6. Determination of intracellular ATP content

Cells were centrifuged and resuspended in pure DMSO (D2650 Sigma), at a concentration of 4×10^7 cells/ml. After a 4 min incubation at room temperature, they were kept at −20°C until ATP was measured. For this, cells were mixed (10 s, Genie 2 vortex, Scientific Industries, USA) and centrifuged. Fifty μl of supernatant was added to a 250 μl luciferin–luciferase solution (4 mg/ml, L0633 Sigma) (pH 7.4). ATP was measured in a luminometer (LKB 1250 Luminometer, Sweden). Calibration of results was performed by using ATP standard solutions.

All experiments were repeated at least three times at 2 or 3 day intervals in order to avoid possible fluctuations due to different physiological states of the cells, mainly due to the age of the culture.

3. Results

Effect of DNase activity of serum on DNA. Serum is known to exhibit an intrinsic DNase activity [14]. Plasmid DNA was incubated with cells for 10 min on ice (i.e. under conditions used in the transfection experiments) in the presence or not of serum and analyzed by agarose gel electrophoresis. Plasmid in pulsing buffer was present in supercoiled and relaxed

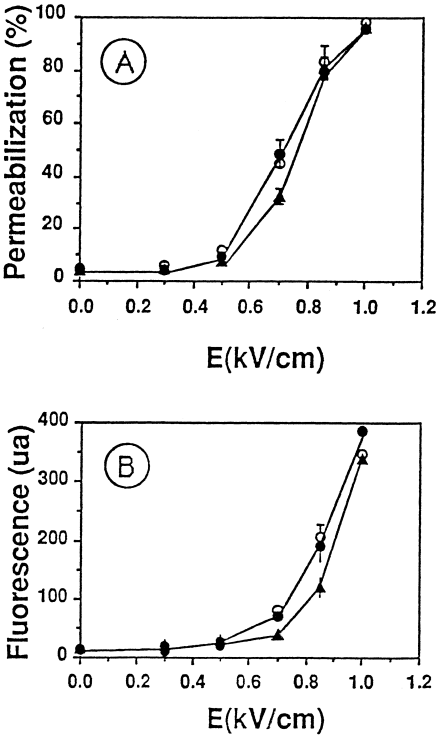

Fig. 1. Effect of serum on the electropermeabilization efficiency of CHO cells, observed by PI uptake. Ten pulses lasting 5 ms at a 1 Hz frequency were applied. Serum (5 or 20%) was added to the cell suspension 10 min before pulsation. PI penetration was quantified by flow cytometry measurements. (○) represents the control cells, (●) represents cells in the presence of 5% serum, and (▲) represents cells in the presence of 20% serum. The percentage of permeabilized cells (A) and the amount of fluorescence associated with this permeabilization (B) are reported as a function of electric field intensity.

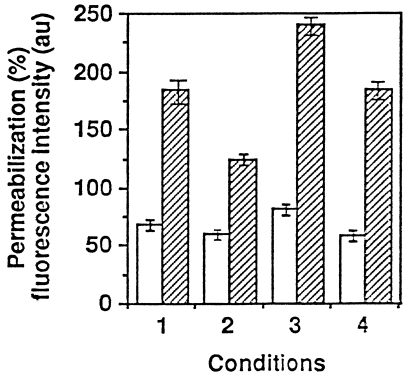

Fig. 2. Effect of the addition sequence of serum on permeabilization of CHO cells. PI fluorescence was the assay of permeabilization as in Fig. 1. Serum at 20% was added to the cell suspension either: (1) and (2) before pulsation, or (4) after pulsation. (3) represents the control with no serum. Ten pulses lasting 5 ms at a 1 Hz frequency and 0.8 kV/cm were applied after 10 min incubation of the cells. Percentage of permeabilized cells (white symbol) and the associated fluorescence intensity (dashed symbol) of electroloaded PI are reported. In condition (1), PI was added to the cell suspension 10 min before addition of serum, pulses being immediately applied after serum addition. In condition (2), PI was added to the cell suspension 10 min after the addition of serum, pulses being applied immediately after PI addition.

form (55% and 45%, respectively). The proportion of the supercoiled form was 55%, 22% and 14% with 5%, 20% and 50% serum, respectively; in a corresponding way, the relaxed form of the plasmid was 45%, 70% and 75% with 5%, 20% and 50% serum, respectively. Linear plasmid was detected; it represented 8% for 5% and 20% serum and 10% for 50% serum. No fragments were visible, a result in agreement with previous observations [14]. Under our experimental conditions, plasmid DNA is not dramatically degraded by the presence of serum.

Effect of serum on cell electropermeabilization. The effect of the electric field intensity on cell permeabilization is reported in Fig. 1. As soon as the electric field was higher than a threshold of 0.3 kV/cm, membrane permeabilization was detected both by an increase in the percentage of permeabilized cells and in the intracellular PI fluorescence. The fluorescence intensity is related to the number of molecules incorporated into electropermeabilized cells. The threshold of 0.3 kV/cm was not affected by the presence of serum. No significant difference could be detected between cells pulsed in the presence of serum or in pulsing buffer alone. A slight but significant decrease in efficiency was observed for field values between 0.7 and 1 kV/cm only at 20% serum. The effect of the addition sequence of serum and PI to cells was therefore studied at 0.8 kV/cm and 20% serum. The results reported on Fig. 2 show that serum present during pulsation only slightly decreased permeabilization by decreasing both the percentage of permeabilized cells and the number of electroloaded molecules. This effect was more pronounced when cells were incubated with serum before the mixing with PI. When serum was added just after pulsation, PI being present during pulsation, the same results were obtained, i.e. a slight decrease in permeabilization efficiency. This result is in agree-

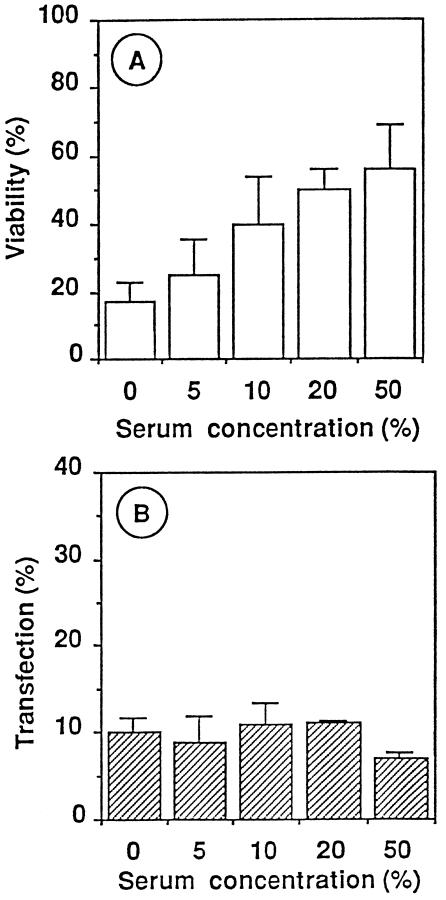

Fig. 3. Effect of the presence of serum during electric field application on transfection efficiency and viability of CHO cells. Increasing percentages of serum were added to the cell suspension already in contact with DNA 10 min before pulsation. Ten pulses lasting 5 ms at a 1 Hz frequency and 0.8 kV/cm were applied. Cell viability (A) and transfection efficiency (B) were measured 24 h later.

ment with the previous conclusion that penetration of PI into electropermeabilized cells occurred also after pulsation [13].

Effect of serum on ATP leakage. CHO cells have an intracellular concentration of ATP of 1 mM. Electropulsation performed under conditions of gene transfer (10 pulses, 5 ms duration, 0.8 kV/cm intensity) induced a decrease of 40% in ATP content of cells which is in agreement with previous studies [15]. In the presence of 20% serum (added before the pulse), the associated ATP leakage was limited to 10%.

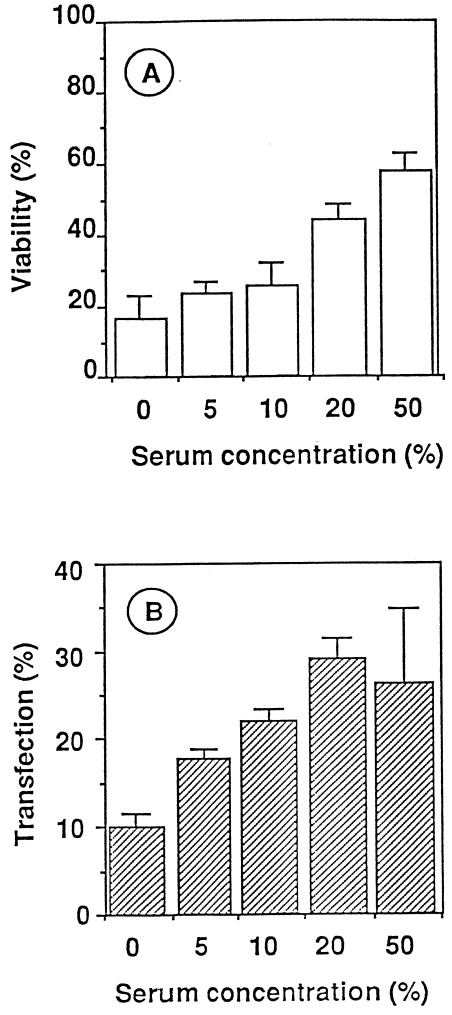

Fig. 4. Effect of the post-pulse addition of serum on transfection efficiency and viability of CHO cells. Increasing percentages of serum were added to the cell suspension just after pulsation. Ten pulses lasting 5 ms at a 1 Hz frequency and 0.8 kV/cm were applied. Cell viability (A) and transfection efficiency (B) were measured 24 h later.

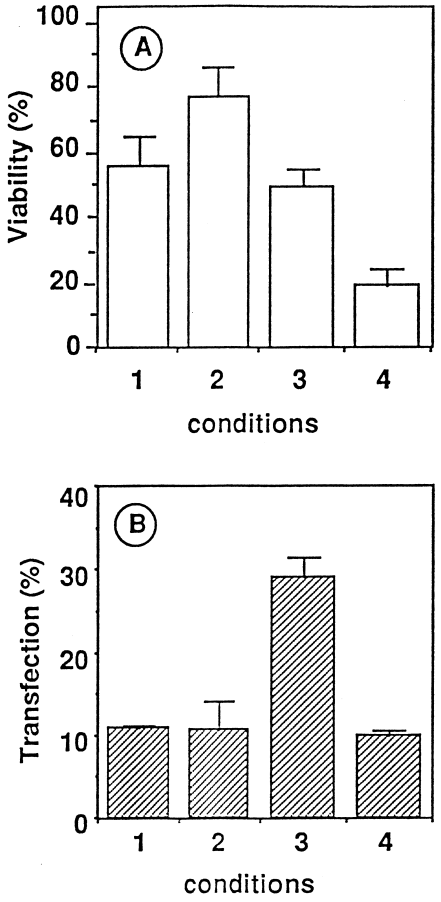

Fig. 5. Effect of the addition sequence of serum on transfection efficiency and viability of CHO cells. 20% serum was added to the cell suspension either: (1) and (2) before pulsation, (3) after pulsation; (4) represents the control with no serum. Ten pulses lasting 5 ms at a 1 Hz frequency and 0.8 kV/cm were applied after a 10 min incubation of the cells on ice. Cell viability (A) and transfection efficiency (B) were measured 24 h later. In condition (1), DNA was added to the cell suspension 5 min before addition of serum, pulses being applied 5 min later. In condition (2), DNA was added to the cell suspension 5 min after the addition of serum, pulses being applied 5 min later. In conditions 3 and 4, DNA was added 10 min before pulsing.

Effect of serum on cell viability, gene transfer and expression. As shown in Fig. 3, addition of serum before the pulses led to an increase in cell viability in a dose dependent manner. Whatever the concentration used, serum had no significant effect on cell transfection efficiency, when measured by the percentage of surviving cells expressing the reporter gene activity. But, taking into account the positive effect of serum on cell viability, transfection efficiency, i.e. the percentage of total pulsed cells ex-

pressing the reporter gene activity, was in fact increased up to 4-fold. When serum was added just after electric pulse application, a viability enhancement was dependent on serum concentration. In those conditions, cell transfection was increased up to3-fold (Fig. 4), and the total number of cells expressing β-galactosidase increased 10 times. These results are in agreement with previous results on murine myelomonocytic leukemia cells showing that 20% of horse serum added after electric field application while modestly improving cell survival significantly improved cell transfection [16].

The effect of the sequence of addition of plasmid DNA and serum to the cell suspension with respect to pulsation was then tested. DNA must be present during pulsation [17]. Results are reported in Fig. 5. Serum induced an increase in cell viability, the serum being present during the electric pulses or added after their application. Interestingly, the effect was more pronounced when serum, present during electric pulses, was added before plasmid DNA. When serum present during field application was added after plasmid DNA addition, viability was increased in a way similar to that obtained when serum was added after pulses application. Transfection efficiency was not increased when serum was present during electric field applications, plasmid DNA being added before or after serum addition.

4. Discussion

The present results show that serum has no negative effect on electrically mediated gene transfer and expression in mammalian cells, while the opposite observation was reported for cationic lipids [1,2]. Indeed, serum present during electric pulses has a positive effect on cell viability and does not decrease cell transfection efficiency. Moreover, serum added after electric pulses induces an increase both in cell viability and in cell transfection. Such an increase in cell viability could be due to a protection of cells by serum against the free exchange across the membrane due to the associated long-lived permeabilization of cells. The ATP leakage and the PI penetration were less pronounced when serum was present. Serum addition after the pulses has a positive effect on cell transfection. In the case of serum added before pulses, serum as a macromolecule could limit the flow of exchange of molecules across the permeabilized membrane. This could have a negative effect on cell transfection, but this is not the case due to the concommitant protective effect of the cell viability. In contrast to small size molecules that can diffuse across the permeabilized membrane both during the pulses and in the minutes following it, the transfer of plasmid DNA across the permeabilized membrane is only detected when the plasmid is present during the pulses [17]. This process depended on the physiology of the cells, the more their viability was preserved the more efficient the transfection obtained [15].

Electropermeabilization leads to efficient transfection in vitro even in the presence of serum. In vivo, the method has already been used to transfect cells [7–12]. A decrease in transfection efficiency by one order of magnitude is generally observed even though in the liver [8] and melanoma in mice [9] efficiencies up to 20% and 5% have been obtained. This decrease in transfection efficiency is not due to serum. The sequence of events is: serum bathing cells, DNA addition and pulse application. This is shown in vitro to increase the number of cells expressing β-galactosidase activity. Other factors can be responsible for the decrease in transfection observed in vivo. One can suggest the heterogeneity of the electric properties of tissues leading to a heterogeneity in the field strength, the diffusion of molecules which is slowed down due to steric hindrances inherent in the cell contacts and the local decrease in DNA concentration due to washing effect when the delay between plasmid injection and pulse application is too long [18,19].

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