

Syringe loading introduces macromolecules into living mammalian cell cytosol

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Summary

We describe a simple, efficient, gentle and inexpensive technique for the introduction of normally impermeant macromolecules into the cytosol of living mammalian cells growing in suspension or attached to the culturing substratum. Loading is achieved by the production of transient, survivable plasma membrane disruptions as cells are passed back and forth through a standard syringe needle or similar narrow orifice. The loading volume required, which contains cells and the macromolecule to be loaded, can be as little as 5 μ l, thus

minimizing the use of valuable reagents. In addition, we report that the surfactant molecule, Pluronic F-68, is capable of altering the physical properties of the plasma membrane in such a way as to increase loading efficiency and the long-term survivability of cells loaded by this and other mechanically based cell-loading techniques.

Key words: syringe loading, cell loading, mechanical disruption, plasma membrane.

Introduction

Now commonplace in cell and molecular biology are experimental strategies that require the introduction of normally impermeant molecules into the cytosol of living cells. Numerous "cell loading" techniques have been developed for accomplishing this, including microinjection, electroporation and several membrane fusion-based methods (see for review, McNeil, 1989). Not surprisingly, however, no technique has been found that is ideal for all cell loading applications.

Our interest has been in developing simple and inexpensive technologies that allow one to load macromolecules into cell populations. We have previously described three such technologies, namely scrape (McNeil et al., 1984), scratch (Swanson and McNeil, 1987) and bead loading (McNeil and Warder, 1987), that are applicable to adherent, cultured mammalian cells. Each of these techniques transiently permeabilizes cells by disrupting their plasma membrane through the imposition of mechanical force. Here we describe a fourth such method, "syringe loading", that will be applicable to mammalian cells growing in suspension, as well as those growing attached to a substratum. This novel method is simple, efficient, inexpensive, gentle and can be extremely economical in its use of the macromolecule to be loaded. As such, it should find a large number of applications in cell and molecular biology.

Moreover, we report here that a surfactant molecule, Pluronic F-68, which has been used as a culture medium supplement (Mizrahi, 1983; Ramirez and Mutharasan,

1990), enhances the efficiency and gentleness of this new technique, as well as that of scrape and bead-loading. Pluronic F-68 may be of general usefulness in enhancing all mechanically based cell loading techniques including microinjection.

Materials and methods

Cell culture media and reagents

Bovine aortic endothelial cells (BAEC) (passage 18) obtained from Dr. P. D'Amore (Childrens Hospital, Boston, MA) and NIH.3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% calf serum, 1 mM glutamine, 100 i.u./ml penicillin and 200 μ g/ml streptomycin (referred to as culture medium) at 37°C in a 5% CO₂ humidified atmosphere. The non-ionic surfactant, Pluronic F-68 (PF-68) (Sigma, St. Louis, MI), was prepared as an aqueous stock (20%, w/v) and sterilized by steam autoclave. Sterile 1 ml syringes and 30 gauge hypodermic needles were obtained from Becton-Dickinson, Rutherford, NJ.

Fluorescently labelled dextrans

A range of fluorescein isothiocyanate (FITC)-labelled dextrans (Fdx) (M_r 10,000, M_r 43,000, M_r 67,000 and M_r 150,000) were purchased from Sigma. A fixable FITC-dextran conjugated to lysine (M_r 10,000) (Fdx-Lys) was produced in house as described by Gimlich and Braun (1985).

Syringe loading

BAEC and NIH.3T3 cells were trypsinized, collected by centrifugation (700 g for 5 min), resuspended in culture medium, counted and then diluted to produce a cell

suspension of 500,000 cells/ml. Samples (1 ml) of this cell suspension were dispensed into sterile 5 ml plastic centrifuge tubes and the cells were allowed to recover for 20 min at 37°C. FITC-labelled dextran (50 μ l at 200 mg/ml in serum-free culture medium) and PF-68 stock, if appropriate, were added, and the cells were then gently agitated by flicking the end of the tube. The cell suspension was drawn up into a sterile 1 ml syringe through a sterile 30 gauge needle by pulling the plunger to the 1 ml level and allowing the barrel of the syringe to fill. The cell suspension was then expelled by a steady pressure on the plunger. This procedure we define as 2 strokes. To ensure reproducibility, the same syringe and needle were used throughout each experiment, being washed with serum-free culture medium between different samples. Control cells were incubated in a 37°C water-bath for the same period of time it took to carry out the syringe loading procedure (approx. 2 min) and were used to determine the amount of fluorescence signal due to pinocytosis of the Fdx-Lys probe. Culture medium (2 ml) was then added to each sample (including controls) and the cells were collected by centrifugation (700 *g* for 5 min). The cell pellet was gently resuspended in 2 ml of culture medium, and the number of cells determined using a Coulter Counter (Model ZB 1). Cell viability was assessed by trypan blue exclusion. The remaining cell suspension was divided into equal portions and plated into one well of a 6-well plate or onto a glass coverslip. After 16 h for cell attachment, the 6-well plates were washed three times with warm PBS, the cells removed by trypsinization and the number of cells that had survived and reattached, expressed as a percentage of the control, was determined using a Coulter Counter. Cells plated onto glass coverslips were washed as above and viewed with a Zeiss Axioskop fluorescence microscope as a live preparation (in the case of non-fixable Fdx), or after fixation for 10 min at room temperature, using 4% paraformaldehyde dissolved in PBS (in the case of Fdx-Lys).

Endothelial cell growth assay

BAEC, suspended in culture medium, were seeded at 10,000 cells/well in 24-well plates and allowed to attach overnight. The medium was then removed and replaced with 1 ml of fresh culture medium or 1 ml of culture medium plus 2% (w/v) PF-68. Each condition was run in replicates of three and cell number was determined at 0, 1, 2 and 3 days using a Coulter Counter.

Scrape and bead loading

Scrape loading was carried out as described by McNeil et al. (1984); bead loading was carried out as described by McNeil and Warder (1987).

Flow cytometry

BAEC or NIH.3T3 cells were loaded with Fdx-Lys as described above and replated into 6-well plates. Cells were allowed to reattach for 16 h, washed three times with warm PBS and removed from the culture substratum by trypsinization. Trypsin was inhibited by the addition of calf serum, the cell number was determined and the remaining cells were placed on ice until analysis by flow cytometry (kindly carried out by Mr. Peter Lopez, Dana Faber Cancer Institute, Boston, MA).

Micro-volume syringe loading

BAEC were prepared as above to produce a cell suspension of 5×10^6 cells/ml in culture medium containing PF-68 (2%, w/v) and Fdx-Lys (20 mg/ml). A 25 μ l sample of cells was removed and syringe loaded using a sterile Hamilton Microsyringe (25

μ l volume, 25 gauge fixed needle) to generate 80 strokes. The cell suspension was then diluted with 1 ml of culture medium and the cells were collected by centrifugation, gently resuspended in 500 μ l of culture medium and plated onto a glass coverslip. The cells were allowed to attach for 16 h, and prepared for microscopy as described above. Alternatively, a 5 μ l sample was removed and loaded using a Ependorf Pipetman (1-10 μ l capacity) and sterile, disposable, MC-50 apex pipette tip (West Coast Scientific, Inc., Hayward, California) to generate 60 strokes. The cells were then processed and prepared for fluorescence microscopy as above.

Results

A 1 ml sterile, disposable syringe was used to force a BAEC suspension back and forth through a narrow gauge hypodermic needle. Initial trials of this "syringe-loading" procedure were carried out in the presence of the normally membrane-impermeant, fixable, fluorescent probe, Fdx-Lys (M_r 10,000), and the treated cells were viewed 16 h after replating under the fluorescence microscope. As can be seen in Fig. 1A, cells subjected to syringe loading in Fdx-Lys frequently contained this molecule in their cytosol and nuclei, but not organelles, a pattern of staining distinct from the punctate, vesicular localization characteristic of pinocytosis of Fdx-Lys. In addition, microscopic observation suggested that the presence during loading of the surfactant molecule, PF-68, increased the number of BAEC that survived the procedure, and increased the proportion of cells cytoplasmically loaded with Fdx-Lys (Fig. 1B). Fdx-Lys staining of the endothelial cell surface (or that of other cell types) was never observed (data not shown), indicating that this probe did not bind to the cell surface.

Fluorometric flow cytometry was used to evaluate quantitatively syringe loading of Fdx-Lys, and the effects of PF-68 on this loading technique. Fig. 2 depicts representative flow cytometry profiles of BAEC (Fig. 2A) and NIH.3T3 cells (Fig. 2B) incubated in Fdx-Lys, but not passed through a syringe and hypodermic syringe needle. These profiles show the fluorescence signal arising from pinocytosis of the label (Fdx-Lys). Cells that were incubated in medium alone (not shown) did not significantly differ in fluorescence signal from those incubated in PF-68 (Fig. 2A and B), indicating that PF-68 neither altered the rate of pinocytosis of the probe nor permeabilized the plasma membrane to the probe in either cell type. Fig. 2C and D depicts flow cytometry profiles of BAEC and NIH.3T3 cells, respectively, syringe loaded in medium alone. The cytoplasmically loaded cell population was identified by: firstly, defining a fluorescence threshold value using the pinocytosis control population, so that 95% of the cell population fell below the threshold; and secondly, determining what percentage of cells in the syringe-treated population fell above this threshold value. In both the BAEC and NIH.3T3 cell populations, 75% of the syringe-loaded cells were above the pinocytosis threshold, i.e. were loaded cytoplasmically with Fdx-Lys. PF-68 increased the percentage of BAEC and

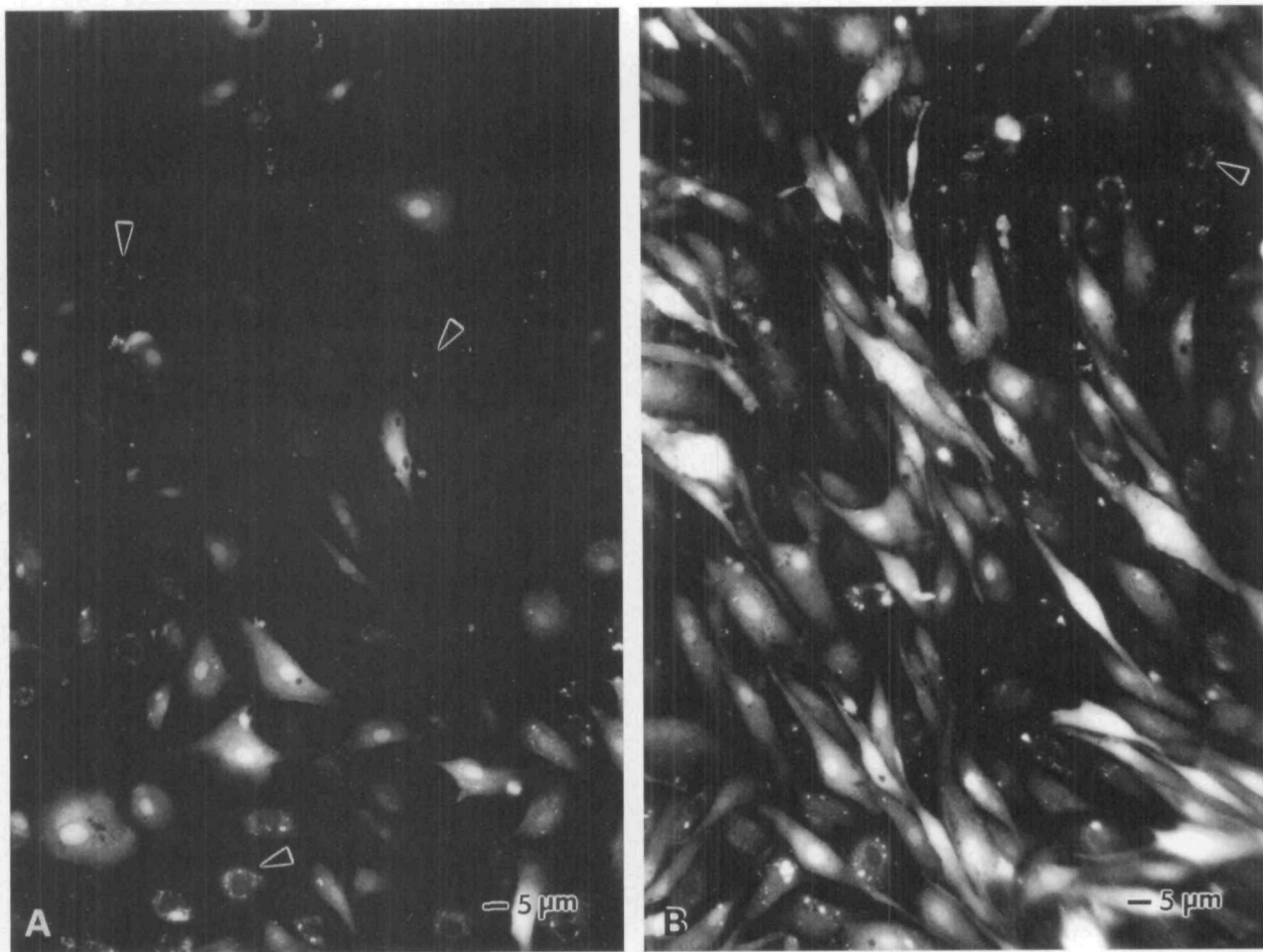


Fig. 1. Fluorescence micrographs of BAEC syringe loaded in the presence of Fdx-Lys. Equivalent numbers of BAEC were syringe loaded in Fdx-Lys (10 mg/ml) using 8 strokes of a 1 ml syringe and 30 gauge hypodermic needle in culture medium alone (A), or in the presence of 2% (w/v) PF-68 (B). The cells were then resuspended in fresh culture medium and allowed to reattach for 16 h on a glass coverslip before fixation in formaldehyde and observation under the fluorescence microscope. Micrographs were taken at equivalent exposure times. As can be seen in A, cells loaded in this manner frequently contained Fdx-Lys in their cytoplasm, indicating that these cells had been transiently wounded at their plasma membrane, compared to cells that have only pinocytosed the label (arrowheads). In the presence of 2% PF-68 (B), the proportion, as well as the fluorescence intensity, of the cells loaded with Fdx-Lys was increased. Bars, 5 μ m.

NIH.3T3 cells above the pinocytosis threshold to 85% and 84%, respectively (Fig. 3E and F). In addition, the mean fluorescence value of both populations was increased by the presence of PF-68 during loading, from 195 to 216 in the case of BAEC, and from 225 to 243 in the case of NIH.3T3 cells.

To determine whether syringe loading is capable of loading larger molecules into the cytoplasm of living cells, a range of non-fixable FITC-linked dextrans (M_r 43,000, M_r 67,000 and M_r 150,000) were used as test molecules. Fig. 3 illustrates the fact that efficient cytoplasmic loading of all three different dextrans was possible.

As the number of syringe strokes was increased, so was the mechanical damage inflicted on the cells (Fig. 4A and B). However, the increased cell loss observed with increasing number of strokes was reduced by PF-68: both BAEC viability, measured as the percentage of trypan blue-excluding cells (Fig. 4A), and recovery,

measured as the percentage of cells recovered from the starting population (Fig. 4B), were significantly increased by PF-68 over levels observed in the controls lacking this agent. The effect of PF-68 on BAEC survival appeared to be slightly more complex when assessed 16 h after syringe loading. Untreated control cells that were not syringe loaded were replated and allowed to recover for 16 h. The number of untreated control cells recovered after this time was referred to as 100% (Fig. 4C). PF-68 (2% + 0.5%) increased the number of cells recovered 16 h after syringe loading, as compared to those cells syringe loaded in medium alone. Surprisingly, the number of cells recovered from cultures syringe loaded in 2% PF-68 was greater than 100%, indicating that substantial growth had occurred in these cultures, relative to the untreated controls in the 16 h interval after syringe loading. Thus, syringe loading in the presence of 2% PF-68 appeared to have a mitogenic effect on BAEC. In summary, under optimal

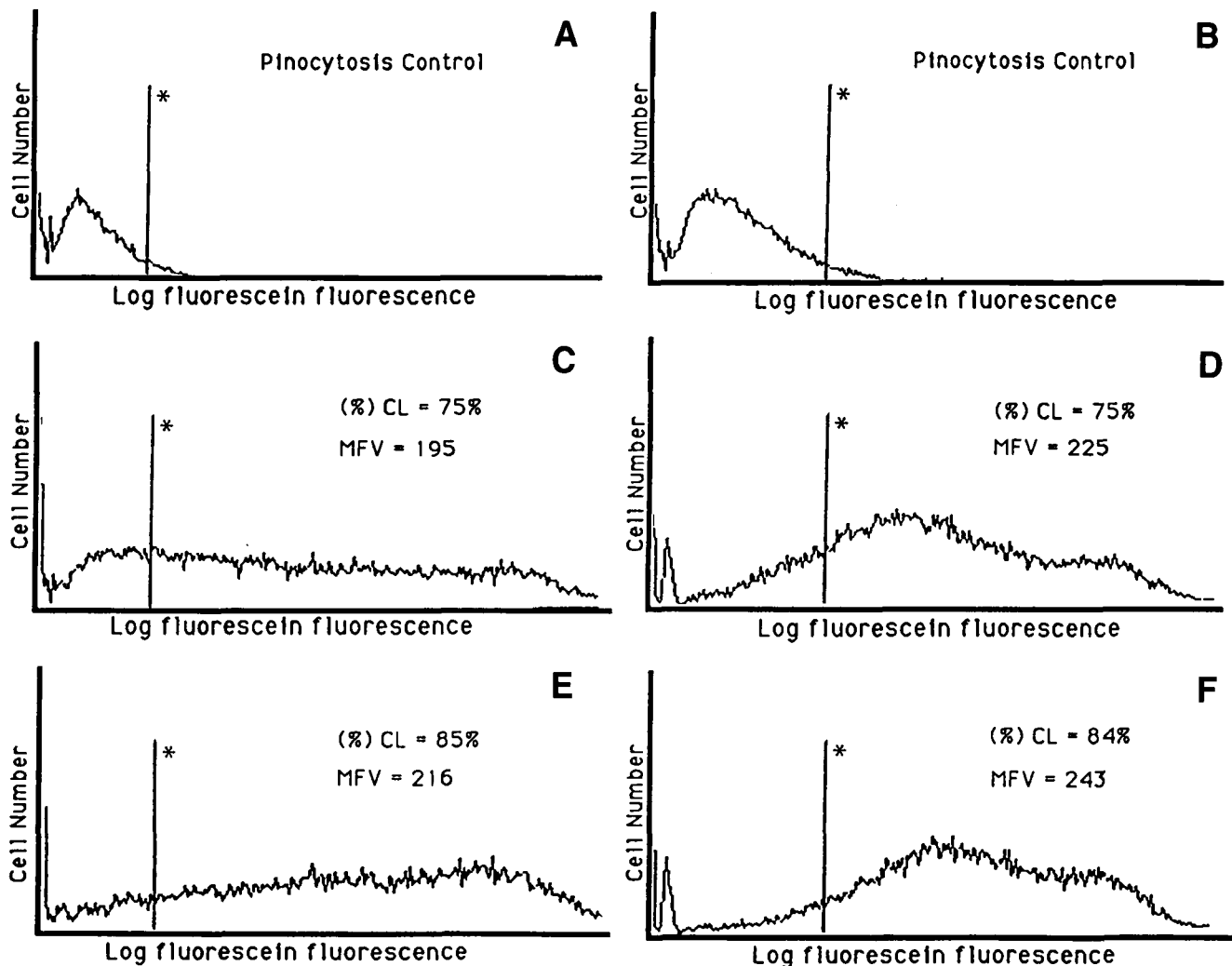


Fig. 2. Analysis of syringe loading of Fdx-Lys (M_r 10,000) using flow cytometry. BAEC (A, C and E) and NIH.3T3 cells (B, D and F) were incubated in PF-68 (2%, w/v) along with Fdx-Lys (10 mg/ml) but not subjected to syringe loading (pinocytosis control) (A and B), or syringe loaded using 8 strokes in culture medium alone (C and D), or syringe loading using 8 strokes in culture medium containing 2% PF-68 (E) and 0.5% PF-68 (F), respectively. A fluorescence threshold value (*) was identified below which 95% of the pinocytosis population fell. The cytoplasmically loaded population was defined as that which was above this threshold value after syringe loading. As indicated, not only did the mean fluorescence value (MFV) increase when cells were loaded in the presence of PF-68, but so did the (%) of cells that had been cytoplasmically loaded ((%) CL).

conditions for loading - 2% PF-68 and 8 strokes - syringe loading is an extremely gentle technique: >80% of syringe-loaded BAEC are viable and can be successfully replated.

In order to rule out the possibility that PF-68 has a direct mitogenic effect on BAEC, and to investigate its long-term effects on cell viability, we measured the effect on BAEC numbers of continuous exposure to PF-68 over a period of three days (as compared to a 2 min exposure during syringe loading). The growth rate of BAEC was not found to be significantly different in PF-68 than in medium alone (Fig. 5). This result indicates that PF-68 is not a direct mitogen for BAEC, or a growth inhibitor. We believe that the "mitogenic" effect observed when BAEC are syringe loaded in the presence of PF-68 may be due to membrane wound-

induced release of growth factors (Muthukrishnan et al., 1991).

In order to provide a useful reflection of the overall efficiency of the different syringe loading conditions, a "loading index" was calculated as follows: (%) cells surviving (at 16 h) \times (%) cells cytoplasmically loaded \times mean fluorescence value (MFV) of the cytoplasmically loaded population. Fig. 6 illustrates the relationship between number of syringe strokes, concentration of PF-68 and loading index for BAEC (Fig. 6A) and NIH.3T3 cells (Fig. 6B). As can be seen in Fig. 6A, the loading index increased with increasing syringe strokes and concentration of PF-68. Optimal loading of BAEC occurred at a concentration of 2% PF-68, using 8 syringe strokes. The amount of cytoplasmic loading of NIH.3T3 cells also increased as the number of syringe

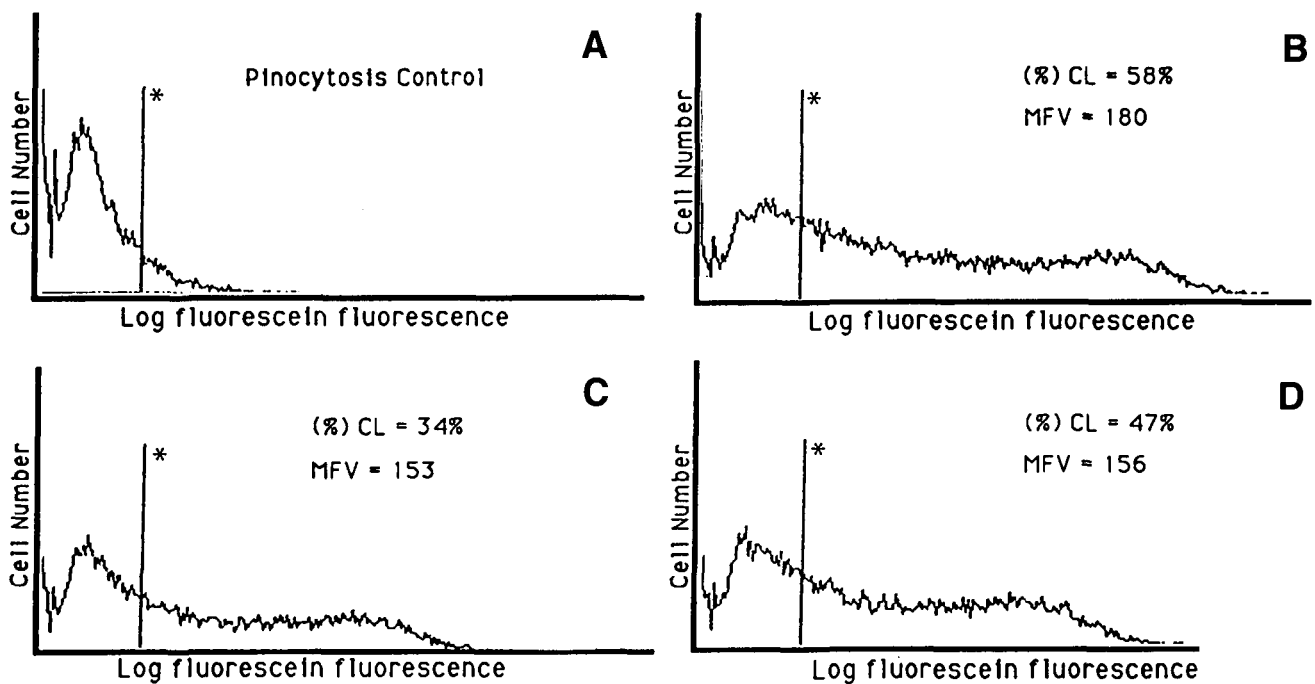


Fig. 3. BAEC syringe loaded in the presence of a range of dextrans of different relative molecular mass and analysed using flow cytometry. The cells were incubated in culture medium containing Fdx (M_r 10,000) and PF-68 (2%, w/v) and not syringe loaded (pinocytosis control) (A), or syringe loaded using 8 strokes in culture medium containing 2% PF-68 and Fdx (M_r 43,000) (B), Fdx (M_r 67,000) (C) and Fdx (M_r 120,000) (D), Fdx at 10 mg/ml. The cytoplasmically loaded population was obtained by using the fluorescence threshold value (*) obtained from the pinocytosis control. The proportion of cytoplasmically loaded cells ((%) CL), as well as the mean fluorescence value (MFV) of the loaded population, are significantly increased over the levels observed in the pinocytosis control, illustrating that syringe loading is an efficient method of loading a range of large macromolecules into the cytosol.

strokes increased. For this cell type, however, 0.5% rather than 2% PF-68 resulted in more efficient syringe loading (Fig. 6B).

The effect of PF-68 on the survival of BAEC subjected to two other mechanically based cell loading techniques, namely scrape and bead loading, was also evaluated. Fig. 7 shows that PF-68 significantly increased the numbers of cells surviving all three loading techniques ($P < 0.01$ for scrape and bead loading, $P < 0.005$ for syringe loading; Student's *t*-test).

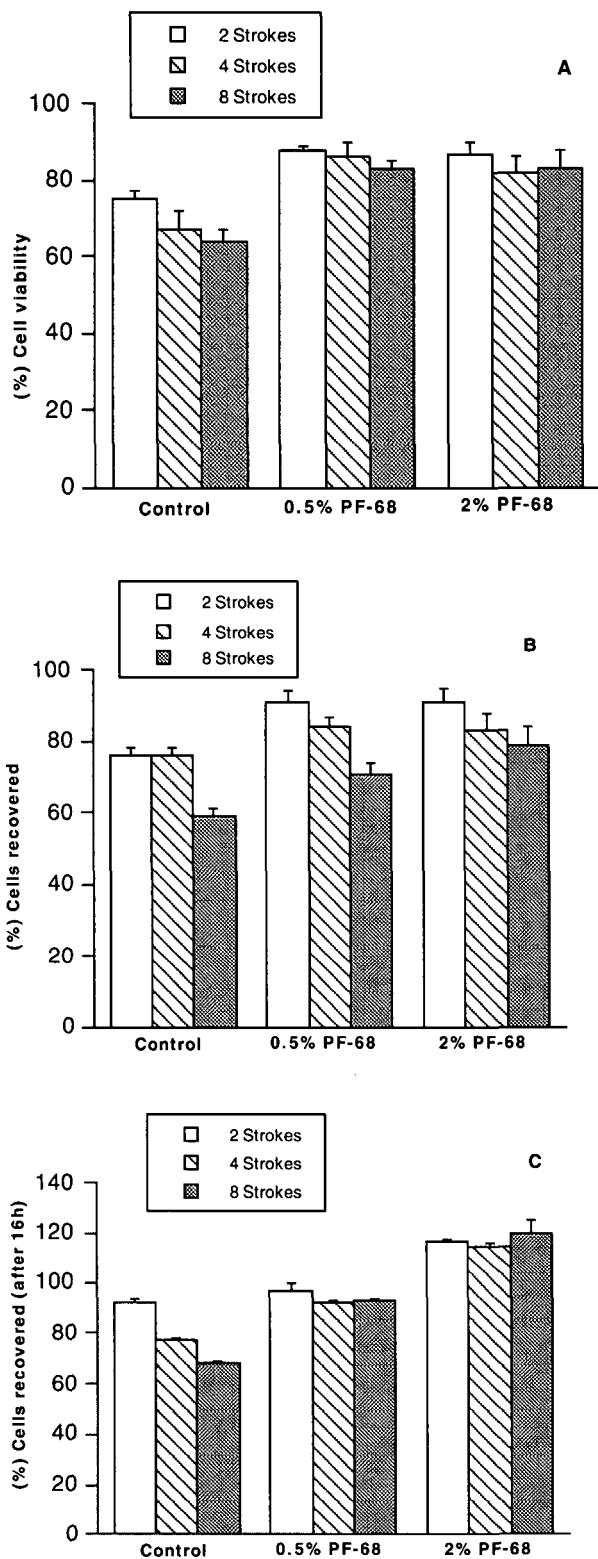
The relative efficiencies of these three loading techniques were next compared under conditions determined to be optimal for each one. Syringe loading, based on its higher loading index, was the most efficient of the three tested (Fig. 8).

Although the syringe loading technique, as just described, is a comparatively highly efficient means of loading macromolecules, it required a relatively large volume (0.25 ml - 1 ml using a 1 ml syringe) of loading solution. To decrease the volume of loading solution and hence the required amount of the macromolecule to be loaded, we substituted a Hamilton microsyringe for the conventional 1 ml syringe, and obtained successful loading of Fdx-Lys into BAEC using 25 μ l of loading solution (Fig. 9A). Moreover, using a specialized ultra low-volume plastic micropipette tip attached to a conventional, hand-held pipettor, we succeeded in loading BAEC using only 5 μ l of loading solution (Fig.

9B). PF-68 appeared, on the basis of microscopic observation, to increase the amount of Fdx-Lys loaded into the cytoplasm of BAEC using these two modifications to the syringe loading technique.

Discussion

The non-ionic surfactant molecule, PF-68 ($M_r \sim 8000$), has previously been described in the literature as a "survival factor" that acts on the plasma membrane of cells growing in suspension, protecting them from the shear forces generated during culture in suspension (Mizrahi, 1983; Ramirez and Mutharasan, 1990). Here, we report that PF-68 increases cell survival during syringe loading, and that, in addition, it increases the loading efficiency of this and other mechanically based techniques. However, it should be noted that PF-68 has been reported to have a number of toxic effects *in vitro*, as well as *in vivo*. These include the induction of phospholipidosis in the rat when administered daily over a period of one month (Magnusson et al., 1986), the impairment of neutrophil delivery to inflammatory sites in a mouse model, with a consequent increase in infection mortality (Lane and Lamkin, 1986), and the inhibition of random migration and chemotaxis of human polymorphonuclear cells (PMN) *in vitro* (Lane and Lamkin, 1984). The effects of PF-68 on PMN reported by Lane and Lamkin (1984) were totally



reversible if the compound was washed away within 1 h of application. Our own data and that of others (Mizrahi, 1983; Ramirez and Mutharasan, 1990) suggest that PF-68 has no effect on the long-term viability of certain cell types. More detailed studies will be required to determine whether PF-68 will effect specific cellular functions and/or the viability of other cell types, when

Fig. 4. The effect of PF-68 on cell recovery after syringe loading of Fdx-Lys (10 mg/ml). BAEC were syringe loaded in the presence or absence of PF-68 (0.5% and 2% w/v). Cell viability (A) and the number of cells recovered (B) were determined immediately after syringe loading.

Measurement of initial recovery levels ensured that damage to cells that is so severe as to make them unrecognizable in the microscope did not go undetected, as is possible when using trypan blue exclusion as a measurement of cell viability alone. The cells were then allowed to reattach to the culture substratum for 16 h, after which time the numbers of cells that had replated and could be recovered was determined (C). All data are expressed as percentage of the number of cells present in the control cell population at each stage. Control cells were incubated in 2% PF-68 and 10 mg/ml Fdx-Lys but were not subjected to syringe loading. When syringe loading was carried out in culture medium alone, increasing the number of syringe strokes resulted in a decrease in cell viability and number of cells remaining. The presence of PF-68 minimized these effects, on both cell viability and the number of cells recovered. When the number of cells remaining after 16 h (C) was determined, a significant increase in the cell number was observed in those cultures that had been syringe loaded in the presence of 2% PF-68, not only over that found in those cultures syringe loaded in culture medium alone, but also over that found in the untreated controls.

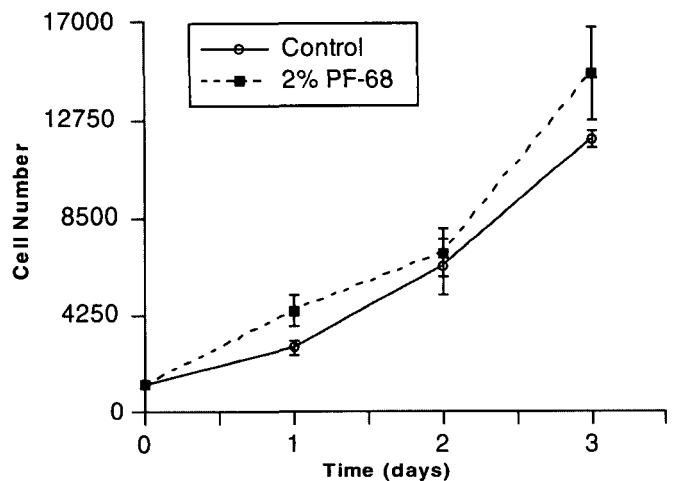


Fig. 5. The effect on BAEC growth of continuous exposure to 2% PF-68. Cells were plated in culture medium, at a cell density of 10,000 cells/well in a 24-well plate, and allowed to attach for 16 h. The medium was then removed and replaced with either 1 ml of fresh culture medium or 1 ml of culture medium containing 2% (w/v) PF-68, each condition being run in replicates of three. The number of cells that had reattached after 16 h was determined using a Coulter Counter and this was designated as day 0. The numbers of cells present under each culture condition was assessed again on days 1, 2 and 3. No significant difference in cell growth rate was observed between the two conditions over this time period.

introduced into the cell cytoplasm, as presumably occurs during syringe loading.

Our data show that PF-68 is responsible for an increase in BAEC survival and loading efficiency in

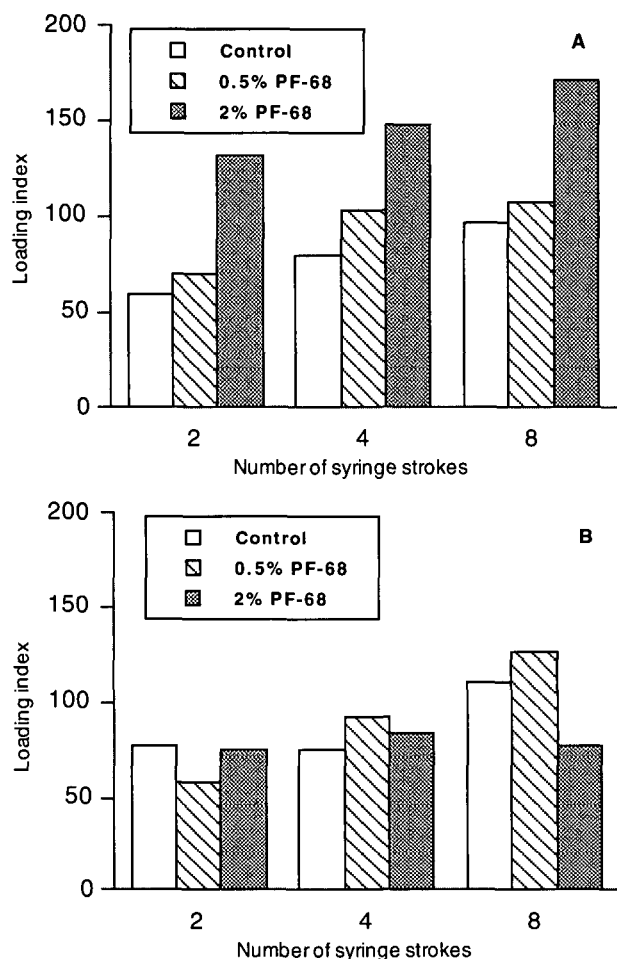


Fig. 6. The efficiency of the syringe loading technique. A loading index, was calculated as follows: (%) cells surviving (at 16 h) \times (%) cytoplasmically loaded cells \times mean fluorescence value (MFV) of the loaded population. In the case of BAEC (A), a linear relationship was seen between the number of syringe strokes, the concentration of PF-68 and the loading index. Optimal loading of BAEC was obtained using 8 syringe strokes in the presence of 2% PF-68. Optimal loading was obtained in the case of NIH.3T3 fibroblast cells (B) using 0.5% PF-68 and 8 strokes, indicating that the effects of PF-68 may be cell type-, as well as concentration-dependent.

mechanically based, cell loading techniques. As yet, we have no conclusive evidence to indicate how PF-68 exerts this effect. Cells loaded in the presence of PF-68 have a greater mean fluorescence value than those loaded in its absence, suggesting that for a given amount of mechanical stress inflicted on a cell, PF-68 allows the formation of larger, or a greater number of, survivable plasma membrane wounds. One possible explanation for this is that PF-68 may act as an antidote to the toxic effects of membrane wounding, such as calcium influx into the cytosol. However, there are no theoretical or experimental reasons that can be given in support of this putative protective mechanism. Alternatively, PF-68 could act to promote the efficiency of membrane resealing, allowing the PF-68-treated cell to reseal successfully larger, or more numerous, membrane wounds. Ramirez and Mutharasan (1990)

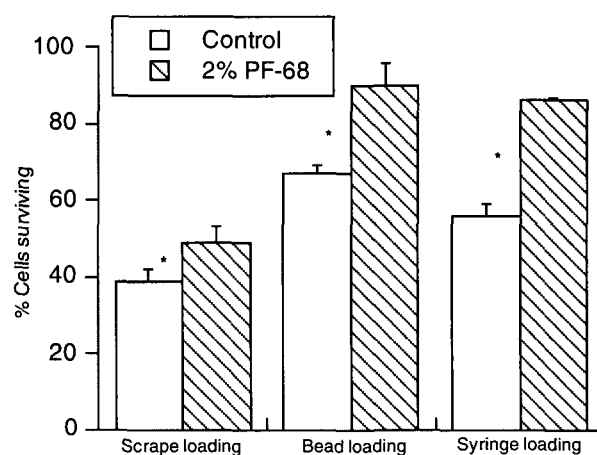


Fig. 7. The effect of PF-68 on BAEC survival after loading with Fdx-Lys, using three different mechanically based loading techniques: scrape, bead and syringe loading. Equivalent numbers of BAEC were loaded in the presence of Fdx-Lys (10 mg/ml) and in the presence or absence of 2% (w/v) PF-68. Loading was carried out under conditions determined to be optimal for each technique, and the cells were then immediately replated. The numbers of cells that survived each procedure after 16 h is expressed as a percentage of the untreated control population (cells simply incubated in Fdx-Lys (10 mg/ml) and 2% (w/v) PF-68). A significant increase in the numbers of cells that survived in all three loading technique was observed when loading was carried out in the presence of PF-68 (* - $P < 0.01$ for scrape loading, $P < 0.005$ for bead and syringe loading, Student's *t*-test).

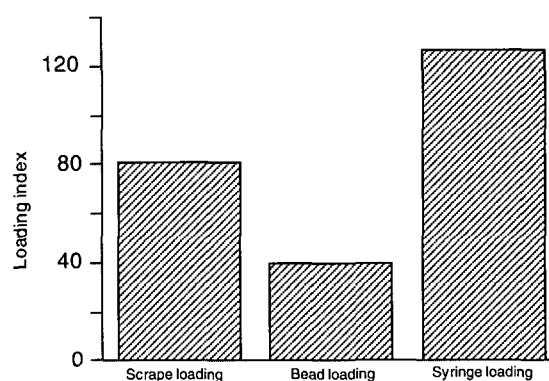


Fig. 8. Comparison of the relative loading efficiencies of scrape, bead and syringe loading. BAEC were cytoplasmically loaded with Fdx-Lys (10 mg/ml) at conditions determined to be optimal for each technique. All procedures were carried out on the same day, using the same stock solution of Fdx-Lys. The results illustrate that syringe loading is the most efficient of the three techniques for cytoplasmically loading Fdx-Lys into the cytosol of this cell type.

reported that PF-68 altered the physical properties of the plasma membrane, making it less fluid. We suggest that such an alteration could conceivably favour resealing of the ruptured plasma membrane.

In summary, syringe loading is a novel method for introducing macromolecules into the cytosol of cells

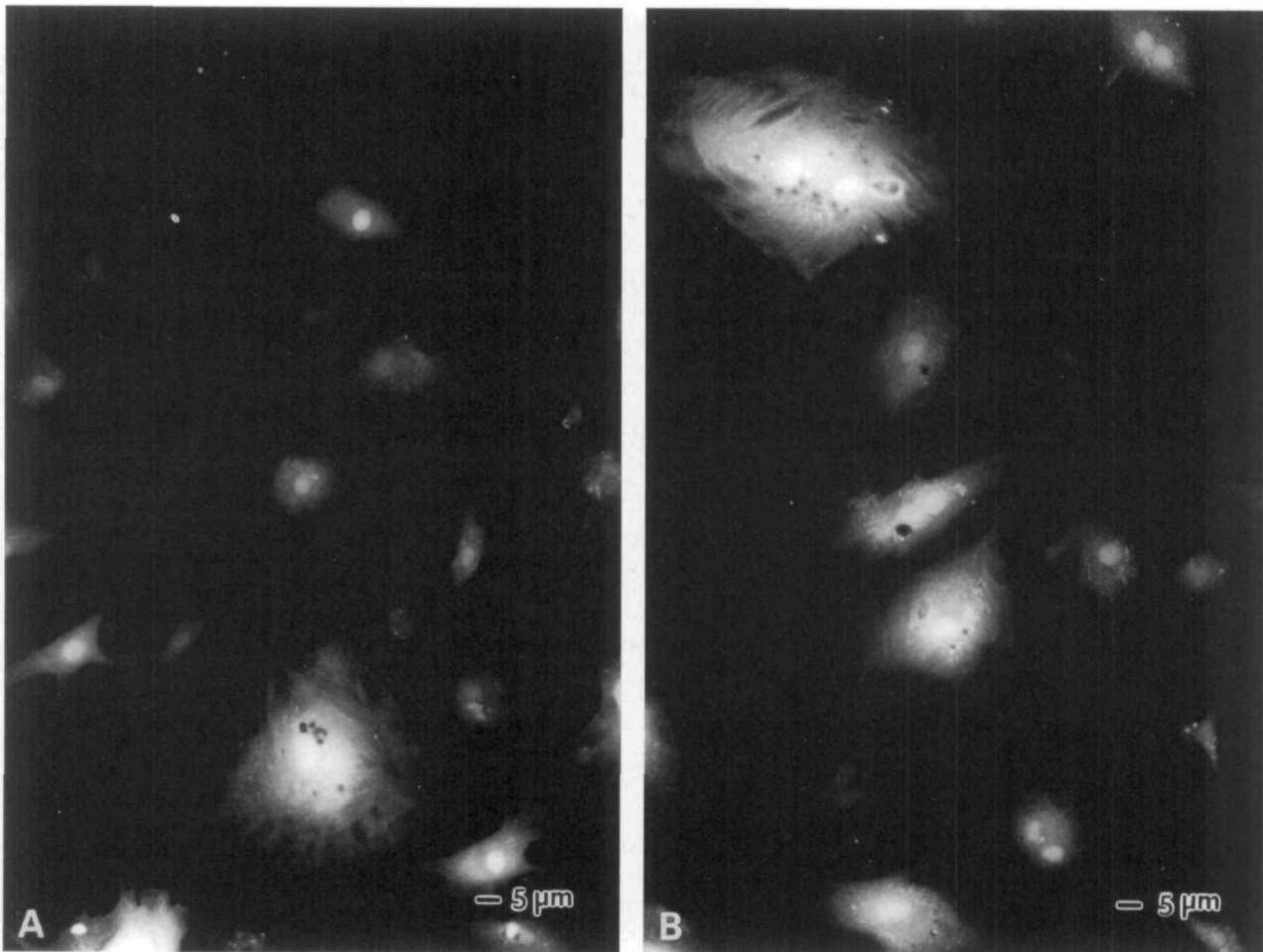


Fig. 9. Syringe loading using microvolume loading solutions. Fluorescence micrographs of BAEC syringe loaded in culture medium containing PF-68 (2%, w/v) and Fdx-Lys (20 mg/ml) using (A) a Hamilton microsyringe (loading volume of 25 μ l) and (B) a standard, hand-held pipettor, with an attached low-volume pipette tip (loading volume of 5 μ l). Micrographs were taken at equivalent exposure times. Bars, 5 μ m.

that is highly efficient, gentle and reproducible: 85% of a cell population could be loaded under conditions in which 85% of the original number of cells survived and were replated. It can also be extremely economical in its use of the macromolecule to be loaded: a volume of loading solution of 5 μ l can be used if a micropipette tip, or microsyringe, is substituted for a conventional syringe and needle. PF-68 demonstrably increases the efficiency of syringe loading and of several other mechanically based loading techniques, and may facilitate microinjection and electroporation as well. The mechanism of action for PF-68 remains to be completely elucidated, but the plasma membrane effects reported here raise several interesting questions with regard to the role of biological surfactants in physiological situations where the imposition of mechanical stress is commonplace, such as in the lungs and gastrointestinal tract.

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