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Permeabilization of the Plasma Membrane of L1210 Mouse Leukemia Cells Using Lithotripter Shock Waves

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Abstract. Permeabilization of L1210 cells by lithotripter shock waves in vitro was monitored by evaluating the accumulation of fluorescein-labeled dextrans with a relative molecular mass ranging from 3,900–2,000,000. Incubation with labeled dextran alone caused a dose- and time-dependent increase in cellular fluorescence as determined by flow cytometry, with a vesicular distribution pattern in the cells consistent with endocytotic uptake. Shock wave exposure prior to incubation with labeled dextran revealed similar fluorescence intensities compared to incubation with labeled dextran alone. When cells were exposed to shock waves in the presence of labeled dextran, mean cellular fluorescence was further increased, indicating additional internalization of the probe. Confocal laser scanning microscopy confirmed intracellular fluorescence of labeled dextran with a diffuse distribution pattern. Fluorescence-activated cell sorting with subsequent determination of proliferation revealed that permeabilized cells were viable and able to proliferate. Permeabilization of the membrane of L1210 cells by shock waves in vitro allowed loading of dextrans with a relative molecular mass up to 2,000,000.

Permeabilization of tumor cells by shock waves provides a useful tool for introducing molecules into cells which might be of interest for drug targeting in tumor therapy in vivo.

Key words: Membrane permeabilization — Tumor cells — Lithotripter shock waves — Labeled dextrans — Flow cytometry

Introduction

Permeabilization of the plasma membrane is a valuable tool for gaining access to the cytosol. Various methods have been used, e.g., treatment of cells with adenosine 5′-triphosphate (Rozengurt & Heppel, 1975), and with detergents such as saponin (Bangham & Horne, 1962) or digitonin (Zuurendonk & Tager, 1974), incubation with *Staphylococcus aureus* α-toxin (Arbuthnott, Freer & Bernheimer, 1967), Streptolysin O (Duncan, 1974), or Sendai virus (Pasternak & Micklem, 1973), exposure to hypotonic (Hoffman, 1962) or hypertonic medium (Castellot, Miller & Pardee, 1978), scrape-loading (McNeil et al., 1984), and exposure to an electrical field (*reviewed in* Zimmermann, 1986). We previously used propidium iodide to demonstrate a transient increase in cell membrane permeability to small molecules by exposure of tumor cells to shock waves (Gambihler, Delius & Ellwart, 1992).

Lithotripter shock waves are pressure pulses of high amplitude and short duration used for the disintegration of urinary and biliary calculi. Various studies have demonstrated disruption of tumor cells by shock waves in vitro (Russo et al., 1986; Brümmer et al., 1989; Oosterhof et al., 1989; Gambihler, Delius & Brendel, 1990; Steinbach et al., 1992). In the present study, experiments were performed in an attempt to demonstrate permeabilization of tumor cells for macromolecules by means of shock wave exposure. Fluorescein-labeled dextran (FD) was used as tracer probes since dextrans are available in a wide range of molecular mass, and determination of fluorescence is easily performed by flow cytometry (McNeil, 1989).

Materials and Methods

Cell Culture

L1210 mouse leukemia cells were grown at 37°C as suspension culture in Roswell Park Memorial Institute 1640 medium, supplemented with 15% heat-inactivated fetal calf serum, 2% sodium pyruvate,

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and 1% antibiotic-antimycotic solution containing 10,000 U/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml Fungizone® (GIBCO, Eggenstein, FRG) in a humidified atmosphere containing 5% CO_2. Log phase single cell suspensions, harvested without trypsinization, and with a viability greater than 98% were used for the experiments.

Shock Waves and Exposure Vials

Shock waves were generated with a XL1 lithotripter (Dornier Medizintechnik, Germering, FRG) by underwater spark discharge between the two tips of an electrode located in a metal hemi-ellipsoid which was used as focusing device. The generator was operated at 80 nF capacitance, a voltage of 25 kV, and a discharge rate of 60/min. The water in the lithotripter tank, maintained at 37°C, was degassed by a vacuum pump; oxygen content as determined with an oximeter OXI 96 (WTW, Weilheim, FRG) was 0.5–1 mg/liter. The pressure field of the shock waves has been described elsewhere (Mueller, 1990).

Polypropylene vials (Nunc, Roskilde, Denmark) with a volume of 3 ml were filled with cell suspension and closed with a silicone stopper (Bender & Hobein, Munich, FRG) perforated with a butterfly canula (size 19G/1.1 mm, Pfrimmer, Erlangen, FRG). The vials were positioned into the focus of the shock waves.

Fluorescein-Labeled Dextran

Relative molecular mass, Stokes' radius, mol fluorescein per mol glucose residue (FITC per glucose), and the molar dye/dextran ratio of the fluorescein-labeled dextrans (Sigma, Taufkirchen, FRG) are summarized in Table 1. The stability of FDs solubilized at 1 mg/ml 0.05 M Tris-HCl (pH 7.0) was tested after exposure to 250 shock waves. Control solutions were not exposed. After high performance gel permeation chromatography of 20 μl each on a TSK G3000SW column (LKB, Bromma, Sweden) at a flow rate of 1 ml/min, fluorescence was recorded at 515 nm with excitation provided at 490 nm with a fluorescence monitor RF-530 (Shimadzu, Tokyo, Japan). Detection limit for FITC was below 0.25 pmol. For the experiments, FD solutions were prepared in culture medium.

Flow Cytometry

Fluorescence of cells was determined with a FACS Analyzer I and a Consort 30 data management system (Becton Dickinson, Heidelberg, FRG). An Argon laser provided excitation at 488 nm. Following calibration using CaliBRITE benchmark beads (Becton Dickinson) list mode data from at least 10,000 cells per sample were recorded by gating on Coulter volume and 90° side scatter.

Fluorescence was quantified with high level quantitative fluorescein microbead standards (diameter 4.4–9.0 μm; Flow Cytometry Standards, Research Triangle Park, NC) as described previously (Bartoletti, Harrison & Weaver, 1989). The known number of equivalent soluble fluorescence molecules (ESFM) per bead provided the basis to calibrate the mean fluorescence intensity detected with the flow cytometer in terms of ESFM.

Cell sorting was performed with a FACSTAR Plus and a Lysis II data management system (Becton Dickinson). Excitation at 488 nm was provided by an Argon laser. The treated samples were sorted from two windows, defined in a two-dimensional plot of forward scatter and fluorescein fluorescence, distinguishing between FD-negative and FD-positive cells. The untreated control was sorted from only one window. 1×10^5–1.5×10^6 cells were sorted in Hank's balanced salt solution (HBSS), centrifuged, and resuspended in culture medium.

Table 1. Characteristics of the fluorescein-labeled dextrans used in this study

Dextran	M_r	Stokes' radius (Å)	FITC per glucose	Dye/dextran ratio
FD 4	3,900	16	0.004	0.09
FD 20	18,900	45	0.004	0.42
FD 40	35,600	59	0.003	0.59
FD 70	71,600	86	0.004	1.59
FD 150	147,800	87	0.004	3.28
FD 500	487,000	205–210	0.007	18.92
FD 2000	2,000,000	280–290	0.007	77.69

Relative molecular mass, Stokes' radius, mol fluorescein per mol glucose residue (FITC per glucose), and the molar dye/dextran ratio are presented.

Cell Number and Proliferation Assay

The number of cells was determined using trypan blue dye exclusion. Equal amounts of cell suspension and trypan blue (2 mg/ml 0.9% NaCl solution; Fluka, Buchs, Switzerland) were mixed. After 3 min, the number of unstained cells was counted in a hemocytometer.

Proliferation of cells was tested with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Carmichael et al., 1987). Cell suspension (100 μl), containing 9×10^2 cells, and 50 μl culture medium were plated into each well of 96-well round bottom microtiter plates (Nunc) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. At least eight replicate wells were used to determine each data point. After 72 hr, providing time for at least six cell duplications of untreated control cells, 50 μl MTT solution (2.5 mg/ml 0.9% NaCl solution; Sigma) was added to each well. After a further incubation of 4 hr, the supernatant fluid was removed, 100 μl dimethylsulfoxide (E. Merck, Darmstadt, FRG) was added to each well, and absorbance at 492 nm was measured within 5 min using a 400 AT plate reader (SLT Labinstruments, Overath, FRG). Proliferation of sorted samples was calculated as a fraction of the proliferation of untreated, unsorted control cells.

Background Fluorescence

Samples of 5×10^5 L1210 cells were incubated with 0.1, 1, or 10 mg/ml FD 40 at 37°C for 10–240 min. Cells were washed four times with a fourfold volume HBSS and resuspended in culture medium. Cell concentrations, determined with the trypan blue assay, were adjusted to 1×10^5/ml, and the intensities of cellular fluorescein fluorescence assessed by flow cytometry. The experiment was performed in triplicate for each FD concentration and incubation time. The efficiency of the washing procedure was tested after incubation of L1210 cells with 10 mg/ml FD 40 at 37°C for 60 min. At the end of each washing step, the cellular fluorescence was detected by flow cytometry.

Exposure to FDs and Shock Waves

Permeabilization of L1210 cells was monitored using FDs (M_r 3,900–2,000,000). Three or more samples of 3 ml cell suspension, containing 5×10^5 L1210 cells and the appropriate FD at various concentrations, and one sample containing no FD were transferred into the exposure vials. The latter sample received no shock wave treatment and served as the untreated control. Two samples were exposed

to 250 shock waves, either before, or during incubation with FD. A third sample was incubated with FD alone. Incubation with 0.01–10 mg/ml FD was at 37°C for 60 min. All samples were washed four times with a fourfold volume HBSS, resuspended in culture medium, and cellular fluorescence was assessed by flow cytometry. The experiment was performed in triplicate for each FD and each FD concentration.

The time course of the decay of fluorescence following permeabilization was monitored by exposure of four samples of L1210 cells to 10 mg/ml FD 40 to 60 min and 250 shock waves as described above. All samples were washed with HBSS, and resuspended in culture medium. Cell concentrations were adjusted to 4×10^3/ml, the suspensions were transferred into culture flasks, and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Every 24 hr, samples were drawn from the culture flasks, the number of cells was determined with the trypan blue assay, and cellular fluorescence was assessed by flow cytometry. The experiment was performed in triplicate.

The viability following permeabilization was investigated with L1210 cells exposed to 250 shock waves during the 60 min incubation with 10 mg/ml FD 40, and with untreated controls. The samples were washed with HBSS, resuspended in culture medium. The desired cell populations were obtained by fluorescence-activated cell sorting, and proliferation was determined with the MTT assay. The experiment was performed twice.

Localization of FD

Samples of L1210 cells were incubated for 60 min with 10 mg/ml FD 40, with or without exposure to 250 shock waves during the incubation time. Cells were washed with HBSS, resuspended in culture medium, pelleted by centrifugation, and transferred on microscope slides. The slides were examined with a MRC 600 confocal laser scanning microscopy system (Bio-Rad Microscience Division, Hempstead, UK) attached to a Nikon Optiphot microscope (Zeiss planapo oil immersion objective 63×; NA = 1.25). The laser source was an Argon laser.

Data Analysis

Four parameters were used: mean fluorescein fluorescence intensity and percentage of FD-postive cells, both obtained from frequency histograms of fluorescence intensities using the Consort 30 data management system, the estimated number of FD molecules per cell introduced by shock waves, and the number of FD molecules per cell per mM extracellular FD.

To obtain the percentage of FD-positive cells, a threshold was set in the frequency histogram of fluorescence intensity of cells that had been incubated with FD alone below which 98% of cells fell. The samples exposed to shock waves before or in the presence of the respective FD at the same concentration were evaluated with the corresponding fluorescence intensity threshold.

The background fluorescence resulting from incubation of cells with FD alone was subtracted from the mean fluorescence intensity of cells exposed to shock waves in the presence of the label. The number of FD molecules loaded into the cells was estimated from the mean fluorescence intensities calibrated in terms of ESFM using the molar dye/dextran ratio according to

$$FD\ molecules\ per\ cell = \frac{ESFM\ (shock\ waves + FD) - ESFM\ (FD\ alone)}{molar\ dye\ /\ dextran\ ratio} \quad (1)$$

For the direct comparison of the number of FD molecules obtained per cell with FDs of different molecular mass, this parameter was normalized for an extracellular FD concentration of 1 mM. To test whether FD diffuses across the membrane of cells permeabilized by shock waves, the normalized number of FD molecules was plotted against the square and the cube root of M_r, since diffusion coefficients are inversely proportional to M_r^β, where β has a value between $-1/2$ to $-1/3$ for diffusion in water (Stein, 1981).

Results

Stability of FDs

In this study, FDs were used to monitor the permeabilization of the plasma membrane of tumor cells as a result of shock wave exposure. To confirm the stability of the FDs during shock wave exposure, gel permeation chromatography was performed. Without (Fig. 1*A*) and following shock wave exposure of FD solutions (Fig 1*B*) neither a difference of the shape of the chromatograms, nor free FITC, both indicating degradation of FD, were detected.

Background Fluorescence

The increase of background fluorescence of L1210 cells due to an incubation with 0.1, 1, and 10 mg/ml FD 40 for 10–240 min was nonsaturable over the tested concentration range (Fig. 2). The increase was initially rapid but after 30–60 min reached a slower, almost linear increase with incubation time. The inset in Fig. 2 shows the efficiency of the washing procedure. During the first steps, a large amount of fluorescence was washed away from the cells. After the fourth wash, no further decrease in cell-associated fluorescence was observed.

Exposure to FD and Shock Waves

The overlay histogram from a single experiment of the fluorescence intensities of L1210 cells incubated with 1 mg/ml FD 40 with or without exposure to shock waves is shown in Fig. 3*A*. Fluorescence of cells incubated with FD alone, the background fluorescence, was again increased. Relative to this background fluorescence, the intensity threshold was set (arrow) so that 98% of these cells were rated as FD negative and 2% as FD positive. Fluorescence of cells exposed to shock waves before incubation with FD was almost identical with 2.7% positive cells (Fig. 3*A*). Cells exposed to shock waves in the presence of FD revealed a further increase in fluorescence with 20.3% of the cells evaluated as FD positive. The wide distribution of fluorescence intensity indicated a heterogeneous amount of loaded FD.

Mean fluorescence of cells, incubated with in-

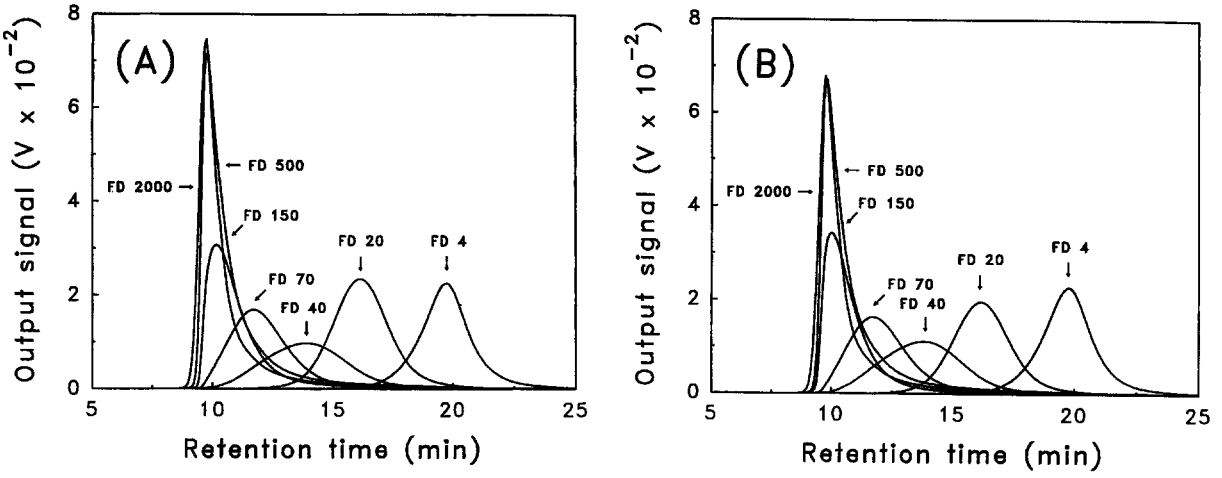

Fig. 1. Stability of FDs solubilized in 0.05 M Tris-HCl (pH 7.0) and either exposed or not exposed to shock waves. Following gel permeation chromatography, fluorescence was recorded as described in Materials and Methods. Overlay histograms of the fluorescence signals are plotted against the retention time of FDs (*A*) without and (*B*) after shock wave exposure.

creasing concentrations of FD 40 with or without exposure to shock waves is shown in Fig. 3*B*. Background fluorescence of cells incubated with FD alone and fluorescence of cells exposed to shock waves before incubation with FD increased with the concentration but with no difference between the two groups. Fluorescence intensity of cells exposed to shock waves in the presence of FD was up to 6.1-fold higher than background fluorescence.

Localization of FD

Fluorescence of FD 40, detected with confocal laser scanning microscopy, was found to be located with a vesicular pattern in cells incubated with FD alone (Fig. 4, *Top*). Cells exposed to shock waves in the presence of FD showed a diffuse intracellular distribution of fluorescence with some vacuole-like structures and the nucleus apparently spared (Fig. 4, *Bottom*).

Viability of Cells and Decay of Fluorescence Following Permeabilization

The viability of L1210 cells after loading with FD 40 was investigated by fluorescence-activated cell sorting and subsequent determination of the proliferation of the sorted cells (Table 2). Cell sorting by itself reduced the proliferation of L1210 cells. FD-negative and FD-positive cells proliferated at almost the same rate as untreated, sorted L1210 cells.

Fluorescence of cells exposed to shock waves in the presence of 10 mg/ml FD 40 decreased slowly during the first day after exposure (Fig. 5). The exponential decrease of the number of FD molecules per cell from day 1 to day 4, estimated according to Eq. (1) from the data presented in Fig. 5, revealed a half-time of 10 ± 1 hr

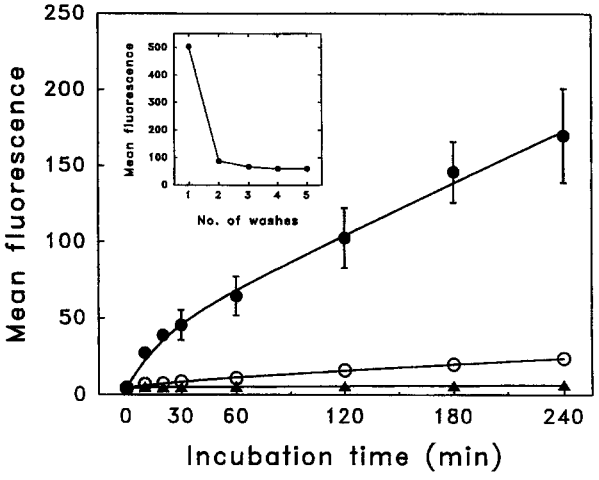

Fig. 2. Background fluorescence of L1210 cells incubated with FD 40 at 37°C for 10–240 min at 0.1 (▲), 1 (○), or 10 mg/ml (●). The samples were washed four times with a fourfold volume HBSS, resuspended in culture medium, and the intensity of cellular fluorescein fluorescence was detected as described in Materials and Methods. Each data point is the mean ± SD of three separate determinations. The efficiency of the washing procedure after incubation with 10 mg/ml FD 40 at 37°C for 60 min is shown in the inset.

(mean value ± SEM obtained from three independent experiments). This corresponds within experimental error to the estimated doubling time of the cells of 9 ± 1 hr from day 1 to day 4 (*see inset* Fig. 5). Fluorescence of cells incubated with FD alone and of cells exposed to shock waves before incubation with FD initially decreased at a similar rate.

Uptake of FDs with Different Molecular Mass

The percentage of L1210 cells that was FD positive after incubation with the different FDs at 10 mg/ml with

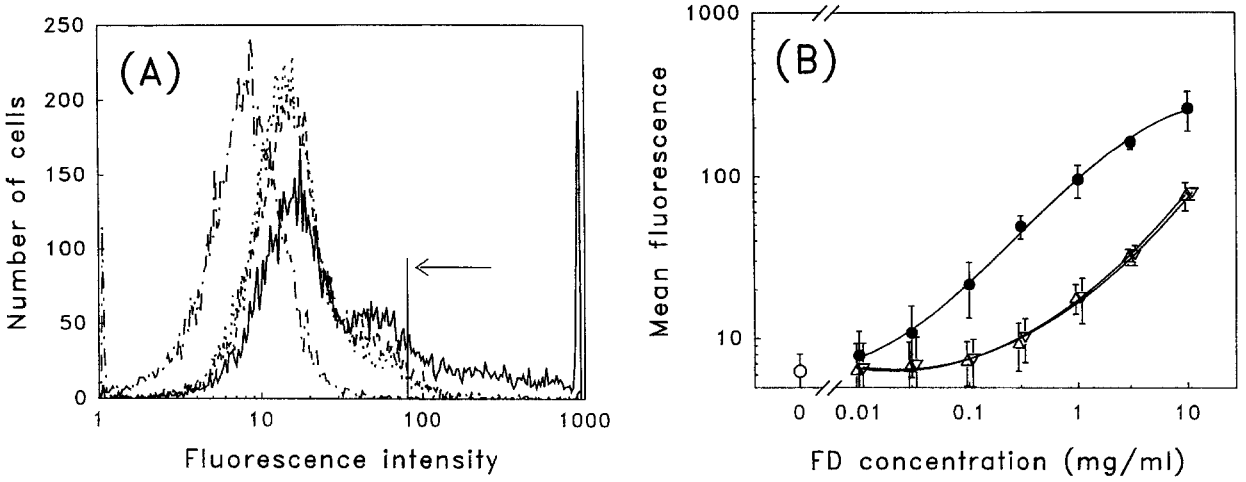

Fig. 3. Fluorescence of L1210 cells incubated with 0.01–10 mg/ml FD 40 at 37°C for 60 min, and exposed to 250 shock waves either before or in the presence of FD. Control cells were not treated. All samples were washed with HBSS, resuspended in culture medium, and cellular fluorescence was assessed by flow cytometry. (*A*) Overlay histogram of the frequency distribution of the fluorescence of control cells (dash-dotted line), of cells incubated with FD alone (narrow dots), and exposed to shock waves either before (wide dots) or during (unbroken line) incubation with FD. The arrow points at the fluorescence intensity threshold. The data presented are from one representative experiment of three with a FD concentration of 1 mg/ml. (*B*) Mean fluorescence of control cells (○), of cells incubated with FD alone (△), and exposed to shock waves either before (▽) or during (●) incubation with FD. The mean values ± SD of three independent experiments are shown. For clarity, some of the points are slightly offset.

or without exposure to shock waves (Fig. 6) was determined relative to a fluorescence intensity threshold. Thus, all samples incubated with FD alone were rated as containing 2% FD-positive cells. Exposure of L1210 cells to shock waves before incubation with FD did not increase this proportion. When L1210 cells were exposed to shock waves in the presence of FD, the percentage of FD-positive cells was increased up to 27%. Even in the presence of FD 2000, shock wave application yielded 10.0 ± 0.1% FD-positive cells (mean value ± SD obtained from three independent experiments).

The number of FD molecules per cell introduced due to the permeabilization of cells by exposure to shock waves in the presence of increasing concentrations of different FDs with a molecular mass ranging from 3,900 to 2,000,000, estimated according to Eq. (1) increased with the extracellular concentration of the FDs (Fig. 7). It was highest, with 2×10^6 FD molecules per cell, after exposure of L1210 cells to shock waves in the presence of 10 mg/ml FD 4. When FD 4 and FD 500 are compared at equimolar concentrations, e.g., 0.003 mM, corresponding to approximately 0.01 mg/ml FD 4 and 1 mg/ml FD 500, respectively, the number of dextran molecules that entered the cells was reduced from 1.5×10^4 for FD 4 to 1.3×10^3 for FD 500. Yet, between the FD species with higher molecular mass no clear difference could be directly derived from this dose-response curve.

To further investigate a molecular mass dependence of the introduction of FDs, the data presented in Fig. 7 were normalized for an extracellular FD concentration of 1 mM and plotted as a function of the square and the cube root of M_r(FD). Figure 8 shows a sharp decrease in the number of intracellular FD molecules with increasing relative molecular mass, yet apparently not inversely proportional when related to either the square or cube root of M_r.

Discussion

The study describes experiments that were performed to characterize the permeabilization of the membrane of tumor cells to macromolecules by exposure to lithotripter shock waves. Cells exposed to shock waves in the presence of labeled dextran revealed an increase in cellular fluorescence indicating internalization of the probe. Labeled dextran was located intracellularly with a diffuse distribution pattern. Permeabilized cells were viable and able to proliferate. Permeabilization of the membrane of L1210 cells by shock waves in vitro allowed loading of dextrans with a relative molecular mass up to 2,000,000.

FDs are particularly suitable for measuring permeability changes in cells since they are available in a variety of molecular mass, are stable under physiologic conditions, and can be quantified by flow cytometry (McNeil, 1989). Degradation of dextrans has been shown to result from sonication with ultrasound (Lorimer, 1991) under conditions where cavitation, the generation and movement of bubbles in a fluid (Crum, 1982), is expected. Shock waves also produce acoustic cavitation (Coleman et al., 1987), yet this did not affect the integrity of the FDs.

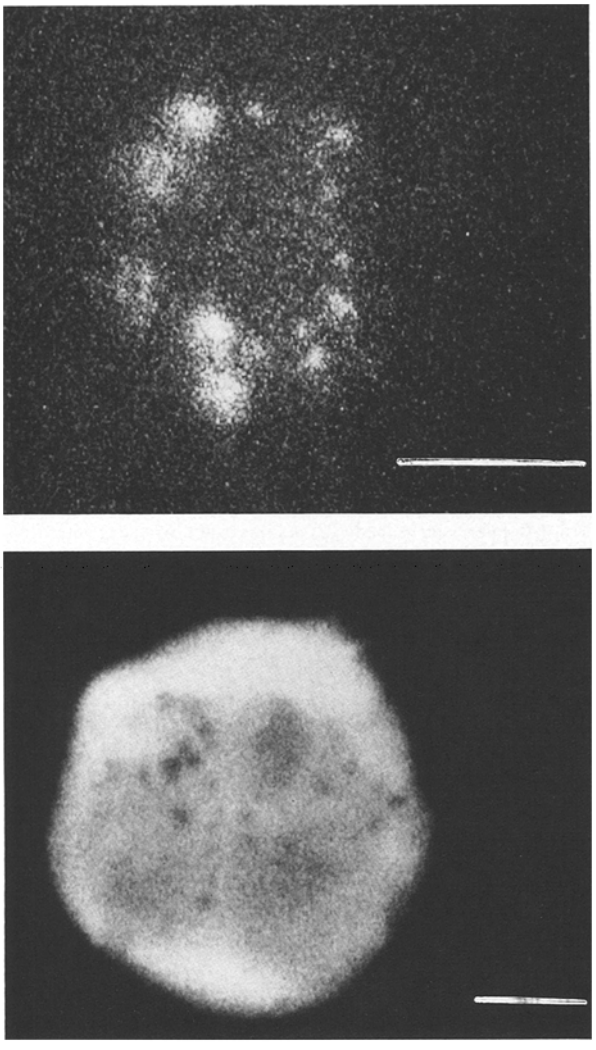

Fig. 4. Localization of FD in L1210 cells incubated with 10 mg/ml FD 40 alone at 37°C for 60 min (*Top*), or exposed to shock waves in the presence of FD (*Bottom*). The samples were washed with HBSS, resuspended, and fluorescence of equatorial sections of the cells was detected with a confocal laser scanning microscope as described in Materials and Methods. Bars, 5 μm.

Table 2. Viability of L1210 cells following permeabilization by shock waves

Treatment	Relative proliferation
Sorted control	0.67 ± 0.09
FD 40 and shock waves, FD negative	0.58 ± 0.14
FD 40 and shock waves, FD positive	0.51 ± 0.02

L1210 cells were incubated with 10 mg/ml FD 40 at 37°C for 60 min and exposed to shock waves. Control cells were not treated. Samples were processed, and the viability of the cells was investigated by fluorescence-activated cell sorting and subsequent determination of the proliferation of the sorted cells as described in Materials and Methods. The relative proliferation of sorted control cells, and of treated cells that were either FD negative, or FD positive, is expressed as a fraction of the proliferation of unsorted control cells. Mean values ± SD obtained from two independent experiments are presented.

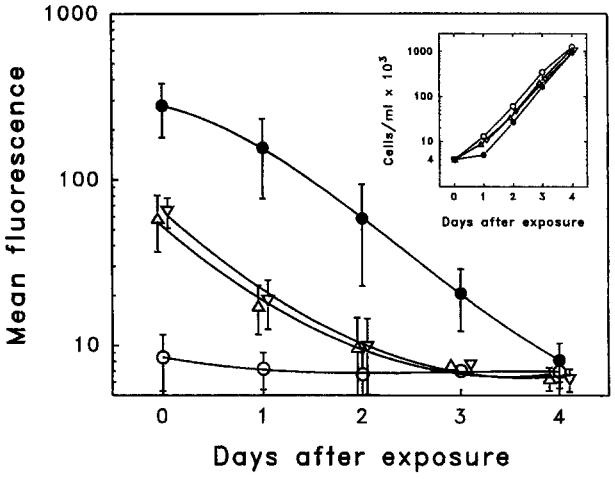

Fig. 5. Decay of FD 40 from L1210 cells following permeabilization by shock waves. L1210 cells were incubated with 10 mg/ml FD 40 at 37°C for 60 min, and exposed to shock waves either before or in the presence of FD. Control cells were not treated. All samples were washed with HBSS, resuspended in culture medium, and transferred into culture flasks as described in Materials and Methods. At the times indicated on the abscissa, cellular fluorescence was assessed by flow cytometry. The data shown are the mean ± SD of three independent experiments. For clarity, some of the points are slightly offset. The cell concentrations determined with the trypan blue assay are shown in the inset.

When L1210 cells were exposed to FD, it was demonstrated that incubation of cells with FD alone increases cellular fluorescence and that exposure of cells to shock waves in the presence of FD further increases cellular fluorescence, whereas exposure of cells to shock waves before incubation with FD does not.

In a control experiment, the increase of background fluorescence as a result of incubation with FD alone was investigated. FDs are considered to be markers for the detection of fluid-phase endocytosis (Swanson, 1989). The concentration dependence and curvi-linear increase of background fluorescence during incubation with FD, and the vesicular distribution of FD fluorescence within the cells is consistent with an endocytotic uptake of the probe during incubation with cells.

The higher cellular fluorescence after exposure to shock waves in the presence of FD was found to be located in the cytosol of the cells. Thus, a marked amount of labeled dextran had entered the cells due to shock wave exposure. Confocal laser scanning microscopy revealed no FD associated with the cell membrane after shock wave exposure, as it has been described to occur after electroporation (Michel et al., 1988; Glogauer & McCulloch, 1992).

Since lithotripter shock waves disrupt tumor cells

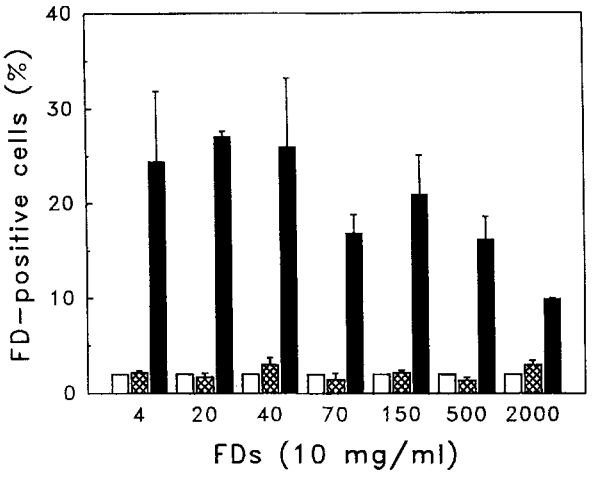

Fig. 6. Percentage of FD-positive L1210 cells incubated with 10 mg/ml FDs alone at 37°C for 60 min (□), incubated with FDs after exposure to shock waves (⊠), or exposed to shock waves in the presence of FDs (■). The samples were washed with HBSS, resuspended, and cellular fluorescence was assessed by flow cytometry. The percentage of FD-positive cells was evaluated according to a fluorescence intensity threshold as described in Materials and Methods. The data shown are the mean ± SD of three independent experiments.

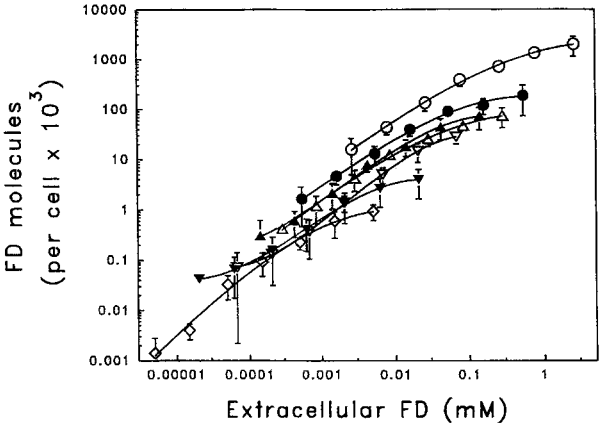

Fig. 7. Loading of FDs with different molecular mass. L1210 cells were incubated with 0.01–10 mg/ml FD 4 (○), FD 20 (●), FD 40 (△), FD 70 (▲), FD 150 (▽), FD 500 (▼), or FD 2000 (◇) at 37°C for 60 min, and either exposed to shock waves or not. The samples were washed with HBSS, resuspended, cellular fluorescence was assessed by flow cytometry, and the number of FD molecules loaded into the cells was estimated according to Eq. (1). The data shown are the mean ± SD of three independent experiments.

in vitro (Russo et al., 1986; Brümmer et al., 1989; Oosterhof et al., 1989; Gambihler et al., 1990; Steinbach et al., 1992), the contribution of cells that have permanently lost the integrity of their cellular membrane to the markedly increased fluorescence had to be considered. Propidium iodide, one of the most commonly used fluorescent dyes to determine viability was not used, since from previous experiments (Gambihler et al., 1992) we

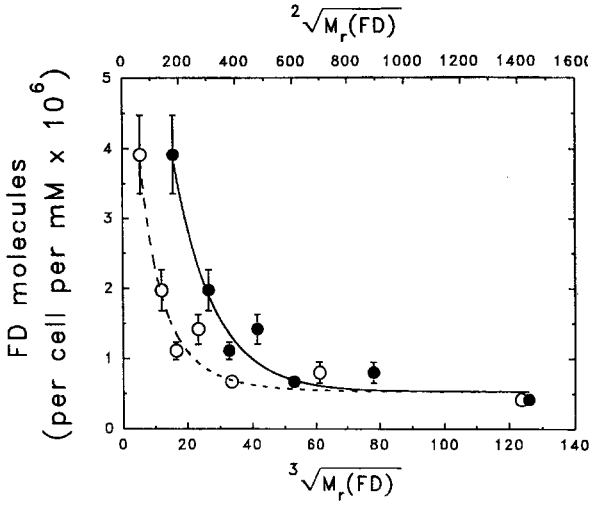

Fig. 8. Molecular mass dependence of loading. The mean number of FD molecules per cell presented in Fig. 7 was normalized for an extracellular FD concentration of 1 mM, and is plotted as a function of the square root of M_r (top abscissa, ○ and dashed line), and of the cube root of M_r (bottom abscissa, ● and unbroken line). Mean values ± SEM for each FD were obtained from 21 determinations of three independent experiments.

knew that propidium iodide is not a reliable measure of irreversible loss of membrane integrity due to shock wave exposure. Nevertheless, a major contribution of permanently permeable cells could be ruled out for two reasons. First, exposure of cells to shock waves before FD incubation (the time interval was typically 2–3 min) did not result in an increase to the same degree of fluorescence. Second, proliferation of FD-positive cells that had been exposed to shock waves, as determined with an in vitro assay after fluorescence-activated cell sorting, was similar to that of sorted control cells.

The fluorescence histograms of cells loaded with FD showed an extremely wide distribution of fluorescence intensities, similar to the more than 100-fold range of intensities after scrape-loading (McNeil et al., 1984). This indicates a heterogeneous amount of molecules loaded into individual cells. Following electroporation of cells, the extent of individual cell loading varies to a lower degree (Bartoletti et al., 1989; Glogauer and McCulloch, 1992).

The amount of intracellular FD, internalized as a result of permeabilization of the cell membrane by shock waves, was stable, and fluorescence was detectable for several days, similar to scrape-loading (McNeil et al., 1984) and electropermeabilization (Zimmermann, 1986; Lambert et al., 1990). Once the probe had entered the cells, its decay was primarily determined by the doubling time of the proliferating cells. This indicates that intracellular components stay intact, and cell function is not heavily affected by the labeled dextran, whereas the cell membrane had transiently lost its integrity, enabling entry of molecules with an M_r of up to 2,000,000.

In fact, a recent report found the cell membrane to be the most sensitive cell component to shock wave exposure (Steinbach et al., 1992).

The amount of internalized FD was proportional to the probe concentration in the medium and decreased with increasing molecular mass. A sharp decline in the number of molecules loaded per cell with increasing molecular mass has also been reported for scrape-loading (McNeil et al., 1984), loading (Wilson, Horwitz & de Lanerolle, 1991; Glogauer & McCulloch, 1992), and release (Dimitrov & Sowers, 1990) of large molecules by electropermeabilization.

The diffuse appearance of the intracellular fluorescence and the lack of a comparably increased fluorescence when the labeled dextran was added after shock wave exposure argues against the stimulation of endocytosis as described for the treatment of cells with electric field pulses (Zimmermann et al., 1990). Another possible explanation is cavitation generated by shock waves (Coleman et al., 1987). It produces shear forces from fluid movement around moving cavities which have been recently quantified (Vogel & Lauterborn, 1988). These shear forces may cause perturbations, and thus open pores in the cell membrane. Extracellular macromolecules would therefore enter the cytoplasm of permeabilized cells by diffusion. The current interpretation of our results (Fig. 8) is that either the probability for the formation of small pores is higher than for larger pores, or that sieving of larger molecules occurs due to a graded reduction of the initial pore radius which had been demonstrated during the resealing of hemoglobin-free ghosts prepared by osmotic lysis (Lieber & Steck, 1982).

The efficacy of the permeabilization of cells by shock waves in the present study was lower when compared to electroporation where the reported numbers of internalized molecules per cell range from 1.4×10^5 dextran molecules (M_r 70,000) at an extracellular concentration of 500 μM (Bartoletti et al., 1989) to 7×10^8 dextran molecules (M_r 150,000) at 8 μM (Glogauer & McCulloch, 1992). The average number of FD 70 molecules loaded into L1210 cells by exposure to shock waves was 7×10^4 at 140 μM. At 30°C, the life span of the high permeation state of the membrane is already very short when compared to 4°C (Zimmermann, 1986). The temperature of the medium used during the electroporation procedure is typically 0–4°C, whereas the exposure of cells to shock waves and FD was performed at a temperature of 37°C in the present study. Thus, the uptake of substances across the permeabilized membrane is expected to be lower at 37°C when compared to 4°C, which might explain the different efficacy.

The size of the molecules introduced, on the other hand, was comparable. Introduction of 20 nm gold particles has been described to occur during electroporation of cells (Lambert et al., 1990). The Stokes' radius of FD 2000, the largest dextran loaded by shock waves, is 28–29 nm.

In conclusion, we demonstrate that permeabilization of L1210 mouse leukemia cells with lithotripter shock waves results in the intracellular internalization of fluorescein-labeled dextrans with a relative molecular mass ranging from 3,900–2,000,000. Since shock waves can be applied in vivo and can be focused well, even deep in body tissue, a combination of shock waves with suitable anticancer drugs might be of special interest for local drug targeting in vivo. Other methods for loading macromolecules into cells, however, are restricted to in vitro situations.

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