A NEW METHOD FOR PERMEABILIZATION OF CULTURED CELLS WITHOUT CELL DAMAGE

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Transient permeabilization was readily achieved by vortex-stirring cultured mammalian cells for a few seconds in the presence of 1 to 10 $\mu\text{g/ml}$ of high molecular weight polyacrylic acid (PAA). This was demonstrated by an appreciable uptake by murine leukemia L1210 cells of Lucifer Yellow (LY), which is known to be a non-permeant except under conditions in which micropores are induced in the plasma membrane. In addition, the cytotoxicity of bleomycin (BLM), a very poor permeant, was markedly enhanced by vortex-stirring with PAA. Scope and limitation of this permeabilization procedure is preliminarily described.

KEY WORDS permeabilization; Lucifer Yellow; polyacrylic acid; culture cell; bleomycin

Permeabilization of the plasma membrane is a valuable means for introducing substances into the cell. Various methods have been reported including techniques involving electric pulses¹⁻³⁾ and shock waves^{4,5)}, both of which are known to cause transient micropores in the plasma membrane. The present study describes transient permeabilization that was readily achieved by vortex-stirring cultured mammalian cells for a few seconds in the presence of 1 to 10 $\mu\text{g/ml}$ of high molecular weight polyacrylic acid (PAA). This was demonstrated by an appreciable uptake by murine leukemia L1210 cells of Lucifer Yellow (LY), which is known to be a non-permeant except under conditions in which micropores are induced in the plasma membrane.^{1,2)} This was further supported by the finding that the cytotoxicity of bleomycin (BLM), a very poor permeant^{6,7)}, was markedly enhanced by vortex-stirring with PAA. It is worth noting that the viability of cultured cells remained unaffected with this procedure.

METHODS AND RESULTS

Materials Bleomycin hydrochloride was purchased from Nippon Kayaku Co., Ltd. (Tokyo). High molecular weight polyacrylic acid (PAA), sodium polyacrylate Aronflock A-119 (MW ca. 9×10^6), was a gift from Toa Gosei Co., Ltd. (Tokyo). A set of fluorescein isothiocyanate-dextrans with various sizes of molecular weight were purchased from Sigma Chemical Co. (St. Louis, MO).⁴⁾

Cell Culture Murine leukemia L1210 cells were maintained in suspension culture with RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 0.1 mg/ml kanamycin at 37°C in a humidified atmosphere containing 5% CO₂. The cells were passaged twice each week. Each experiment was conducted at a cell-density of 2×10^5 cells/ml in an exponential growth phase.

Uptake of LY into Murine Leukemia L1210 Cells after Vortex-Stirring with PAA and LY (Fig. 1) L1210 cells at an early log-phase (2×10^5 cells/ml), PAA (10 $\mu\text{g/ml}$) and LY (1 mM) in 2 ml of culture medium (RPMI 1640 supplemented with 10% fetal bovine serum and 0.1 mg/ml kanamycin) were vortex-stirred at 25°C for 10 s and immediately maintained for 10 min at either 37°C (curve B), 25°C (curve C), or 0°C (curve D). Curve A is that of the control in which the cells were vortex-stirred with LY alone and maintained at 25°C for 10 min. The cells collected by

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centrifugation of the treatment mixture at 4°C were washed four times with 1 ml of PBS each time and then subjected to flow cytometric analysis with a FACSort flow cytometer (Becton Dickinson, Mountain View, CA).

Temperature-Dependent Uptake of LY into L1210 Cells Pretreated with PAA under Vortex-Stirring (Fig. 2)

L1210 cells and PAA in culture medium were vortex-stirred at 25°C for 10 s, followed by immediate addition of LY and incubation for 10 min at either 37°C (curve B), 25°C (curve C), or 0°C (curve D). Curve A is that of the control in which the cells were vortex-stirred without PAA, followed by immediate addition of LY, and then maintained at 25°C for 10 min. Cell density, concentrations of PAA and LY, and other experimental conditions were the same as those described above.

Time-Dependent Decrease in LY-Uptake Capacity of L1210 Cells Treated with PAA under Vortex-Stirring (Fig. 3)

L1210 cells and PAA in culture medium were vortex-stirred at 25°C for 10 s, followed by either immediate addition of LY (curve B), addition 30 s later (curve C), or addition 300 s later (curve D), and then immediately maintained at 25°C for 10 min. Curve A is that of the control, in which the cells were vortex-stirred without PAA, followed by immediate addition of LY, and maintained at 25°C for 10 min. After several washings, the cells were subjected to flow cytometric analysis. Cell density, concentrations of PAA and LY, and other experimental conditions were the same as those described above.

Enhancement of BLM Cytotoxicity with PAA under Vortex-Stirring (Fig. 4)

L1210 cells, PAA and various concentrations of BLM in culture medium were vortex-stirred at 25°C for 10 s and then immediately maintained at 37°C for 10 min (curve A). The cells and PAA were vortex-stirred at 25°C for 10 s, followed by immediate addition of BLM and then maintained at 37°C for 10 min (curve B). The cells, BLM, and PAA were gently mixed by means other than vortex-stirring, followed by maintenance at 37°C for 10 min (curve C). The cells were then washed with 3 ml of PBS and resuspended in 2 ml of culture medium. After incubation at 37°C for 48 h under 5% CO₂-humidified air, growth rates (%) vs. the control were determined by counting cells which excluded trypan blue dye.

DISCUSSION

The results of flow cytometry in Fig. 1 show distribution patterns of cells carrying various amounts of fluorescent LY after they were vortex-stirred at 25°C for 10 sec with LY in the presence of PAA and immediately maintained for 10 min at either 37°C, 25°C, or 0°C. The control cells treated with LY alone carried negligible amounts of fluorescent LY. Taking the results of Fig. 2 into account, LY-uptake was accelerated not only during the vortex-stirring with PAA but also after the vortex-stirring, and the amount of LY-uptake depended on the temperature at which the cells were maintained after the stirring. The flow cytometry results of Fig. 3 show fluorescent cell populations after cells were vortex-stirred at 25°C with PAA alone, followed by adding LY either immediately, 30 s later, or 300 s later, and then maintained at 25°C for 10 min.

This shows that the permeabilized state of cells induced by vortex-stirring with PAA was only transient and disappeared almost completely at 25°C within a few minutes after stirring. Recovery from permeabilized state was prolonged when cells were held at lower temperatures after vortex-stirring with PAA, as shown in Figs. 1 and 2. It is known that with electric pulses, more rapid resealing of micropores occurs at higher temperatures on electroporabilized cells.⁸⁾ Our findings are consistent with these facts.

Taking into account the similarity between LY incorporation by PAA treatment with vortex-stirring and that achieved by electroporabilization, the mechanism likely involves transient formation of micropores on the plasma membrane, possibly due to shearing stress caused by rapid translational flow of long-chained polyanionic PAA molecules, although more experimental evidence is required on the detailed mechanism.

It was reported^{2,6,9)} that the cytotoxicity of the anticancer agent bleomycin (BLM) was highly enhanced by electroporabilization. Now, Fig. 4 illustrates that enhancement of BLM cytotoxicity was achieved either by a few seconds of vortex-stirring in the presence of PAA^{10,11)} or by adding BLM immediately after vortex-stirring with PAA. It is worth noting that a similar extent of enhanced cytotoxicity of BLM was also achieved by vortex-stirring with PAA toward Chinese hamster V79 cells, murine lymphoblastoma L5178Y cells, and its BLM-resistant strain L5178Y/BLM cells.¹⁰⁾ The similarity in cytotoxicity enhancement between the electroporabilization and the PAA-vortex stirring procedure further supports the idea that the mechanism involves ready incorporation of BLM through transient formation of micropores in the plasma membrane of cells, as reported for the electroporabilization. It is worth noting that vortex-stirring of the cell with PAA was not cytotoxic at all: cell growth rates after vortex-stirring for 10 s, 60 s, and 120 s were 108, 107, and 119%, respectively, relative to the control vortex-stirred without PAA.

Preliminary study by flow cytometric analysis of fluorescent dextrans (FDs) indicated that those with MW ranging from 4,400 to 40,000 became appreciably permeabilized by the procedure described here. Cytotoxicity of paraquat, a cationic non-permeable viologen, was also extraordinarily enhanced by vortex-stirring with PAA. Details of the scope and limitation of this permeabilization procedure will be published in a forthcoming paper.

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