Electrofusion between Heterogeneous-Sized Mammalian Cells in a Pellet: Potential Applications in Drug Delivery and Hybridoma Formation

L. H. Li, M. L. Hensen, Y. L. Zhao, and S. W. Hui
Membrane Biophysics Laboratory, Roswell Park Cancer Institute, Buffalo, New York 14263 USA

ABSTRACT High-efficiency electrofusion between cells of different sizes was achieved by application of fusing electric pulses to cells in centrifuged pellets. Larger target cells (Chinese hamster ovary or L1210 cells) were stacked among smaller human erythrocytes or erythrocyte ghosts by sequential centrifugation at 700 g to form five-tier pellets in a specially designed centrifugation-electrofusion chamber. The membranes of erythrocytes and ghost were labeled with fluorescent membrane dye (1,1′ dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI)), and the contents of ghosts were loaded with water-soluble fluorescent dye (42-kDa fluorescein isothiocyanate dextran (FITC-dextran)), to monitor heterogeneous cell fusion. Fusion efficiency was assayed by the extent of either membrane dye mixing or contents (FITC-dextran) mixing with target cells. Four rectangular electric pulses at 300 V and 80 μs each were found to give the optimal fusion results of ~80% heterogeneous fusion by the content-mixing assay and ~95% by the membrane-dye-mixing assay. Cell viability remained greater than 80% after electrofusion. Because of the electric breakdown of cell membranes at the beginning of the pulse, the pellet resistance and hence the partial voltage across the pellet reduced rapidly during the remaining pulse time. This voltage redistribution favored the survival of fused cells. The limited colloidal-osmotic swelling of cells in pellets enhanced cell–cell contact and increased the pellet resistance after each pulse. As a result, the partial voltage across the pellet was restored when the next pulse was applied. This redistribution of pulse voltage in the pellet system permitted the breakdown of cell membranes at a lower applied voltage threshold than that required for electrofusion of cells in suspension or in dielectrophoretic cell chains. The cell viability and soluble dye retention within cells (FITC-dextran) remained at the same high levels for 3 h when the cells were incubated in respective culture media with serum at 37°C. Viability and dye retention decreased significantly within 30 min when cells were incubated in phosphate-buffered saline without serum. The pellet technique was applied to form hybridomas by fusion of larger SP2/0 murine myelomas with smaller naive mouse lymphocytes. An optimum of 173 ± 70 hypoxanthine aminopterin thymidine (HAT)-selected clones of the hybridomas was obtained from 40,000 SP2/0 cells and 1.5 × 10^6 lymphocytes used in each trial. This high-efficiency fusion technique may be adapted to mediate drug and gene transfer to target cells *ex vivo* as well as to form hybrid cells with limited cell sources.

INTRODUCTION

An important biotechnological application of cell fusion is hybridoma formation and introduction of exogenous molecules, such as drugs and genetic materials, into cells. Much effort has been focused on the development of methods for reversibly permeabilizing membranes to facilitate fusion. Usually, application protocols call for fusion of different-sized cells (Furusawa et al., 1974; Kao and Michayluk, 1974; Vienken and Zimmermann, 1985). Virus-mediated (Yoshihiro, 1993) or poly(ethylene glycol) (Boni and Hui, 1987) and other chemically mediated fusion methods (Ahkong et al., 1973) have been developed. These methods have certain limitations, such as cell-line dependency, low efficiency, potential contamination, and cytotoxicity (Poste and Pasternak, 1978; Ahkong et al., 1975).

These problems may be overcome by the recent development of electrofusion (Sowers 1984; Stenger et al., 1991; Vienken and Zimmermann, 1985). Under suitable conditions, transient and reversible electropores are formed by application of an electric pulse, and cell viability is not significantly affected (Rols and Teissie, 1992; Stenger et al., 1988). However, inasmuch as the permeabilizing threshold of the electric-pulse-induced transmembrane potential is cell-size dependent (Garry et al., 1993; Zimmermann 1980), it is difficult to electroporate and fuse cells of different sizes simultaneously. Typically, the larger cells, which are usually the targets for loading and transfection, are damaged by a given field strength, whereas smaller cells remain intact (Hui and Stenger, 1993; Stenger et al., 1991). To overcome this obstacle, we developed a new and simple method based on our understanding of the electric properties of cell pellets (Abidor et al., 1994a,b) to fuse cells of different sizes in a pellet. We formed cell pellets consisting of alternating tiers of cell types and applied low-strength pulses to fuse cells of heterogeneous sizes within the pellet. This method can be used to fuse cells of different sizes and to transfer macromolecules into desired cells efficiently. In the first part of this study, two different cell lines, Chinese hamster ovary (CHO) cells and L1210 lymphocytes, were used as target

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Address reprint requests to Dr. Sek Wen Hui, Department of Physics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263-0001. Tel.: 716-845-8595; Fax: 716-845-8683; E-mail: roswhui@ubvms.cc.buffalo.edu. L. H. Li's permanent address: Department of Biomedical Engineering, Hunan Medical University, Changsha, People's Republic of China.

Abbreviations used: DiI, 1,1′dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; FITC, dextran-fluorescein isothiocyanate dextran; PBS, phosphate-buffered saline; rPBS, potassium-rich PBS; CHO, Chinese hamster ovary; FBS, fetal bovine serum.

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cells, and the smaller human erythrocytes and their ghosts were chosen as carrier vehicles to introduce exogenous macromolecules into the target cells. In the second part, fusion experiments to form hybridomas between mouse myeloma SP2/0 and naive BALB/c mouse lymphocytes were performed. High fusion efficiency for cells of different sizes, with high viability of the target cells and high yield of hybrid clones, was achieved at optimum electric settings.

MATERIALS AND METHODS

Cell preparation

CHO, L1210, and SP2/0-Ag14 myeloma cells

CHO cells were cultured in F10 medium with 15% newborn calf serum and 1% PSN antibiotic mixture (100×) (Life Technologies Inc., Grand Island, NY) in 100-mm culture dishes (Costar Corporation, Cambridge, MA). L1210 leukemic cells were cultured in RPMI-1640 with 10% NU-serum and 0.1 mg Gentamicin/ml as the final concentration (Life Technologies, Inc., Grand Island, NY) in 25-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY). SP2 myeloma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 20 μg/ml 8-azaguanine, 1.5 g/L $NaHCO_3$, 10% fetal bovine serum (FBS), and 0.6% PSN antibiotic mixture (100×) (Life Technologies, Grand Island, N.Y.). Only cells in the exponential growth phase, with ≥95% viability as determined by the trypan blue exclusion test, were used. L1210, SP2, and trypsinized (0.25%, GIBCO, Grand Island, NY) CHO cells were washed with F10 medium, DMEM with 10% FBS, and RPMI-1640 medium with 10% NU-serum, respectively. Cell concentration was determined by use of a hemacytometer. Before pulse application, cells were kept at room temperature until postpulse incubation. All cells were cultured with 5% CO_2.

Human erythrocytes and loaded human erythrocyte ghosts

Human erythrocytes were isolated from 7-day-old blood from healthy donors. Erythrocytes were washed in sodium-rich phosphate-buffered saline (PBS) (150 mM NaCl, 3 mM KCl, 5 mM NaPi, pH 7.4) at 700 × g to remove plasma proteins, platelets, and white cells. 42-kDa FITC-dextran (Sigma, St. Louis, MO) was encapsulated in ghost according to a method previously reported (Yoshihiro, 1993). Briefly, one volume of potassium-rich PBS (rPBS; 3 mM NaCl, 150 mM KCl, 5 mM NaPi, pH 7.4) washed erythrocyte pellet (0.1 ml, 10^{10}/ml) and three volumes of FITC-dextran medium (10 mg/ml in rPBS, 42 kDa, 0.3 ml) were placed in a dialysis bag (molecular weight cutoff 12,000–14,000; Spectrum Medical Industries Inc., Los Angeles, CA) and immersed in 500-ml six-times-diluted rPBS (1/6 rPBS) for 0.5 h at 4°C, followed by another 0.5 h in 500 ml PBS at 25°C to reseal the osmotic pores on the ghost membranes. The loaded and resealed ghost were pipetted into an Eppendorf tube and washed six times in PBS and again in a balanced salt solution (125 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2.5 mM $MgCl_2$, and 5 mM TRIS, pH 7.4). The loaded ghost were kept at 4°C. It was found that the loaded ghost could be used for up to three days without significant leakage or change of loading efficiency.

For some experiments, ghosts were further labeled with 1,1′ dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI) (Molecular Probes, Eugene, OR) as follows (Abidor et. al, 1994c): while the mixture of 100 volumes of PBS and 10 volumes of ghost pellet (or erythrocyte pellet) was being vortexed, 1 volume of DiI (3.5 mg/ml ethyl alcohol) was added. One minute later, ghosts were washed as described above.

Lymphocytes

Spleens from BALB/c naive mice were asepticly isolated, ground, sieved, treated (10 min) with lysing medium to lyse erythrocytes (0.825% NH_4Cl, 0.1% $KHCO_3$, 0.0035% EDTA), and resuspended in DMEM with 10% FBS to isolate lymphocytes (Stenger et al., 1988). The lymphocytes were kept at room temperature throughout the experiments until postpulse incubation.

Pellet formation

The method for cell electrofusion in pellets has been reported (Abidor et al., 1993; 1994a,b). The chamber for holding the pellet is shown in Fig. 1*A*. The chamber was placed within a centrifuge tube in a tabletop centrifuge (IEC HN-S Centrifuge, Needham Heights, MA) (Abidor et al., 1994a). A cylindrical hole measuring 3 mm in diameter and 3.5 mm in height was positioned directly over the bottom electrode. Pellets were formed on the bottom electrode. After one type of concentrated cell (10 μl or 20 μl in volume) was introduced into the hole, 20 s of centrifugation at 700 g was used to form a tier of cells. Here a tier of cells means one type of cell occupying a certain thickness in the cylindrical hole. Extra medium outside the cylindrical hole was withdrawn each time before another type of cell was added. After the pellet was formed, 0.5 ml of PBS was carefully added to the chamber to submerge the top electrode (shaded in Fig. 1 *A*).

According to the number of cells added at each time, (1.5×10^6 cells/tier for erythrocyte (HE) or ghost (HEG), $3–4 \times 10^4$ cells/tier for CHO, or $6–8 \times 10^4$ cells/tier for L1210), the number of cell layers per tier could be roughly estimated to be one for CHO or L1210 and four for erythrocyte or ghost, as schematically shown in Fig. 1 *B*. In the present research, pellets containing five alternating tiers, (HE+CHO+HE+CHO+HE), (HEG+CHO+HEG+-CHO+HEG), or (HEG+L1210+HEG+L1210+HEG), were formed.

Pulse application and postpulse incubation

After the five-tier cell pellet was formed, the sample was subjected to an additional 1 min of centrifugation. Four pulses (at 1-min intervals) of various strengths were applied to the cell pellet. The voltage of the generator output, *V*, was given as the electrical field strength because it was

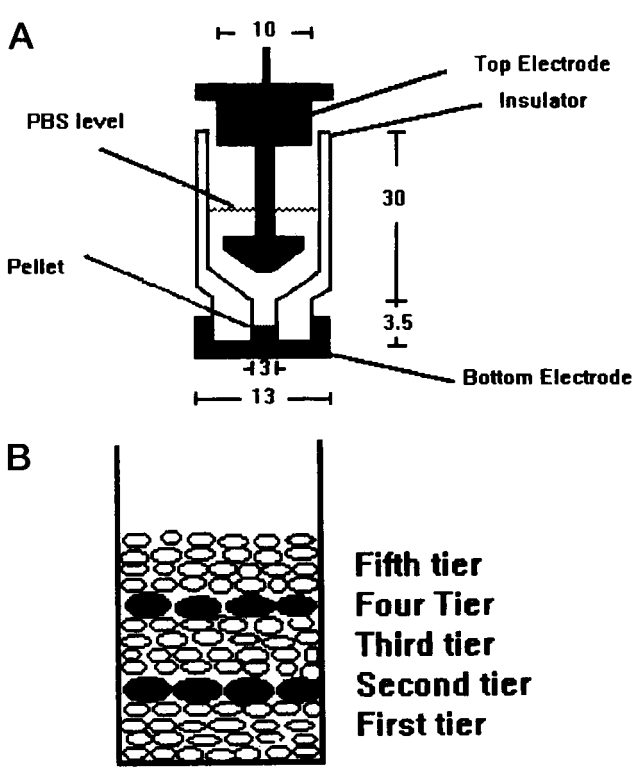

FIGURE 1 (*A*) Schematic drawing of the fusion chamber. Units are millimeters. (*B*) Schematic drawing of the pellet in the fusion chamber.

difficult to determine the exact electrical field across cell pellet owing to uncertainty in estimating the pellet thickness and the number of cell layers. The pellet resistance R_p was in series with an external load resistance R_L = 500 Ω, and the resistance of PBS in the chamber was R_0 = 300 Ω, as shown in the equivalent circuit in Fig. 2. The pellet resistance R_p was monitored at each step and compared with the total number of cells added. After four pulses were applied, the sample was centrifuged at 700 g for an additional 5 min. Cells were then resuspended in culture medium with serum (unless specified) and observed under a fluorescence microscope (Model CHT, Olympus, Japan) either immediately or after various incubation times at 37°C.

Cell counting

To quantify the efficiency of content mixing and membrane dye mixing of targeted CHO or L1210 cells, we counted these cells under a fluorescence microscope. The efficiency was indexed as $(N_{labeled}/N_{total}) \times 100\%$, where $N_{labeled}$ is the number of target cells with fluorescence obtained from content mixing and membrane dye mixing and N_{total} is the total number of target cells. Cell viability was determined by trypan blue exclusion.

Hybridoma formation

Essentially, the previously published procedures (Galfre and Milstein, 1981; Schmitt et al., 1988; Stenger, et al., 1988) for culturing and counting hybrid clones were used. From our preliminary experiments, we found that we had to increase the centrifugation force above that used for erythrocyte–CHO fusion because of the convoluted and more durable membranes of lymphocytes. Cells were kept at room temperature, instead of at 37°C, to yield a higher number of hybrid clones, in agreement with previous findings (Schmitt, et al., 1988). 10 s of centrifugation at 2200 g was used to form each tier of lymphocytes (Lym) (5×10^5 cell/tier) and SP2 cells (2×10^4 cell/tier) for a five-tier pellet (Lym-SP2-Lym-SP2-Lym). After formation the pellet was immersed in B&K medium (125 mM KCl, 15 mM NaCl, 1.2 mM $MgCl_2$, 25 mM Hepes, 3 mM glucose, pH 7.4) with 20% FBS and pulsed three times at 0.5-min intervals. The pulsed cells were incubated at 37°C for 20 min and then resuspended in 0.5% HAT medium in four wells (24-cell culture well, Corning Glassware, Corning, NY) with mouse peripheral macrophage feeder layer cells. We found that use of 0.5% HAT medium as the postpulse cell culture medium was optimal for fused cell recovery and for unfused SP2 cell growth suppression. After 24 h, 100% HAT medium (100-fold dilution of 10 mM hypoxanthine, 40 mM aminopterin, 1.6 mM thymidine in DMEM with 10% FBS, 20 μg/ml 8-azaguanine, 0.6% PSN antibiotics) was added to select hybridomas. On day 7, fresh 100% HAT medium was added again. The number of clones was counted on days 7–14 after pulse. Clones with more than 10 healthy cells were counted as hybrid clones, and the vigorous growth of clones in HAT medium was confirmed after 10–20 days.

All experiments were repeated at least three times. For the hybridoma experiment with the 150-V pulse treatment, nine repetitions of the experiment were performed. The standard error was approximately 8% unless specified otherwise.

RESULTS

The fusion efficiency of the five-tier cell pellet electrofusion technique is field-strength dependent. When the membrane dye (DiI) mixing method is used to monitor the fusion yield, the percentage of fused CHO (15 μm in diameter) with erythrocytes (6 μm in diameter) increases with the increase of field strength, as shown in Fig. 3. When V = 300 V, fusion efficiency can be as high as 80%, whereas when V = 0 V no fusion is observed (Fig. 3, curve 1). Another interesting fact is that the viability is not significantly affected by the pulses. As shown by curve 2 of Fig. 3, the viability stays as high as 90%. CHO–CHO fusion, as checked by phase contrast microscopy, is very low (≈5%). Erythrocyte–erythrocyte fusion yield has the same trend of increasing fusion yield with increasing field strength (data not shown here).

Because there is a possibility of membrane dye (DiI) mixing without membrane fusion, especially in cell pellets in which very close membrane contact can be created owing to the colloidal osmotic swelling after pulses (Abidor et al., 1994a,b), we designed a content-mixing experiment to verify fusion between heterogeneous-sized cells. In this experiment, erythrocytes were replaced by ghosts preloaded with 42-kDa FITC-dextran and the same experiments as in Fig. 3 were performed. As demonstrated in Fig. 4, the curve of fused CHO with ghost shows the same trend as in Fig. 3. At V = 0 V, no content mixing between CHO and ghost was observed, whereas at V = 300 V or 500 V, more than 80% of CHO cells showed content mixing with ghost.

To demonstrate further that fusion occurs during content mixing between ghost and CHO, doubly labeled ghosts with membrane dye (DiI) and water-soluble dye (42-kDa FITC-

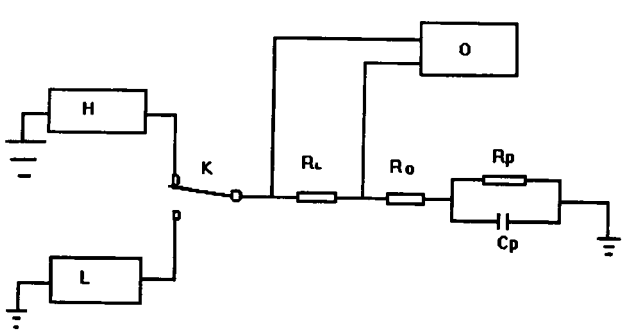

FIGURE 2 Equivalant electrical circuit for applying pulses and measuring the resistance of the cell pellet: H, high-voltage pulse generator; L, low-voltage function generator; O, oscilloscope; R_L, load resistance (500 Ω); R_p, pellet resistance; C_p, pellet capacitance; R_0, chamber resistance with PBS only (310 Ω); k, switch.

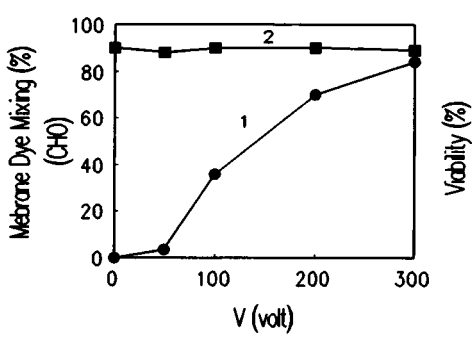

FIGURE 3 Effect of pulse strength on the fusion efficiency of CHO with erythrocytes, as monitored by membrane-dye (DiI) -mixing assay. Four pulses (80 μs) were applied at 1-min intervals. Curves 1 and 2 represent, respectively, the fusion efficiency of CHO cells with erythrocytes and the viability of CHO cells.

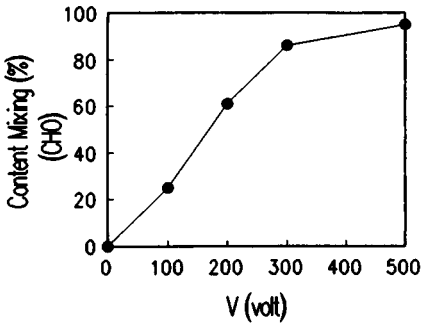

FIGURE 4 Effect of pulse strength on the fusion efficiency of CHO with ghosts, as monitored by a content-mixing assay of 42-kDa FITC-dextran. Four pulses (80 μs) were applied at 1-min intervals. The curve represents the fusion efficiency of CHO cells with ghosts.

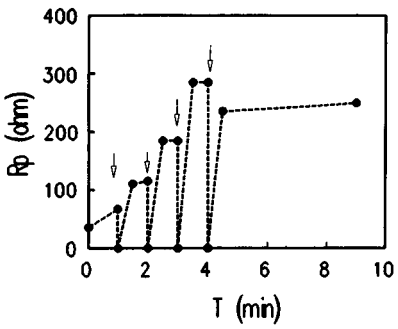

FIGURE 5 Effect of pulses on the change in pellet resistance R_p. Four pulses (300 V, 80 μs) were applied at the points indicated by the arrows.

dextran) were used, and the same experiments were carried out. Table 1 shows the viability and the fusion efficiency measured by membrane dye mixing and content mixing between CHO and ghost, respectively. It shows that the fusion efficiency measured by the membrane-dye-mixing assay is consistently higher than that by the content-mixing assay, and both efficiencies are as high as 80–90%. The control experiment with the same conditions, but without pulse application, shows no membrane dye mixing or content mixing.

There may be advantages in applying multiple pulses to electrofuse cells in a pellet, because of better membrane contact caused by preceding pulse-induced colloidal osmotic swelling of cells (Abidor 1994a,b). To optimize the benefit of multiple pulses, the effect of multiple pulses on pellet resistance R_p was recorded (Fig. 5). At time t = 0, 0.5 ml PBS was added to the chamber. Four pulses were applied to the pellet at the arrow points during 9 min of continuous centrifugation. During pulses, R_p dropped to zero, but within seconds after pulses R_p rose even higher each time to hundreds of ohms until the fourth pulse, when R_p did not recover completely, indicating that there was irreversible membrane breakdown that impeded colloidal-osmotic swelling of cells (Abidor et al., 1994a,b).

The kinetics of pulse voltage across the pellet during a pulse is shown in Fig. 6. The change of R_p during pulse was recorded when a five-tier cell pellet (with pellet resistance just before pulse R_{p0} = 283 Ω) was subjected to a 300-V, 80-μs pulse. The pulse voltage across the pellet, V_p, was calculated by $(V \times R_p)/(R_L + R_0 + R_p)$. V_p (at t = 0) was calculated from the voltage distribution, assuming that no membranes were broken down in the cell pellet, i.e., V_p (at t = 0) = $(V \times R_{p0})/(R_L + R_0 + R_{p0})$. V_p dropped to a significantly low level within microseconds after the initiation of the pulse.

We examined the importance of postpulse recovery for fused heterogeneous-sized cells by measuring postpulse restoration of membrane permeability and retention of fluorescent content obtained from content mixing. Incubation in different media resulted in different viability and the ability to retain fluorescence in CHO cells. The content-mixing efficiency of CHO cells was 71%, and the viability was 80%, when the cells were measured immediately after pulse in PBS. However, the content-mixing efficiency dropped to 16%, and viability to 20%, after 30-min incubation in PBS at 37°C. But if F10 medium with 15% newborn calf serum was used as the incubation medium, as shown in Fig. 7, the content-mixing efficiency (filled circles, curve 1) and the viability (filled diamonds, curve 2) of CHO cells remained at the same high level of ~80–~90%, even after 2.5-h incubation at 37°C. In both cases, control experiments under the same conditions but without pulse application showed no content mixing, as Fig. 7 shows (filled squares, curve 3).

TABLE 1 Cell viability and fusion efficiency of CHO with ghost monitored by both membrane-dye-mixing and content-mixing assays

Condition	Content Mixing [%]	Membrane Dye Mixing [%]	Viability [%]
Pulsed	82	90	83
Control	0	0	95

Four pulses (300 V, 80 μs) were applied. Control represents the result obtained under the same conditions but with no pulse applied.

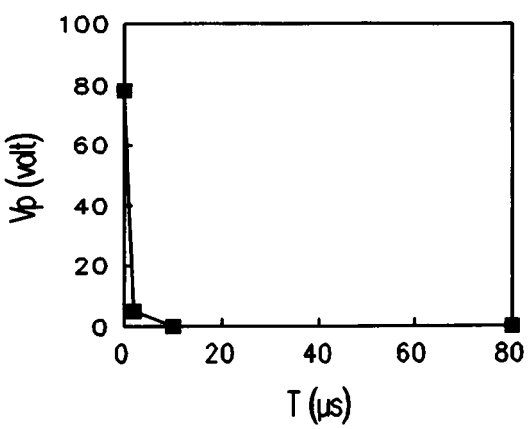

FIGURE 6 Pulse voltage across the pellet, V_p, during the pulse (300 V, 80 μs) with prepulse pellet resistance R_{p0} = 283 Ω.

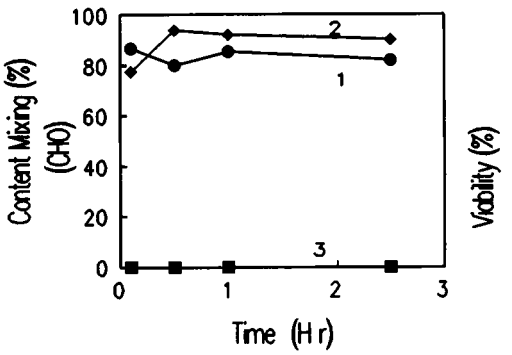

FIGURE 7 Effect of postpulse incubation at 37°C in F10 medium with 15% newborn calf serum on the loading efficiency and the viability of CHO cells. Four pulses (300 V, 80 μs) were applied. Curves 2 and 1 represent the viability and the content-mixing efficiency of CHO with different incubation time at 37°C, whereas curve 3 shows the loading efficiency of the control, i.e., the experiment under the same conditions but with no pulse applied.

To test the general applicability of the pellet fusion technique, another cell line, L1210 cells, with a much smaller size and different growth characteristics, was used. In Fig. 8 the content-mixing efficiency with ghosts and the viability of L1210 cells, after 300-V 80-μs pulses, are represented by filled circles (curve 1) and filled diamonds (curve 2), respectively. RPMI-1640 with 10% NU-serum was used in postpulse incubation. The content-mixing efficiency and viability of L1210 cells remained ~70% and ~90%, respectively, in a 3-h incubation period at 37°C. Control experiments under the same conditions but without pulse application showed no content mixing (filled squares, curve 3).

We tried using higher field pulses (4 pulses, 400 V, 80 μs) to increase the content-mixing efficiency of L1210 cells. Although the initial content-mixing efficiency and viability were as high as 90%, they decreased to the respective lower levels of 36% and 52% after 30-min incubation in RPMI-1640 with 10% NU-serum at 37°C. Control cells under the same conditions but with no pulse applied showed no content mixing.

Doubly labeled ghosts were also used to demonstrate that fusion did occur between L1210 cells and ghost. Table 2 shows that the fusion efficiency of L1210 cells, given by membrane-dye-mixing assay, was 100%. The fusion efficiency given by content-mixing assay was 76%. The viability was 82%, and the control cells under the same conditions but without pulse application showed no content mixing or membrane dye mixing.

In further testing the application feasibility, we tried hybridoma formation between SP2/0 and naive mouse lymphocytes. As shown in Fig. 9, the yield of hybrid clones was field-strength dependent. 100 and 150 V gave the optimum hybrid forming efficiency, approximately 1 of every 200–400 SP2 cells. However, when the field was too low (<50 V), or too high (>200 V), the clone number decreased significantly. For each hybridoma experiment the control had no hybrid clone.

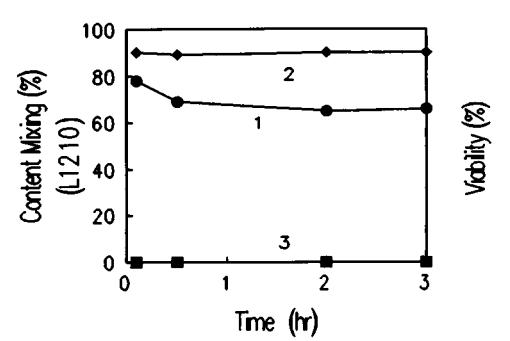

FIGURE 8 Effect of postpulse incubation in RPMI-1640 medium with 10% NU-serum on the viability and the content-mixing efficiency of L1210 cells. Four pulses (300 V, 80 μs) were applied. Curves 2 and 1 represent the viability and the content-mixing efficiency, respectively, of L1210 at different incubation times at 37°C, whereas curve 3 shows the loading efficiency of the control, i.e., the content-mixing efficiency under the same conditions but with no pulse applied.

DISCUSSION

Most fusion applications involve fusion between cells of heterogeneous sizes (Stenger et al., 1988, Vienken and Zimmermann, 1985; Yoshihiro, 1993). Two of the most practical cases are hybridoma formation, such as fusion between myelomas and lymphocytes (Garry et al., 1993) and the transferring of exogenous molecules into cells by fusion of loaded erythrocyte ghosts with target cells (Yoshihiro 1993). The latter case has practical potential because erythrocytes can be obtained in large quantity, can be loaded easily, and pose little possibility of contamination. Using autologous erythrocytes also circumvents the host's immunoresponse. Unfortunately, applying the electrofusion method to these cases has the problem of cell-size dependency on the electric pulse effect (Stenger et al., 1991; Zimmermann, 1980), which inhibits the full advantageous effect of electrofusion. To expand the application potential of electrofusion, we developed a new and simple procedure that overcomes the size problem. Under suitable conditions described in this paper, high fusion and loading efficiency are achieved.

As Figs. 3 and 4 show, CHO can be fused efficiently with erythrocytes or ghosts up to 80% by four low-strength square electric pulses (80 μs) and still retain high viability,

TABLE 2 Cell viability and fusion efficiency of L1210 with ghost monitored by both membrane-dye-mixing and content-mixing assays

Condition	Content Mixing [%]	Membrane Dye Mixing [%]	Viability [%]
Pulsed	76	100	82
Control	0	0	95

Four pulses (300 V, 80 μs) were applied. Control represents the experiment under the same conditions but with no pulse applied.

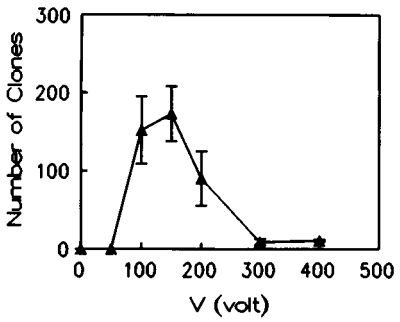

FIGURE 9 Effect of pulse strength on the yield of hybrid clones between mouse lymphocytes and SP2 myeloma cells, as selected by HAT medium. Three pulses (80 μs) were applied at 0.5-min intervals on five-tier cell pellets. The curve represents the number of hybrid clones formed by 4×10^4 SP2 and 1.5×10^6 lymphocytes.

as monitored by membrane-dye-mixing and content-mixing assays. This experiment simulates fusion between different-sized cells for hybridoma formation and ghost-mediated loading. In the experiment, the ratio of the number of erythrocytes or ghosts to that of CHO is approximately 50. We attempted to decrease this ratio by reducing the number of ghost per tier but increasing the total number of tiers from 5 (HEG+CHO+HEG+ CHO+HEG) to 11 (HEG+CHO+HEG+ . . . +HEG), to keep the pellet resistance R_p almost the same. This method results in almost the same fusion efficiency and viability (data not shown). Keeping the same number of tiers of cells (five tiers) but increasing the CHO cell number per tier results in undesirable fusion among CHO cells (data not shown). Therefore, in all later experiments, five-tier cell pellets with a ratio of erythrocytes:CHO of 50 were used.

To confirm that electrofusion of heterogeneous-sized cells did occur in the pellet system, we designed further experiments by monitoring electrofusion with both membrane-dye-mixing and content-mixing assays simultaneously. By using ghosts prelabeled with DiI and preloaded with 42-kDa FITC-dextran, we found that the fusion efficiency of CHO cells monitored by membrane-dye-mixing and content-mixing assays was 90% and 82%, respectively (Table 1). Most CHO cells have double labels (both DiI and 42-kDa FITC-dextran). However, the fusion efficiency by membrane-dye-mixing assay is always higher than that by content-mixing assay. During counting, an intensive effort was made, but we failed to find any CHO cell with content mixing but without membrane dye mixing. The discrepancy between fusion efficiency assayed by content mixing and that by membrane dye mixing is well known (Sowers, 1988; Song et al., 1993). The discrepancy may in part be due to the leakage of content dye during and after fusion. Nevertheless, both assays in our experiments support the occurrence of high-efficiency fusion between CHO and ghost.

What distinguishes this system from other electrofusion systems is the use of alternating tiers of different cells in a cell pellet and the use of multiple low-voltage pulses. The former feature artificially brings the desired cells into contact by centrifuging. However, the reason that low-voltage pulses (300 V) can lead to high fusion efficiency of CHO cells with erythrocytes or ghosts is worth discussing. As shown in Fig. 5, the postpulse pellet resistance R_p rises to higher levels after each of the first three pulse applications because of tighter membrane contact as a result of colloidal-osmotic swelling of cells in the pellet (Abidor et al., 1994a). Because pellet resistance R_p is in series with R_L and R_0, an increase of pellet resistance R_p means a voltage redistribution, according to the formula for pulse voltage across the pellet, $V_p = (V \times R_p)/(R_L + R_0 + R_p)$. If we estimate the approximate transmembrane potential in the pellet by assuming that $L = 14$ layers of cells in the pellet, the initial transmembrane potential, $V_p/2L$, could be as great as 2.7 V after three pulses (see Fig. 6). Usually a transmembrane potential of 0.5–1 V is enough to break down the membrane (Teissie and Rols, 1993). Therefore, the postpulse increase of membrane contact and the consequent increase of the pulse voltage across the pellet facilitate membrane fusion at lower applied voltages. In this sense, a significant increase of cell fusion in pellets may be induced by multiple low-voltage pulses. However, after the fourth pulse was applied, R_p did not increase further but rather was reduced from the level attained after the third pulse. This signaled that irreversible breakdown had begun to occur, as discussed in detail elsewhere (Abidor et al., 1994a,b). From R_p measurements it is known that, for this system, four pulses are the maximum number that can be applied to the pellet before fusion efficiency and viability begin to suffer.

Furthermore, as Fig. 6 shows, V_p drops to a significantly low level within microseconds during the pulse. Once cell membranes on a cell layer are broken down, the voltage across the cells on that layer decreases because of the decrease of R_p. This quick decrease of voltage across cells after membrane breakdown allows the fused cells to suffer much less damage than the cells pulsed and fused in suspension; in the latter case, although the transmembrane potential will decrease after the membranes are broken down, the same high voltage across cells is maintained during the pulse. For this reason a highly conductive pulsing medium can be used in the pellet system. Also, large target cells can be fused with small cells and still maintain high viability.

Like other electrofusion methods, postpulse recovery and resealing of fused cells depends on the incubation procedures (Rols and Teissie, 1992; Stenger et al., 1988). Only two postpulse incubations, in PBS and in F10 medium with newborn calf serum, were tested. The reason that we chose F10 medium with 15% newborn calf serum is that it is the growing medium for CHO cells. It was found that most CHO cells incubated in F10 medium with newborn calf serum retain both their viability and their fluorescent content after 2.5 h of incubation at 37°C, as shown in Fig. 7, whereas CHO cells incubated for 0.5 h in PBS at 37°C would lose most of their viability and fluorescent content (from 80% and 71% to 20% and 16%, respectively). Obviously, 42-kDa FITC-dextran inside CHO cells as a result of

fusion-induced content mixing leaks out during incubation in PBS at 37°C. F10 medium with 15% newborn calf serum helps to restore the impermeability of CHO cell membranes. However, if the membrane dye DiI was used to monitor fusion, no decrease in the fusion efficiency of CHO cells was found, even if the cells were incubated in PBS. Apparently the DiI method of monitoring fusion does not discriminate between live and dead fused cells.

To test whether the pellet fusion system for fusing heterogeneous-sized cells works for other cells, L1210 cells were used. As Fig. 8 shows, after 3 h of incubation, viability and fusion efficiency of L1210 cells with ghosts measured by content mixing stay as high as 90% and 70% respectively. Inasmuch as the viability (Fig. 8) was excellent under the pulse condition, higher-voltage pulses were tried. Although the initial fusion efficiency of L1210 cells and ghosts by content mixing reaches 90% if higher-voltage pulses (400 V) were used, most L1210 cells lose their viability and the ability to retain 42-kDa FITC-dextran after 30 min of incubation at 37°C, even in RPMI-1640 with 10% NU-serum (from 90% to 52% and 36%, respectively). The double-labeling ghost method was also used to monitor the fusion of L1210 cells with ghosts in a pellet. As shown in Table 2, again the membrane-dye (DiI)-mixing assay gives a higher fusion efficiency of L1210 cells than that given by the content-mixing assay. It is reasonable to say that fusion-induced content mixing of L1210 cells and ghosts did occur in the pellet system.

Apparently, our fusion efficiency assayed by microscopically counting the percentage of target cells with membrane dye mixing and content mixing cannot distinguish the number of smaller cells fused to one target cell. The multicell fusion may pose some disadvantages for hybridoma formation. To further show the feasibility in application, we electrofused SP2 myeloma cells and mouse lymphocytes in this system to form hybridomas. 0.25–0.5% of SP2 cells were found to be able to survive the HAT medium selection at optimum field (100 or 150 V), as shown in Fig. 9. We also observed that 60–70% SP2 cells were fused with prelabelled lymphocytes (data not shown) by the membrane-dye-mixing assay. Obviously, there is a large difference between fusion percentage and hybridoma formation. In this pellet method we may further enhance the hybridoma formation by optimizing pulse parameters, pulse medium effects, and postpulse recovery medium effects (Stenger et al., 1988), such as ionic strength and presence of serum and cations (Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}). Unlike for the dielectrophoresis method, we can use any pulse medium and change the desired postpulse recovery medium at any time without affecting the pellet. For this study we used B&K medium with 20% FBS as pulse and postpulse recovery media, based on the experimental result that using B&K medium as the pulse medium causes less pulse-induced SP2 cell lysis than using DMEM medium (SP2 culture medium) (data not shown). Other media may prove more effective.

The high ratio of small lymphocytes to large SP/2 cells required for building up pellet resistance (not for fusion partners) is not favorable for hybridoma production in the case of limited lymphocyte sources. But the significant fusion efficiency and the potential for modification give us possibilities for improving this method. We are currently working on ways to reduce the number of lymphocytes required.

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