Cell poration and cell fusion using an oscillating electric field

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ABSTRACT It has been shown in previous studies that cell poration (i.e., reversible permeabilization of cell membrane) and cell fusion can be induced by applying a pulse (or pulses) of high-intensity DC (direct current) electric field. Recently we suggested that such electro-poration or electro-fusion can also be accomplished by using an oscillating electric field. The DC field relies solely on the dielectric breakdown of the cell membrane to induce cell fusion. The oscillating field, on the other hand, can produce not only a dielectric breakdown, but also a sonicating motion in the membrane that could result in a structural fatigue. Thus, a combination of a DC field and an oscillating field is expected to enhance the efficiency of cell poration and cell fusion. This study is an experimental test of such an idea. Here, pulses of high-intensity, DC-shifted RF (radio frequency) electric field were used to induce cell poration and cell fusion. The fusion experiments were done on human red blood cells. The poration experiments were done on a fibroblast cell line using a molecular probe (which is a DNA plasmid containing the marker gene chloramphenicol acetyltransferase, CAT) and assayed by a gene transfection technique. It was found that the pulsed RF field is highly efficient in both cell fusion and cell poration. Also, in comparison with electro-poration using a DC field, the RF field results in a higher percentage of cells surviving the exposure to the electric field.

INTRODUCTION

Cell poration and cell fusion are important biological methods which have a wide variety of applications, including gene transfection, drug delivery, antibody production, and cell hybridization. In recent years, it was discovered that cell poration and cell fusion can be induced by applying a pulse of intense electric field (Kinosita and Tsong, 1977; Zimmerman et al., 1980; Zimmerman and Vienken, 1982; Neumann et al., 1982; Knight and Baker, 1982), such processes are called "electro-poration" or "electro-fusion". The electroporation method has been shown to be useful in introducing genes into mammalian cells (Neumann et al., 1982; Wong and Neumann, 1982; Potter et al., 1984; Smithies et al., 1985), plant cells (Fromm et al., 1985; Yang, 1985) and bacteria (Miller et al., 1988). Electro-fusion also has been used successfully for hybridoma production (Lo et al., 1984; Onishi et al., 1987) and for making new species hybrid of plants (Bates, 1985; Bates et al., 1987). Because the electric field methods are physical methods, they can avoid many of the biological and chemical side effects that might appear in the more conventional methods, such as the PEG-induced fusion (Davidson et al., 1976) or the virus-induced transfection (Williams et al., 1984). In many cases, the fusion yield and transfection yield using the electric field methods are also higher than those using the chemical or biological methods (Zimmermann, 1986; Chu et al., 1987; Potter, 1988).

In the currently used electro-poration or electro-fusion methods, the applied high-intensity electric field is usually a direct current (DC) field. The field is applied either in the form of a short (e.g., 10 μs) rectangular pulse (Kinosita and Tsong, 1977; Neumann et al., 1982; Zimmermann et al., 1980), or a pulse of exponential decay, generated by discharging a capacitor (e.g., see Sowers, 1984; Fromm et al., 1985). The time constant of the exponential pulse is usually of the order of milliseconds.

At this point, the mechanisms by which the electric field induces cell poration or cell fusion are not well understood. It is generally assumed that the fusion or poration is caused by an electrical breakdown of the cell membrane (Zimmermann and Vienken, 1982) (Also, see Discussion). Consider a spherical cell with radius r. When this cell is placed in an external electric field of strength E, the potential induced at the cell membrane is (Cole, 1968):

$$V_m = (3/2)\, rE \cos\theta. \quad (1)$$

Where θ is the angle between the electric field and the normal vector of the membrane. V_m is maximum near the cell poles where $\theta = 0°$ and $180°$. When E is very large, so

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that V_m reaches a critical value V_c (on the order of 1 V), the field within the cell membrane is high enough to cause an electrical breakdown of the lipid bilayer (Zimmermann et al., 1974; Kinosita and Tsong, 1977; Chernomordik et al., 1987). Membrane pores could be created as a result of such breakdown.

Recently, we investigated a second breakdown mechanism of the cell membrane, which is caused by an electro-mechanical coupling effect (Chang, 1989). It is well known that cell membranes can be disrupted by forced oscillation using ultrasound. The sudden application of an intense electric field may produce a mechanical shock that could also disrupt the structure of the membrane at a local area. When the applied field is high enough to produce a membrane potential on the order of 1 V, the electric field within the cell membrane will exceed 10^6 V/cm. (Note: The thickness of the membrane is on the order of 6×10^{-7} cm). Such a high electric field produces two effects. First, it compresses the lipid bilayer (this effect is related to the electrical breakdown of the membrane) (Crowley, 1973; White, 1974). Second, it forces the charged groups of the membrane (such as the phosphate head groups of lipid molecules and ions absorbed to the membrane) to move in the direction of the field. This movement produces a mechanical stress in the membrane. When this stress is large enough, it can mechanically disrupt the membrane structure. The effect of this stress may be more important if the applied field is an oscillating electric field instead of a DC field. In this case the membrane at the poles is forced to undergo an oscillating motion (which has the same effect as a localized sonication). Using a planar lipid bilayer as a model system, we have actually observed this type of forced mechanical oscillation using light microscopy (Chang and Hunt, unpublished data).

Thus, because of the effect of electro-mechanical coupling, it would be more effective to apply an oscillating electric field to induce cell fusion or cell poration than to apply a DC field. Even when the amplitude of the oscillating motion of the membrane may not be high enough to directly result in a mechanical breakdown, the alternating stress produced by the oscillating electric field can still cause a structural fatigue of the membrane, and thus making the membrane more susceptible to an electrical breakdown.

The main objective of this study is to examine experimentally if cell fusion and cell poration can be effectively induced by a pulsed oscillating electric field. Also, we would like to compare the efficiencies of cell poration and cell fusion obtained using the oscillating electric field with those obtained using a DC field.

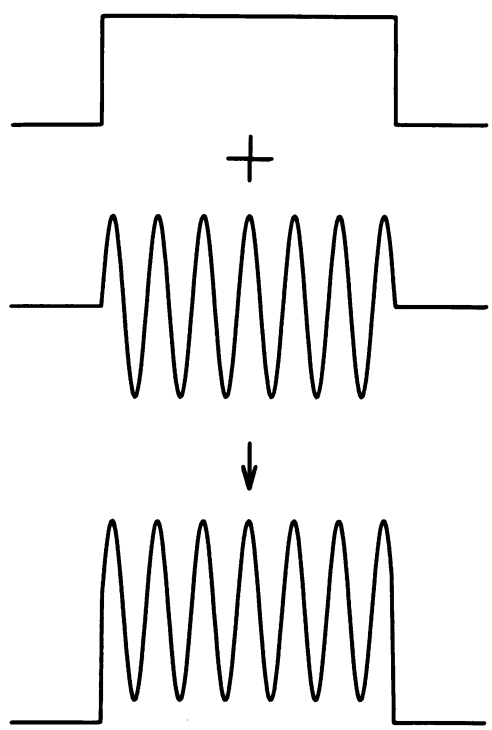


FIGURE 1 The electrical pulse applied in this study is a superposition of a pulse of radio-frequency (RF) field and a DC pulse with the same pulse width.

METHODS

1. Applied electric fields

The waveform of the oscillating electric field applied to induce cell poration and cell fusion is shown in Fig. 1. This field is a pulsed radio-frequency (RF) electric field with a DC shift. This field was generated by combining a DC pulse with an RF pulse of the same pulse width. It has been shown in our earlier experiments that such a combined waveform is more effective in inducing cell fusion than an unshifted RF pulse (Chang, 1989). Also, in consideration of equipment design, it is simpler to raise the peak field strength by a DC shift than to increase the amplitude of the oscillating field.

The oscillating frequency of the applied field varied from a few kHz to 1 MHz. The pulse width varied from 100 μs (in the case of red cell fusion) to 2 ms (in the case of gene transfection). The peak amplitude of the oscillating field typically varied from 0.5 to 5 kV/cm, depending on the cell type used. The RF pulse can be applied in single pulse or in a multi-pulse train. In the case of electro-poration, the only electric field required is that shown in Fig. 1. However, in the case of electro-fusion, a second electric field is needed. Before the cells can be fused, they must be brought into close contact by a low-intensity (100–400 V/cm) alternating current (AC) electric field. This process, called "dielectrophoresis" (Pohl et al., 1978), utilizes the induced dipole moment of the cells to align them in pearl chains (see also Fig. 4).

The block diagram of the apparatus which generated the dielectrophoretic field and the high intensity RF field are shown in Fig. 2. In the cell fusion experiment, the dielectrophoretic field was applied before and after the high intensity RF field. The field switching was controlled by a mercury wetted relay. In the cell poration experiment, no dielectrophoretic field was needed. Only the high-intensity RF field (as shown in

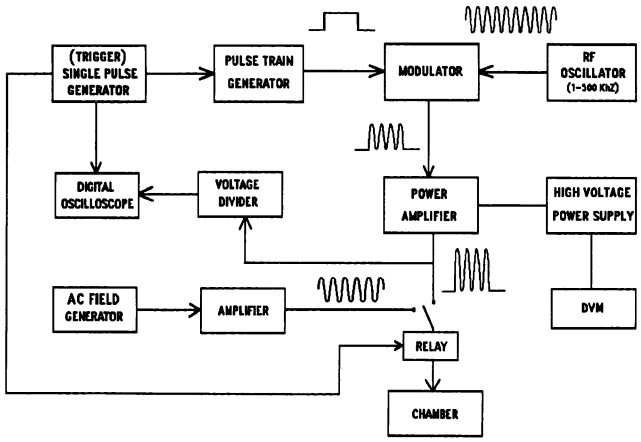


FIGURE 2 Block diagram of the electronic apparatus used here. Basically, a modulator circuit was used to gate the output of an RF oscillator to provide an RF pulse, which was later amplified by a power amplifier. This apparatus was designed and built in this laboratory specially for this work. The output waveform was monitored using a Tektronix digital storage scope (model 2210).

Fig. 1) was applied. The electronic apparatus used in the cell fusion and cell poration experiments were designed and built in our laboratory.

2. Cell fusion

Human red blood cells (RBC) were used to study cell fusion induced by the oscillating electric field. The red cells from whole human blood were suspended in isotonic Na-phosphate buffer (Dodge et al., 1963) and then collected by centrifugation. Cells were washed twice in phosphate buffer and then washed three times in the RBC fusion medium, which was composed of 20 mM Na-phosphate and 150 mM Sucrose (pH 7.5). Unlike the previous study of electro-fusion between intact red cells (Stenger and Hui, 1986), no pronase treatment was used here.

In the experiment where fluorescence microscopy was used to observe fusion between RBC, ~10% of the RBC were prelabeled with a lipophylic fluorescent dye, $DiIC_{16}$ (1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbacyanine perchlorate, obtained from Molecular Probes, Eugene, OR), following the procedure of Sowers (1984). Then the prelabeled cells were mixed with the unlabeled cells before being injected into the fusion chamber.

The design of the cell fusion chamber is shown in Fig. 3 *A*. Two parallel platinum wires (0.4 mm apart, measured between edges) were sandwiched between two glass coverslips which were separated by a nonconducting spacer. The platinum (Pt) wires served as electrodes. Cells suspended in fusion medium were placed in the space between the parallel Pt wires via an inlet tubing. At the end of the fusion experiment, suspended cells were ejected via the outlet tubing. The chamber could be washed by passing solution from the inlet to the outlet.

3. Cell poration

The cell poration induced by the oscillating electric field was studied using a molecular probe method, in which a plasmid DNA, pSV2-CAT (containing the Chloramphenicol acetyltransferase gene from *Escherichia coli*), was introduced into the mammalian cell. Since the plasmid DNA normally cannot penetrate the cell membrane to enter the cell, efficiency of gene transfection depends directly on the ability of the

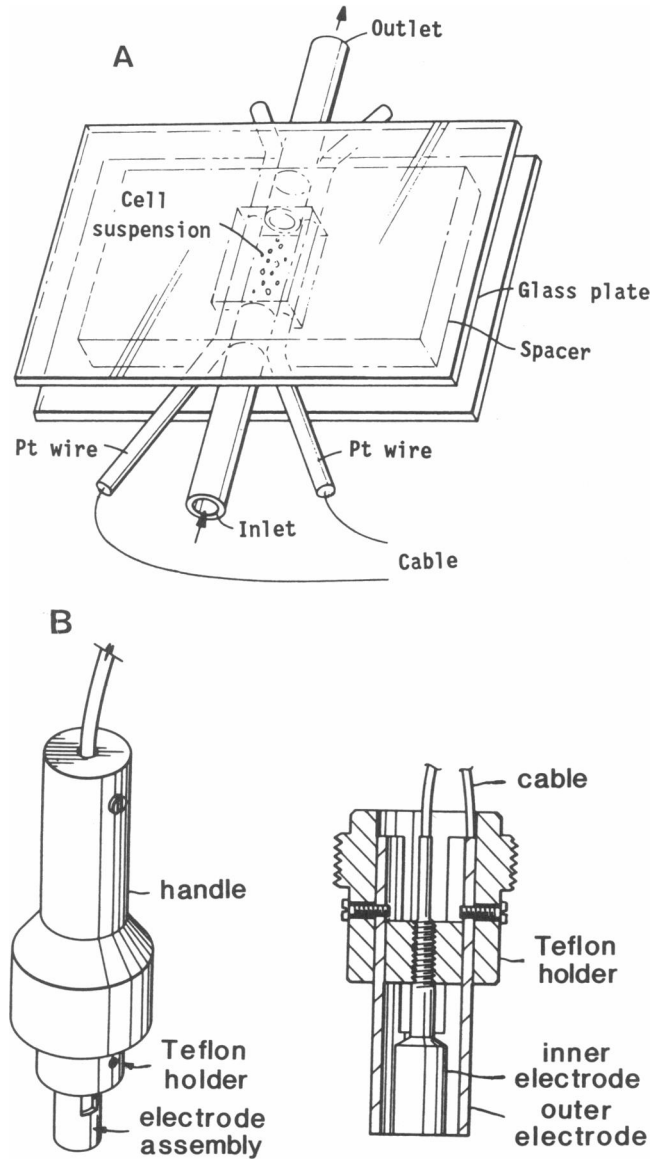


FIGURE 3 (*A*) Cell chamber for studying electro-fusion of red blood cells. The entire chamber is ~ 1-mm thick and can be fitted easily under the objective of a light microscope to allow direct observation of fusion events. (*B*) Design of a hand-held probe for the cell poration experiment. This probe fits into the well of a 96-well cell culture plate. The cell suspension fills the space between the inner and outer electrodes. Up to 5 million cells can be porated simultaneously using this probe.

oscillating electric field to porate the cell membrane. The cell model used here was a cultured eukaryotic fibroblast cell line COS-M6 (M6). The CAT enzyme is not endogenously produced in mammalian cells, such as M6. Thus, the amount of CAT gene incorporated into the target cells can be assayed by monitoring the amount of CAT enzyme produced by the M6 cells after the transfection.

The CAT assay has been previously described by Gorman et al. (1982). Basically, after the pSV2-CAT gene was introduced into the M6 cells by electro-poration, the cells were allowed to grow for two days. The cells were then harvested, and cellular proteins were extracted. Part

of these extracted proteins (25 μg) were reacted with 3.33 nM of ^{14}C labeled chloramphenicol for 90 min (at room temperature). The reaction products were extracted with ethyl acetate, separated by thin layer chromatography and autoradiographed using an x-ray film for visualization. The enzyme activity then was quantified by counting the ^{14}C radioactivities of the acetylated- and the unacetylated-chloramphenicol.

Because a large number of cells (usually two million) were used in the electro-poration studies, the chamber designed for the electro-fusion studies was no longer adequate. Hence, we have designed a hand-held probe especially for this poration work. This probe basically consists of two co-axial cylinders made of stainless steel (with a gap of 0.7 mm) which serve as electrodes (see Fig. 3 *B*). These cylinders were made to fit into the well of a 96-well culture plate (No. 25860; Corning Glass Works, Corning, NY), where the cell suspension was contained.

In each cell poration measurement, ~100 μl of cell suspension was put in one well. The cells were mixed with 0.1 μg of CAT DNA. Pulsed electric fields with various intensities and frequencies were used to porate the cells. After poration, the cells were allowed to post-incubate for 1 min before plated in culture medium. The poration efficiency was later determined by the CAT assay after 2 d of cell growth.

RESULTS

1. Electro-fusion of human RBC

Using intact RBC from human as a cell model, we found that the oscillating electric field is highly effective in inducing cell fusion. Fig. 4 shows the event at different stages of the electro-fusion process. RBC were suspended between two platinum electrodes and were observed under a light microscope. When there was no electric field applied across the electrodes, the RBC distributed randomly as shown in Fig. 4 *A*. After a low-intensity AC field (200 V/cm, 60 kHz) was applied for 0.5 min, the cells were lined up by a dielectrophoretic process to form pearl chains (Fig. 4 *B*). At this point, three pulses of RF electric field (100 μs wide, 3 kV/cm, 100 kHz) were applied; the RBC generally became more rounded in shape; and some neighboring cells began to fuse with each other. Many pairs of fused cells can be seen in Fig. 4 *C*, which was recorded at 1.5 min after applying the electrical pulses.

It is clear from Fig. 4 that an oscillating electric field can induce fusion between RBC. To better quantify the fusion events, it is advantageous to use a fluorescent dye transfer method to examine the fusion process (Sowers, 1984). Fig. 5 shows fluorescence micrographs taken at different times before and after applying the fusion pulses. About 10% of the suspended RBC were prelabeled with a lypophilic fluorescent dye (DiI), the rest of the RBC were unlabeled. This dye concentrated in the cell membrane and gave a brilliant fluorescent image. Within each pearl chain, the labeled and unlabeled cells were randomly mixed. Because of their low density, the labeled RBC were usually separated by the unlabeled cells. They appeared as isolated monomers viewed under a fluorescence microscope (Fig. 5 *A*). Because the membranes of the RBC are coated with extracellular glycoproteins and thus maintaining a small gap between neighboring cells, the dye normally could not transfer from one cell to another. When pulses of the high intensity radio-frequency (RF) field were applied to the RBC, fusion was induced between neighboring cells. Such fusion allowed the dye to diffuse from the membrane of labeled cells to the unlabeled cells (Fig. 5 *B*). With time the dye became evenly distributed between the prelabeled and the newly-labeled cells; they appeared as dimers under a fluorescence microscope. As the fusion process progressed, many newly-labeled cells fused with their next neighbors, and chains of cells stained with the fluorescent dye could be observed (Fig. 5 *C*).

Using the fluorescent dye method, two types of fusion events were seen. The first type is "membrane fusion", in which the fluorescent dye can freely pass between two fusing cells, but the cytoplasms of the two cells do not merge; each cell still retains its individual shape (see cells marked by arrow heads in Fig. 5 *C*). The second type of fusion is "cytoplasmic fusion," in which the cytoplasms of the fusing cells merge together, and the fused cells finally coalesce to form a larger, rounded cell. Obviously, only the latter type of fusion (i.e., cytoplasmic fusion) could lead to cell hybridization. The membrane fusion may be regarded as an abortive fusion process, in which the fusion partners are able to fuse their membrane lipid bilayers but fail to establish a cytoplasmic bridge. The membrane cytoskeletons are probably intact in this case and the fused cells cannot change their individual shapes.

Using the flourescent dye-transfer method, we have studied the effect of oscillating frequency of the applied field on electro-fusion. The yield for cytoplasmic fusion was determined at 5 min after applying three pulses of RF electric field (100 μs wide, 3.5 kv/cm). The results are shown in Fig. 6 *A*. The fusion yield is found to be strongly dependent on the frequency of the applied field, with the highest yield at a frequency near 80 kHz. The fusion yield is sharply reduced at lower frequencies, and gradually declines as the frequency increases from 100 kHz to 1 MHz. (The fusion yield was measured by counting the number of labeled cells undergoing cytoplasmic fusion divided by the total number of prelabeled cells).

Thus, it seems that the frequency of the applied field has a strong effect on the electro-fusion yield. The only uncertainty with the dye-transfer method is that under the fluorescence microscopy, one cannot differentiate intact cells from lysed cells. The data shown in Fig. 6 *A* may involve statistics contributed by cells that lysed during the fusion process. Hence, we have repeated the study of frequency dependence using DIC (differential

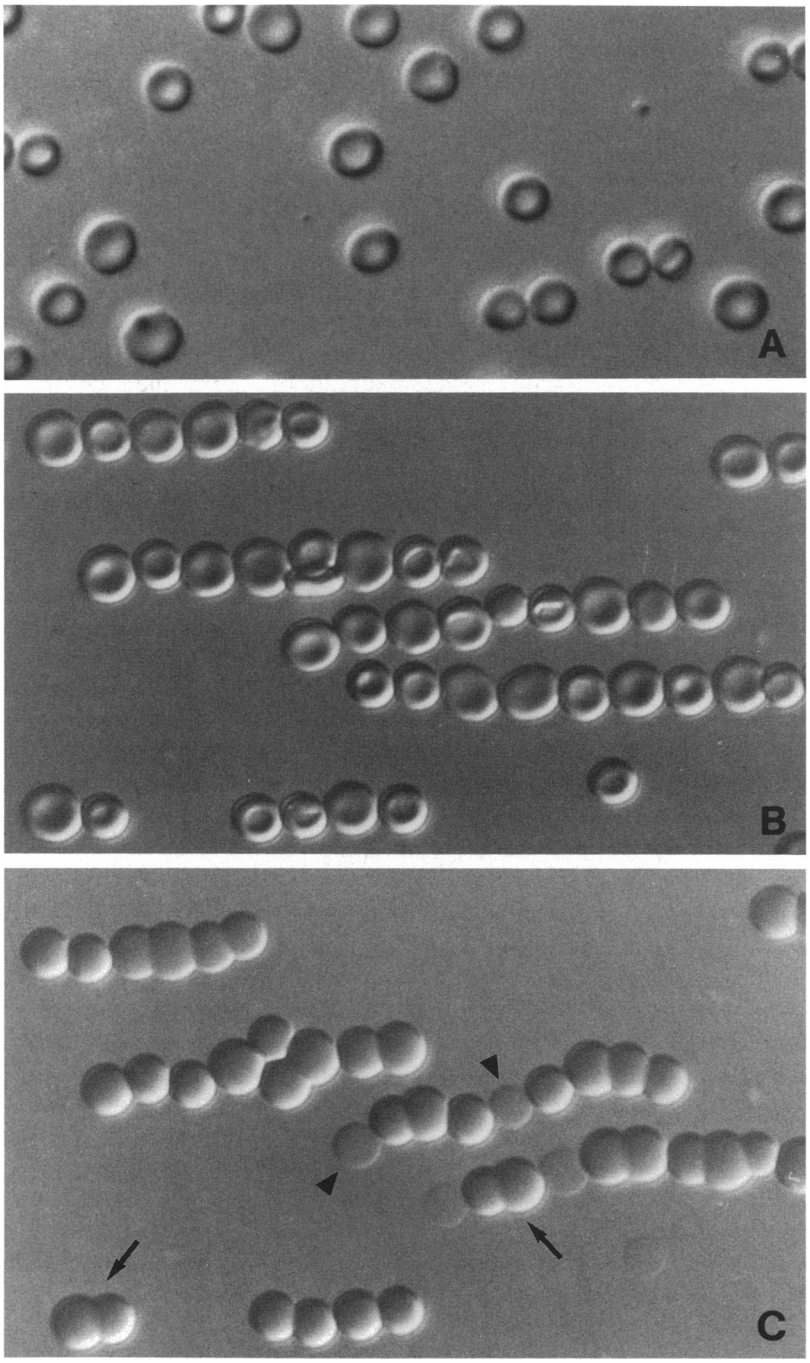
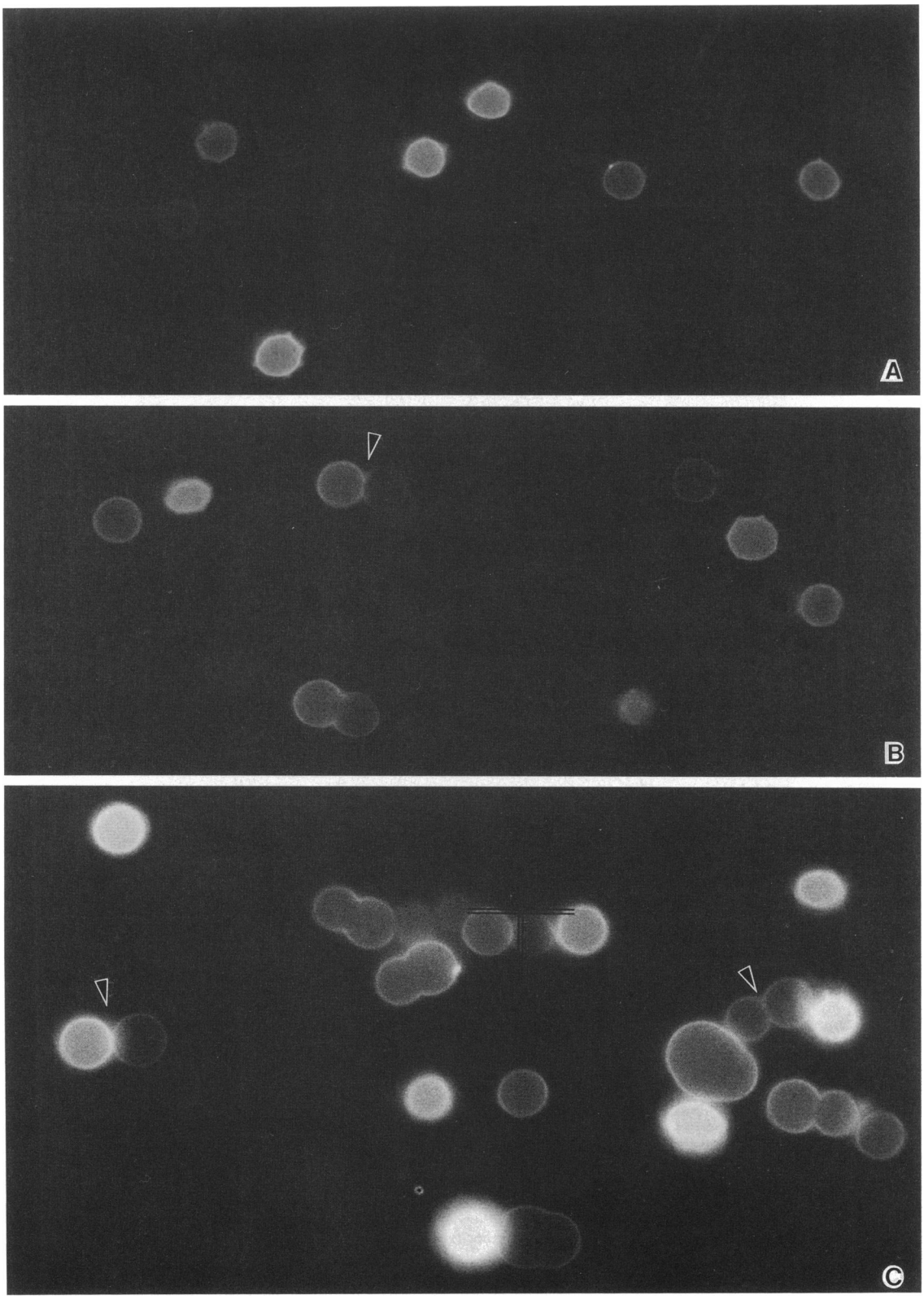


FIGURE 4 Fusion of human red blood cells (RBC) by the RF field as observed using DIC (differential interference contrast) optics: (*A*) Before any electric field was applied, RBC were distributed randomly. (*B*) After a low-intensity, continuous AC field (200 V/cm, 60 kHz) was applied RBC were lined up in "pearl chains" by dielectrophoresis. (*C*) One and one-half minutes after applying three pulses of high-intensity RF field (3.5 kV/cm, 100 kHz, 100 μs wide). Some of the neighboring RBC began to fuse together (see arrows). Some cells also lysed (indicated by arrow heads) after the electrical pulsation.


A
B
C

interference contrast) optics (see Fig. 4). With the DIC microscope, one can easily distinguish lysed and nonlysed cells (See Fig. 4 *C*). At 5 min after applying three pulses of high- intensity RF field, the number of fused and unfused cells among the RBC which remained unlysed were measured. The results of these measurements are shown in Fig. 6 *B*. Again, we found a strong effect of frequency of the applied field on the fusion yield. The fusion yield appears to be highest near a frequency of 100 kHz.

The results shown in Fig. 6, *A* and *B*, are basically similar; except that the fusion yields observed with DIC optics are higher than that observed using the fluorescent dye method. Part of this difference may be due to the fact that, in Fig. 6 *B*, the fusion yield was determined only from cells that are resistant to lysis. Apparently, the cells which lysed soon after the electrical pulsation could not fuse with their neighboring cells by cytoplasmic fusion.

It is evident from the data presented in Fig. 6, *A* and *B*, that oscillating electric fields of certain frequencies are highly effective in inducing cell fusion. The fusion yield presented here is by no means the optimal value. By slightly increasing the field strength or lengthening the pulse width, one could improve the fusion yield even further. Sometimes we have observed fusion yields exceeding 80%.

How effective is the RF field method in comparing with the more conventional DC field? We have conducted a study in which RBC were divided into two groups; one group was fused using RF electric field pulses and the other group was fused using DC electric field pulses. The pulse protocol, pulse width, and pulse amplitude were identical in these two cases (100 μs width, 3.7 kV/cm, 3 pulses applied 1 s apart). The results are summarized in Table 1. Using the fluorescent dye method, the average fusion yield obtained by the DC field is 3.4%, while the average fusion yield obtained by applying the RF field is 18.3%.

The difference between the RF field and the DC field becomes even more prominent when the fusion yield was measured among the RBC which remained unlysed after 5 min (Note: such measurement was done using the DIC optics). Over four experiments, the average fusion yield obtained using the RF field (at 100 kHz) was 55.6%, while no cytoplasmic fusion was observed using a DC field with the same pulse width and amplitude (see Table 1). Hence, the RF field appears to be more effective than the DC field in electro-fusion. We may also mention that the fusion yield can be affected by the pulse protocol used. The preceding results were obtained by applying three consecutive pulses of electric field at 1 s intervals. The pulse width was 100 μs. If one applies one long pulse with three times the pulse width (i.e., 300 μs), the result is different (Table 2). The average fusion yield obtained using a longer pulse of DC field is higher than that obtained from three short DC pulses. On the other hand,

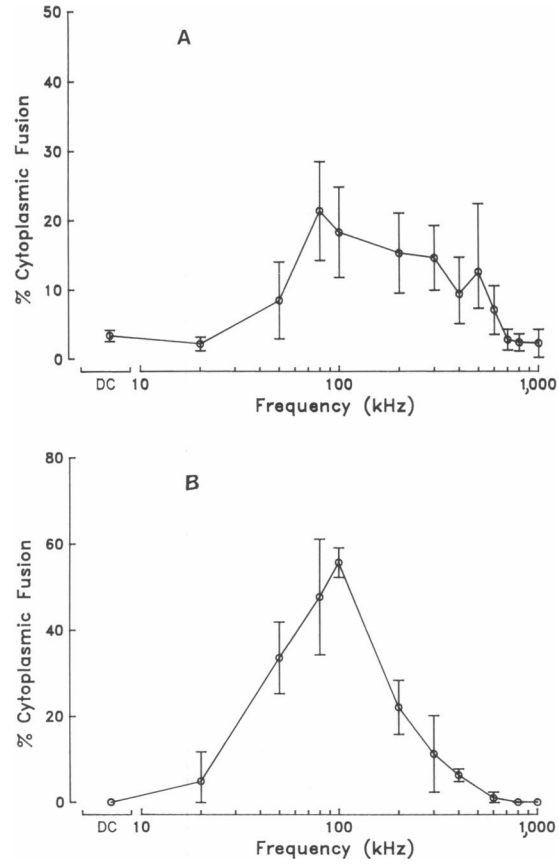


FIGURE 6 (*A*) The fusion yield of RBC obtained using the RF field method is plotted as a function of the frequency of the applied RF field (3 pulses, 100 μs wide, 3.5 kV/cm). The fusion events were assayed using the fluorescent dye transfer method. (*B*) Fusion yield versus the frequency of the applied RF field. The pulse protocol was the same as Fig. 6 *A*. The fusion events were assayed using DIC optics 5 min after the electrical pulsation. Only unlysed cells were counted in this measurement.

FIGURE 5 Observations of RBC fusion using the fluorescent dye transfer method. About 10% of the RBC were prelabeled with DiI. The other cells were unlabeled. (*A*) Before the high-intensity RF field was applied, the labeled cells appeared as isolated cells under a fluorescence microscope, although in reality they were embedded within pearl chains made up of many unlabeled cells. (*B*) One minute after applying three high-intensity pulses. Some of the labeled cells started to fuse with their unlabeled neighbors (*arrowhead*). The fluorescent dye now could be transfered from the labeled cell to the unlabeled fusing partner, making both cells fluorescent. (*C*) Four minutes after application of the RF pulses. Some of the fused cells had merged their cytoplasms to form larger cells. Some cells fused their membranes but did not merge their cytoplasms (see *arrowheads*).

TABLE 1 Electro-fusion of red blood cells using multiple pulses

Experiment No.	Fusion yield (%)		Method
	RF PULSE	DC PULSE	
050388	50.5	3.3	Fluorescent dye transfer
051088	16.0	0.0	
051188	13.5	4.9	
051988	9.0	6.0	
052488	9.0	3.0	
052588	12.0	3.0	
MEAN ± SD	18.3 ± 15.9	3.4 ± 2.0	
070788	58.3	0.0	DIC optics
070988	58.6	0.0	
071188	53.8	0.0	
071288	51.6	0.0	
MEAN ± SD	55.6 ± 3.4	0.0 ± 0.0	

Fusion was induced by applying 3 pulses (pulse width 0.1 ms, 100 kHz, 3.7 kV/cm).

the fusion yield using the RF field is higher with the multiple-pulse protocol. Nevertheless, by comparison, the RF field is still significantly more effective than the DC field in inducing fusion even in the condition that only one single long pulse was applied.

2. Study of electroporation using a marker gene, pSV2-CAT

This study was designed to serve two purposes: (*a*) to demonstrate the usefulness of the RF electro-poration method as a tool for gene transfection, and (*b*) to provide a quantitative assay to measure the effectiveness of using an oscillating field to porate cells. The molecular probe to be introduced into the target cells is a marker gene chloramphenicol acetyltransferase (CAT). The target cell is a cultured eukaryotic fibroblast cell line COS-M6. Because the CAT enzyme is not endogenously produced in the M6 cells, incorporation of the CAT gene into the target cells by electro-poration can be detected by monitoring the activity of the CAT enzyme in the transfected cells.

TABLE 2 Electro-fusion of red blood cells using a single pulse

Experiment No.	Fusion yield (%)		
	RF pulse	DC pulse (full height)	DC pulse (1/2 height)
080388*	50.0	13.5	0.0
080388‡	38.5	21.3	0.0
080588*	16.7	0.0	0.0
080588‡	31.7	0.0	—
MEAN ± SD	34.2 ± 13.9	87 ± 10.5	0.0 ± 0.0

*Fusion was induced by applying a single pulse (pulse width 0.3 ms, 100 kHZ, 3.7 kV/CM).
‡Observed using DIC optics.

A typical record of a CAT assay is shown in Fig. 7, which is an autoradiograph showing the separation of chloramphenicol and acetylated chloramphenicol by thin layer chromatography. In each column, 25 μg of cell protein extract were used to catalyze the reaction. A higher rate of acetylation of chloramphenicol (i.e., a higher CAT enzyme activity) implies a more effective gene transfection. From Fig. 7, it is clear that the pulsed oscillating electric field can be used to effectively porate the M6 cells to insert the CAT gene. Here, two million cells were transfected using only 0.1 μg of CAT DNA. The efficiency of gene transfection apparently depends on the strength of the applied field. The relationship between the CAT enzyme activity and the field strength is plotted in Fig. 8. Under the given experimental condition, the most effective gene transfection (and thus the optimal electro-poration) occurs when the electric field strength is between 1.8 to 2.1 kV/cm. Because the field strength can also affect the number of cells killed by the poration pulses, we have measured the survivability of the transfected cells at different field strengths (see Fig. 8). At $E = 1.8$ and 2.1 kV/cm, the cell survivability is ~80 and 70%, respectively. Thus, with the oscillating electric field it is possible to maintain the viability of the majority of cells even at a field strength high enough to provide the maximum poration efficiency.

An important question in this study is that whether the oscillating electric field can offer an advantage over the more conventional DC field in cell poration. We have done a series of comparative studies. First, in regards to cell survivability, the RF electric field is apparently more gentle to the cells than the DC field. Using electric pulses with the same pulse width and pulse amplitude, more cells can survive the exposure to five pulses of RF field than a single pulse of DC field (see Fig. 9). Secondly, the efficiency of gene transfection (which is proportional to the percentage of cells porated by the electric field) is higher using the RF field than the DC field. Table 3 summarizes the results of four experiments which compare the transfection efficiency using five pulses of RF field with that using five pulses of DC field. Measured on the basis of constant cell mass of the surviving cells, the transfection rate obtained from the RF field method is about twice that obtained from the DC field method (see column 1, Table 3). However, because the cell survivability in the RF field is almost three times that in the DC field (see column 2, Table 3), the net transfection rate (which is equal to the product of cell survivability and the

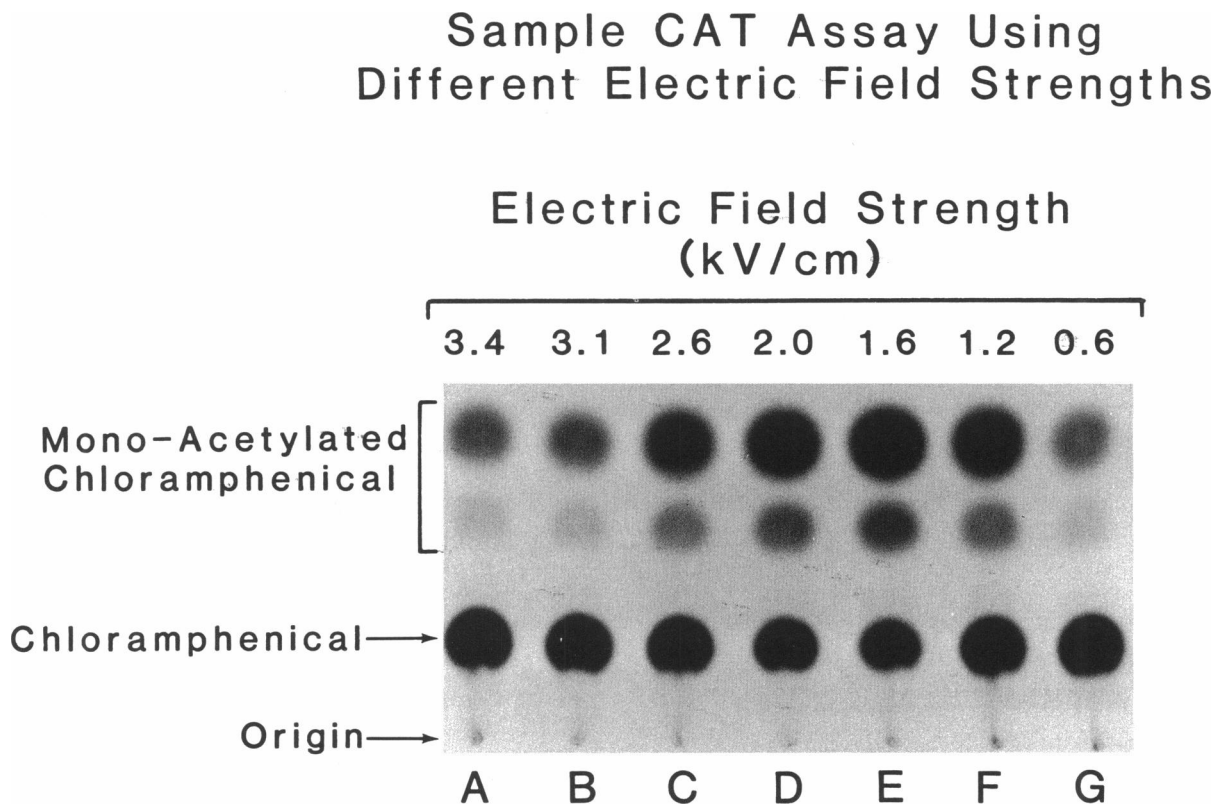


FIGURE 7 Sample result of the CAT assay, which measured the efficiency of gene transfection (see text). The CAT genes were introduced into the M6 cells by electro-poration using the RF field (40 kHz, five pulses with pulse width of 2 ms). The transfection efficiency appeared to depend strongly on the intensity of the applied RF field.

transfection rate of the surviving cells) is more than five times higher in the RF field method than that in the DC field method (see column 3, Table 3). Hence, it seems that there is a significant advantage of using the RF electric field to porate the cells than a single DC field.

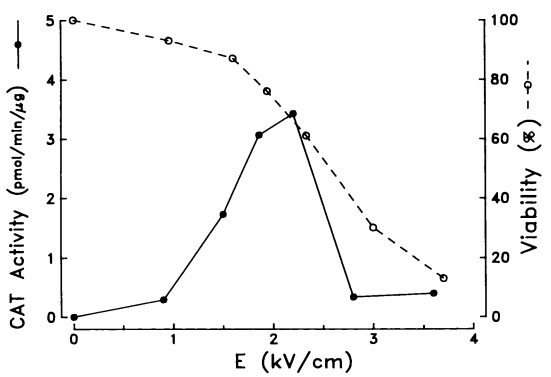


FIGURE 8 CAT activity and cell survivability are plotted as a function of the field strength of the applied RF field. The pulse protocol was similar to that of Fig. 7.

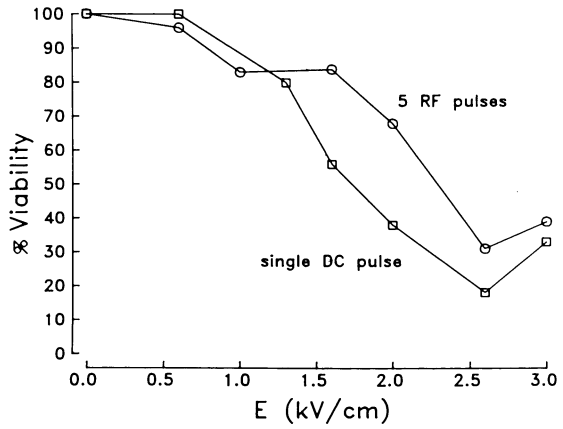


FIGURE 9 The effects of the RF field and the DC field on the cell viability are compared. Cell survivability after electro-poration was determined as a function of the field strength of the applied field. In one experiment, the M6 cells were exposed to five pulses of RF field, while in the paired experiment, cells were exposed to one pulse of DC field. (The pulse width was 2 ms and the RF frequency was 50 kHz).

TABLE 3 Comparison of two electro-poration methods

Method	Viability	CAT activity	Net transfect rate*
		pmol/min/μg	
RF field	0.61 ± 0.07	0.52 ± 0.19	0.309 ± 0.07
DC field	0.22 ± 0.04	0.28 ± 0.09	0.062 ± 0.03

*NTR = Viability times CAT activity.
Sample size $n = 4$.
Transfection of COS-M6 cells using 0.1 μg of pSV2-CAT.

DISCUSSION

Here, we have demonstrated that one can apply a pulse or pulses of DC-shifted oscillating electric field in the radio-frequency range to induce cell fusion or cell poration. In comparison to the results obtained with the DC field applied in the same protocol, the RF field appears to produce a higher yield of fusion in human RBC, and is more effective in transfecting M6 cells with the CAT gene.

Furthermore, the use of an oscillating field provides better cell viability. As shown in Table 3, under the same field strength, the cell survivability is higher with the RF field than with the DC field. This result is probably due to the fact that an oscillating electric field can avoid polarizing the cell membrane beyond the critical potential for an extensive period, therefore preventing the cell membrane to be irreversibly ruptured over a significantly large area. Earlier studies of electro-poration had indicated that when a cell is exposed to a high intensity DC field, the pulse length is a critical factor for the reversibility of the field induced effects in the cell. For example, Zimmermann et al. (1980) showed that when thymocytes were exposed to a DC field of 4 kV/cm for 40 μs, they suffered irreversible damage. However, when the exposure time was reduced to 0.5 μs, no irreversible damage was observed even when the field strength was increased to 14 kV/cm. Thus, the RF field would have the advantage that prevents the cell from being continuously exposed to a high intensity field for an extensive period.

Our observations that an oscillating electric field was able to fuse or porate cells with higher efficiency are particularly interesting. When one compares an RF pulse (such as the one shown in Fig. 1) with a DC pulse, it is apparent that the applied energy (i.e., the integration of V^2/R over the pulse width, where R is the load resistance of the cell sample) is less in the RF pulse than in the DC pulse with the same pulse height. Yet, as shown in both our fusion experiments (Tables 1 and 2) and poration experiments (Table 3), the RF pulse is able to provide higher fusion yield and higher gene transfection efficiency. If fusion or poration depends purely on an electrical breakdown of the membrane, then a DC field should be far more effective than an RF field. Hence, we feel that the results of this study suggest that the sonicating effects of the oscillating field have a significant contribution in causing the membrane breakdown.

Another intriguing observation of this study is the frequency dependency in cell fusion using the RF field. At this point, the underlying mechanisms which give rise to such a frequency dependence are not fully understood. The reduced fusion efficiency at high frequency could be attributed to the bandwidth limiation of the induced membrane potential of the cell. Eq. 1 describes the induced membrane potential in a case where the external field changes very slowly with time. If the applied field is an oscillating field, the amplitude of the induced membrane potential is (Holzapfel et al., 1982):

$$V_m = \frac{3rE\cos\theta}{2[1 + (\omega\tau)^2]^{1/2}},$$

where ω is the angular frequency and τ is the relaxation time of the cell. (τ is proportional to the cell radius. In the case of RBC, τ is in the order of 1 μs). Thus, when ω is larger than $1/\tau$, V_m is almost inversely proportional to ω. Hence, the induced electric field within the membrane decreases as the oscillating frequency is increased. Such a bandwidth consideration thus, could at least partially explain the reduced fusion yield at higher frequency.

The reduced fusion yield at lower frequency is more difficult to explain. One possibility is that there are certain characteristic frequencies that may be related to the molecular structure of the membrane or to the normal modes of motion of the cell membrane; the matching of the frequency of the applied RF field to one of these characteristic frequencies may produce a resonance effect (and thus become more effective in generating a structural fatigue). Another possibility is that certain frequencies may be particularly effective in maintaining a constant dipole orientation of the suspended cells. Some aspects of the natural and induced electrical resonances in cells have been previously investigated (Pohl, 1982; Holzapfel et al., 1982).

It is generally believed that cell poration and cell fusion are caused by a local membrane breakdown. At this point, the exact nature of the membrane breakdown in association of electro-poration or electro-fusion is still unclear. Membrane breakdown is related but different to membrane rupture; the latter by definition is irreversible. Most of the mechanistic studies of membrane breakdown employed the lipid bilayer as a model (Benz and Zimmermann, 1980; Chernomordik et al., 1983). When a lipid bilayer is charged with an increasing electrical potential, a reversible high conductance state occurs before the

bilayer becomes ruptured (Benz and Zimmermann, 1980; Chernomordik et al., 1983). This high conductance state has been attributed to a "reversible electrical breakdown" of the bilayer. During such reversible breakdown, it was believed that many small, resealable pores are formed at the bilayer membrane. This reversible electrical breakdown of lipid bilayer has been used as a standard model to explain the process of electro-poration and electro-fusion in the cell membrane (Zimmermann and Vienkin, 1982; Chernomordik et al., 1987). However, we feel that this standard model may not be entirely adequate. First, the size of pores created by a reversible electrical breakdown in the lipid bilayer is extremely small (estimated to be ~2 nm in diameter) (Glaser et al., 1988). The electro-pores created in the plasmalemma of porated cells, on the other hand, are significantly larger. For example, the pores are large enough to allow free passage of large DNA molecules. Recently, we used a rapid-freezing electron microscopy technique to study the formation of membrane pores in human red blood cells. We found that the diameters of membrane pores induced by electric fields are in the range of 25–120 nm (Chang and Reese, 1989). Secondly, the lifetime of the pores in the lipid bilayer is relatively short (in the range of 2–10 μs) (Benz and Zimmermann, 1981). The electro-pore lifetime of the cell membrane, however, is much longer. The large membrane pores of RBC observed in the rapid-freezing EM study reseal on a time scale of 10 s. Resealing time of smaller electro-pores observed in other studies are even longer (from minutes to hours) (Kinosita and Tsong, 1977; Zimmermann et al., 1980).

Thirdly, the cell membrane is supported by a mesh work of cytoskeleton (or membrane cytoskeleton) (Branton et al., 1981), while the artificial lipid bilayer is not. The cell membrane has considerable mechanical strength and could allow a hole to be plunged through without rupturing the whole membrane. (For example, consider the case of microinjection using a glass pipette). The lipid bilayer is very fragile and cannot support pores of significant size. It thus appears that a structural breakdown in the membrane that may result in a rupture of the bilayer may not cause a rupture in a real cell membrane.

Hence, we think the membrane breakdown involved in cell-poration does not necessarily correspond to a reversible breakdown of the lipid bilayer. In fact, the electro-pore in the cell membrane may be more likely to be caused by an irreversible breakdown of the lipid bilayer. Such breakdown may be regarded as a localized mechanical breakdown over a small region of the cell membrane; it is prevented from causing a rupture of the entire membrane by the support of the membrane skeletal proteins. Such breakdown could account for the creation of large membrane pores. These pores may later be resealed either by the diffusion of lipid molecules into the damaged region or by the membrane regeneration mechanisms of the cell.

So far we have not separated the type of membrane breakdown involved in cell fusion from the membrane breakdown involved in cell poration. Although the effects of electric field on the membrane must be similar in these two cases, the breakdown events which lead to fusion and those which lead to poration may not be the same. There has been evidence indicating that the fusion process may not necessarily involve membrane pores (Sowers, 1986). Our observations that the frequency of the oscillating field has a stronger effect on cell fusion than cell poration are also consistent with the suggestion that the mechanisms of electro-fusion and the mechanisms of electro-poration could be significantly different.

At this point we have just begun to evaluate the potential of cell fusion and cell poration using the oscillating electric field method. There are several important parameters involved in this method that need to be thoroughly tested. These parameters include the pulse width, field strength, number of repetitive pulses, waveform, pulse shape, temperature, pH, osmolarity, ionic concentration, etc. Many of these parameters may be interrelated. Here, we can only vary a few of these parameters to show that the oscillating field method is potentially useful and, in certain cases, it already has some advantages over the conventional method of cell fusion and cell poration. For example, with the conventional methods, such as the chemical method or electro-fusion using a DC field, the fusion yield in making hybridomas is generally $<10^{-5}$. In our experiments, electro-fusion of RBC using an oscillating field sometimes can provide a fusion yield >80%. Thus, there seems to be a tremendous potential in improving the hybridoma yield. Particularly, this RF field cell fusion method may be potentially useful in developing technology for producing human hybridomas. Also, in the case of gene transfection, conventional methods (including the Ca-PO_4 method and electro-poration using a DC pulse) usually requires 10 to 40 μg of DNA for each transfected cell sample. Here, we used only 0.1 μg of DNA and still obtained a very high transfection rate. Hence, the method of using the oscillating field seems to be more efficient and more economical in terms of DNA consumption. In the future, it would be of interest to extend this method to study other cell types, such as plant cells, yeast, or bacteria.

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