Hybridization Frequencies of Different Mammalian Cell Types by Electrofusion

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Abstract. The efficiency of electrofusion of four types of cells: CHO, HeLa, mouse melanoma cells and human skin fibroblasts has been studied. The frequencies of fusion products were determined 1) directly in a closed flow-through fusion chamber after dielectrophoresis and pulsation; 2) after short-term post-fusion cultivation period of 5 to 10 minutes; and 3) in various intervals up to 30 hours after fusion induction. No substantial differences were found in the rates of formation of heterokaryons and synkaryons between the individual cell types, and this confirmed the uniformity of the effects of electric fields on diverse cell membranes. After 5 hours of culture the yield of fusion products reached 15 to 35 % in various cell combinations and the frequencies of synkaryons reached up to 7 % in almost all the combinations studied 24 to 30 hours after fusion.

Key words: Electrofusion – Heterokaryon – Homokaryon – Synkaryon – Cultured somatic cells

Introduction

Recently, cell fusion methods based upon effects of electric field on cell membranes have been developed (Zimmermann 1982; Zimmermann et al. 1984, 1985). Intimate cell-to-cell contacts are achieved by applying a non-uniform alternating electric field of low intensity to a cell suspension, which brings about the formation of electric dipoles within the cells. Thus cells are moving to one another and establish mutal contacts due to electrostatic forces (Zimmermann 1982). The fusion of adjacent cell membranes is initiated by applying a short direct current pulse of a field intensity of kilovolts per cm. This leads to perturbations of lipid molecules within adjacent membranes and to the formation of apposed pores in the contact zone (electrical breakdown) (Zimmermann

et al. 1985). Although the events leading to fusion of neighbouring membranes in dielectrophoretically collected cells have been extensively studied (Ohno-Shosaku and Okada 1985; Stenger and Hui 1986, 1988; Sowers and Lieber 1986), not all the mechanisms have been well understood as yet. Nevertheless, the electrofusion technique shows many advantages when compared to chemical or virus-induced cell-to-cell fusion. Among these advantages the high yield of fusion products seems the most prominent. Since electrofusion is based on unified physical mechanisms applied to biomembranes, it allows not only cells but also artificial membrane structures (e. g., ghosts, liposomes) and cell organells to be fused as well. Further enhancement of the efficiency of electrofusion is believed to be achieved by the direct fusion of nuclear plasmalemas within the polynuclear cell, as shown recently by Bertsche et al. (1988).

In the present paper the fusion yields of various cell types are compared. Using pre-labelling of the cells by latex beads of two sizes, we were able to follow the formation of heterokaryons and synkaryons as well as the fate of polynuclear cells in culture.

Materials and Methods

Cells and media: In fusion experiments four types of mammalian and human cells were studied: established cells of Chinese hamster ovary (CHO), HeLa cells, mouse melanoma cells (MM) and human skin fibroblasts (SF). Mouse melanoma cells were obtained by primary culture of explants from transplated tumor B16, human skin fibroblasts were cultured from a stillborn male with multiple congenital defects.

In the course of experiments, the skin fibroblasts reached the 24th passage. All the cell types, except for the human skin fibroblasts, were cultured in RPMI 1640 medium (Gibco) supplemented by 10 % fetal calf serum (HyClone), penicillin and streptomycin. The skin fibroblasts were cultured in McCoy's 5A medium (Gibco) with 10 % fetal calf serum and antibiotics. All cell lines were maintained as monolayers in T 25 flasks (Corning) and subcultured every 2nd or 3rd day using 0.05 % trypsin and 0.53 mmol . l^{-1} EDTA in Hanks' balanced salt solution.

Labelling of cells with polystyrene beads: In order to distinguish between homokaryons, heterokaryons and synkaryons, the cells were prelabelled with latex beads of two different sizes: 1.069 and 0.497 μm (Ted Pella, CA). Polystyrene beads were added to the cell cultures in an amount of 20 μl of the stock bead suspension per ml of culture medium, and incubated overnight. This yielded uniformly labelled cell populations, 95 to 98 % of the cells being labelled. As a rule, individual cells contained more than ten beads; a small proportion of cells (less than 1 %) contained smaller bead numbers. Cells containing three or less beads were considered as non-labelled and were not included in the analyses.

Electrofusion procedure: For the purpose of fusion experiments, cells of the monolayer cultures were trypsinized, washed in 5 ml Hanks solution and resuspended in 10 ml of fusion medium, which consisted of 0.3 mol . l^{-1} manitol (Sigma), 0.05 mmol . l^{-1} $MgCl_2$ and 0.05 mmol . l^{-1} $CaCl_2$ dissolved

Table 1. Frequencies of fusion in fusion chamber and after short-term culture

Cell combination	ELECTRICAL PARAMETERS		FUSION CHAMBER			SHORT-TERM CULTURE		
	Dielectrophoresis field strength[1] ($V \cdot cm^{-1}$)	El. breakdown field strength[2] ($kV \cdot cm^{-1}$)	Frequency of fusion in the chamber[3] (%)	Average chain length[4] (No. of cells)	n	Fused cells[5] (%)	n[5]	Total cells counted
HeLa × CHO	200	2.6	89.53	17.08 ± 8.10	(12)	25.95 ± 6.51	(5)	637
MM × CHO	200	2.6	84.58	19.83 ± 8.55	(12)	41.77 ± 2.91	(7)	1173
SF × CHO	320	2.0	87.50	18.15 ± 9.29	(13)	51.55 ± 18.08	(4)	486
SF × HeLa	200	2.6	91.43	16.69 ± 7.94	(13)	45.58 ± 12.11	(7)	759
SF × MM	280	2.0	87.56	17.46 ± 7.40	(13)	20.92 ± 9.42	(5)	579
CHO × CHO	220	2.6	88.47	13.35 ± 7.26	(14)	28.30 ± 13.32	(11)	1289
HeLa × HeLa	200	2.6	92.16	14.90 ± 7.58	(10)	43.35 ± 4.41	(4)	547
MM × MM	320	2.6	97.45	15.91 ± 9.04	(11)	45.79 ± 7.25	(6)	612
SF × SF	200	2.0	86.30	15.36 ± 6.65	(11)	45.07 ± 12.47	(5)	468

[1] Dielectrophoresis was performed at 620 kHz.

[2] Pulse duration was 30 μs in each experiment.

[3] The frequencies of fusion were determined as the ratio of cells forming chains to all cells in the fusion chamber.

[4] Average chain lengths were determined in fields of photographed samples and are expressed as $\bar{X} \mp s\bar{x}$; n denotes number of samples.

[5] The percentages of fused cells were determined on photographs taken 5 to 10 minutes after the fusion and collection of $3 \cdot 10^5$ cells into prewarmed medium in Petri dishes; the percentages are expressed as $\bar{X} \mp s\bar{x}$, s.d. shows the variation within fields photographed, n = number of fields photographed.

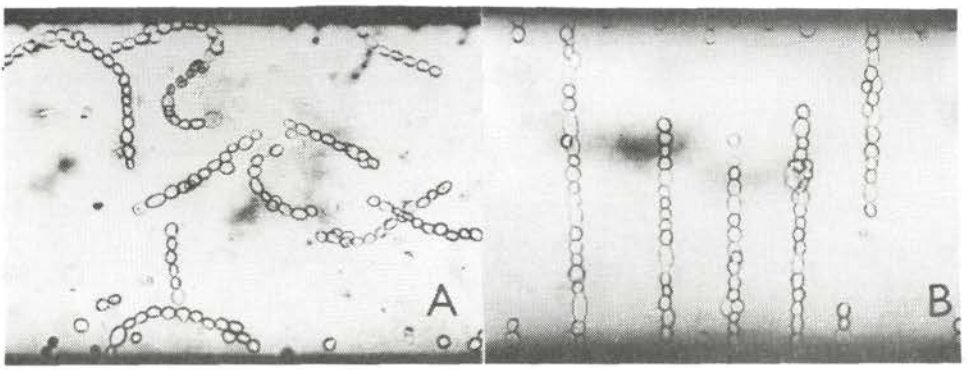

Fig. 1. CHO cells after dielectrophoresis in a flow-through fusion chamber (*B*) and after pulsation (2.6 kV/cm, 30 μs.) and displacement by hydrodynamic force (*A*). Sticking of cells demonstrated fusion initiation. Living cells.

in low ion water. The cells were pelletted at 600 RPM for 3 min, resuspended and centrifuged again. Cells in final pellets were counted and mixed in a 1:1 ratio at a final density of 1.10^6 cells per ml. All these steps were done at room temperature since lowering of the temperature of the solutions markedly inhibited the fusion. The suspension of the cells was drawn into a 1 ml syringe and, using a microburet syringe device, the cells were slowly injected into a closed flow-through electrofusion chamber connected to a FP 001003 Cell Fusion Processor (D.E.P. Systems, Metamora, MI) in four-microliter increments. After dielectrophoretic alignment the cells were fused using one DC pulse.

During each experiment, four sets of photographs were taken: aligned cells in fusion chamber, the chains of fused cells after the DC pulse and displacement by a slow stream of fusion medium flowing through the chamber, after collecting the cells to the Petri dish containing medium and subsequent 5 minutes' cultivation, and after long term culture of 24 to 30 hours in preparations fixed on slides and stained with Giemsa.

Results

In order to reveal potential differences in the fusion capacity of various cells, four types of cells of different origin were used in the experiments. Basically, the experiments involved two types of fusion: autofusion, with the same cells being fused to obtain homokaryons, and five various combinations of heterofusion, where cells of different origin were fused. The combinations used in the experiments are summarized in Table 1. After dielectrophoretic collection of cells at 620 kHz (maximal frequency available) and AC field 200 to 320 $V.cm^{-1}$, the cells were fused using one DC pulse of 30 μs duration of a field intensity

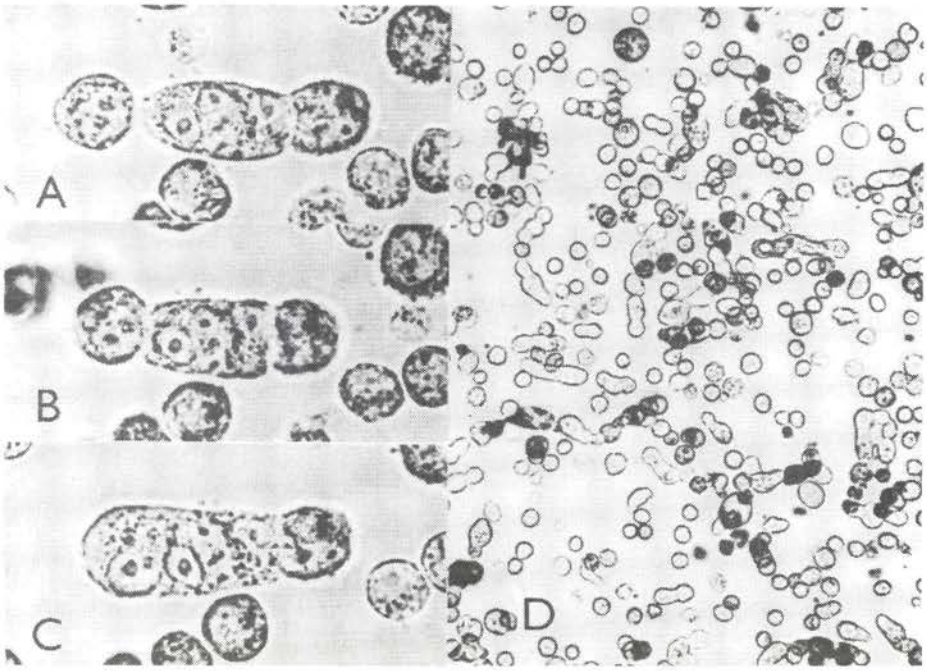

Fig. 2. Sequence of fusion progression of CHO cells in a medium in Petri dish. The photographs were taken at intervals after pulsation: 5 min (*A*), 8 min (*B*) and 13 min (*C*). Mixed culture of CHO and MM cells undergoing fusion in culture medium at 37 °C (D). Living cells.

2.6 kV . cm $^{-1}$. In some cases, i.e. with skin fibroblasts, a field strength of 2 kV . cm^{-1} was chosen to prevent cell lysis. These electrical parameters were standardized after preliminary experiments and were used in all the experiments. As seen in Table 1, at the given electrical parameters, the chains of cells undergoing fusion initiation consisted of 13 to 20 cells in average, 84 to 97 % of cells in the suspension being fused. At a sufficient duration of dielectrophoresis (10 to 15 s) and at 1.10^6 cells per ml, only small proportions of cells in the chamber escaped chain formation. Typical illustrations of dielectrophoresis and fusion initiation are shown in Fig. 1.

When cells were collected into Petri dishes after dielectrophoresis and DC pulse application, and cultured in an incubator for five minutes, a rapid fusion of neighboring cells was evident (Fig. 2). By photographing randomly chosen areas in dishes, the frequencies of cell fusion were estimated (Table 1). The percentages of cell fusion are total cell number in a particular sample. In some

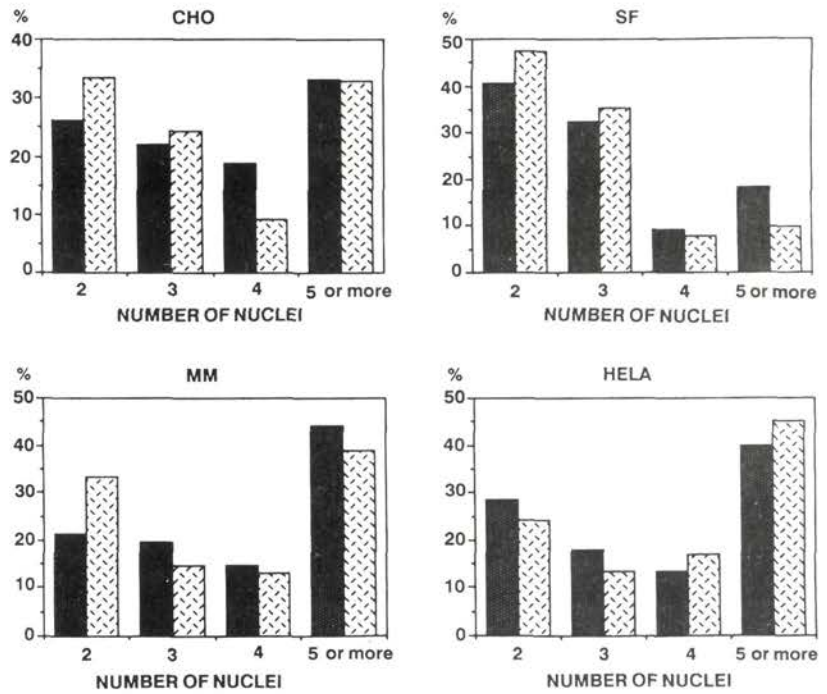

Fig. 6. Relative frequencies of numbers of nuclei in homokaryons in autofusion experiments. Cells were fixed and stained 6 hours after fusion (heavy shadowed columns) and 24 hours after fusion (lightly shadowed columns).

and fixed directly on the slides 6, 11, 24 and, in some cases, 30 hours after the fusion induction. This method led to the attachment of cells and allowed the determination of heterokaryon, homokaryon, and synkaryon frequencies (Fig. 3).

The frequencies of fusion products in all the nine combinations studied are shown in Figs. 4 and 5. For autofusion, the cells were also prelabelled in order to monitor subsequent "nuclear fusion", i.e. formation of synkaryons. The frequencies of fusion products were expressed as absolute values determined by microscopic analysis; occasional polynucleation in control preparations (cells not subjected to electrical field) were subtracted. The total frequencies of fusion products in fixed preparations reached 30 % in autofusion as well as in heterofusion with only two exceptions (HeLa × SF and MM × SF), whre slightly smaller fusion frequencies were detected (approx. 20 %). The frequencies of fused cells shown in Figs. 4 and 5 are numbers of fusion products related to all

the cells in a sample, regardless of the numbers of nuclei in the respective homokaryons and heterokaryons.

To illustrate the situation in polynucleation six and twenty-four hours after fusion induction, data from autofusion were used in constructing graphs in Fig. 6, where percentages of polynuclear cells are plotted against numbers of nuclei in these fusion products. Differences were found in polynucleation between cells of malignant and non-malignant origin. In SF × SF fusion, homokaryons with lower nubmers of nuclei were generated while in combinations involving CHO and malignant cells, large homokaryons with five and more nuclei were favoured.

When comparing the frequencies of binuclear homokaryons after 6 h and 24 h cultivation time, the numbers of binuclear cells were higher at 24 hrs than the numbers of polynuclear cells.

Discussion

In the experiments cells of various provenience were used to explore variations in fusion efficiency. The cells were not pretreated by any proteolytic enzymes which are used to form protein free lipid domains (Ohno-Skosaku and Okada 1984; 1985) by degrading membrane-associated proteins (Stenger and Hui 1988). The use of proteolytic enzymes has been reported to enchance the fusion yield (Ziemmermann et al. 1985). After dielectrophoresis of sufficient duration at appropriate breakdown voltage the majority of cells in a chamber (85—97 %) initiated fusion (Table 1). Agitation of chains of cells demonstrated that the cells had initiated fusion, but the typical rounding up (Zimmermann et al. 1985) of fusion products did not occur in the chamber. Both, the appropriate temperature and the presence of bivalent cations were shown to be inevitable for fusion completion (Zimmermann et al. 1985); Ohno-Shosaku and Okada 1984). The conditions were met by transferring the cells from fusion chamber to a Petri dish with prewarmed medium.

When comparing the numbers of nuclei in fusion products over time (cf. Table 1 and Fig. 6), a decrease in the bulk of fusion products was observed. In the fusion chamber the fusion products consisted of 13 to 20 cells on the average. After long-term culture polynuclear cells containing lower numbers of nuclei prevailed. This phenomenon might be explained by the breaking of the chains due to mechanical forces during the flow through the chamber outlet, by osmotic changes during the transfer of cells from mannitol solution to culture medium, or even by lysis at supracritical field strengths (Zimmermann et al. 1985) of a cell within the chain which results in chain fragmentation. In autofusion differences were observed in polynucleation patterns between cells of

different origin (Fig. 6). Binuclear products were predominant over polynuclear ones in SF × SF fusion. In malignant-cell fusion (HeLa × HeLa and MM × MM) and partially in CHO × CHO, three and more nuclei containing homokaryons were most common. With a prolongation of the culture time, these differences became more prominent. It is likely that the low frequencies of polynucleated cells in SF × SF fusion are due to their preferential detachment off the surface of the culture vessel. Multinuclear cells were only rarely observed (0.05 %) in control, non-fused SF preparations.

In long term cultures a constant decrease in the number of both homokaryons and heterokaryons (Fig. 5) as well as in the multinuclear fusion products in autofusion (Fig. 4) was observed with the increasing culture time. Contrary to this, the frequencies of synkaryons increased in all the cell combinations studied reaching up to 4—7 % in mixed cell populations. No "mononuclear" cells labelled with beads of both sizes were found in control, non-fused preparations. Thus, it is unlikely that such cells can be formed by uptake (e. g. phagocytosis) of beads from the culture medium after occasional cell death and/or lysis. The decreases in heterokaryon and homokaryon frequencies can easily be explained by a shorter survival of polynuclear cells in the culture and by "nuclear fusion" i. e. formation of synkaryons.

In conclusion, although cells of rather different origin were fused, various cell combinations were observed to behave in a similar way. In spite of lower yields of fusion products from SF × HeLa and SF × MM combinations, and despite some differences in polynucleation after fusion, all the cell types formed heterokaryons with high frequencies; and after 24 h period, electrofusion seems highly efficient, regardless of the cell type used.

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