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HOMOKARYON PRODUCTION BY ELECTROFUSION : A CONVENIENT WAY TO PRODUCE A LARGE NUMBER OF VIABLE MAMMALIAN FUSED CELLS

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Summary: A convenient technique to obtain homokaryons is described that provides large amounts of fused mammalian cells. Chinese hamster ovary cells grown in monolayers on a Petri dish are submitted to square wave electric pulses. Viability of cells is observed not to be affected by this electric treatment. The yield of fusion is strongly dependent on the strength of the field (KV/cm range) and on the duration of the pulse (microsecond range). The yield is not improved by accumulation of pulses. Yields up to 80 % are obtained and under our experimental conditions 200 000 cells are fused per assay.

Cultured mammalian cell fusion provides heterokaryons, a research tool widely used in cell biology. Different techniques were developed in the 60's and 70's where the cell fusion is obtained by using the fusing properties of viruses (1), of polyethyleneglycol (PEG) (2) and of lipid liposomes (3). In each case, the major shortcoming of these techniques is linked to the presence of exogenous reagents which are lethal for the cells. Furthermore in the case of PEG some recent papers demonstrate that the real fusing agent was not well characterized (4). As a consequence, the yield of viable hybrids was always very low and the success of an experiment was problematic.

Recently, a new technique, the so called "electrofusion method" has been described where the disadvantages linked to the external reagents are avoided (5, 6). In a very similar way, but by using neuramidase-treated cells clustered by an inhomogeneous AC field, rather impressive results were described when working on a very small volume (7).

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This report shows that repetitive microsecond square wave electric pulses, when applied to chinese hamster ovary cells (CHO) grown in monolayers in Petri dishes, are able to generate large amounts of viable polynucleated cells with a very high efficiency. Besides this advantage, this technic is demonstrated to be under the control of physical parameters. A systematic investigation of the dependence of the fusion process on : 1) the magnitude of the field, 2) the pulse duration and 3) the number of applied pulses is reported. This provides firstly information on the molecular mechanism which induces fusion and secondly describes the optimal conditions for obtaining viable fused cells.

MATERIALS AND METHODS

- Cell culture:Chinese hamster ovary (CHO) cells were grown in suspension at 37°C in a 5 % CO_2/95 % air atmosphere in Eagle's minimum essential medium (MEM 011, Eurobio, France) (8). The solution was supplemented with 8 % new born calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml) and L-glutamine (1.16 mg/ml).The cells were plated on Petri dishes (5 cm in diameter) and kept for 24 hours in the CO_2 atmosphere before electrical treatment. After this overnight incubation, the cell density was 815 cells per mm^2. The volume of medium per dish was 5 ml. Another technique was used when the fused cells' viability was tested. In that particular case, the cells were plated on sterile 18 x 18 mm glass coverslips in a 35 mm Petri dish. Here again, overnight incubation was performed before pulsing.
- Fusion protocol:All the experiments were performed under sterile conditions in a vertical laminar flow hood .Just before pulsation, the culture medium was substituted by 2 ml of 250 mM sucrose, 1 mM $MgCl_2$ in 10 mM phosphate buffer (pH = 7.75) ("pulsing buffer"). This buffer with a low ionic content has a low conductance and is thought to reduce the joule effect associated to the electric field. The basic design of the electrical set-up for cell fusion was similar to the one described previously (5). High voltage pulses with a short duration generated by a pulse generator (Cober 605, USA) were applied to two thin parallel stainless steel electrodes. The distance between the electrodes was 3.5 mm and their length was 30 mm. The electrodes were dipped in the buffer and seated on the bottom of the culture dish. The initial temperature was 21 ± 1°C. The temperature increase due to joule heating was computed to be less than 2°C. The shapes and durations of the pulses were monitored through a voltage divider on a memory scope .Pulses were repeated at an interval of about 3 sec to avoid the accumulation of heat.After pulsation, the "pulsing buffer" was substituted by culture medium and the Petri dishes were reincubated for about 2 hours. Then the cells were washed and fixed in ethanol,formaldehyde, acetic acid 6/3/1 v/v/v. They were directly observed in the Petri dish under an inverted phase contrast microscope. A slightly different procedure was used when the viability of pulsed cells was monitored. The electrodes were 18 mm apart and 18 mm long. This allows to pulse all the cells growing on a glass coverslip (as described above). The coverslip was then removed from the Petri dish and put in another dish. In this way, all the cells contained in the new dish had been pulsed. Their growth at 37°C was monitored for several weeks with replating in small Falcon flasks when needed. For each experiment, a control was made by substituting culture medium by pulsing buffer for about 2 minutes without applying a pulse. No effect was observed on the level of polynucleation of the cell culture.

-Rate of fusion : the rate of fusion is given as

$$R = \frac{\sum_{n=2}^{k} n C_n}{\sum_{n=1}^{k} n C_n}$$

(expressed in % where C_n is the number of cells containing n nuclei).

RESULTS AND DISCUSSION

As shown in fig. 1, electric pulses induce very efficient cell fusion. In our experimental conditions, the pulsed cells were close to the unpulsed ones so visual control under the microscope was very easy. The yield of fusion was very high and the process was much more efficient than the one we described previously for 3T3 cells (6).

Electrically-mediated CHO cell fusion depends on the amplitude, the duration and the number of applied pulses. As shown in fig. 2, a background level in polynucleation was present in the culture. The rate of spontaneous polynucleation was systematically counted in all experiments. It ranged from

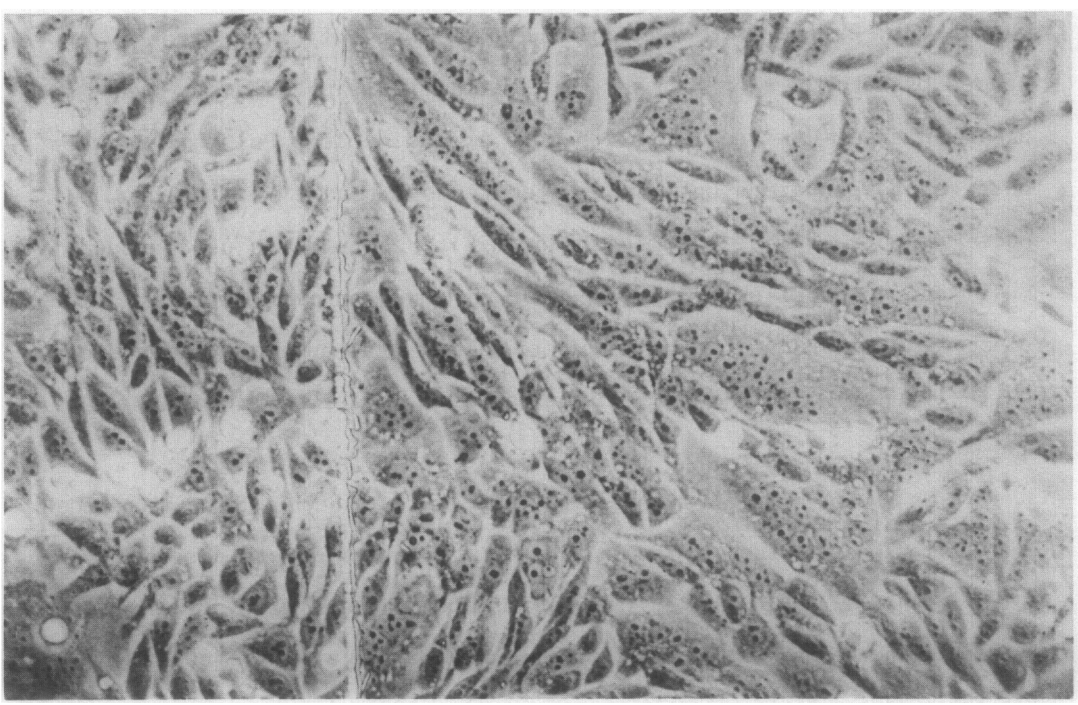

Fig. 1 : Micrographic pictures of CHO cells after electric treatment. Control cells (on the left) are separated from pulsed ones (on the right) by the print of an electrode. Fusion was induced by five pulses of 1700 V/cm lasting 100 μS.

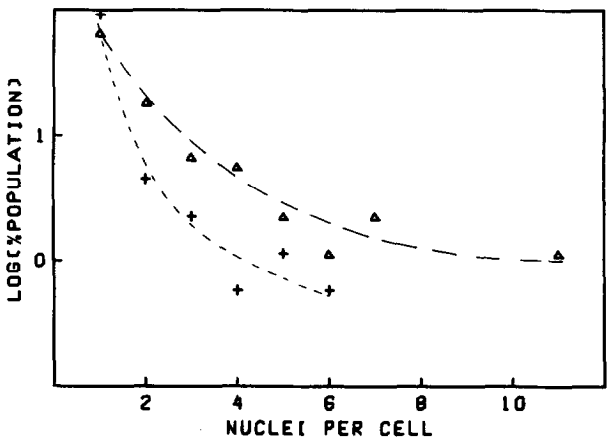

Fig. 2 : Population distribution change induced by "electrofusion". The extent of polynucleation is plotted for control (+) and pulsed (Δ) cells (5 pulses of 1430 V/cm lasting 200 μS). The percentage of polynucleation is always larger for the pulsed cells.

10 to 25 %. As reported in Methods, it was not affected by mere washing of the cells by the pulsing buffer. This polynucleation is a well known property of CHO cells (F. Amalric, personal communication).

Fig. 2 shows that electric induced polynucleation is strongly enhanced by both the magnitude and the duration of the pulses. Untreated samples contained almost no cells with more than 3 nuclei. Pulsed ones were observed to contain a noticeable amount (more than 1 %) of cells with 7 nuclei or more. This is clearly shown in Fig. 2 where the percentage of multinucleated cells is displayed for unpulsed and pulsed samples. After electrical treatment, it was possible to observe that some cells contained up to 10 nuclei. In one particular experiment, a cell containing 25 nuclei was observed.

As shown in fig. 3 a threshold value of the electric field magnitude must be reached in order to induce cell fusion. No fusion was observed for fields smaller than 900 V/cm lasting 100 μS. Then, the yield of fusion increases with the magnitude of the pulses. A plateau value in fusion yield was observed for field intensities greater than 1200 V/cm.

In a similar way, fig 4 shows that pulse duration must be longer than 20 μS to promote cell fusion (for a pulse intensity of 1400 V/cm).

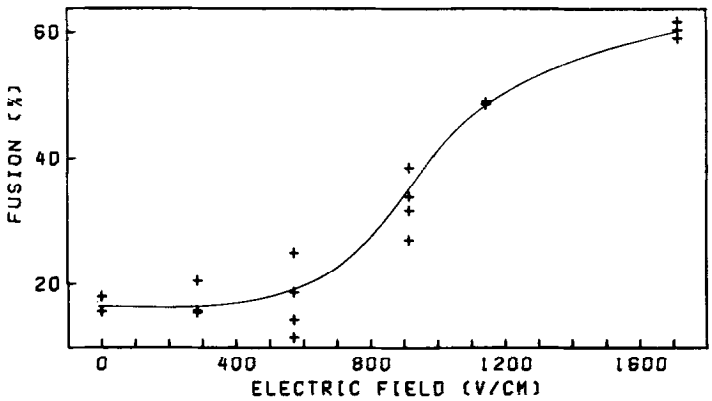

Fig. 3 : Percent polynucleation as a function of the electric field strength (five pulses of 100 μS).

Above this limit, the fusion yield increases up to a maximum observed for pulse durations longer than 100 μS.

The dependence of cell fusion on the number of pulses is shown in fig. 5. Pulses of 1570 V/cm lasting 100 μS were applied to the monolayer. It can be noted that both parameters (intensity and duration) were chosen to give maximum fusion. After only one pulse, the extent of fusion is already very high; about 60 % of the maximal effect is observed, the plateau value was reached after only five repeated pulses. No cell disruption was noted under the microscope. Even when drastic conditions were applied to the CHO cells, i.e. 5 pulses of 2700 V/cm lasting 300 μS. It should be emphasized

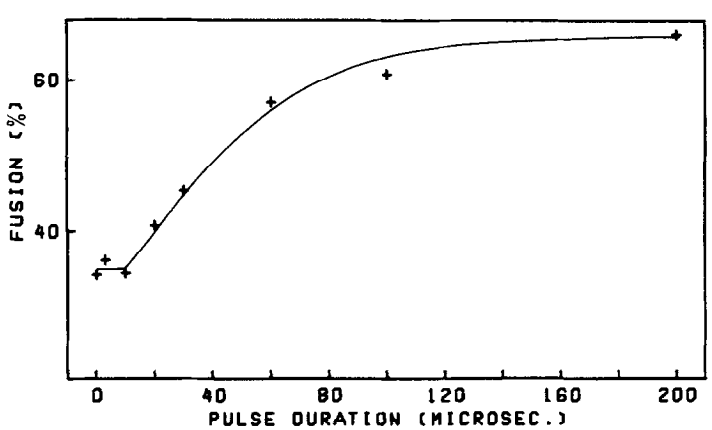

Fig. 4 : Percent polynucleation as a function of the duration of the pulse (5 pulses of 1400 V/cm).

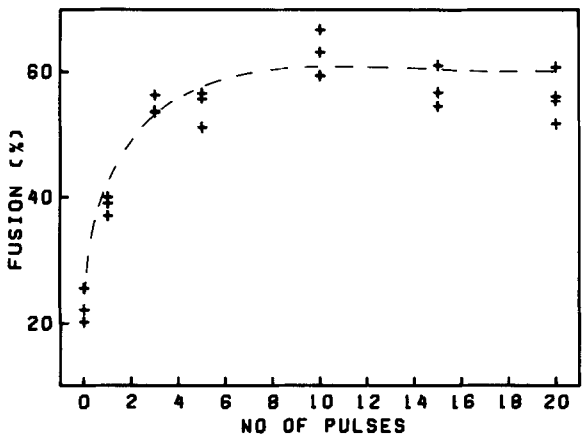

Fig. 5 : Percent polynucleation as a function of the number of applied pulses (pulse of 1570 V/cm lasting 100 μS).

that this was not the case in the previous work with 3T3 cells (6) with a similar experimental set-up but with cells at confluence. CHO cells were observed to stand up to 20 repetitive pulses (1600 V/cm, 100 μS) without damage.

The viability of the pulsed cells was tested a) by checking the integrity of the cell membrane and b) by observing the growth of the pulsed cells for a long period of time. When heterogeneous dyes (Trypan blue, eosin, acridine orange) were added to the medium ten minutes after the pulse, no incorporation was observed. Cultures grown on coverslips were replated in Falcon flasks and cell proliferation was monitored for about 3 weeks. The pulsed cells grew in a similar way to the control cultures; changing the pulsing conditions did not alter this.

CONCLUSION

In this report, electric pulse induced cell fusion is demonstrated to be a very effective procedure. Up to 80 % of the parent cells were fused under optimal conditions. The viability of the obtained homokaryons is totally unimpaired as shown by direct observation, exclusion of exogenous dyes and ability to grow.

We should emphasize that this method is fast and very simple to operate as the treatment is applied directly in the Petri dish. No additives

were present thus no chemical modification (incorporation of components as with Sendai virus, interaction with polymers using PEG, modification of the cell surface as with the dielectrophoresis) of the cells is involved.

Our work provides experimental evidence that this process may be easily controlled by the parameters of the external electric shock (amplitude, duration). The occurence of an intensity threshold suggests that this process may be a direct consequence of the perforation of the lipid layer. This phenomenon was shown to occur only when the applied field was greater than a threshold value (7). Present investigations in our laboratory are looking for a correlation between the two phenomena.

The amount of fused cells obtained is large and it is possible to routinely produce 200 000 fused cells per Petri dish. This number shows that when fusion is used to obtain hybrid cells, selection may be operated just after pulsing by using a FACS cells sorter with suitably labeled cells (9). This way, hybridization would be obtained very quickly with a high yield as observed between Chinese hamster CH cells and mouse C1D cells (Finaz, Lefevre, Teissié, submitted) and with considerable amounts of viable hybrids.

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