Electrofusion of myeloma cells on the single cell level

Fusion under sterile conditions without proteolytic enzyme treatment

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A technique is presented which allows electrofusion of single cells under sterile conditions. The electrofusion chamber is placed in a Petri dish. Before a droplet of the fusion medium is pipetted between the electrodes, the chamber is completely covered with vaseline, which prevents the fusion medium evaporating. Additionally, the fusion chamber is treated with solutions containing poly(L)-lysine and pronase which results in a decreased movement of the cells on the glass between the electrodes and which allows electrofusion without any proteolytic pretreatment.

Electrofusion *Single cell* *Sterility* *Pronase-carpet* *Myeloma cell* *Hybridoma cell*

1. INTRODUCTION

Electrofusion allows controlled fusion of a preselected number of cells under the microscope and leads to a high yield of viable fused cells [1–4]. The electrofusion technique is based on two steps: establishment of a close membrane contact between at least two cells by dielectrophoresis in the presence of a weak inhomogeneous alternating field and subsequent electrical breakdown in the membrane contact zone in response to a very short-field pulse of high intensity. Electrofusion is performed in weakly conductive solution in order to avoid heat development. Pretreatment with 1 mg/ml pronase (or 10 μg/ml dispase) very often facilitates electrofusion of animal cells and sea-urchin eggs [5,6]. The addition of these enzymes leads to an increased field stability of the cells so that pulses of higher intensity and longer duration can be applied without irreversible damage to the cells. This action of pronase (as well as dispase) on the field stability of cells can be explained in a number of different ways [3–6]. It is conceivable that the proteolytic activity of the enzymes causes a partial degradation of some of the membrane-integral proteins so that the mobility of the remaining proteins is increased. This increase in mobility could favour the emergence of lipid domains in the electric field which, on the one hand, facilitates electrofusion and, on the other hand, explains the field stability [3,4].

It is also conceivable that the charge density and distribution of these enzymes are responsible for the observed effects, so that, generally speaking, other proteins with the appropriate charge and hydrophilic structure, but without any enzyme activity, could lead to similar reactions. Such proteins could, for example, change the water structure or viscosity in the membrane contact zone of dielectrophoretically adhering cells in such a way as to favour fusion. On the other hand, by establishing an electrostatic contact between neighbouring cells in an otherwise non-conducting environment, they could prevent the membrane contact from becoming too loose as a result of the cell movements elicited by the electrical breakdown of the membrane in the contact zone. Movement arises from electrophoresis during field pulse ap-

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plication and from repulsion, due to the field-induced increase in membrane permeability in connection with the slightly higher hydrostatic pressure within the cells (see [4]). Movement of the cells would of course prevent the cell membranes of the two cells from intermingling.

We here report on a new experimental arrangement for electrofusion of cells which shows that the proteolytic activity of these enzymes is not the primary cause, but rather that the protein character (charge) of these enzymes is responsible for the observed effects, so that, generally speaking, pronase (or dispase) can be dispensed with in the future in the electrofusion of animal/mammalian cells.

2. MATERIALS AND METHODS

The electrode arrangement and the electronic equipment used were similar to that described in [2–6]. However, the two platinum wires were glued in parallel, 200 μm apart, onto a glass microslide, which was pretreated in order to modify the adhesion between the glass surface and the cells. For this purpose the microslide was submerged for 15 min in a solution containing 50 μg/ml poly(L)-lysine (Serva GmbH, Heidelberg). After washing with distilled water, the slide was rinsed with solutions containing 1 mg/ml pronase (Serva GmbH, Heidelberg) or FCS (Fetal calf serum, 10%). The microslide was then dried in air, placed in a Petri dish and completely covered with sterile vaseline (Vaseline Parlax, Laboratoires Lefranc, Romainville). With the aid of a micropipette, 20 μl of a solution containing 0.3 M mannitol, 0.5 mM $MgCl_2$ and $CaCl_2$ were injected into the electrode gap under the vaseline cover, so that a cavity filled with solution is formed. Those cavities are protected from evaporation by the film of vaseline (fig.1). Sterility was provided by closing the Petri dish with a covering plate. The use of vaseline was described for single cell experiments in [7,8]. After cooling the Petri dish filled with vaseline/mannitol to 4–8°C, two cells were introduced to the mannitol cavity using a micropipette in such a way that the cells were located close to one another on the glass between the two electrodes [9]. Immediately after the injection of the electric field-pulse the temperature was raised to 37°C to enhance the fusion process.

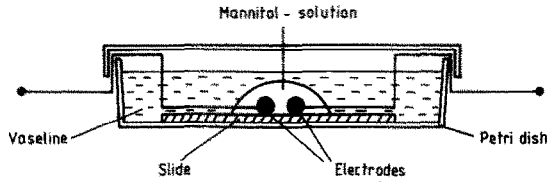

Fig.1. Schematic cross-section of the experimental arrangement used for electrofusion under sterile conditions. Two platinum electrodes (diameter: 100 μm) are glued in parallel on a slide, which is then treated with poly(L)-lysine/pronase (for details see text). The microslide is placed in a Petri dish which is filled with sterile vaseline. By means of a micropipette a droplet of a solution containing 0.3 M mannitol, 0.5 mM $CaCl_2$ and $MgCl_2$ is injected into the vaseline in order to form a small cavity over the electrodes. Thus the vaseline prevents evaporation of the mannitol solution. The cells to be fused are introduced to the mannitol droplet with a micropipette. The electrodes are then connected to the electronic equipment, and the Petri dish is closed with a covering plate. For reasons of clarity cells are not shown.

The cells used were murine myeloma cells (SP_2 and X 63) which were not pre-treated with pronase.

3. RESULTS AND DISCUSSION

For both SP_2 and X 63 myeloma cells, the application of an alternating sinusoidal field of 200 V cm^{-1} and of 800 kHz caused the cells to form a cell-pair oriented along the field lines with the establishment of close membrane contact (dielectrophoresis). Injection of an external field-pulse of 20 μs, 2.5 kV.cm^{-1}, which was sufficiently high to induce reversible electrical breakdown, led to fusion within 5 min. However, efficient fusion was only observed if the microslide of the fusion chamber had been pre-treated with poly(L)-lysine/pronase, but not with poly(L)-lysine/FCS. When using microslides pre-treated with poly(L)-lysine/pronase, it was often observed that the two myeloma cells moved about 1–2 μm apart after the application of the breakdown pulse, although the alternating current was switched off. The cells then approached each other again under the same orientation and fused, probably due to the local non-uniform fields, arising from the anisotropic membrane potential after breakdown [4] and from the charged pre-treated glass surface.

On the other hand, when poly(L)-lysine/FCS-

treated microslides were used, the mobility of the cells was very much greater after the application of the breakdown pulse. The distance between the cells thus became so great that they were no longer able to get close enough for their membranes to intermingle. The adhesion between the cells and the pre-treated surface of the microslides is apparently no longer sufficient under these conditions.

Since under the conditions described above pronase is only present between the glass surface and the cells, but not between the cells themselves, one can conclude that pronase, when present in the solution and hence in the membrane contact zone between adjacent cells, prevents the cells from moving too far apart (see discussion of the mechanism of electrofusion in [4]).

This occurs due to the repulsive forces resulting from the increase in the membrane permeability in response to the field pulse. It is thus not the enzymatic properties of pronase which are responsible for the action of pronase in electrofusion. This was also confirmed in further experiments adding PMSF (phenylmethyl–sulfonyl fluoride) to the solution. PMSF is an inhibitor which suppresses the proteolytic activities of proteases. Under these conditions, optimal fusion of cells was also observed.

Furthermore, we believe that the charge of pronase is responsible for the action of these molecules in the former fusion experiments. It can be argued, that in the procedure described the net charge of the pronase is altered due to its interaction with positively charged poly(L)-lysine.

The procedure described above thus not only explains the mechanism of pronase action, but also permits electrofusion of cells to be carried out without any danger of pronase molecules being taken up during the fusion process. In the conventional procedure [3–6], in which pronase was added to the solution or still adhered to the external membrane surface, following pre-treatment of the cells, some pronase molecules could be taken up by the newly-formed cells from the volume of solution present between the two membranes in the contact zone during the fusion process.

Because of their enzymic action, the presence of pronase molecules in the cell interior could lead to the death of the cells, thus reducing the yield of viable hybrids.

On the other hand, uptake of pronase molecules into the cell interior during the fusion process is avoided under the conditions described here.

The single cells fused can be removed with the aid of micropipettes and then cloned, as demonstrated in the meantime for a large number of cells of different species, in particular for the production of hybridoma cells (in preparation).

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