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Enhanced hybridoma production by electrofusion in strongly hypo-osmolar solutions

J.J. Schmitt and U. Zimmermann

Lehrstuhl für Biotechnologie, Universität Würzburg, Würzburg (F.R.G.)

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Electrofusion of mammalian cells in strongly hypo-osmolar media containing sorbitol, small amounts of divalent cations and albumin resulted in high yields of hybrids. The number of viable hybrids was higher than any value for chemically- or electrically-mediated fusion reported in the literature. Optimum clone numbers were obtained for fusion of osmotically-stable subclones of murine myeloma cells with DNP-Hy-stimulated lymphocytes provided that the osmolarity of the fusion medium was as low as 75 mosmol/l. Similar results were obtained for fusion of osmotically stable subclones of myeloma cells with the murine hybridoma cell line G8. Due to the dramatic increase in volume the field strength of the breakdown pulse (leading to fusion of the dielectrophoretically aligned cells) has to be reduced, as predicted by theory. The efficacy of hypo-osmolar electrofusion allowed the use of very few cells (about 10^5 lymphocytes or G8 cells per fusion chamber). This figure is considerably smaller than that reported in the literature for iso-osmolar electrofusion. It is significant that, in contrast to iso-osmolar conditions, the fusion yield in hypo-osmolar electrofusion was reproducible over long periods of time and less dependent of variations between cultures. At suspension densities of about 10^6 cells per fusion chamber (normally used in iso-osmolar electrofusion) hypo-osmolar electrofusion of homogeneous cell suspensions resulted in the formation of many giant cells when the appropriate field conditions were applied. Similar high or, at some field strengths, even higher numbers of clones at low cell suspension density were obtained when G8 and myeloma cells were first exposed during the washing procedure to strongly hypo-osmolar media, but then transferred to iso-osmolar solutions for electrofusion. Similar experiments with lymphocytes and myeloma cells failed because of destruction of many lymphocytes by the two osmotic shock steps in rapid succession. Volume distribution measurements of G8 and myeloma cells showed that after re-incubation of the osmotically pre-stressed cells the original volume distribution is largely, but not completely re-established. This and other results indicate that osmotic pressure gradients and associated tensions in the membrane do not play a primary role in the initiation of the electrofusion process. The experiments suggest that due to the osmotic (pre-) stress the membrane permeability is slightly and uniformly increased presumably due to the dissolution of membrane- and cell-skeleton proteins. Obviously, this facilitates electrofusion in hypo-osmolar or subsequently in iso-osmolar solutions.

Introduction

The potential of electrofusion in genetic engineering and membrane research has now been widely accepted [1–3]. The electric field technique has permitted the successful production of a variety of viable somatic cell hybrids of bacteria [4], yeast [5,6], plant [7–9] and mammalian cells [10,11] as well as of eggs [12,13]. In recent years electrofusion has also been applied to the generation of antibody producing hybridomas of both murine and human origin [14–20]. Schmitt et al. [19] showed that modification of the electrofusion protocol by pretreatment of the fusion partners with proteolytic enzymes or the use of antigen bridging (see Ref. 1) is not required for electric field-mediated production of hybridomas. Although the yields of hybridomas obtained by electrofusion were much higher than those obtained by the conventional fusion techniques, improvements in current electrofusion protocols are still required in order to meet the considerably higher fusion efficiencies demanded by fusion of very small numbers

Correspondence: U. Zimmermann, Lehrstuhl für Biotechnologie, Universität Würzburg, Röntgenring 11, D-8700 Würzburg, F.R.G.

of cells and other specialised applications.

Modifications of the present electrofusion protocol can be envisaged in the light of observations of Zimmermann et al. [21] and Lucy and Ahkong [22–65]. These authors have shown for both freely suspended and also dielectrophoretically aligned erythrocytes that swelling occurred due to the colloid-osmotic pressure when the cells were exposed to field strengths exceeding the breakdown voltage of the membrane. In addition, Lucy and Ahkong [23–25] showed that intermingling of dielectrophoretically aligned erythrocytes or myeloma cells occurred more readily when the cells were incubated in slightly hypo-osmolar media than in iso-osmolar solutions. These authors also postulated that osmotic pressure gradients primarily drive cell fusion when chemical steps were taken to free the plasma membrane from the restraining influence of its membrane skeleton.

If osmotic forces supplement the electrical compressive forces in electrofusion we should expect that electrofusion of myeloma cells with lymphocytes or other mammalian cells in hypo-osmolar solutions should enhance the yield of hybrids. However, experiments in this direction failed. This is particularly obvious from the very recent work of Stenger et al. [20]. These authors observed a 2-fold increase in the number of hybridomas when the fusion partners were transferred to media of 250 mosmol/l although the total number of 11 hybridoma cells per $5 \cdot 10^5$ lymphocytes recorded at this osmolarity was very small. A further decrease in the osmolarity to 180 mosmol/l did not cause a further increase in efficiency.

In this communication we demonstrate that the failure of such experiments resulted primarily from the use of inappropriate conditions for osmotic pressure shock, as well as from the use of unfavourable field pulse parameters and suspension densities. (Pre-) treatment of osmotically stable mammalian cells in strongly hypo-osmolar solutions indeed leads to a dramatic increase in hybrid yield at relatively low cell numbers if the associated changes in the field pulse parameters are taken into account. It is most interesting that our studies demonstrate that osmotic pressure gradients are not primarily involved in electrofusion as postulated by Lucy and Ahkong [22–25]. They are apparently only required for the dissolution of the membrane- and cell-skeleton proteins. This presumably increases membrane permeability and thus facilitates the intermingling process of attached cell membranes in response to the electrical breakdown pulse.

Furthermore, the modified electrofusion protocol described here does not only allow the gentle and reproducible production of high yields of hybridoma and other mammalian hybrids at relatively low cell numbers and field strength, but also the reproducible production of huge numbers of giant cells [1,26] when slightly higher cell suspension densities are used.

Materials and Methods

Fusion partners and growth medium. The murine hybridoma cell line G8 was prepared as follows [27,28]: immune spleenic B cells derived from a C57Bl/6 mouse were fused to the non-secreting myeloma cell line SP2/0-Ag14 [29] using PEG (poly(ethylene glycol)). The resulting hybridomas were selected in hypoxanthine-aminopterin-thymidine (HAT) supplemented medium as described elsewhere [19]. A thymidine-kinase deficient variant of one of these cells was grown for 1 week followed by subcloning in complete growth medium supplemented with 20 μg/ml of bromo-deoxyuridine (BdUR). HAT-sensitive clones were selected and passaged in BdUR-supplemented complete growth medium. Control experiments to verify continued HAT sensitivity were performed at regular intervals.

A subclone of the non-secreting myeloma cell line, SP2/0-Ag14 was selected as a fusion partner for the G8 cells or B-lymphocytes [28]. Subclones (SP2/0-UZ) were used which could be maintained in media of low osmolarity (75 mosmol/l) for 1 h without bursting. HAT sensitivity of the selected clones was verified in experiments performed at regular intervals.G8 and SP2/0-UZ cells were cultured in completed growth medium (CGM), i.e. RPMI 1640 (Seromed, F.R.G.) supplemented with 10% fetal calf serum (FCS) (Boehringer, Mannheim, F.R.G.), 2 mM L-glutamine, 2 mM sodium pyruvate, 2.0 g/l $NaHCO_3$, non-essential amino acids (Boehringer, Mannheim), 50 μM 2-mercaptoethanol and 100 units/ml 100 μg/ml penicillin-streptomycin (Seromed) at 37°C in an atmosphere supplemented with 5% CO_2.

One day prior to fusion, the cells of the logarithmic growth phase were split and grown to a density of $(3–5) \cdot 10^5$/ml in CGM supplemented with 10% or 20% FCS. then harvested and fused as described below.

Mice and immunizations. Mice (Balb/C-AnNCrlBR) were obtained from the Charles River Wiga GmbH FRG. Dinitrophenol (DNP) was conjugated to *Limulus polyphemus* hemocyanin (Hy) as described elsewhere [19] with a final coupling ratio of 10–14:1. The mice received two intraperitoneal (i.p.) injections of 100 μg of DNP-Hy, the first in complete Freund's adjuvant (CFA) and the second in normal phosphate-buffered saline (PBS) in a volume of 0.2 ml. At least 4 weeks after the second i.p. injection, the mice were boosted with 100 μg of DNP-Hy in saline intravenously (i.v.). Three days after the i.v. boost, the mice were killed and their spleens removed aseptically.

Preparation of B-lymphocytes. The spleens were crushed and single cell suspensions were made. The cells were washed once in CGM and resuspended in 10 ml of 0.8% ammonium chloride and washed in CGM to lyse the red blood cells (RBC). Spleenic T cells were then lysed as follows: the cells were resuspended in 100 μl of 1:200 anti-murine Thy 1.2 (ICT) in PBS at 37°C

for 30 min then washed in RPMI 1640. They were then suspended in 500 µl of 1 : 10 diluted guinea pig complement (Flow, McLean, VA) in CGM at 37°C for 45 min to lyse the T-lymphocytes and then washed three times in CGM and counted.

Fusion media. The control iso-osmolar fusion medium was as follows [30]: 0.28 M sorbitol, 0.1 mM calcium acetate, 0.5 mM magnesium acetate and 1 or 2 mg/ml bovine serum albumin (BSA) (Serva 11930). The pH was adjusted to 7.0.

The various hypo-osmolar fusion media were obtained by appropriate reduction of the sorbitol concentration. The osmotic pressure of the solution was cryoscopically determined by using the Osmomat 030 (Gonotec, F.R.G.).

Fusion procedure. SP2/0 cells were mixed with lymphocytes or G8 cells and washed twice in the appropriate fusion medium immediately prior to fusion. The cells were then resuspended in 200 µl (or in 340 µl in the case of G8 × SP2/0 fusion) of either hypo- or iso-osmolar fusion media (see below) and pipetted into a helical chamber with electrode spacing of 200 µm [1,14,30]. The chamber was closed and connected to the electrofusion apparatus (Biojet CF, Braun-Melsungen, F.R.G.) by means of two platinum-plated electrodes. An alternating alignment field (1.5 to 2 MHz at 300 V/cm) was immediately applied for 30 s followed by three square pulses at 1 s intervals, if not otherwise stated. The duration of a pulse was 15 µs. The field strength of the pulses was varied between 1.00 to 2.25 kV/cm; the alignment off time (AOT) of the alternating field during each pulse was adjusted to 10 ms. After the final pulse the alternating field was applied for a further 30 s. In order to complete the fusion process of the cells and the resealing of the membranes, the helical chambers were kept at the fusion temperature of 25°C without disturbance for 10 to 30 min [1,2,19]. After this the chambers were opened gently and rinsed with 1 to 2 ml of CGM. 0.5 to 0.6 ml of this diluted cell suspension was added to 1.0 ml of CGM in each of four wells of a 24 well cloning plate (Greiner). $2 \cdot 10^4$ peritoneal macrophages were added to each well as feeder cells. The hybrids were allowed to grow at 37°C in a 5% CO_2 atmosphere. Fusion products of SP2 × G8 were cultured without addition of feeder cells. After 24 h the growth medium was replaced by CGM supplemented with 6.8 mg/l hypoxanthine, 0.088 mg/l aminopterin and 1.938 mg/ml thymidine (HAT, Boehringer, Mannheim, F.R.G.).

5 to 7 days later, the number of viable hybridoma colonies was determined by visual inspection with an inverted microscope (Leitz, F.R.G.). Growing controls (i.e., SP2/0 without spleenocytes and lymphocytes without myeloma cells) were performed in order to ensure that no HAT-resistant revertants emerged, and to determine the time required for decease of the unfused SP2/0.

When individual hybridoma colonies were large enough to view them with the naked eye, they were transferred with a small bore pipette to individual wells of 96 well plates and allowed to grow to confluence. At this stage, supernatants were assayed for EIA.

Enzyme immunoassay (EIA). 96-well polyvinyl ELISA plates (Flow, McLean, VA) were coated with 100 µl DNP-hemocyanin or alternatively with 100 µl goat-anti-mouse IgG + M antibody (Dianova, U.S.A.) per well. The coating buffer was mixed with 0.05 M Na_2CO_3 and 0.05 M $NaHCO_3$, until a pH of 9.6 was reached, including 0.05% sodium azide. Incubation overnight at a temperature of 4°C was followed by washing and addition of 200 µl CGM to each well. After 1 h at 37°C the plates were washed three times with EIA-buffer (750 mM NaCl, 200 mM Tris-HCl (pH 7.4) to which Tween 20 had been added to a concentration of 0.2%). 100 µl of individual hybridoma supernatants were placed on the wells and allowed to incubate for 1 h at 37°C following by washing three times in EIA buffer. 100 µl of a goat-anti-mouse-IgG + M antibody conjugated with peroxidase (Dianova, U.S.A.) was added then to each well. After incubation for 1 h at 37°C the wells were washed three times in EIA buffer and once in citric acid buffer. The buffer was prepared by adding solid citric acid to 0.02 M Na_2HPO_4 until a pH of 5 was reached. Substrate for peroxidase (*o*-phenylenediamine, 0.4 mg/ml in citric acid buffer and 0.012% H_2O_2) was added then and the plates were allowed to incubate for 30 min at room temperature. Development of colour was stopped by adding 50 µl/well of 25% H_2SO_4. Antibody production of the hybridoma clones was detected by visual inspection for colour in the EIA tests.

Measurement of volume distribution. The volume distribution of the three cell types was measured before and after osmotic treatment by means of a hydrodynamically focussing particle analyser with an orifice of 60 µm or 100 µm in length and diameter (AEG Telefunken). The analysis of the volume distribution curves is described in detail in Ref. 5.

Results

Fusion in media of low osmolarity

Formation of giant cells. In iso-osmolar solutions electrofusion yielded maximum fusion and hybrid yield for myeloma/lymphocyte and myeloma/G8 cells if aligned cells were subjected to three consecutive pulses of 15 µs duration and of 2.00 and 2.25 kV/cm strength, respectively. The suspension density was adjusted to $2 \cdot 10^6$ cells per chamber in order to obtain a reasonable number of clones [19,28].

Similar conditions were used for hypo-osmolar elec-

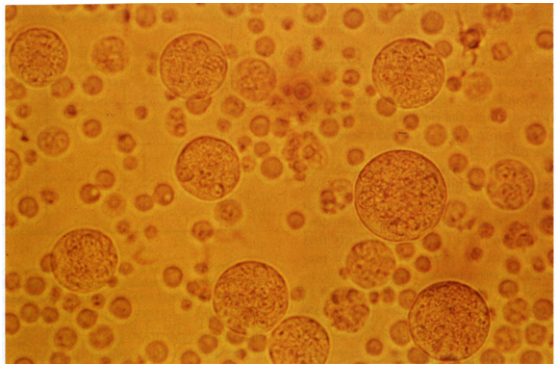

Fig. 1. Giant myeloma cells obtained by electrofusion in 100 mosmol/l sorbitol solutions containing 0.1 mM calcium acetate, 0.5 mM magnesium acetate and 1 mg/ml bovine serum albumin. $2 \cdot 10^6$ cells were aligned in an alternating inhomogeneous field of 300 V/cm strength and 2 MHz frequency and then fused with a single pulse of 1.75 kV/cm strength and 15 μs duration. After fusion was completed the cells were transferred to nutrition medium and photographs were taken (Magnification was about × 328).

trofusion of homogeneous cell suspensions of SP2/0, G8 or lymphocyte cells. Decrease of the osmolarity in the washing and fusion solutions to 150 mosmol/l resulted in a dramatic increase in the fusion frequency of myeloma cells. After injection of three consecutive pulses fusion was observed not only between cells in a given chain, but also between cells of adjacent chains. The final result was the formation of many giant cells which could be removed from the helical chamber and transferred into nutrition medium. For comparison, in iso-osmolar solutions formation of giant cells could only be obtained after application of more than three field pulses and/or after pre-treatment of the cells with proteolytic enzymes [26]. In addition, much higher suspension densities were required [1,26].

Exposure of myeloma cells to 100 mosmol/l or 75 mosmol/l media enhanced the fusion frequency further to nearly 100% between the electrodes, if a single pulse was applied and the field strength was adjusted to 1.75 kV/cm. Correspondingly, the yield of giant cells exceeded that estimated from fusion in 150 mosmol/l solutions (Fig. 1).

Control experiments with myeloma cells showed that spontaneous fusion in these hypo-osmolar solutions was a very rare event. A significant number of spontaneous fusions was only observed after incubation of the cells in distilled water, provided that the suspension density was high enough to give random collision. However, under these conditions bursting of most of the cells occurred after about 10 to 30 min. In contrast, in solutions of more than 75 mosmol/l the cells could be kept for at least 30 min without significant deterioration. On average, 10% of the cells were destroyed as indicated by measurements of staining the dead cells by Trypan blue. The survival of most of the myeloma cells in these strongly hypo-osmolar solutions for 1 h was also obvious from growth experiments of osmotically pre-stressed cells which were not subjected to the electric field treatment. No significant changes in the growth rate of myeloma cells could be detected after 1 h incubation in 75 mosmol/l solution and subsequent re-suspension in nutrition medium. In most electrofusion experiments the cells were not exposed for more than 60 min to the low osmolarity, so we could safely conclude that this treatment was not harmful to the cells (see also below).

The absence of spontaneous fusion suggests that the preferential formation of giant cells observed after injection of the breakdown pulse(s) resulted from facilitated electrofusion in hypo-osmolar solutions. One reason may be the increase of volume when the cells were transferred into the hypo-osmolar solutions. The modal volume of the myeloma cells increased from about 828 μm^3 in iso-osmolar solutions to about 1720 μm^3 or 2380 μm^3 after incubation in 150 mosmol/l or 75 mosmol/l solutions, respectively. This corresponds to an increase in the radius from 5.9 μm to 8.3 μm. The cells behaved nearly as ideal osmometers. Due to the radius- and

angular-dependence of the generated membrane potential (see the Laplace equation in the footnote * and in Ref. 31) field pulses of the same strength will induce larger perturbations in the membrane of the differently sized cells in the population under hypo-osmotic than under iso-osmotic conditions. This is also the reason why in 75 mosmol/l solutions the field strength must be decreased to 1.75 kV/cm in order to avoid irreversible deterioration of the cells [1,2].

The radius- and angular-dependence of the generated membrane potential also explains the following. In contrast to iso-osmolar work, fusion still occurred at a high rate in hypo-osmolar media when the suspension density was lowered. However, at a suspension density that gave $2 \cdot 10^5$ cells per chamber fusion events were predominantly observed between two or three cells. Microscopic observation of the fusion process showed that at this low cell density adjacent cell chains were a rare event. In addition, the number of cells in a given chain was reduced.

Similar results were obtained for homogeneous fusion of G8 or of lymphocyte cells. The modal volume of the G8 and stimulated lymphocyte cells also increased dramatically when the cells were transferred from iso-osmolar solutions into the various hypo-osmolar solutions (from about 885 μm^3 to about 2890 μm^3 for G8 cells and from about 320 μm^3 to about 1120 μm^3 for lymphocyte cells after transfer to 75 mosmol/l media). These osmotically induced changes in volumes are expected for cells which behave like a ideal osmometer.

Hybrid production. From the foregoing considerations it is evident that high hybrid yields can only be obtained in hypo-osmolar solutions at suspension densities below 10^6 cells per chamber. Otherwise the possible formation of giant cells interferes with the two-cell fusion events required for hybridisation. In screening experiments using SP2/0 × G8 fusion a cell ratio of 1 : 1 and a total suspension density of $2 \cdot 10^5$ cells per chamber yielded best results. On the other hand, with the differently-sized lymphocyte and myeloma cells, maximum fusion and hybrid yield was always obtained when $2 \cdot 10^5$ lymphocytes were mixed with $4 \cdot 10^5$ myeloma cells. This cell ratio in hypo-osmolar solution is opposite to that reported for other electrofusion protocols (see, for example, Refs. 19 and 20).

The histogram in Fig. 2 shows the yield of clones obtained after electrofusion of myeloma with G8 cells in 75 mosmol/l media as a function of the field strength of the fusion pulses. The clones were counted after selection in HAT media for 5 days. The yield was maximum at a field strength of 1.75 kV/cm. However,

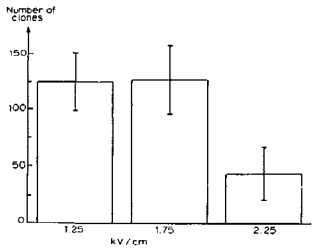

Fig. 2. Hybrid yield as a function of the strength of the fusion pulse obtained after electrofusion of G8 hybridoma with SP2/0 myeloma cells in 75 mosmol/l sorbitol solution. The field conditions were as described in Fig. 1 except that three field pulses were applied at 1-s intervals. The ratio of the cell densities was 1:1 and the total suspension density $2 \cdot 10^5$ cells per fusion (helical) chamber. The columns and error bars represent the means of 6–9 measurements performed in different fusion chambers and/or on different days.

comparable yields were also obtained at 1.25 kV/cm whereas at 2.25 kV/cm the number of clones decreased considerably. The yield of 125 clones/10^5 G8-cells was significantly higher than under optimum conditions in iso-osmolar electrofusion [28]. In addition, in hypo-osmolar electrofusion the hybrid yield was also reproducible within about 20% when experiments were performed with different cultures over a long period of time. In comparison, the yield of hybrids varied considerably from culture to culture when electrofusion was carried out in iso-osmolar solutions (between about 200 and 1000 clones per 10^6 cells which corresponded to 20 to 100 cells per 10^5 cells, Ref. 28 and unpublished results). The occurrence of a high number of clones represented further support for the conclusion that the viability of the cells and the hybrids was apparently not affected by hypo-osmolar treatment.

Fig. 3 shows the yield of hybridomas obtained by electrofusion of DNP-Hy-stimulated lymphocytes and myeloma cells washed and fused in solutions of various osmolarities. An osmolarity of 75 mosmol/l seemed to be appropriate for optimum production of hybridomas at a field strength of 1.5 kV/cm. The yield was a factor of eight higher than that reported by Stenger et al. [20], if the yield is referred to the same number of lymphocytes. Higher or lower field strengths resulted in less clones (Fig. 4), even though in all cases the number of hybrids exceeded the maximum obtained by iso-osmolar electrofusion (Fig. 3). Most of the hybridoma cells obtained with hypo-osmolar electrofusion secreted antibodies.

The finding that optimum hybrid yields in hypo-osmolar fusion of G8 × SP2/0 and lymphocytes ×

* The generated membrane potential is given by $V_m = 1.5 \cdot a \cdot E \cdot \cos \alpha$ [1], where a is the radius of a spherical cell, E the applied external electrical field and α the angle between the normal vector of a given membrane site and the direction of the external field vector.

TABLE I

Comparison of the field strength calculated to give breakdown of the cell membrane and that field strength required experimentally for optimum hybrid yield

The additional field strength (E_a) is the difference between the experimentally found optimum fusion field strength (E_f) and the theoretical breakdown field strength (E_{th}) of the smaller fusion partner in a fusion experiment. For the calculation of E_{th} according to the Laplace equation ($\cos \alpha = 1$) a membrane breakdown voltage of 1 V was assumed [1].

Cell line	Medium: 300 mosmol/l				Medium: 75 mosmol/l			
	Mean cell radius (μm)	Field strength			Mean cell radius (μm)	Field strength		
		E_{th} (kV/cm)	E_f (kV/cm)	E_a (kV/cm)		E_{th} (kV/cm)	E_f (kV/cm)	E_a (kV/cm)
G8	5.9	1.13	2.25	1.10	8.8	0.76	1.75	0.950
SP2/0	5.8	1.15	2.00	0.41	8.3	0.80	1.50	0.460
Lymphocytes	4.2	1.59			6.4	1.04		

SP2/0 were obtained at lower field strengths than under iso-osmolar conditions (Figs. 2–4) can be explained by the radius-dependence of the generated membrane potential difference. Using the appropriate values for the cell radii (as derived from the volume distribution measurements, see above) the critical field strength theoretically required for breakdown at membrane sites oriented in field direction is easily calculated by the Laplace equation (Table I, see also the footnote on p. 46). The critical field strength required for breakdown of the smaller fusion partner determines the field strength which must be applied for fusion with a larger partner. Experience has shown that the field strength for fusion must be somewhat higher than this critical field strength. This is due to the angular dependence because appreciable membrane areas of the spherical cells must be permeabilised [1,2]. According to Table I the experimental field strength for fusion of G8 × SP2/0 or lymphocytes × SP2/0 is about 1 kV/cm or 0.4 kV/cm, respectively, higher than the theoretical critical field strength of the smaller fusion partner. It is important to note that these additional field strengths are independent of the osmolarity. Thus, facilitation of fusion and hybridisation in hypo-osmolar solutions cannot be explained (as assumed above) by a more favourable ratio of field-pulse to cell radius compared to iso-osmolar fusion. A possible explanation may be an increase in membrane permeability associated with the emergence of lipid domains. This assumption was supported by staining experiments using DAPI (4',6-diamidino-2-phenylindole · 2 HCl, 0.1 mg/ml) as a mem-

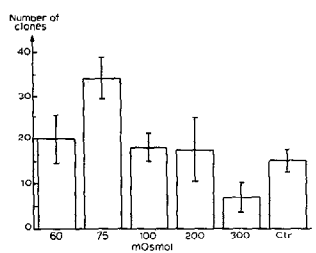

Fig. 3. Yield of hybridomas obtained by electrofusion of $4 \cdot 10^5$ myeloma cells with $2 \cdot 10^5$ DNP-Hy-stimulated lymphocytes per fusion chamber as a function of the osmolarity of the fusion medium. The strength of the fusion pulses was 1.5 kV/cm and the frequency of the alternating field was 1.5 MHz; otherwise the field conditions were the same as in Figs. 1 and 2. 10 minutes after the pulses the cells were transferred into nutrition medium and cultured as described in Materials and Methods. The fusion conditions for optimum hybridoma yields in iso-osmotic fusion medium [19] were used in parallel control experiments (Ctr: 3 pulses of 2.00 kV/cm in iso-osmolar medium). The columns and error bars represent the means of four measurements performed in different fusion chambers and/or on different days.

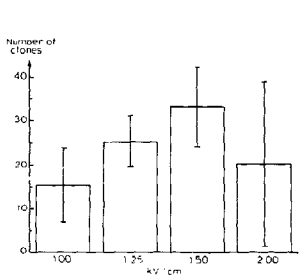

Fig. 4. Hybridoma yield as a function of the strength of the field pulses. Fusion was performed in 75 mosmol/l sugar solutions; field and other conditions were the same as in Figs. 1 and 3. The columns and error bars represent the means of four measurements performed in different fusion chambers and/or on different days.

brane permeability probe. This dye stains the DNA, but is not readily taken up through membranes of untreated cells. Most of the cells were not stained in iso-osmolar solutions within 10 min, whilst most of the osmotically-stressed cells were stained.

Fusion of osmotically pre-treated cells in iso-osmolar solutions

In the following set of experiments, different cell types were washed twice in 75 mosmol/l sugar solutions for about 30 min. However, about 10 min before field application the cells were returned to the iso-osmolar fusion medium. Measurements, using the particle analyser, showed that the original volume distribution of the cells were nearly re-established, although a few somewhat larger cells still remained even after a re-incubation time of about 30 to 60 min (see right-hand side of the size distributions in Fig. 5). Despite of this decrease in volume, high yields of hybrids were obtained from the fusion partners G8 and SP2/0 when the suspension density was adjusted to 10^5 cells of each type per chamber. It is most interesting that the hybrid yield was always higher for pre-stressed cells than for the control cells over the field strength range of 1.25 to 2.25 kV/cm (Fig. 6). Maximum yield was obtained at a field strength of 2.25 kV/cm. This shift of the optimum field strength towards the optimum value for iso-osmolar fusion of un-treated cells is expected because of the comparable volume distributions of the cells after re-incubation.

As in hypo-osmotic electrofusion the hybrid yield was very reproducible as above and more independent of cell culture conditions.

On the other hand, hybridoma production was always less with pre-stressed cells than with control cells

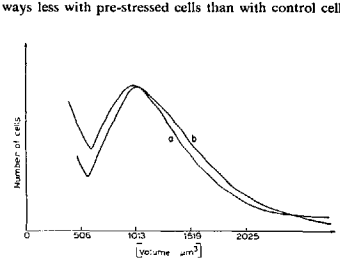

Fig. 5. Size distributions of SP2 cells incubated in iso-osmolar fusion solutions (curve a) and in iso-osmolar solutions 10 min after pre-treatment in 75 mosmol/l solutions (curve b). Note, that the volume distribution of the osmotically pre-treated and un-treated cells were very similar. However, comparison of the right-hand sides of the two size distributions indicate the presence of some slightly larger cells in the osmotically pre-treated cell suspension. G8 cells yielded the same result.

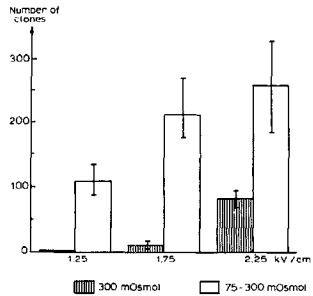

Fig. 6. Hybrid yield obtained from electrofusion of G8 hybridoma with SP2 myeloma cells in iso-osmolar medium after osmotic pre-stress in 75 mosmol/l sorbitol solution (white columns). The dark columns represent the control fusion experiments in iso-osmolar fusion medium without hypo-osmolar pre-treatment of the cells. Field and other conditions has been as in Fig. 2. The columns and error bars represent the means of 4–6 measurements performed in different fusion chambers and/or on different days.

(data not shown). Careful inspections of the viability of the lymphocytes showed that the lymphocytes were partly destroyed by the two osmotic shocks following each other in a short time interval. This clearly resulted in an unfavourable ratio of lymphocytes to myeloma cells (see above).

It is further interesting to note that the formation of giant cells by multiple fusion of pre-stressed cells using a suspension density of 10^6 cells per chamber was not possible using the gentle field pulse parameters optimum in hypo-osmolar media. Formation of giant cells by fusion of osmotically pre-stressed cells required the same conditions as un-treated cells in iso-osmolar solutions [1,2]. Apparently the slight increase in membrane permeability by osmotic pre-stress was sufficient to facilitate two-cell fusion, but not multiple fusion between cells in adjacent chains. It is well-known [26] that multiple cell fusion using electric field pulses requires perturbations over the entire membrane surface in order to proceed. Under the present conditions this can obviously only be achieved (as in un-treated cells) by injection of pulse trains of high field strengths.

Discussion

Lucy and Ahkong [22–25] have suggested a general model for chemically- and electrically-induced fusion based on the assumption that osmotic pressure gradients are primarily responsible for fusion of biomembranes. These authors proposed that damage to mem-

brane skeleton has to preceed the osmotically-driven fusion process in many cells. The restraining influence of membrane skeleton proteins is assumed to be diminished by interactions of fusogenic chemicals or by the breakdown pulse. These interact with membrane components resulting in both an increase of membrane permeability and in the emergence of protein-free lipid areas at the fusion sites. The increase in membrane permeability is responsible for swelling of the attached cells due to the colloid osmotic pressure within the cells. Lucy and Ahkong [22–25] further postulated that under the influence of the osmotic pressure gradients established by water uptake the two phospholipid bilayers are forced together at the fusion site to give formation of an intermediate, single bilayer that is common to both of the fusing membranes. This bilayer is assumed to be ruptured by the stretching force within the membrane plane generated by further osmotic swelling.

Although there is a body of evidence for the occurrence of secondary osmotic processes induced by electropermeabilisation [21,32] it is difficult to envisage how osmotic pressure gradients can primarily initiate cell fusion. In contrast to the osmotic gradient-driven fusion of phospholipid vesicles with planar phospholipid bilayers [33] the two cell compartments swell simultaneously in hypo-osmolar solutions. Thus the osmotic pressure gradient between the attached cells should always be zero or at least negligible. In addition, the osmotic pressure gradient between the cell compartments and the external solution must be very small because, at least for the cells used here, a new equilibrium state was reached very quickly under hypo-osmolar conditions. Lysis of the cells occurred readily only in distilled water. Because of these difficulties Lucy and Ahkong [22–25] assumed that the increase in the absolute pressure within the cells generated by water uptake forces the membrane together. However, this seems to be unlikely in our opinion because of the very small magnitude of these pressures in the swollen cells. From the data presented in this communication we have to conclude that neither pressure nor osmotic gradients, nor the induced increase of cell volume including the associated tensions in the membrane plane are per se responsible for fusion of attached membranes. Otherwise, it can not be explained why the fusion and hybrid yields remained high or exhibited even higher values when the cells were returned from hypo-osmolar to iso-osmolar conditions before electrofusion. The measurements of the volume distributions showed that the osmotic-induced volume changes were reversible to a large extent, although the distribution became slightly slewed to the right-hand side. This result is expected if the permeability of the cell membranes has increased in response to osmotic stress [34–36]. Thus, we have to assume that the membrane structure has been changed by the tensions created in the membrane plane of swollen cells, but only to a small extent. The slight increase in permeability seems to be sufficient to facilitate subsequent two-cell electrofusion without significant effects on membrane transport properties and cell viability. Such small changes in the membrane structure can be envisaged if the membrane and/or cell skeleton proteins are (partly) dissolved by the osmotic stress resulting in a higher mobility of membrane components and probably in the emergence of protein-free lipid domains [26]. It is likely that only a few lipid molecules must be rearranged [37] in order to achieve the observed permeability changes.

The removal of restraining membrane proteins can, in principle, be achieved both by the tension created in the plane of the membrane by osmotic pressure gradients during the swelling process in hypo-osmolar solutions, or by the compressive and tangential forces created by the electric field pulses in the membrane. However, the perturbations created in the membrane by these two forces must be different. The tangential components of the electric field vector can only interact with charged molecules [38,39] whereby the magnitude of the tangential component depends on the orientation of a given membrane site in relation to the field (see the Laplace equation in the footnote on p. 46 and Ref. 39). The compressive force of the electric field arising from the normal component of the field shows a similar, but reversed, angular dependence. In contrast, the osmotic forces and the associated tensions in the membrane plane interact with charged and un-charged membrane components uniformly over the whole membrane surface. Thus, different effects on membrane structure are expected in response to these two physical forces. The interaction of the breakdown pulse(s) with the cells was apparently identical for iso- and for hypo-osmolar conditions. This is evident from the comparison of the experimental field strengths required for fusion of both cell systems with the theoretical values (Table I), if the radius-dependence of the field strength is taken into account. Thus, the additional changes induced osmotically in membrane permeability must be responsible for the facilitation of electrofusion. Stenger et al. [20] and Lucy and Ahkong [22–25] apparently overlooked the important point that the field strength for fusion must be adjusted for the change in cell radii when the osmolarity of the medium is altered. The finding of Stenger et al. [20] that the hybridoma yield could not be further enhanced when the osmolarity was lowered to 180 mosmol/l is due to the inappropriately high field strength of the fusion pulse.

It can also be argued that secondary osmotic processes are involved in iso-osmolar and hypo-/iso-osmolar electrofusion as mentioned above [21] and that it is this process that is indirectly responsible for the re-arrangement of membrane components and the intermingling processes of the membranes rather than the

electric field. This possibility cannot be excluded at the present stage of information.

The experiments reported here force the conclusion, that disruption of restraining proteins by osmotic means is more advantageous than by field pulse(s) alone. Fusion yields were very high and reproducible and more independent of changes of the fetal calf serum used in the growth medium as well as of the general culture conditions and number of cell passages. This is in contrast to the experience gathered using electrofusion in iso-osmolar solutions. Under the latter conditions it often occurred that optimum field conditions changed once the cells have been passaged several times or when the ingredients of the culture medium were changed. The reason for this variability in fusion yield is evident from the explanation given above. Slight changes for example in the cell and membrane skeleton proteins during growth and passage of cells (e.g., induced by overgrowth of a subpopulation) can alter the interaction of the field vector with the membrane components in and close to the flattened membrane zone of two attached cells [26].

It is obvious that success in the re-arrangement of membrane components induced only by electric field pulses requires a profound knowledge of the physical background of electrofusion and much experience in order to find optimum conditions for iso-osmolar electrofusion once the properties of the cells have changed due to altered growth conditions.

The present protocol for the formation of hybrids and hybridomas as well as of giant cells avoids these complications and allows the production of high yields of fused products in a very simple and reproducible manner.

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