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Membrane fusion without cytoplasmic fusion (hemi-fusion) in erythrocytes that are subjected to electrical breakdown

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There are many reports of hemi-fusion in phospholipid vesicles but few published studies on hemi-fusion in cells. We report evidence from both fluorescence microscopy and freeze-fracture electron microscopy for hemi-fusion in the electrofusion of human erythrocytes. We have also characterised the conditions that favour hemi-fusion as opposed to complete fusion, and discuss the possibility that hemi-fusion might precede complete electrically-induced cell fusion. A membrane probe ($DiIC_{16}$) and a cytoplasmic probe (6-carboxyfluorescein) were used to investigate the behaviour of doubly-labelled human erythrocytes which were aligned in chains by dielectrophoresis and then exposed to high voltage breakdown pulses. Some of the cells were fused by the pulses, as shown by diffusion of both membrane and cytoplasmic probes from labelled to unlabelled cells. With other cells, the membrane probe diffused into unlabelled cells after the breakdown pulses, without the cytoplasmic probe diffusing into unlabelled cells or leaking into the medium. Membrane fusion (hemi-fusion) thus occurred without cytoplasmic fusion in these erythrocytes. Such cells were irreversibly, but fragilely, attached to their neighbours by the breakdown pulses. There was an inverse relationship between conditions that permit complete fusion and those that favour hemi-fusion, with respect to breakdown pulse length, breakdown voltage and, in particular, osmolarity and temperature. The incidence of hemi-fusion in 250 mM erythritol was twice that in 150 mM erythritol, and hemi-fusion was 5-fold greater at 25°C than at 20°C. Hemi-fused erythrocytes occasionally fused completely on heating to 50°C, demonstrating that hemi-fusion can proceed to complete cell fusion. Freeze fracture electron micrographs of preparations of hemi-fused cells revealed long-lived, complementary depressions and protrusions on the E- and P-fracture faces, respectively, of tightly apposed cells that may mediate hemi-fusion. The possibility that the fusion of closely adjacent human erythrocytes by electrical breakdown pulses may involve an intermediate, shared bilayer structure, which is stable in certain conditions but which can be ruptured by osmotic swelling of the permeabilised cells, is discussed.

Introduction

Membrane fusion is a fundamental phenomenon that occurs in many biological processes. However, the molecular mechanisms that govern membrane fusion reactions are still unclear, and consequently they are currently the subject of numerous investigations [1]. An aspect of the fusion of biomembranes that is of particular interest is the possible participation in the process of intermediate structures, such as a shared bilayer (reviewed in Ref. 2). Ultrastructural investigations have indicated that an intermediate single bilayer occurs in, for example, exocytosis of vascular endothelial vesicles [3,4], zoospore secretion in *Phytophthora palmivora* [5], myoblast fusion [6], and the fusion of carrot protoplasts by poly(ethylene glycol) [7]. Related work with fluorescent probes on the fusion of phospholipid vesicles has shown that, under certain conditions, an intermixing of lipid probes can occur without mixing of the vesicular contents [8]. This phenomenon has been observed by a number of investigators (reviewed in Refs. 9 and 10), and it has been termed 'hemi-fusion' [11]. Hemi-fusion may also involve fusion of the outer monolayers of the

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vesicles into an intermediate, shared bilayer, or possibly the formation of a transient H_{II} hexagonal phase as suggested in Ref. 8.

In previous work on the fusion of human erythrocytes induced by poly(ethylene glycol) 6000, it was found that carbocyanine and rhodamine membrane probes diffused from labelled to unlabelled cells when they were dehydrated by 40% poly(ethylene glycol), but there was no corresponding movement of the cytoplasmic probe, 6-carboxyfluorescein, until the polymer solution was replaced by an isotonic buffer [12]. The rapid diffusion of carboxyfluorescein that then occurred was thought to be consistent with the rupture by osmotic stretching of an intermediate, shared bilayer.

In the present paper, experiments are reported on the exposure to electrical breakdown pulses of human erythrocytes which were also labelled with a membrane probe and a cytoplasmic probe. The membrane probe, but not the cytoplasmic probe, diffused from labelled to unlabelled cells (i.e. hemi-fusion occurred) when the cells were attached to each other, but were not completely fused by the breakdown pulses. As hemi-fusion in cells exposed to electrical pulses has not previously been characterised, we have investigated the conditions that favour hemi-fusion as opposed to complete fusion. The observations made indicate that the complete electrofusion of erythrocytes, which are in close contact before exposure to breakdown pulses, is mediated by an intermediate structure that is stable in certain circumstances. This intermediate may be a shared bilayer that is comparable to the transient structures which have been observed ultrastructurally in some naturally-occurring fusion reactions of biological membranes, and to the more stable bilayer intermediates which are formed when planar phospholipid bilayers fuse in model systems [13–17].

A preliminary report on this work was published in a review article [18].

Materials and Methods

6-Carboxyfluorescein diacetate, and the carbocyanine, DiI-C_{16}(3) (1,1-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) were from Molecular Probes, Eugene, OR, U.S.A. Pronase was from Serva, erythritol was from Sigma Chemical Co., and histidine was from BDH.

Human erythrocytes were washed and freed from leucocytes as described in [19], and resuspended in a buffered salt solution (124 mM NaCl/40 mM Hepes buffer at pH 7.4). The cells were doubly-labelled with carboxyfluorescein and DiI-C_{16}(3), and then mixed with unlabelled erythrocytes in the ratio of 1:9 (v/v) as described in Ref. 12. For electrical breakdown, 5 μl of a packed suspension of the cells was then added to 950 μl of a solution of erythritol (150 or 250 mM), containing 1 mg ml^{-1} pronase [20] and 10 mM histidine, which had a conductivity of 286 μS cm^{-1}. The presence of pronase stabilises cells against the alternating field and d.c. field pulses: this appears to result from proteolytic action on the cells and the presence of contaminating Ca^{2+} ions in commercial preparations of pronase [21]. 40 μl of the erythritol suspension of erythrocytes was placed in a chamber in which the electrodes were 260–280 μm apart, unless otherwise stated. After 1.5 min the cells were aligned by dielectrophoresis in an a.c. field of 0.38 kV cm^{-1} at 1.5 MHz from a function generator (type TE7702, Toellner Electronic, Frankfurt). After a further 0.5 min, three square wave, electric field pulses (2.3–3.1 kV cm^{-1}, 10–30 μs, as described in the text) were applied to the cells at 1-s intervals from a pulse generator (type 214B, Hewlett-Packard). The a.c. field was removed 1 min later. For cells exposed to the breakdown pulses at 25°C, cell counts were commenced after a further 2 min by which time diffusion of the DiI lipid probe had reached equilibrium. For cells treated at 20°C, cell counts were commenced 5 min after exposure to the breakdown pulses.

Diffusion coefficients were computed similarly to the method described in Ref. 22. Video images of the microscopic field, which were obtained with a Sofretec SIT camera and recorded with a Sony U-matic recorder, were digitized by a Pericolor 1500 computer. Frame-by-frame analyses, starting from the contact point between pairs of labelled and unlabelled erythrocytes, were undertaken of changes with time (after the electric pulse) of the fluorescence intensity at the periphery of the originally, unlabelled cells to estimate the diffusion coefficient, D, of the membrane probe, DiI, using the formula $D = x^2/4t$.

Fluorescence microscopy for estimating the incidence of hemi-fusion was undertaken with a Zeiss Standard microscope, fitted for epi-fluorescence, with a Neofluar 40/0.75 Ph2 objective and an HBO 50 W mercury lamp. Two filter cassettes (BP 450–490/BP 520–560, and BP 546/12/LP/5900 were used to view the carboxyfluorescein and DiI-C_{16}(3) probes, respectively. The electrode chamber was mounted on a heating stage, connected to a circulating water bath, the temperature of the solutions used was controlled by a heating block, and room temperature was controlled by air-conditioning.

Visual fields were selected randomly for cell counting. Hemi-fused cells were inspected under both phase contrast and fluorescence, and lysed cells were excluded from the counts. Single cells (i.e. cells not in chains) were also excluded. Between 100 and 200 cells (usually > 150) were counted under each condition. The incidence of hemi-fusion, $\pm$ S.D. for 4–13 separate experiments (see Table I), was calculated by the formula $[(A - B)/A] \times 100$, where A was the number of

cells containing the DiI-C_{16}(3) probe, and B was the number of cells containing the carboxyfluorescein probe.

For freeze-fracture electron microscopy, a stainless steel pipetting annular electrode chamber (Kruss GmbH, Hamburg) with an electrode gap of 250 μm was used with a Zimmermann Cell Fusion System (GCA Corp., Chicago) at room temperature. After 1 min the cells were exposed to an a.c. field of 0.32 kV cm^{-1} at 1.5 MHz. After a further 0.5 min, a single square wave pulse (10 kV cm^{-1}, 60 μs) was applied. The a.c. field was removed 0.5 min later, and the cells were fixed after a further 5 min by incubation for 2 h at room temperature with an equal volume of 4% (v/v) glutaraldehyde in the erythritol pulsing medium. The fixed cells were gently centrifuged (600 $\times g$ for 5 min), and resuspended in a solution of glycerol (30%) in water. Samples were subsequently frozen in Freon 22 and fractured in a Balzers BAF 301 freeze etch unit. The replicas were examined with a Philips 201 electron microscope.

Results

Cell attachment induced by electrical breakdown

Human erythrocytes are reversibly aligned in pearl chains, perpendicular to the electrodes, by dielectrophoresis in an a.c. field. The cells are not fused by the a.c. field provided the field strength is below 0.7 kV cm^{-1} [23]. Exposure of erythrocytes in such chains to high voltage d.c. breakdown pulses induces some of the cells to fuse. When the a.c. field is subsequently removed, the pearl chains are normally dispersed by thermal currents in the fusion chamber, yielding randomly distributed fused and unfused cells. Occasionally, however, cells in the pearl chains are irreversibly attached to one another by the breakdown pulses, even though they are not fused. Such chains, which contain both fused and unfused cells, do not disperse when the a.c. field is removed. Instead, they drifted randomly in the chamber, as shown by phase contrast microscopy (Fig. 1A).

Membrane fusion without cytoplasmic fusion (hemi-fusion)

The diffusion of the carbocyanine membrane probe DiI-C_{16}(3), from labelled to unlabelled human erythrocytes that are fused by electric field pulses, has been investigated previously by Sowers [24]. He recorded the movement of the fluorescent label by micrography, and obtained average lateral diffusion coefficients of $3.8 \cdot 10^{-9}$ and $8.1 \cdot 10^{-9}$ cm^2 s^{-1} in isotonic phosphate buffer at pH 7.4, at 23–25°C and 35–37°C, respectively. In our experiments, the diffusion coefficient at 20°C for the movement of this probe from unlabelled to labelled erythrocytes that were fused by the electrical breakdown pulses was approx. $5 \cdot 10^{-9}$ cm^2 s^{-1}.

It is well established that exposure of erythrocytes to an electrical breakdown voltage is followed by the almost-instantaneous diffusion of fluorescent cytoplasmic probes, e.g. FITC-dextran [25], and 6-carboxyfluorescein [26] from labelled to unlabelled cells. The fluorescent membrane probe, DiI, also diffuses into unlabelled cells, but more slowly, after the electrical breakdown [24,25]. This behaviour, which was reproduced in the present study, is consistent with the induction of cell fusion by electrical breakdown pulses.

Fluorescent probes in the erythrocytes that were irreversibly attached to their neighbours by breakdown pulses, without being fused, behaved in a different way. Video microscopy with a low-light sensitive camera showed that the membrane probe, DiI, diffused from labelled to unlabelled cells without the cytoplasmic probe diffusing into unlabelled cells or leaking into the medium. An example of this behaviour is illustrated by the fluorescence micrographs shown in Figs. 1B–1D. These micrographs were taken, in the absence of the a.c. field, 10–15 min after the erythrocytes had been exposed to breakdown pulses. In Fig. 1B, the cytoplasmic label is restricted to two adjacent cells in a stabilised chain, but the membrane probe in Fig. 1C has diffused into at least one cell to the left and into two cells to the right of the cytoplasmically-labelled pair. The erythrocytes into which the membrane probe has diffused, but which do not contain the cytoplasmic probe, are clearly visible by phase contrast microscopy in Fig. 1D and they were thus not lysed by the breakdown pulse.

The behaviour of fluorescent probes in the stabilised pearl chains of unfused cells indicates that membrane fusion (hemi-fusion) occurred without cytoplasmic fusion in response to the breakdown pulses. Hemi-fusion, as monitored by movement of the two fluorophores, typically gave rise to hemi-fused chains of cells that contained up to three or four erythrocytes (labelled by the membrane fluorophore only), whereas complete cell fusion yielded much longer chains (labelled by both cytoplasmic and membrane fluorophores). However, phase-contrast microscopy of erythrocytes that were irreversibly attached to one another by the breakdown pulses revealed the presence of chains containing up to 12 cells [18]. Some of these attached, but not hemi-fused, cells may have been linked via their glycocalyx [25].

Effects of changes in the electrical parameters

With erythrocytes at 25°C in 250 mM erythritol that were subjected to an applied voltage of 2.7 kV cm^{-1}, it was observed that an increase in duration of the breakdown pulses from 20 μs to 30 μs resulted in a small decrease in hemi-fusion (Table I). This decrease was

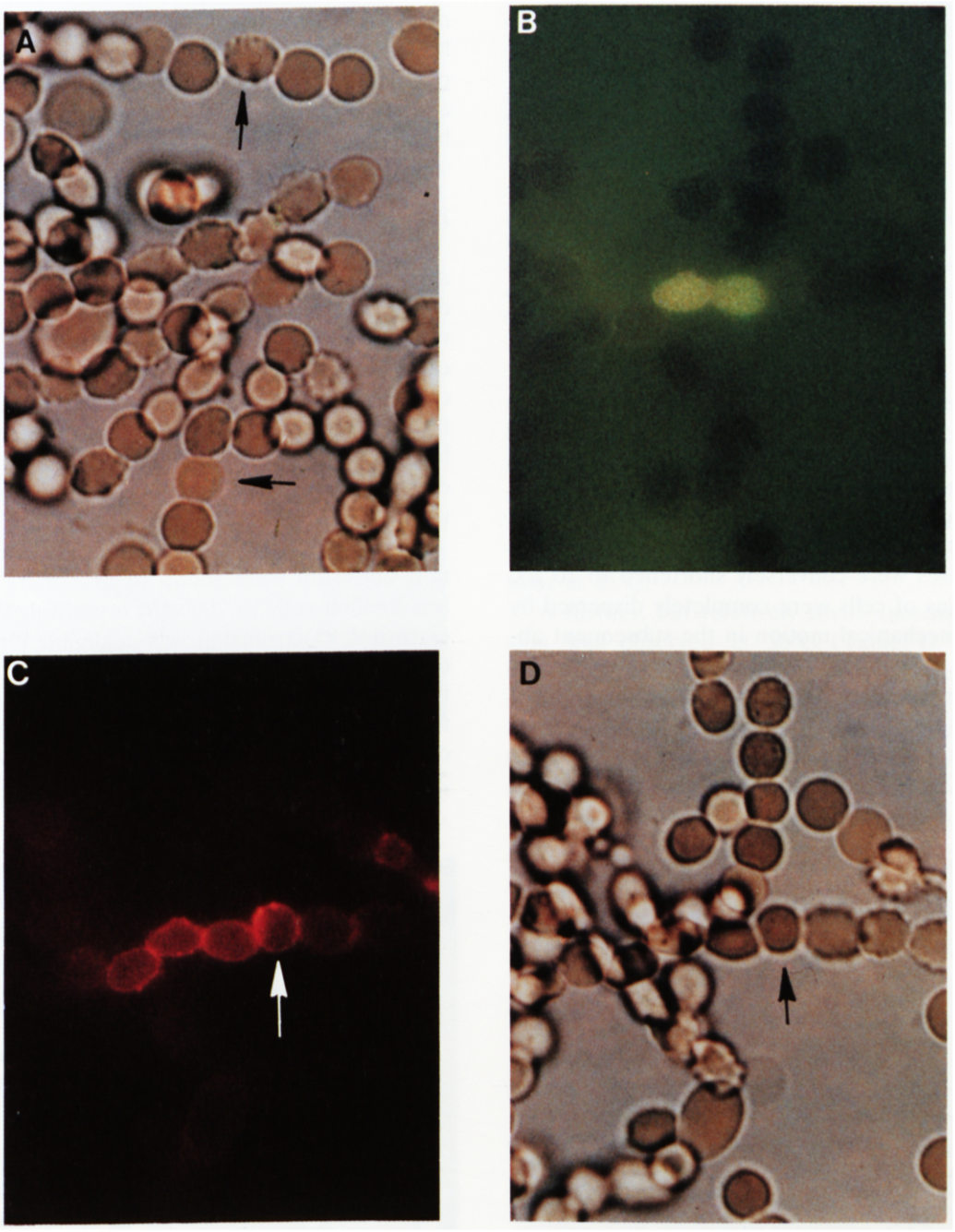

Fig. 1. Fluorescence and phase-contrast micrographs of human erythrocytes which were photographed, in the absence of the a.c. field, about 15 min after the application of three square wave, 28-μs d.c. pulses (at 3.2 kV cm^{-1}) at 1-s intervals (in a chamber in which the electrodes were 280 μm apart). The cells were initially aligned in pearl chains, perpendicular to the electrodes, by dielectrophoresis in a solution of erythritol (250 mM) containing 1 mg ml^{-1} pronase and 10 mM histidine, at 25°C. Panel A shows that, after exposure to a d.c. breakdown pulse and subsequent removal of the a.c. field, the cells remained attached to one another in randomly orientated chains (arrows). Panel B shows cells labelled with the cytoplasmic fluorophore, carboxyfluorescein; this fluorophore is restricted to two cells. Panel C shows cells labelled with the membrane fluorophore, DiI. The arrowed cell, which is immediately to the right of the carboxyfluorescein-labelled pair, (and other cells that similarly do not contain carboxyfluorescein) are labelled with DiI. Panel D (a phase contrast micrograph) shows that carboxyfluorescein has not been lost from the arrowed cell as a consequence of lysis. Magnification: ×440.

TABLE I

Hemi-fusion of human erythrocytes induced by electrical breakdown pulses

The incidence of hemi-fusion, ±S.D., was determined as described in Materials and Methods. The numbers in brackets represent the number of separate experiments. Between 100 and 200 (usually > 150) unhaemolysed, fluorescent erythrocytes were counted in randomly-selected fields in each experiment.

Applied voltage ($kV cm^{-1}$)	Duration pulses of (μs)	Osmolarity (mM erythritol)	Temp. (°C)	Incidence of hemi-fusion
2.7	10	250	25	negligible
2.7	20	250	25	50.9 ± 3.8 (13)
2.7	30	250	25	42.0 ± 4.1 (7)
2.3	20	250	25	negligible
3.1	20	250	25	40.6 ± 3.3 (11)
2.7	20	150	25	23.4 ± 2.8 (8)
2.7	20	250	20	10.6 ± 1.3 (4)
2.7	20	250	30	59.2 ± 1.2 (5)
2.7	20	250	37	approx. 60

necessarily paralleled by a corresponding increase in complete fusion (see Materials and Methods for the formula used to calculate the incidence of hemi-fusion). When the pulses were conversely shortened to 10 μs, the pearl chains of cells were completely dispersed by thermal and mechanical motion in the subsequent absence of an a.c. field, and hemi-fusion was negligible. Pulses of approximately 20 μs were therefore optimal for inducing hemi-fusion.

With 20-μs pulses, an increase in the breakdown voltage from 2.7 to 3.1 $kV cm^{-1}$ slightly decreased the incidence of hemi-fusion, and increased complete fusion (Table I). When the breakdown voltage was decreased to 2.3 $kV cm^{-1}$ with 20-μs pulses, the pearl chains were readily dispersed in the absence of the a.c. field and hemi-fusion was again negligible. It thus appears that an increase in the duration of the pulses, or in the applied voltage, results in an increase in the number of completely fused cells and a decrease in hemi-fusion. Conversely, if the value of either of these parameters is decreased below threshold values, the applied electrical energy is insufficient even to induce hemi-fusion.

Effects of osmolarity and cell swelling

Potassium ions leak from human erythrocytes within a few seconds of their exposure to an electrical breakdown pulse [27]. This causes cell shrinkage which is rapidly followed by cell swelling of the permeabilised cells. In the above experiments it was observed that cells that were fused by breakdown pulses, as judged by the transfer of carboxyfluorescein from labelled to unlabelled cells, were usually extensively swollen after electrical breakdown. By contrast, hemi-fused cells were usually much less swollen, or even shrunken.

In previous work, swollen human erythrocytes in 150 mM erythritol were found to fuse more readily than less swollen cells in 200 mM erythritol. With 400 mM erythritol, electrofusion was negligible [26]. The effect of a decrease in the osmolarity of erythritol, from 250 to 150 mM, on the incidence of hemi-fusion has therefore been determined at 25 °C, under optimal electrical conditions for hemi-fusion (viz., breakdown pulses of 20 μs, and a voltage of 2.7 $kV cm^{-1}$). The incidence of

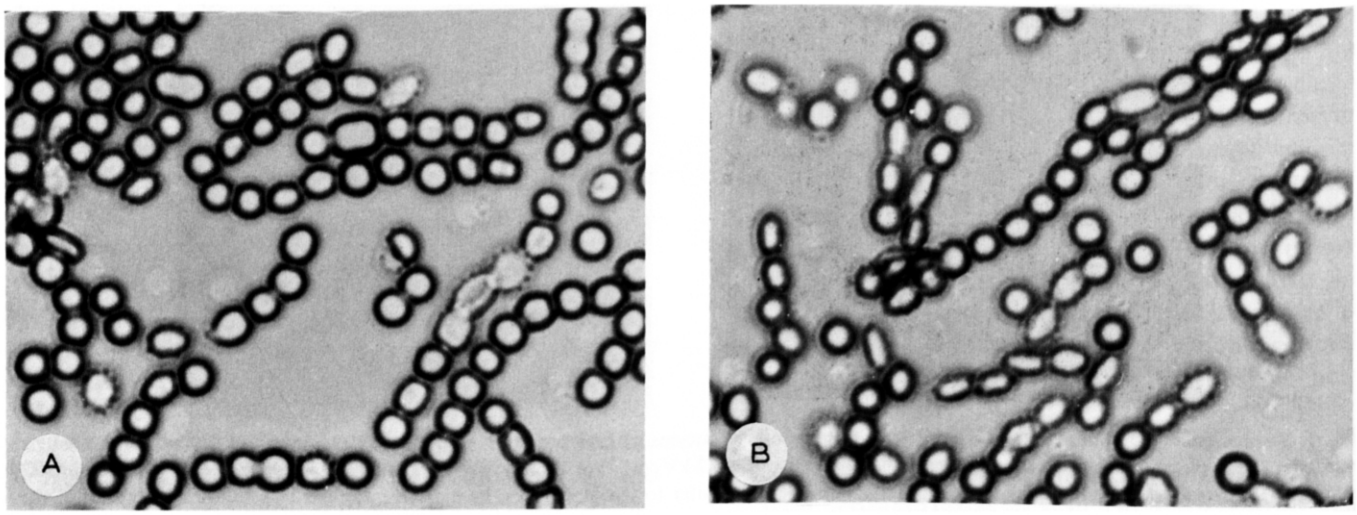

Fig. 2. Bright-field light micrographs of human erythrocytes, in the absence of the a.c. field, 10 min after the application of three square wave, 28-μs d.c. pulses (at 3.2 $kV cm^{-1}$) at 1-s intervals (with the electrodes 280 μm apart). The cells were initially aligned by dielectrophoresis in the medium used in Fig. 1. Cells in panel A were exposed the breakdown pulses at 20°C, and a high proportion of them (both fused and unfused) are swollen. Cells in panel B (most of which are hemi-fused) were exposed to the breakdown pulses at 25°C, and they are extensively shrunken. Magnification: ×200.

Fig. 3. An electron micrograph of freeze-fractured human erythrocytes that are closely apposed and may be hemi-fused, which was prepared as described in Materials and Methods. Patchwork-like structures (curled arrows) are present where the fracture plane has jumped from the E-face down to the P-face, and back again. Irregular protrusions occur on the P-face floor of the patches. More defined, small protrusions (arrow heads), approx. 18 nm in diameter, are also visible on the P-face of the lower cell. Magnification: ×53000.

hemi-fusion with cells in 150 mM erythritol was found to be approximately half that with the less swollen cells in 250 mM erythritol (Table I). This observation further indicates that there is an inverse relationship between the conditions that favour hemi-fusion and those that permit complete fusion.

Effects of temperature

The incidence of hemi-fusion was influenced most strongly affected by changes in temperature. Under optimal electrical and osmotic conditions (viz., breakdown pulses of 20 μs, a voltage of 2.7 kV cm^{-1}, and 250 mM erythritol), the incidence of hemi-fusion at 25°C (50.9 ± 3.8) was approx. 5-fold greater than that at 20°C (10.6 ± 1.3) (Table I). It was also noteworthy that a high proportion of the cells (both fused and

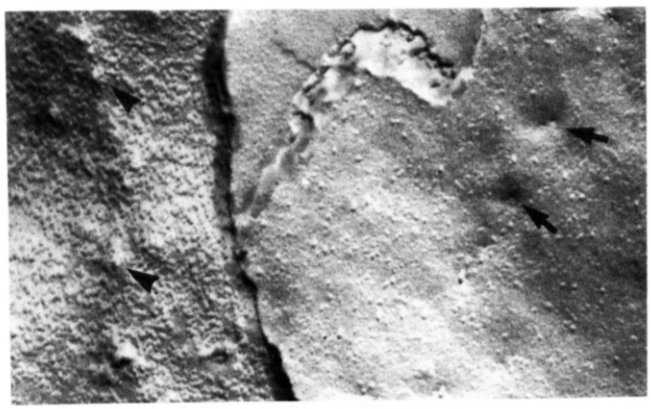

Fig. 4. An electron micrograph of another pair of freeze-fractured human erythrocytes that may be hemi-fused. The protrusions (arrow heads), approx. 18 nm in diameter, that are present on the P-face of the lower cell appear to match depressions (arrows) seen on the apposed E-face of the upper cell. These features may represent the sites of attachment and membrane continuity in hemi-fused cells. Magnification: ×82000.

unfused) were swollen 10 min after the exposure to breakdown pulses at 20°C (Fig. 2A), whereas the cells (most of which were hemi-fused) were extensively shrunken 10 min after exposure to breakdown pulses at 25°C (Fig. 2B). With time, the shrunken cells swelled slowly, and they exhibited extensive swelling after 1 hour at 25°C.

A further, but relatively small, increase in hemi-fusion was observed at 30°C (Table I). Considerable bleaching of the fluorescent probes occurred at 37°C, and it was therefore difficult to estimate the incidence of hemi-fusion accurately at this temperature. However, hemi-fusion appeared to be no greater at 37°C than at 30°C.

It has been reported that the membrane skeleton of human erythrocytes inhibits the electrofusion of human erythrocytes, and that the incidence of fusion increases sharply when the proteins of the skeleton are denatured by heating the cells to 50°C [28]. The effect of heating erythrocytes, which were hemi-fused at 25°C, was therefore studied. Carboxyfluorescein was observed to diffuse at about 50°C from labelled, hemi-fused cells into adjacent unlabelled cells, showing that hemi-fused cells proceed to complete, cytoplasmic fusion at this temperature. However, this was not a frequent occurrence. Probe bleaching and movements of the erythrocytes at 50°C also prevented micrographs of the phenomenon from being obtained.

Ultrastructural changes associated with hemi-fusion

To investigate the ultrastructure of the hemi-fused cells, human erythrocytes in 250 mM erythritol were aligned by dielectrophoresis in an annular electrode chamber, exposed to a single breakdown pulse (10 kV cm^{-1}, 60 μs), and then prepared for freeze-fracture electron microscopy as described in Materials and

Methods. Under these conditions, very few cells were completely fused by the breakdown pulses.

During these experiments it was observed that the linkages between hemi-fused cells are easily disrupted, and it was therefore necessary to centrifuge chains of hemi-fused cells very gently in order to preserve them for electron microscopy. Figs. 3 and 4 are electron micrographs which show the appearance of freeze-fractured cells that are closely apposed and may be hemi-fused. In Fig. 3, patchwork-like structures (curled arrows) are present where the fracture plane has jumped from the E-face down to the P-face, and back again. Irregular protrusions occur on the P-face floor of the patches. More defined, small protrusions (arrow heads), approx. 18 nm in diameter, are visible on the P-face of the lower cells in both Figs. 3 and 4. The dimensions of these protrusions appear to match depressions (arrows) that are present on the apposed E-face of the upper cell in Fig. 4. These protrusions and depressions may represent sites of attachment and membrane continuity in hemi-fused cells.

Discussion

Although there are many reports of hemi-fusion in phospholipid vesicles which are widely employed as models for biological membranes [8–11], there are few published studies on hemi-fusion in cells. The present paper reports evidence from both fluorescence microscopy and freeze-fracture electron microscopy for the occurrence of hemi-fusion in the electrofusion of human erythrocytes, characterises the conditions that favour hemi-fusion as opposed complete fusion, and discusses the possibility that hemi-fusion might precede complete electrically-induced cell fusion.

The hemi-fusion reported here is a different phenomenon from the long-lived fusogenic state that has been observed by Sowers [29] in random suspensions of erythrocyte ghosts which are exposed to electric field pulses and then brought into contact by dielectrophoresis. Sowers [25] also reported that human erythrocyte ghosts, orientated in pearl chains by an a.c. field, were occasionally attached to another by breakdown pulses since subsequent removal of the a.c. field gave rise to randomly orientated, but indefinitely stable, pearl chains. However, in his experiments, neither the cytoplasmic fluorophore (FITC-dextran) nor the membrane probe (DiI) diffused from labelled to unlabelled ghosts in the stabilised pearl chains. It was therefore suggested that the glycocalyx and cytoskeleton were involved in the attachment of the ghost plasma membranes to one another. By contrast, both the octadecyl rhodamine probe used in our preliminary experiments on electrically-induced, hemi-fusion [18], and the DiI carbocyanine probe employed in the present work, diffused from labelled to unlabelled cells in pearl chains that were stabilised by breakdown pulses. It seems likely that one or more of the experimental differences between our experiments and those of Sowers (e.g. the use of intact erythrocytes in non-ionic media instead of resealed ghosts in sodium phosphate buffer) is responsible for the differing observations made. It may be relevant that small quantities of electrolytes prevented the formation of tightly adhering aggregates of human erythrocytes that was observed when the cells were exposed to high voltage a.c. fields [30]. These aggregates were stable when the a.c. field was removed. However the cells were probably not hemi-fused since the aggregates dissociated into individual erythrocytes when electrolyte was added [31], whereas in our experiments hemi-fused cells were unaffected by the addition of Na^+ or Ca^{2+} ions. Furthermore, hemi-fusion was observed in our work only when erythrocytes, aligned by dielectrophoresis, were exposed to d.c. breakdown voltages.

Recently it has been reported that membrane fusion and cytoplasmic fusion are both observed when electric field pulses of radio-frequency are applied to human erythrocytes that are aligned in chains by dielectrophoresis [32]. In this work DiI-C_{16} was used to monitor membrane continuity, and membrane fusion was 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. Cell fusion was, however, determined by visual observation of the cytoplasmic merging of adjacent cells, and experiments with FITC-dextran have shown that it is possible for cytoplasmic continuity to be induced between adjacent cells by a d.c. breakdown pulse in the absence of visible cytoplasmic merging [25].

The electrical breakdown of cell membranes has been interpreted in terms of an electro-mechanical model which postulates that membrane thickness depends on the voltage across the membrane and the applied pressure [33,34]. Zimmermann [31] has also suggested that pores in plasma membranes, which are produced by electrical breakdown, mediate cell fusion when they are induced in closely apposed membranes. However, from ultrastructural observations on the electrically-induced fusion of human erythrocytes aligned by dielectrophoresis, Stenger and Hui [23] have concluded that fusion sites arise where electrically damaged regions of membrane (point defects) make contact. It has also been suggested that a breakdown pulse, which is just below the threshold that is necessary to induce sufficient membrane thinning for complete fusion, may nevertheless have enough energy to compress the adjacent plasma membranes of aligned cells into shared bilayers at the points of very close contact, i.e. induce hemi-fusion [18]. It is possible that osmotic swelling of the permeabilised cells, and rupture of the shared bilayer at the point of contact, may

then occur so quickly that cell fusion is usually virtually instantaneous with electrical breakdown [35]. Schmitt and Zimmermann [36] have recently concluded, however, that neither pressure, nor osmotic gradients, nor the increase in volume of cells in hypo-osmolar solutions, contribute directly to the mechanism of electrofusion since they found that treatment of myeloma cells with hypo-osmotic solutions enhanced the electrofusion yield even when the cells were returned to iso-osmolar conditions before being subjected to electrofusion. They suggested that the increase in fusion yield is due, instead, to the small increase in membrane permeability associated with the swelling process and/or to the dissolution of membrane skeletal proteins caused by osmotic stress.

Whether or not the electrofusion of cells is normally mediated via a transient, hemi-fused state, it is apparent that hemi-fused cells can give rise to completely fused cells since hemi-fused erythrocytes occasionally fused completely on heating to 50°C in our experiments. The presence of pronase may have contributed to this finding. However, a delayed cell fusion has also been observed (monitored by the diffusion of carboxyfluorescein) when electrically-permeabilised erythrocytes are subsequently allowed to swell in 200 mM erythritol [35].

If osmotic swelling is normally responsible for converting a short-lived, hemi-fusion state to complete cell fusion, resealing of electrically-permeabilised erythrocytes would be expected to inhibit complete fusion. Pores that are induced in cell membranes by an electrical breakdown pulse re-seal increasingly rapidly between 10°C and 37°C. It has been reported that the electrically-induced leak permeability of human erythrocytes decreases by 85% within 15 min at 25°C and, at breakdown voltages of approx. 3 $kV\,cm^{-1}$, the number of erythritol-permeable pores may average only one per cell [37,38]. The marked increase in electrical hemi-fusion (and decrease in complete fusion) at 25°C, by comparison with that seen at 20°C in our experiments, may therefore have been due to rapid (although incomplete) resealing of the permeabilised cells at the higher temperature. This interpretation is supported by our observation that, 10 min after exposure to the breakdown pulses, erythrocytes at 20°C were mostly swollen whereas those at 25°C were mostly shrunken.

The depressions on the E-face and protrusions on the P-face of the membranes in the present work are closely similar to the transient point defects (diameter 20–50 nm) that were observed by Stenger and Hui [23] between 2 and 10 s after exposing aligned human erythrocytes to a breakdown pulse. These investigators suggested that the defects were related to a transient form of membrane contact or adhesion that led, within 10 s, to the formation of permanent lumina between fusing cells. Virtually no point defects were observed 10 s after the breakdown pulse. The corresponding structures observed in our experiments were, however, still present 5 min after the breakdown pulse. They may mediate hemi-fusion by breakdown pulses that are insufficient to induce complete fusion, and also mediate complete cell fusion (whether it occurs 10 s after the breakdown pulse or, under certain conditions, many minutes later).

It is relevant that patchwork-like structures where the fracture plane jumped reversibly between two closely apposed membranes, as in the present work, were observed in freeze-fractured preparations of mitochondria that were quenched from 37°C [39]. Bulges and pits (diameter 25 nm) were also seen that may have represented attachment sites of the inner and outer membranes. Contact points between the two mitochondrial membranes were interpreted in terms of a semi-fusion model, but it was not possible to discriminate between semi-fusion with, or without an inverted micelle (or H_{II}-tube), at the points of contact.

Ultrastructural studies on the fusion reactions of biomembranes [3–7] have indicated that an intermediate single bilayer (hemi-fusion) may occur, but such intermediates are very transitory structures. In the present investigation it has been shown that the electrical breakdown of human erythrocytes under specific experimental conditions favours stable, hemi-fused cells at the expense of complete cell fusion, and that heating the hemi-fused cells occasionally results in complete fusion. This behaviour resembles that of human erythrocytes when they exhibit membrane fusion without cytoplasmic fusion on dehydration with 40% poly(ethylene glycol), followed by complete fusion on subsequent swelling in isotonic buffer [12]. In each case, complete cell fusion is preceded by the formation of stable, hemi-fused cells. The intermediate structure present in these cells may be some form of shared bilayer, which is structurally similar to the transient bilayers that have been observed in naturally-occurring fusion reactions of biological membranes. However, unlike these intermediates, it is apparently not subjected to the immediate stress that rapidly results in a completion of the fusion reaction. In this respect, the intermediate in the hemi-fused erythrocytes resembles the more stable bilayer structures which mediate the fusion of planar phospholipid bilayers in model systems [13–17], but the possibility that a transitory H_{II} hexagonal phase or other non-bilayer configuration may occur in the hemi-fused erythrocytes cannot be excluded.

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