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Evidence that electrofusion yield is controlled by biologically relevant membrane factors

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Rabbit + rabbit and human + human combinations of erythrocyte ghost membranes were fused under the same conditions with an electric pulse. Storage at 4 °C of ghost membranes from both rabbit and human erythrocytes showed no change with time but storage of the erythrocytes for various periods before ghost preparation showed consistent storage-dependent changes in fusion yield.

Fusion of two membranes in close contact is an all-or-nothing event. To measure fusion requires that the number of fusion events in a population of membranes in close contact be compared with the number of unfused membranes in the same population. However, neither the mechanism of membrane fusion nor the factors which allow fusion between one given pair of membranes in contact but not another pair is well understood. In past studies, exogenously added chemical fusogens (e.g., Ca^{2+}, fat-soluble compounds, and PEG) and biological fusogens (e.g., naturally-occurring fusion proteins, proteins with fusogenic properties, and fusogenic peptides) have been used to induce fusion in both lipid bilayer systems and cell membranes (for reviews, see Refs. 1–10).

It is reasonable to expect different fusion yields from the use of different chemical fusogens. However, these fusogens can be added initially in equal amounts to a given membrane preparation, but they may bind or interact with the membrane in a way which is dependent on both the chemistry of the fusogen and the composition, structure, or properties of the membrane. Moreover, slow secondary processes may interfere with critical primary processes. Thus the contributions from: the fusogen, the chemical conditions in the medium, and the membrane may all influence the fusion process but are difficult to separate from one another and identify.

In this study, a single electric field pulse was used as the fusogen [12–14] because of four unique characteristics. First, it is non chemical. Second, it is present only transiently. Third, it can induce fusion in high yields. Fourth, all fusion events occur simultaneously within 10–20 ms, or less. Membrane-membrane contact, needed for any study of membrane fusion, was induced before and maintained during the application of the fusogenic electric pulse using the phenomenon of dielectrophoresis [15]. The significance of this is that it is mild, reversible, and nonchemical. Thus neither the induction of membrane-membrane contact nor the introduction of the fusogen involves the addition of an exogenous chemical entity. This allows membrane-membrane contact, the fusogen, and the chemistry of the medium to be manipulated independently of one another.

Artificial membranes containing a few lipid species have been used as membrane models and have permitted much progress in understanding membrane fusion, but it has been pointed out that fusion phenomena in these membranes may not represent fusion in cell membranes [11]. Conversely, cell membranes and their preparations can be used as more natural model membranes but intrinsic factors which contribute to the fusion event are difficult to study because of: natural cell to cell heterogeneity, influences on the membrane from sources external to the membrane, and overall complexity in cell membrane structure. For this study the ghost membrane from the erythrocyte were used as convenient and simple, but relatively well understood plasma membrane model in which the influences from factors exogenous to the membrane should be highly reduced or nearly absent.

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Two questions were asked in this study. First, if the same buffer and the same fusogenic electric pulse are used, would different yields be found for human + human and rabbit + rabbit ghost membrane fusions? Second, would manipulation of the mileau of the erythrocyte before ghost membrane preparation modulate the fusion yield?

Erythrocyte sources and storage. Rabbit erythrocytes were obtained from whole blood (15 ml) of New Zealand White rabbits by collection into vacutainers containing 0.3 ml of sodium citrate (1 M) as anti-coagulant. The rabbit diet was the NIH-09 open formula [16]. Human erythrocytes were obtained as packed cells from whole blood by centrifugation at $300 \times g$ for 10 min and removal of the supernatant and buffy coat by expression.

Three kinds of in vitro erythrocyte incubation experiments were performed. First, the rabbit whole blood and the packed human erythrocytes (the pellet) were stored at 4°C for up to 3 weeks. Second, rabbit whole blood was washed in isotonic phosphate buffer (pH 7.4) and, after removal of the buffy coat and supernatant, the pelleted erythrocytes were resuspended and stored in an incubation medium (Table I of Ref. 17) composed of (in mM, unless otherwise indicated): NaCl (116.4), KCl (5.4), $CaCl_2 \cdot 2H_2O$ (1.8), $MgSO_4 \cdot 7H_2O$ (0.8), $NaH_2PO_4 \cdot H_2O$ (1.0), glucose (5.6), $NaHCO_3$ (15), Hepes buffer (18), streptomycin (100 units/ml), penicillin (100 units/ml), bovine serum albumin (10 g/l); the following vitamins (in mg/l): D-biotin (0.2443), calcium D-pantothenate (0.4765), choline chloride (0.1396), folic acid (0.4414), nicotinamide (0.1221), pyridoxal · HCl (0.2036), riboflavin (0.0376), thiamine · HCl (0.3373); and 0.5 mM of each of cysteine, glycine, glutamic acid, and glutamine. All reagents were obtained from Sigma. Third, rabbit erythrocytes were resuspended and stored in the incubation medium but without glucose. Aliquots of blood were withdrawn aseptically at one week intervals and ghosts were prepared as described below.

Erythrocyte ghost preparation. Human erythrocyte ghosts (HEG) were obtained by washing the packed cells in isotonic sodium phosphate buffer (pH 7.4), and then hemolyzing them in 5 mM sodium phosphate buffer (pH 8.5) for 20 min. All operations were conducted at 0–4°C unless otherwise specified. The ghosts were then washed three times ($10\,000 \times g$, 20 min) in 20 mM sodium phosphate buffer (pH 8.5). The HEG were stored as pellets in the 20 mM sodium phosphate buffer for various lengths of time at 4°C until samples were withdrawn for the fusion assay. Rabbit erythrocyte ghosts (REG) were obtained by washing rabbit whole blood with isotonic sodium phosphate buffer (pH 7.4) with centrifugation at $270 \times g$, 10 min. The buffy coat and supernatant were removed by aspiration and the pellet was resuspended with 5 mM sodium phosphate buffer (pH 8.5) for 30 min at 0–4°C. The REG were centrifuged ($10\,000 \times g$, 20 min) and resuspended with 20 mM sodium phosphate and centrifuged again. The ghost membranes and intact blood cells withdrawn for ghost preparation were always checked by phase optics for overall morphology and presence of bacterial contamination.

The HEG were labeled with 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanide perchlorate (DiI) using ethanol as previously described [13]. The REG were similarly labeled with DiI except that the wash centrifugation was at $3600 \times g$ for 10 min. DiI was obtained from Molecular Probes (Eugene, OR 97402). All other reagents were from Sigma.

Observation, prefusion alignment of membranes into contact, fusion, and fusion yield calculations were conducted at 20–24°C as previously described [18,19]. Fusion was induced with an exponentially-decaying electric pulse from a charged capacitor. The decay half-time of the pulse was monitored by a storage screen oscilloscope. The details of the circuit of this pulse source are described elsewhere [14]. Fusion yield (in %) is calculated from count in a sample area of the number of fluorescent membranes before the pulse (N_u) and then, after the pulse, the count of those events in which the fluorescence moved from an originally labeled membrane to at least one adjacent but originally unlabeled membrane (N_f), and use of the formula:

$$\text{Fusion yield} = (N_f/(N_f + N_u)) \times 100$$

The strength of the sodium phosphate buffer in which the membranes were assayed was changed from 20 mM to 60 mM by one or two washes in 60 mM sodium phosphate buffer.

Two experiments were conducted. First, HEG were prepared from packed human erythrocytes and REG were prepared from rabbit whole blood and stored in 20 mM sodium phosphate buffer (pH 8.5) at 4°C for up to 4 to 5 days. Aliquots were taken daily from these preparations and assayed for fusion yield. Second, the same unit of packed human erythrocytes, the same sample of rabbit whole blood, and the same rabbit erythrocytes in the artificial incubation medium were stored for up to 3 weeks at 4°c. After 1, 2, and 3 weeks, aliquots were taken for ghost membrane preparation and fusion yields were measured one day after preparation.

Fig. 1 shows the yields in HEG + HEG fusions for ghosts from units of packed erythrocytes from two human blood donors. The rabbit erythrocyte ghosts data are from four different rabbits. As can be seen, the fusion yields are nearly constant up to about the fourth day after preparation but are markedly different for the two erythrocyte sources. Rabbit ghosts are more than two times more fusible than human ghosts for the same pulse in the same 20 mM sodium phosphate buffer.

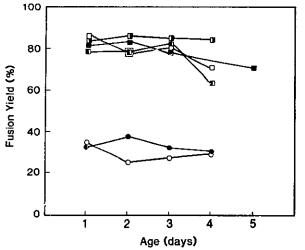

Fig. 1. Fusion yield in two different samples of human (●, ○) and four different samples of rabbit (◨, ■, □, ◧) erythrocyte ghosts as function of storage at 4°C in 20 mM sodium phosphate (pH 8.5) after they were prepared. Assays were performed at 20–24°C. In all cases a single 500 V/mm, 1.0 ms decay half-time pulse was applied to ghosts aligned into pearl chains.

Fusion yield drops off considerably and in an inconsistent manner after five or more days of storage (data not shown). Swimming bacteria were usually noticeable as early as the fourth or fifth day after ghost preparation.

Fig. 2 shows that the yield in REG + REG fusions increases almost uniformly from 57% to 80% when REG were made from the same rabbit whole blood after storage at 4°C for a 2 week period. However, when the rabbit erythrocytes were removed from the whole blood

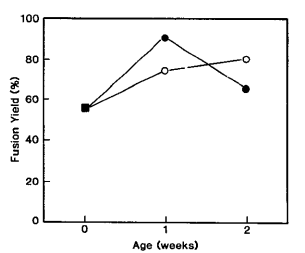

Fig. 2. Fusion yield as a function of erythrocyte storage before ghost preparation. Fusion yield was measured in ghosts membranes one day after preparation from: (■), erythrocytes of rabbit whole blood on same day blood was collected; (●), erythrocytes from rabbit whole blood washed on day of collection in isotonic sodium phosphate buffer (pH 7.4) and stored (4°C) in a complete incubation medium (see text) for up to 2 weeks; (○), erythrocytes stored in the rabbit whole blood (4°C) for up to 2 weeks. Aliquots were taken for ghost preparation after 1 and 2 weeks of storage. A single 400 V/mm, 1.0 ms decay half-time pulse was applied to ghosts aligned into pearl chains.

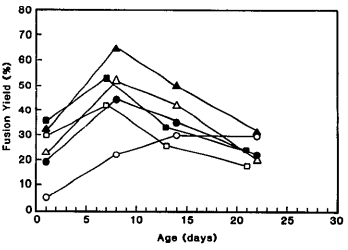

Fig. 3. Fusion yield measured in ghost membranes one day after preparation from human packed red cells stored at 4°C for up to 3 weeks. Aliquots were taken after one, two, and three weeks of storage and ghosts prepared on the same day. A single 700 V/mm, 0.75 ms decay half-time pulse was applied to ghosts aligned into pearl chains. Different symbols represent three different human blood donors. Sodium phosphate buffer (pH 8.5) strengths: open symbols, (20 mM); solid symbols, (60 mM).

and resuspended in the defined medium and stored at 4°C, then the fusion yield in ghosts increased from 57% to 92% after one week of storage but then dropped back to 65% after the second week of storage. There was no significant difference in the data for the experiment where glucose was omitted from the defined medium (data not shown).

Fig. 3 shows how the yield in HEG + HEG fusions change as a function of the interval over which the human erythrocytes were stored beefore the ghosts were prepared. It is striking that if the erythrocytes were stored for one week before ghost preparation, the HEG + HEG fusion yields went up by a factor of at least 1.4 (open square, Fig. 3). However, for longer storage periods, the yields were much closer to what would have been obtained with ghosts from freshly collected blood. In one case (open circle, Fig. 3), however, storage of the packed erythrocytes for 3 weeks caused the fusion yield to increase by a factor of 5 over the yields measured in ghosts made from fresh erythrocytes pelleted and stored for one day before ghost preparation. Except for one data point (circle, Fig. 3) for one unit of human erythrocytes stored for 3 weeks, a buffer strength of 60 mM, caused the fusion yields for HEG + HEG fusions to be uniformly shifted upwards and parrallel to those at 20 mM.

Repeat determinations of the fusion yield on the same preparation of ghost membranes were usually within 2–3 percentage points (data not shown). This indicated that the electric pulse fusogen, the buffer conditions, and the methodology of the fusion assay produced consistent and reproducible data. The absence of significant time-dependent changes in the yields of

either the REG + REG fusions or the HEG + HEG fusions when the ghosts were stored at 4°C for up to 4 days is independent evidence of reproducibility in the data. It also showed that simple storage-induced aging in ghosts over the 4 day period had no significant effect on the fusibility of the membranes during the storage period. Since the ghost membranes were at least highly depleted if not essentially free of cytoplasmic elements the different fusion yields for REG + REG fusions and HEG + HEG fusions must have been due to specific differences in the membranes.

The changes in the yield of REG + REG fusions caused by the storage of rabbit erythrocytes either as whole blood or as rabbit erythrocytes in a chemically defined medium before ghost preparation suggests that metabolic or storage-induced processes alter the plasma membrane in a manner which can substantially change the fusion yield. The fact that absence of glucose in the medium had no effect on fusion yield suggested that a metabolically derived membrane potential was not a significant factor in fusion yields. Storage of the same units of packed human erythrocytes for one week also showed a significant increase in yields for HEG + HEG fusions. However, longer storage was accompanied by lower fusion yields.

That the fusion yield was proportional to buffer strength is consistent with previous observations [18]. This suggests the presence of buffer strength-dependent variables which influence yield in the HEG + HEG fusions. However, with the exception of the data from one blood donor (circle, Fig. 3) the general uniformity of the shift suggests an electrochemical rather than a biological origin.

The HEG fusion yield data shown in Fig. 1 were from two different units of donated human blood which were close in fusion yield at the time of preparation and were thus chosen for additional assay over the 4 day storage period. Yields in HEG + HEG fusions generally [19] show a much larger individual to individual difference (see Fig. 3) compared to the narrower range of yields seen in REG + REG fusions. The larger range in the HEG data suggest that naturally occuring variables but largely uncontrolled factors are present while the narrower range in the REG data suggest better control of the variables, including diet.

The above data suggest that intrinsic membrane factors are: (i) present in membranes alone (ghosts) and can have significant effects on membrane fusibility, but that (ii) these factors can be modulated by processes exogenous to the membrane. Also, these data originated from the use of fundamentally new methods (electrofusion and dielectrophoresis) to study fusion mechanisms and fusion-modulating influences. A related line of thinking was also used in studies which showed that mammalian cell lines exposed to increasing concentrations of PEG became fusion resistant to PEG and that this may have come about because the cells showed new lipid compositions [20]. While it was noted in an earlier publication (Table 1 in Ref. 21) that optimum fusion yield (of interest in applications work) in a variety of cells required *different* pulse characteristics, the data and experimental emphasis in the present paper suggests, conversely, that the *same* fusogenic electric pulse and buffer could be used on different membranes to help identify those membrane components and properties which have major influences of the fusibility of membranes. The results of this paper suggest that correlation of changes in measured fusion yields in a given membrane system with changes in other measurable membrane properties in the same system *and conducted under conditions such that the aqueous medium and fusogen are identical* – as is possible with the approach described in this paper – may eventually lead to new understandings not only of fusion mechanisms but also the factors which allow fusion between one pair of membranes in contact but not another pair.

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A nearly identical range in electrofusion yields in erythrocyte membranes has also been recently found in another laboratory (Chang et al. [22]).

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