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# Electrofusion of dissimilar membrane fusion partners depends on additive contributions from each of the two different membranes

Arthur E. Sowers

*Jerome H. Holland Laboratory for the Biomedical Sciences, Rockville, MD (U.S.A.)*

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**Rabbit erythrocyte ghost (REG) membranes and human erythrocyte ghosts (HEG) were aligned into contact by dielectrophoresis and fused with an electric pulse in REG + REG, HEG + HEG, and REG + HEG combinations. REG + HEG fusion yields were approximately midway between fusion yields for REG + REG and HEG + HEG over a wide range of pulse characteristics.**

When membrane fusion takes place between two membranes, the two membranes are either identical or dissimilar. Myotube formation is an example where membrane fusion takes place between identical membranes [1]. Cellular infection by enveloped viruses [2,3] and sperm-egg fusion [4] are examples of fusion between dissimilar membranes. In somatic cell genetics [5,6] and biotechnology applications such as hybridoma formation [7], fusion is often induced between two populations of dissimilar membranes. However, the analysis of the mechanistic details of these fusions has only recently been seriously studied [8–10].

In this paper we compare the fusion of two different kinds of membrane (i.e. A + B) with the fusion of similar kinds of membrane (i.e., A + A, B + B). Rabbit erythrocyte ghosts (REG) and human erythrocyte ghosts (HEG) were used because they are simple but well studied plasma membranes which have no complicating influences from the cytosolic compartment and are also nearly identical in size. An electric field pulse was used as an artificial fusogen [11–14] because it is a nonchemical fusogen, it is present only transiently, and it can induce fusion in high yields. The phenomenon of dielectrophoresis [15] was used to induce and maintain membrane-membrane contact during the application of the fusogenic electric pulse because it is a mild, reversible, and nonchemical effect. 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. In this study we tested the hypothesis that if the same buffer and the same fusogenic electric pulse are used, then different fusion yields between dissimilar membranes may be due to membranes intrinsic factors or other membrane influences.

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. Human erythrocytes were obtained as packed cells from whole blood by centrifugation at 300 × *g* for 10 min and removal of the supernatant and buffy coat by expression.

Human erythrocyte ghosts (HEG) were obtained by washing the packed erythrocyte 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 (10000 × *g*, 20 min) in 20 mM sodium phosphate buffer (pH 8.5). These membranes 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 (collected in vacutainers containing sodium citrate) with isotonic sodium phosphate buffer (pH 7.4) and centrifugation at 270 × *g*, 10 min. The buffy coat and supernatant were removed by aspiration and the pellet was resuspended

Correspondence: A.E. Sowers, Jerome H. Holland Laboratory for the Biomedical Sciences, 15601 Crabbs Branch Way, Rockville, MD 20855, U.S.A.

with 5 mM sodium phosphate buffer (pH 8.5) for 30 min at 0–4°C. The REG were centrifuged (10000 × g, 20 mins) 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 the fluorescent lipid analog 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) using ethanol as previously described [16,17]. The REG were similarly labeled with DiI except that the wash centrifugation was at 3600 × g for 10 min. DiI was obtained from Molecular Probes (Eugene, OR 97402). All other reagents were from Sigma.

Prefusion alignment of membranes into contact and fusion were induced at 20–24°C as previously described [18,19]. Fusion was induced with a single pulse, having an exponentially-decaying waveform, with a capacitor discharge circuit which is described elsewhere [14]. The decay half-time, $T_{1/2}$, of the pulse was monitored by a storage screen oscilloscope. Fusion yield was calculated as follows. Mixtures of unlabeled ghost membranes and DiI-labeled ghost membranes (15 : 1, in numbers) were placed in the chamber, aligned into pearl chains with the weak alternating current, and then the strong fusogenic electric pulse was applied. After the pulse, all fluorescent membranes in a standard sample volume were counted an placed in one of two categories: (i) single fluorescent membranes (= unfused), and (ii) multiple and adjacent fluorescent membrane (= at least one fusion with an adjacent but originally unlabeled membrane). The fusion yield was calculated from these two numbers, $N_u$ and $N_f$, respectively, according to the formula

$$FY = (N_f)/(N_u + N_f) \times 100$$

Thus FY is restricted to the ranges from 0 to 100%.

The strength of the buffer in which the membranes were assayed was changed from 20 mM to other buffer strengths by one or two washes in the buffer of the appropriate strength.

Fusion partner convention: fusion of similar (i.e., A + A) membranes will be referred to as homofusions while fusion of dissimilar (i.e. A + B) membranes will be referred to as heterofusions. The fusion partner which was labeled with DiI was designated with an asterisk in the superscript position.

Fig. 1 shows that fusion yield in REG + REG * partners was proportional to pulse field strength, $E$, and *proportionally dependent* on $T_{1/2}$ when the $T_{1/2}$ was between 0.04 and about 0.12–0.2 ms, but *independent* of $T_{1/2}$ when the $T_{1/2}$ was between 0.12–0.2 and 1.0 ms.

Fusion yield in HEG + HEG * fusion combinations was always proportional to both pulse field strength

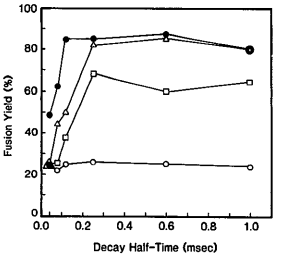


Fig. 1. Fusion of rabbit erythrocyte ghosts with rabbit erythrocyte ghosts (REG + REG) as function of pulse field strength and pulse decay half-time in 20 mM sodium phosphate buffer (pH 8.5) at 20–24°C. See text for fusion assay details. Pulse field strength: ○, 300 V/mm; □, 400 V/mm; △, 500 V/mm; ●, 600 V/mm.

and pulse decay half-times over the ranges of the two variable (Fig. 2). While no threshold effect was observed such as that seen in Fig. 1 it must be stated that, compared to Fig. 1, measurements for $T_{1/2}$ values less than 0.4–0.6 ms were not attempted because yields in HEG + HEG * fusions were anticipated to be much lower than the lowest yields observed in Fig. 1 (Measurements are usually not attempted when fusion yields are expected to be less than 15–20% because the mean value of the measured quantity becomes small relative to the scatter in the data).

Figs. 1 and 2 clearly show not only that REG + REG * fusion partners are significantly more fusible for a given pulse than HEG + HEG * fusion partners,

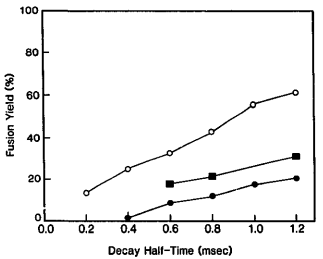


Fig. 2. Fusion of human erythrocyte ghosts with human erythrocyte ghosts (HEG + HEG) as function of pulse field strength and pulse decay half-time in 20 mM sodium phosphate buffer (pH 8.5) at 20–24°C. See text for fusion assay details. Pulse field strength (V/mm): ●, 400; (■), 600; (○), 800.

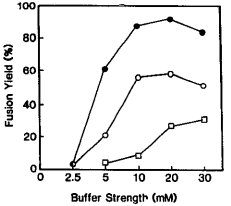


Fig. 3. Fusion yield in REG + REG (○, ●) and HEG + HEG (□) fusions partners at pH 8.5 and 20–24°C as function of sodium phosphate buffer strength. Pulse field strength (V/mm): □, ●, 600; ○, 400. Pulse decay half-time was 1.0 ms.

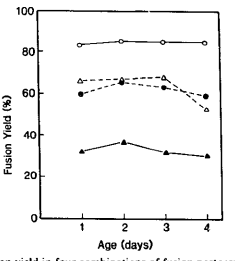


Fig. 4. Fusion yield in four combinations of fusion partners in 20 mM sodium phosphate buffer (pH 8.5) at 20–24°C as function of time stored at 0–4°C. Fusion partners are: ○, REG + REG *; ●, REG + HEG *; △, REG * + HEG; ▲, HEG + HEG *. Asterisk superscript indicates the membrane labeled with DiI. Pulse field strength: 500 V/mm. Decay half-time: 1.0 ms.

but that, over the range of pulse voltage and decay half-times, the fusion yield dependence is qualitatively different in the two membrane types. This suggests that different variables are involved in the fusion process and that these variables may be linked in different ways to the two pulse characteristics ($E$ and $T_{1/2}$).

Fusion yield was strongly dependent on buffer strength (Fig. 3. Over the buffer strength range of 2.5–30 mM, fusion of REG + REG * was highest at a buffer strength of about 20 mM while fusion of HEG + HEG * was highest at 30 mM. In all cases, fusion yield dropped off to zero as the buffer strength was reduced to 2.5 mM for REG + REG * and 5 mM for HEG + HEG * partners. A similar fusion yield dependence on buffer strength has also been observed previously in other cells by Sukharev et al. [18] but just the opposite effect has been reported by another labcratory [19,20].

Our qualitative observation of dielectrophoretic alignment of REG into pearl chains (long rows of ghost membranes in single rows parallel to one another) suggests that the forces which cause these membranes to come into contact may not be as strong as for HEG when the buffer strength is 20 mM because it takes somewhat longer for the alignment to become complete. Although this may be the cause of the lower fusion yields in REG + REG * fusion partners at buffer strengths above 20 mM (see Fig. 3), further quantitative investigation of this possibility was beyond the scope of this paper but will be addressed in a future paper.

Storage of either human or rabbit erythrocyte ghosts at 0–4°C for up to four days has no effect on fusion yields for any partner combination (Fig. 4) and shows that a given membrane preparation should be useful for several days of valid experiments.

Heterofusions resulted in fusion yields which were slightly closer to that for REG + REG * but were otherwise nearly midway between both homofusion partner combinations (Fig. 4 and 5) over the range of pulse voltages used. Moreover, the fusion yield was independent of which partner contained the fluorescent label. This indicates that the presence of the label had little or no effect on the process being measured.

Fusion yields in HEG + HEG *, HEG + REG *, HEG * + HEG, and REG + REG * fusion partners are roughly proportional to pulse field strength (Fig. 5). As can be seen heterofusion yields are about halfway between homofusions for the entire range of field strengths used while still being roughly proportional to field strengths.

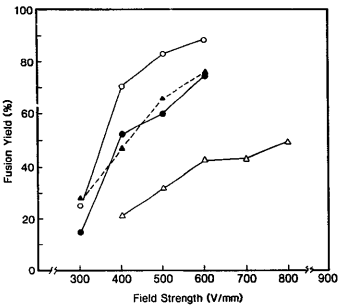


Fig. 5. Fusion yield in various combinations of fusion partners in 20 mM sodium phosphate buffer (pH 8.5) at 20–24°C as function of pulse field strength. Fusion partners are: ○, REG + REG *; ●, REG + HEG *; ▲, REG * + HEG; △, HEG + HEG *. Asterisk superscript indicates which partner was labeled with DiI. Pulse decay half-time: 1.0 ms.

Natural fusogens, such as viruses, and artificial fusogens, such as chemicals and electric pulses, are commonly used in biotechnology applications as well as in fundamental studies of membrane fusion mechanisms. It has been recently observed that different cell lines and cell lines exposed to selection pressure from fusogens will have different fusibilities and that the fusibilities can be different for the same fusion partners when different fusogens are used [8,9]. Biochemical analysis indicated that membrane composition may play a significant role in the fusion process [8–10].

The fact that heterofusion yields were always nearly halfway between either homofusion combination indicates that the fusion process depends on additive contributions from both membranes. This is in marked contrast to results of heterofusion and homofusion yields obtained using adult chicken erythrocyte ghosts (ACEG) and chicken embryo erythrocyte ghosts (CEEG) in three fusion partner combinations using ammonium acetate buffer (pH 7.4) (see Fig. 7 in Ref. 21). In those experiments, use of a buffer strength of 120 mM caused the fusion yields for the heterofusion of ACEG + CEEG partners to be nearly the same as for the homofusion of CEEG + CEEG partners. The ACEG + ACEG homofusion combination gave the lowest fusion yields. In contrast, the use of a buffer strength of 20 mM caused the heterofusion yield to be about halfway between the two homofusion combinations. The chicken erythrocyte ghost study is not completely comparable with the present study because the chicken erythrocyte ghost nucleus requires divalent cations for maintaining its integrity [22]. Therefore chicken erythrocyte ghost fusion experiments cannot be conducted in sodium phosphate buffer. Conversely, human erythrocyte ghosts do not fuse in the presence of ammonium acetate buffer (Sowers, unpublished). Also, dielectrophoretic alignment of rabbit and human erythrocyte ghosts in sodium phosphate buffer (pH 8.5) is not practical above concentrations of approx. 30 and 60 mM, respectively. Hence the comparison of electrofusion in chicken erythrocyte ghosts with either rabbit or human erythrocyte ghosts under identical conditions is not feasible. However, results of this study indicate that future studies involving various combinations of erythrocyte ghosts from other *mammalian* species may be feasible.

Taken together the above results suggest that, during electrofusion, dissimilar membranes in close contact may, on the one hand, contribute equally and in an additive way towards the fusion event, or, as in the case of chicken erythrocyte ghosts (see Ref. 21), the fusibility of one membrane may dominate the fusion event. The discovery of more examples of these two possibilities may shed new light on how fusion takes place between two membranes in close contact and the effect when these membranes are similar or dissimilar. It may be possible to use many combinations of similar and dissimilar fusion partners from a variety of species to correlate the effects of, for example, membrane composition of fusion yield. This offers the possibility of dissecting the fusion mechanism in terms of both how the fusogen and the membranes contribute to the fusion event. In the present study we have used the same buffer and the same fusogen on three combinations of fusion partner. The results of this approach suggest that, while the fusogen initiates the fusion event, membrane factors are totally responsible for controlling the completion of the fusion event. Isolating and identifying those factors which play major roles in the fusion process may make a significant contribution to the understanding of membrane fusion.

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