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Survival of sucrose-loaded erythrocytes in the circulation

In an attempt to clarify the mechanism of cell lysis under intense electric fields[1–5], we have found that aqueous pores are introduced into human erthrocyte membranes when an isotonic suspension of red cells is exposed to an electric field of a few kV cm^{-1} for a duration in μs range. These pores are formed when the transmembrane potential induced by the externally applied field exceeds a critical value of 1 V. The effective radius of the pores is several Å, and can be varied by the adjustment of field intensity, field duration, and the ionic strength of the medium. The pores remain open at low temperatures but close completely on incubation at 37 °C. In a proper medium, the resealing of perforated cells takes place without haemolysis, allowing us to prepare erythrocytes (not ghosts) of altered intracellular composition. In particular, foreign molecules such as sucrose have successfully been incorporated into resealed erythrocytes, which were apparently intact at least in terms of cell volume, cell shape, glucose transport, and Na–K pump activity[4]. Thus we have suggested that erythrocytes loaded with a drug by this technique might serve as intravenous drug reservoirs which slowly release the drug molecules into the circulation. Here we demonstrate that erythrocytes loaded with sucrose survive in the circulation with a lifetime almost indistinguishable from that of normal cells, and that the sucrose remains entrapped within the cells. For drugs that slowly permeate the erythrocyte membranes, therefore, our technique offers a means of sustaining a low plasma level for a long period of time, and this could be advantageous in clinical and other situations.

Mice, AKR/J female, were used throughout the experiments. The response of mouse erythrocytes to pulsed electric fields is quite similar to that of human erythrocytes, as is shown in Fig. 1 and Table 1. Figure 1 shows that an electric field greater than about 2.5 kV cm^{-1} induces haemolysis of mouse red cells. The haemolysis is the result of the osmotic imbalance caused by the destruction of the permeability barrier, and is directly correlated with the formation of pores[3,5]. From the data in Fig. 1 and the dimension of mouse erythrocytes (5.8 μm in diameter, ref. 7, and 49 μm^3 in volume, ref. 8), the critical transmembrane potential at which pores are formed in mouse erythrocyte membranes was calculated to be 1.0 V for the 20-μs pulse and 0.9 V for the 80-μs pulse, in good agreement with the values for human erythrocytes[5]. The rather high level of haemolysis in untreated cells is because these mouse erythrocytes are more fragile than human erythrocytes. If the incubation medium in Fig. 1 does not contain Ca, Mg and glucose, untreated mouse erythrocytes almost completely haemolyse within 20 h, whereas fresh human erythrocytes can be kept in isotonic NaCl for days.

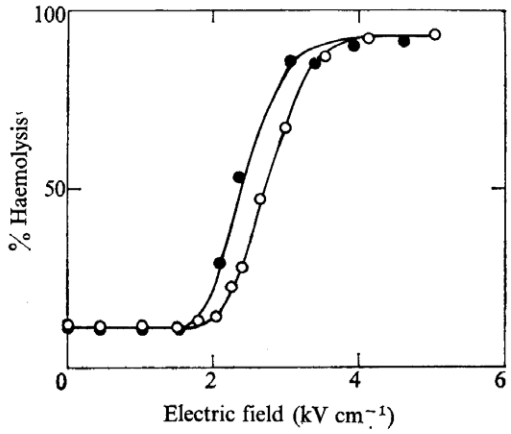

Fig. 1 Haemolysis of mouse erythrocytes as a result of voltage-induced pore formation. ○, 20-μs pulse; ●, 80-μs pulse. Blood was collected from eyesockets of AKR/J female mice into heparinised capillary tubes. Erythrocytes were washed by centrifugation at 4 °C twice with a washing medium (NaCl 150 mM, KCl 8 mM, Na-phosphate buffer 6 mM, $MgSO_4$ 2 mM, D-glucose 10 mM, adenosine 1 mM, inosine 1 mM, penicillin G 500 units ml^{-1}, chloramphenicol 0.1 mg ml^{-1}, *p*H 7.5) and once with an incubation medium (washing medium plus bovine serum albumin at 3.5% v/v), and finally resuspended in the incubation medium at a volume concentration of 6%. An aliquot of the suspension was warmed to 25 °C and exposed to a single square-wave electric pulse of indicated intensity and duration in a device described elsewhere[3]. Immediately after the pulse treatment, the suspension was diluted with 12 volumes of ice-cold incubation medium containing 2 mM $CaCl_2$, and kept at 4 °C with occasional mixing. After 20 h, the sample was centrifuged and the extent of haemolysis determined from the concentration of haemoglobin in the supernatant (cyanmethaemoglobin method[6]). The value corresponding to 100% haemolysis was obtained by hypotonic lysis in water.

In the experiment shown in Table 1, erythrocytes were treated with an electric pulse in the presence of [^{14}C]-sucrose. As in the case of human erythrocytes[4,5], the size of the voltage-induced pores was found to be strongly dependent on pulse duration, and pores which admit sucrose molecules were obtained when the duration exceeded 20 μs. Subsequent incubation at 37 °C allowed resealing of the leaky erythrocytes, while stachyose was added to the medium to prevent haemolysis[4]. Thus, at the end of the incubation, we obtained apparently intact erythrocytes which had entrapped sucrose inside. Again, a considerable degree of haemolysis was observed during the resealing procedure, in contrast to the nearly 100% survival of human erythrocytes through a similar treatment[4].

The erythrocytes loaded with [^{14}C]-sucrose were injected back into mice from a tail vein. The top curve in Fig. 2 represents the level of sucrose remaining in circulating erythrocytes. The curve starts from 100% recovery, which was calculated from the injected amount and the total erythrocyte volume in a mouse, and declines with a half life of 14 d, or approximately 18 d after correction for the loss by bleeding; this is within the range of the known half life of mouse erythrocytes of 15–20 d (ref. 9). The data show, therefore, that the loaded erythrocytes were essentially intact, and that the erythrocyte membranes had completely regained their impermeability to sucrose. In the second curve, the resealing period was reduced to 1 h, whereupon half of the injected sucrose had disappeared from the circulation by 12 h. This is probably due to lysis of, rather than to leakage of sucrose molecules from,

Table 1 Incorporation of sucrose in mouse erythrocytes

Pulse duration (μs)	0	10	20	40	80	160
Sucrose content (mmol l^{-1} cells)	0.1	0.3	0.5	1.4	3.5	6.9
% cells surviving	98	98	97	96	91	78

Washed erythrocytes were prepared as described in the legend to Fig. 1. The cells were suspended at 20% v/v in a mixture of 76 volumes of the incubation medium, 15 volumes of 270 mM stachyose, and 9 volumes of 290 mM sucrose containing [^{14}C]-sucrose at a final specific activity of 0.4 mCi $mmol^{-1}$. The suspension was treated with a 3.5 kV cm^{-1} pulse of indicated duration at 25 °C, immediately transferred into a plastic tube, and incubated at 37 °C in a shaker bath. After 1 h, the cells were centrifuged down, resuspended at 5% v/v in a mixture of 85 volumes of the incubation medium containing 2 mM $CaCl_2$ and 15 volumes of 270 mM stachyose, and incubated at 37 °C for an additional 5 h. Aliquots of the resealed erythrocytes were packed in microhaematocrit tubes, bleached with perchloric acid/hydrogen peroxide, mixed with ascorbic acid and Hydromix (Yorktown Research), and counted in a Beckman liquid scintillation counter. The % of cells which survived the loading procedure was estimated from the amount of haemoglobin released during the two incubations. The procedure described above is a simplified version of the method in ref. 4 (in Table 1 of this ref., Na and K contents in untreated erythrocytes were interchanged by mistake).

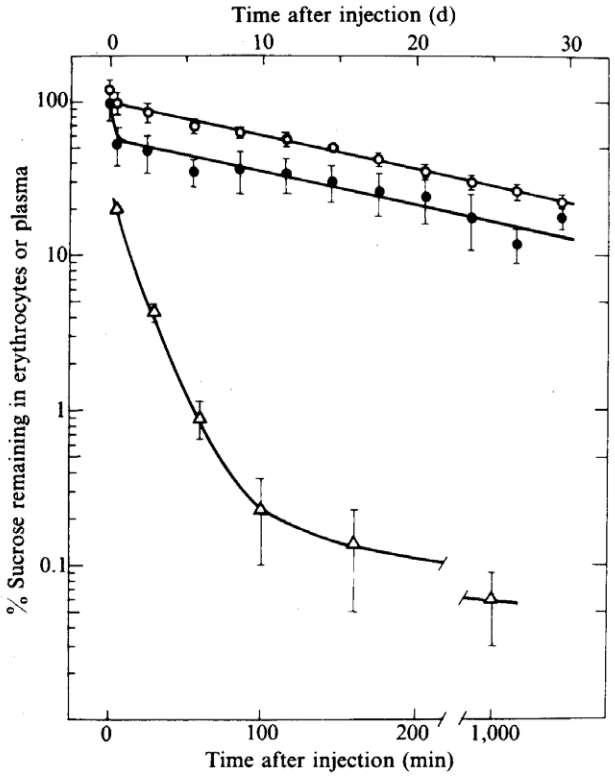

Fig. 2 Elimination of sucrose from circulation. ○, sucrose entrapped in fully resealed erythrocytes; ●, sucrose entrapped in partially resealed erythrocytes; △, free sucrose. In the upper two curves (upper abscissa) erythrocytes were loaded with [^{14}C]-sucrose as described in Table 1, using an 80-μs pulse and incubation periods of 1+5 h (full resealing) or 0.5+0.5 h (partial resealing). The loaded erythrocytes were washed twice with the washing medium and resuspended at about 35% v/v in the same medium. Mice weighing 24–30 g were injected with 150–250 μl of the final suspension which contained approximately 0.3 μmol or 2 μCi of [^{14}C]-sucrose (in one experiment, a lower specific activity of 0.4 mCi mmol^{-1} was used, giving the same result). In the bottom curve (lower abscissa) 7 μmol or 3 μCi of free [^{14}C]-sucrose in 250 μl of the washing medium was injected into each mouse. At intervals after the injection, approximately 40 μl of blood was withdrawn from an eye socket of each mouse, and radioactivities in erythrocytes and plasma were determined as described in Table 1. Total activities in a mouse were calculated by assuming that 30 μl of erythrocytes and 50 μl of plasma were contained per g body weight[10]. Each point is an average of 3–8 mice, the vertical bar being the standard deviation. The mice were apparently in a state of mild shock for a few hours after the injection (haematocrits were abnormally high), and this probably accounts for the slightly over 100% recovery in the top curve at 5 min.

incompletely resealed cells, because *in vitro* experiments on human erythrocytes showed that incubation at 37 °C for 1 h is sufficient to reseal the membranes against sucrose, mannitol or xylitol, but not to small ions such as Na^+ or K^+, which, although very slowly, leak through the membranes (K.K. and T.Y.T., unpublished). The other half of the injected erythrocytes, however, survived in the circulation with the normal half life. When free sucrose was injected into a vein, it quickly disappeared from the plasma, as shown in the bottom curve of Fig. 2. In contrast, the plasma concentration of sucrose in the presence of loaded erythrocytes (the upper two curves) was held at roughly 1/1000 the concentration in erythrocytes for the whole 30-d period.

Many systems, including erythrocytes, liposomes and nylon microcapsules, have been proposed as vehicles of drugs, the aims being prolonged plasma level, possible targeting, or protection against inactivation (for a review, see ref. 11). Of these, erythrocytes seem to be the most attractive because of their natural abundance and long life span (120 d in human[12]) in the circulation. Although past proposals on erythrocytes[13–16] have almost exclusively focused on the use of ghosts (which are difficult to reseal completely) as vehicles of macromolecules such as enzymes, intact erythrocytes could be used as reservoirs of small-sized drugs, as we have shown above. Many drugs are known to be rapidly removed from the circulation with a time course more or less similar to that of free sucrose above[17], and so entrapment in erythrocytes could increase the efficiency of the drugs by sustaining the plasma level for a longer period of time. Specifically, (1) frequency of injection could be reduced; (2) for a drug that is toxic at high concentrations, the entrapment assures a low plasma level under a relatively high dosage; inflammation at the site of injection, for example, could be avoided; (3) the relatively constant plasma level might enable accurate targetting if target cells have a somewhat lower threshold than other cells; (4) for a drug that does not permeate erythrocyte membranes, targeting of the reticuloendothelial system is possible. Loading by the present voltage-pulsation technique is applicable to those drugs with a molecular weight of the order of several hundreds (large pores would be difficult to reseal), those which are soluble in water, those which permeate erythrocyte membranes only slowly, and those which do not impair the viability of erythrocytes.

Finally, we point out that the loading technique described here, which could be applicable to cells other than erythrocytes[5], may be useful not only for medical application, but also for basic researches such as membrane transport and intracellular metabolism.

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