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ELECTRO-INSERTION OF XENO-GLYCOPHORIN INTO THE RED BLOOD CELL MEMBRANE

Youssef Mouneimne[1,2], Pierre-François Tosi[1], Yair Gazitt[1], and Claude Nicolau[1,2]

[1]Biophor Corporation
Texas A&M University Research Park
College Station, Texas 77840

[2]Division of Molecular Virology
Baylor College of Medicine
Houston, TX 77030

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The electroporation technique, with field strengths slightly below the critical value Ec for electroporation of red blood cells (RBC), enables the insertion of xeno-proteins into the RBC membrane without damaging the cells. The electro-insertion has been used to insert biotinylated human glycophorin into human RBC membrane and human glycophorin into murine RBC membrane. Binding anti-human glycophorin antibody (10F7) to the murine RBC bearing human glycophorin indicates extracellular orientation of inserted glycophorin. Insertion of about 10^5 glycophorin molecule per cell has been estimated by whole cell ELISA.

Red blood cells, subjected to a sequence of high electric field square pulses in the presence of a xeno membrane protein and suspended in an insertion buffer, can insert in their plasma membrane up to 3.5×10^5 molecules of xeno-protein per cell without apparent damage to the cells. Thus, a potentially long lived carrier for membrane antigens is obtained which may prove to be useful in different diseases.

The non-destructive insertion of xeno-proteins into red blood cell (RBC) membranes may constitute a way of introducing a long-lived carrier with targeting proteins into circulation. Provided the epitopes present on the inserted protein are properly exposed after insertion and their surface density is sufficient, this carrier system could present some advantages. Work done on low-pH association of the CD4 receptor and of glycophorin in human and mouse RBC has permitted studies concerning the interaction of $CD4^+$-RBC with HIV infected H9 cells and with the HIV-I virus itself (1).

Electrical breakdown of membranes has been studied, based on the assumption that transient pores are generated in cell membrane and using electrical field strength equal to or higher than a critical value Ec (2). This technique is used in order to induce cell fusion (3) or gene transfer (4). We present evidence that field strengths slightly below the critical value Ec are capable of inducing sufficient perturbation in the membrane to enable insertion of a very high number of membrane protein molecules in the RBC membrane.

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MATERIALS AND METHODS

Electro-Insertion Instruments:

The pulse generator used was a 606 Cober device. The teflon chamber used in electro-insertion experiments is cylindrical, 1.2 cm diameter, with each end formed by 1.2 cm x 2.5 cm stainless steel electrodes, the electrode gap being 0.2 cm. Potential and current are monitored by a Nicolet 2090 digital oscilloscope.

Chemicals:

The electroporation medium (EM) contained 0.3 M Mannitol and 6 mM Histidine, pH=7.8 . The incubation medium (IM) consisted of 4.844g/l KCl, 2g/l glucose, 2g/l BSA, 4.08g/l NaCl buffered with 5mM phosphate buffer pH 7.4. The anti-human glycophorin monoclonal antibody 10F7 was kindly provided by Dr. R.H. Jensen from the Biomedical Sciences Division, Livermore National Laboratory, University of California (5). Fluorescein conjugated Rabbit Anti-Mouse IgG F(ab')2 Fragment was obtained from Accurate Chemical and Scientific Corporation (Westbury, N.Y.).

Biotinylation of Glycophorin:

Human glycophorin MN (MW=31,000 (6) (Sigma,St. Louis,MO)) is dissolved in sodium bicarbonate 0.1 M to 0.5 mg/ml. At 4°C and while stirring, equal volume of 1 mg/ml of succinimidyl D-biotin, (Molecular Probes Inc., Eugene, OR), in dimethylformamide is added. The solution is incubated overnight at 4°C with stirring, and then dialysed four times against one liter of 0.15 M KCl and once against one liter of 0.3 M Mannitol, within 24 hours at 4°C (7).

Whole Cell Enzyme-Linked Immunosorbent Assays (ELISA):

In order to determine the number of inserted glycophorin molecules per red blood cell, an ELISA test was used to detect the glycophorin-biotin molecule by alkaline phosphatase-avidin conjugate (ICN Immunobiologicals, Lisle, IL). ELISA plates (Costar, Cambridge, Ma) were used and color intensity was quantified by a DYNATECH Mr 700 device (Chantilly, Va) with a 410 nm filter.

First, a standard curve of optical density versus concentration of biotinylated glycophorin is obtained and corrected for the background of unbiotinylated glycophorin. A serial dilution of suspended cells bearing glycophorin-biotin in PBS buffer pH 7.4 was made; plates were kept at 37°C for 90 minutes with the lid open to allow evaporation of PBS and cell adherence. The cells were washed three times with a washing buffer (PBS(5 mM phosphate,145 mM NaCl pH 7.4), 1% BSA, 0.5% Tween 20). The plates were blocked by adding a given volume of washing buffer and incubating them 1 h at 37°C. After three washes in the washing buffer, Alkaline phosphatase-avidine conjugate was added at a dilution of 1:10000 and incubated for 1 hour at 37°C. After three washings with the washing buffer and three additional washings with distilled water, the substrate buffer (0.4 mg/ml of para-nitrophenyl phosphate in diethanolamine DEA buffer pH 9.8) was added and incubation followed 1 hour at room temperature. Finally, the reaction was stopped with 2.5 N NaOH and the plates were read at 410 nm. Color intensity was corrected for cell number per well, following cell count in a Coulter Counter (ZM Channalyzer 256).

ELISA, similar to that described above, was performed in order to assay the human glycophorin molecules inserted in mouse RBC. The 10F7 anti-human glycophorin antibody was added (1:10000 dilution) for 1 hour incubation at 37°C, followed by washing of unbound antibody. A 1:10000 dilution of Alk. phos.AP F(ab')2 Fragment goat anti-mouse IgG (Jackson Immuno Research Lab. Inc. West Grove, PA) was added, and followed by 1 hour incubation at 37°C. The final steps of washing and substrate addition were the same as above.

Flow Cytometry:

Flow cytometry was performed on a Coulter EPICS Profile instrument. Fluorescein was measured with a 525 nm band pass emission filter. Alignment of the instrument was performed using 5 micrometer diameter Immunocheck beads (Coulter,Hialeah,Fl). Using Immunobright beads (Coulter) with different fluorescence intensity, the high voltage of the photomultipliers was set up to obtain the characteristic fluorescence peak at the channels of these fluorescent standards. The histograms collected for analysis were: 1) 90°-side scatter versus forward angle scatter; 2) log of green fluorescence versus number of cells.

Electro-Insertion:

Red cells from freshly drawn human or murine blood were washed three times in PBS buffer pH 7.4 and two times with EM. After addition of 0.5 mM of $CaCl_2$ and 0.5

mM of $MgCl_2$, the cells were incubated 5 minutes on ice and 5 minutes at 37°C (Hematocrit 90%). Immediately, a given volume of the solution of glycophorin-biotin was added to the human red blood cells (or a solution of 4 mg/ml of human glycophorin MM (Sigma) in 0.15 M KCl and a given volume of EM was added to the murine red blood cells) in a ratio of 5 micrograms glycophorin to 10^6 cells. This suspension was then centrifuged and the supernatant removed to get a hematocrit of 16%. Fourty microliters of the final suspension was subjected to electrical field pulses at 4°C. The electro-insertion was accomplished by exposing the cell suspension to eight successive electric field square pulses at 5 seconds interval. Each pulse was 400 sec long and 1.3 kV/cm height for human red blood cells or 1.5 kV/cm for murine red blood cells. Both field intensity values were lower than the critical field intensity Ec for electroporation of human or mouse RBC (8,9,10).

Immediately after the pulse, the cells were incubated at 37°C for one hour, plus one more hour at 37°C, after addition of IM. The cells were then washed one time with the IM and two times with PBS buffer pH 7.4.

After insertion of biotinylated glycophorin into the human RBC membrane, the washed cells were incubated with Avidin-FITC (Molecular Probes Inc. Eugene, OR) for 30 minutes at room temperature and then washed three times in PBS buffer pH 7.4.

After insertion of the glycophorin in the murine RBC membrane, the washed cells were incubated with 2 microliters of 0.17 mg/ml 10F7 anti-human glycophorin monoclonal antibody (5) for 30 minutes at room temperature and then washed three times with PBS buffer pH 7.4 to be incubated finally with 6 microliters of 0.7 mg/ml fluoresceine conjugated affinipure F(ab')2 fragment Rabbit Anti-mouse IgG (Jackson Immunoresearch Laboratories Inc, West Grove,PA) at room temperature for additional 30 minutes. The cells were finally washed three times in PBS buffer pH 7.4. Control samples where human or murine RBC have been subjected to all the steps, but without electrical pulses, were used as references for each corresponding case.

RESULTS

Examination of glycophorin-inserted RBC under the fluorescence microscope, with blue light excitation, shows highly fluorescent agglutinated cells (Fig.1), while cells of the control sample not subjected to electrical pulses reveal weak punctual fluorescence

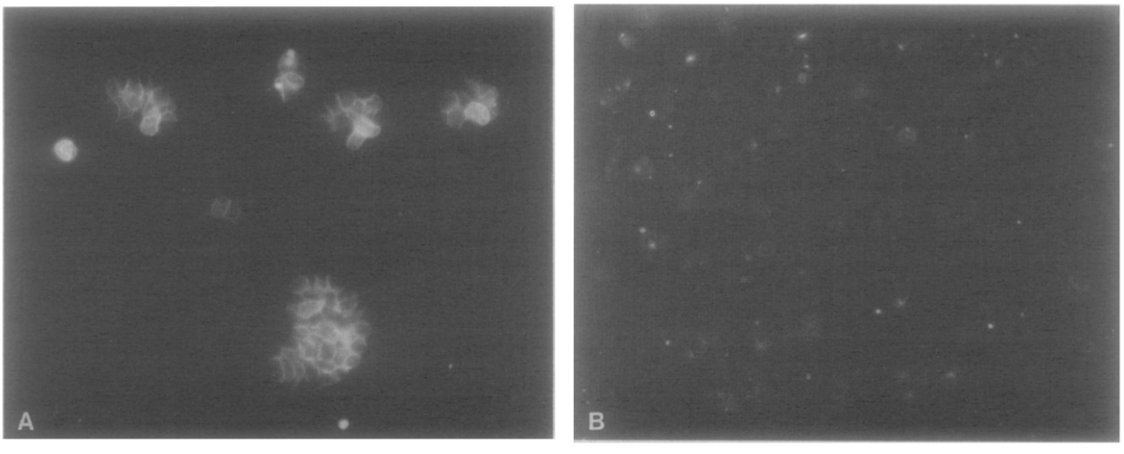

Fig.1 : A: Agglutination of human RBC bearing electro-inserted biotinylated lycophorin after reaction with avidin-FITC, due to cell cross linking via avidin.
B: Human RBC incubated with biotinylated glycophorin,not subjected to electrical pulses,after reaction with avidin-FITC.

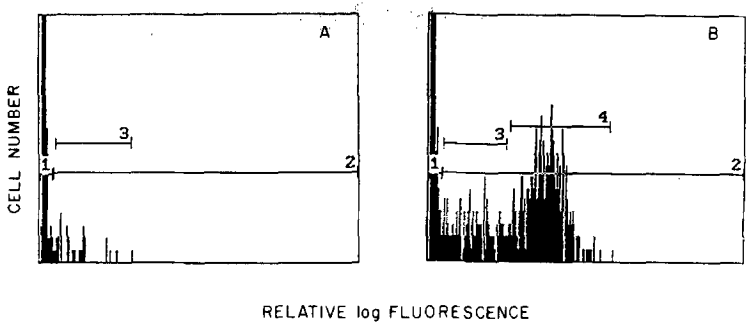

Fig.2 : Flow cytometry histograms:
A: Human RBC incubated with biotinylated glycophorin not subjected to electrical pulses reacted with avidin-FITC.
B: The same sample subjected to electrical pulses indicating the presence of $5x10^4$ fluorescein molecules per cell.

on their membrane. The agglutination is due to the bivalent avidin molecule bridging between cells having integrated biotinylated glycophorin. This agglutination indicates that integrated glycophorin is stably inserted in the RBC membrane. The results of the whole cell ELISA indicate that about $3.5x10^5$ glycophorin molecules per cell are inserted.

Using the flow cytometry, the efficiency of insertion or the estimation of protein-integrated per cell is difficult to conduct on the whole sample because of the agglutination of these cells. For single cells, however, the average fluorescence intensity per cell corresponded to a mean value of about $5x10^4$ fluorescent molecules (fluoresceine) per cell (Fig.2). This number is in agreement with the estimated level of glycophorin insertion necessary to induce agglutination. In order to rule out nonspecific binding of Avidin-FITC, an additional control experiment has been performed where the RBC have been electropulsed exactly in the same condition but in the absence of glycophorin-biotin. No fluorescence or agglutination could be observed upon addition of Avidin. This indicates that the agglutination is not due to an artifact.

We have used the agglutination of RBC bearing glycophorin-biotin in order to get a rough estimation of the percentage of RBC which inserted the protein. From the sample subjected to the electrical breakdown, two aliquots of equal volume were collected. To the first, a given volume of Avidin-FITC was added, while the same volume of PBS buffer pH 7.4 was added to the second. After 30 minutes' incubation at room temperature, the cells were counted using a Coulter Counter (ZM Channelyzer 256). The count of the sample where Avidin-FITC was added yields a main channel count which is lower than that where PBS was added. The difference is due to the agglutination of cells bearing glycophorin-biotin. This corresponds to 30% of the whole cell population. The agglutinated cells are shown in a second new peak (Fig.3).

This shows that 30% of the red blood cells have inserted the glycophorin with at least 10^5 molecules per cell, the other cells which do not agglutinate have inserted less or not at all.

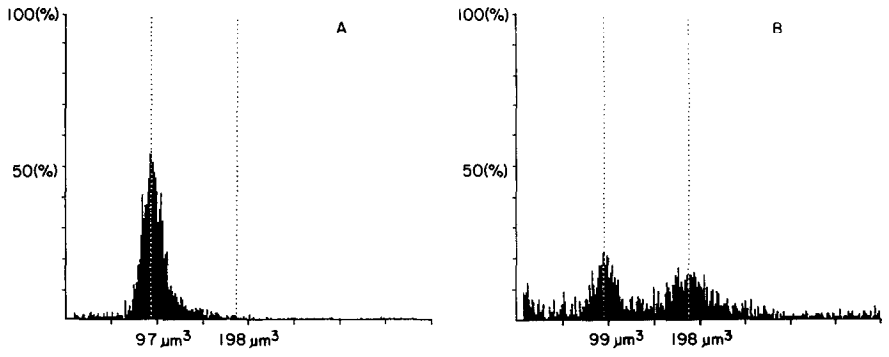

Fig.3 : Channelyzer histogram of human RBC subjected to electro-insertion of biotinylated glycophorin:
A: Addition of phosphate buffered saline pH 7.4 .
B: Addition of avidin-FITC.

Murine Red Blood Cell-Glycophorin

The observation of the fluorescence of the sample under the fluorescence microscope with blue light excitation shows also highly fluorescent cells which agglutinate upon addition of anti-human glycophorin antibody 10F7 (Fig.4). Binding of 10F7 to the glycophorin inserted murine RBC indicates extracellular orientation of inserted glycophorin (5). On the other hand, no fluorescence or agglutination have been observed upon electroporation of murine RBC in the absence of glycophorin, following addition of antibody .

The ELISA results indicate that about $4x10^5$ glycophorin molecules per cell were inserted, whereas the flow cytometry analysis of single cells fluorescence indicates a mean value of about $2.5x10^5$ fluorescent molecules (fluorescein) per cell (Fig.5.).

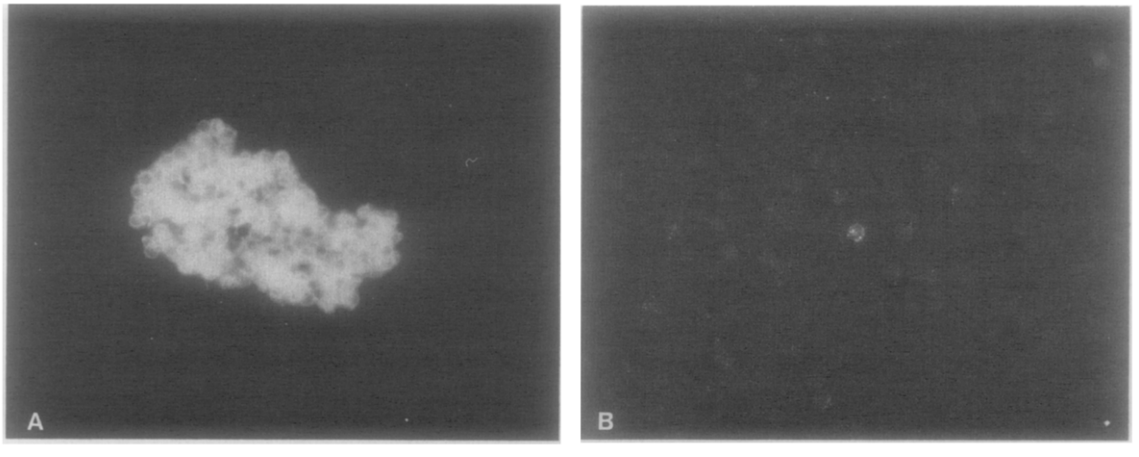

Fig.4 : A: Agglutination of murine RBC bearing electro-inserted human glycophorin after reaction with 10F7 anti-human glycophorin antibody and a secondary fluorescent antibody (rabbit antimouse-FITC).
B: Murine RBC incubated with human glycophorin,not subjected to electrical,pulses reacted with fluorescent antibodies.

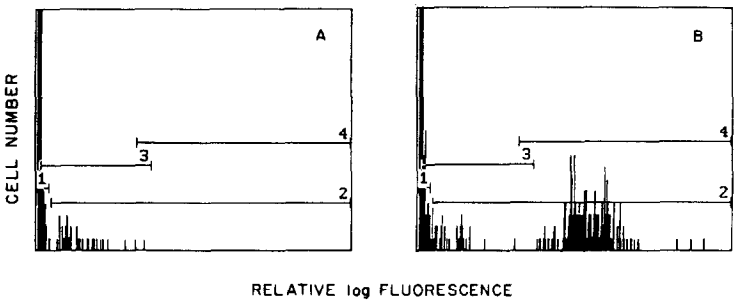

Fig.5 : Flow cytometry histograms:
A: Murine RBC incubated with human glycophorin, not subjected to electrical pulses,reacted with 10F7 anti-human glycophorin antibody and rabbit antimouse-FITC antibody.
B: The same sample subjected to electrical pulses indicating the presence 2.5×10^5 fluorescein molecules per cell.

DISCUSSION

The process of electro-insertion we described is a new technique based on a specific phenomenon corresponding to a direct effect of the electric field on the RBC membrane. In order to get unlysed cells, the electric field intensity must be lower than the critical field for electroporation, Ec. If it is below this value, the electric field induces perturbations in the structure of the RBC membrane (11,12,13,14); these transient perturbations may be the result of the insertion. To attain the insertion, preincubation of RBC with calcium and magnesium ions at low concentration with low monovalent ions concentration is crucial. Ca^{++} and Mg^{++} are responsible for the glycophorin association to the plasma membrane of RBC via electrostatic interactions (15,16). Thus, the protein molecules, having hydrophobic sequence and being very close to the membrane, may be inserted.

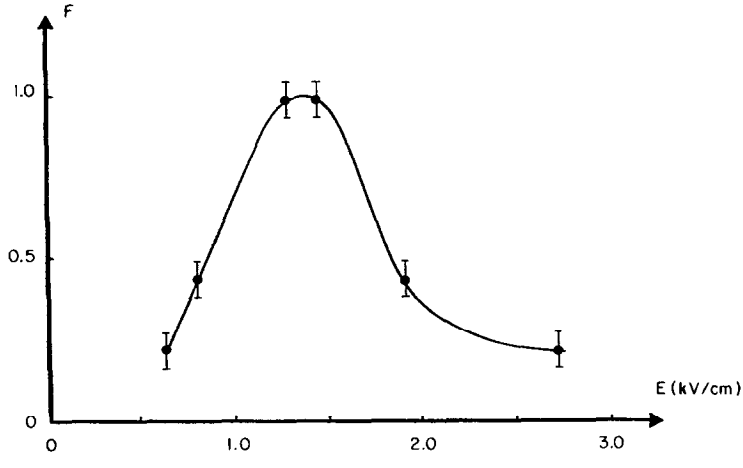

Fig.6 : Determination of the optimal electric field intensity (E) needed to induce electro-insertion of glycophorin in murine RBC membrane. F is the ratio of the number of inserted cells at a given field to the number obtained at the optimum field (E=1.5 kV/cm) measured by flow cytometry.

Working below the electroporation field intensity Ec is necessary for insertion, but it is not sufficient to reach optimum efficiency. A narrow range of field strengths has to be used. In the case of murine RBC-glycophorin, the best insertion efficiency is obtained in the range of 1.5 kV/cm, as measured by flow cytometry (Fig.6). Murine RBC subjected to such conditions show normal lifetime when injected in mouse (results not shown).

The insertion is not due to heat because the energy involved in the electropulsation is negligible, and neglecting heat dissipation, the increase of the temperature does not exceed 1°C. Moreover, heating the cells in the presence of glycophorin up to 40°C does not lead to the glycophorin insertion.

This technique could be applied to many animal cells with different proteins presenting hydrophobic sequence, especially the CD4 receptor (manuscript in preparation).

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