Biochimica et Biophysica Acta, 1027 (1990) 53–58
Elsevier

BBAMEM 74934

Electroinsertion of full length recombinant CD4 into red blood cell membrane

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(Received 15 January 1990)

Key words: Electroinsertion; Antigen CD4; Recombinant CD4; Glycophorin; Erythrocyte membrane

Electroinsertion is a novel technique of protein implantation in cell membranes using electrical pulses, of field strength between 1.3 kV/cm and 2.1 kV/cm and up to 1 ms duration. The full length recombinant CD4 receptor could thus be inserted in human and murine red blood cell (RBC) membranes. 100% of the RBC subjected to this procedure were shown to expose different CD4 epitopes after electroinsertion. An average of 5000 epitopes per cell has been detected by immunofluorescence assay using flow cytometry and whole cell ELISA. CD4 electroinserted in red blood cell membranes showed upon reaction with monoclonal antibody significant patching similar to that observed in T4 cells expressing CD4. Furthermore, the fluorescent enhancement coming from accumulation of immune complex phycoerythrin-antiphycoerythrin was similar for both native CD4 on T4 cells or CD4 electroinserted into erythrocyte membrane. Attempts to electroinsert proteins without a membrane spanning sequence have consistently failed, suggesting that adsorption is not responsible for the observed phenomena.

Introduction

The electroinsertion process consists of the application of pulsed electrical field, of microseconds duration, on a suspension of cells, in the presence of a selected membrane protein having a membrane spanning sequence [1]. It results in the implantation of the protein in the cells' plasma membrane.

In previous studies we reported the electroinsertion of human glycophorin into mouse RBC membranes. Insertion of 10^5 molecules per cell was observed, but only 30% of the cells had inserted glycophorin [1]. On the basis of these results, we further developed the electroinsertion technique with the aim to insert membrane proteins in 100% of the RBC subjected to the electrical pulses. The rationale behind these experiments is that insertion of CD4 into RBC membrane by a non damaging method may provide a long lived CD4 carrier in circulation. These RBC-CD4$^+$ might attach circulating HIV or gp120-expressing HIV-infected cells and form aggregates with them, subsequently phagocyted by the reticuloendothelial system [2].

After brief exposure at low pH (4.7), CD4 associates with human RBC [3] and such RBC-CD4$^+$ were shown to bind specifically to gp120-coated wells and to aggregate with gp120-expressing cells (Arvinte et al., submitted to J. AIDS). Nevertheless, the low pH treatment of the RBC, under conditions of maximum CD4 association, leads to a significant reduction in the RBC lifespan in mouse (results not shown). RBC subjected to pulsed electric fields have been shown to maintain a normal life span [4,5], and the results previously obtained with glycophorin [1] indicated that significant levels of insertion may be attained. We report here the electroinsertion of CD4 in RBC membranes leading to an immunologically active CD4-carrier.

Abbreviations: CD4, antigen on helper-inducer T lymphocytes, monocytes; RBC, red blood cells; rCD4, recombinant CD4; RBC-CD4$^+$, red blood cells with CD4 inserted in their membrane; HIV, human immunodeficiency virus; gp120, surface antigen of HIV; PBS 7.4, 5 mM sodium-phosphate buffer, 145 mM NaCl (pH 7.4); OKT4A, OKT4C, OKT4D, Leu 3a, BL4/10T4, anti-CD4 monoclonal antibodies; Gam-IgG-PE, goat antimouse immunoglobulin G, phycoerythrin labelled; Anti-PE, antiphycoerythrin monoclonal antibody (clone PE-85); PEAPE, phycoerythrin-antiphycoerythrin complex; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; 10F7, anti-human glycophorin monoclonal antibody; Tris, tris(hydroxymethyl) aminomethane; CEM, human acute lymphoblastic leukemia cells expressing CD4 on their surface; ELISA, enzyme-linked immunosorbent assays; mAb, monoclonal antibody; β2-m, β_2-microglobulin.

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Materials and Methods

Chemicals

The electroinsertion medium consisted of 0.14 M NaCl and 10 mM Na_2HPO_4/NaH_2PO_4 adjusted to pH 8.8 by NaOH. The washing buffer contained 0.145 M NaCl and 5 mM phosphate at pH 7.4 (PBS 7.4). The anti-CD4 monoclonal antibodies including OKT4A, OKT4C and OKT4D were provided by P. Rao from Ortho Diagnostic Systems, Inc. (Raritan, NJ, U.S.A.), Leu 3a was from Becton Dickinson Immuno-Cytometry System (Mountain View, CA, U.S.A.) and the BL4/10T4 was from AMAC, Inc. (Westbrook, ME, U.S.A.). Phycoerythrin conjugated affinipure F(ab')2 fragment goat anti-mouse IgG (Gam-IgG-PE) and alkaline phosphatase AP F(ab')2 goat anti-mouse IgG was obtained from Jackson Immuno Research Laboratories (West Grove, PA, U.S.A.). Antiphycoerythrin monoclonal antibody, clone number PE-85 (Anti-PE), fluorescein isothiocyanate isomer I (FITC), bovine serum albumin (BSA), human glycophorin type MM and human β_2-microglobulin were from Sigma (St. Louis, MO, U.S.A.). FITC-labeled rabbit-anti-human β_2-microglobulin antibodies was from Accurate Chemical and Scientific Corporation (Westbury, NY, U.S.A.). The anti-human glycophorin monoclonal antibody 10F7 was kindly provided by Dr. R.M. Jensen from the Biomedical Sciences Division, Livermore National Laboratory, University of California, and it has been described [6].

Electroinsertion

The pulse generator used was a 606 Cober device. The teflon chamber used in electroinsertion experiments is cylindrical, 1.2 cm diameter, with each end formed by 1.2 cm × 2.5 cm stainless steel electrodes, the electrode gap being 0.2 cm. Potential and current were monitored by a Nicolet 2090 digital oscilloscope. During the experiment, the electroinsertion chamber was held at 37°C using a circulating water thermostat.

Human erythrocytes were separated from fresh whole blood of healthy donor with citrate buffer as anticoagulant. Mouse erythrocytes were separated from heparinized fresh whole blood obtained from BALB/C strain mouse by retro orbital sinus puncture. The blood was washed three times with the electroinsertion medium, and an erythrocyte stock suspension was prepared.

Highly purified, lyophilized, full length recombinant CD4 obtained in our laboratory [7] was dissolved into the electroinsertion medium. The CD4 solution, at 8 mg/ml concentration, was added to the erythrocyte suspension. After 20 min incubation on ice, the temperature was raised to 37°C and four square electrical pulses, 1 ms long, were applied at 15-min intervals. The field intensity was 1.3 kV/cm for human RBC and 2.1 kV/cm for mouse RBC. After pulse application, the cells were washed three times in PBS 7.4 and kept at 4°C.

A control sample where RBC were subjected to all steps of the described procedure but CD4 was replaced by BSA, was used as reference. Another control for the possibility of insertion of protein without membrane spanning sequence was performed replacing CD4 by β_2-microglobulin.

In a control experiment aimed at showing that patching of electroinserted protein occurred only upon reaction with monoclonal antibodies, FITC was covalently bound to human glycophorin [8]. Briefly, glycophorin was dissolved at 10 mg/ml in 20 mM Tris-HCl (pH 8.9), and FITC was added as a powder at a ratio of 100 μg per mg protein. After 4 h on ice, free fluorescein was removed by chromatography on Sephadex G-25. The FITC-glycophorin fractions were collected and desalted by four washings with distilled water (Amicon micropartition system). The immune reactivity of FITC-glycophorin was verified by immunoblotting. The FITC-labeled glycophorin was electroinserted into mouse RBC membrane using the same procedure as above.

Immunofluorescence assay

To assay different epitopes of the CD4 glycoprotein at the surface of RBC membranes, $1 \cdot 10^7$ cells were incubated, at room temperature during 30 min, with 2 μg of different monoclonal antibodies, OKT4A, OKT4C, OKT4D, BL4/10T4 or Leu 3a in a total volume of 50 μl PBS 7.4, washed twice and incubated with 3 μg of secondary antibody Gam-IgG-PE in 50 μl PBS 7.4. After two washings with PBS 7.4, cells are kept at 4°C prior to observation by fluorescence microscopy and flow cytometry analysis.

To compare the inserted CD4 into the red blood cells and the native CD4 into human cells (acute lymphoblastic leukemia cells, line CEM-CM3), we studied the possibility to accumulate successive immune complex as previously described [9]. Briefly, RBC-$CD4^+$ or CEM cells already stained with OKT4D and Gam-IgG-PE were incubated with a mouse monoclonal antibody anti-PE (clone PE-85), washed and then incubated with Gam-IgG-PE. The enhancement of the fluorescent signal was quantified by flow cytometry after application of every new cycle of PEAPE complexes.

Flow cytometry

Flow cytometry was performed on a Coulter EPICS profile instrument. The phycoerythrin fluorescence emission was measured with a 575 nm band pass emission filter. Alignment of the instrument was performed using 5 μm Immunocheck beads (Coulter). A standard curve of fluorescence intensity versus the number of fluorescent molecules is obtained at the same photomultiplier-voltage used to measure the fluorescence of

labeled RBC-CD4$^+$. The histograms collected for analysis were:

(1) 90°-side scatter versus forward scatter;

(2) log of red fluorescence versus number of cells.

Whole cell enzyme-linked immunosorbent assays (ELISA)

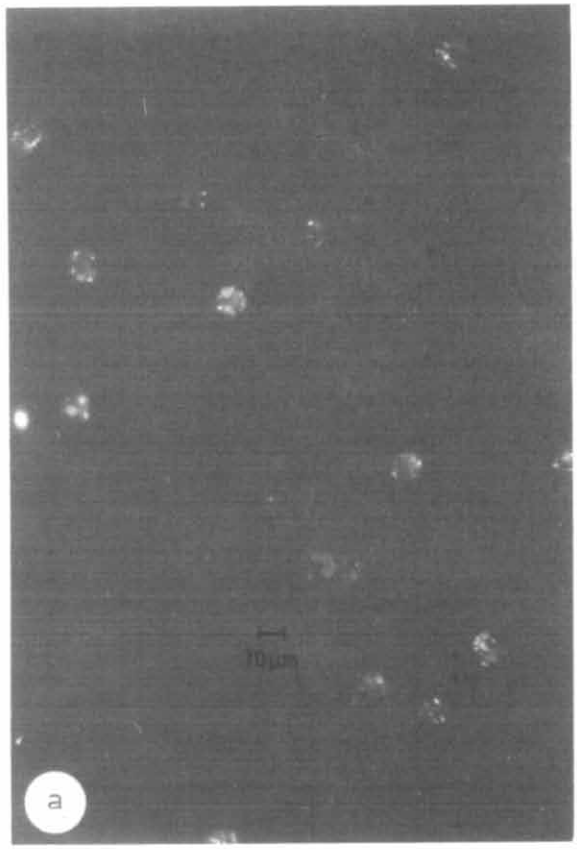
Besides the flow cytometry technique, an ELISA test was developed in order to estimate the number of inserted CD4 molecules in the red blood cell membrane. ELISA plates (Costar, Cambridge, MA, U.S.A.) were used and the color intensity was quantified by a DYNATECH MR 700 device (Chantilly, VA, U.S.A.). First, a standard curve of optical density versus concentration of CD4 is obtained and corrected for the background of BSA. A serial dilution of suspended cells bearing CD4 in PBS 7.4 was made. Serial dilutions of CEM (a human T-lymphoblast cell line exposing CD4 on its plasma membrane) cells and reference RBC were made too. Plates were kept at 37°C for 90 min with the lid open to allow evaporation of water and cell adherence. The cells were washed three times with a washing buffer (PBS 7.4, 1% BSA, 0.5% Tween 20). The plates were blocked by adding 100 μl of washing buffer and incubating 1 h at 37°C. After three washes with the washing buffer, OKT4A monoclonal antibody was added at a dilution of 1 : 5000 and incubated for 1 h at room temperature. The cells were washed thrice with washing buffer, and Alkaline phosphatase AP F(ab')2 Goat Anti-Mouse IgG was added at a dilution of 1 : 5000. After one hour incubation at room temperature the plates were washed three times with the washing buffer and three additional times with distilled water. Then the substrate buffer (0.4 mg/ml of *p*-nitrophenyl in diethanolamine DEA buffer (pH 9.8)) was added and incubation followed one hour at room temperature. Finally the reaction was stopped with 2.5 M NaOH and the plates were read at 410 nm filter. Color intensity was corrected for cell number per well, following cell count in a Coulter Counter (ZM Channelyzer 256).

Results and Discussion

The CD4 molecules inserted into the red blood cell membrane were able to react with the following different anti-CD4 monoclonal antibodies: OKT4A, OKT4C, OKT4D, Leu 3a, and BL4/10T4. These monoclonal antibodies are specific for different epitopes of the CD4 molecule [10,11]. It appears that the electroinserted CD4 exposes the different active epitopes in the proper orientation including the Leu 3a and OKT4A epitopes characteristic for the binding of the viral envelope protein gp120 [12,13]. Knowing that these epitopes are distributed over all the external sequence of CD4 [11,12,14,15] and that the fluorescence intensity, measured by flow cytometry, upon reaction of each monoclonal antibody separately with the same RBC-CD4$^+$ sample was identical, it appears that electroinserted CD4 exposes the different active epitopes in the proper orientation and ratio. This further supports the view that adsorption of CD4 is unlikely to be the result of subjecting RBC and CD4 to electrical fields. Should the CD4 molecules have been adsorbed, some epitopes would have been less detectable than others. When ^{125}I-labelled CD4 is electroinserted in RBC membranes, only $\approx$ 70% of the radioactive material shows also the epitopes when assayed with monoclonal, anti-CD4 antibodies (Nicolau et al., manuscript in preparation). This observation would suggest that $\approx$ 30% of electroinserted CD4 is in the 'wrong' orientation. Finally, the presence of the different epitopes indicates that the CD4 molecule was not denatured during the electroinsertion procedure.

Examination of the RBC-CD4$^+$ under the fluorescent microscope, with blue light excitation (488 nm), yielded the images shown in Figs. 1a–1c. The reference sample, where CD4 was replaced by BSA, did not show any fluorescence (figure not shown). The punctate fluorescent pattern observed on the RBC-CD4$^+$ after immunofluorescence reaction is due to the patching of the inserted CD4 receptors upon reaction with monoclonal antibodies (Leu 3a and Gam-IgG-PE). The same phenomenon is observed with CEM cells (a human T-lymphoblast cell line) upon reaction with mAb (Leu 3a and a Gam-IgG-PE) (Fig. 1e). In order to check that patching occurred only after reaction with mAb, FITC-labeled human glycophorin was electroinserted in mouse RBC membrane. It showed uniformly fluorescent cells under the fluorescent microscope. Upon reaction with 10F7 anti-human glycophorin monoclonal antibody, patching was observed (data not shown). This indicates that the patchy distribution does not occur upon insertion of the antigen into the RBC membrane, it only happens due to antibody cross linking and is possible because of the lateral mobility of the inserted antigen. Fluorescence recovery after photobleaching studies have shown that fluorescence probes adsorbed on cell surfaces [16] did not display lateral mobility. Patching of CD4 may be considered as a clear indication of insertion of CD4 molecules in the RBC lipid bilayer. The inserted receptor diffuses in the plane of the bilayer, just as it does in its natural environment, the T4 lymphocyte plasma membrane [17,18].

After reaction of RBC-CD4$^+$ with mAB Leu 3a and Gam-IgG-PE, the flow cytometry analysis shows single peak of fluorescence with a complete shift of the cell population towards the higher fluorescence intensity (Fig. 2). This means that the red blood cells in the population subjected to the electroinsertion procedure expose CD4 molecules. Fig. 2 indicates a mean concentration of 4800 epitopes per mouse RBC. The reference sample showed the normal background level of the flow cytometry measurements.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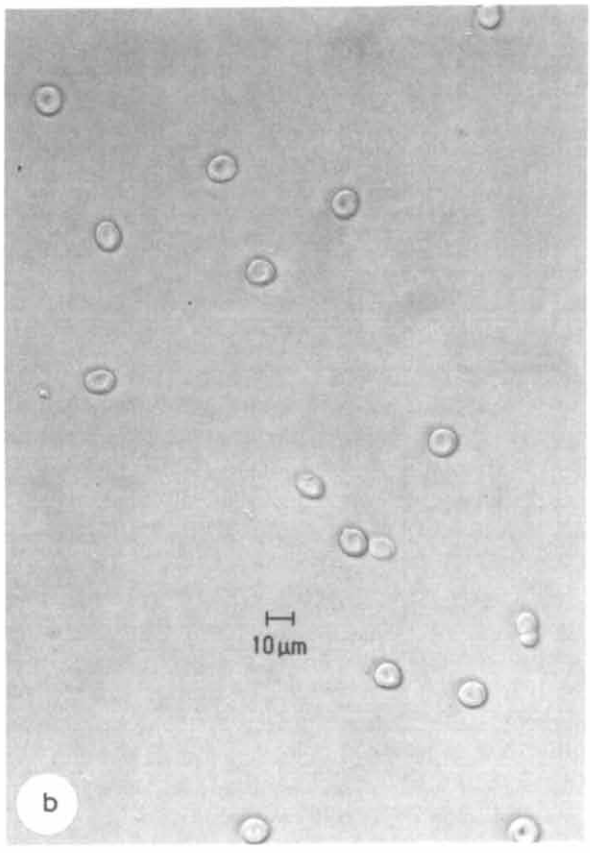
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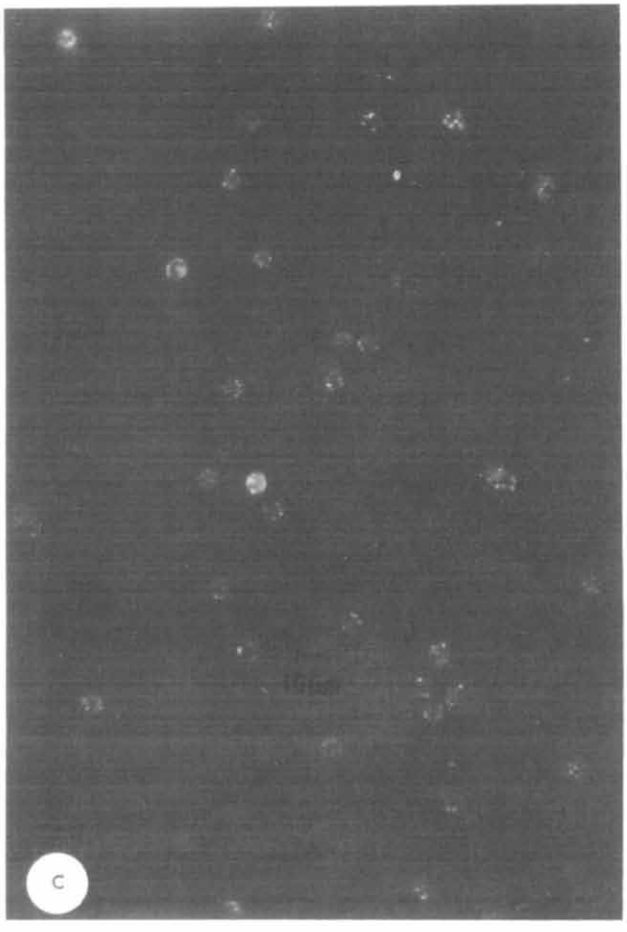
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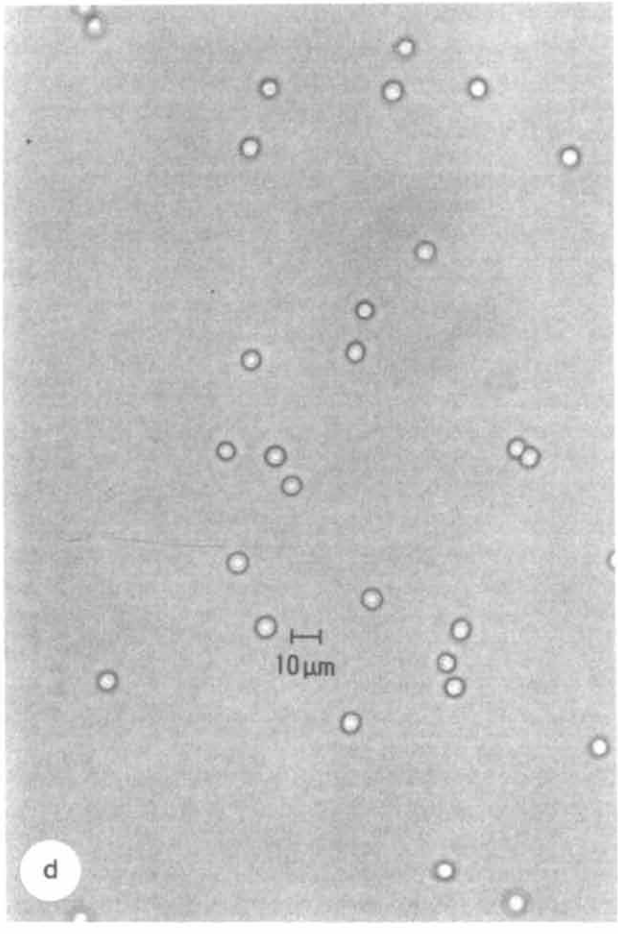
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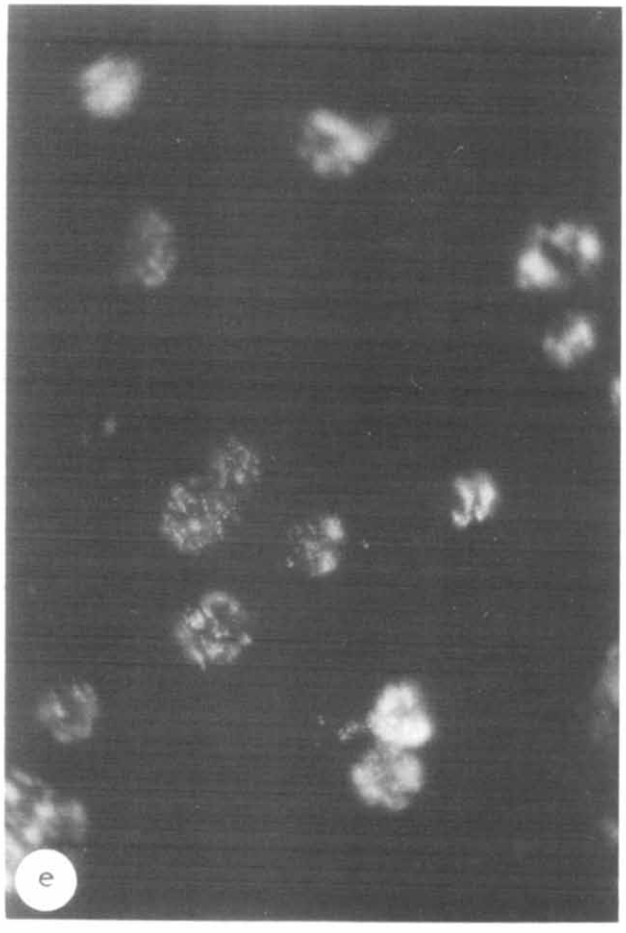

Fig. 1. Immunofluorescence imaging of RBC-CD4$^+$ (a,c) and CEM cells (e). RBC-CD4$^+$ and CEM cells were stained with monoclonal antibody Leu 3a then Phycoerythrin-conjugated goat anti-mouse. (a) Human RBC-CD4$^+$. Excitation wavelength 488 nm. (b) The same human RBC-CD4$^+$. Direct light. (c) Mouse RBC-CD4$^+$. Excitation wavelength 488 nm. (d) The same mouse RBC-CD4$^+$. Direct light. (e) CEM cells. Excitation wavelength 488 nm. Notice the capping of CD4 upon reaction with monoclonal antibodies in both cases (RBC-CD4$^+$ and CEM cells). Fluorescence microscope VANOX0T, Olympus Inc. (San Antonio, TX, U.S.A.).

The results of the whole cell ELISA indicate that an average of 5300 CD4 molecules were inserted in the membrane of mouse cells. The reference sample had the background level, and the CEM cells tested showed $5 \cdot 10^4$ CD4 molecules. The 10% difference between the ELISA and the flow cytometry results could be due to differences inherent to these techniques; the ELISA measures the average of the whole sample while the flow cytometry gives a statistical average of the individually measured cell where the sensitivity is poorer for fluorescence intensities corresponding to less than 1000 epitopes.

Fig. 3 shows the variation of fluorescence of RBC-CD4$^+$ when successive immune complex were accumulated. The similarity between this curve and that of CEM cells treated with the same procedure, shows that

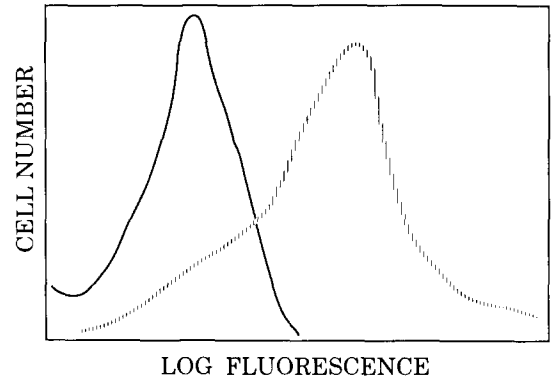

Fig. 2. Flow cytometry histograms. Mouse RBC subjected to electroinsertion with BSA (solid line) or CD4 (broken line) were stained with monoclonal antibody Leu 3a and subsequently with phycoerythrin conjugated goat anti-mouse secondary antibodies. Histograms were analyzed for red fluorescence intensity and cell number. The mean peak channel of the control RBC and RBC-CD4$^+$ were respectively 5.3 and 48.1. This corresponds to a mean peak of 4800 epitopes per cell. Epics Profile flow cytometer; Coulter Epics flow cytometer (Hialeah, FL, U.S.A.).

the inserted CD4 molecule into the RBC membrane behaves like the naturally expressed CD4 on CEM cells, where three cycles PEAPE can be formed before saturation. This may be additional proof that CD4 molecules are inserted into the RBC membrane and not adsorbed. Adsorbed CD4 on the surface of RBC would not withstand the mechanical stress caused by the four successive immune complex accumulated and would very probably be desorbed from the RBC surface.

In our experience, only membrane proteins, for example full length rCD4 or glycophorin, could be electroinserted. Proteins without membrane spanning sequence such as human β_2-microglobulin could not be electroin-

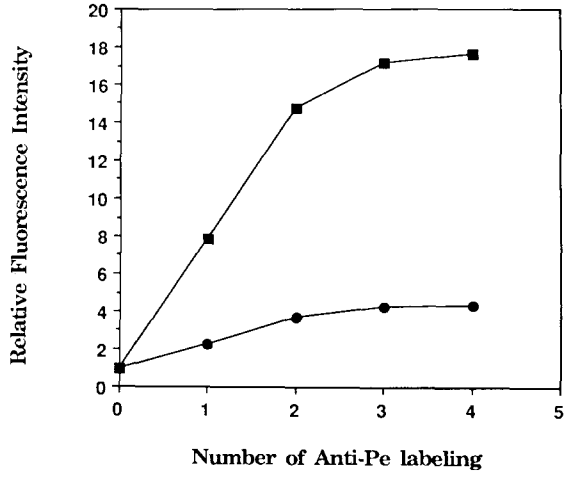

Fig. 3. Variation of fluorescence intensity with the number of Anti-PE successive labeling of RBC-CD4$^+$ (●) and CEM cells (■). Mouse RBC-CD4$^+$ and CEM cells, labeled with OKT4D and Gam-Ig-PE, were labeled successively with Anti-Pe and Gam-IgG-PE till saturation. The fluorescence intensity was plotted relatively to the intensity of the first Gam-IgG-PE antibody.

serted into RBC membrane. β_2-Microglobulin (β2-m) is a soluble protein (M_r 11 800) that was found associated with cell surface antigens of the major histo compatibility complex [19, 20]. Attempts to electroinsert β2-m as a control (similar conditions as for CD4, see Materials and Methods) failed. No β2-m epitopes following interaction with anti-β_2 antibodies were ever detected by flow cytometry analysis or by fluorescent microscope observations (data not shown) on the RBC subjected to this procedure in the presence of the protein. Our failure to associate β2-m at the surface of RBC which allowed insertion of both CD4 and glycophorin suggests that this technique does not favor nonspecific adsorption of proteins on the plasma membrane of RBC.

The electroinsertion technique provides a procedure which enables insertion of the CD4 and other membrane proteins into the RBC membrane, without damage to the cell and with a substantial fraction of the molecules properly exposed. The inserted CD4 is immunologically active and capable of reaction with several monoclonal antibodies, well established for functional epitopes on the native molecule. The number of functional epitopes detected by flow cytometry or ELISA had an average of about 5000.

The patching phenomena observed upon reaction with monoclonal antibodies and secondary fluorescent antibodies, as well as the ability to accumulate several immune complex, indicate that the identified epitopes are from membrane inserted CD4.

The electroinsertion of specific receptors into the RBC membrane may offer a way to construct long-lived, targeted carriers or cellular agents with potential therapeutic use, besides its possible contribution to the understanding of protein–membrane interaction.

Acknowledgement

The excellent technical help of P. Clark Mertes is gratefully acknowledged.

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