Stability and immunological reactivity of recombinant membrane CD4 electroinserted into the plasma membrane of erythrocytes

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Abstract Concentration-dependent electroinsertion of recombinant human membrane CD4 in human erythrocytes shows a saturation at an average of about 3,500 inserted CD4 epitopes per cell, detectable by flow cytometry. The erythrocyte recovery drops to 10% at this high level of electroinsertion. Experimentally an optimum for cell recovery and insertion rate was found at about 2,500 CD4 epitopes per red blood cell. In vitro stability assays by flow cytometry indicated a temperature- and medium-dependent decrease in the number of CD4 epitopes inserted per cell. This decrease is biphasic, with an exponential part during the first 24 h after electroinsertion followed by a much slower linear decay.

Key words: Electroinsertion; Recombinant CD4; Erythrocyte; Number of epitopes; Stability; Flow cytometry

1. Introduction

Human recombinant membrane CD4 (rmCD4), the high affinity receptor for the HIV-1 envelope glycoprotein gp-120 on the surface of T-lymphocytes, electroinserted into human erythrocytes (RBC) and injected into the patient might be used as a therapy against AIDS [1–3]. Soluble forms of CD4 (sCD4) lacking the transmembrane portion did not fulfill the expectation of blocking infection by patient isolates in vitro [4–7]. The short half-life of sCD4 in circulation [8,9] and the absence of membrane environment for the CD4 epitopes, among other reasons, led to the failure of sCD4 as a therapeutic agent against AIDS [10,11].

Subjected to a pulsed electric field, erythrocytes retain unchanged life spans in circulation [12]. RBCs bearing electroinserted rmCD4 (RBC-rmCD4) or human glycophorin injected in mice and piglets also have the same half-life as normal RBC [13]. The RBC-rmCD4 were shown to bind gp-120 [14], to attach and internalize HIV-1 particles in vitro, and form aggregates with HIV-infected Chinese hamster ovary fibroblasts expressing gp-120 [14] or with HIV-infected CEM cells [12]. It appears thus, that RBC-rmCD4 as a long-lived carrier for CD4 might provide a significant advantage over sCD4.

A Phase I clinical trials pilot study in which autologous RBC-rmCD4 was injected into HIV-1 positive patients revealed a complex life span of the RBC-rmCD4 in circulation [3]. ^{125}I-labeled rmCD4 was electroinserted into RBC and the RBC-rmCD4 were labeled with ^{51}Cr after the electroinsertion process. Radioactivity measurements of the in vivo stability of this system were made at different time points after infusion [3]. The ^{51}Cr-data confirmed an only slight decrease in the survival of the RBC-rmCD4s. In contrast, the ^{125}I signal showed a rapid decrease during the first 3–4 days followed by a slow decrease parallel to the ^{51}Cr decay. This observation raised the question of whether the ^{125}I decrease reflects clearance of ^{125}I from the rmCD4 or loss of rmCD4 from the RBC-rmCD4.

The potential efficiency of RBC-rmCD4 as a therapeutic agent depends on the total amount of inserted rmCD4 available in the circulation after infusion, as a potential decoy for HIV-1. A high number of CD4 epitopes in circulation may be achieved by either: (i) increasing the number of CD4 epitopes per RBC-rmCD4, or (ii) increasing the number of reinfused RBC-rmCD4. Since the volume of RBC-rmCD4 to be reinfused will be limited, the final number of reinfused RBC-rmCD4 depends on the percentage of cells 'surviving' the process of electroinsertion.

We report here stability measurements on RBC-rmCD4, using flow cytometry, and rmCD4 concentration data leading to the optimization of the number of CD4 epitopes on the RBC-rmCD4 along with the highest achievable recovery of cells.

2. Materials and methods

2.1. Chemicals

Chemicals were obtained from Sigma (St. Louis, MO, USA), ACS grade. The electroinsertion medium consisted of 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 adjusted to pH 8.8 with NaOH. The wash buffer had the same composition but was adjusted to pH 7.2 with HCl (PBS 7.2).

The anti-CD4 monoclonal antibodies Leu3a (mouse IgG1) and fluorescein isothiocyanate isomer I conjugated (Leu3a-FITC) were from Becton Dickinson Immunocytometry Systems (Mountain View, CA, USA). Negative control mouse antibodies IgG1 were provided by Amac Inc. (Westbrook, ME. USA). FITC-conjugated affinipure rabbit anti-mouse antibodies (RAMF) were from Jackson Immuno Research Laboratories (West Grove, PA, USA).

Quantum simply cellular microbeads (QSC-beads, diam. 8 μm) for the quantitation of antibodies on red blood cells (RBC) were provided by Flow Cytometry Standards Corp. (San Juan, Puerto Rico). Alignment of the flow cytometer was carried out with Immunocheck beads (IC-beads, diam. 10 μm) from Coulter Corp. (Hialeah, FL, USA).

Adsol red cell preservation solution (PM) was from Baxter Healthcare Corp. (Fenwal Div., Deerfield, IL, USA). RPMI 1640

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Abbreviations: RBC, red blood cell; rmCD4, recombinant membrane CD4; sCD4, soluble CD4; RBC-rmCD4, RBC bearing electroinserted rmCD4; QIFA, quantitative immunofluorescent assay; PBS 7.2, phosphate buffered saline at pH 7.2; -FITC, fluorescein isothiocyanate conjugated; QSC-beads, Quantum Simply Cellular micro-beads; PM, Adsol red cell preservation solution; $PM_{high, low}$, high and low cell density in PM; FBS, fetal bovine serum; RPMI/FBS, RPMI 1640 medium with 5% heat-inactivated FBS; RAMF, FITC-conjugated rabbit anti-mouse antibody.

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medium with L-glutamine (RPMI) and heat-inactivated fetal bovine serum (FBS) were from JRH Biosciences (Lenexa, KS, USA).

2.2. Electroinsertion

The electroinsertion device was the BTX T100 electroporation system and the pulse chamber consisted of the disposable sterile Electroporation cuvette plus with either 2 mm or 4 mm electrode gap and safety stand (all BTX Inc., San Diego, CA, USA). The applied voltage and the time constant ($t_{1/e}$) of the exponential decay pulse were monitored by a BTX pulse checker electroporation monitor.

Human erythrocytes (RBC) were separated from fresh whole blood of healthy donors by centrifugation with citric acid and dextrose (ACD) as anticoagulant. The plasma was collected, 1.2 μm filtered and later on used for RBC-rmCD4 cultures. The RBCs were washed three times with PBS 7.2 and once with electroinsertion medium. The RBC stock suspension had a final hematocrit of 0.82 ± 0.02.

Affinity purified, recombinant membrane CD4 obtained in our laboratory [15,16] was concentrated to final concentrations of 1–8 mg/ml (Bradford assay [17]) in PBS, pH 7.2, and stored at −70°C. The immunological activity of each rmCD4 sample was characterized in arbitrary fluorescence units per volume (units/μl) by a quantitative immunofluorescent assay (QIFA) [18].

The rmCD4 stock was thawed for 1 h on ice, mixed with the RBC stock suspension (1:10, v/v) and incubated for 20 min on ice. The mixture was then transferred into the electroporation chamber and the temperature was raised over 10–15 min to 37°C, in a water bath. Four exponentially decaying electrical pulses were applied at 15 min intervals. Between the pulses the chamber was kept at 37°C in the water bath. The field strength used was 2.0 ± 0.1 kV/cm and the pulse length was 1.1 ± 0.1 ms. After the last pulse application the cells were kept 20 min at 37°C and then washed several times with PBS 7.2 until the supernatant was hemoglobin free.

The final pellet of rmCD4 bearing RBC (RBC-rmCD4) was resuspended in 2 vols. of PM and stored at 4°C for further analyses and stability measurements. The entire procedure was performed under sterile conditions. Cell recovery was determined by using a Coulter MAXM (Coulter Corp., Miami, FL, USA) and is expressed as the percentage of the number of RBC in the initial RBC/rmCD4 mixture.

As a control sample for the immunofluorescence assays, an aliquot of the RBC stock suspension was subjected to all steps of the described procedure without the addition of rmCD4. The mean fluorescence of the control RBC was the same whether the electric pulses were applied or not. Further control measurements concerning the quality of the electroinsertion, e.g. orientation of the rmCD4 in the membrane, were already described elsewhere [1,12].

2.3. Immunofluorescence assay

To assay the stability of the immunologically active rmCD4 in the RBC membrane, 2×10^6 cells were washed once, incubated with 50 ng of Leu3a or isotypic control mouse IgG1 in a total volume of 50 μl, washed, incubated with ≈ 1 μg of the second antibody RAMF in 50 μl, washed and resuspended in buffer for flow cytometric analysis. Leu3a competes for the same CD4 epitope as gp-120, the HIV envelope glycoprotein [19]. For the determination of the total number of CD4 epitopes per RBC, control RBC, RBC-rmCD4 and QSC beads were labeled directly with 50 ng of Leu3a-FITC in a total volume of 50 μl.

All incubations were performed at room temperature for 30 min and under saturation conditions. Every step was carried out in PBS 7.2.

2.4. Flow cytometry

Flow cytometric analysis was performed with a Coulter Epics Elite instrument (Coulter Corp., Hialeah, FL, USA). The FITC fluorescence emission was measured using a 525 nm band-pass filter after alignment of the instrument with IC beads. The standardization curve of fluorescence intensity vs. the number of antibody binding sites was obtained at three different photomultiplier (PMT) voltages, one of which was the same used to measure the fluorescence of the labeled RBC-rmCD4. The histograms collected for analysis were: (i) 90° side scatter vs. forward scatter, and (ii) log of green fluorescence vs. number of cells.

2.5. RBC-rmCD4 stability

The stability of the immunologically active rmCD4 in the RBC mem-

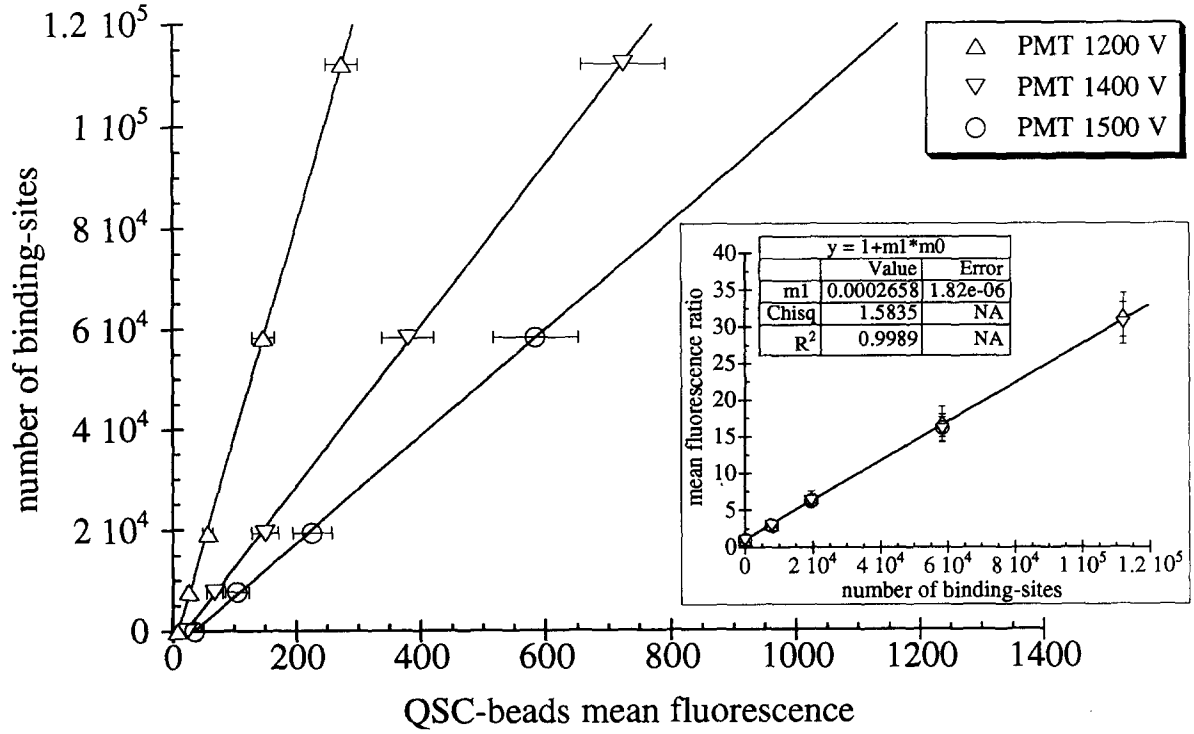

Fig. 1. Number of binding-sites on Quantum simply cellular microbeads labeled with Leu3a-FITC vs. their mean fluorescence as measured at difference PMT voltages △, 1500 V; ▽, 1400 V; ○, 1200 V. The inset shows the PMT voltage-independent linear relationship between the mean fluorescence ratio and the number of binding sites that was used to determine the number of CD4 epitopes on similarly labeled RBC-rmCD4. The ratio was derived through division of each single mean fluorescence value by the mean fluorescence of the control beads population without any specific binding. The error bars represent the standard deviation.

brane was followed over several days by flow cytometric analysis. For this purpose the RBC-rmCD4 were stored under different conditions: (i) stock suspension in PM, hematocrit around 0.30, 4°C; (ii) diluted in PM, 4°C; (iii) diluted in RPMI/FBS, 4°C; (iv) diluted in RPMI/FBS, 23°C; (v) diluted in RPMI/FBS, 37°C; (vi) diluted in plasma, 37°C. For samples ii–vi 5×10^7 cells were taken from the stock suspension, washed once with the appropriate medium, resuspended in 1 ml of the medium, transferred to 12-well plates and stored or cultured as described. Samples were taken at certain time intervals and labeled with Leu3a or control IgG1 and RAMF.

3. Results

3.1. Quantitation of epitopes and recovery

The quantitation of CD4 epitopes on RBC-rmCD4 by flow cytometry is not straightforward since all standards available are not able to simulate RBCs exactly since they lack hemoglobin. The Quantum Simply Cellular standard used consists of a population of four highly uniform microbeads of the same size, having varying capacities to bind mouse monoclonal IgG antibodies. Included in the mixture is a blank population of microbeads that has no specific binding capacity for mouse IgG. Measurement of these beads labeled with Leu3a-FITC similar to the RBC in the flow cytometer, using the same PMT-voltage, shows five distinct peaks in the green histogram channels. The blank beads, with no binding capacity, have a higher mean peak channel than control mouse IgG1-FITC-labeled RBC-rmCD4. Thus, the determination of the number of binding-sites on the RBC, using the linear extrapolation of binding sites against mean peak channels of the similarly labeled QSC beads, as suggested by the manufacturer, is not possible. Lowering the PMT voltage in order to match the mean fluorescence of blank beads and non specific labeled RBC-rmCD4 does not increase accuracy. This is due to the decreased slope in the linear dependency of the mean peak channel upon the number of binding-sites (see Fig. 1).

Assuming that the quenching of fluorescence due to light-absorbing, disk-shaped cells is proportional to the mean peak

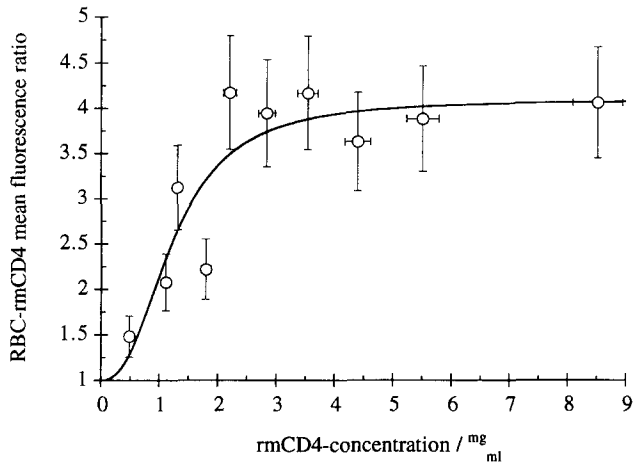

Fig. 2. RBC-rmCD4 mean fluorescence ratio as a function of the rmCD4 concentration. The mean fluorescence ratio was calculated by dividing the mean fluorescence of specifically labeled RBC-rmCD4s by those of unspecifically labeled RBC-rmCD4s (Leu3a or IgG1 and RAMF). The extrapolated curve is not based on a theoretical equation but ease the view of the two contrasting relationships (see Fig. 3). The error bars represent the standard deviation of at least three independent electroinsertion experiments and duplicate labelings.

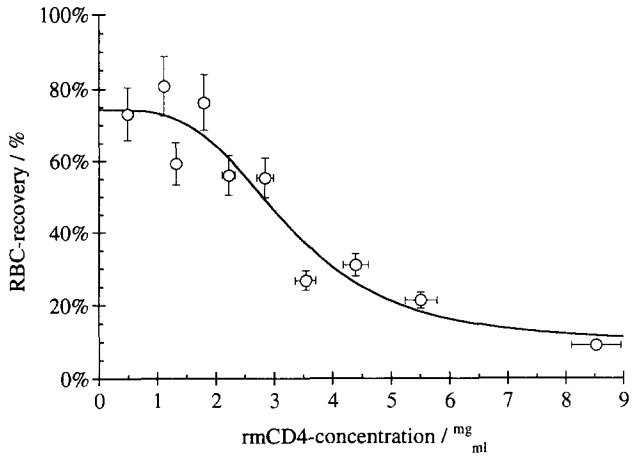

Fig. 3. Percentage RBC recovery as a function of the rmCD4 concentration. The extrapolated curve is not based on a theoretical equation but ease the view of the two contrasting relationships (see Fig. 2). The error bars represent the standard deviation of at least three independent electroinsertion experiments and duplicate labelings.

channel of either specific or non-specific labeled RBC-rmCD4, we calculated the number of binding sites using the ratio of the mean fluorescence, e.g. RBC-rmCD4$_{Leu3a\text{-}FITC}$/RBC-rmCD4$_{IgG1\text{-}FITC}$. By doing so, we obtained with the beads' standards a linear dependency of the number of binding sites upon the mean fluorescence, independent of the PMT voltage (see inset in Fig. 1). Using this standard curve we determined the number of CD4 epitopes on the RBC-rmCD4 up to 5,000.

The number of CD4 epitopes determined by a quantitative immunofluorescent assay (QIFA) [18] increased linearly with the rmCD4 concentration in the range from 1 mg/ml to 8 mg/ml as measured by Bradford protein assay [17] using purified human IgG as a standard (data not shown). In addition, the number of CD4 epitopes electroinserted (RBC suspension : rmCD4 solution = 9 : 1, v/v) into the RBC plasma membrane was dependent on the rmCD4 batch used. Fig. 2 shows that the correlation between the number of CD4 epitopes on RBC-rmCD4 expressed in mean fluorescence ratios and the rmCD4 concentration yields a saturation effect at about 3,500 rmCD4 epitopes per RBC. Here we used the more sensitive indirect staining with Leu3a or IgG1 and RAMF.

At the same time, the recovery of the RBC after the electroinsertion process decreases with higher CD4 concentrations (see Fig. 3). The recovery was determined by cell counts and is expressed as a percentage of the initial number of RBC.

The extrapolated curves help us to understand the two processes that occur during the electroinsertion, namely implanting rmCD4 into the RBC membrane on the one hand and RBC lysis on the other. rmCD4 concentrations between 2 and 3 mg/ml led to the insertion of an optimal number of CD4 epitopes per RBC (between 2,000 and 3,000) to the best possible cell recovery.

3.2. Stability of RBC-rmCD4 in vitro

In order to enhance the sensitivity of the in vitro stability assays, the RBC-CD4 were also indirectly labeled with Leu3a or control IgG1 and RAMF. We used the ratio of the mean fluorescence (RBC-CD4$_{Leu3a/RAMF}$ to RBC-CD4$_{IgG1/RAMF}$) in order to eliminate sensitivity fluctuations in the flow cytometer

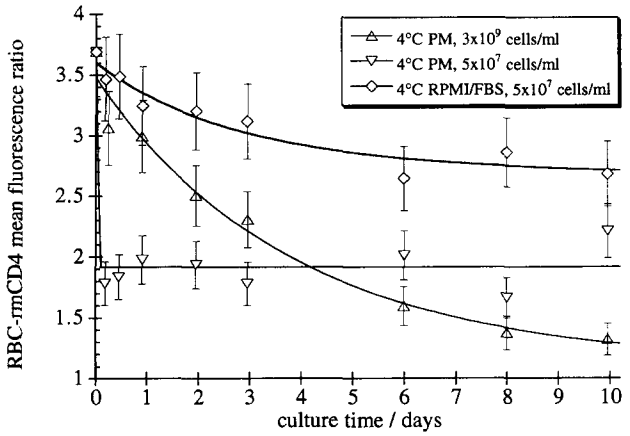

Fig. 4. CD4 epitopes on a single representative RBC-rmCD4 population at 4°C in different media as a function of time, described by the mean fluorescence ratio after indirect labeling (see Fig. 2). The error bars indicate the standard deviation of duplicate labeling, both specific and unspecific. The extrapolated curve is fitted using $y = a \times \exp(-b \times t) + c + d \times t$. △, RBC-rmCD4 in PM, cell density 3×10^9 cells/ml; ▽, in PM, 5×10^7 cells/ml; ◇, in RPMI/FBS, 5×10^7 cells/ml.

signal. Every labeling was duplicated and averaged (inclusive standard deviation). At the beginning, the stability of the CD4 electroinsertion was checked at 4°C in Adsol red cell preservation solution (PM), a commonly used solution for long-term storage in blood banks, and at 37°C in RPMI 1640 medium supplemented with fetal bovine serum (5%, RPMI/FBS). Seeing a significant difference in the rate of disappearance of exposed CD4 epitopes on RBC-rmCD4 under these conditions, we investigated the stability of insertion under different storage or culture conditions. In a final series of experiments the temperature, the nature of the medium and the cell density dependence of the insertion stability were investigated.

RBC-rmCD4 were stored at 4°C in PM at a hematocrit of around 0.30 (ca. 3×10^9 cells/ml, PM_{high}) and in PM (PM_{low}) or RPMI/FBS at a density of 5×10^7 cells/ml. Fig. 4 shows the decrease of the mean fluorescence ratio of a RBC-rmCD4 population with time. In PM_{high} and in RPMI/FBS this dependence is curvilinear. The best fit of these data points is obtained by using a combination of a single exponential and a linear decay. The stability of insertion appears completely different when RBC-rmCD4 are stored diluted in PM to the same density as in the RPMI/FBS storage. After an immediate drop, the mean fluorescence ratio remains constant during the observation period. In several experiments this constant value was about 30 ± 3% of the value at $t = 0$. In RPMI/FBS the stability was always better than in PM_{high} and the mean fluorescence ratio never dropped below the 30% value seen in PM_{low}. Moreover the observed number of CD4 epitopes in PM_{high} always dropped below this 30% value after 3–5 days.

Fig. 5 shows the loss of CD4 epitopes on RBC-rmCD4 in RPMI/FBS at the same densities (5×10^7 cells/ml) but different temperatures. The combination of a single exponential and a linear decrease also led to the best fit of the data points. With increasing temperature the exponential part becomes increasingly prevailing but even at 37°C a single exponential curve fit is never satisfying.

Culture of the RBC-rmCD4 at 37°C in plasma improved the stability slightly in comparison to RPMI/FBS (see Fig. 5). The rmCD4 in vitro stability in both media is clearly better than that of sCD4 in vivo [8], mainly as the entire signal does not disappear exponentially. The [^{125}I]rmCD4 stability in vivo [3] is even better than the flow cytometric data in vitro suggest.

Considering the complex nature of this system and that the flow cytometer measurements are performed 1.5 h after the sample has been taken, we cannot expect to clearly separate the two phases of the decay in a mathematical way. Therefore we characterize the in vitro stability by using a 'pseudo' half time, $t_{50\%}$. These $t_{50\%}$ values indicate the time, in days, when the mean fluorescence ratio has dropped to 50% of the initial value. Due to the thereby neglected linear phase the stability of the rmCD4 appears smaller than it is in reality but enables us to look at a single parameter for comparison. Table 1 summarizes the $t_{50\%}$ values of the different RBC-rmCD4 storage/culture conditions.

4. Discussion

Based on a standard microbeads population having different numbers of binding sites, we determined the number of CD4 epitopes on RBC-rmCD4 produced by electroinsertion. It was assumed that the fluorescence-quenching effect of the RBC is proportional to the total emitted fluorescence light of the cells. We eliminated this correction factor by calculating the mean fluorescence ratio of specifically and non-specifically labeled cells. It appears that this assumption leads to an underestimation of the number of epitopes. The visibility of the fluorescence emitted by labeled rmCD4 that has migrated into the grove of the disk is definitely lower than the fluorescence of labeled rmCD4 on the rest of the RBC plasma membrane [20]. Therefore the average effective fluorescence per protein ratio is smaller on Leu3a-FITC bound to RBC-rmCD4 than bound to the white spherical standards beads. Although this method is an underestimation it provides us with a minimum number of CD4 epitopes on the cell surface.

The optimization of the rmCD4 electroinsertion in RBC in the direction of a high number of CD4 epitopes per cell and high RBC recovery shows a very interesting relationship. The reason for the saturation effect in the number of CD4 epitopes with increasing rmCD4 concentration is most likely the limited expandability of the RBC membrane, as suggested by the following calculation: one CD4 molecule requires an area of 1.54 nm^2, assuming a radius of $r = 0.7$ nm for the transmembrane helix [21]. 5,000 CD4 epitopes need then 8×10^3 nm^2. The average surface area of an RBC is about 140×10^6 nm^2 [22]. The maximal inserted 5,000 CD4 molecules increase the RBC surface area by 0.006%. In addition we have to consider two facts: (i) the number of CD4 epitopes per RBC determined by flow

Table 1
'Pseudo' half-decay time $t_{50\%}$ for the loss of immunologically active rmCD4 on RBC-rmCD4 stored under different conditions, as measured by flow cytometry after indirect labeling with Leu3a and RAMF

Medium	T (°C)	$t_{50\%}$ (days)	Number of experiments
PM_{high}	4	4.2 ± 1.2	35
RPMI/FBS	4	> 10	4
RPMI/FBS	23	2.8 ± 0.9	4
RPMI/FBS	37	0.71 ± 0.16	33
Plasma	37	0.93 ± 0.21	6

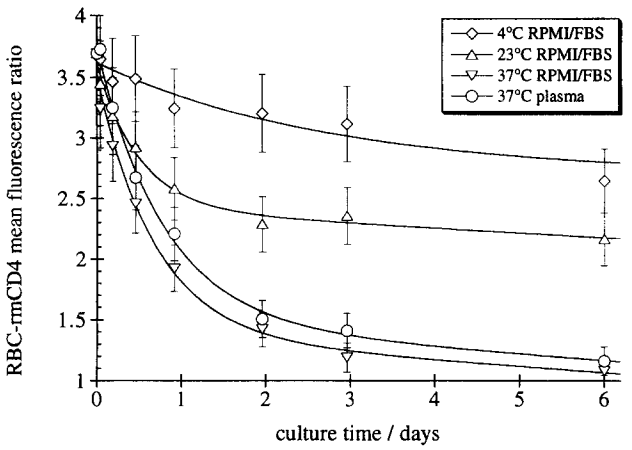

Fig. 5. CD4 epitopes on a single representative RBC-rmCD4 population at different temperatures and media as a function of time, expressed by the mean fluorescence ratio (see Fig. 4). ◇, 4°C; △, 23°C; ▽, 37°C; all in RPMI/FBS; ○, 37°C in plasma; cell density for each is 5×10^7 cells/ml.

cytometric analysis is an underestimation for the reasons shown, and (ii) we detect only the immunologically active rmCD4. The protein is purified and detergent free, and therefore we must assume a percentage of denatured protein in the rmCD4 concentrates. Some of it may not be amenable to insertion due to denatured transmembrane structure, but along with active rmCD4 we also insert rmCD4 with denatured antibody binding sites. We also have to consider that the tertiary structure of the extracellular part of the rmCD4 has to squeeze itself amongst the very dense glycocalyx of the RBCs and every integral membrane protein is usually surrounded by a number of closely adjacent lipid molecules [23]. Therefore we believe the increase in the RBC surface by inserted rmCD4 may reach up to 1%. Investigations to determine the elasticity of the erythrocyte membrane have been described in the literature: the best comparable model to rmCD4 electroinsertion may be the detergent-like auto-insertion of apolipoprotein into the bilayer membrane [24]. Even though this study focusses on many different quantitative aspects, the occurence of lysis is never related to the percentage extension of membrane expansion. The majority of the articles describe shape changes induced in RBC by expanding only the outer leaflet of the bilayer through the insertion of lipids or lipid analogs, e.g. monopalmitoyl phosphatidylcholine [25]. Others tried to mechanically expand small areas of the membrane of osmotically preswollen RBC. They found immediate lysis with about a 3% [26] or 2.6% [27] area increase, and lysis within 20–30 s with a 2% area increase [26]. The RBC-rmCD4 are washed after electroinsertion for more than 1 h. This period of time gives even those RBC-rmCD4s which experience a lesser expansion of their membrane enough time to lyse. Gradual reduction of the volumes of RBC-rmCD4 pellets observed throughout the post-wash process, until no further lysis occurs, supports our explanation. Therefore, the existance of an upper limit of the number of inserted proteins, without destruction of the integrity of the membrane, is most likely. The RBC, into the plasma membrane of which more than 5,000 CD4 epitopes have been inserted, lyse. This evidence is also supported by the decrease in the RBC recovery with higher rmCD4 concentrations used during the electroinsertion. It seems that the majority of the RBC population withstands rmCD4-insertion at the level of 2,000–3,000 epitopes per cell. At the appropriate rmCD4 concentrations we obtain a high RBC recovery.

The immunofluorescence data do not give information about the fate of the lost rmCD4. The reason why about 70% of the inserted CD4 epitopes are lost instantly when we store the RBC-rmCD4 diluted in PM at 4°C is not clear. Possibly, part of the cells lose rmCD4 spontaneously under these conditions, until somehow an equilibrium within the medium has been reached. In this case the equilibrium should shift to a different value at different densities, but we found the same instant loss of CD4 epitopes (about 70%) at double and quadruple cell densities (data not shown). Performing standard blood bank storage at a high hematocrit (30%) and 4°C, the decrease appears linear for a portion of the curve and exponential for the other. The Adsol red cell preservation solution used is certainly optimized in its chemical composition to maintain transfusionable conditions of RBCs during storage for up to 30 days at 4°C. Nonetheless it may only be effective at standard high hematocrits but not in dilute suspensions.

The temperature dependence of the rmCD4 stability in RPMI/FBS suggests, on the other hand, that the loss of the rmCD4 signal may be related to the metabolism of the cells. The kinetics of the loss of CD4 epitopes increases with higher temperatures. This indicates an active process removing the rmCD4 out of the membrane. A reason therefore may be the above-discussed extension of the membrane due to the inserted rmCD4. The higher the temperature the more RBCs are able to enforce a relaxation of the membrane by removing rmCD4, as seen in the exponential part of the stability curves. However if this 'active' process is delayed by a lower temperature or a more native culture condition, as in plasma for example, a higher percentage of inserted rmCD4 remains stable in the membrane and the signal decreases only due to cell loss. Fig. 5 clearly shows that the in vitro studies seem to confirm the [^{125}I]rmCD4 measurements conducted in vivo [3]. Due to this similarity we conclude that the flow cytometric observations indeed reflect a loss of rmCD4 from the membrane and not just denaturation of its epitopes. The amount of rmCD4 that is stably inserted in vivo, is even better than in vitro [3]. We believe this to be caused by the optimal conditions for the RBC-rmCD4 that keep the cells more tolerant to long-term membrane expansion. Further studies to understand this phenomenon of rmCD4 loss from RBC-rmCD4 better are under investigation in our laboratory.

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