



Full-length CD4 electroinserted in the erythrocyte membrane as a long-lived inhibitor of infection by human immunodeficiency virus

(life span of CD4 in erythrocytes/human immunodeficiency virus type 1 inactivation/fluorescence dequenching/reverse transcriptase/p24 core antigen)

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ABSTRACT Recombinant full-length CD4 expressed in *Spodoptera frugiperda* 9 cells with the baculovirus system was electroinserted in erythrocyte (RBC) membranes. Of the inserted CD4, 70% was "correctly" oriented as shown by fluorescence quenching experiments with fluorescein-labeled CD4. The inserted CD4 displayed the same epitopes as the naturally occurring CD4 in human T4 cells. Double-labeling experiments (^{125}I -CD4 and ^{51}Cr -RBC) showed that the half-life of CD4 electroinserted in RBC membrane in rabbits was approximately 7 days. Using the fluorescence dequenching technique with octadecylrhodamine B-labeled human immunodeficiency virus (HIV)-1, we showed fusion of the HIV envelope with the plasma membrane of RBC-CD4, whereas no such fusion could be detected with RBC. The dequenching efficiency of RBC-CD4 is the same as that of CEM cells. Exposure to anti-CD4 monoclonal antibody OKT4A, which binds to the CD4 region that attaches to envelope glycoprotein gp120, caused a significant decrease in the dequenching of fluorescence. *In vitro* infectivity studies showed that preincubation of HIV-1 with RBC-CD4 reduced by 80–90% the appearance of HIV antigens in target cells, the amount of viral reverse transcriptase, and the amount of p24 core antigen produced by the target cells. RBC-CD4, but not RBCs, aggregated with chronically HIV-1-infected T cells and caused formation of giant cells. These data show that the RBC-CD4 reagent is relatively long lived in circulation and efficient in attaching to HIV-1 and HIV-infected cells, and thus it may have value as a therapeutic agent against AIDS.

Although soluble CD4 (sCD4) acts efficiently in blocking human immunodeficiency virus (HIV) infection *in vitro* (1–5) and simian immunodeficiency virus (SIV) infection in rhesus monkeys (6), it appeared that very high amounts of sCD4 may be required in humans to achieve "therapeutic concentrations" (6), largely due to its short half-life (7). There might be, therefore, a significant advantage in having a CD4 carrier that is long-lived in circulation and capable of both clearing free HIV and binding to HIV-infected cells (8). The latter could form aggregates that would be removed from circulation by phagocytosis by the reticuloendothelial system (8).

The human erythrocyte (red blood cell; RBC), with a half-life of about 2 months, appears to be a good candidate for a long-lived CD4 carrier in the circulation (9). We have developed a method to electroinsert xenoproteins into RBC membranes that preserves protein epitopes and stability in circulation of the RBC-protein conjugate (10, 11). We then expressed the full-length CD4 molecule (which includes the membrane-spanning sequence) in insect cells (*Spodoptera frugiperda* isolate 9), using the baculovirus expression vector

system (12). Recombinant CD4 was isolated by immunoaffinity chromatography (12) and was inserted in rabbit RBC membranes by electroinsertion (11). It showed patching upon monoclonal antibody (mAb) binding and displayed the same anti-CD4 monoclonal epitope map as CD4 expressed by CEM cells (a line of human T leukemia cells), just as was the case with CD4 inserted in mouse or human cells (11).

We report here that when inserted into rabbit RBC membranes, CD4 is relatively long-lived in circulation. Moreover, we show that human RBC-CD4 can attach to HIV-1 particles and fuse with their envelopes, inhibit HIV infection of susceptible target cells, and aggregate HIV-infected cells that expose the HIV envelope glycoprotein gp120 on their plasma membranes.

MATERIALS AND METHODS

Expression and Purification of Full-Length CD4. A cDNA encoding full-length CD4 (gift from R. Axel, Columbia University, New York, NY) was inserted into the genome of *Autographa californica* nuclear polyhedrosis virus under transcriptional regulation of the viral polyhedrin gene promoter. The recombinant virus was used to infect insect cells, resulting in the abundant expression of CD4 as evaluated by flow cytometry and immunoblot analysis (12). Recombinant CD4 expressed on the surface of infected insect cells was immunologically indistinguishable from human T-cell surface CD4 when tested with 11 different anti-CD4 mAbs. Sequential phase-transition separation with Triton X-114, immunoaffinity chromatography, NaDodSO₄/PAGE, and silver staining revealed that infected cells express a single CD4 protein (12). Immunoblot analysis under nondenaturing conditions showed that the purified protein reacted with the anti-CD4 mAb Leu-3a (12).

Electroinsertion of CD4 in the RBC Membrane. The procedure was performed as described (11). Briefly, we employed a 606 Cober pulse generator, a cylindrical Teflon chamber 1.2 cm in 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 maintained at 37°C by using a circulating water thermostat. RBCs, obtained from a blood bank, were separated from fresh whole blood of healthy donors with citrate buffer as anticoagulant. The RBCs were washed three times with the electroinsertion medium (0.14 M

Abbreviations: RBC, red blood cell; HIV, human immunodeficiency virus; CD4, receptor for HIV-1 on human T4 cells; sCD4, soluble CD4; R 18, octadecylrhodamine B chloride; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; DQ, dequenching.

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NaCl and 10 mM $\text{HNa}_2\text{PO}_4/\text{H}_2\text{NaPO}_4$ adjusted to pH 8.8 with NaOH), and a RBC stock suspension of 2×10^9 cells per ml was prepared. Highly purified, lyophilized, full-length CD4 obtained in our laboratory (12) was dissolved in the electroinsertion medium. The CD4 solution, at 1 mg/ml, was added to the RBC suspension. After 20-min incubation on ice, the solution was brought to 37°C and four square electrical pulses of 1 ms each were applied at 15-min intervals. The field intensity was 1.3 kV/cm for human RBCs and 2.1 kV/cm for mouse RBCs. After pulse application, the cells were washed three times with phosphate-buffered saline (PBS), pH 7.4, and stored at 4°C.

Orientation of CD4 Electroinserted in the RBC Membrane. To assess the orientation of the CD4 molecules in the RBC membrane after electroinsertion, CD4 was conjugated with fluorescein isothiocyanate (FITC) prior to insertion. Briefly, CD4 at 4 mg/ml in 0.25 M borax/boric acid, pH 8.0, was incubated with FITC (Sigma) at 100 $\mu\text{g}/\text{mg}$ of protein. After 4 hr on ice, free fluorescein was removed by chromatography on Sephadex G-25 (Pharmacia). The FITC-CD4 fraction was collected, concentrated (Amicon Micropartition System, Amicon), and lyophilized. For the quenching studies, 10^7 RBC-CD4-FITC were incubated with anti-FITC antibodies (Molecular Probes) for 20 min then diluted and analyzed for their green fluorescence. Flow cytometry was performed in a Coulter Epics Profile instrument. The percentage of quenching by anti-FITC antibodies was estimated according to the mean peak fluorescence of the different samples.

Life-Span Measurements of CD4 Inserted in RBC Membranes in Rabbits. Purified CD4 (250 μg) was labeled with 500 μCi (1 μCi = 37 kBq) of Bolton-Hunter ^{125}I reagent (13) for 2 hr at 4°C. Labeled CD4 was purified on a Sephadex G-25 column. Glycophorin (1 mg) was labeled with 250 μCi of ^{125}I by following the same procedure. ^{125}I -CD4 or ^{125}I -glycophorin was electroinserted into 700 μl of packed rabbit RBC membranes (11). ^{125}I assay indicated that ≈ 5000 molecules of CD4 were inserted per RBC. Epitopes were assayed by flow cytometry using unlabeled CD4 as a control. After insertion, the RBC-CD4 were labeled (14) with 5 μCi of ^{51}Cr , resuspended in plasma, and transfused with a 22-gauge Teflon-coated needle catheter into autologous New Zealand White rabbits weighing 2.7–2.9 kg that were anesthetized with ketamine at 25 mg/kg and xylazine at 2 mg/kg. At the indicated time, 1.5 ml of blood was drawn from the marginal ear vein of the rabbit.

Measurement of Fusion of HIV-1 Envelope with RBC-CD4 by Using the Membrane Fluorescence Dequenching (DQ) Technique. HIV-1 was propagated in chronically infected H9 cells and infection was monitored by reverse transcriptase activity and by immunofluorescence assay of HIV-1 antigens. Concentrated virus was purified by centrifugation through a 20–60% sucrose gradient. Intact virions were labeled with octadecylrhodamine B chloride (R 18; Molecular Probes) as described (15); 0.6 μl of a 1 mg/ml ethanolic solution of R 18 was rapidly added to 50 μg of HIV-1. After 15-min incubation at room temperature, virus was separated from free dye by centrifugation. Labeled virions (R-18-HIV-1) were resuspended in PBS to a final concentration of 0.5 mg of viral protein per ml. R-18-HIV-1 was adsorbed to target cells in 100 μl of RPMI 1640 medium/5% fetal bovine serum for 5–7 min at 4°C followed by washing off unadsorbed virus and incubation at 37°C for an additional 25 min. Cells were then diluted to 3 ml with RPMI 1640 medium and analyzed for fluorescence by using a Perkin-Elmer LS-3B fluorescence spectrophotometer (560-nm excitation, 590-nm emission), first without any treatment to obtain the actual fluorescence value and then with 0.15% Triton X-100 to elute the R 18 from membranes and to determine the maximum fluorescence associated with these membranes. The background fluorescence value was measured on a mixture of R-18-HIV-1 and

target cells at zero time. Specific DQ was calculated as a proportion of actual system fluorescence to 100% fluorescence value, after subtracting the background fluorescence. A 2.5- μg sample of R-18-HIV-1 was incubated with the designated amounts of RBC-CD4, RBC-CD4 saturated with OKT4A mAb, control RBC, or CD4-positive cell lines CEM and CR10 as controls. When fusion between the labeled virus and the target cells occurred, an increase in fluorescence signal (i.e., fluorescence DQ) was observed that was directly proportional to the extent of virus–cell fusion (15–19).

Infectivity of HIV-1 after Preincubation with RBC-CD4. RBC-CD4, RBC-CD4/OKT4A, or untreated RBCs (1.5×10^9 cells each), or 1.5×10^8 CEM cells were suspended in 0.5 ml of RPMI-1640 medium and mixed with 20 μl of concentrated HIV-1_{IIIIB} (8.85 μg of viral p24 per ml), or the same amount of virus was mixed with 0.5 ml of PBS. The mixtures were incubated for 15 min at 4°C for viral adsorption, followed by 30 min at 37°C for viral fusion. Cells were removed by centrifugation; supernatants containing free virus were filtered through 0.45- μm filters to ensure sterility, and 0.25 ml of each was used for infection of 0.2×10^6 C-8166 (HTLV-I-immortalized cord blood lymphocytes) or CEM cells according to a standard protocol (19). Infection was evaluated on days 6, 9, and 12 after addition of supernatant by measuring (i) the levels of HIV-1 reverse transcriptase activity and HIV-1 p24 core antigens in culture supernatants; and (ii) the proportion of cells expressing intracellular HIV-1 antigens, as determined by HIV-1-specific immunofluorescence assay (19). HIV-1 p24 capsid protein was detected by using Coulter HIV Ag Assay (Coulter).

RESULTS

Orientation of Electroinserted CD4 Molecules. Electroinsertion is not expected to result in all CD4 molecules being exposed in the native orientation. To determine the frequency of “correctly” oriented CD4 molecules on the surface of the RBC, FITC was covalently attached to CD4 prior to electroinsertion. The number of FITC-CD4 molecules per cell was determined by cytofluorimetric assay, then the RBC-CD4-FITC were exposed to anti-FITC antibodies. Cytofluorimetric assay of the fluorescence emission indicated that 73% of the fluorophores were quenched by the anti-FITC

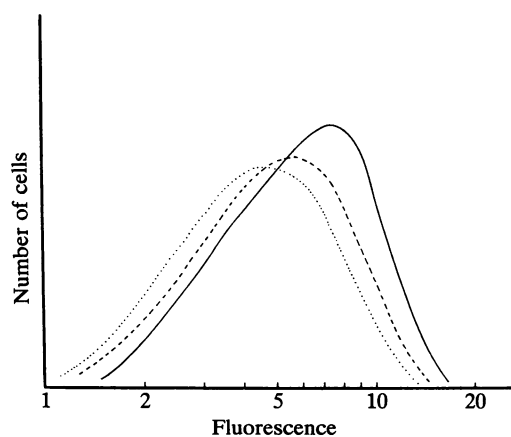


FIG. 1. Quenching of RBC-CD4-FITC fluorescence by anti-FITC antibodies. Flow cytometry histograms (green fluorescence) of RBCs with FITC-CD4 inserted in their membrane (—); quenching of the same RBC-CD4-FITC after incubation with anti-FITC antibodies (- - -); and control RBCs (· · ·). FITC was covalently bound to CD4. Flow cytometry was performed in a Coulter Epics Profile instrument. The percentage of quenching by anti-FITC antibodies was estimated according to the mean peak fluorescence of the different samples (RBC control, 4.78; RBC-CD4-FITC–anti-FITC, 5.74; and RBC-CD4-FITC, 7.09).

antibodies (Fig. 1), indicating that most of the inserted CD4 was available for interactions with ligands.

Life-Span Measurements of RBC-CD4 in Rabbits. To perform the life-span measurement, full-length CD4 was labeled with ^{125}I and inserted into rabbit RBC membranes. The RBCs were then labeled with ^{51}Cr so that the life span of the cell and that of the inserted protein could be measured simultaneously. As a control, human glycoprotein A was labeled with ^{125}I and inserted by the same technique. Radioactivity measurement indicated the presence of about 6000 molecules of glycoprotein per RBC. Autologous transfusions of ^{51}Cr -RBC- ^{125}I -CD4 (two animals) and ^{51}Cr -RBC- ^{125}I -glycoprotein (two animals) were performed and the ^{125}I and ^{51}Cr radioactivity were measured at different time points. Fig. 2 shows these radioactivities as a function of time. Whereas the radioactivity measurements of ^{51}Cr indicate a half-life of about 16 days for the rabbit RBC, the ^{125}I measurements indicate a half-life of about 7 days for the inserted CD4 or glycoprotein (Fig. 2). Both proteins show the same half-life, shorter than that of the "carrier" but significantly longer than the values obtained for free sCD4, hybrid sCD4 injected i.v. (7), or sCD4 injected intramuscularly (6). During 30 days of observation, no immune response against either inserted CD4 or glycoprotein could be detected in the injected rabbits, whereas the free proteins injected i.v. in the same amounts elicited a strong immune response (data not shown).

Fusion of HIV-1 Envelopes with the Membrane of RBC-CD4. To determine whether CD4 molecules inserted into the RBC membrane are functionally equivalent (with regard to their interaction with HIV-1) to native CD4 expressed on the T-cell surface, we evaluated three functions normally associated with membrane-bound viral receptors: (i) their ability to mediate HIV-1 entry; (ii) their ability to inhibit infection of CD4-positive T cells with cell-free HIV-1; and (iii) their ability to mediate the aggregation with HIV-1-infected T cells.

The potential of CD4 exposed on the RBC surface to mediate HIV-1 binding and entry was determined by using the membrane fluorescence DQ technique (15–19). HIV-1 fused in a cell-number-dependent manner with human RBC-CD4 but not with control human RBCs (Fig. 3). Results of two separate experiments using different preparations of RBC-CD4 and labeled virions are shown. The DQ value obtained with 25×10^6 RBC-CD4 was equivalent to that obtained with 2×10^6 CD4-positive CEM or CR10 cells (Fig. 3). This is consistent with the 2000 CD4 epitopes per RBC

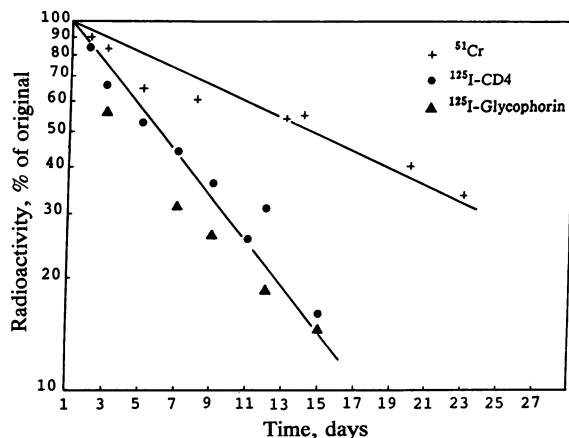


FIG. 2. Life-span measurements, in rabbits, of ^{125}I -CD4 or ^{125}I -glycoprotein inserted in RBC membrane. The RBCs were labeled with ^{51}Cr . Epitopes were assayed by flow cytometry with unlabeled CD4 as control. At the indicated time points, 1.5 ml of blood was drawn from the marginal ear vein of the rabbit and its radioactivity was measured in a γ counter (Packard model 5002).

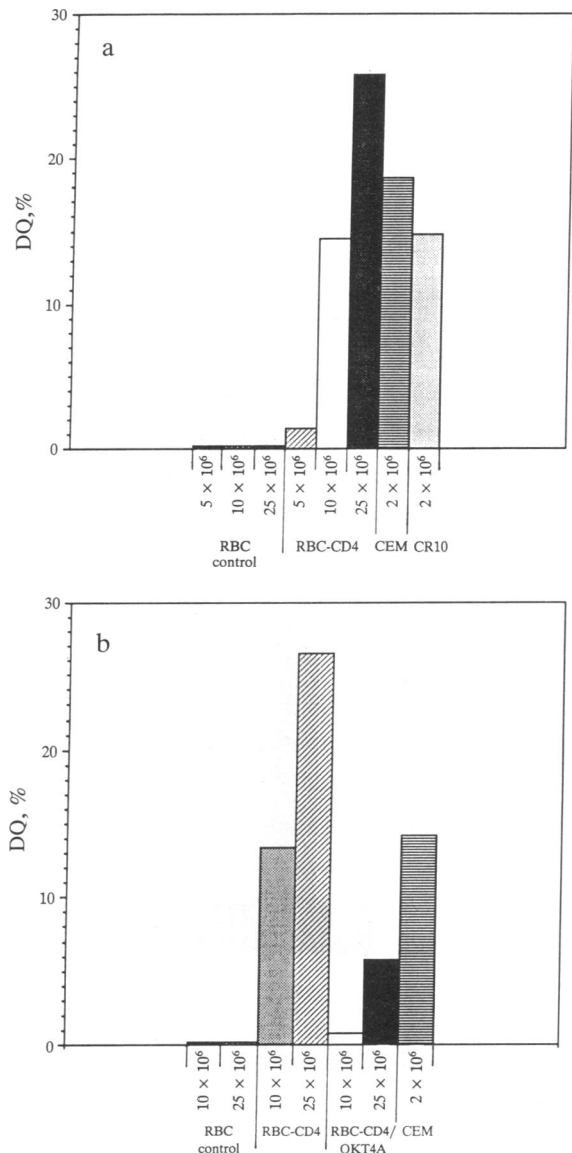


FIG. 3. Measurement of fusion between HIV-1 and RBC-CD4 by the membrane fluorescence DQ. Purified R-18-labeled HIV-1 was incubated with the designated amounts of RBC-CD4, RBC-CD4 saturated with OKT4A mAb, control RBC, or CD4-positive cell lines CEM and CR10 as controls. (a and b) Results of two separate experiments.

versus about 50,000 CD4 molecules per CEM cell (12). Fusion of HIV-1 with RBC-CD4, like that with CEM, occurred at 37°C but not 4°C and had a half-DQ time of 5–8 min at 37°C . It is noteworthy that pretreatment of RBC-CD4 with OKT4A antibodies significantly reduced the DQ (Fig. 3b). This suggests that the binding and fusion of HIV-1 with RBC-CD4 are mediated by the same functional region(s) of the CD4 molecule that serve as the HIV receptor on T cells.

Inhibition of HIV Infection of Target Cells by RBC-CD4. We next examined the capacity of RBC-CD4 to block infection *in vitro* with cell-free HIV-1. RBC-CD4, RBC-CD4/OKT4A, RBCs, or CEM cells were incubated with HIV-1_{IIIIB} for 30 min at 37°C , the cells were removed by low-speed centrifugation, and supernatants were filtered through $0.45\text{-}\mu\text{m}$ Millipore filters. Filtered supernatants were then used for infection of target CD4-positive T cells by a standard protocol (19). Fig. 4 shows the results of experiments of inhibition of HIV-1 infection by RBC-CD4. Clearly, pretreatment with either RBC-CD4 or CEM cells reduced the infectious titer of HIV-1

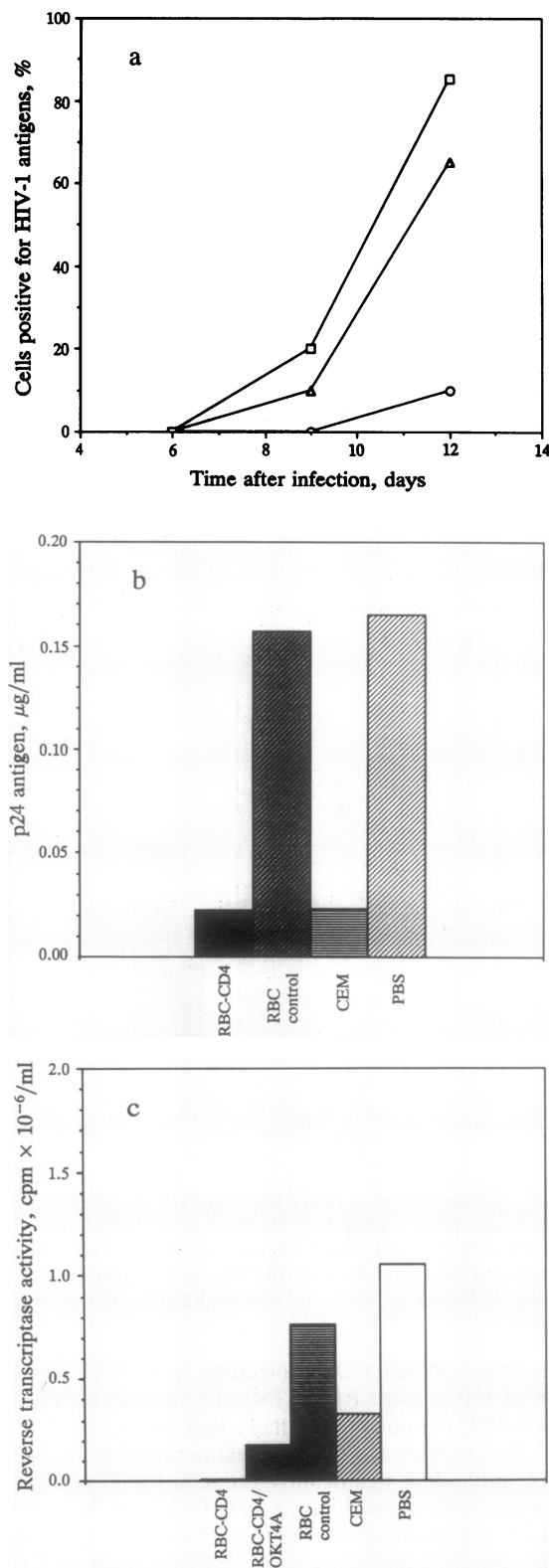


FIG. 4. Reduction in the infectivity of HIV-1 by preincubation with the RBC-CD4. (a) Kinetics of intracellular HIV-1 antigen expression after exposure of CEM cells to HIV-1 preincubated with RBC-CD4 (○), RBC-CD4 blocked with OKT4A (△), or untreated RBC (□), as measured by immunofluorescence. (b) Supernatant p24 antigen concentrations in parallel experiment using C-8166 cells as targets, assayed 6 days after infection. (c) Supernatant reverse transcriptase on day 12 after infection of CEM cells.

present in the virus preparation 80–90% as assayed by immunofluorescence of p24 or production of reverse tran-

scriptase. RBC-CD4 in which HIV-1-binding CD4 epitopes were blocked with OKT4A antibodies were significantly less effective in “clearing” infectious HIV (Fig. 4). Similar results were obtained when C-8166 cells, which are exquisitely susceptible to HIV infection, served as targets in these experiments (Fig. 4b).

Aggregation of RBC-CD4 with HIV-Infected, gp120-Expressing CEM Cells. To evaluate whether RBC-CD4 can interact with HIV-1-infected T lymphocytes, RBC-CD4 were mixed with the chronically infected CEM/NIT-E (19) cells at a ratio of 100:1 and examined under a light microscope after 5–10 min of incubation at 37°C. Numerous aggregates between CEM/NIT-E cells and RBC-CD4, but not control RBCs, were seen within a short time of incubation. Fusion was observed between RBC-CD4 and T cells and occasionally among T cells themselves (Fig. 5). RBC-CD4 do not aggregate with uninfected CEM cells (not shown). Thus, RBC-CD4 appear to recognize the native gp120 molecules on the surface of HIV-1-infected cells and thus aggregate with these cells. These observations confirm those made with CHO cells constitutively expressing gp120 and RBC-CD4 (20).

DISCUSSION

We have constructed a long-lived vehicle for the adsorption and possible clearance of HIV-1 virions or infected gp120-expressing cells present in the circulation, RBCs bearing electroinserted CD4. The electroinserted CD4 molecules are accessible to interactions at the surface membrane, a requirement for binding HIV-1 (Fig. 1). In addition, by using autologous RBCs as carriers, we have overcome one major limitation which has been found in therapy with sCD4—its relatively short lifetime *in vivo* (7). When inserted in rabbit RBC membranes, the CD4 molecule acquires a half-life in the circulation of 7 days (Fig. 2). The RBCs’ half-life time in circulation is not affected by the electroinsertion procedure,

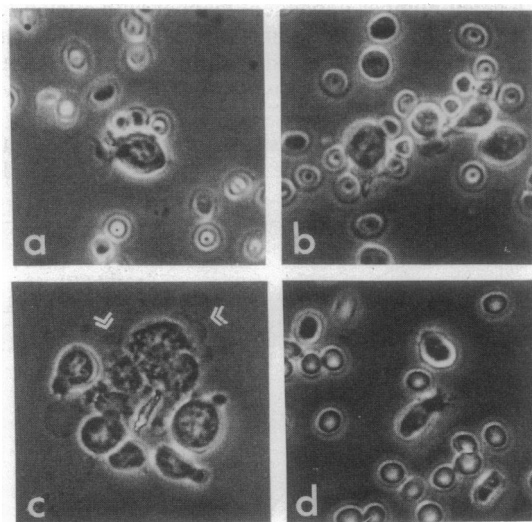


FIG. 5. Interaction of RBC-CD4 with HIV-1-infected T lymphocytes. RBC-CD4 were mixed with chronically infected CEM/NIT-E cells at 100:1 ratio and examined under a light microscope after 5–10 min of incubation at 37°C. (a, b, and c) RBC-CD4 mixed with infected CEM/NIT-E. Note in a and b the “rosetting” of RBC-CD4 around the HIV-1-infected T cells. The picture shown in c was taken after 30-min incubation at 37°C. At this time giant cells began to form, possibly after RBC-CD4 fused with CEM/NIT-E cells and the latter were then able to fuse among themselves. Lysed erythrocyte “ghosts” by arrowheads. (d) Control RBCs mixed with infected CEM/NIT-E cells and incubated for 30 min. (Phase contrast, $\times 350$.)

similar to the observation of unchanged life spans of RBCs subjected to electroporation (21).

Although we have previously characterized electroinserted CD4 as retaining the same epitope display as T-cell surface CD4 (11), it was necessary to demonstrate that such CD4 remained able to serve as an HIV-1 receptor. We evaluated this by assay of virus-RBC membrane fusion through membrane fluorescence DQ (Fig. 3). This method has been previously demonstrated to accurately measure HIV-1 binding and internalization (15–18). Indeed, HIV-1 fused specifically with RBC-CD4 but not with control RBCs. That these interactions preserve the specificity of the native CD4-gp120 recognition was demonstrated by the ability of OKT4A mAb to block the entry of HIV-1 into RBC-CD4, analogous to its ability to block HIV-1 binding to T lymphocytes (18). The fact that HIV-1 may be internalized by the RBC carrier offers an additional advantage to ultimate therapeutic application. It may be envisioned that RBC-CD4 can absorb and internalize HIV-1, physically removing the particle from interactions with cells susceptible to infection.

We have shown that, consistent with the findings above, RBC-CD4 absorb and thus reduce the infectious titer of HIV-1, suggesting their potential as an efficient competitor with target T lymphocytes for gp120 binding (Fig. 4). Finally, another attribute of the RBC carrier of CD4 we have demonstrated here is its ability to agglutinate HIV-1-infected cells, through CD4-gp120 binding (Fig. 5). These RBC-lymphocyte aggregates would likely suffer phagocytosis in the circulation.

In summary, RBCs as carriers for CD4 for neutralization of HIV *in vivo* maintain the critical features of sCD4 that recommended its clinical evaluation: the abilities to bind HIV-1 and effectively compete with T cells for this binding (2–7). Moreover, they have three distinct advantages over sCD4: (i) their lifetime in the circulation is 7 days in rabbits, compared to hours for adhesins (7); (ii) they internalize and thus, for the life span of the RBC, irreversibly remove HIV-1 from potential interaction with susceptible cells; and (iii) through their multivalent display of CD4 they can agglutinate gp120-displaying HIV-1 infected cells, which may result in their clearance from the circulation. The latter finding is noteworthy in that the currently available treatments for HIV-1 infection depend upon blocking the infection of new, uninfected cells (2–6); this agent also has the potential for eliminating HIV-1-infected cells.

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