JOURNAL OF VIROLOGY, Jan. 1995, p. 528–533 Vol. 69, No. 1
0022-538X/95/$04.00+0

Human Immunodeficiency Virus Type 1 Nef Induces Accumulation of CD4 in Early Endosomes

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Received 27 June 1994/Accepted 23 September 1994

We have studied the fate of CD4 in CEM T cells expressing a human immunodeficiency virus type 1 HIV-1 Nef protein. Nef triggered a rapid endocytosis and a degradation of CD4, while most of the $p56^{lck}$ was upheld at the cell membrane. In the presence of Nef, CD4 accumulated in acidic intracellular vesicles that were not stained by antibodies against rab6, a marker of the Golgi apparatus complex. Detection of transferrin in CD4-containing vesicles showed that CD4 was trapped in early endosomes, without significant accumulation of CD4 in late endocytic compartments. Internalization pathways taken by CD4 in Nef^+ cells may therefore be different from those observed after treatment with phorbol esters.

The primate lentiviruses human and simian immunodeficiency viruses (HIV and SIV, respectively) possess a gene referred to as *nef* (for negative factor). It is expressed early in the viral cycle and encodes a 27- to 30-kDa myristoylated protein, which is predominantly localized in the cytoplasm and associated with membranes (12, 20). In $CD4^+$ cells, Nef expression is associated with a reduction of CD4 levels at the cell surface (13, 16, 27). Nef-induced CD4 down-regulation may have different implications on viral multiplication, by protecting T cells from envelope-induced cytopathic effects (27) or by conferring resistance to superinfection (5). It has also been shown that Nef modulates T-cell activation, possibly leading to enhanced viral production (30).

In cells constitutively expressing Nef, surface CD4 has been shown to be rapidly endocytosed (2), sequestered in an uncharacterized cytoplasmic compartment (13), and degraded (2, 13, 25). Here, we have studied the cellular mechanisms of Nef-induced CD4 modulation in a population of CEM lymphoblastoid cells expressing the *nef* gene from a primary HIV type 1 (HIV-1) isolate (CEM Nef) (27). These cells display 10- to 20-fold-reduced levels of surface CD4 in comparison to those of CEM control cells (27), and as recently reported by others (2, 13, 25), we have observed that this down-regulation is associated with a decreased half-life of the molecule, while de novo synthesis is not altered. Pulse-chase experiments indicated that CD4 half-life is greater than 8 h in control CEM cells and is reduced to 3.5 h when Nef is present (data not shown).

The fate of CD4 molecules present at the cell surface was monitored by using a fluorocytometric assay. CEM Nef and control cells were labeled at 4°C with the anti-CD4 monoclonal antibody (MAb) NUTH-I (26), washed, transferred to 37°C for different periods of time, cooled to 4°C, and stained with a fluorescent anti-immunoglobulin G (IgG) antibody (Ab). Surface levels of NUTH-I-bound CD4 molecules were then measured with a fluorescence-activated cell sorter (FACS). Data were plotted as the means of fluorescence of the peak at different time points (Fig. 1a). In CEM control cells, high levels of surface CD4 were detected at time zero (mean fluorescence, 580 arbitrary units [a.u.]). Over 90% of the staining was still detectable in control cells after 60 min at 37°C, corresponding to a decrease of 50 a.u. in 1 h. This confirmed that anti-CD4 MAb does not induce a significant modulation of CD4 cell surface levels in the absence of cross-linking (22). As expected, the amount of CD4 detected at time zero in CEM Nef cells was 15-fold lower than in control cells (mean fluorescence, 38 a.u.). The amount of NUTH-I MAb bound to the cell surface rapidly decreased over time, and after 60 min at 37°C, 50% of the surface fluorescence had disappeared, corresponding to a decrease of 18 a.u. in 1 h. To establish that this phenomenon was actually due to an internalization of surface CD4, rather than to antibody shedding, CEM Nef and control cells were labeled at 4°C with ^{125}I-OKT4, washed to remove unbound MAbs, and incubated at 37°C for 30 min. The amounts of surface and internalized OKT4-CD4 complexes were determined by treating the cells with pronase and measuring the amount of radioactivity remaining associated with the cells. Data are presented as the ratio of pronase-resistant cell counts to total cell-associated counts (Fig. 1b). As expected, after the binding period on ice, the total amount of cell-associated radioactivity was ninefold higher in the CEM control than in CEM Nef cells (respectively, 45,000 and 5,000 cpm). However, in CEM control cells, less than 10% of the labeling was internalized after 30 min at 37°C, while a mean value of 37% was measured in CEM Nef cells. Uptake was abrogated when cells were kept at 4°C (not shown).

Taken together, these data indicated that in cells stably expressing Nef, in which CD4 surface levels are constitutively low, 50% of surface CD4 molecules are endocytosed in 1 h. However, the absolute number of CD4 molecules endocytosed per unit of time was higher in CEM controls than in CEM Nef cells (50 and 18 a.u. in 1 h, respectively). In lymphoid cells, the endocytic machinery continuously recycles 5 to 10% of all CD4 molecules (21). Since in CEM Nef cells CD4 surface levels are constitutively reduced to 10% of normal levels, the rapid endocytosis that we observed could have simply been due to this normal recycling activity. To address this issue, we checked that Nef-induced endocytosis was also effective in cells express-

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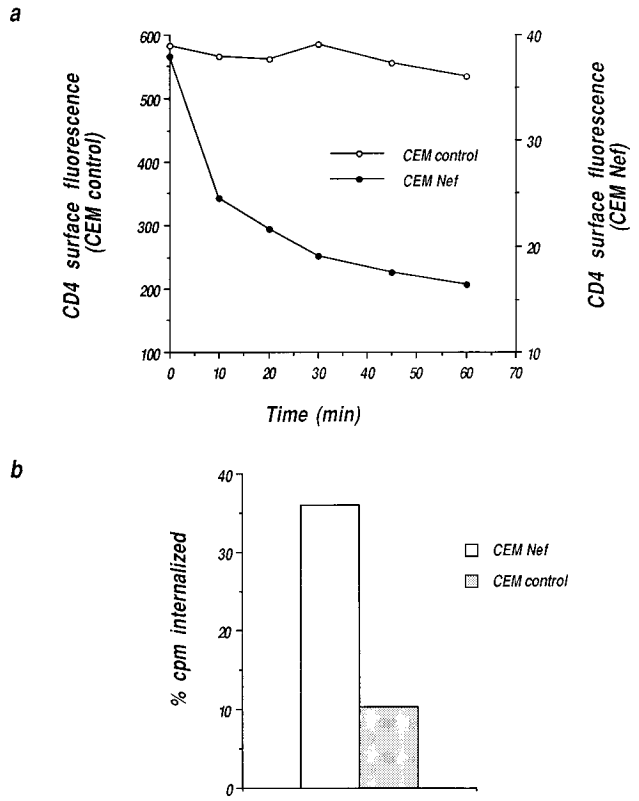

FIG. 1. CD4 internalization in CEM Nef and control cells. (a) Kinetics of CD4 decrease from the surface of cells constitutively expressing Nef. CEM Nef and control cells were labeled for 30 min at 4°C with NUTH-I anti-CD4 MAb (0.5 μg/ml; Nichreï Corp., Tokyo, Japan), washed, and warmed to 37°C for the indicated periods of time. Cells were then cooled to 4°C and stained with a labeled anti-mouse IgG Ab (GAM-PE, 1:100 dilution; Southern Biotechnology). Surface levels of NUTH-I–CD4 complexes were analyzed with a FACScan cytofluorometer (Becton Dickinson). Data are plotted as the mean fluorescence of the peak, at different time points. Results of one experiment representative of three are shown. (b) CD4 internalization. CEM Nef and control cells were labeled on ice with ^{125}I-OKT4 anti-CD4 MAb (0.75 nM) for 30 min, washed, and incubated at 37°C, as described elsewhere (11). After 30 min, discrimination between intracellular and surface CD4 was performed by treating cells with the proteolytic enzyme pronase. Cells were incubated for 30 min in 0.5% pronase (Boehringer) at room temperature and pelleted. Radioactivity present in supernatants (pronase sensitive) and pellets (pronase resistant) was quantified in a gamma counter (Pharmacia). Pronase-resistant radioactivity corresponds to internalized CD4 molecules. Background pronase-resistant activity after the binding period on ice was between 9 and 12% for both cell types. Data are presented as the proportion of pronase-resistant counts to total cell-associated activity, subtracted from the background pronase-resistant activity. Binding of ^{125}I-OKT4 was reduced to less than 1% by preincubating CEM cells with a 100-fold excess of cold MAb (not shown). Total cell-associated radioactivity values after binding with ^{125}I-OKT4 were, respectively, 5,000 and 45,000 cpm for CEM Nef and control cells. Results of one experiment representative of two are shown.

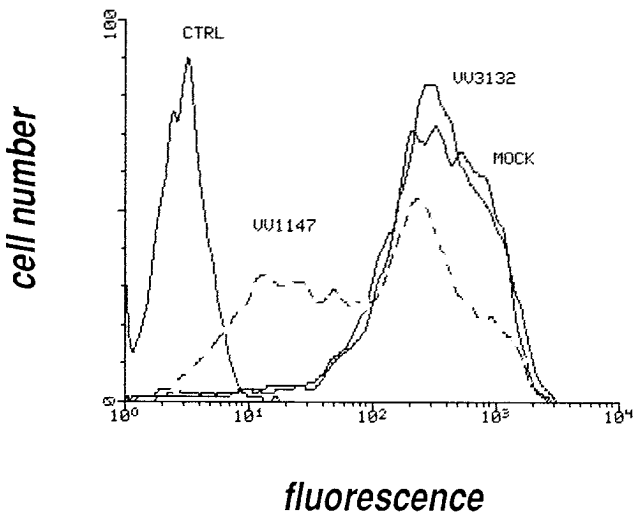

FIG. 2. Surface CD4 levels after transient expression of Nef. CEM cells were labeled at 4°C with NUTH-I anti-CD4 MAb and washed. Cells were then infected with VV vectors as previously described (27). Fifteen hours after infection, surface levels of NUTH-I–CD4 complexes were measured by flow cytometry after a staining with a labeled anti-mouse IgG Ab. Two vectors were used: VV1147 encodes a wild-type HIV_{LAI} Nef (dashed line) and VV3132 encodes an inactive mutant (with a deletion of RFDS residues at positions 184 to 187 [solid line]). Mock-infected cells were also analyzed (solid line). Nonspecific binding on VV-infected cells was measured with anti-keyhole limpet hemocyanin IgG1-phycoerythrin MAb (CTRL curve). Results of one experiment representative of two are shown.

ing normal levels of CD4 at the surface. CEM cells were first stained with NUTH-I and then infected with a recombinant vaccinia virus (VV) encoding the HIV_{LAI} *nef* gene (16). The presence of the preformed CD4-anti-CD4 complexes at the cell surface was analyzed by FACS 15 h after infection (Fig. 2). When cells were mock infected or infected with a VV encoding an inactive Nef mutant (17), high CD4 surface levels were detected. These levels were decreased when the active Nef was expressed, indicating that CD4 endocytosis occurred even in the presence of high CD4 surface levels. These data also showed that Nef can act on CD4 molecules after their transport to the cell surface.

In order to localize the site of CD4 accumulation in Nef-expressing cells, CEM Nef and control cells were stained for indirect immunofluorescence microscopy. The anti-CD4 MAb NUTH-I stained the cell surface of control CEM cells and gave a weak additional intracellular signal probably corresponding to newly synthesized CD4 molecules engaged along the secretory pathway (Fig. 3, upper right panel). In CEM Nef cells incubated with NUTH-I, the surface staining was abrogated, and multiple intracytoplasmic fluorescent dots were observed (Fig. 3, upper left panel), as initially reported for HPBALL cells (13). In $CD4^+$ T cells, up to 95% of CD4 molecules are associated with the tyrosine kinase $p56^{lck}$ (18), and this association inhibits CD4 endocytosis by preventing its entry into coated pits (23). In the CEM cells used in this study, at least 50% of the total $p56^{lck}$ was found to be associated to CD4 by coimmunoprecipitation with OKT4 (data not shown). Staining of these cells with affinity-purified anti-$p56^{lck}$ Abs resulted in a strong membrane-associated signal that was not modified in the presence of Nef (Fig. 3, lower panels). Therefore, CD4 internalization was not accompanied by a significant relocalization of $p56^{lck}$, and it is likely that the kinase was upheld at the membrane. This is in agreement with the observation that the levels of CD4-associated $p56^{lck}$ are decreased in cells producing Nef (2, 4).

Double staining and confocal microscopic analysis were performed to identify the intracellular compartments in which CD4 molecules accumulated in Nef-expressing cells (Fig. 4). Anti-CD4 NUTH-I (green fluorescence) and specific markers of subcellular compartments (red fluorescence) were used. The Golgi apparatus complex was visualized with Abs directed against rab6, a small GTP-binding protein concentrated on the medial and *trans* cisternae of the Golgi apparatus complex (14). Figure 4a shows one representative horizontal medial section of CEM control and Nef cells. The left and center

FIG. 3. CD4 and p56^{lck} localization in CEM Nef and control cells. CEM Nef (left panels) and control cells (right panels) were centrifuged on polylysine-coated glass coverslips, fixed, and permeabilized with saponin. Cells were then labeled with anti-CD4 NUTH-I MAb and then fluorescein isothiocyanate-conjugated sheep anti-mouse Abs (upper panels) or with immunoaffinity-purified anti-p56^{lck} rabbit Abs (8) followed by Texas red-conjugated donkey anti-rabbit Abs (lower panels). The specificity of the affinity-purified Abs for p56^{lck} has been described elsewhere (8). Cells were analyzed with a Nikon microscope equipped for epifluorescence, and photographs were taken with Kodak Ektachrome 400 ASA film.

columns represent stainings with anti-CD4 (green) and anti-rab6 (red) MAbs, respectively, while the right column is a superposition of the two stainings, in which costained regions appear in yellow. Anti-rab6 Abs stained CEM control and Nef cells in a region next to the nucleus, which is typical of the Golgi apparatus complex (14). This region was also stained with the anti-CD4 MAb in both cell types, likely corresponding to neosynthesized CD4 molecules transiting through the Golgi apparatus complex. However, most CD4 molecules were located at the surface of control CEM cells. In Nef cells, the intracellular vesicles positive for CD4 were not stained by the anti-rab6 MAb, indicating that the Golgi apparatus complex is not the site of CD4 accumulation in Nef-expressing cells.

We observed in CEM Nef cells a colocalization of CD4 and DAMP, a probe for acidic organelles, including early and late endosomes and lysosomes (3) (not shown). To determine in which acidic organelle CD4 accumulated, CEM Nef and control cells were stained with transferrin-rhodamine (RITC-Tf), a specific marker of early endosomes (10). Cells were deprived of transferrin by incubation for 30 min at 37°C in serum-free medium and were stained with RITC-Tf for 15 min. After fixation and permeabilization, they were counterstained with NUTH-I MAb (Fig. 4b). Early endosomes appeared as clusters of vesicles in the juxtanuclear region of both control CEM and Nef cells (red fluorescence). In Nef-expressing cells, CD4-specific staining overlapped with that of RITC-Tf, indicating that CD4 molecules accumulated in early endosomes. CEM Nef and control cells were also stained with Abs against the cation-independent mannose-6 phosphate receptor (CI-MPR), which is mostly located in late endosomes or prelysosomes (15, 24). In both cell types, staining with anti-CI-MPR Abs produced a pattern of large and diffuse vesicles typical of late endosomes (Fig. 4c, red fluorescence). These vesicles were not stained with the anti-CD4 MAb in CEM Nef cells. Thus,

FIG. 4. Confocal microscopic analysis of CD4 localization. CEM control (upper rows) and Nef cells (lower rows) were centrifuged on polylysine-coated glass coverslips, fixed, and permeabilized with saponin. Cells were then labeled with anti-CD4 NUTH-I MAb (green fluorescence) and with different markers of subcellular compartments (red fluorescence). Cells were analyzed by confocal microscopy on a Wild Leica CLSM instrument based on a Leitz diaplan microscope. Series of optical sections at 1-μm intervals were recorded. Photographs were taken with Kodak Ektachrome 100 ASA film. One representative optical medial section of 1 μm is shown. For each subcellular marker, the third column represents a superposition of green and red fluorescence, with costained regions appearing in yellow. (a) Stainings with Abs against CD4 and against rab6, a marker of the Golgi apparatus complex (14); (b) stainings with Abs against CD4 and with RITC-Tf, a marker of early endosomes (10); (c) stainings with Abs against CD4 and CI-MPR, a marker of late endosomes (15).

Nef promotes an accumulation of CD4 in an acidic compartment that includes early, but not late, endosomes.

We have observed here that in the presence of Nef, cell surface CD4 molecules are actively endocytosed, accumulate into early endosomes, and are degraded, while CD4 synthesis is not modified. The enhancement of endocytosis was observed both in cells constitutively expressing Nef and displaying low CD4 surface levels and in cells with high CD4 surface levels in which Nef was transiently expressed. Our data suggest that most CD4 molecules retained into early endosomes had transited through the cell surface. Endocytosis was very effective, with 50% of surface CD4 being internalized in 1 h. During the natural course of HIV infection, it is therefore likely that a rapid endocytosis of the viral receptor takes place shortly after Nef is expressed and before *env* and *vpu* gene products act on newly synthesized CD4 molecules (9, 32).

Brady et al. have previously reported the colocalization of CD4 with a Golgi apparatus-specific marker in T cells of *nef* transgenic mice (7). However, because of the small size and large nucleus of T cells, precise intracellular localization in organelles by immunolabeling was difficult. Our finding that CD4 accumulates in early endosomes was deduced from confocal microscopic analysis showing colocalization with RITC-Tf but not with CI-MPR or rab6. Transferrin is indeed internalized in early endosomes and recycled to the cell surface, without reaching late compartments (10). The reduced half-life of CD4 in Nef-expressing cells indicated that CD4 molecules accumulating in endosomes end up being degraded. This degradation can take place in acidic early endosomes, which contain active proteolytic enzymes implicated in the processing of hormones, growth factors, toxins, or antigens (6). Alternatively, a fraction of endocytosed CD4 molecules could be routed to late endosomes and lysosomes and be rapidly degraded. This process has been recently documented for interleukin-2, which is internalized upon binding to its receptor, accumulates in early endosomes, and is then degraded in lysosomes without detectable accumulation in the late compartments (11).

Down-modulation of CD4 by activation of the endocytic pathway is a physiological event observed when T cells are stimulated by antigen-presenting cells and which can be mimicked by treatment of cells with phorbol esters (1). Phorbol ester treatment of T cells first induces a dissociation of CD4 from $p56^{lck}$, before CD4 internalization, and then promotes a redistribution of CD4 from the recycling to the degradative pathway (19, 24, 31). Although both phorbol esters and Nef induce CD4 endocytosis, it is likely that they act according to different mechanisms. This is suggested by the fact that CD4 mutants that do not, or only partially, respond to phorbol esters retain their sensitivity to Nef (2, 13). Here we further document the difference between the two modulation pathways by showing that in contrast to phorbol esters, when CD4 is internalized under the action of Nef, it accumulates in early endosomes and is not massively targeted in late compartments. Phorbol ester-induced endocytosis involves the phosphorylation of three serines in the cytoplasmic tail of CD4 (29). Yet, in the absence of these residues, a minor phosphorylation-independent pathway has been observed (28). It is possible that Nef, which does not require the presence of the serines (13), acts along this phosphorylation-independent pathway. Further investigations on the interaction of Nef with the endocytosis machinery are now required to understand the specificity of the phenomenon and how it relates to the enhancement of virus production in the infected cell.

We thank Raymond Hellio for confocal microscopy analysis, Sigmund Fisher and Anne-Marie Cardine for discussions and for the gift of anti-$p56^{lck}$ antibodies, Bernard Hoflack for the gift of anti-CI-MPR antibodies, and Yves Rivière and Marie-Paule Kieny for the gift of VV vectors.

O.S. is a fellow of the Institut Pasteur-CANAM. This work was supported by grants from the Agence Nationale de Recherche sur le SIDA (ANRS) and the Institut Pasteur.

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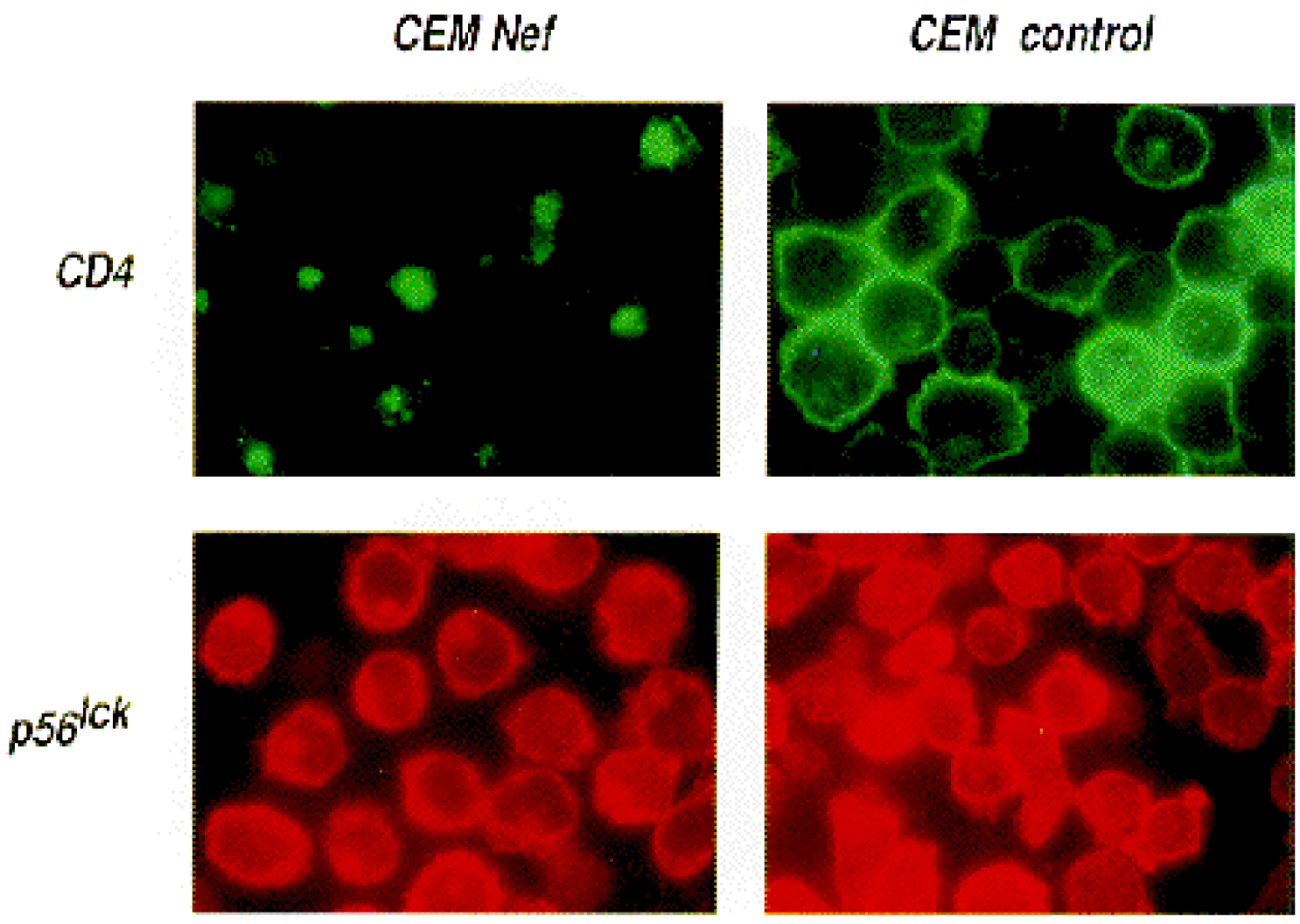
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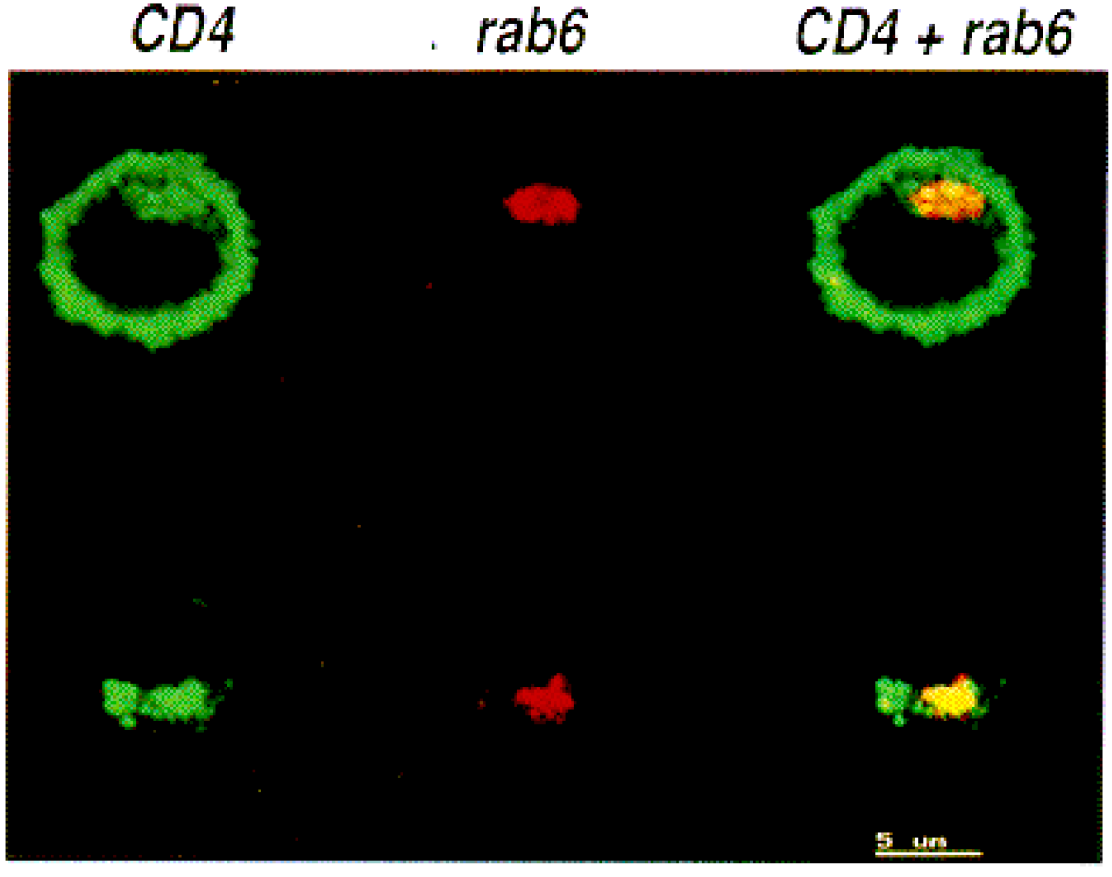

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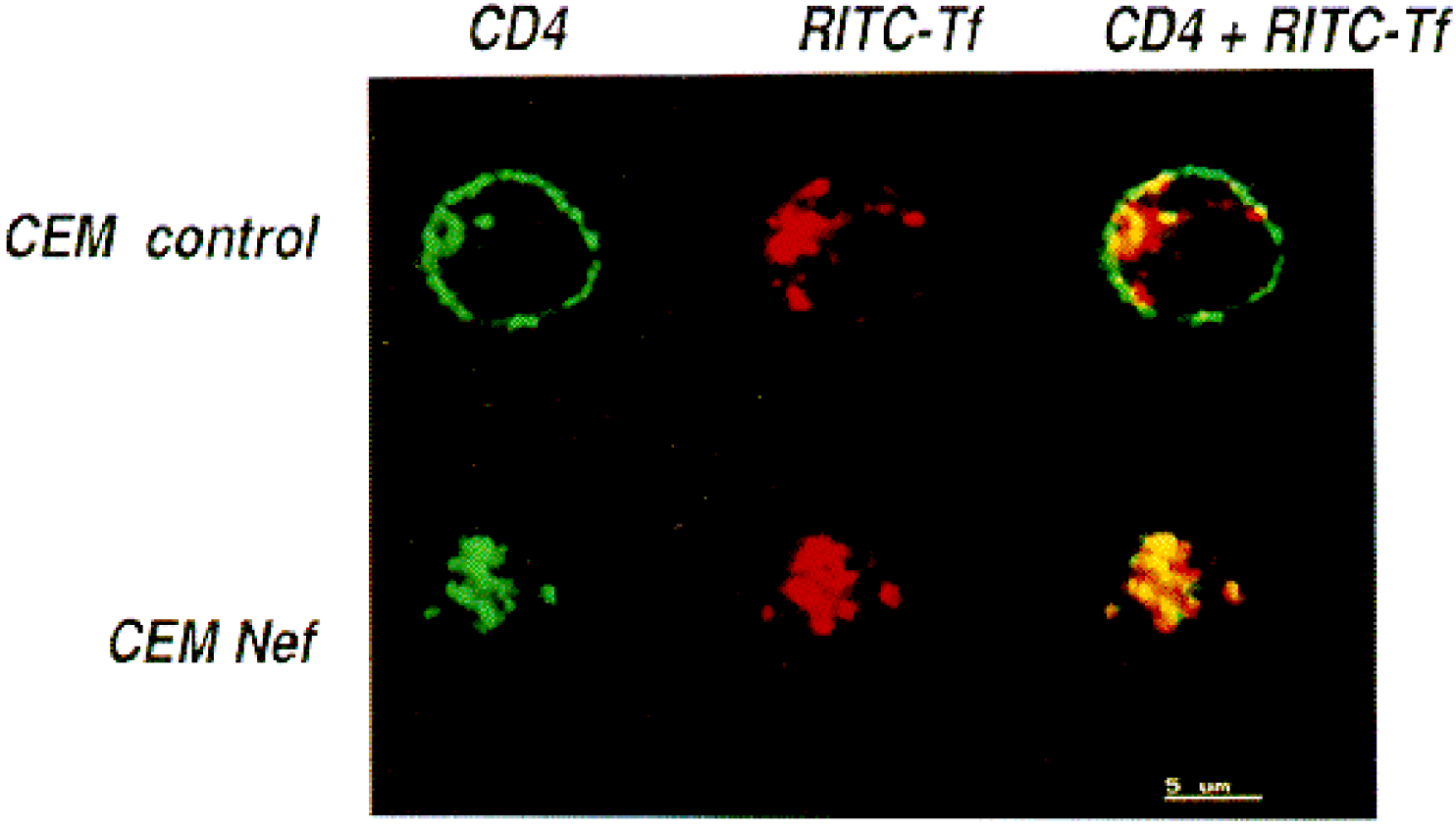

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