

murine cells only weakly support HIV replication even if the entry blocks are bypassed (3, 4). In contrast, mink cells readily replicate HIV-1 if entry is facilitated as an amphotropic MLV pseudotype (3) or if nonpseudotype HIV-1 is adsorbed to mink cells expressing human CD4 and then treated with the nonspecific fusogen, polyethylene glycol (5). Moreover, we have shown that mink Mv-1-lu cells and feline CCC cells can be infected by several strains of HIV-2 and simian immunodeficiency without the presence of human factors other than CD4 (6).

We used the same CD26 cDNA clone as did Callebaut *et al.* (1). Stable, dual expression of human CD4 and human CD26 did not render these cells susceptible to infection by the LA1 and RF strains of HIV-1 (Table 1). Flow cytometry revealed that more than 99% of mink CD4-CD26

cells expressed substantial amounts of both human antigens at the cell surface, whereas approximately 75% of cat cells expressed both antigens. The CD26 was enzymatically active. In conclusion, our results, obtained with the use of an assay system for CD4-dependent HIV infection in permissive carnivore cell lines, indicate that human CD26 is not required for HIV-2 entry and is not sufficient for HIV-1 entry.

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Callebaut *et al.* conclude that the human T cell activation antigen CD26 is a cofactor for HIV infection of CD4<sup>+</sup> cells (1). Their data indicate that murine NIH 3T3 cells are permissive to infection by HIV only when human CD4 and CD26 are present. If correct, this observation has important im-

plications for HIV tropism in human cells, as it has previously been reported by ourselves and others that CD4 is necessary but not sufficient for HIV infection of human lymphoblastoid T cell lines and mononuclear phagocytes. Furthermore, it has been shown that human CD4 is not sufficient for

HIV infection of mouse (NIH 3T3) cells (2) or of African green monkey cells (COS) (3). Because one of us (D.C.) was involved in the isolation of a cDNA encoding the human CD26 antigen (4), we tested the role of CD26 in HIV-1 infection of murine and nonhuman primate cell lines.

**Table 1.** CD26 function in HIV infection. Light units are the result of luciferase assay of lysates of the indicated cell lines and transfectants after incubation with HIV-1-luciferase transducing particles coated with the indicated envelope. Syncytia data indicate the number of blue syncytia formed when the same cells were transfected with a T7-promoter-lacZ construct and mixed with HeLa cells expressing HIV-1 envelope and T7 RNA polymerase. The percentages of cells expressing CD4, CD26, and both antigens are shown. Values are the average of four luciferase assays with LAI HIV-1 envelope, two luciferase assays with amphotropic envelope, and three syncytium assays; they represent at least 2500 cells analyzed by flow cytometry. For the luciferase assay the background was 113 ± 7 light units for eight measurements of buffer only, and the background values were all within one standard deviation of that value. For the syncytium assay the background values were all less than four. Bckg, background; ND, not determined. COS cells were co-transfected with an envelope-deleted JR-CSF HIV-1 genome bearing the firefly luciferase gene and an LAI HIV-1 envelope or murine amphotropic envelope expression vector (7), by electroporation or with lipofectamine (Gibco/BRL, Bethesda, Maryland). Pseudotype HIV-1-luciferase transducing

particles were harvested 2 days later and incubated with the cells listed. The cells were lysed 3 days later and assayed for luciferase activity with a Monolight 2010 luminometer and Promega luciferase assay kit. The syncytium assay was done essentially by the protocol of Berger *et al.* (6). Briefly, 2 × 10<sup>5</sup> of the cells per well of each indicated cell type were infected with wild-type vaccinia and transfected with the lacZ gene under the control of the T7 promoter (8) with the use of lipofectamine. They were then mixed with HeLa cells infected with recombinant vaccinia encoding LAI HIV-1 envelope and T7 RNA polymerase (9). The cells were fixed 1 day later and incubated with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Sigma, St. Louis, Missouri); then syncytia were counted in one-eighth of each well of a 24-well plate. Surface expression of CD4 and CD26 was determined by incubation with Leu-3A-FITC (Becton Dickinson, Mountain View, California) and Ta1-RD1 (Coulter, Hialeah, Florida) or unconjugated 4ELIC7, CB.1, and TS145 ascites, and then with phycoerythrin conjugated goat antibody to mouse IgG (Caltag, South San Francisco, California), respectively, with the use of a FACScan flow cytometer (Becton Dickinson). The percentages of mixed HeLa and HeLa-CD4 cells positive for CD4 were calculated.

Cell line (transfected plasmid)	%CD4 <sup>+</sup>	%CD26 <sup>+</sup>	%CD4 <sup>+</sup> CD26 <sup>+</sup>	Syncytia	HIV-luciferase + LAI HIV-1 envelope (light units)	HIV-luciferase + amphotropic envelope (light units)
HeLa-CD4	>98	>98	>98	Confluent	ND	ND
HeLa	0	>98	0	0 ± 0	ND	ND
HeLa-CD4/HeLa = 0.1	10	>98	10	98 ± 13	1719 ± 47	149 ± 10
HeLa-CD4/HeLa = 0.02	2	>98	2	21 ± 1	1525 ± 1215	206 ± 9
HeLa-CD4/HeLa = 0.004	0.4	>98	0.4	Bckg	631 ± 81	163 ± 58
HeLa-CD4/HeLa = 0.0008	0.08	>98	0.08	Bckg	Bckg	766 ± 29
COS-CD4 (CDM7-CD26)	>98	81.3	81.3	Bckg	Bckg	18983 ± 5311
COS-CD4 (CDM8)	>98	<2	<2	Bckg	Bckg	10235 ± 2207
A9 (CDM8-CD4, CDM7-CD26)	21.6	39.8	19.6	Bckg	Bckg*	ND
A9 (CDM8-CD4)	27.2	<2	<2	Bckg	Bckg*	ND
NIH 3T3-CD4 (CDM7-CD26)	>98	2.3	2.3	ND	Bckg	4634 ± 95
NIH 3T3-CD4 (CDM8)	>98	<0.1	<0.1	ND	Bckg	4280 ± 276

\*These values were determined in a separate assay in which 1 HeLa-CD4 cell in 10 HeLa cells could be detected, but 1 in 100 could not.

The ability of the HIV-1 *envelope* gene products to form syncytia is correlated with infectivity of HIV-1 strains with respect to species and cell type (5). Simian and murine cells bearing human CD4 and CD26 were tested in a syncytium assay with the use of vaccinia-expressed *envelope* from the LAI isolate of HIV-1, essentially as described by Berger *et al.* (6). In this assay, HeLa-CD4 cells form abundant blue syncytia, detectable in a 50-fold excess of syncytium-resistant HeLa cells (Table 1). In contrast, we found that COS cells or A9 cells expressing human CD4 and CD26, or CD4 alone, did not form detectable HIV-1 envelope-mediated syncytia.

To more directly test the role of CD26 in HIV-1 infection, we constructed an *envelope*-defective molecular clone of the JR-CSF isolate of HIV-1 that bears the firefly luciferase gene in place of the *nef* gene. With this clone, luciferase activity could be used as a sensitive assay for HIV-1 infection. We prepared pseudotype defective HIV-1-luciferase virions by cotransfecting this modified HIV-1 genome with an LAI HIV-1 envelope expression vector, or with a murine amphotropic retroviral envelope expression vector, and then used them to infect human, simian, and murine cell lines that express both human CD4 and CD26. This assay is not only sensitive, but because it measures intracellular expression of luciferase, potential problems with residual input of HIV-1 [which Callebaut *et al.* address with trypsin treatment (1)] are obviated. We incubated HIV-1-luciferase virions bearing LAI HIV envelope glycoproteins with simian (COS) and mouse (NIH 3T3 and A9) cells expressing CD4 alone or CD4 and CD26. We assayed cells for luciferase activity 3 days after exposure to the HIV-1 pseudotype. As expected, COS, NIH 3T3, and A9 cells expressing CD4 alone were not infectable. Co-expression of CD26 with CD4 in these cells did not render them infectable by HIV-1-luciferase virions bearing LAI HIV-1 envelope glycoproteins as determined by luciferase activity (Table 1). In a reconstruction experiment, infection of HeLa-CD4 cells mixed with a 250-fold excess of HeLa cells resulted in detectable luciferase activity. Therefore, the sensitivity of the assay was sufficient to detect infection of the murine and simian cells expressing human CD4 and CD26 had they been susceptible to HIV-1. Furthermore, all these cells expressed luciferase after infection by HIV-1-luciferase virions bearing murine amphotropic retrovirus envelope glycoproteins, which indicates that the transfected cells were competent to support HIV-1 infection and luciferase expression, given a functional envelope-receptor pair.

One potential explanation for the positive NIH 3T3 infection observed by Calle-

baut *et al.* (1) is that CD26 with CD4 may indeed confer HIV susceptibility very inefficiently, detectable only after incubation with CEM cells. In such an assay, even a few infectious virions may be amplified, by viral spread in CEM cells, to detectable amounts. However, if this is the case, one must question the biological relevance of a cofactor that does not confer susceptibility to the majority of target cells harboring it.

In summary, we used sensitive, quantitative assays of HIV-1-mediated syncytium formation and HIV-1 infection to test the role of CD26 in these processes. We are unable to confirm the report of Callebaut *et al.* and conclude that human CD26 does not confer susceptibility to HIV-1 syncytium formation or infection in murine or simian cell lines that express human CD4.

*Note added in proof:* We recently made an NIH 3T3 cell line that stably expresses human CD4 and CD26. Although 45% of the cells expressed both receptors, we were unable to detect luciferase activity after infecting the cells with HIV-1-luciferase coated with LAI HIV-1 envelope.

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Callebaut *et al.* identify the cell surface protease CD26 as the long-sought human cofactor that allows HIV entry into CD4<sup>+</sup> cells (1). Their principal experimental evidence is the complementation of murine NIH 3T3 cells for productive HIV infection by transient transfection with CD4 and CD26 cDNAs. However, an undetermined and probably minor fraction of the murine

cells coexpressed CD4 and CD26, and virus entry could not be measured directly. Hence, virus production was detected after amplification on human CD4<sup>+</sup> cells.

We sought to perform more direct assays to evaluate the possible role of CD26 in HIV entry. We stably transfected a CD26 expression vector (2) into CD4<sup>+</sup> NIH 3T3 cells [SC6 cell line (3)]. We performed experiments on a clone (SC6-CD26) that expresses large amounts of surface CD26 (similar to activated human lymphocytes), and on an uncloned population that we had sorted for CD26 expression (SC6-CD26P). Surface CD4 expression was similar in parental and CD26<sup>+</sup> cells; DPP IV activity was only detected in CD26<sup>+</sup> cells (4).

Because HIV entry in murine CD4 cells is blocked at the stage of membrane fusion with the viral envelope (5), we tested the ability of CD4<sup>+</sup>CD26<sup>+</sup> murine cells to form syncytia with cells that express HIV envelope proteins. The SC6 cells contain a LTR-*lacZ* transgene induced after fusion with cells that express the viral protein Tat, such as HIV-infected cells (3). With the use of this highly sensitive assay, we could not detect fusion between CD26<sup>+</sup> SC6 cells and different HIV-1 infected cell lines. We also did not detect virus production after contact of parental or CD26<sup>+</sup> SC6 cells with HIV-1 (one infectious unit, IU, per cell), either directly or after coculture with human CD4<sup>+</sup> cells (4). Because the small amount of HIV-1 expression in murine cells might have limited the sensitivity of assays that are based on virus production, we sought proviral DNA as a marker of virus entry (Fig. 1). HIV-1 DNA was not amplified by polymerase chain reaction (PCR) in lysates from parental or CD26<sup>+</sup> SC6 cells after contact with 5,000 IU of HIV-1 (6). Proviral DNA was readily detected in parallel CD4-independent infections with 500 IU of HIV-1 coated with the envelope of human T cell leukemia virus type 1 (HTLV-1) (6).

In these experiments, we did not confirm the role of CD26 as a cofactor that allows HIV-1 entry into murine CD4<sup>+</sup> cells. Callebaut *et al.* did not observe HIV production from murine cells in the presence of zidovudine (AZT), which led them to suggest that HIV replication was necessary and, hence, that virus entry occurred. It can be argued that AZT also has effects at the cell surface, as it reduces the ability of uninfected CD4<sup>+</sup> cells to form syncytia (7). However, a simpler explanation could be that calcium phosphate transfection of murine cells allowed artifactual virus entry. Indeed, HIV was retrieved by Callebaut *et al.* (1) from murine cells transfected with CD4 alone, or with CD26 alone. A simple additive effect could explain the apparently higher quantity of virus rescued from cotransfected cells.

## CD26 antigen and HIV fusion?

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