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37. White blood cells from the family members were lysed and the DNA denatured by heating in aqueous suspension for 30 min at 95°C. Eight polymorphic DNA microsatellite markers (bold font, larger size in Fig. 1) were amplified by PCR and analyzed by electrophoresis in denaturing acrylamide gels (*18*). *IL4-R1*, a complex $(TG)_n$-$(TA)_m$ repeat in intron 2 of *IL4* (*35*) was also analyzed by agarose gel electrophoresis (*19*). The length for the shortest, most common allele of *IL4-R1* corresponds to that reported (*35*).
38. We wish to thank the Amish families for their willingness to participate in this study, W. Bias for providing cell and plasma samples from certain Amish families, R. Hamilton for help with the total IgE analyses, J. York-Blasser, RN, and S. Beiler for fieldwork, C. Stewart and G. Hansen for technical assistance, S.-K. Huang and B. Catipovic for discussions, and CIBA-Corning for provision of equipment. This research was supported by NIH grant AI20059. The program package S.A.G.E. is supported by USPHS resource grant 1 P41 RR03655 from the Division of Research Resources.

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TECHNICAL COMMENTS

CD26 Antigen and HIV Fusion?

The human CD4 molecule generally must be expressed on a human cell type in order to support the membrane fusion reactions involved in human immunodeficiency virus type 1 (HIV-1) infection and syncytium formation (*1–7*). Studies of cell fusion mediated by recombinant CD4 and HIV-1 envelope glycoprotein (env) indicate that this species restriction is unidirectional: CD4 must be present on a human cell type, whereas env functions comparably when expressed on human or nonhuman cells (*2*). Furthermore, the fusion defect in CD4-expressing nonhuman cells can be overcome by forming stable (*5*) or transient (*6, 7*) hybrids with human cells. These findings suggest the essential involvement of a human-specific accessory component in the $CD4^+$ cell rather than the presence of a fusion inhibitor in the nonhuman cells or an inherent fusion incompatibility between human and nonhuman cell types. C. Callebaut *et al.* recently proposed that the human CD26 antigen, also known as dipeptidyl peptidase IV (DPP IV), serves as an essential cofactor for HIV entry into $CD4^+$ cells (*8*). This report prompted us to analyze the role of CD26 in HIV-1 env/CD4-mediated cell fusion. A preliminary summary of our findings has been presented (*9*).

One of our experimental approaches was based on the report by Callebaut *et al.* that transient co-expression of human CD4 and CD26 rendered murine NIH 3T3 cells permissive for HIV infection (*8*). We used a well-characterized expression system based on vaccinia vectors to study directly fusion between cells expressing recombinant HIV-1 env and cell types of several species expressing recombinant human CD4; the objective was to test whether co-expression of human CD26 could overcome the fusion defect in nonhuman cells that express CD4. For sensitive quantitative analysis, we used a newly developed assay (*10*) in which the cytoplasm of one cell population contains a transfected plasmid with the *Escherichia coli LacZ* gene linked to a T7 promoter and the cytoplasm of other cell population contains vaccinia-encoded bacteriophage T7 RNA polymerase. Cell fusion results in activation of the reporter gene selectively in the cytoplasm of the fused cells; β-galactosidase activity is quantitated in detergent cell lysates with a colorimetric assay. We also scored cell fusion using a standard assay of syncytia formation (*7, 11*). We used vaccinia vectors to induce the expression of T7 RNA polymerase, with or without CD4, in HeLa (human), NIH 3T3 (murine), and BS-C-1 (simian), cells (Fig. 1); each cell type was also transfected with either a control plasmid or a plasmid containing the CD26 complementary DNA (cDNA) linked to a strong vaccinia promoter. Flow cytometry analysis (Fig. 1A) indicated efficient expression of vaccinia-encoded CD4 on the surface of all three cell types. When CD26 cDNA was included (Fig. 1A, right panels), cell surface CD26 was readily detected in each case. The amounts of vaccinia-encoded CD26 produced in the murine and simian cells were comparable to the amounts present in the human cells without or with augmentation by the vaccinia expression system. Furthermore, separate analyses of intact cells and cell lysates showed that vaccinia-mediated CD26 expression greatly elevated DPP IV enzymatic activity, thereby confirming the functionality of the expressed protein (not shown).

To analyze cell fusion, we mixed the cell populations described above with separate populations of cells expressing vaccinia-encoded HIV-1 env and also transfected with a plasmid containing the *LacZ* gene linked to the T7 promoter. We examined functional wild-type envs from two distinct HIV-1 isolates (IIIB and SF2); as a negative control, we used a mutant uncleavable env (Unc-IIIB, derived from IIIB) that had been rendered fusion-incompetent by deletion of the normal gp120/gp41 cleavage site (*12*). After cell mixing and incubation, the detergent NP-40 was added to one set of samples and the amounts of β-galactosidase were measured (Fig. 1B). A second set of samples was analyzed microscopically for syncytia formation (Fig. 1C). When HeLa cells (Fig. 1B, top panel) expressing CD4 were mixed with cells expressing active envs from either the IIIB or the SF2 isolates, large amounts of β-galactosidase were produced. We observed only low background amounts in the negative controls when the mutant uncleavable env was used or when CD4 was omitted from the T7 RNA-polymerase–containing HeLa cells, or both. These results demonstrate that the β-galactosidase signals with the wild-type envs represented env/CD4-mediated cell fusion. Expression of vaccinia-encoded CD26 on the CD4-expressing HeLa cells had no significant effects on fusion. When we examined the murine (Fig. 1B, center panel) or simian cells (Fig. 1B, bottom panel), expression of CD4 yielded only background amounts of β-galactosidase, independent of the fusogenic nature of env on

the partner cell. Most important, co-expression of CD26 did not confer fusion competence to the CD4-expressing murine or simian cells (Fig. 1B). With the syncytia assay, we observed extensive cell fusion when HeLa cells co-expressing vaccinia-encoded CD4 and CD26 were mixed with cells expressing vaccinia-encoded env (IIIB) (Fig. 1C, top panel). Analysis of other samples (not shown) indicated that syncytia formation was independent of vaccinia-mediated CD26 expression on the HeLa cells, but did not occur when CD4 was omitted from the HeLa cells or when the mutant uncleavable env was expressed on the partner cell, consistent with previous findings (*13*). In contrast with the results with HeLa cells, we did not observe syncytia when either the murine (Fig. 1C, center panel) or simian (Fig. 1C, bottom panel) cells co-expressing vaccinia-encoded CD4 and CD26 were mixed with the same env-expressing cells. The pattern of syncytia formation with env from the SF2 isolate paralleled that observed for the IIIB env (not shown). Thus, two distinct fusion assays indicated that CD26 expression does not render nonhuman cells competent for env/CD4-mediated cell fusion.

To examine whether the block to fusion in the CD4-expressing nonhuman cells could potentially be overcome, we tested the effects of forming transient hybrids with human cells. The experiment was conducted in two phases (Fig. 2). In the first phase, Sendai virus was used to generate transient cell hybrids in mixtures of cells expressing vaccinia-encoded CD4 and cells expressing vaccinia-encoded T7 RNA polymerase. After 6 hours, the cells were detached from the plate by trypsinization, washed, and divided into three portions. Each portion was infected at an moi of 10 with the following vaccinia recombinants containing the indicated HIV-1 *env* gene linked to a synthetic strong early–strong late vaccinia promoter (*23*): vSC60, wild-type env, HTLV-IIIB BH8 isolate (*23*); vCB-34, wild-type env, SF2 isolate (*24*); vCB-16, mutant uncleavable env, HTLV-IIIB BH8 isolate (*24*). After infection, the cells were suspended to a density of 5×10^5 cells per milliliter in Eagle's minimal essential medium supplemented with 2.5% fetal bovine serum (MEM-2.5% FBS) and incubated overnight (14 hours) to allow accumulation of the recombinant envs. For the second fusion partner, monolayers of HeLa, NIH 3T3, or BS-C-1 cells in 75-cm^2 flasks were co-infected with vaccinia recombinants vCB-3 [encoding CD4 linked to the strong early–strong late vaccinia promoter (*7*)], and vTF7-3 [encoding bacteriophage T7 RNA polymerase linked to the p7.5 vaccinia promoter (*19*)]; for CD4$^-$ controls the same cell types were co-infected with wild-type vaccinia virus strain WR and vTF7-3. The moi was 10 for each virus. After 1 hour, one portion of each infected cell type was transfected with 15 μg of control plasmid pSC59 containing the vaccinia strong early–strong late vaccinia promoter followed by a multiple cloning site (*23*); a second portion was transfected with 15 μg of plasmid pCB47 containing the human CD26 cDNA linked to the vaccinia strong early–strong late promoter. Plasmid pCB47 was prepared by digesting plasmid pKG5-CD26 (*20*) with Xho I and isolating the 2.9-kbp fragment; the 3′ recessed ends were filled in with the Klenow fragment of *E. coli* DNA polymerase I and cloned into the Stu I site of pSC59. At 4.5 hours after infection the cells were detached from the plate by trysinization, washed, suspended at a density of 5×10^5 cells per milliliter in MEM-2.5%, and incubated overnight (14 hours) to allow accumulation of the vaccinia-encoded proteins. After the incubations, each cell sample was washed and suspended in medium to a density of 1×10^6 cells per milliliter. Aliquots of some cell samples were analyzed by flow cytometry, and mixtures of aliquots of various samples were prepared for cell fusion assays. (**A**) Flow cytometry analysis. HeLa (top panels), NIH 3T3 (middle panels), or BS-C-1 (bottom panels) cells co-infected with vTF7-3 and vCB-3 and transfected with either control plasmid pSC59 (left panels) or the CD26-encoding plasmid pCB47 (right panels) were analyzed by indirect immunofluorescence. The monoclonal antibodies (all murine IgG_{2a}) used in the first step were antibody to CD26 (solid line) (BA5, Biosource International, Camarillo, California); antibody to CD4 (dashed line) (OKT4A, Ortho Diagnostics, Raritan, New Jersey); control antibody to env (dotted line) [D20, (*21*)]. After 1 hour of incubation at 4°C, the cells were washed twice and stained with fluorescein isothiocyanate–labeled goat antibody to mouse IgG (H+L), F(ab′)$_2$ (Boehringer Mannheim). The cells were incubated for 1 hour at 4°C, washed twice, and fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS). Surface fluorescence was analyzed with the use of a Becton Dickinson FACScan flow cytometer (San Jose, California), using identical conditions for all samples. Cell fusion analysis with (**B**) reporter gene activation and (**C**) syncytia. Duplicate cell mixtures (0.1-ml aliquots of each of the two cell types) were prepared in individual wells of two 96-well flat-bottom tissue culture plates (Costar, Cambridge, Massachusetts). Cultures were kept at 37°C in a humidified CO_2 incubator. For analysis of reporter gene activation on one plate (B), HeLa (top panel), NIH 3T3 (middle panel), or BS-C-1 (bottom panel) cells expressing vaccinia-encoded T7 RNA polymerase, with or without CD4 and CD26 expression as indicated, were mixed with cells transfected with plasmid pG1NT7β-gal and expressing the indicated env (Wt-IIIB, wild-type IIIB isolate; Wt-SF2, wild-type SF2 isolate; Unc-IIIB, mutant uncleavable IIIB isolate). After 2.5 hours, the fusion reactions were terminated by addition of 20 μl of 10% (v/v) NP-40. Aliquots (50 μl) of each detergent cell lysate were assayed for β-galactosidase activity in assay buffer containing 0.06M $Na_2HPO_4 \cdot 7H_2O$, 0.04 M $NaH_2PO_4 \cdot H_2O$, 0.01 M KCl, 0.001 M $M_gSO_4 \cdot 7H_2O$, 0.05 M 2-mercaptoethanol, and 8 mM substrate (chlorphenol red-β-D-galactopyranoside). The rates of substrate hydrolysis were determined by measuring A_{590} with the use of a microplate absorbance reader. Data are expressed as OD/min × 1000, where OD is optical density; error bars represent population standard deviation. For analysis of syncytia formation (C), samples on the second plate were examined 5 hours after mixing with the use of an inverted tissue culture microscope with a ×20 phase contrast objective and a ×10 ocular objective. Equivalent results were obtained 20 hours after cell mixing. Only selected samples are shown: namely, cells expressing wild-type env, IIIB isolate, mixed with HeLa (top panel), NIH 3T3 (middle panel), or BS-C-1 (bottom panel) cells expressing both CD4 and CD26. Scale bar in the bottom pane, 100 μm.

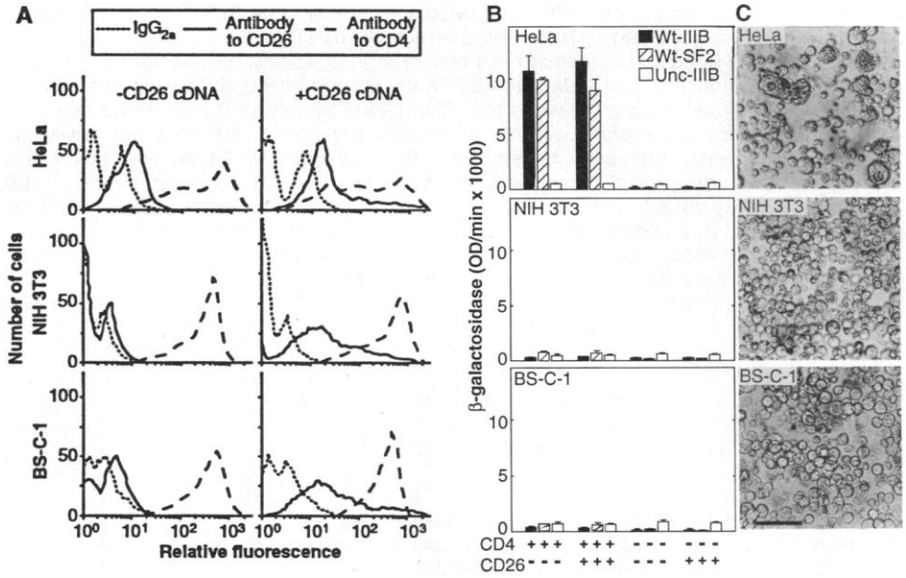

Fig. 1. Effect of CD26 expression on HIV-1 env/CD4-mediated cell fusion. For one fusion partner, HeLa cell monolayers (two 150 cm^2 flasks, 2×10^7 cells per flask) were transfected using DOTAP (Boeringher Mannheim, Indianapolis, Indiana) with 25 μg of plasmid pG1NT7β-gal, which contains the *E. coli LacZ* gene linked to the bacteriophage T7 promoter and the 5′ untranslated sequence of encephalomyocarditis virus (*22*). After 6 hours, the cells were detached from the plate by trypsinization, washed, and divided into three portions. Each portion was infected at an moi of 10 with the following vaccinia recombinants containing the indicated HIV-1 *env* gene linked to a synthetic strong early–strong late vaccinia promoter (*23*): vSC60, wild-type env, HTLV-IIIB BH8 isolate (*23*); vCB-34, wild-type env, SF2 isolate (*24*); vCB-16, mutant uncleavable env, HTLV-IIIB BH8 isolate (*24*). 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For analysis of reporter gene activation on one plate (B), HeLa (top panel), NIH 3T3 (middle panel), or BS-C-1 (bottom panel) cells expressing vaccinia-encoded T7 RNA polymerase, with or without CD4 and CD26 expression as indicated, were mixed with cells transfected with plasmid pG1NT7β-gal and expressing the indicated env (Wt-IIIB, wild-type IIIB isolate; Wt-SF2, wild-type SF2 isolate; Unc-IIIB, mutant uncleavable IIIB isolate). After 2.5 hours, the fusion reactions were terminated by addition of 20 μl of 10% (v/v) NP-40. Aliquots (50 μl) of each detergent cell lysate were assayed for β-galactosidase activity in assay buffer containing 0.06M $Na_2HPO_4 \cdot 7H_2O$, 0.04 M $NaH_2PO_4 \cdot H_2O$, 0.01 M KCl, 0.001 M $M_gSO_4 \cdot 7H_2O$, 0.05 M 2-mercaptoethanol, and 8 mM substrate (chlorphenol red-β-D-galactopyranoside). The rates of substrate hydrolysis were determined by measuring A_{590} with the use of a microplate absorbance reader. 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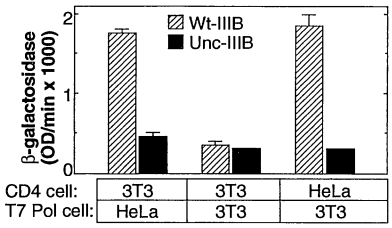

Fig. 2. Fusion of env-expressing cells with CD4-expressing transient cell hybrids. In the first phase cells (HeLa or NIH 3T3) expressing vaccinia-encoded T7 RNA polymerase (vTF7-3–infected, moi 10, incubated overnight) were washed and suspended at 1.5 × 10^6 cells per milliliter in serum-free Opti-MEM I (Gibco) with 2.5 mm of $CaCl_2$ added. Sendai virus (5 μg of protein) was added to 1 ml of each cell suspension; the suspensions were incubated at 4°C for 15 min, then at 37°C for 30 min with gentle mixing to minimize cell fusion. Mixtures (in the same medium, 0.4-ml total volume) were prepared containing equal numbers (5 × 10^5) of the indicated Sendai virus-treated T7 RNA polymerase-containing cells and the indicated cell types (HeLa or NIH 3T3) expressing vaccinia-encoded CD4 (vCB-3 infected, moi 10, incubated overnight). The cell mixtures were incubated at 4°C for 15 min, then at 37°C for 20 min; during this time the cells were allowed to settle without shaking to facilitate transient hybrid formation. Dithiothreitol was added (5 mM final concentration), and cells were mixed and incubated at 37°C for 20 min. Control experiments (not shown) indicated that dithiothreitol treatment efficiently inactivated the Sendai virus to eliminate subsequent nonspecific cell fusion and had negligible effect on normal env/CD4 mediated fusion. The treated cells were washed twice and suspended at a density of 1 × 10^6 cells per milliliter (on the basis of the original cell numbers) in MEM-10% FBS. In the second phase, duplicate samples were prepared in individual wells of 96-well flat bottom plates by mixing 0.1 ml of the indicated transient hybrid cell suspensions with 0.1 ml of cells (HeLa, 1 × 10^6 cells per milliliter in MEM-10% FBS) which had been transfected with plasmid pG1NT7β-gal and which also expressed the designated vaccinia-encoded env: Wt-IIIB, wild-type env, IIIB isolate (encoded by vSC60); Unc-IIIB, mutant uncleavable env (encoded by vCB-16). Cultures were kept at 37°C in a humidified CO_2 incubator. After 2.5 hours, the fusion reactions were terminated with NP-40, and the β-galactosidase levels were determined. Data are expressed as OD/min × 1000; error bars represent population standard deviation. Methods for vaccinia infection, plasmid transfection, and enzyme assay were similar to those described in the caption for Fig. 1.

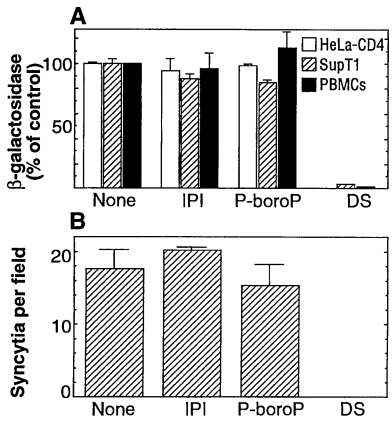

Fig. 3. Effects of DDP IV inhibitors on cell fusion. (**A**) Reporter gene activation assay. Vaccinia-encoded T7 polymerase (vTF7-3 infection, moi 10) was expressed in three distinct $CD4^+$ cell populations: HeLa cells infected at moi 10 with vCB-3 (HeLa-CD4); SupT1 cells (SupT1); and Ficoll-Hypaque enriched human peripheral blood mononuclear cells (PBMCs) from pooled blood of HIV seronegative donors, stimulated with phytohemagglutinin. Vaccinia infections were performed overnight for HeLa cells and for 6 hours for SupT1 cells and PBMCs. After the cells were washed, duplicate samples (1 × 10^5 cells in 0.18 ml of Opti-Mem I with 2.5 mM $CaCl_2$) were incubated in individual wells of 96-well flat-bottom plates with the indicated agents at the following concentrations: IPI, 10 mM; P-boroP, 0.1 mM for HeLa cells, 1 mM for SupT1 cells, and PBMCs; DS, 100 μg/ml (DS was not tested with the vCB-3-infected HeLa cells in this experiment, but was shown repeatedly in other experiments to markedly inhibit fusion with these cells). Control samples contained no addition. After incubation at 37°C for 30 min, fusion partner cells were added to each well [1 × 10^5 cells in 0.02 ml; HeLa cells expressing vaccinia-encoded env (wild-type IIIB isolate, encoded by vSC60) and transfected with plasmid pG1NT7β-gal]. Cultures were kept at 37°C in a humidified CO_2 incubator. The fusion reactions were terminated after 2.5 hours by addition of NP-40 (0.5% final concentration), and the amounts of β-galactosidase in the cell lysates were determined. For each CD4-expressing cell type, results are expressed as the percentage of β-galactosidase obtained with the indicated agent as compared with the control samples with no addition. In all cases parallel experiments using the uncleavable mutant env verified that the β-galactosidase produced in the control samples resulted from env/CD4-mediated cell fusion (not shown). Methods for vaccinia infection, plasmid transfection, and enzyme assay were similar to those described in the caption for Fig. 1. (**B**) Syncytia formation assay. Duplicate samples of SupT1 cells (1 × 10^5 cells in 0.18 ml of RPMI medium supplemented with 10% FBS) were incubated in individual wells of a 96-well flat bottom plate with the indicated agents at the following concentrations: IPI, 10 mM; P-boroP, 1 mM; DS, 100 μg/ml. Control samples contained no addition. After incubation at 37°C for 30 min, 8E5 cells were added to each well (1 × 10^5 cells in 0.02 ml, same medium). Cells were cultured at 37°C in a humidified CO_2 incubator. After 5 hours, additional aliquots of each agent were added at the original concentrations. Syncytia formation was scored after 24 hours by microscopic examination with an inverted tissue culture microscope with the use of ×10 phase contrast objective and a ×10 ocular objective. Results are expressed as the number of syncytia per field and represent the mean of quadruplicate fields (two fields in each of two duplicate wells for each condition). In (A) and (B), error bars represent population standard deviation.

ter inactivation of the Sendai virus and washing of the cells, the second phase was initiated by adding cells expressing vaccinia-encoded env (wild-type or uncleavable mutant) and also containing the plasmid with the *LacZ* gene linked to the T7 promoter. After the addition of the fusion partner and incubation, NP-40 was added to each sample and β-galactosidase activity was quantitated. In this experimental design, enzyme accumulation dependent on env/CD4-mediated fusion could arise only from fusion of env-expressing cells with transient hybrids containing both T7 RNA polymerase and surface CD4. The results demonstrate that fusion competence could be conferred to the CD4-expressing NIH 3T3 cells by forming transient hybrids with human (HeLa) cells, but not with murine (NIH 3T3) cells. The lower background amounts of β-galactosidase that we observed when the fusion partner expressed the mutant uncleavable env verified that the signal with the wild-type env reflected primarily env/CD4-mediated cell fusion. The simplest interpretation is that the human cells provided an accessory component (or components) essential for env/CD4-dependent fusion, a conclusion reached by us earlier (*7*) and by others (*5*, *6*) using different hybrid cell systems and assay methods. The signals (Fig. 2) were comparable whether in the first phase CD4 was expressed in the NIH 3T3 cells and T7 RNA polymerase in the HeLa cells, or vice versa. This result suggests that the fusion signal was limited by the efficiency of transient hybrid formation during the first phase, rather than by the functional efficiency of unknown molecular component or components provided by the formation of transient cell hybrids. We conclude that CD4-expressing murine cells could be rendered fusion competent by the formation of transient hybrids with human cells, under experimental conditions similar to those in which CD26 expression proved ineffective.

As a second experimental approach to test the involvement of CD26 in HIV-1 env/CD4-mediated cell fusion, we examined the effects of inhibitors of the DPP IV activity of CD26. Callebaut *et al.* reported (*8*) that the tripeptide IPI (diprotin A), an inhibitor of DPP IV (*14*), markedly impaired HIV entry at concentrations of 10 mM and higher (*8*). We therefore examined the effects of DPP IV inhibitors on fusion between env-expressing and CD4-expressing cells, using several alternative cell systems and assay methods. In experiments using a vaccinia-based system, one cell partner expressed vaccinia-encoded env (IIIB) and also contained the plasmid with the *LacZ* gene linked to the T7 promoter (Fig. 3, top). As the other cell partner, T7 RNA polymerase was expressed

in three types of $CD4^+$ cell populations: HeLa cells expressing vaccinia-encoded CD4, the SupT1 $CD4^+$ human T cell line, and phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMCs) from healthy human donors. Controls experiments (not shown) indicated that in all cases, significant β-galactosidase was produced with wild-type env, but not with the fusion-defective uncleavable mutant env. To examine the effects of DPP IV inhibitors, we preincubated the indicated $CD4^+$ cell types without or with the designated inhibitor before mixing with the env-expressing fusion partner. In the reporter gene activation assay, IPI had no effect on cell fusion at 10 mM, a concentration greater by $>10^3$ than the reported Ki for DPP IV inhibition (2.2 μM) (*14*). Similarly, we observed no effects with the extremely potent inhibitor L-Pro-L-boroPro (P-boroP), even at concentrations (0.1 to 1 mM) greater by 10^6 to 10^7 than the corresponding Ki value for inhibition of DPP IV, 16 pM (*15*). In contrast, strong inhibition was observed with 100 μg of dextran sulfate (DS) per milliliter, indicating that fusion monitored by the reporter gene activation assay was sensitive to a well-known inhibitor of HIV env/CD4-mediated fusion (*16*).

We also tested the effects of the DPP IV inhibitors on cell fusion in vaccinia-free systems, using mixtures of one continuous cell line expressing endogenous CD4 and another expressing endogenous env (Fig. 3, bottom). The results with mixtures of $CD4^+$ $SupT_1$ cells and HIV-1 persistently infected 8E5 cells (Fig. 3, bottom) indicate no significant inhibition by the DPP IV inhibitors IPI (10 mM) or P-boroP (1 mM); under parallel conditions DS (100 μg/ml) abolished syncytia formation. Additional experiments (not shown) using mixtures of various combinations of HIV-1 persistently infected cell lines (8E5 or H9/HTLV-IIIB), and $CD4^+$ continuous human cell lines (A3.01 T cell line, HeLa-CD4 transfectant cell line) similarly showed no inhibition of syncytia formation by the DPP IV inhibitors.

Taken together, these findings suggest that CD26 does not play a critical role in HIV-1 env/CD4-mediated cell fusion. It is possible that the discrepencies between our results and those of Callebaut *et al.* (*8*) reflect differences in the biological processes studied and the assays used. Arguments have been raised that env/CD4-mediated virus-cell fusion may be mechanistically different from cell-cell fusion (*17*). However this notion has been directly challenged by experimental findings (*11, 18*). A wide diversity of criteria has shown that the specificity of env/CD4-mediated cell fusion in the vaccinia-based system closely parallels that observed for HIV infection and syncytia formation (*13*). Most critically, both processes require CD4 to be expressed on a human cell type, and the defects with CD4-expressing nonhuman cells can be overcome by the formation of hybrids with human cells. We therefore believe that the discrepancies between our findings with cell fusion and those of Callebaut *et al.* with virus entry do not reflect a differential involvement of CD26 in these two processes. Definitive resolution of this problem will come when and if one or more factors are identified that render CD4-expressing nonhuman cells permissive for both virus-cell and cell-cell fusion mediated by HIV-1 env.

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Callebaut *et al.* (*1*) report that CD26 may act as an accessory receptor to CD4 for HIV infection. We have previously investigated the possible role of cell surface proteinases cleaving the V3 loop of HIV gp120 as a pathway to entry into the cell (*2*). We have therefore tested whether human CD26 co-expressed with human CD4 on the surface of mink (Mv-1-lu) and cat (CCC) cell lines would provide the missing factor required for HIV-1 entry. We chose these cell lines in preference to mouse NIH 3T3 cells because

Table 1. Titration of HIV on cells expressing human CD4 and CD26 antigens. Data are shown in $\log_{10}$ infectious units per milliliter. HeLa, Mv-1-lu (mink lung), and CCC (cat kidney) cells were transduced with human CD4 with the use of a retroviral vector and selected in neomycin as described by Clapham *et al.* (*5*). To express human CD26, cells were co-transfected (Lipofectamine; Gibco BRL, Paisley, Scotland) with CD26 cDNA in pCDM8 and pSV_2-puro and selected in puromycin (3 μg/ml). All (100%) of CD4-transduced cells expressed CD4 as assessed by fluorescence-activated cell sorting analysis with monoclonal antibody (mAb ADP 318). All (100%) of Mv-1-lu-CD4/CD26 cells co-expressed human CD26 (detected with mAb TA5.9; Eurogenetics, Teddington, United Kingdom) in amounts some 20-fold higher than are present on H9 human T cells. Approximately 75% of CCC-CD4/CD26 cells expressed human CD26 in amounts similar to those expressed by Mv-1-lu. Cells were seeded and HIV titrated by focal antigen assay as described previously (*5*). Undetectable infection (−) indicates that no infectious units were found in 250 μl of undiluted virus stock. Infection of cell mixtures indicates that a mixture of HeLa-CD4 cells and Mv-1-lu-CD4 cells in a ratio of 1:10,000 yielded detectable infection with the same RF HIV-1 stock.

Cells	HIV-1 LAI	HIV-1 RF	HIV-2 ROD*	HIV-1 (HTLV-I) pseudotype†
HeLa-CD4	4.3	5.2	4.2	2.6
Mv-1-lu	−	−	−	2.5
Mv-1-lu-CD4	−	−	2.8	2.7
Mv-1-lu-CD4/CD26	−	−	2.6	2.5
CCC	−	−	−	2.6
CCC-CD4	−	−	3.5	2.5
CCC-CD4/CD26	−	−	3.6	2.6

*ROD virus stock was rescued from the pACR23 molecular clone. †The HIV-1(HTLV-I) pseudotype was prepared by propagating HIV-1 RF in C91/PL cells. Because Mv-1-lu and CCC cells express receptors for HTLV (*7*), the pseudotype was used as a positive control for HIV-1 replication in these cells, provided that entry occurred.

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 cytofluorometry 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 $CD4^+$ 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 implications 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, 2×10^5 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)	%$CD4^+$	%$CD26^+$	%$CD4^+$ $CD26^+$	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 Callebaut *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$^+$ 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$^+$ 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$^+$ 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$^+$ cells; DPP IV activity was only detected in CD26$^+$ 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$^+$CD26$^+$ 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$^+$ SC6 cells and different HIV-1 infected cell lines. We also did not detect virus production after contact of parental or CD26$^+$ SC6 cells with HIV-1 (one infectious unit, IU, per cell), either directly or after coculture with human CD4$^+$ 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$^+$ 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$^+$ 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$^+$ 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.

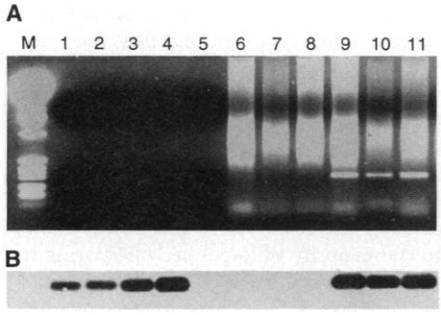

Fig. 1. Detection of HIV-1 entry into murine cells by PCR amplification of an HIV-1 *pol* gene fragment. Ethidium bromide staining of a 1.5% agarose gel (**A**) and hybridization to an HIV-1 *pol* probe after membrane transfer (**B**). DNA size markers, M (BRL 1 kb, ladder). Uninfected human T cells lysates containing 12, 25, 50, and 100 copies, respectively, of HIV-1 proviral DNA (lanes 1–4). Uninfected human T cells cells (lane 5). Lysates of murine cells SC6 (*6, 9*), SC6-CD26 (*7, 10*), or SC6-CD26P (*8, 11*), infected with wild-type HIV-1 (*6–8*) or HIV-1 with mixed HIV-1–HTLV-1 envelope phenotype (*9–11*) (lanes 6–11). After overnight contact with DNase-treated viral supernatants, the cells were washed and trypsinized to remove the inoculum and grown for 48 hours before lysis for PCR (*8*).

Finally, Callebaut *et al.* detected (*1*) CD26 expression in the MOLT-4, U937, and in Jurkat human cell lines, which were previously characterized as CD26-negative (*2*). Although variability among subclones cannot be ruled out a priori, it should be noted that CD26 expression was sought by Callebaut *et al.* with the use of monoclonal antibody BA5 (mAb, Immunotech, Marseille, France). Some background signal can be observed with BA5 in human cell lines, including CEM, that are not stained with other mAbs against CD26 and do not express DPP IV activity (*4*). The effect of agents aimed at blocking an eventual interaction between HIV and CD26 should therefore be confirmed by standard virus neutralization techniques, in authentic $CD26^+$ cells. Meanwhile, the cofactors of HIV entry have yet to be identified.

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Response: The HIV-1 *envelope* gene codes for a precursor polyprotein gp160 which, through proteolytic cleavage, yields extracellular (gp120) and transmembrane (gp41) envelope glycoproteins; gp120 binds to the CD4 receptor, whereas gp41 is involved in the fusion process (*1*). Both gp120 and gp41 are associated in a noncovalent manner to generate a complex that has two major functions in HIV infection. For the HIV particles, this complex is essential to the virus/cell membrane fusion that allows viral entry; whereas in HIV-infected cells, the gp120-gp41 complex expressed on the membrane of one cell interacts with CD4 molecules on another cell to initiate cell/cell membrane fusion, which results in the formation of syncytia. By investigating the virus/cell fusion process, we found that another cell surface antigen, CD26, serves as a cofactor for CD4, probably by interacting with highly conserved motifs in the V3 loop of gp120 (*2*).

Broder *et al.* use an assay for cell/cell fusion, to argue against the involvement of CD26 in the HIV envelope-mediated fusion process. Although virus/cell and cell/cell fusion processes require the interaction of gp120-gp41 complex with the CD4 receptor, there are subtle differences between these two events.

1) Cell surface adhesions molecules (such as LFA-1) have been demonstrated to be essential for cell/cell, but not for virus/cell, fusion: HIV-1 infection can occur in $LFA\text{-}1^-$ lymphocytes or in $LFA\text{-}1^+$ lymphocytes treated with antibody to LFA-1, whereas in a syncytium assay the antibody inhibits cell/cell fusion (*3, 4*).

2) HIV isolates from seropositive individuals manifest a significant variability in their capacity to induce syncytium formation. Nonsyncytium-inducing (NSI) isolates have been detected throughout the course of HIV-1 infection, whereas syncytium-inducing (SI) isolates tend to predominate in AIDS patients (*5*). Both NSI and SI isolates of HIV-1 must be able to induce virus/cell fusion because they can infect cells; however, they differ significantly in their capacity to initiate cell/cell fusion. ROD HIV-2 and EHO HIV-2 are two virulent isolates of HIV-2 (6); however, they manifest different phenotypes in the cell/cell fusion process: ROD is SI whereas EHO is NSI (*7*).

3) HIV-1 isolates of SI phenotye do not systematically initiate cell/cell fusion in all human cells permissive to virus/cell fusion. For example, it has been reported that infection of CEM cells (a $CD4^+$ T cell line) with a SI HIV-1 isolate results in single cell killing in the absence of cell/cell fusion (*8*). We have routinely used a clone of CEM cells characterized by the expression of a high density of CD4 (CEM clone 13, prepared in the laboratory of L. Montagnier). Infection of these cells with SI HIV isolates resulted systematically in the formation of syncytia (*9, 10*). In contrast, others have reported that this specific clone is resistant to cell/cell fusion. However, when conjugate formation is increased by the lectin wheat germ agglutinin, then cell/cell fusion has occurred (*11*). Slight differences in cell culturing conditions, therefore, could generate modifications in the surface structure of cells and may account for such discrepancies.

4) Cell death by apoptosis occurs during infection of CEM cells by SI and NSI HIV isolates; apoptosis is initiated by the interaction of cell membrane–expressed gp120-gp41 complex with the CD4 receptor (9). HIV entry (that is, virus/cell fusion) does not initiate apoptosis, so there might be some structural and conformational differences in the gp120-gp41 complex when it is presented by virus particles or by infected cells.

5) Cell/cell fusion has been reported to require Mg^{2+}. Accordingly, the use of EDTA as a chelating agent results in the suppression of cell/cell fusion without affecting HIV infection (*11*).

6) HIV-1–infected H9 cells (because of their membrane presentation of gp120-gp41 complex) are used routinely to induce cell/cell fusion when they are cocultured with uninfected $CD4^+$ cells (*11*). By comparing this cell/cell fusion assay with virus/cell fusion (that is, HIV infection), we have observed that (i) the monoclonal antibody mAb OKT4A against the gp120 binding site in the CD4 molecule (*12*) blocks both virus/cell and cell/cell fusion;

(ii) the mAb 110/4 against the V3 loop (*13*) inhibits virus/cell, but not cell/cell, fusion; and (iii) the synthetic double-stranded RNA, poly(A).poly(U) inhibits virus/cell fusion (*14*), but has no effect on the cell/cell fusion process.

Altogether, these observations emphasize that besides the interaction of the gp120-gp41 complex with the CD4 receptor, there might be independent parameters of viral or cellular origin, or both, implicated in these two events. The V3 loop plays a critical role in both processes; thus, the inability of anti-V3 loop antibody to inhibit cell/cell fusion is perhaps a consequence of the more complex nature of the cell/cell, as compared with virus/cell, fusion process. For example, because cell-surface adhesion molecules are essential for cell/cell fusion, they might cause circumvention of a step that implicates the V3 loop. Point mutations in the GPG conserved motif in the V3 loop (into GAG or GSG) have been shown to inhibit virus/cell and cell/cell fusion (*15*). A tenfold increase in the expression of such envelope mutants, however, results in cell/cell fusion, which indicates that overexpression of the envelope could circumvent the step that involves the V3 loop in the fusion process. Thus, it becomes evident that cell/cell fusion assays should not be used as a means to study virus/cell fusion. A similar conclusion was reached by A. Fauci and his co-workers (*4*), who stated that cell/cell fusion cannot be used as the sole parameter to evaluate virus/cell fusion. Furthermore, they concluded that (*4*, p. 1774)

(a) Inhibition of syncytia formation does not necessarily reflect suppression of HIV infection; (b) syncytia formation is not necessary for efficient spreading of HIV or for virus replication; and (c) based on these findings, syncytia and syncytia-forming assays should not be used as the sole indicators of HIV infection.

We reported that the CD26 inhibitor, the tripeptide IPI, inhibits HIV entry and infection at a concentration of 10 mM (*2*); at 20 mM it has no effect on cell/cell fusion. We have now developed new CD26 inhibitors that inhibit HIV entry, infection, and even syncytium formation at concentrations of 10 to 50 μM. In view of this and the role of the V3 loop in virus/cell and cell/cell fusion (*15*), it remains possible that CD26 is also implicated in the cell/cell fusion process. Consistent with the more complex nature of cell/cell fusion as compared with virus/cell fusion, inhibition of syncytia requires at least a fivefold higher concentration of this inhibitor as compared with that of virus infection.

There may be at least two major obstacles that hinder HIV entry and replication in most nonhuman cells that express the human CD4 molecule: (i) the presence of a cell-surface associated protein that should be species specific and may also have a peptidase activity, and (ii) the proper functioning of viral transactivator proteins Tat and Rev (*16–18*). These requirements are probably not absolutely rate limiting in HIV-1 and HIV-2 infection.

In agreement with the first hypothesis, we reported that DPP IV may serve as the cofactor of CD4 for HIV entry into cells (*2*). In contrast, Patience *et al.* report that mink and cat cells that express both human CD4 and CD26 are not infected by HIV-1, whereas expression of human CD4 alone in these cells makes them permissive to HIV-2 infection. A differential permissivity of cells toward infection by different isolates of HIV-1 and HIV-2 has been reported in nonhuman cells that express human CD4 (*19*). This, in part, may be the consequence of differences in the structures of envelope glycoproteins, as the deduced amino acid sequence of HIV-2 envelope glycoproteins is as much as 60% divergent from that of HIV-1 (*20*). Also, as the functioning of Tat and Rev is dependent on their association with cellular factors (*21*), there may be differences in the mechanism of transactivation necessary for the replication of HIV-1 and HIV-2 isolates. Indeed, significant differences have been reported between HIV-1 and HIV-2 for the capacity of Tat and Rev to reciprocally transactivate HIV gene expression (*21*). Other differences have also been reported for the requirement of cellular transactivators that interact with HIV LTR (*22*). In view of these findings, the fact that a cell line is permissive to HIV-2 entry or replication is not straightforward evidence that it should also be permissive to HIV-1. In addition, the observation that the expression of human CD4 alone in these cells was sufficient for infection by HIV-2 indicates that these are probably particular cases because not all nonhuman cells that express human CD4 are permissive to HIV-2 infection. The absence of HIV-1 infection in mink and cat cells that express both human CD4 and CD26 might be a result of nonreplication after entry into these cells. It might also be a question of detecting small amounts of virus produced by mink and cat cells. Finally, if HIV-1 replication is restricted in these cells, then the use of high-titered virus is recommended (*2*) in order to increase the probability of virus replication.

Patience *et al.*, by citing already published papers (*19, 23*), state that "mink cells readily replicate HIV-1" when HIV-1 particles are fused to mink cells that express human CD4 in the presence of polyethylene glycol (*19*) or when cells are infected by an amphotropic MLV pseudotype (*23*). What could be the significance of these observations in connection with normal HIV-1 infection? The fusion data experiment of Clapham *et al.* (*19*) does not demonstrate convincingly that HIV replication was occurring and does not include controls such as the use of mink cells that do not express CD4. These results (*19*) may have been due to contamination by the input HIV-1 adsorbed on the surface of cells. Concerning the experiment with the amphotropic MLV strain pseudotype (*23*), the HIV-1 preparation should represent a mixture of viruses such as MLV, HIV, MLV/HIV hybrids, and perhaps HTLV and HTLV/HIV hybrids, because virus production was carried out in HUT-78 cells, a cell line established from a patient with adult T cell leukemia. Accordingly, HIV-1 replication might have been assisted by favorable conditions induced by MLV and HTLV infection. A similar situation might have occurred in the experiment in which Canivet *et al.* (*23*) show, with the use of the HIV-1/HTLV pseudotype, that nonhuman cells are permissive to HIV-1 replication. However, as this preparation also contains HTLV, these results may perhaps be due to the transactivation of HIV LTR by the HTLV transactivator protein Tax, that is, restriction of HIV-1 replication in nonhuman cells might be circumvented in the case of superinfection with HTLV.

The statement by Patience *et al.* that CD26 is not required or sufficient for HIV-1 entry is not supported by the available evidence. As far as HIV-2 entry, there might be exceptions that result from the specific strain of HIV-2, the particular preparations of virus stocks, or the cell line used for infection. Accordingly, one should not generalize from the results obtained from a particular experiment. Mink and cat CD26 may be functional for HIV-2 infection, as expression of human CD4 in these cells was sufficient for rendering them permissive to virus infection. In our hands, CD26 or a CD26-like molecule appears to be implicated in the infection of cells with different HIV-related viruses. For example, the same tripeptide inhibitor of the human CD26 can prevent HIV-1, HIV-2, and SIV infection in $CD4^+$ T cell lines (*24*). However, we cannot eliminate the possibility that other cell-surface proteins may be operational along with or independent of CD26 in the entry process in some particular cells.

To verify the role of CD26 as a cofactor for HIV entry in $CD4^+$ cells (*2*), Camerini *et al.* used two different experimental approaches. First, they used a syncytium formation assay between human cells that express HIV envelope glycoproteins and

heterologous cells that express human CD4 and CD26 molecules. As emphasized previously by several investigators (*3*) and by us here, such a cell/cell fusion process (formation of syncytia) does not necessarily reflect the virus/cell fusion (viral entry) and therefore should not be used as a means to monitor HIV infection.

In the second assay, Camerini *et al.* used an HIV-1 construct that expresses the firefly luciferase gene to infect nonhuman cells that express human CD4 and CD26 molecules. This latter approach, although attractive, has severe limitations. First, because of the low virus titer of such construct preparations, infection of cells will be much less efficient; the amount of HIV particles that becomes associated with cells does not exceed 1% of the input virus particles (*14*). Second, as the expression of the luciferase gene is under the control of HIV gene replication, any species specific restriction on LTR HIV activation or on Tat and Rev functioning (*17*) will also be effective on the expression of the luciferase gene. In murine cells, independent restrictions of Tat and Rev functioning have been reported (*18*). Such limitations probably account for the negative results observed when COS, A9, and NIH 3T3 cells that express human CD4 and CD26 molecules were challenged with the HIV-luciferase construct. In addition, the low luciferase activity assayed in the cell lysates from HeLa cells (table 1 of the comment by Camerini *et al.*) may suggest that the HIV-luciferase titer in the infection or expression of the HIV and the luciferase genes was weak even in human cells that express both CD4 and CD26. Furthermore, there was no linear correlation between the proportion of HeLa cells positive for both CD4 and CD26 and luciferase activity, which should indirectly reflect viral entry and replication.

Finally, in order to overcome any restriction during HIV entry, Camerini *et al.* used HIV-luciferase virions bearing murine amphotropic retrovirus envelope glycoproteins to infect human and nonhuman cell lines. Independent of viral entry, however, the expression of the luciferase gene in this amphotropic virus is still under the control of LTR HIV and is dependent on the functioning of Tat and Rev. Camerini *et al.* found that luciferase activity (that is, luciferase expression) in human cells was low, with the mean value between the four HeLa cell samples being 321 against the background value of 117 (table 1 of their comment). Thus, the net value in human HeLa cells was 204, which is lower than that in murine NIH 3T3 cells by a factor of 21, an observation which argues against the restriction of HIV replication in murine cells. Camerini *et al.* do not explain the discrepancy between their observation and results in the literature that emphasize the restriction of HIV-1 replication in murine cells (*18, 25*). Unknown parameters are implicated when HIV-luciferase virions with amphotropic envelope glycoproteins are used, which suggests that this test is not the best control for monitoring HIV replication in nonhuman cells.

Previously, we reported that murine NIH 3T3 cells that expressed human CD4 and CD26 could become permissive to HIV-1 and that such infection was inhibited by AZT (*1*). Because of the restriction of HIV replication in murine cells, the amount of virus produced under such experimental conditions is small. For this reason, we demonstrated the presence of virus by amplification with $CD4^+$ human T cells. We have now used a murine T cell hybridoma cell line that expresses human CD4 constitutively (*26*) and human CD26 by transfection to demonstrate that after HIV-1 infection, viral DNA can be detected in the nucleoplasm of such cells (Fig. 1). This DNA, which corresponds to the unintegrated HIV DNA (*10*), was abolished when the infection was carried out in the presence of AZT, thus confirming that replication had taken place in these cells. No HIV DNA was detectable in the murine cells that expressed only the CD4 molecule (Fig. 1). Under our experimental conditions, high doses of virus were used to infect the murine cells, and the HIV DNA was detected after amplification with PCR and assay by Southern (DNA) blot. Because of the poor replication of HIV in nonhuman cells, it is essential to use optimal conditions for the infection of cells and for the detection of the produced virus. Nevertheless, our results (Fig. 1) confirm that the expression of CD26 permits HIV entry into murine cells. Because of restrictions on both viral entry and replication, it is important to investigate the permissivity of several nonhuman cells toward HIV replication before establishing cell lines that express CD4 and CD26.

Alizon and Dragic attempt to verify the role of CD26 in HIV infection (i) with an assay based on syncytium formation, and (ii) by challenging murine cells that express human CD4 and CD26 with HIV-1. As we have said, syncytium formation should not be used as a systematic means to monitor HIV entry. As for the fact that murine cells that express human CD4 and CD26 did not become infected, this result could be a consequence of the lower effective doses of HIV used as compared with our experiments. The dose of HIV-1 we used was defined as one synchronous infectious dose capable of infecting the majority of CEM cells by the input virus (*27*). Although murine cells that express human CD4 and CD26 become susceptible to HIV infection (*2*) (Fig. 1), the replication of virus is poor in these cells as a result of restrictions exerted on HIV replication in nonhuman cells. Consequently, it is essential to use higher doses of HIV (defined by the effective infectious dose) in order to obtain an optimum infection. Alizon and Dragic treated their HIV preparation with DNase, during which time the infectious titre of HIV might have also been reduced as a result of the instability of HIV particles. It has been reported that HIV particles undergo massive shedding of their external envelope glycoprotein when incubated at 37°C, resulting in a significant reduction of their infectious titer (*28*).

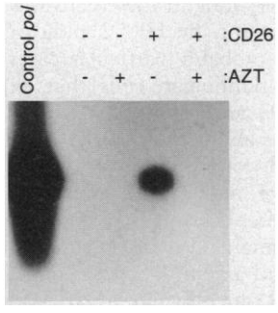

Fig. 1. Accumulation of unintegrated HIV-1 DNA in the nucleoplasm of murine cells that express human CD4 and CD26. The murine T cell hybridoma cell line expressing constitutively human CD4 (*26*) was transfected by electroporation with the CD26 expression plasmid vector (*2*) and, as a control, with the plasmid vector (pKG5) without the CD26 cDNA. Cells were infected 3 days later with LAI HIV-1 in the absence or presence of 5 μM AZT. The dose of virus used corresponded to one synchronous infectious dose (*10*) that resulted in more than 90% of the CEM cells being positive, as shown by immunofluorescence 48 hours after infection. After 3 and 5 days, respectively, cytoplasmic and nuclear extracts were prepared (*10*) and 5-μl aliquots were used to amplify with PCR a 334-bp fragment corresponding to the pol gene of HIV-1. The primers, each 22-mers, were derived from the pol gene. The 5′ primer: GGAATCATTCAAGCACAACCAG [nucleotide (nt) 3641 to 3662]; the 3′ primer: GAAGCCATGCATGGACAAGTAG (nt 3953 to 3974). These primers generated a 334-bp fragment in positive samples, that is, in cells that express CD4 and CD26 that were infected in the absence of AZT. PCR products were analyzed on a 1.5% agarose gel and assayed with a Southern (DNA) blot with the use of a ^{32}P-labeled 36-mer synthetic oligonucleotide in the *pol* gene (nt 3732 to 3767). The autoradiogram shows the results in the nucleoplasm 3 days after infection. Similar results were observed in the nucleoplasm 5 days after infection. No HIV DNA was detectable in the cytoplasm of cells (not shown). The lane control *pol* corresponded to hybridization of the ^{32}P-labeled 36-mer synthetic probe with the 334-bp amplified fragment with the use of HIV-1 cDNA.

Alizon and Dragic use "HIV-1 coated with the envelope of human T cell leukemia virus type 1" to produce a CD4-independent infection, but this result should not be interpreted as evidence for the capacity of HIV to replicate in nonhuman cells. Such a mixture of virus preparation is poorly characterized and represents HIV-1 and HTLV-1 pseudotypes produced by coculturing HIV-1– and HTLV-1–infected cell lines.

We have demonstrated that AZT blocks HIV infection of murine cell that express human CD4 and CD26 (*2*). Alizon and Dragic imply that this effect might have been the consequence of AZT on HIV entry. However, in contrast to a single report that AZT inhibits syncytium formation (*29*), several other reports have shown that AZT has no effect on syncytium formation (*10, 30*), or on the adsorption of HIV particles on permissive cells, or on viral entry (*9, 14*). Their comment that cotransfection might have allowed artifactual virus entry is not logical, as the same experimental conditions were used for the transfection of cells with either CD4 or CD26 plasmids alone. In addition, we have also shown that besides AZT, the presence of tripeptide IPI (inhibitor of the human CD26) and heparin also results in inhibition of HIV-infection of the murine $CD4^+$ $CD26^+$ cells (*2*).

The monoclonal antibody 1F7 raised against the human CD26 does not cross-react with the murine CD26, and it has been widely used in many studies (*31*). All the human cell lines (MOLT-4, U937, CEM, and Jurkat) that we have used in our experiments (*2*) expressed CD26 on their surface, which was revealed by the use of monoclonal antibodies BA5 (Immunotech, Marseille, France) (*2*) and 1F7 (*32*). All of these cells expressed DPP IV-like peptidase activity on their cell surface (*2*), thus confirming the results we observed with monoclonal antibodies.

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27. By one synchronous infectious dose of HIV-1, more than 95% of cells become producers of HIV proteins at 48 hours after infection. Evidence that all cells become infected by the input virus can be provided by the observation that AZT, when added at 8 hours after infection, does not affect the kinetics of infection nor the amount of virus production (*10*).
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32. Fluorescence-activated cell sorting analysis of cell lines with the use of the monoclonal antibody 1F7 was carried out at a dilution of 1:100 with ascitic fluid preparation. At a dilution of 1:20 to 1:40, HIV-1 entry into CEM cells was inhibited by at least 80% (*2*).
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