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SHORT COMMUNICATION

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Expression of a soluble CD4 molecule (sCD4-KDEL) containing a specific retention signal for the endoplasmic reticulum was shown previously to block propagation of the HIV-1_{MN} prototype strain in a transformed T cell line. However, the virus present in HIV-1-infected individuals is more closely represented by primary HIV-1 isolates which, unlike the HIV-1_{MN} strain, have not been adapted to growth in cell lines. To determine if sCD4-KDEL could block replication of primary isolates we used the PM1 cell line that has been shown to propagate primary isolates without adaptation. Here we show that the replication of four primary HIV-1 isolates was strongly inhibited in PM1 cells that expressed sCD4-KDEL under control of the HIV-1 LTR. Infection with primary HIV-1 isolates induced sCD4-KDEL expression driven by the LTR, HIV-1 spread was dramatically reduced, and reverse transcriptase activity in the cell culture supernatants was greatly diminished. sCD4-KDEL, therefore, represents a potent inhibitor of HIV-1 replication for gene therapy-based approaches for the treatment of AIDS.

Gene therapy offers a promising approach for the treatment of the acquired immunodeficiency syndrome (AIDS) (for discussion and review of strategies see Baltimore, 1988; Pomerantz and Trono, 1995) The basic scheme envisioned is that genes encoding RNAs or proteins that dominantly interfere with HIV-1 replication would be introduced into the hematopoietic stem cells of HIV-1-infected individuals. After reintroduction of these cells into patients, the transduced stem cell progeny, which include $CD4^+$ lymphocytes and cells of the monocyte/macrophage lineage, would then express the transduced genes and inhibit the replication of HIV-1. Although techniques are not yet available to obtain efficient repopulation of humans with genetically modified stem cells, recent work has established that transfection of $CD4^+$ peripheral blood lymphocytes with a gene inhibiting HIV replication can prolong survival of these cells after introduction into HIV-1-infected persons (Woffendin *et al.,* 1996). These results provide encouragement for continuing studies on means for inhibiting HIV replication.

We have previously reported that sCD4-KDEL, a soluble CD4 molecule that is retained in the endoplasmic reticulum (ER) by the retention signal Lys-Asp-Glu-Leu (K-D-E-L) (Munro and Pelham, 1987), effectively binds to and retains HIV-1 gp160 in the ER (Buonocore and Rose, 1990). We have also described a retroviral vector, LHSN/sCD4-KDEL that encodes sCD4-KDEL under control of the HIV-1 long terminal repeat (LTR). In H9 cells transduced with this vector, only a very low level of sCD4-KDEL is expressed. Following infection of the cells with HIV-1, expression of the HIV Tat protein transactivates the LTR and induces high level expression of sCD4-KDEL (Buonocore and Rose, 1993). This high level expression of sCD4-KDEL completely blocks propagation of the HIV-1 prototype strain MN. Following the initial infection, p24 is released into the culture medium, but no infectious HIV particles are released, the infection does not spread, and after 4 to 6 weeks the cells infected initially can no longer be detected in the culture. In addition, no syncytium formation was observed, as would be expected if gp160 transport were blocked in the ER. This approach to inhibiting the spread of HIV-1 infection is particularly attractive because HIV-1 should not be able to avoid the block through mutations in gp120 that would block CD4 binding. Although such mutants could occur, they would no longer be capable of infection via CD4.

HIV-1_{MN} and other HIV-1 isolates that have been extensively passaged in and adapted to growth in transformed T-cell lines are commonly referred to as "laboratory" HIV-1 isolates. Such isolates have been found to differ significantly from "primary" HIV-1 isolates which have been minimally passaged only in peripheral blood mononuclear cells (PBMCs). Many primary isolates cannot be propagated initially in transformed T-cell lines (Asjo *et al.,* 1986) and must be passaged extensively before they become adapted. This adaptation has been correlated with mutations of the viral envelope protein and alterations of viral phenotype (Harrowe and Cheng-Mayer,

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1995; Moore *et al.,* 1993; Orloff *et al.,* 1995; Wang *et al.,* 1995; Wrin *et al.,* 1995). As a result of these mutations, laboratory isolates have an abnormal sensitivity to neutralization by sera obtained from HIV-1-infected individuals (Moore *et al.,* 1995). Moreover, laboratory isolates are 100 to 1000 times more sensitive to neutralization by recombinant soluble CD4 (sCD4), compared to primary HIV-1 isolates (Daar *et al.,* 1990). However, the decreased sensitivity of primary HIV-1 isolates to neutralization by sCD4 does not appear to be related to a decreased affinity of HIV-1 envelope protein for CD4 (Ashkenazi *et al.,* 1991; Turner *et al.,* 1992). Rather, structural alterations of the oligomeric envelope protein complex displayed on the HIV-1 virion surface appear to be responsible for the different phenotypes of laboratory and primary HIV-1 isolates (Moore *et al.,* 1992).

Because gp160 molecules from primary and laboratory isolates differ, and since primary isolates are more likely to reflect the virus present in HIV-1-infected individuals (Moore *et al.,* 1995), we felt it was important to determine whether the replication of primary HIV-1 isolates would be inhibited in cells transduced with a retroviral vector that encodes sCD4-KDEL. This was also especially important because of the requirement of high concentration of sCD4 for neutralization of primary isolates (Daar *et al.,* 1990).

PM1 cells, a human T-cell line which has a unique susceptibility to infection by primary HIV-1 isolates (Lusso *et al.,* 1995), were transduced with the recombinant retroviral vectors LHSN and LHSN/sCD4-KDEL (Buonocore and Rose, 1993) by incubating the cells with supernatants of PA317 cell clones that produced the recombinant vectors. The PM1 cells were then cultured in complete medium with 1.5 mg/ml G418 (Gibco) and G418-resistant PM1 cell pools were cloned by limiting dilution.

To determine initially if functional sCD4-KDEL was being expressed in the PM1 cell line, individual clones were challenged with the laboratory isolate HIV-1_{IIIB}. At 1 week postinfection, 5 of 16 of the LHSN/sCD4-KDEL transduced PM1 cell clones showed a complete protection from HIV-1_{IIIB}-induced syncytium formation (Fig. 1). This result suggested that these five clones expressed sufficient sCD4-KDEL to retain the HIV-1_{IIIB} gp160 in the ER. In contrast, all subclones of LHSN-transduced PM1 cells showed syncytium formation when infected with HIV-1_{IIIB}.

To assay for sCD4-KDEL production, HIV-1-infected PM1 cell clones were fixed (McLean and Nakane, 1974) and permeabilized with 0.1% Triton X-100 and then analyzed by indirect immunofluorescence for the presence of HIV-1 antigens and sCD4-KDEL. The cells were washed and resuspended in a 1:100 dilution of HIVIG (Prince *et al.,* 1988) a purified immunoglobulin reactive with HIV-1 structural proteins (obtained through the AIDS Research and Reference Reagent Program, ARRRP), and a 1:100 dilution of OKT4 ascites, reactive with CD4 (Reinherz *et al.,* 1979). Secondary antibodies were FITC-conjugated anti-human IgG (Murex) and rhodamine conjugated anti-mouse IgG (Jackson Immunoresearch). The cells were then observed with a Nikon Microphot-FX microscope.

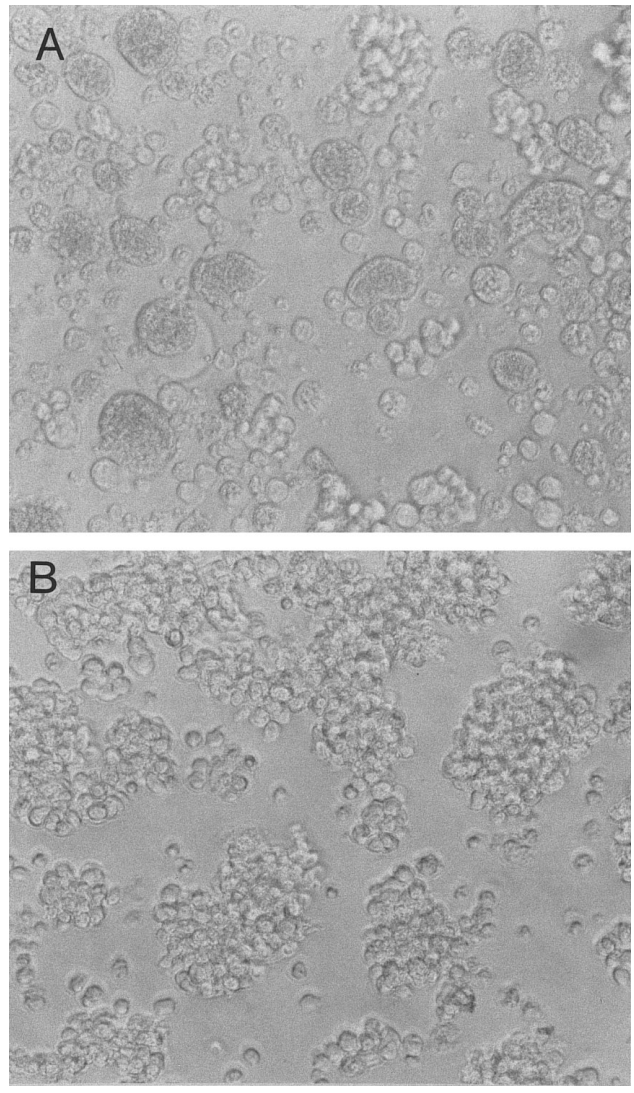

FIG. 1. Inhibition of syncytium formation in a LHSN/sCD4-KDEL-transduced PM1 cell clone. LHSN-transduced PM1 cells or LHSN/sCD4-KDEL-transduced PM1 cells were infected with HIV-1_{IIIB}, cultured for 1 week, and observed by light microscopy. (A) Syncytium formation in a PM1 cell clone transduced with the control LHSN vector. (B) Lack of syncytium formation in a PM1 cell clone transduced with LHSN/sCD4-KDEL.

HIV-1-positive cells were found in the cultures of the five PM1 clones that did not form syncytia, and as observed previously in H9 cells (Buonocore and Rose, 1993) every HIV-1-positive cell examined also expressed high levels of sCD4-KDEL. In contrast, LHSN-transduced control cells infected with HIV-1_{IIIB} showed only HIV-1-positive staining and no sCD4-KDEL staining (data not shown).

To determine if the replication of primary HIV-1 isolates

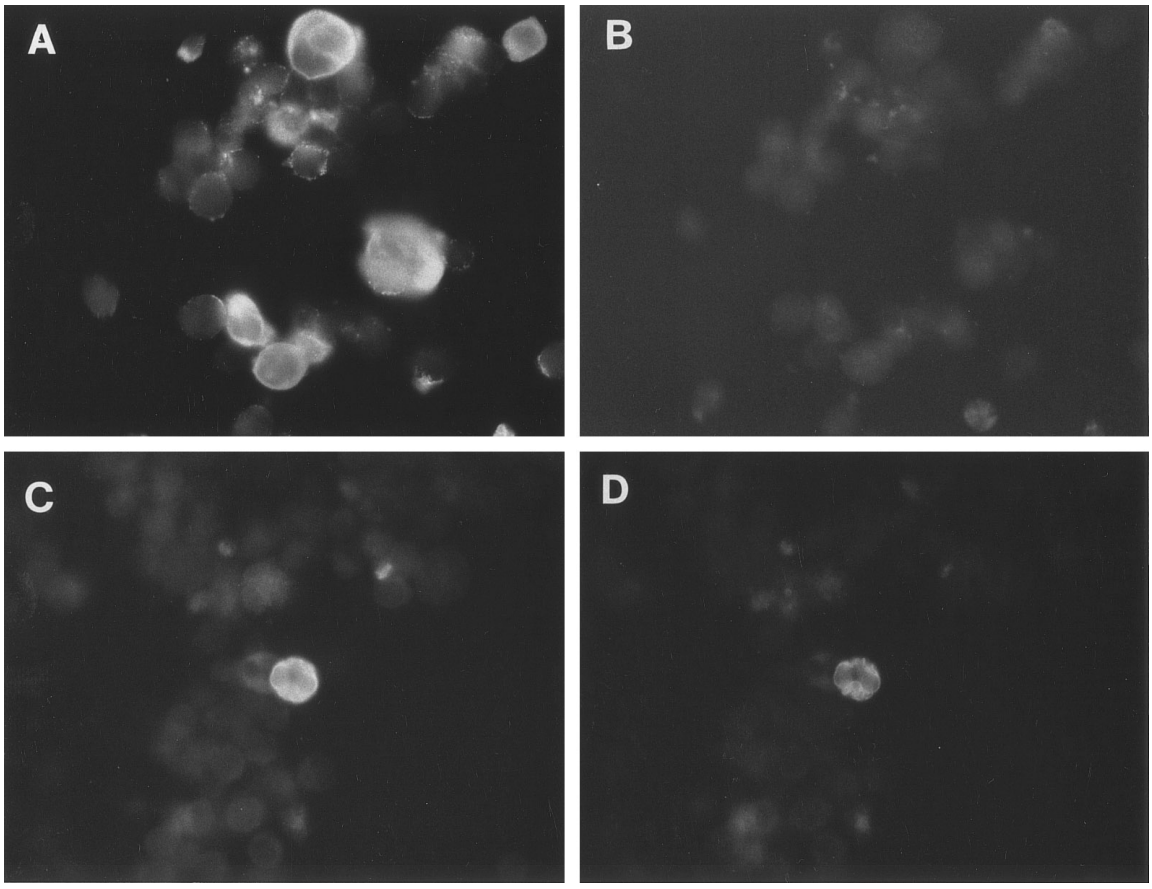

FIG. 2. Detection of HIV-1 antigens and sCD4-KDEL in HIV-1-infected cells by immunofluorescence microscopy. An LHSN-transduced PM1 cell clone was infected with HIV-1_{301657}. At 2 weeks postinfection a typical field showed many cells staining positively for HIV-1 antigens (A). The same cells showed only background staining for sCD4 (B). At 2 weeks postinfection the LHSN/sCD4-KDEL transduced PM1 cell clone showed very few HIV-1_{301657}-positive cells (C). Every HIV-1-positive cell also showed a strong cytoplasmic staining for sCD4-KDEL (D).

was inhibited in PM1 cell clones that expressed sCD4-KDEL, four different primary isolates of HIV-1 (obtained through the ARRRP) were used to infect a LHSN/sCD4-KDEL-transduced PM1 clone as well as an LHSN-transduced control PM1 cell clone. The HIV-1 isolates included HIV-$1_{Ba\text{-}L}$, a macrophage tropic, nonsyncytium inducing (NSI) isolate, HIV-1_{301657}, a syncytium inducing (SI) isolate, HIV-1_{302056}, and HIV-1_{302151}, an SI infant isolate. The PM1 cells were challenged with the HIV-1 isolates at a multiplicity of infection (m.o.i.) of 0.02 to 0.06 infectious particles per cell. Using an immunofluorescence assay for both HIV-1 and sCD4-KDEL at 4 days postinfection, we found that the LHSN vector transduced PM1 cells and the sCD4-KDEL expressing cells were equally infected at low levels by all four primary isolates (data not shown). To determine if the spread of infection was inhibited by sCD4-KDEL as observed previously with the HIV-1_{MN} strain, we analyzed infection and sCD4-KDEL expression by indirect immunofluorescence at 2 weeks and 6 weeks postinfection (Fig. 2).

In the control, LHSN-transduced PM1 cells, HIV-1-infected cells were observed in the cultures of all four viral isolates at 2 weeks postinfection. Many of the cells infected with HIV-1_{301657} and HIV-1_{302151} appeared as multinucleated syncytia. The cells exhibited a strong cytoplasmic and surface staining for HIV-1 proteins compared to uninfected control cells, and only a diffuse background CD4 staining (Figs. 2A and 2B). Note that endogenous CD4 is expressed at only very low levels and is undetectable by immunofluorescence. In contrast, only a small number of HIV-1-infected, LHSN/sCD4-KDEL-transduced PM1 cells were found but every HIV-1-positive cell found showed a strong ER staining pattern for sCD4-KDEL (Figs. 2C and 2D). This strong staining pattern indicated the expression and ER retention of sCD4-KDEL as previously described (Buonocore and Rose, 1990; Buonocore and Rose, 1993).

The numbers of HIV-1-infected, LHSN-transduced, and LHSN/CD4-KDEL-transduced PM1 cells were then quantitated at 2 weeks postinfection (Fig. 3A). At 2 weeks postinfection, approximately 30% of LHSN control cells were infected with HIV-$1_{Ba\text{-}L}$ while 100% of the cells were infected with HIV-1_{301657}. The levels of infection of control cells with HIV-1_{302056} and HIV-1_{302151} were 0.8 and 20%, respectively. In contrast, in the LHSN/sCD4-KDEL-transduced cells, HIV-1-infected cells were either no longer

detectable (HIV-1_{302056} and HIV-1_{302151}) or there were 100- to 1000-fold fewer HIV-1-positive cells compared with LHSN-transduced cells (HIV-1_{Ba-L} and HIV-1_{301657}). The loss of HIV-1-infected cells in the sCD4-KDEL transduced lines was seen previously in H9 cells infected with HIV-1_{MN}. Infected cells are eliminated from infected cultures, either because they grow more slowly than uninfected cells or are eventually killed by the infection (Buonocore and Rose, 1993).

At 6 weeks postinfection, the number of LHSN control cells infected with HIV-1_{Ba-L} did not change significantly. However, we did observe an increase from 0.2 to 1.5% in the PM1 cells expressing sCD4-KDEL. In addition, there was a more significant increase from 0.2 to 10% in the HIV-1_{301657}-infected cells. These data suggest that a low level of infectious virus is being released from sCD4-KDEL expressing cells infected with these two isolates, presumably because a low level of gp160 is able to escape the sCD4-KDEL trap.

To follow the kinetics of primary HIV-1 infection in LHSN-transduced and LHSN/sCD4-KDEL-transduced PM1 cells we also used a reverse transcriptase (RT) assay performed essentially as described (Hoffman *et al.,* 1985), using supernatants of the HIV-1-infected cell cultures. Both HIV-1_{Ba-L} and HIV-1_{301657} productively infected LHSN-PM1 cells as shown by a rapid peak in culture supernatant RT activity (Figs. 4A and 4B). The rapid loss of RT activity at later time points was due to extensive cell death in the infected cultures. In contrast, only background RT activity was detected in the supernatants of HIV-1_{Ba-L} or HIV-1_{301657}-infected, LHSN/sCD4-KDEL-transduced PM1 cells, for up to 3 weeks in culture. The supernatant of HIV-1_{301657}-infected, LHSN/sCD4-KDEL-transduced PM1 cells did eventually show a low but significant RT activity at 21 days postinfection. This small amount of RT activity is consistent with the spread of HIV-1_{301657} observed in LHSN/sCD4-KDEL-transduced cells at 6 weeks postinfection (see Fig. 3B). Collectively, these data suggest that HIV-1_{301657}-infected, LHSN/sCD4-

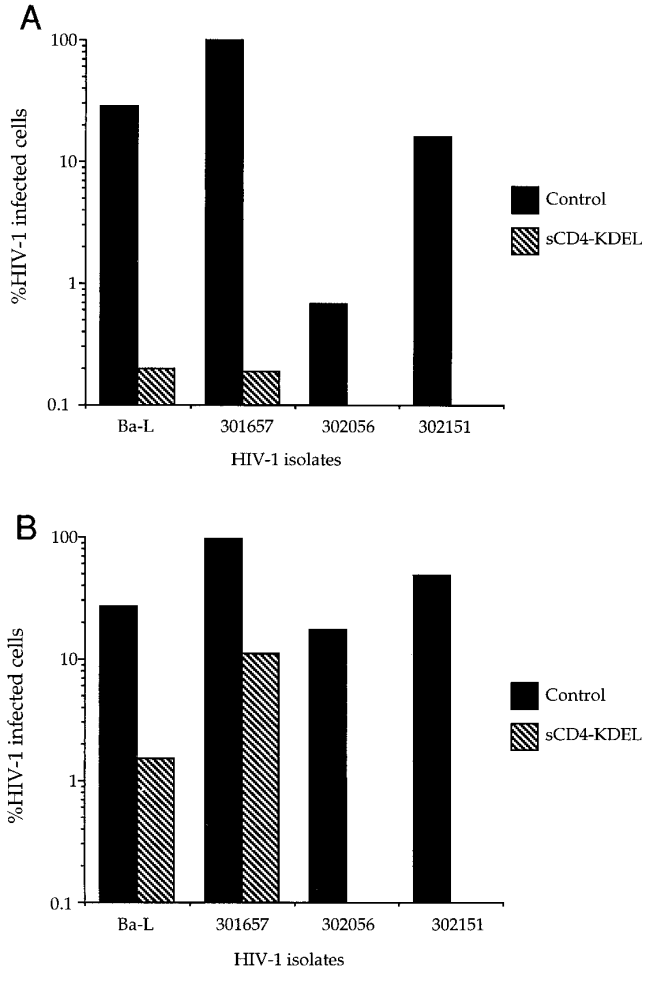

FIG. 3. Percentage of HIV-1-infected cells determined by immunofluorescence microscopy. The percentage of infected cells was determined at 2 weeks (A) and 6 weeks (B) postinfection.

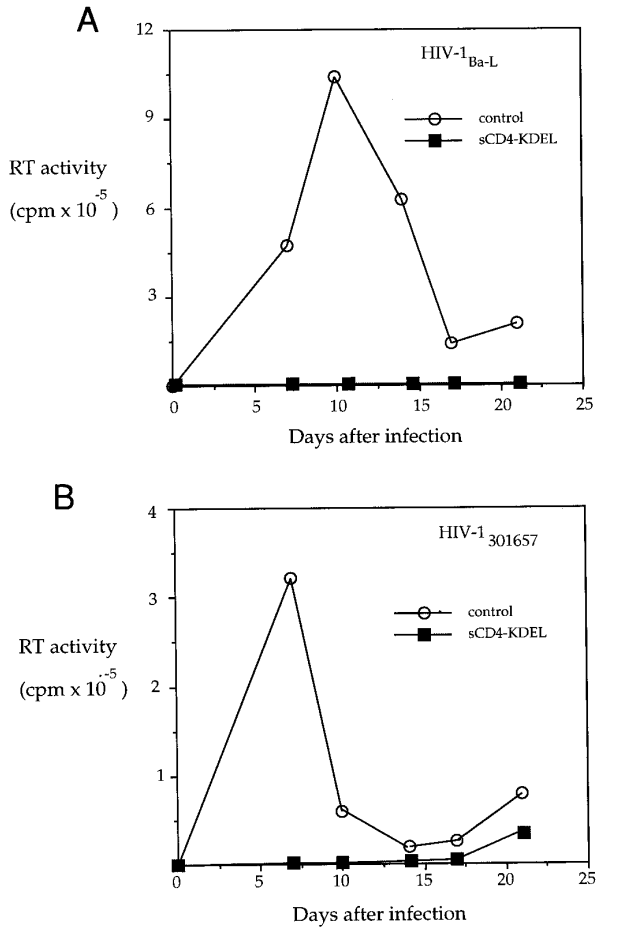

FIG. 4. Reverse transcriptase assay of supernatants of LHSN control-transduced or LHSN/sCD4-KDEL-transduced PM1 cells. Cells were infected with HIV-1_{Ba-L} (A) and HIV-1_{301657} (B) and supernatants assayed for reverse transcriptase at the indicated times postinfection. Background counts were subtracted from all samples. The drop in RT activity in the control cells corresponded with a severe cytopathic effect of the infection and a temporary drop in cell numbers.

KDEL-transduced PM1 cells can produce a low level of infectious virus.

The retention of HIV-1 gp160 in the ER by sCD4-KDEL prevents cleavage to gp120/gp41 and its subsequent export to the HIV-1-infected cell surface (S.D. and J.R., unpublished results). In several different systems it has been shown that the HIV-1 envelope protein is not required for the assembly and budding of retroviral particles, although it is required for infectivity (Gheysen *et al.,* 1989; Jacobs *et al.,* 1989; Karacostas *et al.,* 1989; Sadaie *et al.,* 1992). We have found that LHSN/sCD4-KDEL-transduced H9 cells infected with HIV-1_{IIIB} also produce retroviral particles which lack envelope protein (S.D. and J.R., unpublished results). Therefore, the detection of RT activity and low level spread of HIV-1_{301657} after an extended period in culture is likely to reflect the incorporation of a small amount of mature HIV-1 envelope protein into assembling particles. Based on our studies of spread of some laboratory HIV-1 isolates in the presence of the sCD4-KDEL block, the spread does not appear to be due to selection of mutants, since virus harvested after spread shows a lag in propagation equivalent to the starting virus when reinoculated into cells expressing sCD4-KDEL (S.D. and J.R., unpublished results).

The potential clinical significance of a slow but detectable spread of some primary isolates from cells expressing sCD4-KDEL is unclear. If a large fraction of $CD4^+$ cells in HIV-1-infected individuals could be made to express sCD4-KDEL, it is likely that the viral burden would be reduced dramatically, even if cells infected with some strains produced a low level of infectious virus.

An important finding described here is the use of the PM1 cell line to assay a gene therapy strategy against primary HIV-1 isolates. Previous studies designed to assay primary HIV-1 isolates utilized peripheral blood mononuclear cells (PBMCs) as the target cell and it was not possible in these studies to obtain expression of the dominant negative gene in all of the cells (Leavitt *et al.,* 1994; Woffendin *et al.,* 1994). PBMCs are difficult to maintain in culture for the long periods of time required for retroviral transduction, G418 selection, limiting dilution cloning, and HIV-1 challenge. Furthermore, we have found PBMCs to be very difficult to transduce using retroviral vectors, with either the murine amphotropic or the vesicular stomatitis virus G protein as the envelope protein. In contrast, PM1 cells are much easier to culture, and more amenable to retroviral transductions.

Overall, the results presented here suggest that sCD4-KDEL is a potent inhibitor of the replication of primary HIV-1 isolates and should be further pursued as a potentially useful gene therapy approach for the treatment of AIDS.

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