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# Blockade of human immunodeficiency virus type 1 production in $CD4^+$ T cells by an intracellular CD4 expressed under control of the viral long terminal repeat

(intracellular immunization/retroviral vector)

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ABSTRACT **A retroviral vector was constructed in which a gene encoding a mutated soluble CD4 protein that is retained in the endoplasmic reticulum (sCD4-KDEL) is expressed under control of human immunodeficiency virus type 1 (HIV-1) regulatory elements. HIV-1 infection of a human T-cell line transduced with this vector led to induction of sCD4-KDEL synthesis and a block in transport of the HIV envelope protein to the cell surface. There was a complete block to maturation of infectious HIV-1 in the transduced cells, no viral spread, and little or no syncytium formation. Infected cells gradually disappeared from the culture over a period of 2 months. This intracellular trap for HIV has potential application in gene therapy for AIDS.**

The magnitude of the worldwide AIDS epidemic and the difficulty of developing and testing an effective vaccine against human immunodeficiency virus (HIV) have led to proposals for alternative therapies that might prove applicable to infected patients. Because the $CD4^+$ T cells that are a major target for HIV infection are thought to be derived from a common precursor cell in the bone marrow, it may be possible to introduce protective genes into most or all of the T cells in an individual and greatly reduce HIV replication. This type of genetic therapy has been called intracellular immunization (1), and several therapies of this type have been suggested for AIDS. These include dominant negative mutants derived from HIV genes (2–4), HIV-induced expression of $\alpha_2$-interferon (5), specific ribozymes (6), decoy RNAs (7), antisense RNAs (8), and selective killing of HIV-infected cells (9). In studies where these strategies have been used to inhibit HIV-1 growth, the inhibition was often transient or incomplete or was followed for only a short period of time. Eventual selection of viral mutants able to evade specific blocks is also likely to occur in many cases. It is therefore desirable to find alternative methods that block viral propagation and that cannot be eluded through viral mutation.

The envelope glycoprotein of HIV-1 (gp120/41) is incorporated into virions during budding from the cell surface and mediates binding and fusion of the virus with T cells through the cellular CD4 receptor (10–12). Expression of gp120/41 on the surface of T cells also causes cytopathic fusion of infected T cells (13–15). Blockage of gp120/41 surface expression is therefore an excellent target for genetic therapy because it would prevent formation of infectious virions and cytopathic fusion of infected cells.

Like many other viral membrane proteins, the HIV envelope protein precursor (gp160) is inserted into the endoplasmic reticulum (ER), transported through the Golgi cisternae, and cleaved to its functional form (gp120/41) before arriving at the cell surface (reviewed in ref. 16). We reported previously (17) that transport of the HIV envelope protein from the ER could be blocked by a soluble CD4 protein (sCD4-KDEL) containing a tetrapeptide retention signal, Lys-Asp-Glu-Leu (KDEL), for the ER (18). We show that when the DNA encoding sCD4-KDEL is placed in a retroviral vector under HIV-1 control, it can be delivered efficiently into a T-cell line and provide a complete block to production of infectious HIV-1.

## MATERIALS AND METHODS

**Vector Construction and Preparation of Retroviral Stocks.** To construct the pLHSN vector diagramed in Fig. 1, a 252-nucleotide portion of the HIV-1 LTR was excised from pHenv (19) with *Xho* I and *Hin*dIII and ligated to pBluescript KS(+) (Stratagene) that had been cleaved with *Xho* I and *Hin*dIII. The HIV-1 LTR was then excised with *Xho* I and *Bam*HI and ligated into the *Xho* I and *Bam*HI sites of the vector pLXSN (20) to generate the vector pLHSN. To construct pLHSN-CD4K, a DNA fragment encoding sCD4-KDEL was first excised from pBS-CD4KDEL (17) with *Hin*dIII and *Bam*HI and then ligated into the *Hin*dIII and *Bam*HI sites of pHenv. The *Xho* I–*Bam*HI fragment containing the LTR and sCD4-KDEL sequences was then ligated into the *Xho* I and *Bam*HI sites of pLXSN.

Helper-free recombinant virus stocks were derived in two stages as described (20). Briefly, the plasmids described above were transfected into the PE501 ecotropic retrovirus packaging cell line (21). Virus harvested from this line was used to infect the PA317 amphotropic packaging line. G418-resistant clones of the infected PA317 cells were isolated and supernatants from these cells were assayed for viral titer. Virus was harvested by adding fresh culture medium to confluent dishes of cells for 16 hr, removing the medium, and filtering through 0.45-$\mu$m filters. The titers obtained from individual clones ranged from $8 \times 10^3$ to $10^6$ colony-forming units (cfu)/ml.

HIV-1 (strain MN) was prepared by infecting H9 cells with a frozen virus stock provided by G. Miller (Yale University). At 2 weeks after infection, when there was considerable cytopathic effect and all of the cells were found by immunofluorescence microscopy to be infected, a cell-free virus supernatant was prepared and used directly to infect other cells. The titer of this supernatant was estimated to be $\approx 5 \times 10^4$ infectious HIV-1 particles per ml, based on the number of H9/LHSN-CD4K cells infected (determined by fluorescence microscopy) after 3 days (see text).

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Abbreviations: cfu, colony-forming units; endo H, endoglycosidase H; ER, endoplasmic reticulum; HIV, human immunodeficiency virus; LTR, long terminal repeat; sCD4-KDEL, soluble CD4 protein containing a tetrapeptide signal (Lys-Asp-Glu-Leu) for retention in the ER.

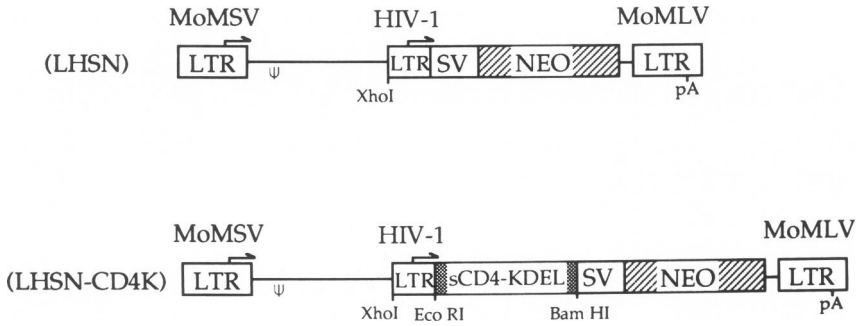


FIG. 1. Diagram of the retroviral vectors. Construction of the vectors is described in the text. The vectors contain long terminal repeat (LTR) sequences from Moloney murine sarcoma virus (MoMSV), HIV-1, and Moloney murine leukemia virus (MoMLV), as well as regulatory elements from simian virus 40 (SV) upstream from a neomycin-resistance marker (NEO). $\psi$, Packaging signal; pA, polyadenylylation signal.

**Transduction of H9 Cells with Retroviral Vectors.** H9 cells ($5 \times 10^5$ per ml) were washed and resuspended in 2.5 ml of LHSN or LHSN-CD4K viral stocks ($2 \times 10^5$ cfu/ml) containing Polybrene (4 $\mu$g/ml). The cells were incubated overnight at 37°C, washed, and suspended in medium containing the neomycin analogue G418 (1.4 mg/ml). Individual clones were derived from the initial pool by limiting dilution.

**Indirect Immunofluorescence Microscopy.** Cells were harvested by centrifugation, washed, and fixed for 1 hr by a modified paraformaldehyde procedure (22). Briefly, cells were washed, permeabilized for 3 min with 0.1% Triton X-100, washed again, and incubated for 30 min at 37°C with 0.05 $\mu$g of OKT4 antibody and pooled sera from AIDS patients (diluted 1:100). Cells were washed and then incubated for 20 min at 37°C with rhodamine-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch) and fluorescein-conjugated sheep anti-human antibody (Wellcome). Cells were photographed with a Nikon Microphot-FX microscope fitted with a ×40 objective.

## RESULTS

In initial experiments designed to determine whether constitutive expression of sCD4-KDEL could block production of infectious HIV, we employed a retroviral vector (LXSN) known to yield constitutive protein expression (20). This vector was used to express the DNA encoding sCD4-KDEL in the human T-cell line H9, which was derived from a T-cell lymphoma (23, 24). The H9 derivatives prepared with this vector expressed levels of sCD4-KDEL that were at best only 2–3 times greater than the low level of endogenous CD4 ($\approx 10^4$ molecules per cell) found in H9 cells. When these lines were infected with HIV-1, infection spread throughout the culture, just as in untransduced H9 cells, and infectious HIV-1 was recovered from the medium (data not shown).

**Retroviral Vector Containing the HIV-1 LTR.** We suspected that this initial failure to achieve a block of HIV-1 production resulted from sCD4-KDEL levels that were insufficient to complex with all of the HIV gp160 protein molecules produced during viral infection. We therefore adopted the strategy of using part of an HIV-1 LTR to drive expression of sCD4-KDEL so that high-level expression would occur soon after HIV infection due to production of the HIV transactivating protein Tat. HIV Tat protein promotes a several hundred-fold activation of transcription from the HIV LTR and can effectively transactivate expression of foreign genes under HIV LTR control (25–27).

To obtain an efficient system for delivery of the sCD4-KDEL gene under Tat control, we constructed the retroviral vector designated LHSN (LTR, HIV, SV40, Neo), which has 252 nucleotides of an HIV-1 LTR located within a murine retroviral vector containing a selectable gene (Fig. 1). The DNA encoding sCD4-KDEL was cloned downstream of the HIV-1 LTR to generate the construct designated LHSN-CD4K (Fig. 1). These constructs were then used to obtain helper-free retroviral stocks from the amphotropic packaging line PA317 (21).

To determine whether the virus stock expressed the sCD4-KDEL molecule under HIV-1 Tat regulation, we performed the transient expression assay shown in Fig. 2. HeLa cells and a HeLa cell line expressing Tat (19) were infected with the viral stock and labeled with [$^{35}$S]methionine along with control uninfected cells. Cell lysates were then immunoprecipitated with anti-CD4 antibody. Only the infected HeLa-Tat cells showed an immunoprecipitable protein band (Fig. 2, lane 4) that comigrated with a marker of sCD4-KDEL, indicating that sCD4-KDEL was under HIV Tat regulation.

**Expression in T Cells.** To prepare T-cell lines expressing sCD4-KDEL, H9 cells were transduced with the LHSN-CD4K virus stock, and G418-resistant cells (carrying the neomycin-resistance marker from the vector) were selected and then cloned. Seven independent clones were selected, and all expressed low constitutive levels of sCD4-KDEL (data not shown). To verify that the sCD4-KDEL expressed was retained in the ER, we performed the experiment shown in Fig. 3 to analyze carbohydrate processing in one of the

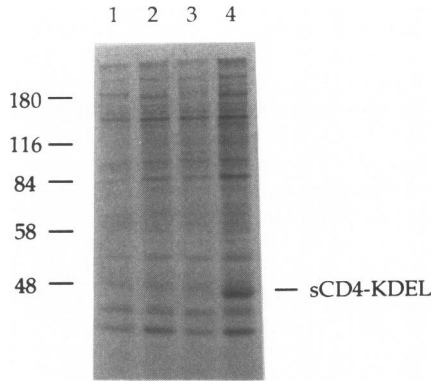


FIG. 2. Expression of sCD4-KDEL is specifically induced in HeLa-Tat cells. HeLa cells (lanes 1 and 3) and HeLa-Tat cells (28) (lanes 2 and 4) were plated at $5 \times 10^5$ cells per 6-cm dish. After 24 hr the medium was replaced with medium containing Polybrene (4 $\mu$g/ml), and the cells were either mock-infected (lanes 1 and 2) or infected (lanes 3 and 4) with 2 ml of LHSN-CD4K recombinant virus ($10^5$ cfu/ml) for 18 hr. After a 6-hr incubation at 37°C, the medium was replaced and the cells were further incubated for 40 hr at 37°C. Three days later, cells were metabolically labeled by incubation for 7 hr in 2 ml of methionine-free Dulbecco's modified Eagle's medium containing 75 $\mu$Ci of [$^{35}$S]methionine (1 $\mu$Ci = 37 kBq). Proteins were immunoprecipitated from cell lysates with 0.05 $\mu$g of OKT4 monoclonal antibody (Ortho Diagnostics). Immunoprecipitated proteins were analyzed by electrophoresis in SDS/10% polyacrylamide gels (29) and visualized by autoradiography. Positions of markers indicating apparent molecular mass (kDa) are shown at left.

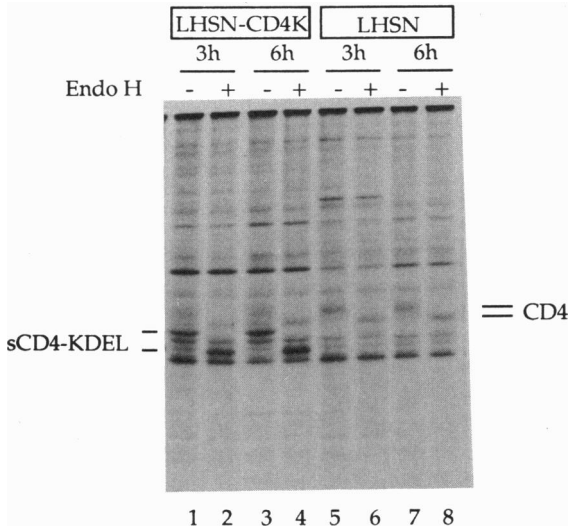


FIG. 3. sCD4-KDEL retains endoglycosidase H (endo H)-sensitive oligosaccharides when expressed in H9 cell lines. H9/LHSN or H9/LHSN-CD4K cells ($10^7$ cells per sample) were labeled by incubation for 3 hr in 1 ml of methionine-free DMEM containing 100 $\mu$Ci of [$^{35}$S]methionine. The cells then were lysed (3h) or were washed and incubated for 3 hr in DMEM containing 2 mM unlabeled methionine (6h). Proteins were immunoprecipitated from cell lysates with a rabbit anti-CD4 serum (a gift from Paul Maddon, Progenics, Tarrytown, NY). Immunoprecipitates were divided in half and either mock-treated (−) or treated (+) with 1 milliunit of endo H (30) in 0.15 M sodium citrate buffer (pH 5.5). Immunoprecipitated proteins were analyzed by electrophoresis in SDS/10% polyacrylamide gels.

lines. When sCD4-KDEL is retained in the ER, neither of its two N-linked glycans is processed by Golgi enzymes and they remain sensitive to digestion by endo H (17). In contrast, one of the glycans on CD4 is processed to endo H resistance with a half-time of about 45 min (17, 31). One of the subcloned H9 lines expressing sCD4-KDEL and a control line transduced with the LHSN vector were labeled for 3 hr with [$^{35}$S]methionine and lysed, or incubated in chase medium for an additional 3 hr before lysis. Proteins immunoprecipitated from lysates with anti-CD4 antibody were then digested with endo H or left untreated as indicated. The band corresponding to a sCD4-KDEL marker (Fig. 3, lanes 1–4) was judged to be fully sensitive to endo H even after the 3-hr chase period, because it migrated with the marker of fully deglycosylated sCD4-KDEL after endo H treatment. The cells containing vector alone did not express sCD4-KDEL (lanes 5–8) but did express a protein about 6 kDa larger that comigrated with a CD4 marker. This protein was almost certainly the endogenous CD4, since it showed the smaller shift in mobility corresponding to removal of one N-linked glycan after endo H treatment. This CD4 band was also apparent in the cells expressing sCD4-KDEL.

From densitometric scans we estimated that the basal level of sCD4-KDEL expression was 2–3 times that of the endogenous CD4. Earlier experiments (17) and the data in Fig. 3 suggested that expression of sCD4-KDEL did not interfere with transport of CD4 in HeLa cells. Using flow cytometry we also observed normal cell surface expression of CD4 in the T-cell lines expressing sCD4-KDEL (data not shown).

**HIV-1 Propagation Is Blocked.** To determine whether sCD4-KDEL production from the LHSN vector could interfere with HIV-1 propagation in the H9 cells, we infected these cells with the MN strain of HIV-1 (32). This strain has been adopted as the standard for vaccine testing in the United States because it is closely related to a major group of isolates commonly seen in patients (33). As a control we used H9 cells transduced with the vector LHSN alone, and infection was monitored by indirect immunofluorescence. Three days after HIV-1 infection of control cells, about 5–10% of the cells stained brightly for surface and internal HIV proteins. In addition, about half of these infected cells had fused to form large syncytia. Typical fields of cells are shown in Fig. 4*A*. Considerable cell death was observed in subsequent days and the infection progressed gradually through the culture. After 2 weeks, immunofluorescence microscopy showed that >90% of the cells were infected, and at later times all cells in the population were infected. Identical results were obtained with H9 cells (data not shown).

The results with the cells transduced with the LHSN-CD4K vector were strikingly different (Fig. 4*B*). Initially, 5–10% of the cells stained internally for HIV antigen, but antigen expression at the cell surface was not observed (Fig. 4*B*) and there was little or no formation of syncytia. Based on these and earlier results (17), we conclude that transport of HIV glycoprotein to the cell surface was blocked effectively by sCD4-KDEL expression. After 2 weeks we observed <1% HIV antigen-positive cells in the population, and this number gradually declined to the point (60 days) where HIV antigen-positive cells were undetectable (<1 infected cell per $10^6$ cells). The same results were obtained with two independent H9/LHSN-CD4K lines. The HIV-infected cells were apparently either killed by the HIV infection or divided more slowly than the uninfected cells.

To verify that sCD4-KDEL expression was induced in the HIV-1-infected cells, we carried out double-label immunofluorescent staining for both CD4 antigen and HIV antigens in the same cells. In every cell that expressed HIV proteins we also observed clear internal staining with anti-CD4 (Fig. 4*B*). The pattern observed was the nuclear membrane and reticular cytoplasmic staining expected for sCD4-KDEL retained in the ER. Note that there was no staining for CD4 antigen in uninfected or infected control cells (Fig. 4*A*) because endogenous surface CD4 expression levels are insufficient to be detected by indirect immunofluorescence, although surface CD4 was detected by flow cytometry.

After the initial infection of cells carrying the LHSN-CD4K vector, we did not observe any spread of HIV infection, suggesting that little or no infectious virus was being produced. In addition, culture medium taken from these cells at 7 days postinfection was incubated with H9 cells. The H9 cells were then examined for HIV infection after 10 and 30 days by indirect immunofluorescence, and no HIV antigens were observed. In contrast, HIV production from H9/LHSN cells was readily detected by this method. These results indicate that production of infectious HIV-1 was prevented in cells expressing the sCD4-KDEL gene under HIV LTR regulation.

**Release of HIV-1 p24 Protein.** If this intracellular trap for the HIV envelope protein were functioning as expected, we anticipated that tests for the major HIV capsid protein (p24) in the medium would be positive initially, because cells expressing only the HIV-1 *gag* and *pol* genes produce HIV-like particles that bud from cells and contain p24 (34). To test for p24 production, we infected two independent H9/LHSN-CD4K cell lines with HIV-1 and a control cell line transduced with LHSN vector only. Three days after infection the cells were washed to remove unadsorbed virus, and the medium was assayed for p24 antigen at various times over a period of 60 days (Fig. 5). The cultures were diluted 5- to 10-fold into fresh medium (cell numbers were kept nearly equal in the three cultures) and were allowed to grow for 4–5 days before each medium sample was taken for the p24 assay. The p24 amounts therefore approximate a rate of p24 release per cell over each 4- to 5-day period. The results show that in the two cell lines transduced with the LHSN-CD4K vector, the rate of p24 production was initially about half that observed in the control cells but then gradually declined to nearly undetect-

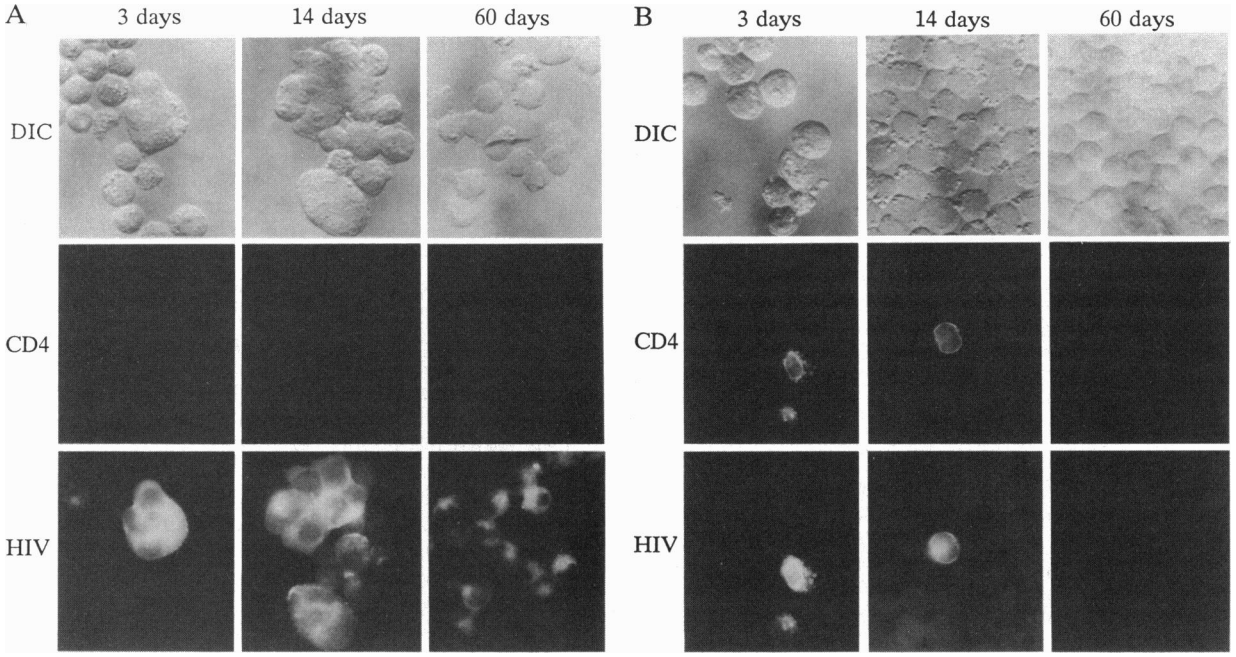


FIG. 4. Immunofluorescence microscopy of H9/LHSN (*A*) or H9/LHSN-CD4/ (*B*) cells infected with HIV-1. Approximately $10^6$ H9/LHSN or H9/LHSN-CD4K cells were infected in 1 ml of cell-free culture supernatant from HIV-1-infected H9 cells ($\approx 5 \times 10^4$ infectious HIV-$1_{MN}$ particles). Three days post-infection, the cells were washed and suspended in fresh medium. At the indicated number of days after infection, samples of cells were fixed and were made permeable with detergent before labeling with antibodies to HIV proteins or CD4 for immunofluorescence microscopy as described in *Materials and Methods*. Micrographs were taken with differential interference contrast (DIC) optics so that all cells could be visualized, or for rhodamine (CD4) or fluorescein (HIV antigens) fluorescence.

able levels. In contrast, p24 production continued in the control cells throughout the 60-day period. These results parallel the number of infected cells observed by immunofluorescence microscopy.

It should be noted that the H9/LHSN-CD4K cell lines are useful for estimating titers of infectious HIV because, once infected, they express large amounts of viral antigens readily detectable by immunofluorescence microscopy but do not produce virus able to infect surrounding cells. For example, when we infect $10^6$ H9/LHSN-CD4K cells with 1 ml of an HIV sample and find 5% of the cells infected (maximal infection is seen after 3–4 days), we can estimate that the titer must have been at least $5 \times 10^4$ infectious particles per ml. A similar procedure is not possible with H9 cells, because infected cells rapidly fuse with uninfected cells to produce large syncytia, and infectious virus is being released to infect uninfected cells.

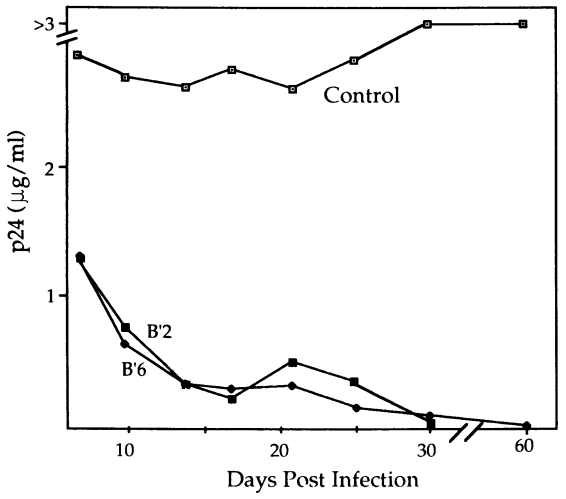


FIG. 5. Time course of HIV-1 p24 production in H9/LHSN and H9/LHSN-CD4K cells. H9/LHSN cells or two independent clones of H9/LHSN-CD4K cells (B'2 and B'6; $10^6$ cells per sample) were infected with HIV-$1_{MN}$ as in Fig. 4. Three days postinfection, the cells were washed free of input virus and suspended in fresh medium. Cells were fed by dilution to $\approx 10^5$ cells per ml every 4–5 days. The amount of p24 antigen in a cell-free supernatant at the end of each 4- to 5-day period (beginning at day 7 after infection) was determined by an assay for HIV-1 p24 protein (Abbott). □, Infected H9-LHSN cells; ●, infected H9-B'2 clone; ■, infected H9-B'2 clone.

## DISCUSSION

The intracellular trap for HIV described here is presumably effective in part because of the high affinity of gp160 for CD4 in the ER (31, 35, 36) and because sCD4-KDEL is concentrated in the ER (17). Recent results show that primary isolates of HIV often have 10- to 30-fold reduced binding of sCD4 (37), and the reduced affinity may explain their relative resistance to neutralization by sCD4. We argue that such reductions in affinity are unlikely to prevent the intracellular trapping of HIV envelope protein by sCD4-KDEL. After HIV infection of H9/LHSN-CD4K lines and induction of sCD4-KDEL synthesis, the protein should be expressed at a level of at least $10^6$ molecules per cell, and expression of gp160 from the HIV-1 LTR should also be at high levels. Assuming a volume of $\approx 10^{-10}$ cm$^3$ for the ER (ref. 38; this number was obtained in BHK cells and is probably an overestimate for T cells, which are smaller than BHK cells), we estimate that the concentration of sCD4-KDEL in the ER would be at least 50 $\mu$M. Given the high affinity of CD4 for gp120 ($K_d$ = 0.9 nM; ref. 39), and the high concentrations of sCD4-KDEL and gp160 in the ER, gp160 with 30-fold reduced affinity for CD4 would still most likely be trapped. Indeed, in recent experiments (unpublished results) we have found that mutations reducing the affinity of CD4 for gp120 as much as 500-fold (39) have little effect on the ability of sCD4-KDEL to retain gp120 in the ER. It therefore seems unlikely that mutated HIV could escape this intracellular trap without losing the ability to bind and infect CD4$^+$ cells.

Two promising strategies for gene therapy in AIDS have been described recently. One employed a retroviral vector overexpressing an RNA containing the TAR sequence, which binds the HIV Tat protein (7). The results obtained with T cells in that system are similar to what we have observed here, with initially infected cells eventually declining in number, although virus apparently persisted through low-level reinfection. More recently, the herpes simplex virus thymidine kinase gene was expressed in T cells under HIV LTR control. Upon HIV-1 infection in the presence of acyclovir, these cells were killed and HIV production was prevented (9). The system we have described here also apparently leads to elimination of infected cells but has the advantage that acyclovir is not required.

In future experiments it will be critical to address the ability of the LHSN-CD4K vector to transduce Tat-regulated sCD4-KDEL into primary T lymphocytes and to prevent production of infectious HIV in these cells. If HIV maturation is prevented in primary cells, the effectiveness of this intracellular trap on a panel of HIV primary isolates, which typically do not grow in T-cell lines, could be assessed. If the outcome of these experiments is successful, then development of effective methods for introducing the vectors into all or a large fraction of the $CD4^+$ cells in an HIV-infected individual is a logical next step.

**Note Added in Proof.** After this paper was accepted for publication, we learned that a cloning anomaly caused reversal of the orientation of the HIV-1 LTR in the LHSN vector relative to that shown in Fig. 1. The orientation in the LHSN-CD4K vector is correct as shown.

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