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High Level of Surface CD4 Prevents Stable Human Immunodeficiency Virus Infection of T-Cell Transfectants

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CD4 is the principal receptor for the human immunodeficiency virus (HIV). We have isolated and studied CD4-expressing tumor cell clones made by expressing CD4 in the T-cell tumor line HSB. Two clones, one designated HSBCD4, a clone expressing low levels of CD4, and the other, HSB10×CD4, a high-expresser CD4$^+$ clone, were studied for their ability to bind and replicate HIV. In contrast to many other CD4$^+$ cells that down-modulate CD4 following HIV infection, the HSB10×CD4 clones continued to express high levels of surface CD4 following infection with HIV. Unlike infection of HSBCD4 or many other human CD4$^+$ cells, HIV infection of HSB10×CD4 clone was short lived: p24 antigen, provirus, or coculturable virus was present for less than 14 days following infection with several strains of HIV-1 or with HIV-2. When infection was initiated by transfection of proviral DNA, high and low CD4 expressers initially produced p24 antigen at approximately the same level. However, high CD4 expressers produced coculturable virus only during the first few days following transfection, whereas low CD4 expressers transfected with HIV continued to produce virus beyond 6 weeks. Monoclonal antibody-mediated down-modulation of CD4 surface expression on HSB10×CD4 clones permitted these formerly HIV-resistant cells to become persistently infected with HIV. Thus, high concentrations of CD4 on the surface of an HIV-infected cell prevent persistent HIV infection of CD4$^+$ cells.

CD4 is a 55-kDa transmembrane glycoprotein that functions as a major histocompatibility complex receptor and in cellular signaling (3, 14, 25, 38). CD4 exists in close association with the p56^{lck} protein kinase (38), and this may allow it to affect many cellular processes. In addition, CD4 is closely associated with the CD3–T-cell receptor (TCR) complex (29), which provides the basis for antigen recognition by T cells and the activation of the T cell (3, 29). The transfection of the CD4 gene into non-CD4-expressing human cells renders them susceptible to human immunodeficiency virus (HIV) infection (21).

HIV infection of a variety of CD4$^+$ cells results in decreased cell surface expression of CD4 (9, 13, 17, 31, 32, 35). Several mechanisms have been proposed to explain the down-modulation of CD4 on HIV-infected cells. First, a number of investigators have studied the interaction of the HIV transmembrane glycoprotein precursor, gp160, with CD4 and have shown an inverse correlation between gp160 production and surface CD4 expression (8, 18), suggesting that intracellular complexes between gp160 and CD4 lead to the observed decreased surface CD4 expression. Second, other investigators have shown that HIV infection affected CD4 mRNA levels and decreased total immunoprecipitable CD4 levels (16, 31). Finally, several of the regulatory proteins of HIV have been proposed to regulate surface expression of CD4. *nef* (5, 13), a myristylated HIV regulatory phosphoprotein, has been reported to decrease CD4 expression by a posttranslational mechanism (13), although this is controversial (5). In addition, *vpu* has been shown to decrease CD4 expression when transfected either with *env* or alone into CD4$^+$ cell lines (41).

There appears to be a direct relationship between down-modulation of CD4 and the successful HIV infection of T cells (9, 16, 17, 31, 35). Simian immunodeficiency virus (African green monkey strain) (SIV_{agm}) infection of the high CD4 expresser SupT1 results in minimal down-modulation of CD4 and a less cytopathic HIV infection (17), compared with results of infection of the low CD4 expresser Hut 78. The redundancy of the viral mechanisms (8, 13, 31, 41) that lead to decreased surface CD4 expression on HIV-infected cells suggests that down-modulation of surface CD4 may be important to the viral life cycle. To explore this possibility, we studied the effect of high levels of constitutive surface CD4 expression on the course of HIV infection in T cells.

MATERIALS AND METHODS

Immunofluoresence assays. The expression of cell surface antigens was assessed by immunofluoresence flow cytometry with a FACscan flow cytometer (Becton-Dickinson, Sunnyvale, Calif.). Cells were labeled in suspension with saturating amounts of either OKT4 or OKT4c (Ortho Diagnostics, Raritan, N.J.) or Leu3a (Becton-Dickinson) all of which are monoclonal antibodies specific for CD4. As a control, other cells were also incubated separately with OKT8 (Ortho), an irrelevant, isotype-matched monoclonal antibody. Labeling with OKT4, OKT4c, OKT8, or Leu3a was followed by incubation with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins G, A, and M (Sigma).

Cells. A Moloney murine leukemia virus (MoLV) vector, MNST4 (33), containing the gene *neo* was used to introduce the gene for transmembrane CD4 (33) into a T-cell tumor, HSB-2, that is CD4-CD3-TCR negative (15). The infection of the CD4-negative T-cell line, HSB, with MNST4 resulted in cells expressing low levels of CD4 on their surface; one of

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these clones was designated HSBCD4.10 (10). A subclone of these cells was obtained and called HSBCD4. This infection with MNST4 also resulted in a series of cells: HSB10×CD4, MS-14, and H; these cells had 10 times the surface expression of CD4 compared with the low expressers. One of these cell populations, HSB10×CD4, was cloned, subcloned, and studied extensively. The H9 (27) cells used to passage HIV and the C8166 cells (30) used in syncytium-forming assays were made available through the AIDS Repository.

Virus. Laboratory isolates of HIV were obtained from the AIDS Repository. Isolates used included HIV-1_{MN}, HIV-1_{IIIB}, and HIV-1_{NL4-3}, as well as HIV-$2_{MVP-15132}$. Virus was passaged in H9 cells fed twice weekly and carried in a mixture consisting of 10% bovine calf serum, 600 μg of L-glutamine per ml, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 μg of ciprofloxacin per ml. Infectious supernatants were obtained by centrifuging cells at 2,000 × *g* followed by filtration through a 0.45-μm-pore-size filter. Some supernatants were stored at −70°C before use.

PCR. Cells were assayed for the *gag* proviral gene by liquid hybridization following 30 cycles of polymerase chain reaction (PCR). Cells were aliquoted from cultures at various times. The cells were washed twice and then resuspended in 100 mM KCl–10 mM Tris HCl (pH 8.3)–2.5 mM $MgCl_2$ to a concentration of 2 × 10^6 cells per ml. An equal volume of 10 mM Tris HCl (pH 8.3)–2.5 mM $MgCl_2$–1% Tween–1% Nonidet P-40–120 μg of proteinase K per ml was added, and the experimental sample was incubated for 1 h at 37°C and then for 10 min at 95°C to inactivate the proteinase K. Total DNA (1 μg) was added to 100 μl of PCR buffer consisting of 2 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus), 50 mM KCl, 10 mM Tris HCl (pH 8.3), 2.5 mM $MgCl_2$, 12.5 pmol of each deoxynucleoside triphosphate, and 50 pmol each of primer SK 38 (ATAATCCACC TATCCCAGTAGGAGAAAT) and primer SK 39 (TTTGGT CCTTGTCTTATGTCCAGAATGC). Samples were overlaid with mineral oil and run on a Gene Machine II Thermal Cycler (USA Scientific, Ocala, Fla.) for 30 cycles: 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. The final cycle was followed by a 10-min extension at 72°C.

Amplified DNA was detected by liquid hybridization to a labeled oligonucleotide probe, SK 19 (ATCCTGGGATTAA ATAAAATAGGTAAGATGTATAGCCCTAC). PCR product (30 μl) was hybridized in 10 mM NaCl–1 mM EDTA–0.2 pmol of ^{32}P-labeled probe as described previously (20). Then 25 μl of each sample was extracted with chloroform and run on a 10% polyacrylamide gel. Gels were autoradiographed overnight by using Kodak X-Omat X-ray film.

The signal generated from experimental samples was compared with signal from 50 proviral copies and 5 proviral copies that were each spiked into an equivalent amount of cellular DNA. Negative controls consisted of amplification product of buffers and media used in the assay. Additionally, amplification was performed on cells immediately postincubation with HIV-containing supernatants (time zero point), which ruled out the presence of input provirus from the infectious supernatants. All HIV viral stock supernatants required pretreatment with RNase-free DNase I (10 μg/ml; Worthington Biologicals) in 0.01 M $MgCl_2$ to eliminate proviral DNA. Samples that were negative for HIV *gag* were subsequently amplified with primers directed at cellular sequences or were spiked with HIV and amplified to rule out the presence of an inhibitor of the PCR.

Transfection of provirus. The provirus was transfected by a modification of the DEAE-dextran procedure (32). Infectious molecular clone pNL4-3U35 (36) was obtained from K. Streibel through the AIDS Repository, and clone HXBC21 was obtained from Joseph Sodroski.

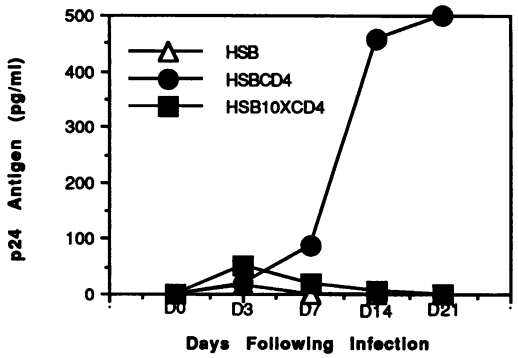

FIG. 1. Cell lines differ dramatically in their production of HIV-1 proteins following infection. A total of 5 × 10^6 cells were infected with filtered supernatants containing 5 × 10^4 $TCID_{50}$ of HIV-1_{MN} (i.e., multiplicity of infection of 0.01) by incubation with virus for 60 to 90 min at 37°C. The CD4-negative parent line (HSB) served as a negative control. The cells were washed, and the amount of p24 in the supernatant was measured. The high CD4 expresser clones showed a transient rise in p24 level that was 5 to 15 times that in negative controls, depending on the experiment. No residual virus or p24 antigen was found in the HSB10×CD4 cells by 2 weeks following infection. This experiment was repeated 10 times.

P24 assay. The presence of p24 antigen in culture and transfectant supernatants was assayed by enzyme-linked immunosorbent assay (ELISA; Abbott).

$TCID_{50}$. The amount of infectious virus (measured as the 50% tissue culture infective dose [$TCID_{50}$]) present in the supernatants of the cell lines that are transfected with HIV was assayed as described previously (44). Briefly, serial dilutions of the supernatants were added to a cell line (either H9 or C8166) susceptible to HIV cytopathic effect. The presence of HIV viral infectious units was determined visually by observing for syncytia by endpoint titration and confirmed by p24 assay.

RESULTS

HSB transfectants differ in their ability to replicate HIV. We have defined a series of clones made by introducing cDNA encoding CD4 into a T-cell tumor line, HSB, that is CD4-CD3-TCR negative (15). A MoLV vector, MNST4, containing the gene *neo* was used to transduce the gene for CD4. The introduction of MNST4 into the CD4-negative T-cell line HSB resulted in cells stably expressing either low or high levels of CD4 on their surfaces. Cells expressing low levels of CD4 were cloned (10), and one was further subcloned and called HSBCD4. This transduction also resulted in a cell line, HSB10×CD4, that had 10 times as much surface CD4 as did the low expressers, as determined by flow cytometry with three anti-CD4 antibodies, Leu3a, OKT4, and OKT4c (data not shown). HSB10×CD4 was cloned and studied extensively.

The results for the HSB10×CD4 clone are shown in Fig. 1. Despite a transient rise in the level of p24 antigen in HSB10×CD4 cell supernatants (Fig. 1), no p24 antigen was detected 2 weeks after infection. This was true over a wide range (10 to 0.001) of multiplicities of infection. This result is in contrast to those for many other CD4-expressing cells

with various amounts of CD4 on their surface (7, 9, 14, 16, 17, 22, 32, 41). Resistance to HIV infection was not a property solely of this clone, HSB10×CD4, but was also true of two other high CD4 expressers, obtained by transducing HSB with MNST4 (data not shown).

Cell lines with high CD4 expression displayed only a transient rise in the p24 antigen level following exposure to several different HIV-1 isolates: HIV-1_{MN}, HIV-1_{IIIB}, and HIV-1_{NL4-3} (only data for infection with HIV-1_{MN} are shown). In addition, HIV-$2_{MVP-15132}$ (data not shown) was able to infect HSBCD4 but not HSB10×CD4. During each infection, cell viability was determined by trypan blue exclusion. In the first few weeks following infection, viability was more than 90% for all cells infected and did not differ between HSB10×CD4 and HSBCD4 cells. Identical results were obtained in more than 10 experiments.

One possible explanation for the lack of stable infection of the HSB10×CD4 clones was that CD4 was shed into the medium and inhibited infection as soluble CD4 does (23). Neither supernatants from HSB10×CD4 nor coculture in the presence of HSB10×CD4 with susceptible HSBCD4 cells was capable of preventing infection and eventual killing of the susceptible HSBCD4 cells (data not shown).

The presence of p24 in the high CD4 expressers could be explained either by transient infection or by passive binding of input virus to CD4 on high expressers, unaccompanied by entry and infection. Failure of virus to enter the cell, despite attachment to CD4, has been demonstrated in mouse lines transfected with human CD4 (21) and, rarely, with CD4-positive human cell lines (6), infected with a limited number of strains of HIV-1 (7). We hypothesized that the very high concentrations of CD4 on the HSB10×CD4 cell surface either might be shed before virus bound to CD4 entered HSB10×CD4 cells or might sterically hinder the subsequent steps thought to be necessary for viral entry (6, 21). Therefore, we investigated whether HIV entry might be inhibited in the HSB10×CD4 clone.

Lack of lasting infection in HSB10×CD4 lines is not due to a block at entry or transcription. As determined by a quantitative technique for the detection of HIV provirus by PCR (18), HIV proviral DNA was present in these high CD4-expressing cells early after infection. The HIV proviral DNA in the HSB10×CD4 (high CD4 expresser) clone was initially present in an amount equal to that in a low expresser of CD4, HSBCD4 (Fig. 2). This implied that viral entry was not significantly blocked since the viral RNA was able to enter and be reverse transcribed into DNA and was detectable by PCR. Several days following infection, however, no viral DNA was detected, coincident with a loss of virus as determined either by coculture with permissive cells or by p24 antigen detection.

Transient p24 antigen production following HIV infection of the high CD4 expressers is not due to a susceptible subpopulation of low CD4 expressers. Low CD4 expressers were susceptible to HIV-induced cytopathic effect and death following entry and infection, whereas the high CD4 expresser, HSB10×CD4, was not susceptible. It was thus possible that the transient p24 antigen and provirus present in the HSB10×CD4 cells following HIV infection arose from a small contaminating subpopulation of low CD4 expresser cells. Therefore, we sought to determine whether this transient presence of p24 in the supernatants of HSB10×CD4 clones and the transient presence of provirus was due to selective infection and elimination of cells susceptible to HIV infection. If a susceptible population existed, this population would have been eliminated by infection. To demonstrate that a susceptible subpopulation was not responsible for the evidence of transient HIV infection in the HSB10×CD4 cells, we cloned HSB10×CD4 twice and then reinfected HSB10×CD4 clones that had previously been transiently infected with HIV.

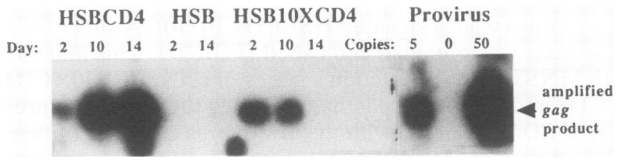

FIG. 2. Entry and reverse transcription of HIV are not blocked by high levels of CD4. The figure shows an autoradiograph of polyacrylamide gel electrophoresis of the PCR product from cell lysates of HIV-exposed cells. The PCR product from lysates was probed with a ^{32}P-labeled oligonucleotide specific for the *gag* region. As controls, proviral DNA at a copy number of 50 (far right lane) and 5 (third from right lane) was spiked into cell lysates of uninfected cells and analyzed by PCR. The lack of detectable DNA in lane 0 (second from right) ruled out contamination of the reagents used in the PCR. The amount of proviral DNA detected in HSB10×CD4 cells (lane HSB10×CD4 2) 2 days postinfection was at least as great as that found in the persistently HIV-infectable low CD4 expresser cell line, HSBCD4. Proviral DNA was absent in the CD4-negative cell line HSB and disappeared from HSB10×CD4 cells by day 14. As controls, uninfected samples were run in parallel (lane 0) to rule out false-positives resulting from HIV contamination of the reagents. Additionally, PCR of cells at time zero, immediately following infection, did not reveal the presence of HIV provirus (data not shown). The viral supernatants used to infect the cells were treated with DNase I to eliminate input provirus in the infectious HIV-1_{MN} supernatants. This experiment was repeated three times.

Two weeks after the initial infection, when HSB10×CD4 clones no longer produced p24 antigen, the HSB10×CD4 clones were rechallenged with HIV. Upon rechallenge, the same phenomenon of transient rise and fall of the level of p24 antigen occurred (Fig. 3). This indicated that the

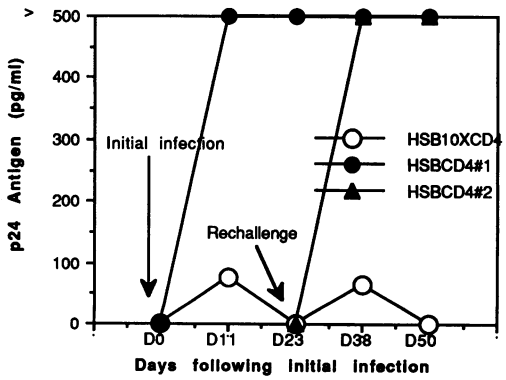

FIG. 3. Reinfection of high expressers. The figure shows p24 antigen expression on days following the initial challenge of high (HSB10×CD4) and low (HSBCD4) expressers with HIV at a multiplicity of infection of 0.1. On day 23 following infection of the HSB10×CD4 line, at a time when the HSB10×CD4 cells were negative for p24 and coculturable virus, the cells were rechallenged with HIV. p24 antigen ELISA and coculture were again performed, with the same result that no virus was present in the HSB10×CD4 cells that were assayed beyond 2 weeks following infection. The HSBCD4 clones that have low levels of surface CD4 were able to produce p24 and virus (as determined by coculture) continuously after challenge with HIV.

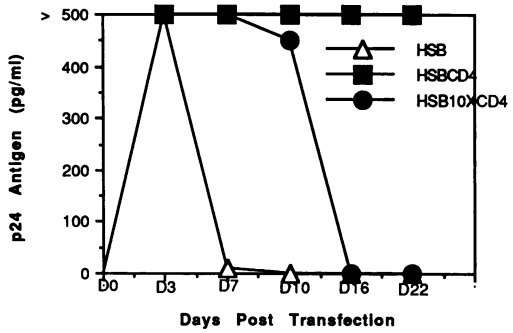

FIG. 4. HIV Transfection of HSB10×CD4 clones results in transient p24 antigen production. The HSB parent and the T-cell clones HSBCD4 and HSB10×CD4 were transfected with HIV provirus on day zero (D0). Supernatants were monitored for the presence of viral p24 antigen. The CD4-negative parent, HSB, only transiently produced p24 antigen (■ overlaps △ on day 3). The cells expressing wild-type CD4 were persistently infected beyond 4 weeks. The high CD4 expresser, HSB10×CD4 (● overlaps ■ on days 3 and 7), does not produce p24 antigen beyond day 16. This experiment was repeated twice.

HSB10×CD4 clones themselves, and not a susceptible subpopulation of cells, were responsible for the transient, small rise in p24.

As a control, HSBCD4 cells were infected at the same time as the HSB10×CD4 clones each time a persistent infection was established in the low CD4 expressers. Cocultures of the HSB10×CD4 clones with H9 cells showed no p24 antigen production at 28 days postinfection, whereas identical cocultures of HSBCD4 cells produced >500 pg of p24 per ml (data not shown), indicating that no viable virus was present in the HSB10×CD4 line.

Successful transfection of high CD4 expressers with HIV rules out an absolute posttranscriptional block to viral replication in high CD4 expressers. We next transfected the HSB10×CD4 clones with HIV provirus to determine whether the failure to establish persistent HIV infection in high CD4 expressers was due to a block in a postentry step of the viral life cycle. One advantage of the transfection approach is that there is no input p24 antigen as there is in infection, so p24 antigen produced must result from the transcription and translation of the viral genome in the transfected cells. The same advantage holds true for quantitation of virus produced by such cells.

As shown in Fig. 4, transfection of the T-cell lines resulted in only transient expression of HIV p24 core protein in the CD4-negative parent cell line, HSB. This rapid clearance of p24 antigen is not surprising because virus produced by transfected HSB cells could not infect untransfected HSB cells in the same culture, since HSB lacks the HIV receptor, CD4. The HSB10×CD4 clones also produced p24 but did not produce any p24 antigen after day 16 posttransfection. This is consistent with the idea that infection is abortive in these cells, as seen with live virus in Fig. 1. However, the production of infectious virus and viral proteins by HSB10×CD4 cells indicates that there was no absolute block to virion formation.

The ratio of p24 antigen to the amount of infectious virus (expressed as $TCID_{50}$ in Table 1) suggests that for a given amount of viral protein, less infectious virus was produced by the high expresser, HSB10×CD4, than by the low expresser, HSBCD4. This implies either a quantitative defect in infectious virion production or a defect in the infectivity of the virions produced by the HSB10×CD4 cells. Either explanation for the lower titer of virus from HSB10×CD4 HIV transfectants is formally possible.

TABLE 1. HSB10×CD4 clones produce infectious virus, although less efficiently[a]

Clone	Amt of p24 antigen (pg/ml)	$TCID_{50}$	No. of IU/pg of p24
HSB10×CD4	483	1:50	0.104
HSBCD4	229	1:500	2.183

[a] Supernatants from cell cultures of the T-cell transfectants were obtained at 18 h following transfection with HIV provirus. The amount of p24 antigen present was determined. The $TCID_{50}$ of dilutions of the virus was determined by the syncytium-forming assay in the C8166 cell line.

HSB10×CD4 cells do not down-modulate CD4 in response to HIV infection. Figure 5 shows the flow-cytometric analysis of HSB transfectants, HSB10×CD4 and HSBCD4, before and after HIV infection. Clone HSB10×CD4 had a surface CD4 expression 1 log unit higher than that of HSBCD4 as determined by flow cytometry with OKT4 monoclonal antibody. The level of CD4 expression on HSBCD4 is comparable to the surface level of CD4 on H9 (27), C8166 (30), and Jurkat cell lines and $CD4^+$ peripheral blood T lymphocytes (flow cytometry data not shown). As has been reported for other cell lines (9, 16, 17, 31, 35), the expression of CD4 following HIV infection remains dramatically suppressed in all other $CD4^+$ cell lines that we examined (data not shown). The expected down-modulation of CD4 following HIV infection was also seen in HSBCD4 (Fig. 5). This property of resistance to down-modulation of CD4 after HIV exposure was observed in two other, uncloned, high CD4-expressing HSB cell lines that also resist persistent HIV infection.

HSB10×CD4 cells do not form syncytia when infected or transfected with HIV. HSB10×CD4 cells that are either transfected with HIV provirus or infected with HIV do not form syncytia (Fig. 6) at a time when the HSB10×CD4 cells are producing HIV. This lack of syncytium formation is in contrast to the behavior of transfected HSBCD4 cells (Fig. 6), and similar syncytium formation was observed following infection of HSBCD4.

Syncytium formation is the result of fusion between infected cells which express functional envelope protein and cells which express cellular receptor (CD4). The absence of visible syncytia does not preclude fusion between infected and uninfected cells. Indeed, the results in Fig. 2 demonstrate that initially HIV entry and, hence, fusion occur equivalently between HIV and the cell membrane of the HSB10×CD4 or HSBCD4 clone. However, the conspicuous absence of syncytium formation in HSB10×CD4, together with the prominent syncytium formation in HSBCD4, suggests that dysfunction of either the viral envelope or cellular receptor on HSB10×CD4 clones (after initial infection with exogenously produced HIV) might be responsible for the abortive infection of the HSB10×CD4 clone. Inhibition of binding or fusion, manifested by a lack of syncytium formation in the transfected HSB10×CD4 clone, might also explain the decreased infectivity of virus produced by HSB10×CD4 (see Table 1). The most straightforward explanation would be that the high levels of CD4 on HSB10×CD4 could directly interfere with the function of the HIV envelope gene product. Thus, we designed an experiment to alter the surface expression of CD4 on the HSB10×CD4 cells and look at the ability of such lower CD4-expressing HSB10×CD4 clones to be infected.

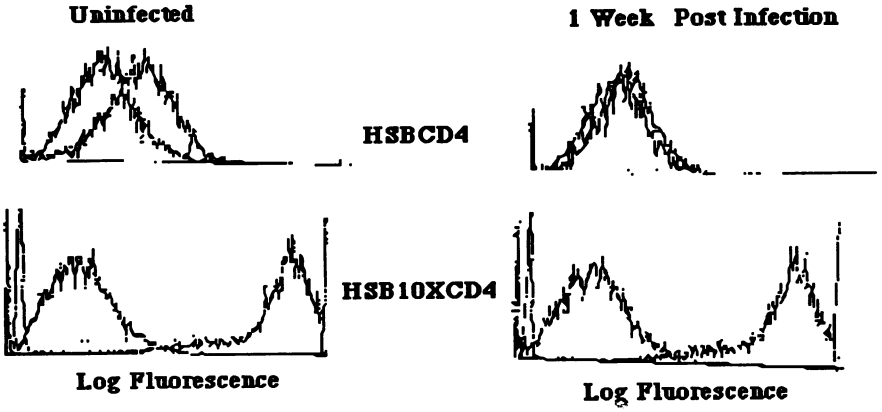

FIG. 5. CD4 expression on HSB clones before and after HIV infection. HSB clones were incubated with saturating amounts of fluorescein isothiocyanate-conjugated monoclonal antibody against CD4, OKT4 (right peak of each pair), and the intensity of cell surface fluorescence was compared with that of OKT8, an irrelevant isotype-matched monoclonal antibody (leftmost peak of each pair). HSB10×CD4 had a 1-log-unit higher surface CD4 expression as determined by flow cytometry. The expected down-modulation of CD4 following HIV infection is seen in HSBCD4 (top two panels). High CD4-expressing clones that are resistant to persistent HIV infection continue to express high levels of CD4 even 1 week postinfection, at a time when the high expressers contain HIV provirus and p24 can be detected in cell culture. Identical results were obtained in three experiments.

Down-modulation of CD4 on HSB10×CD4 cells allows a persistent HIV infection to occur. By using cross-linking of surface CD4 with OKT4 and a second, goat anti-mouse (GAM) antibody, we were able to down-modulate CD4 on some of the high expressers (HSB10×CD4) so that they expressed a low CD4 expresser phenotype (Fig. 7a). We were then able to persistently infect anti-CD4 monoclonal antibody-treated HSB10×CD4 cells (T4GAM [Fig. 7a]) with HIV as shown by serial p24 antigen measurement (Fig. 7c) or as determined by coculture with C8166 cells (data not shown). This result is contrasted with the transient HIV infection in the HSB10×CD4 cells (Fig. 7c) that were treated with second antibody alone and thus did not down-modulate CD4 as determined by flow cytometry (Fig. 7b). Syncytia could be observed in the T4GAM population but not the GAM population of HSB10×CD4 clones. There was no difference in viability in the HSB10×CD4 cells treated with OKT4 and second antibody compared with cells treated with second antibody alone, as determined by trypan blue exclusion. This experiment demonstrates that it is the high surface CD4 expression on HSB10×CD4 cells which conferred HIV resistance.

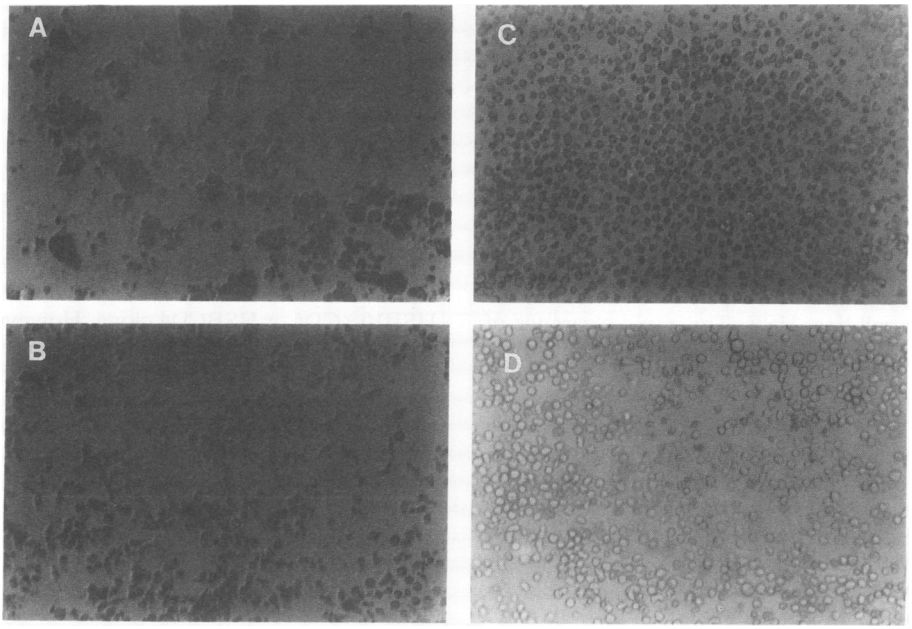

FIG. 6. The HSB10×CD4 clone does not form syncytia following HIV infection. (A and B) Photomicrographs of HSBCD4 (panel A) and HSB10×CD4 (panel B) 48 h following transfection with HIV provirus. Note the prominent multinucleated giant cells (syncytia) in the HSBCD4 transfectants but not the HSB10×CD4 clone 48 h following transfection, at a time when HIV was being produced in the cultures. Viability was more than 95% in panels B to D, as determined by trypan blue exclusion. (C and D) Uninfected HSBCD4 (panel C) and HSB10×CD4 (panel D). Comparable results were obtained for more than 10 infections with HIV-1 and an infection with HIV-2. Magnification, ×200.

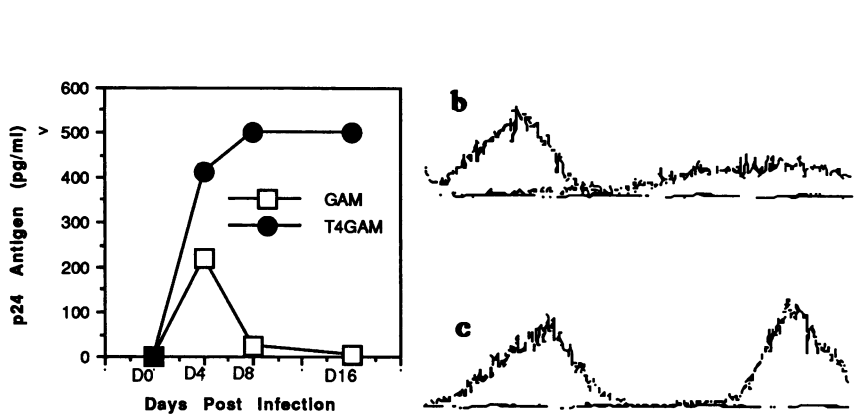

FIG. 7. Persistent HIV infection of HSB10×CD4 occurred only after anti-CD4 monoclonal antibody mediated the down-modulation of CD4 on HSB10×CD4 cells. HSB10×CD4 was incubated with OKT4c and a second, GAM antibody (panel b) or with GAM antibody alone (panel c) and subsequently stained with OKT4-FITC antibody (panels b and c; the rightmost peak on FACS analysis represents $CD4^+$ cells). Incubation with OKT4c and GAM resulted in a shift in the surface CD4 staining of HSB10×CD4 clones as determined by flow cytometry (compare panels b and c). The HSB10×CD4 cells that were incubated with OKT4c and GAM were then able to be persistently infected with HIV (T4GAM in panel a). The non-down-modulated HSB10×CD4 cells were unable to be persistently infected with HIV (GAM in panel a). This experiment was repeated twice.

DISCUSSION

In contrast to many other $CD4^+$ cells that down-modulate CD4 following HIV infection (9, 16, 17, 31, 35), the HSB10×CD4 clones continue to express high levels of surface CD4 following infection with HIV. Unlike many other human $CD4^+$ cells described to date, HIV infection of HSB10×CD4 cells is short lived: p24 antigen, provirus, or coculturable virus is present for less than 14 days following infection with several strains of HIV-1 or with HIV-2. When infection was initiated by transfection of proviral DNA, high and low CD4 expressers initially produced p24 antigen at approximately the same level. However, high expressers produced coculturable virus only during the first few days following transfection, whereas low expressers transfected with HIV continued to produce virus beyond 6 weeks. The implication of these results is that cells which express high levels of surface CD4 in the presence of HIV infection exert a postentry block upon HIV infection. However, such high CD4 expresser cells are transiently capable of replicating virus.

An example of a similar abortive HIV infection has been described with HIV-1 JRCSF infection of A2.01 cells (4). HIV was able to enter the cells as shown by postinfection PCR, and the cells could transiently produce p24 antigen and virus posttransfection. Nevertheless, infection with JRCSF failed to spread throughout the culture, whereas infection with another HIV strain, NL4-3, was persistent (4). This indicated a postentry block in the HIV life cycle, culminating in an abortive infection.

What is striking about the phenomenon of resistance in HSB10×CD4 cells is that not only does it appear to be HIV strain independent, but also the ability of the cell to resist stable HIV infection appears to reside at the cell surface, since it is a decrease in cell surface CD4 expression that allows the high-expresser HSB10×CD4 cells to be persistently infected. The observations of both the absence of CD4 down-modulation and the absence of syncytium formation correlate with the phenomenon of resistance to stable HIV infection. These observations suggest a possible mechanism to explain how these two qualities of the HSB10×CD4 cell may lead to the abortive HIV infection in HSB10×CD4 cells: high concentrations of surface CD4 on the surface of HIV-infected HSB10×CD4 cells may saturate the available viral envelope, making cell-to-cell (and possibly virion-to-cell) spread of the virus difficult. Experiments are under way to determine whether this is so, and our finding that HSB10×CD4 cells form syncytia, once surface CD4 is down-modulated, is consistent with this tentative hypothesis.

It is unclear whether there is a survival advantage for a virus as a result of depressed cell surface expression of viral receptors during lytic infection. Many enveloped viruses alter functional cell surface expression of their receptor determinants during infection (11, 24, 31, 39, 40). There are several ways in which such a mechanism may be important in the life cycle of an enveloped virus which must bud from the surface of the cell in a lytic infection.

Influenza viruses (26) and coronaviruses (39) possess receptor-destroying enzyme activity. Receptor-destroying enzyme activity has been hypothesized to be necessary for viral release (42). This hypothesis was demonstrated conclusively through the use of temperature-sensitive mutants of the influenza virus neuraminidase gene (which possesses the receptor-destroying enzyme activity for the influenza virus receptor sialic acid) that were capable of infection of susceptible cells but were unable to be released efficiently and formed aggregates of virions (26). This observation would be consistent with the observation by electron microscopy of the aggregates of HIV that bud from the surface of infected cells early following HIV infection, while surface CD4 is still being down-modulated (our unpublished observations).

Another possible survival advantage for viruses that down-modulate their receptors is demonstrated by some strains of avian leukosis virus, for which massive second-round reinfection (40) occurs. Because it has been shown that CD4 receptor expression is not rate limiting to HIV infection (2), a similar explanation for the decrease in CD4 expression following HIV infection is less likely. Another possible way in which retroviral interference (40) might convey a survival advantage to HIV would be through prevention of cytolysis in the host cell, allowing continuous viral production. However, one study of cytolysis resistance

induced by HIV envelope interference demonstrated decreased syncytium formation, in $CD4^+$ cells which expressed HIV envelope and were subsequently superinfected with HIV, but this study did not find a difference in single-cell cytolysis (35). Since we did not observe syncytia in the transfected or infected high CD4 expresser (HSB10×CD4 clone), the survival advantage conferred by interference-induced cytolysis resistance via lessened syncytium formation (35) is unlikely to explain the transient HIV infection in HSB10×CD4 cells.

Susceptibility to the cytopathic effect seems to correlate with the level of cell surface expression of CD4 on monocytic cell lines (1, 19). Expression of HIV-1 envelope in T-cell tumors is toxic (34) and leads to decreased expression of CD4 on the cell surface (8). Decreased expression of CD4 on HIV-infected cells might conceivably lessen the susceptibility of the infected cells to the toxic effects of HIV gp120 or gp160 (12) and might convey a survival advantage to viruses capable of down-modulating surface CD4, thus allowing continued virus production. SIVagm infection of SupT1 (which possesses a 1 log unit higher surface expression of CD4 than Hut 78 does) was less cytopathic in SupT1 than was the infection seen in Hut 78. This indicates that the relationship between surface CD4 expression and cytopathic effect is neither linear nor simple. Finally, when we looked for greater cell killing of the high expressers, we were unable to see a difference in cell number or viability, as determined by trypan blue exclusion, following HIV infection or transfection. Some killing of HIV-infected HSB10×CD4 cells must be postulated to explain the loss of HIV-infected HSB10×CD4 cells by 2 weeks following infection or transfection with HIV (Fig. 2 and 4).

Conceivably, high levels of cellular receptor may interfere with the normal function or assembly of viral glycoproteins. In the case of SIVagm infection of the high CD4 expresser SupT1, immunoprecipitates of SIVagm transmembrane envelope protein revealed that envelope proteins appeared to be alternatively processed in the SupT1 cells (17). Immunoprecipitation of HIV proteins in HSB10×CD4 cells reveals no evidence for this type of interference with HIV virion formation in the HSB10×CD4 cell line (data not shown).

Syncytium formation and stable HIV infection in HSB10×CD4 cells occur only when cell surface CD4 is down-modulated; thus it is likely that the mechanism of resistance is due to a cell membrane property of these cells. Since retroviruses are known to incorporate some transmembrane host cellular proteins such as CD4 (43), CD4-gp120 interactions on the virion or HSB10×CD4 cell surface might mask the viral envelope and prevent the attachment of virus produced by HSB10×CD4 cells to receptors on uninfected cells. Alternatively, the high level of non-down-modulated CD4 receptor on HSB10×CD4 cells may bind budding virions and prevent the release of HIV, except as viral aggregates. This mechanism would be analogous to the aggregates of influenza virus particles seen in neuraminidase-deficient mutants that lack the ability to decrease cell surface expression of their sialic acid receptor, and consequently are less infectious (26). Another possibility is that surface CD4 may strip gp120 from the virion and cell surface, as soluble CD4 does (23), resulting in a less infectious viral particle (Table 1).

If it is true that high levels of surface CD4 interfere with virion formation, it thus becomes easier to understand why down-modulation of surface CD4 expression is so common following productive HIV infection. Also, such a requirement for lower levels of CD4 may explain why HIV has evolved several mechanisms for accomplishing this down-modulation.

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REFERENCES

1. **Asjo, B., I. Ivhed, M. Gidlund, S. Fuerstenberg, E. M. Fenyo, K. Nilsson, and H. Wigzell.** 1987. Susceptibility to infection by the human immunodeficiency virus correlates with T4 expression in a parental monocytoid cell line and its subclones. Virology **157**:359–365.
2. **Besansky, N. J., S. T. Butera, and T. M. Folks.** 1991. Unintegrated human immunodeficiency virus type 1 DNA in chronically infected cell lines is not correlated with surface CD4 expression. J. Virol. **65**:2695–2698.
3. **Blue, M. L., D. A. Halfer, J. F. Daley, H. Levine, K. A. Craig, J. Breitmayer, and S. F. Schlossman.** 1988. Regulation of T cell clone function via CD4 and CD8 molecules: anti-CD4 can mediate two distinct inhibitory activities. J. Immunol. **140**:376.
4. **Cann, A. J., J. A. Zack, A. S. Go, S. J. Arrigo, Y. Koyanagi, P. L. Green, Y. Koyanagi, S. Pang, and I. S. Y. Chen.** 1990. Human immunodeficiency virus type 1 T-cell tropism is determined by events prior to provirus formation. J. Virol. **64**:4735–4742.
5. **Cheng-Mayer, C., P. Ianello, K. Shaw, and J. A. Levy.** 1989. Differential effects of *nef* on HIV replication: implications for viral pathogenesis in the host. Science **246**:1629–1632.
6. **Chesebro, B., R. Buller, J. Portis, and K. Wherly.** 1990. Failure of human immunodeficiency virus entry and infection in CD4-positive human brain and skin cells. J. Virol. **64**:215–221.
7. **Clapham, P. R., D. Blanc, and R. A. Weiss.** 1991. Specific cell surface requirements for the infection of CD4-positive cells by HIV-1 and 2 and by SIV. Virology **181**:703–715.
8. **Crise, B., L. Buonocore, and J. K. Rose.** 1990. CD4 is retained in the endoplasmic reticulum by the human immunodeficiency virus type 1 glycoprotein precursor. J. Virol. **64**:5585–5593.
9. **Dalgleish, A. G., P. C. L. Beverly, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential components of the receptor for the AIDS retrovirus. Nature (London) **312**:763–767.
10. **Diamond, D. C., R. W. Finberg, S. Chaduri, B. P. Sleckman, and S. J. Burakoff.** 1990. Human immunodeficiency virus infection is efficiently mediated by a glycolipid-anchored form of CD4. Proc. Natl. Acad. Sci. USA **87**:5001–5005.
11. **Fingeroth, J.** Personal communication.
12. **Fisher, A. G., L. Ratner, H. Mitsuya, L. M. Marselle, M. E. Harper, S. Broder, R. C. Gallo, and F. Wong-Staal.** 1986. Infectious mutants of HTLV-III with changes in the 3′ region and markedly reduced cytopathic effects. Science **233**:655–659.
13. **Garcia, J. V., and A. D. Miller.** 1991. Serine phosphorylation-independent downregulation of cell surface CD4 by *nef*. Nature (London) **350**:508–511.
14. **Gay, D., P. Maddon, R. Sekaly, M. A. Talle, M. Godfrey, E. Long, G. Goldstein, L. Chess, R. Axel, J. Kappler, and P. Marrack.** 1987. Functional interaction between human T-cell protein CD4 and the major histocompatability complex HLA-DR antigen. Nature (London) **328**:626–629.
15. **Hara, J., S. H. Benedict, E. Champagne, T. W. Mak, M. Minden, and E. W. Gelfand.** 1988. Comparison of T cell receptor α, β, and γ gene rearrangement and expression in T cell acute lymphoblastic leukemia. J. Clin. Invest. **81**:989–996.
16. **Hoxie, J. A., J. D. Alpers, J. L. Rackowski, K. Hueber, B. S. Haggerty, A. J. Cedarbaum, and J. C. Reed.** 1986. Alterations in T4 (CD4) protein and mRNA synthesis in cells infected with HIV. Science **234**:1123–1127.
17. **Hoxie, J. A., B. S. Haggarty, S. E. Bonser, J. L. Rackowski, H. Shan, and P. J. Kanki.** 1988. Biological characterization of a

simian immunodeficiency virus-like retrovirus (HTLV-IV): evidence for CD4-associated molecules required for infection. J. Virol. **62:**2557–2568.

18. **Koga, Y., M. Sasaki, K. Nakamura, G. Kimura, and K. Nomoto.** 1990. Intracellular distribution of the envelope glycoprotein of the human immunodeficiency virus and its role in the production of cytopathic effect in $CD4^+$ and $CD4^-$ cell lines. J. Virol. **64:**4661–4671.
19. **Koga, Y., M. Sasaki, H. Yoshida, H. Wigzell, G. Kimura, and K. Nomoto.** 1990. Cytopathic effect determined by the amount of CD4 molecules in human cell lines expressing envelope glycoprotein of HIV. J. Immunol. **144:**94–102.
20. **Krone, W. J. A., J. J. Sninsky, and J. Goudsmit.** 1990. Detection and characterization of HIV-1 by polymerase chain reaction. J. Acquired Immune Defic. Syndr. **3:**517–524.
21. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47:**333–348.
22. **McDougal, J. S., M. S. Kennedy, J. M. Sligh, S. P. Cort, A. Mawle, and J. K. A. Nicholson.** 1986. Binding of HTLV-III/LAV to T cells by a complex of the 110K viral protein and the T4 molecule. Science **231:**382–385.
23. **Moore, J. P., J. A. McKeating, R. A. Weiss, and Q. J. Sattentau.** 1990. Dissociation of gp120 from HIV-1 virions induced by soluble CD4. Science **250:**1139–1142.
24. **Muchmore, E. A., and A. Varki.** 1987. Selective inactivation of influenza C esterase: a probe for detecting 9-O-acetylated sialic acids. Science **236:**1293–1295.
25. **Neudorf, S. M., M. M. Jones, B. M. McCarthy, J. A. K. Harmony, and E. M. Choi.** 1990. The CD4 molecule transmits biochemical information important in the regulation of T lymphocyte activity. Cell. Immunol. **125:**301–314.
26. **Palese, P., K. Tobita, M. Ueda, and R. W. Compans.** 1974. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. Virology **61:**397–410.
27. **Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo.** 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science **224:**497–500.
28. **Rossi, A. D., G. Franchini, A. Aldovini, A. D. Mistro, L. Chieco-Bianchi, R. C. Gallo, and F. Wong-Staal.** 1986. Differential response to the cytopathic effects of human T-cell lymphotropic virus type III (HTLV-III) superinfection in T4+ (helper) and T8+ (suppressor) T-cell clones transformed by HTLV-I. Proc. Natl. Acad. Sci. USA **83:**4297–4301.
29. **Saizawa, K., J. Rojo, and D. Janeway.** 1987. Evidence for a physical association of CD4 and CD3:α:β T-cell receptor. Nature (London) **328:**260.
30. **Salahuddin, S. Z., P. D. Markham, F. Wong-Staal, G. Franchini, V. S. Kalyanaraman, and R. C. Gallo.** 1983. Restricted expression of HTLV in transformed human umbilical cord blood lymphocytes. Virology **129:**51–64.
31. **Salman, P., R. Olivier, Y. Rivere, E. Brisson, J. C. Gluckman, M. P. Kieny, L. Montagnier, and D. Klatzmann.** 1988. Loss of CD4 membrane expression and CD4 mRNA during acute human immunodeficiency virus replication. J. Exp. Med. **168:** 1953–1969.
32. **Sattentau, Q., J. A. G. Dalgleish, R. A. Weiss, and P. C. L. Beverly.** 1986. Epitopes of the CD4 antigen and HIV infection. Science **234:**1120–1123.
33. **Sleckman, B. P., A. Peterson, W. K. Jones, J. A. Foran, J. L. Greenstein, B. Seed, and S. J. Burakoff.** 1987. Expression and function of CD4 in a murine T-cell hybridoma. Science **328:**351–354.
34. **Sodroski, J., W. C. Goh, C. Rosen, K. Campbell, and W. A. H. Haseltine.** 1986. Role of the HTLV-III/LAV envelope in syncytium formation and cytopathology. Nature (London) **322:**470–474.
35. **Stevenson, M., C. Meier, A. M. Mann, N. Chapman, and A. Wasiak.** 1988. Envelope glycoprotein of HIV induces interference and cytolysis resistance in $CD4^+$ cells: mechanism for persistence in AIDS. Cell **53:**483–496.
36. **Streibel, K., T. Klimkait, and M. Martin.** 1988. A novel gene of HIV-1, vpu, and its 16-kilodalton product. Science **241:**1221–1223.
37. **Sussman, D. J., and G. Milman.** 1984. Short-term, high-efficiency expression of transfected DNA. Mol. Cell. Biol. **4:**1641–1643.
38. **Veillette, A., M. A. Bookman, E. M. Horak, L. E. Samelson, and J. B. Bolen.** 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase $p56^{lck}$. Nature (London) **338:**257–259.
39. **Vlasak, R., W. Luytjes, W. Spaan, and P. Palese.** 1988. Human and bovine coronaviruses recognize sialic acid-containing receptors similar to those of influenza C viruses. Proc. Natl. Acad. Sci. USA **85:**4526–4529.
40. **Weller, S. K., A. E. Joy, and H. M. Temin.** 1980. Correlation between cell killing and massive second-round superinfection by members of some subgroups of avian leukosis virus. J. Virol. **33:**494–506.
41. **Willey, R. L., F. Maldarelli, M. A. Martin, and K. Streibel.** 1992. Human immunodeficiency virus type 1 Vpu protein regulates the formation of gp160-CD4 complexes. J. Virol. **66:**226–234.
42. **Wilson, I. A.** 1986. The three dimensional structure of surface antigens from animal viruses, p. 19. *In* A. L. Notkins and M. B. A. Oldstone (ed.), Concepts in viral pathogenesis. Springer-Verlag, New York.
43. **Young, J. A. T., P. Bates, K. Willert, and H. E. Varmus.** 1990. Efficient incorporation of human CD4 protein into avian leukosis virus particles. Science **250:**1421–1423.
44. **Zack, J. A., S. J. Arrigo, S. R. Weitsman, A. S. Go, A. Haislip, and I. S. Y. Chen.** 1990. HIV-1 entry in quiescent primary lymphocytes: molecular analysis reveals a labile latent viral structure. Cell **61:**213–222.