Variants Selected by Treatment of Human Immunodeficiency Virus-Infected Cells with an Immunotoxin

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Summary

An immunotoxin has been made by coupling anti-human immunodeficiency virus (HIV) envelope antibody 907 to ricin A chain (907-RAC). 907 recognizes an epitope within the immunodominant PB-1 loop of gp120. Variant cells were selected by cloning persistently infected H9/human T lymphocyte virus IIIB cells in the presence of the immunotoxin. Clones resistant to 907-RAC arose at a frequency of 0.1–1.0%. Seven clones were selected for intensive analysis. When studied, these clones fell into two distinct groups, members of which appeared to be identical, suggesting that the variation arose before the selection process. In contrast to the parent cells, none of the cloned variants produced infectious HIV. The first set of clones, designated the "E" variants, expressed decreased levels of the HIV envelope on the cell surface. However, levels of intracellular HIV antigens and reverse transcriptase were equal to or greater than that of the parental cell line. Radioimmunoprecipitation demonstrated that the gp160 was truncated to 145 kD (gp120 was normal length), capable of binding to CD4, and, unlike normal gp160, was released in its unprocessed form into the cellular supernatant. Sequence analysis demonstrated that a deletion at codon 687 of the envelope gene resulted in the production of this truncated protein. Ultrastructural analysis of E variants demonstrated some budding forms of virus, but also large numbers of HIV within intracellular vesicles. The second set of variants, the "F" series, produced no HIV antigens, reverse transcriptase, nor was there ultrastructural evidence of virus. However, proviral DNA was present. Virus could not be induced with agents known to activate latent HIV. These cells also lacked cell surface CD4 and could not be infected with HIV. These studies demonstrate that variation in HIV can affect the phenotype of the cells carrying the altered virus, allowing for escape from immunologic destruction. The E variants may serve as prototypes for attenuated HIV, which could be used as a vaccine. We have reconstructed the mutation found in the E variants within the infectious HIV clone HXB-2 and demonstrated that the resulting virus retains its noninfectious phenotype.

We have previously coupled ricin A chain (RAC)[1] to mAb 907, which recognizes an epitope within the immunodominant PB-1 loop of the HIV envelope protein. This immunotoxin can effectively and specifically kill cells infected with HIV (1). Antigenic variation can allow targets to avoid immunologic destruction in both neoplastic and infectious processes (2, 3). To study this phenomenon in HIV-infected cells, we used 907-RAC to select for cells that escape the action of the immunotoxin. Because the epitope recognized by antibody 907 lies within a hypervariable region of the HIV envelope, we expected that variant cells surviving treatment with the immunotoxin would have alterations in the structure of the epitope. Rather, we found that variation elsewhere in the virus decreased or eliminated cell surface expression of the epitope recognized by 907. The study of these variants has revealed information regarding processing and function of the HIV envelope protein, as well as revealing mechanisms of HIV variation.

[1] *Abbreviations used in this paper:* HTLV, human T lymphocyte virus; IC, infectious center; RAC, ricin A chain; SIV, simian immunodeficiency virus.

Materials and Methods

Reagents, Viruses, and Cells. Antibody 907 and the immunotoxin 907-RAC have been described elsewhere (1, 4). 907 binds to an

epitope within the hypervariable PB-1 loop of gp120 and reacts only with the human T lymphocyte virus (HTLV)-IIIB/LAV isolate of HIV. The isotype-matched control antibody T7 is directed against the synthetic polypeptide antigen (Tyr,Glu)-Ala-Lys (5). OKT4a was purchased from Ortho Diagnostic Systems, Inc. (Westwood, MA). Polyclonal anti-HIV antibodies were present in the pooled serum from AIDS patients. Peroxidase-conjugated goat anti-human Ig and fluorescein-conjugated goat anti-mouse Ig were obtained from Organon Teknika (Malvern, PA). Soluble CD4 was obtained from Genentech (South San Francisco, CA) and was immobilized on cyanogen bromide–activated sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). The H9 cells chronically infected with the HIV isolate HTLV-IIIB used in these studies were from E.-M. Fenyo of the Karolinska Institute, Stockholm, Sweden. The cells had been maintained in continuous culture for >1 yr before the time of selection with the immunotoxin. The infectious HIV clone HXB-2 has been described elsewhere (6). Malcolm Martin (NIH, Bethesda, MD) provided the infectious clone NL4-3 as well as the clinical isolate NY-5 (7). HT4-6C cells are HeLa cells that have been transfected with the gene encoding CD4. These cells stably express CD4 on the cell surface (4). A3.01 cells, permissive for HIV infection, were obtained from Tom Folks (8; Center for Disease Control, Atlanta, GA). Cells were maintained in RPMI 1640 medium containing 10% FCS, penicillin, gentamicin, and 2-ME (5×10^{-4} M).

Selection of Immunotoxin-Resistant Cells. Bulk selections were performed by incubating 2×10^5 H9/HTLV-IIIB cells in 2 ml of medium containing 907-RAC at 2.5 or 10 μg/ml. The cells were cultured for 10 d, with fresh immunotoxin being added on days 4 and 8. Cloned variant cells were obtained by culturing 10–1,000 H9/HTLV-IIIB cells per 0.2 ml well in 96-well flat-bottomed tissue culture trays. Cells were cloned in the presence of 907-RAC at 2.5 μg/ml. Fresh immunotoxin was added every 4 d for the first 3 wk of culture.

Focal Immunoassay for the Detection of HIV-secreting Cells. A focal immunoassay was used to quantify the number of cells producing infectious HIV (1, 4). Cells to be tested were plated at different concentrations on a monolayer of adherent HT4-6C cells in 6-well, 35-mm tissue culture plates (Falcon 3046; Becton Dickinson & Co., Mountain View, CA). The test cells were washed off 24 h later, and the monolayer was incubated for an additional 72 h. The HT4-6C cells were then fixed in methanol and stained with human anti-HIV serum followed by peroxidase-conjugated goat anti-human Ig. The substrate, aminoethyl carbazole (Sigma Chemical Co., St. Louis, MO), produces a red precipitate in the presence of peroxidase. Viral foci were identified by the presence of staining and multinucleated cells. The data are presented as the percentage of input cells forming foci (infectious centers [ICs]).

^{35}S-Methionine Incorporation. Protein synthesis in the presence of the immunotoxin was measured as the incorporation of ^{35}S-methionine into cellular protein. Target cells (5×10^4) were plated, in triplicate, in flat-bottomed 96-well tissue culture plates in 100 μl of medium. An additional 100 μl of medium containing the immunotoxin or antibody 907 was added, and the cells were cultured for 72 h. For the final 16 h of the culture, ^{35}S-methionine was added to the cells (0.5 μCi/well in 20 μl). Cells were harvested onto glass filters using an automated cell harvester and washing with methanol. The filters were then placed in 2 ml of scintillation fluid and counted in a scintillation counter (LS8000; Beckman Instruments, Inc., Fullerton, CA). Results are shown as means and SEM of triplicate samples.

Immunologic Detection of HIV Antigens. Cell surface antigens were detected using flow cytometry. Living cells were stained with mAb 907, OKT4a, or T7, followed by fluorescein-conjugated goat anti-mouse Ig. Cells were then fixed in 2% paraformaldehyde and analyzed by one-color flow cytometry on a FACstar (Becton Dickinson & Co.). 5,000 cells were analyzed. Results are plotted with the cell number on the vertical axis and the log fluorescence intensity on the horizontal axis. Intracellular antigens were detected on cells that had first been fixed with methanol. The cells were then stained with human anti-HIV serum, peroxidase-conjugated goat anti-human Ig, and aminoethyl carbazole. The intracellular level of HIV p24 antigen was determined using the Retro-Tek p24 antigen capture ELISA (Cellular Products, Buffalo, NY). Cells were washed in PBS and then lysed with 1% Triton X-100. Serial dilutions of the cell lysate were incubated on plates coated with anti-p24 mAbs. The plates were washed and incubated with biotinylated human anti-HIV serum, followed by streptavidin–horse radish peroxidase, and then the peroxidase substrate tetramethylbenzidine. Antigen levels were determined by comparison with standards provided by the manufacturer.

Assay for Supernatant Reverse Transcriptase. Reverse transcriptase activity was measured in cell supernatants (9) by adding 10 μl of the supernatant to 20 μl of the following mixture: 50 mM Tris HCl, pH 7.9, 75 mM KCl, 2 mM dithiothreitol, 5 mM $MgCl_2$, 0.05% NP-40, 5 μg/ml poly(rA), 2.5 μg/ml oligo(dT), and 20 μCi/ml ^{32}P-dTTP. The supernatant and the mixture were incubated for 2 h at 37°C, spotted onto DEAE paper, washed exhaustively in 2× SSC, and then once in 95% ethanol. Autoradiograms were then performed.

Electron Microscopy. Cells were fixed for 1 h at room temperature in 2.5% glutaraldehyde, 2.0% paraformaldehyde, 0.05% trinitrocresol (Kodak, Rochester, NY), 0.1 M tricine, 0.1% phosphotungstic acid, 1.0% NaCl, 0.1 M Na cacodylate, pH 7.2. The cells were washed twice in 0.1 M Na cacodylate, pH 7.9, and then incubated for 1 h in 2.0% OsO_4 and 0.8% $K_3Fe(CN)_6$ in 0.1 M Na cacodylate. Cells were washed twice and then treated with 0.1% tannic acid for 5 min and washed twice in tap water. The cells were stained for 30 min with 1.0% aqueous uranyl acetate, pH 3.9, and then dehydrated by successive exposure to 30, 60, 90, and 100% acetone. The dehydrated cells were incubated twice in low viscosity Spurr's resin (Polysciences, Inc., Warrington, PA). The cells were then placed in Beem capsules (003; Polysciences, Inc.) in fresh Spurr's and polymerized for 16 h at 70°C. Sections were placed on 300-mesh copper grids and examined in an electron microscope (HU-11E-1; Hitachi, Tokyo, Japan) at 75 KV.

Radioimmunoprecipitation. Cells were incubated in methionine and cysteine-free medium containing 10% dialyzed FCS and ^{35}S-cysteine and methionine (1,200 Ci/mM) to a final concentration of 100 μCi/ml. After an incubation time of 12 h, medium was removed, and the supernatants were spun at 2,000 *g* for 10 min to remove cells and debris. The supernatants were precleared by incubation with normal rabbit serum and protein A–Sepharose beads. Precipitation with antibody was performed by incubating the supernatants with anti-HIV antisera and protein A–Sepharose (Pharmacia Fine Chemicals). Precipitation of CD4 binding proteins was performed with rCD4 immobilized on cyanogen bromide–activated sepharose. After 16 h of incubation, immunoprecipitates were collected by centrifugation, washed repeatedly, resuspended in SDS-PAGE sample buffer, heated for 3 min at 100°C, and analyzed by SDS-PAGE.

Genetic Analyses. Cellular DNA was prepared by incubating 10^7 cells in buffer containing 50 mM Tris, pH 8, 100 mM EDTA in the presence of 0.5% SDS and 500 μg/ml of proteinase K for 16 h at 56°C with agitation. The resulting mixture was then extracted with phenol, phenol/chloroform, and then chloroform. The

DNA was precipitated from the aqueous phase with ethanol. The DNA was spooled onto a hooked pasteur pipette and resuspended in Tris/EDTA buffer. DNA hybridization was performed using a modification of the original method of Southern (10). DNA was blotted onto nitrocellulose paper and then hybridized with either the cloned HIV HXB-2 (6), which had been labeled with ^{32}P using random primers (Pharmacia Fine Chemicals), or with the oligonucleotide 5'-dCCTATCTGTCCCCTCAGCTA (referred to as the internal oligonucleotide) that was 5' end-labeled with T4 kinase.

The DNA encoding the COOH-terminal portion of the envelope protein (beginning at codon 453) and a portion of the 3' open reading frame (a total of 1,344 bp) was cloned from variant 10E using the PCR. DNA was amplified using the oligonucleotides 5'-dGCTGGAATTCACAAGAGATGGTGGTAATAA and 5'-dTTGGTACCTGTGATTGCTCCATGT. The former oligonucleotide is at the 5' end of the amplified sequence and incorporates an EcoRI site, the latter oligonucleotide contains a KpnI site. DNA was amplified using Taq polymerase, and the reagents were supplied with the GeneAmp kit (Perkin Elmer Cetus, Norwalk, CT). DNA was subjected to 40 cycles of 1 min at 94°C, 1 min at 42°C, and 2 min at 72°C. The amplification of the correct target sequence was confirmed by Southern DNA hybridization with the internal oligonucleotide. Amplified DNA was run on a 0.7% low melt agarose gel, excised, and extracted with phenol. The DNA was further purified on an Elutip-D (Schleicher & Scheull, Inc., Keene, NH) before being ligated into pTZ-18R (Pharmacia Fine Chemicals), which had been cut with EcoRI and KpnI. Clones were screened by colony hybridization using the internal oligonucleotide. Plasmid preparations were made from the positive clones, and the presence of HIV sequences was confirmed by restriction mapping. Four independent clones (designated 10E-2, 10E-3, 10E-6, and 10E-7) were obtained containing the PCR amplified sequences from variant cell line 10E. Sequence analysis was performed on double-stranded DNA using Sequenase (United States Biochemical Corp., Cleveland, OH) and the following oligonucleotides: M13 −20 primer 5'-dGTAAAACGACGGCCAGT; M13 reverse 5'-dAACAGCTATGACCATG; and the HIV sequences 5'-dCTGTAGGAGATTCCACC, 5'-dTCACAGTCTGGGGCATCA, 5'-TGGTGAGTATCCCTGCCT, 5'-dGCCTTGGAATGCTAGTTG, 5'-dTCAGCTCGTCTCATTCTT, 5'-dTTCACTAATCGAATGGA, 5'-dCCAGGTCGTGTGATTCC, and 5'-dGCCAAGGATCCGTCAC.

The mutation found in the envelope gene was reconstructed in the infectious HIV clone HXB-2, which contains the HIV sequence introduced into the pSVgpt expression vector (6). The 2,700-bp SalI-BamHI fragment of HXB-2 was subcloned into pGEM-4 (Promega Biotec, Madison, WI) and designated HXB-SB. The PvuII-HindIII fragment from this subclone was ligated to the HindIII-BamHI fragment from the variant clone 10E-2 and cloned into pSP-72 (Promega Biotec). The PvuII-BamHI fragment was excised, ligated to the SalI-PvuII fragment derived from HXB-SB, and inserted into pTZ-18R. This in turn was excised with SalI and BamHI and ligated to the 14KB SalI-BamHI fragment of HXB-2. In this way, the final construct derives entirely from HXB-2, with the exception of a HindIII-BamHI fragment (bases 8140–8474) that encodes the portion of the envelope gene containing the mutation derived from the 10E variant. The presence of this mutation in the new constructions was confirmed by sequence analysis. Two different constructs, designated 7.4 and 7.5, were made. Plasmid DNA from this construction was transfected into COS-1 cells using calcium phosphate. DNA (10 μg) was suspended in 0.2 ml of HBSS containing 125 mM $CaCl_2$. After 30 min, a light precipitate formed and the mixture was added to 5×10^5 COS-1 cells in 35-mm tissue culture plates. 2–4 d later, cells were stained by immunoperoxidase with anti-HIV sera and were assayed for the production of infectious HIV by focal immunoassay.

Results

Treatment of Bulk Populations with 907-RAC. H9/HTLV-IIIB cells were incubated in the presence of 907-RAC at 2.5 μg/ml (low dose) or 10 μg/ml (high dose) for 10 d. There was an initial period of cell death, followed by the outgrowth of a subpopulation of cells. The surviving cells were washed and tested for their response to treatment with 907-RAC and for their ability to form infectious centers. The response of the cells to 907-RAC was measured by the uptake of ^{35}S-methionine into cellular proteins in the presence of the immunotoxin (Table 1). H9/HTLV-IIIB were exquisitely sensitive to the action of the immunotoxin, with >90% inhibition of protein synthesis. No inhibition was seen in the presence of antibody 907 alone. Variant cells treated with a high dose of the immunotoxin were able to resist the action of 907-RAC, while cells treated in the presence of a lower dose were still partially susceptible to the action of the immunotoxin (~50% inhibition). The ability of the treated cells to produce infectious HIV was measured using the focal immuno-

Table 1. *Selection of 907-RAC-Resistant Variants in Bulk Populations*

Cell	Media	907 alone (10 μg/ml)	907-RAC (10 μg/ml)	907-RAC (2.5 μg/ml)
H9/HTLV-IIIB	66,629 ± 1.758	70,543 ± 11,392	4,024 ± 82	5,043 ± 90
High-dose variants	42,420 ± 1,629	35,791 ± 1,122	38,572 ± 922	43,013 ± 2,361
Low-dose variants	52,788 ± 1,366	47,377 ± 516	23,207 ± 9,234	17,764 ± 1,411

Cells were cultured for 72 h in the presence of the indicated reagent. During the final 16 h, ^{35}S-Met was present. Results are the cpm of ^{35}S-Met incorporated into cellular protein. The mean ± SEM of triplicate samples is shown.

Table 2. *Susceptibility of Variant Clones to 907-RAC*

Cell	Media	907 alone (10 μg/ml)	907-RAC (10 μg/ml)	907-RAC (2.5 μg/ml)
H9/HTLV-IIIB	24,502 ± 1,336	25,721 ± 388	1,654 ± 115	2,561 ± 65
3E	44,679 ± 2,338	42,709 ± 1,426	29.949 ± 522	35,045 ± 853
11E	51,308 ± 3,011	48,906 ± 765	35,866 ± 227	44,378 ± 1,029
10E	53,656 ± 1,758	48,999 ± 414	24,629 ± 210	34,791 ± 1,647
4F	34,026 ± 1,055	34,072 ± 583	38,589 ± 536	38,039 ± 2,484

Cells were cultured for 72 h in the presence of the indicated reagent. During the final 16 h, ^{35}S-Met incorporated into cellular protein. The mean ± SEM of triplicate samples is shown.

assay. Surprisingly, the cells treated with the immunotoxin produced extremely low levels of infectious virus (0.5 ± 0.06% ICs) when compared with the parental H9/HTLV-IIB cells (64.7 ± 4.3% ICs). These data suggest that the process allowing for escape from immunotoxin action also renders the cells noninfectious. To explore the molecular mechanisms involved, 907-RAC-resistant clones were derived.

Cloning of H9/HTLV-IIIB Cells in the Presence of 907-RAC. Cells were cloned in 96-well tissue culture plates at a concentration of 10–1,000 cells per well. 907-RAC was added to 2.5 μg/ml, and fresh immunotoxin was added every 4 d. Colonies became visible at 12–16 d of culture. Colonies arose at a frequency of between 0.1 and 1.0%. Seven cloned lines were chosen for further analysis. Clonality was established by frequency analysis (<10% of wells positive). As the data given below will demonstrate, these clones fell into two groups. Members of each group appeared to be identical. These groups of immunotoxin-resistant variants have been designated the E series (3E, 7E, 8E, 10E, and 11E) and the F series (4F and 10F).

Sensitivity of Variant Clones to 907-RAC. Variant cells were tested for their sensitivity to immunotoxin-mediated suppression of protein synthesis. Uptake of ^{35}S-methionine into cellular protein was measured in cells incubated in the presence of media, immunotoxin, or antibody 907 alone (Table 2). H9/HTLV-IIIB cells were inhibited >90% by the immunotoxin at both doses tested. The three E series variants tested were each partially inhibited in a dose-dependent manner by 907-RAC. Variant 4F was not inhibited at all.

Expression of HIV Antigens on Variant Cells. The expression of HIV envelope antigens on the surface of living cells was studied by flow cytometry. Cells were stained with antibody 907 or an irrelevant isotype-matched monoclonal T7, followed by fluorescein-conjugated goat anti-mouse Ig. The results (Fig. 1) demonstrate that both of the E variants tested had markedly reduced levels of envelope antigen expression on their cell surface when compared with the parental cell line H9/HTLV-IIIB. Variant 4F had no detectable envelope antigen on its surface. Similar results were obtained with antibodies detecting other epitopes on the envelope protein (not shown). Viral antigens within the cell were studied by immunoperoxidase staining on methanol-fixed cells. HIV antigens were detected in fixed cells with pooled human anti-HIV antisera and peroxidase-conjugated goat anti-human Ig (Fig. 2). Visual inspection indicates that the E variants contained at least as much viral antigen internal to the cell membrane as the parental H9/HTLV-IIIB cells, possibly more. No viral antigens were seen in uninfected H9 cells and the F variants. These data suggest that the E variants produce HIV proteins, but that these proteins are poorly expressed on the cell surface when compared with the parental H9/HTLV-IIIB cells. There is no evidence that the F variants produce any viral proteins.

Production of Reverse Transcriptase by Variant Cells. Reverse transcriptase activity secreted into cell supernatants by the variant cells was measured (Fig. 3). The E variants produced as much (or more) reverse transcriptase as H9/HTLV-IIIB. The F variants produced no detectable reverse transcriptase activity. These data are consistent with the interpretation of

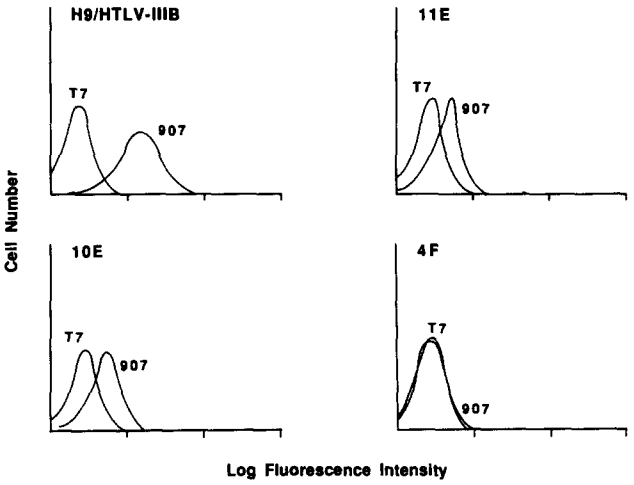

Figure 1. Flow cytometric analysis of gp120 on the surface of variant cells. The parental cell line H9/HTLV-IIIB and three immunotoxin-resistant variant cell lines were stained with anti-gp120 antibody 907 or control antibody T7 followed by fluorescein-conjugated goat anti-mouse Ig. Cells were fixed and analyzed by flow cytometry. When compared with the parental cells, both the E variants had greatly decreased levels of antibody binding. The F variant had no cell surface gp120 expression.

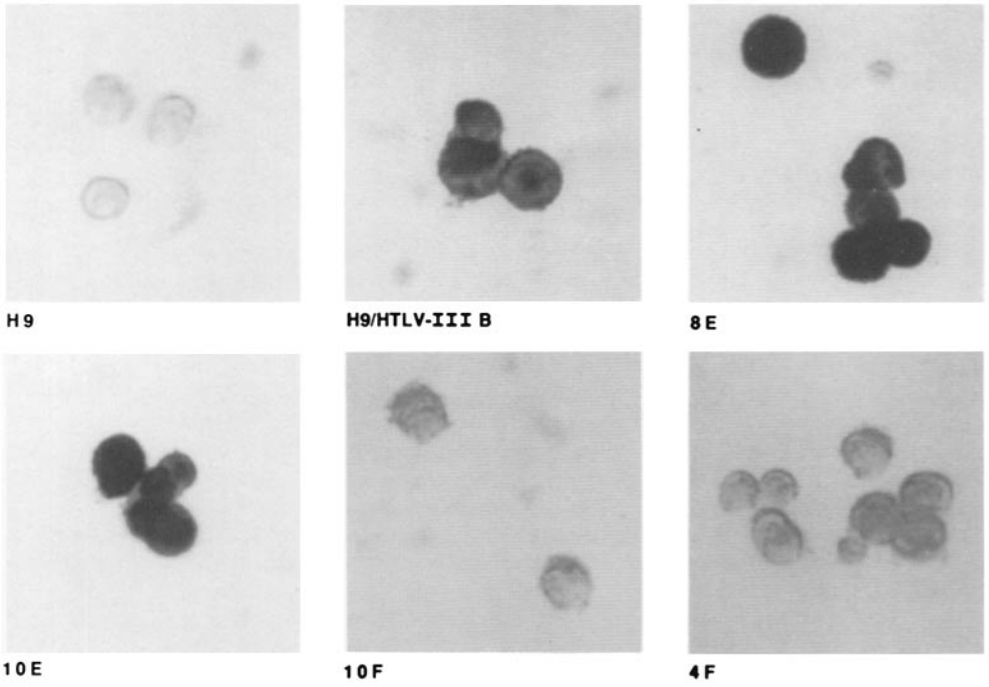

Figure 2. Immunoperoxidase staining of variant cells. Uninfected H9, H9/HTLV-IIIB, and variant cells were fixed in methanol and stained with pooled human anti-HIV antisera followed by horseradish peroxidase– conjugated goat anti–human Ig. Antibody was localized using the chromogenic substrate aminoethyl carbazole. The results demonstrate that viral antigens were detected to an equal degree in the H9/HTLV-IIIB and E variant cells. No HIV antigens were seen in H9 or the F variants.

Fig. 2 that the E variants produce normal levels of viral proteins, while the F variants fail to produce any.

p24 Antigen Levels in Variant Cells. The intracellular concentration of the HIV p24 core antigen was measured in Triton X-100–lysed H9, H9/HTLV-IIIB, 10E, and 4F cells. The p24 levels were measured in an antigen capture assay. Serial dilutions of lysed cells were compared with a standard curve. H9/HTLV-IIIB contained 0.188 ± 0.004 pg/cell of p24 antigen; 10E contained 0.153 ± 0.011 pg/cell. Both H9 and 4F cells contained $<10^{-5}$ pg/cell (that is, 10^5 cells produced a signal equal to background, with a detectable signal at 1.56 pg/well). The exact numbers for the antigen concentration per cell may not be strictly accurate, since this assay was designed and tested on supernatant fluids. However, the data clearly indicate that there are roughly equivalent antigen concentrations in H9/HTLV-IIIB and 10E cells, while there is no antigen detectable in the 4F variants.

SDS-PAGE Analysis of Secreted HIV Envelope Proteins. HIV envelope proteins were assayed in the supernatants of variant cells by radioimmunoprecipitation and SDS-PAGE analysis (Fig. 4). The left-hand lane contains the lysate of COS-1 cells infected with the infectious HIV clone HXB-2, and serves as a marker for the normal length of the envelope proteins gp120 and gp160. H9/HTLV-IIIB cells only secreted the mature gp120. The E variants secreted gp120, but they also released an altered form of the envelope of ~145 kD. This altered protein is most likely a truncated form of gp160 that has not been properly processed into the mature envelope protein gp120. This antiserum did not precipitate gp41 or gag proteins from cell supernatants. The F variants produced no detectable viral protein.

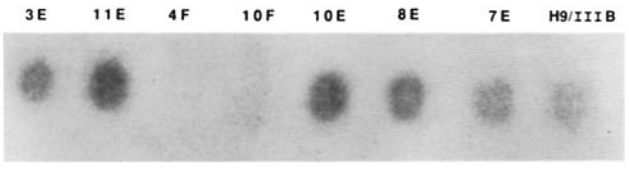

Figure 3. Supernatant reverse transcriptase activity of variant cells. Supernatants from parental and variant cells were examined for the presence of reverse transcriptase activity. The parental H9/HTLV-IIIB and E variant cells produced equivalent amounts of reverse transcriptase, none was produced by F variant cells.

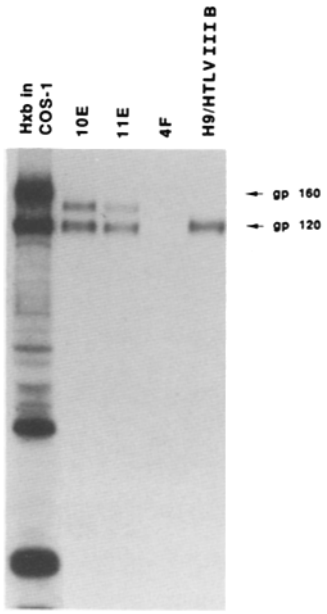

Figure 4. Release of HIV envelope proteins into cell supernatants. A radioimmunoprecipitation was performed using antienvelope antibody and protein A to precipitate envelope proteins secreted by the parental and variant cell lines. A lysate of COS-1 cells transfected with the infectious clone HXB-2 was included for comparison purposes. The parental cell secreted only gp120. The E variants produced gp120 and a 145-kD form of the envelope protein. Variant 4F produced no envelope proteins.

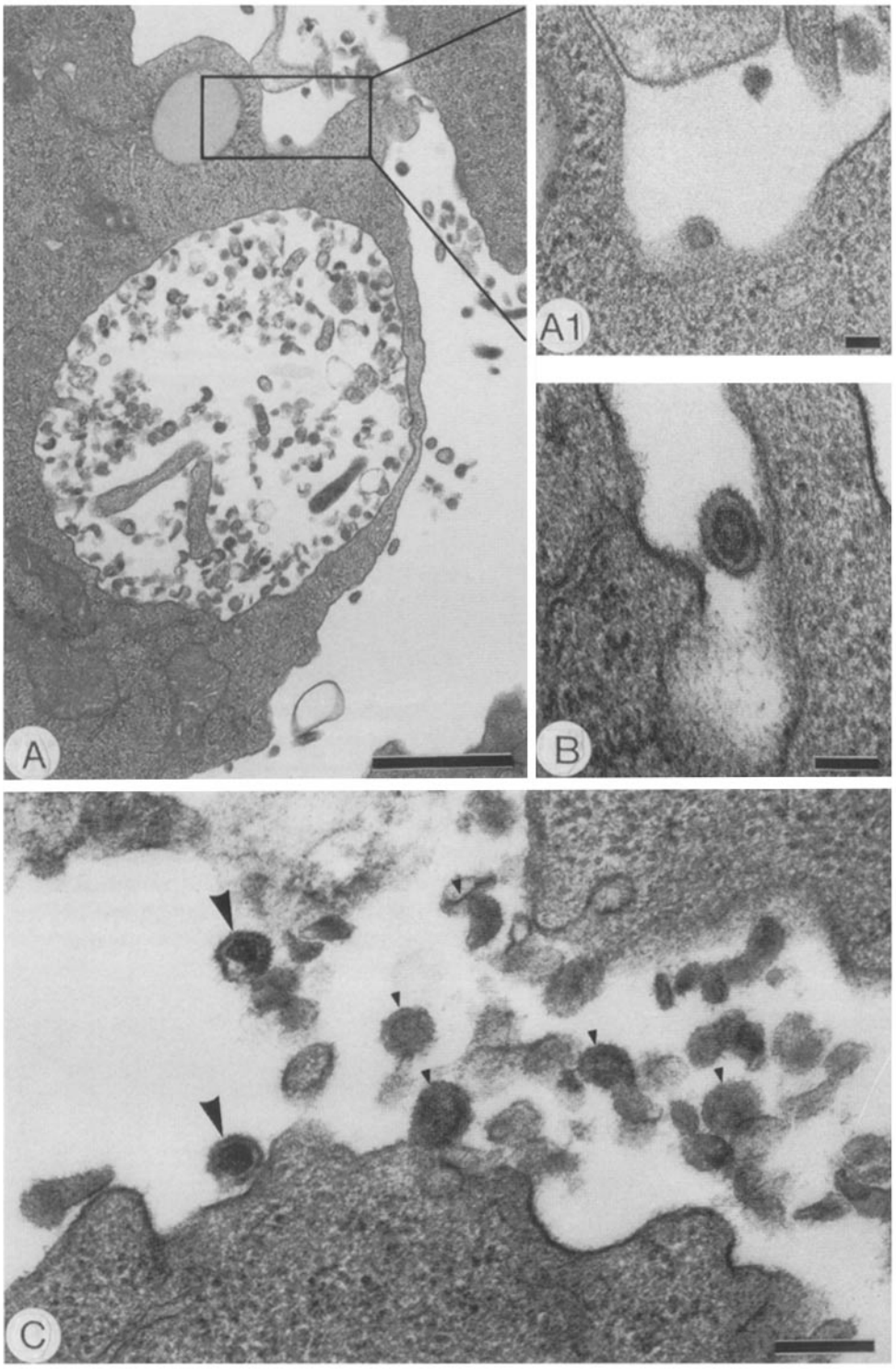

Figure 5. Ultrastructural analysis of the 10E variant cells. Thin section profiles of variant 10E were examined by electron microscopy. (*A*) A vacuole containing viral particles, not typically seen in T cells infected with HIV. A budding virus is seen in the insert of *A* and at higher magnification in *A1*. (*B*) A surface-budding particle exhibiting the early pre-condensing profile of HIV. (*C*) Extracellular viral particles. Large arrows denote typical morphotypes of HIV, while small arrows denote aberrantly formed particles. Bar markers: (*A*) 1,000 nm; (*A1*) 100 nm; (*B*) 100 nm; (*C*) 200 nm.

Ultramicroscopic Analyses of Variant Cells. Thin section profiles of H9/HTLV-IIIB and the variant cells were studied by electron microscopy. Fig. 5 shows the results seen with variant 10E. Although there were a few viral particles demonstrating the typical budding normally seen with lentiviruses (Fig. 5 *A1*), the majority of viral particles were contained within intracellular vesicles (Fig. 5 *A*). In contrast, H9/HTLV-IIIB cells had large numbers of viruses budding from the plasma membrane (not shown). This is consistent with and partially explains the data obtained by flow cytometry. Extracellular viral particles demonstrated typical morphology as well as aberrant forms in both the variant (Fig. 5, *A1*, *B*, and *C*) and HTLV-IIIB (not shown). No viral particles were seen in the F variants.

Table 3. *Ability of HT4-6C Cells to Infect CD4⁺ HeLa Cells*

Cell	Percent ICs
H9/HTLV-IIIB	68.5
3E	<0.01
11E	<0.01
10E	<0.01
8E	<0.01
7E	<0.01

The indicated cell lines were incubated with adherent HT4-6C cells for 24 h and washed off. 72 h later, the HT4-6C cells were fixed and stained with anti-HIV anti-serum and a peroxidase-conjugated secondary antibody. The results are presented as the percentage of input cells forming viral foci.

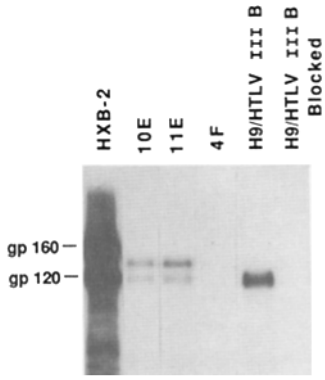

Figure 6. CD4 binding by proteins produced by parental and variant cells. Proteins secreted by cells were analyzed for CD4 binding activity by radioimmunoprecipitation with immobilized CD4. H9/HTLV-IIIB cells produced only a single protein, gp120, that bound to CD4. This binding could be blocked with soluble CD4. Both gp120 and the altered envelope protein (gp145) bound to CD4 in the E variant cells. Variant 4F produced no CD4 binding proteins. A lysate of COS-1 cells transfected with HXB-2 serves a standard for comparison of molecular sizes.

Inability of E Variants to Infect CD4⁺ Cell Lines. The E variants secrete viral particles containing an aberrant envelope protein. To determine if this alteration changes the functional capacity of the virus produced, we measured the ability of these viruses to infect CD4⁺ cell lines (Tables 3 and 4). Table 3 shows infection of HT4-6C cells by H9/HTLV-IIIB and the five E variants. The results, shown as the percentage of input cells forming viral foci, demonstrate the inability of the E variants to cause a productive infection. Infection of T cells was also tested (Table 4). H9/HTLV-IIIB or 10E cells were irradiated (1,200 rad) and mixed in the indicated ratio with the target cells. 5 d later, cells were fixed and stained with anti-HIV antiserum, and the percentage of cells expressing viral antigens was determined. H9/HTLV-IIIB was able to establish infection in both the target cell lines; 10E did not. These data demonstrate that the virus produced by the E variants is not infectious as assayed on these cell systems.

Table 4. *Ability of Variant Cells to Infect CD4⁺ T Cell Lines*

	Target cell	
Infecting cell	A3.01	H9
	%	
None	0	0
H9/HTLV-IIIB		
1:100	0	50
1:10	23	44 (many dead)
10E		
1:100	0	0
1:10	0	0

Irradiated infecting cells were mixed in the indicated ratio with uninfected target cells. Data show the percent target cells staining with anti-HIV antiserum 5 d after coculture with the infecting cells.

CD4 Binding by Variant HIVs. One possible explanation for the inability of the variant HIVs to infect CD4⁺ cells is that the altered envelope cannot bind to CD4. To test this, radioimmunoprecipitation was performed using CD4 immobilized on sepharose to precipitate the labeled proteins in cell supernatants (Fig. 6). The HXB-2 lysate serves as a size standard. H9/HTLV-IIIB cells exported one CD4-binding protein, gp120. This binding was blocked by the presence of soluble CD4. Both secreted forms of the envelope protein of the E variants (gp120 and truncated gp160) bound to CD4. The failure of the E variants to infect CD4⁺ cells is not due to the inability of the altered envelope to bind to CD4.

Analysis of Envelope Genes in E Variant Cell Lines. The production of a normal gp120 and a shortened gp160 led us to search in the portion of the envelope gene encoding gp41 for a mutation. Using the PCR, DNA from codon 453 of the envelope gene and extending 1,344 bp downstream (into the 3′ open reading frame) was amplified. Southern hybridization analysis with the internal oligonucleotide demonstrated that there was no major deletion within this portion of DNA (Fig. 7). The hybridizing fragment was of equal length in the H9/HTLV-IIIB and the 10E variant cells. Amplification of DNA from uninfected H9 cells produced no signal. Fine structural analysis was performed by cloning and sequencing the PCR-amplified fragment. Amplified DNA of the correct length was excised from an agarose gel, cut with KpnI and EcoRI (restriction sites were built into the amplification primers), and cloned into pTZ-18R. The DNA sequence of the amplified fragment showed the presence of a single base deletion after codon 686 of the envelope protein, resulting in three translational stop codons immediately after the deletion (Fig. 8). The predicted length of the protein derived from this mutation is the same as the observed length of the altered envelope protein (145 kD). The same mutation was found in four independently derived clones, ruling out the possibility that the observed deletion was an artifact of PCR amplification. In the 1,344 bp that were sequenced, an additional four single-base substitutions were found, resulting in two amino acid differences: gly to glu in codon 464, and val to leu in 673.

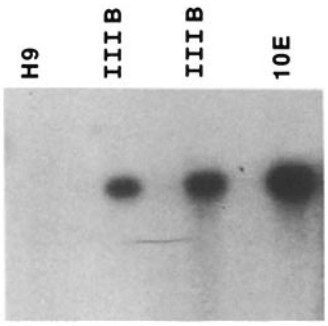

Figure 7. Southern blot analysis of amplified envelope gene. DNA from uninfected H9 cells, H9/HTLV-IIIB, and variant 10-E cells was amplified using the PCR with primers that flank the COOH-terminal portion of the envelope gene. The amplified DNA was sized on a 0.7% agarose gel, blotted onto nitrocellulose, and hybridized against the internal oligonucleotide. The data demonstrate that the shortened envelope protein was not due to a deletion within the proviral DNA.

Demonstration that the Mutation Detected in the E Variants Renders HIV Non-infectious. To test whether the single-base deletion detected in the envelope gene renders the virus noninfectious, the mutation was reconstructed in the infectious HIV clone HXB-2. Sequences derived from the envelope gene of variant 10E (corresponding to bp 8140–8474 of HXB-2) were inserted in place of the corresponding HXB-2 sequences. The variant HXB-2 viruses were transfected into COS-1 cells with calcium phosphate. The efficacy of transfection was measured by immunoperoxidase staining of the transfected cells. The infectivity of the HIV was measured by cocultivation of the transfected cells with the HIV-permissive cell line A3.01. The infection of A3.01 was assayed using the focal immunoassay (Table 5). Five simultaneous transfections were performed using plasmid DNA containing parental HXB-2, two different variant HXB-2s (designated 7.4 and 7.5), the gene transfer vector pSV-neo (containing no HIV sequences), and a mock transfection with no DNA. Immunoperoxidase staining of the transfected COS-1 cells was seen only with HXB-2, 7.4, and 7.5. Only rare cells (<1 in 10^4) contained HIV antigens 2–3 d after the transfection, with two to three times as many cells staining in HXB-2 transfected wells than in wells receiving 7.4 or 7.5. No infection was produced in A3.01 cells cocultured with 7.4- and 7.5-transfected COS-1 cells, while a substantial infection was established in A3.01s cocultured with HXB-2-transfected cells. We have repeated this experiment, this time transfecting by electroporation. In this second experiment, HXB-2, 7.4, and 7.5 produced equal numbers of transfectants staining for HIV antigens by immunoperoxidase. Once again, no infectious virus was made by 7.4 and 7.5 transfectants, while HXB-2 transfectants were able to infect A3.01 cells. Thus, the envelope mutation of variant 10E renders HXB-2 noninfectious.

F Variants Lack CD4 and Cannot be Infected with HIV. By all methods of detection, the F variant cells do not express any viral proteins and appear to be uninfected. Yet, they are derived from cells permissive for HIV infection and have existed within a milieu where they had ample opportunity to be infected. To demonstrate that these cells are indeed resistant to infection with HIV, the variant cell lines 4F and 10F were incubated with two strains of HIV, and then tested for evidence of infection using the focal immunoassay (Table 6). No sustained infection could be produced. The extremely low level of infectious centers seen with NL4-3 is most likely due to carry-over virus nonspecifically adhering to the 4F cells, since at subsequent time points, there were no ICs (data not shown). To explore the mechanism for the resistance of the F variants to infection, we examined the cell surface expression of CD4 on these cells (Fig. 9). Uninfected H9 cells expressed moderate levels of CD4. Consistent with previous observations (11-13), H9/HTLV-IIIB cells did not express

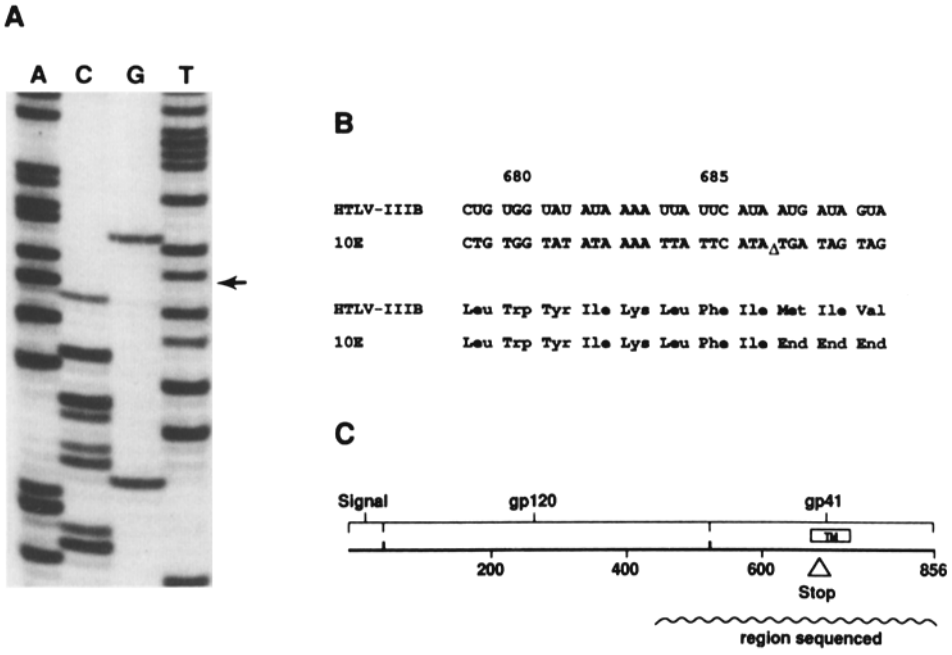

Figure 8. Sequence of mutation causing the truncation of the envelope gene in the E variants. *A* reproduces the sequencing gel performed on the antisense strand of DNA. The position of the deletion is marked. The sequences surrounding the mutation are shown in *B*. The RNA sequence of HTLV-IIIB and its translation are given at the top, and the DNA sequence of the mutant provirus and its translation are given below. The site of the deletion is marked. Identical sequences were detected in four distinct clones derived from the amplified 10E DNA. (*C*) A stick figure of gp160 showing the region sequenced, the site of the deletion, and the major structural characteristics: signal peptide, gp120, gp41, and the transmembrane portion of gp41. Numbers indicate amino acid residues.

Table 5. *Infection of A3.01 Cells by Transfectants*

A3.01 cells	DNA transfected				
	7.4	7.5	HXB	pSVneo	0
Day 2 fresh	0	0	30	0	0
Day 2 cultured	0	0	1,660	0	0
Day 3 fresh	0	0	12	0	0
Day 3 cultured	0	0	368	0	0

COS-1 cells were transfected with the indicated DNA. 2 or 3 d after transfection, the COS-1 cell monolayers were cocultured with A3.01 cells for 24 h. The A3.01 cells were removed and tested for HIV infection with the focal immunoassay either immediately or after 4 additional days in culture. The data are presented as the number of infectious centers per 10^5 input cells.

CD4. Variant 4F also failed to express CD4, thus accounting for their inability to become infected.

F Variant Cells Contain Proviral DNA that Cannot be Induced from Latency. Using the Southern DNA hybridization technique, we examined DNA of variant cell line 10F and the parental H9/HTLV-IIIB for the presence of proviral DNA (Fig. 10). HindIII-digested DNA was blotted against radiolabeled HXB-2. Equivalent amounts of DNA were loaded in each lane. Although there was a less intense signal in the 10F variants and there were differences in the relative intensity of the different bands, all of the restriction fragments present in H9/HTLV-IIIB were also present in 10F, although it is possible that the broad band at 7.5 kb in H9/HTLV-IIIB is a doublet. Thus, the F variants were infected with HTLV-

Table 6. *Infection of F Variants*

HIV strain	Cell	Percent ICs	
		4 d	7 d
None	H9	<0.01	<0.01
	4F	<0.01	<0.01
	10F	<0.01	0.01
NL4-3	H9	2.5 ± 0.52	26.05 ± 11.20
	4F	0.06	0.03
	10F	<0.01	<0.01
NY5	H9	0.81 ± 0.09	3.27 ± 1.10
	4F	<0.01	<0.01
	10F	<0.01	<0.01

Cells were incubated with cell supernatants containing the indicated strains of HIV and 8 μg/ml of polybrene. 4 and 7 d after infection, cells were treated for HIV infection using the focal immunoassay. The results are presented as the percentage of input cells forming viral foci.

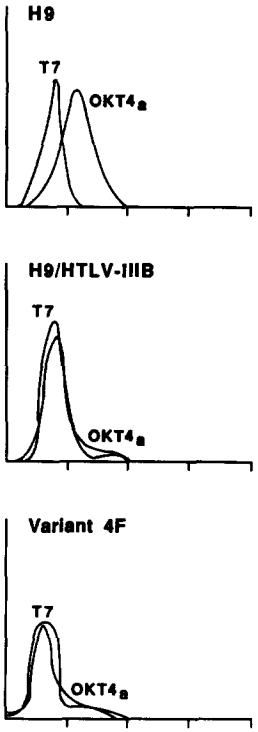

Figure 9. Cell surface expression of CD4 on variant 4F. Uninfected H9, H9/HTLV-IIIB, and variant 4F cells were stained with the anti-CD4 antibody OKT4a or the control antibody T7, and then with fluoresceinated goat anti-mouse Ig. Cells were analyzed by flow cytometry. No CD4 was detectable on H9/HTLV-IIIB or variant 4F.

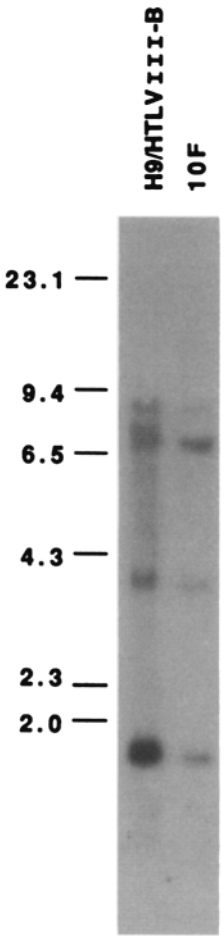

Figure 10. Presence of HIV genome in variant 10F. DNA from H9/HTLV-IIIB or 10F cells was digested with the restriction enzyme HindIII, sized on a 0.7% agarose gel, and blotted onto nitrocellulose. The DNA was hybridized to radiolabeled HXB-2. Although there are differences in the intensity of hybridization, the presence in 10F DNA of bands corresponding to each internal band in H9/HTLV-IIIB can be seen.

IIIB at some point in time. The virus has subsequently either become latent or suffered a debilitating mutation that prevents viral expression. To determine if the virus is in a state of latency, 4F and 10F cells were incubated with agents that are known to activate latent HIV (14, 15). Neither phorbol esters nor various mixtures of ILs and cytokines were able to induce HIV expression by F variant cells, as measured by the focal immunoassay.

Discussion

The resistance of microorganisms to antimicrobial therapy has emerged as a major problem in the treatment of infectious diseases. The phenomenon is well established in bacterial populations and has also begun to emerge as a serious issue with viruses; acyclovir resistant herpes viruses have been reported (16). Variation of HIV is well established, and most likely results from an error-prone reverse transcriptase (17, 18). This diversity results in antigenic variation (19), as well as the emergence of HIV with altered biological properties (20–22). Because of the extreme variability of HIV, variants may arise that might then be amplified under the selective pressure of antiviral therapy. Azidothymidine (AZT)-resistant HIV has been reported, and mutations in the reverse transcriptase gene are thought to be responsible for this resistance (23, 24). In the case of immunologically targeted anti-HIV agents, changes in antigenic structures would allow for escape variants. A single point mutation in the envelope gene can generate HIV resistant to neutralization by immune antisera (25). In this report, we have used an antibody-targeted immunotoxin to generate HIV variants that escape immunologic destruction. The antibody is directed against the hypervariable immunodominant PB-1 loop of the HIV envelope, gp120. Because of the variability of the target structure, we expected that escape variants would have alterations in the epitope recognized by the antibody. Instead, we found that alterations elsewhere in the virus affect the levels of envelope protein expressed on the surface of the HIV-infected cells, and were responsible for immunotoxin resistance.

We have identified two sets of variant cells arising from the immunotoxin-treated HIV-infected cell line. Because the cells within each set appear to be identical, and the first exposure of the cells to the immunotoxin was when they were cloned in its presence, it is likely that the variation preceded the selection process. The F variants escape the action of the immunotoxin because the virus is not expressed in these cells. Without viral envelope expression on the surface of the F variants, the immunotoxin does not bind, and the cells survive. The mechanism of HIV latency in the F variants has not been established. The E variants contain a single base deletion after codon 686 of the envelope gene that results in premature termination of translation and the production of a truncated gp160. This altered envelope protein is not completely processed, which in turn results in abnormal viral secretion and a marked decrease in the expression of envelope on the surface of the infected cell. The mutation in the envelope gene also renders the virus noninfectious, which is consistent with previously reported studies using site-directed mutagenesis (26).

Our results with the E series variants are reminiscent of findings seen with Friend murine leukemia virus. Kabat et al. (27) used antibody and complement to select for Friend virus mutants that express their envelope genes aberrantly and that accumulate unusual intracellular forms of the envelope glycoproteins. Pinter et al. (28) used inhibitors of oligosaccharide processing to study the processing of the Friend envelope protein. Tunicamycin completely inhibited glycosylation of the envelope protein, which led to a failure of proteolytic cleavage. Deoxynojirimycin, an inhibitor of cellular glycosidases involved in the final processing of the carbohydrate moiety, resulted in decreased levels of cell surface envelope protein, and an accumulation of virus within intracellular vesicles. Electron micrographs of deoxynojirimycin-treated cells show the same findings as Fig. 5 in this report (28).

The emergence of immunotoxin-resistant variants has been studied in tumor cells (2). B cell tumors were treated with an antiidiotypic antibody coupled to saporin. Immunotoxin-resistant variants fell into two classes. In the first, cell surface Ig was absent, while in the second, mutations in the V region rendered the Ig nonreactive with the antiidiotypic antibody. The variants that we have detected in this study most closely resemble the Ig-negative B cell tumor variants, although in the case of our E variants, the molecule bearing the target structure is present, but in decreased quantity. Resistance of the E variants to immunotoxin action is relative; the cells are partially susceptible to high doses of immunotoxin. This is an excellent example of correlation between cell surface antigen density and the vulnerability of the cell to an immunotoxin binding the antigen. This finding has implications for the treatment of neoplastic disease with immunotoxins.

The failure of the F variants to express CD4 is surprising. The disappearance of cell surface CD4 in HIV-infected cells is well documented. However, this phenomenon is thought to be due to intracellular binding of CD4 by the viral envelope (11–13). In the case of the F variants, we were unable to find evidence that any viral proteins were produced, suggesting that a different mechanism is responsible for their failure to express CD4. Although these cells have been infected with HIV, we have no data to suggest that this infection is responsible for the failure of the F variants to express CD4. An equally likely possibility is that there has been a mutation in the CD4 gene that prevents it from being expressed. Since we have shown that these $CD4^-$ cells cannot be reinfected with HIV, treatment of the tissue culture cell line with immunotoxin would select for these $CD4^-$, HIV^- variants. Whatever the reason for the failure of the F variants to express CD4, this failure has resulted in the inability of the F variants to become infected by HIV a second time. The configuration of the proviral DNA in the genome of the F variant cells confirms that only a single infection has occurred.

Naturally arising mutations that result in a truncated gp160 have not been previously reported for HIV. However, such

mutations have been seen in the simian immunodeficiency virus (SIV) (29). The most commonly seen SIV mutation results in a truncation of approximately the same size that we have reported. The SIV truncation results from base substitutions rather than the single base deletion that we have seen. The truncated SIV are infectious and appear to result from the passage of SIV through human cells. When SIV with a full-length envelope is passaged in human cells, truncated variants arise; when truncated SIV is passaged through macaques or macaque cells, full-length variants arise. The relationship between these mutations in SIV and the mutation that we have observed is unclear.

The development of vaccines to prevent HIV infection has been problematic (30, 31). According to conventional wisdom, "whole virus vaccines do not represent a practical avenue for a vaccine against AIDS because of the possibility of infectious particles" (31). Thus, reductionist approaches have been taken, including the use of recombinant envelope protein (32), synthetic polypeptides (33, 34), and the introduction of various HIV epitopes into genetically engineered vaccine vectors (35, 36). Although most of these maneuvers can elicit neutralizing antibodies, protection against viral challenge has not been demonstrated. In fact, immunization with recombinant gp120, a more complete immunogen than some of the other proposed vaccines, failed to protect chimpanzees from HIV challenge in the presence of a strong anti-HIV neutralizing antibody and T cell response (32). In contrast, studies utilizing inactivated whole virus preparations have demonstrated protective efficacy in SIV infection of macaques (37, 38). Perhaps it may be worth reconsidering the use of whole virus HIV vaccines. The E variants described in this study could serve as models for viruses that are first rendered noninfectious by genetic modifications and then by chemical or mechanical disruption. The studies described here demonstrate that such genetically altered viruses have morphologic and antigenic characteristics not too different than the native HIV. During several months of passage of the E variant cells, we have not had any reversion to infectious HIV, although a rigorous selection for revertants was not applied. Safety could be increased by engineering additional mutations. We are currently evaluating the immunogenicity of the virus produced by the E variant cells.

The studies presented in this communication address the general issue of evasion of immunologic surveillance as a mechanism of viral persistence. The role of antibody in this phenomenon is well studied in a number of different viral systems (39). The best known example is the antigenic shift and drift that allows the influenza virus to persist in populations. An example of escape from immune surveillance in an individual animal occurs with the lentivirus that causes equine infectious anemia. Recurrent episodes of clinical illness correlate with the emergence of viral variants resistant to neutralization with serum antibodies. Analysis of these variants shows alteration of the envelope glycoproteins (40). In addition to selecting for variant viruses with altered antigens, antibodies may induce changes in the cells that harbor viruses, allowing the virus to persist in these cells. Noncytotoxic antibodies have been shown to cap and remove measles virus antigens from the surface of infected cells, allowing these cells to escape destruction by other immunologic effector mechanisms (41). Similarly, antibodies lacking neutralizing and cytotoxic activity may serve as blocking antibodies, preventing recognition and removal of the virus by the immune system (39). The role of antibody in the progression of HIV infection from acute infection through latency to AIDS is the focus of intense investigation. Evidence exists showing that some specific antibody response is beneficial in inhibiting the disease process (42), and that loss of anti-p24 and anti-gp120 antibodies is associated with progression to outright AIDS (43). Administration of anti-HIV antibodies to AIDS patients appears to have a beneficial effect (44). On the other hand, there is concern that serum antibody may enhance the ability of HIV to infect cells via an Fc receptor–dependent mechanism (45). Understanding the relationship between protective antibody responses, antibodies that enhance infection, and the effects of antibody on HIV persistence and antigenic variation will allow us to design more effective vaccines and antibody therapies for the prevention and treatment of AIDS.

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