JOURNAL OF VIROLOGY, Apr. 1992, p. 1875–1883
0022-538X/92/041875-09$02.00/0

Vol. 66, No. 4

Mutations in the Principal Neutralization Determinant of Human Immunodeficiency Virus Type 1 Affect Syncytium Formation, Virus Infectivity, Growth Kinetics, and Neutralization

RAYMOND J. GRIMAILA,[1] BARBARA A. FULLER,[1] PAUL D. RENNERT,[1] M. BUD NELSON,[2] MARIE-LOUISE HAMMARSKJÖLD,[2] BARBARA POTTS,[1] MICHAEL MURRAY,[1] SCOTT D. PUTNEY,[1] AND GARY GRAY[1]*

Repligen Corporation, One Kendall Square, Cambridge, Massachusetts 02139,[1] and Departments of Microbiology and Oral Biology, State University of New York at Buffalo, Buffalo, New York 14214[2]

Received 17 June 1991/Accepted 31 December 1991

The principal neutralization determinant (PND) of human immunodeficiency virus type 1 envelope glycoprotein gp120 contains a conserved GPG sequence. The effects of a 29-amino-acid deletion of most of the PND, a 3-amino-acid deletion in the GPG sequence, and 16 single-amino-acid substitutions in the GPG sequence were determined in a transient expression assay. All mutant envelope glycoproteins were expressed at levels comparable to that of the wild-type envelope, and mutations in the GPG sequence did not affect processing to gp120 or, except for the 29-amino-acid deletion, binding to CD4. Of all of the mutants, only the GHG and GFG mutants induced formation of syncytia similar in size and number to those induced by the wild-type envelope. When the envelope expression level was increased 10-fold or more, several additional mutants (APG, GAG, GSG, GQG, GVG, and GPF) also induced syncytium formation. Transfection with infectious proviral molecular clones containing the GHG, GFG, APG, GAG, GSG, or GPF mutations induced production of viral particles; however, only the GPG, GHG, and GFG viruses produced active infections in CD4-bearing cells. Furthermore, whereas the wild-type virus was efficiently neutralized by PND polyclonal and monoclonal antibodies, the GHG- and GFG-containing viruses were not. These results show that mutations in the GPG sequence found within the PND do not affect envelope expression and do not significantly affect CD4 binding or production of viral particles but that they do affect the ability of the envelope to induce syncytia and those of the viral particles to infect CD4 cells and be neutralized by PND antibodies.

The predominant route of HIV-1 infection involves binding of external viral envelope glycoprotein gp120 to the surface CD4 molecule on human T lymphocytes. A post-gp120-CD4-binding event facilitates fusion of the viral envelope with the cellular membrane, thereby permitting viral entry. Viral infectivity and fusion of human immunodeficiency virus type 1 (HIV-1)-infected cells with uninfected CD4 cells can be blocked by antibodies directed to the principal neutralization determinant (PND) located on the viral envelope glycoprotein (11, 32, 33, 37).

Several studies have shown that the PND of HIV-1 resides in a 36-amino-acid disulfide loop formed between two invariant cysteines (positions 303 to 338) located in the third variable region of gp120. A comparison of 245 different HIV-1 envelope sequences (19) showed that the PND contains conserved sequences (e.g., over 90% of these PND sequences contain the GPG motif) and that the PND contains the structural motif β-strand–type II β-turn–β-strand–α helix. This suggests that there is selective pressure to maintain these conserved elements. Antibodies elicited by conserved amino acid sequences neutralize divergent HIV-1 isolates (14).

Previous studies (10, 17), using transient envelope expression systems, showed that mutations within the PND can reduce or abolish syncytium formation without affecting gp160 expression, processing, or CD4 binding. This suggests that, even though the PND is distinct from envelope domains directly involved in CD4 binding, elements of the PND are required for the membrane fusion event. These studies did not, however, investigate the effects of PND mutations on virus infectivity, growth kinetics, or neutralization by PND antibodies. Another study (13) determined the effect of the GPG-to-GAG substitution on virus infectivity and neutralization. This single change altered the ability of the virus to replicate in some cell lines and affected the ability of the virus to be neutralized by some PND-specific antibodies.

We made a series of deletions and substitutions in the conserved GPG sequence at the tip of the PND to study the importance of the PND in syncytium formation and virus infectivity, growth, and neutralization. Our data show that even though all mutant envelopes were expressed and processed as the wild-type GPG, all of the mutations studied except two abolished the ability of the envelope to induce syncytia. Each of these two viruses was resistant to neutralization by PND antibodies. These results show that certain mutations in the PND are infectious and confer the property of neutralization resistance.

MATERIALS AND METHODS

Cells, plasmids, and antibodies. Cell lines SW480 (ATCC CCL228) and COS-7 (ATCC CRL1651) were cultured in Dulbecco modified Eagle minimal essential medium (GIBCO Laboratories) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Logan, Utah). $CD4^+$ cell lines CEM and CEM-SS (27) were propagated and maintained in RPMI 1640 medium (GIBCO Laboratories) supplemented with 10% heat-inactivated FBS. *Escherichia coli* JM105, HB101, BW313 (18), and TG1 were used for

* Corresponding author.

subcloning, mutagenesis, and amplification of plasmid DNAs and bacteriophage M13. Infectious provirus pNL4-3, an HIV-1 hybrid containing NY-5 and LAV-1 viral DNAs, has been previously described (1). Transient expression plasmid pLOTSA is derived from previously described plasmid pSVSX1 (36). Its construction will be described in detail elsewhere (31a). pLOTSA contains 2.1 kB of the HIV-1 IIIB envelope gene and expresses a 140-kDa envelope glycoprotein. Expression is driven by the simian virus 40 late promoter. *rev*-expressing plasmid pCMVrev has been previously described as pRev1 (21). It contains a cDNA copy of HIV-1 *rev*, and expression is driven by the simian cytomegalovirus immediate-early promoter.

The neutralizing antisera used were goat anti-RP135 (37) and guinea pig anti-RP174c. (RP174 is RP135 with disulfide cross-bridged cysteines on both the amino and carboxyl termini.) Antiserum G1115 was prepared in goats immunized with baculovirus-insect cell-expressed gp160 which contained a deletion removing the entire PND (gp160 delta loop). Monoclonal antibody (MAb) Cβ1 is a human-murine chimeric antibody constructed from 0.5β (23) that binds the PND, including the sequence GPGRAF (a gift from S. Matsushita). MAb 1C1 (Repligen Corporation) recognizes a conserved epitope in the carboxy terminus of gp120. OKT4 was obtained from Ortho Diagnostics.

Oligonucleotide synthesis and purification. Oligonucleotides were synthesized by using an Applied Biosystems 380A DNA synthesizer and purified by using 10% polyacrylamide gels followed by Sep-Pak C18 column chromatography (Waters, Millipore Corp.). Three oligonucleotide mutagenesis primers were synthesized such that each primer contained one degenerate codon in place of that which encodes amino acid G, P, or G. The oligonucleotide primer used to mutagenize the first glycine (XPG) was 5′-GTATCCGTATCCA GAGAXXSCCCGGGAGAGCATTTG-3′, that used to mutagenize the central proline (GXG) was 5′-CCGTATCCAGA GAGGAXXSGGGAGAGCATTTGTTAC-3′, and that used to mutagenize the second glycine (GPX) was 5′-CCGTATCCA GAGAGGACCCXXSAGAGCATTTGTTACAATAGG-3′. The degenerate codon in each oligonucleotide contains all four nucleotides at the first two codon positions (X) and a roughly equal mixture of G and C at the third position (S). This codon, XXS, can contain 32 different nucleotide combinations, which encode all 20 amino acids and one termination codon. Deleted PND mutants were prepared by using the mutagenic oligonucleotides ΔGPG, 5′-CTAATGTTACAATGTGCGGGTCTT GTACAATTAATTTC-3′, which removed the central GPG, and ΔPND, 5′-GTAACAAATGCTCTTCTCTGGATACG G-3′, which deleted most of the 36 amino acids, to leave a 7-amino-acid PND with the sequence CTRPAHC. Two oligonucleotides, VSP-PCR, 5′-GTAGAAATTAATTGTAC AAGACCC-3′, and XBA-PCR, 5′-CCATTTTGCTCTAGA AATGTTACAATGTGC-3′, were used to sequence PND mutant envelopes from single-stranded M13 DNA and for polymerase chain reaction amplification and sequencing of the entire PND from virus-infected cells.

PND mutagenesis and envelope subcloning. PND mutations were introduced by mutagenesis without phenotypic selection by using the mutagenic oligonucleotides (18). The 2.1-kb *Kpn*I-to-*Bam*HI fragment from pLOTSA, which encodes all but the carboxy-terminal 104 amino acids of gp160, was cloned into the *Kpn*I and *Bam*HI sites of M13 vectors mp19 and mp18. Template single-stranded DNA containing uracil was obtained by growing these bacteriophage in dUTPase- and deoxyuracil-*N*-glycosidase-negative *E. coli* BW313 in the presence of uridine. The mutagenesis reaction products were transformed into *E. coli* to select for bacteriophage progeny derived from the mutagenized strand. Bacteriophage plaques from the mutagenesis reaction were chosen, and the envelope mutations were identified by dideoxy-chain termination sequence analysis. GPG mutant envelopes were subcloned as *Kpn*I-*Bam*HI fragments from the double-stranded replicative form of the appropriate bacteriophage into the corresponding sites of pLOTSA. Following analysis of the mutants by transient expression, selected *Kpn*I-*Bam*HI fragments were cloned into pNL4-3 to generate the proviral plasmids.

Transient expression assay. Rapidly dividing COS-7 cells were trypsinized, washed with RPMI 1640 medium supplemented with 10% FBS, and collected by centrifugation at 1,000 × *g* for 5 min. Transfection conditions consisted of 3×10^6 to 5×10^6 COS-7 cells in 250 μl of ice-cold RPMI supplemented with 10% FBS to which 125 ng of pCMVREV and 5 μg of envelope plasmid DNA were added. The cell-DNA mixture was transferred to a cuvette, and electroporation was performed by using a Bio-Rad Gene Pulsar set at 0.25 kV, 960 μF, and 400 Ω. Following electroporation, the cuvette was incubated on ice for an additional 10 min and the transfected cells were plated onto a 10-cm-diameter plate containing 10 ml of Dulbecco modified Eagle minimal essential medium supplemented with 10% FBS. After overnight incubation, the cells were trypsinized, washed, and counted. Viable cells (10^4) were mixed with a 10-fold excess of $CD4^+$ CEM cells and placed in a well of a 96-well microtiter tissue culture plate. Syncytia were visualized in the positive control wells as early as 4 h after the cells were mixed, although syncytia were not counted until 24 h after mixing.

CD4-binding assay. The ability of the envelope to bind CD4 was assayed by radioimmunoprecipitation of labeled cell lysates with soluble CD4. COS-7 cells were transfected as described above, except that incubation proceeded for 40 h after transfection and before labeling. Each 10-cm-diameter dish of cells was washed twice with phosphate-buffered saline (PBS) (GIBCO-BRL), starved for methionine by incubation in methionine-free medium for 30 min, and metabolically labeled overnight with 200 μCi of high-specific-activity [^{35}S]methionine (New England Nuclear). After being labeled, the cells were washed twice with PBS, scraped from the plates, transferred to Eppendorf tubes, and washed again with PBS. The cells were lysed by addition of 100 μl of RIPA-Lite (150 mM NaCl, 10 mM sodium phosphate, pH 7.2) containing 1% Nonidet P-40 and vigorous vortexing. After dilution with 9 volumes of RIPA-Lite, insoluble material was precipitated by centrifugation for 5 min at 12,000 × *g*. Labeled cell lysates were incubated with 1 μg of soluble CD4 (American Bio-Technologies, Inc., Cambridge, Mass.) per ml at 22°C for 1 h and then incubated with 1 μg of OKT4 per ml at room temperature for 1 h. Immobilized, recombinant protein A-Sepharose (IPA-300; Repligen Corporation) was pretreated by being washed several times with RIPA-Lite–0.1% Nonidet P-40 and then incubated with an unlabeled COS-7 cell lysate in PBS. One-tenth of a volume of a 1:1 slurry of pretreated IPA-300 in RIPA-Lite was added to the cell lysate containing sCD4 and OKT4 and incubated at 22°C for 30 min. The IPA-300 immunocomplexes were precipitated by brief centrifugation at 12,000 × *g*, washed three times with RIPA-Lite containing 0.1% Nonidet P-40, washed three times with RIPA-Lite, and boiled with an equal volume of gel loading buffer. Proteins were separated on sodium dodecyl sulfate–9% polyacrylamide gels and transferred by electroblotting to either nitrocellulose or Immobilon P (Millipore Corp.) membranes for

autoradiography. Cell lysates were also radioimmunoprecipitated as described above with 1 μg of MAb 1C1 per ml.

Cell staining. COS cells transfected with 5 μg of pCMVREV and 5 μg of each envelope expression plasmid were cultured as for the transient expression assay, and the cells were harvested by using EDTA (GIBCO), washed with PBS, and reacted with goat anti-gp160 delta loop (G1115). The cells were stained with fluorescein isothiocyanate-conjugated anti-goat serum and analyzed in a Becton Dickinson FACScan flow cytometer.

Viral infectivity and growth kinetics. Proviral plasmid DNA was transfected into SW480 cells by calcium phosphate precipitation as described previously (1). The infectivity of progeny virions was assessed by infecting CEM-SS $CD4^+$ T cells with cell-free supernatant harvested 48 h after transfection of the SW480 cells. The infections were performed in a 24-well plate by using 0.1 ml of cell-free transfection supernatant and 10^6 CEM-SS cells in 1.9 ml of RPMI supplemented with 10% FBS. Viral growth was determined by reverse transcriptase (RT) activity by using [^{32}P]dTTP (47) measured at 2- to 3-day intervals. Cell viability was monitored by using trypan blue exclusion, and the cell concentration was maintained at 10^6/ml. Infections were terminated when extensive cell death was observed. Cells were taken at day 20 postinfection for polymerase chain reaction amplification and sequencing of the PND region of the mutant HIV-1 envelopes.

Western immunoblot analysis was performed on SW480 cells transfected with proviral clones as described previously, except that the proteins were transferred to Immobilon P membranes. Western blots were probed with either human sera from HIV-1-positive individuals or MAb 1C1.

Virus neutralization assay. The abilities of PND antisera to neutralize the wild-type and mutant viruses were assessed by measuring the ability of a fixed amount of antibody to neutralize virus at 10-fold dilutions. For this assay, MAbs were used at 200 μg/ml and polyclonal antiserum was used at a 1:10 dilution. Viral stocks, stored frozen until use, were serially diluted 10-fold by using RPMI 1640 medium containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES). Starting with the highest viral dilution, 0.1 ml of each virus dilution and the undiluted viral stock were transferred to a labeled 96-well plate. A 0.1-ml volume of serum was delivered to all dilutions of the virus. In addition to the specific antiserum used, controls included virus with nonimmune antiserum and without antiserum. The control lacking antiserum allowed determination of 50% tissue culture infective doses ($TCID_{50}$) (40). The 96-well plate was then incubated at 37°C in 5% CO_2 for 30 min, after which each virus-antiserum mixture was transferred to a 24-well plate containing 10^6 CEM-SS cells and mixed. The volume of the wells in the 24-well plate was then increased to 2 ml by addition of 0.9 ml of RPMI supplemented with 10% FBS, and the plates were incubated at 37°C in 5% CO_2. At 3 days, each well containing cells and medium was mixed, 1 ml was transferred to a new well (to obtain a duplicate of the dilution), and 1 ml of complete medium was added to each well. The wells were split 1:2 by removing 1 ml and adding 1 ml of fresh medium every 3 days thereafter. Medium was harvested for RT assays, and the cytopathic effect was recorded every 3 days.

RESULTS

Mutagenesis and selection of PND mutations. Site-directed mutagenesis without phenotypic selection was performed on the PND of the HIV-1 IIIB (BH10) (34) envelope glycoprotein to study the effects of changes in these conserved amino acids on syncytium formation, CD4 binding, and viral infectivity and neutralization. Briefly, uracil-containing single-stranded template DNAs containing the 2.1-kb *Kpn*I-*Bam*HI envelope fragment from the envelope were mutagenized with oligonucleotides which generate either deletions or single-amino-acid substitutions in the GPG sequence. DNA sequencing of 54 separately isolated bacteriophage clones derived from GPG mutagenesis reactions yielded 49 substitution mutants and 5 nonmutagenized clones for a mutagenesis efficiency of ~91%. Of the 49 mutants obtained, 21 contained unique amino acid substitutions in the GPG sequence. Of the 21 GPG substitutions, 16 were chosen for further study. In addition, two deletion mutations, ΔGPG and ΔPND, were constructed. The 2.1-kb *Kpn*I-*Bam*HI fragments of these 18 mutant envelope genes were cloned into the corresponding sites of transient expression vector pLOTSA.

Syncytium formation of PND mutations. The effects of the mutations on syncytium formation were assayed by mixing envelope-expressing COS-7 cells with $CD4^+$ human T-cell clone CEM. The COS-7 cells were cotransfected with 125 ng of pCMVREV, to provide a source of rev protein required for efficient envelope expression, and 5 μg of the envelope plasmid. COS-7 cells expressing the wild-type envelope and the substitution mutant GHG and GFG envelopes each formed equivalent numbers and sizes of syncytia. All other mutants, including the two deletion mutants (ΔGPG and ΔPND), failed to form syncytia under these transfection conditions (Fig. 1 and Table 1). Although no clearly defined syncytia were generated with the mutants, cells expressing most of the variant envelopes appeared to induce cell association, as visualized by cell clumps (Fig. 1C).

Western blots of transfected COS-7 cells probed with MAb 1C1 (which recognizes a conserved sequence at the carboxyl terminus of gp120) showed that all mutant envelopes were produced at levels equivalent to that of the wild type (GPG) and were processed to the 140- and 120-kDa forms expected in the pLOTSA-based transient expression system (Fig. 2). Since the envelope clones tested contained examples of substitutions at each of the GPG positions, we conclude that any changes made to these positions affect neither envelope expression nor processing and that the mutant envelopes behave like the GPG envelope. Probing of similar Western blots with a MAb specific for the amino-terminal half of gp41 (data not shown) showed that all of the mutants tested showed a band at ~20 kDa (the size expected from cleavage of gp140 to gp120 and the transmembrane fragment).

Cell surface expression of envelope substitution mutants was investigated by staining transfected COS cells with goat antiserum (G1115) raised against a HIV-1 gp160 which lacked a PND loop. This antiserum was used because of the poor reactivity of several of the substitution mutants with antiserum prepared against the entire HIV envelope due to variation in the antigenic PND. Figure 3 shows staining of COS cells transfected with pCMVREV plus pΔGPG (panel A) or pGPR (panel B). Both mutants showed clear and reproducible, albeit low, levels of cell staining, as evidenced by the shift in fluorescence intensity. Similar results were observed for substitution mutants pEPG, pGAG, and pGHG, in addition to wild-type pLOTSA. Control COS cells mock transfected and stained with G1115 antiserum or stained with an irrelevant antibody showed cell staining the same as that of COS cells transfected with plasmid pLOTSA alone. Although the level of staining of transfected COS cells was low, all of the mutants tested repeatedly showed shifts

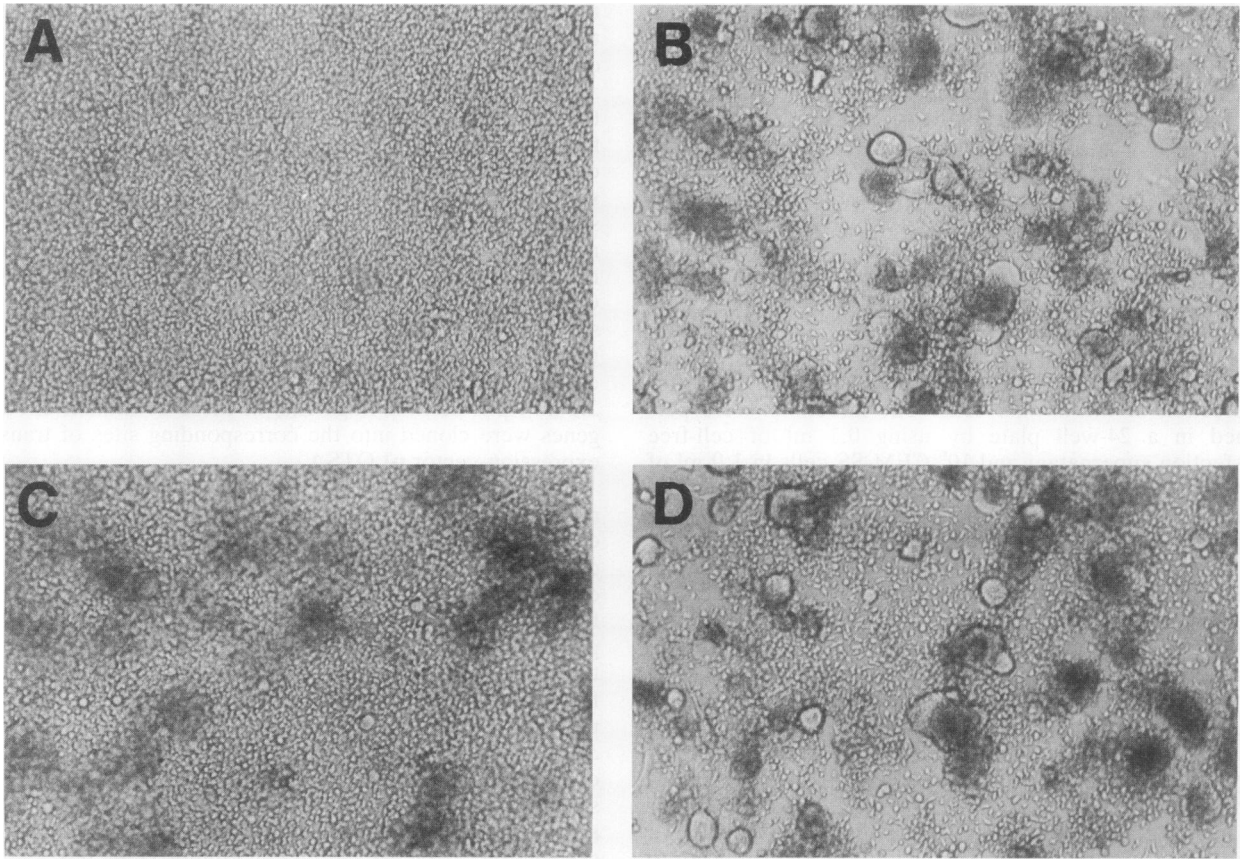

FIG. 1. Syncytium formation in the transient expression assay. COS-7 cells were mock transfected (A, negative control) or transfected with envelope expression plasmid GPG (B, positive control), WPG (C), or GHG (D).

similar to those presented in Fig. 3. Most of the transfected COS cells showed little increase in envelope expression, 10 to 20% showed a less-than-10-fold increase in staining, and 1 to 5% showed an up-to-100-fold increase.

Titration experiments using the GAG envelope in which the amount of pCMVREV was varied showed that the level of envelope expression increased approximately 10- to 50-fold when the amount of pCMVREV was increased from 0.125 to 2 μg (Fig. 4). Transfections using 5 μg of pCM VREV resulted in no additional envelope expression above that found for 2 μg (data not shown). Increasing envelope expression by increasing the amount of pCMVREV in the transient expression assay to 5 μg allowed syncytium formation for mutants GAG, GSG, GVG, GQG, APG, and GPF (Table 1). None of the remaining mutants induced syncytia at the elevated level of envelope expression.

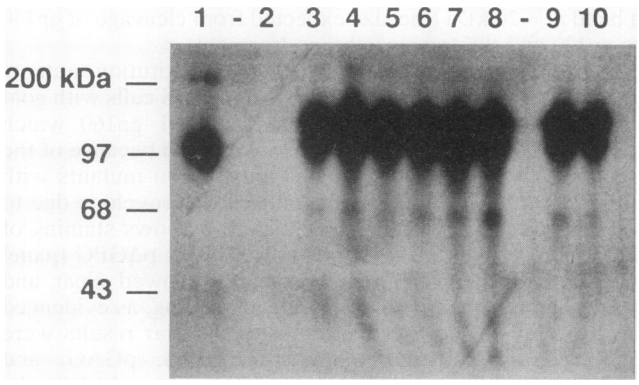

FIG. 2. Western blot analysis of envelope substitution mutants. Cells were transfected with pCMVREV plus expression plasmid pLOTSA (lane 3), pGPF (lane 4), pGPW (lane 5), pGPL (lane 6), pEPG (lane 7), pAPG (lane 8), pGAG (lane 9), or pGFG (lane 10). Lane 1 contained purified recombinant gp120, and lane 2 contained mock-transfected COS cells.

CD4 binding of mutant envelopes. Previous work has shown that an insertion (17) or amino acid substitutions (10) in the PND did not affect gp120 binding to CD4. We assayed the abilities of the wild-type (GPG), ΔPND, ΔGPG, and GFG envelopes to bind sCD4 by incubating labeled COS-7 lysates with sCD4 and determining whether MAb OKT4 could precipitate the gp120-sCD4 complex. Whereas MAb 1C1 effectively precipitated each of these envelopes, sCD4 and OKT4 failed to precipitate the ΔPND envelope (Fig. 5). This indicates that neither the phenylalanine substitution nor the GPG deletion significantly affected the ability of the envelope to bind CD4, but deletion of the bulk of the PND abolished the ability to bind CD4. This could be due to a conformational change in gp120 caused by this large deletion which alters the CD4-binding site. It is known that deletions of other regions of gp120 affect CD4 binding (5, 6, 8, 22).

Analysis of PND mutations for effect on virus infectivity and growth kinetics. Both of the substitution mutants that formed

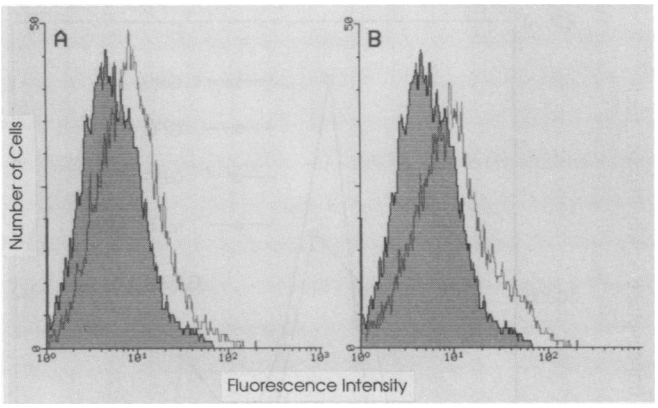

FIG. 3. Cell staining of COS cells transfected with HIV-1 envelope variants. Control COS cells were transfected with pLOTSA alone and lacked the pCMVREV plasmid required for envelope expression. (A) COS cells transfected with pLOTSA (gray) or pΔGPG and pCMVREV (white). (B) COS cells transfected with pLOTSA (gray) or pGPR and pCMVREV (white).

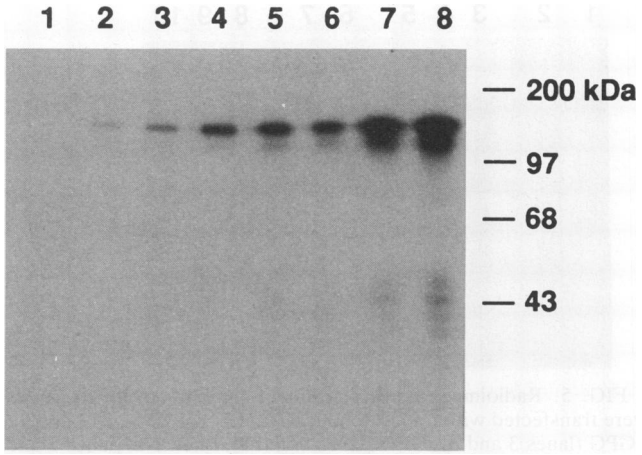

FIG. 4. Titration of PCMVREV and expression of GAG envelope variant. COS cells were transfected with 5 μg of pGAG and various amounts of pCMVREV, i.e., 0 (lane 1), 0.031 (lane 2), 0.062 (lane 3), 0.125 (lane 4), 0.250 (lane 5), 0.50 (lane 6), 1.0 (lane 7), and 2.0 (lane 8).

syncytia in the transient expression assay under conditions of lower envelope expression (GFG and GHG), as well as four mutants which formed syncytia under conditions of high envelope expression (GAG, GSG, GPF, and APG) were cloned into the pNL4-3 proviral vector to determine the effects of these mutations on viral infectivity and growth. SW480 cells were transfected with proviral DNA, and after 40 h, supernatants were assayed for RT. All of the mutants were RT positive, indicating that transfection supernatants contained viral particles (data not shown). These supernatants were used to infect CEM-SS cells. During the subsequent 40 days, virus replication was monitored by measuring the cytopathic effect and culture supernatants were assayed for RT. Viruses NL-GPG (wild type), NL-GHG, and NL-GFG all showed viral replication, while viruses NL-GAG, NL-GSG, NL-GPF, and NL-APG were not infectious by the RT and cytopathic effect assays (Fig. 6). This demonstrated that only the mutant envelopes inducing syncytia in the transient expression assay under conditions of lower envelope expression give rise to infectious virus.

The growth kinetics of the three viable viruses showed that NL-GHG displays kinetics similar to those of the wild-type parental NL-GPG virus in that both reached peak RT activity at day 10 postinfection. However, the number of infectious viral particles of NL-GHG ($TCID_{50}$, $\sim 3 \times 10^3$/ml) was 1/10 of that for NL-GPG ($TCID_{50}$, $\sim 3 \times 10^4$/ml). NL-GFG exhibited both delayed growth kinetics (peak RT at day 22 postinfection) and reduced viral titer ($TCID_{50}$, $\sim 3 \times 10^2$/ml). Cells were taken at day 20 postinfection to obtain template DNA for polymerase chain reaction amplification reactions using PND primers XBA-PCR and VSP-PCR. The three clones which produced infectious virus, NL-GPG, NL-GFG, and NL-GHG, yielded amplified DNA with the corresponding PND sequence, indicating that growth of NL-GHG and NL-GPG was not due to reversion to the wild-type GPG sequence. None of the noninfectious viruses yielded polymerase chain reaction-amplifiable DNA.

Neutralization of mutant proviruses. The ability of PND polyclonal antibodies and MAbs to neutralize virus infectivity is well documented (11, 14, 19, 32, 37). To investigate the effect of PND antibodies on infectivity, the titers of the GPG, GHG, and GFG viruses were determined in the absence or presence of PND polyclonal antibodies and MAbs known to neutralize IIIB virus. The titers of serial dilutions of virus supernatants taken at peak RT (day 10 for GPG and GHG and day 22 for GFG) in the presence or absence of a 1:10 dilution of anti-RP135 or anti-RP174 antiserum or 200 μg of MAb Cβ1 per ml were determined (Fig. 7). Whereas the titer of the wild-type virus was 10^3 $TCID_{50}$/ml in the absence of antibody, the titer was 10/ml in the presence of polyclonal serum and less than 10/ml with the MAb, indicating that 99% or more of the virus infectivity

TABLE 1. Syncytium formation of PND mutants

Envelope	Syncytium formation in transient expression assay[a]:	
	I[b]	II[c]
GPG	+	+
None	−	−
ΔPND	−	−
ΔGPG	−	−
EPG	−	−
WPG	−	−
VPG	−	−
APG	−	+
GAG	−	+
GSG	−	+
GQG	−	+
GVG	−	+
GHG	+	+
GFG	+	+
GPF	−	+
GPR	−	−
GPI	−	−
GPK	−	−
GPW	−	−
GPL	−	−

[a] −, lack of visible syncytia; +, visible syncytia.
[b] Transfection conditions included 125 ng of pCMVREV and 5 μg of envelope expression plasmid.
[c] Transfection conditions included 5 μg of pCMVREV and 5 μg of envelope expression plasmid.

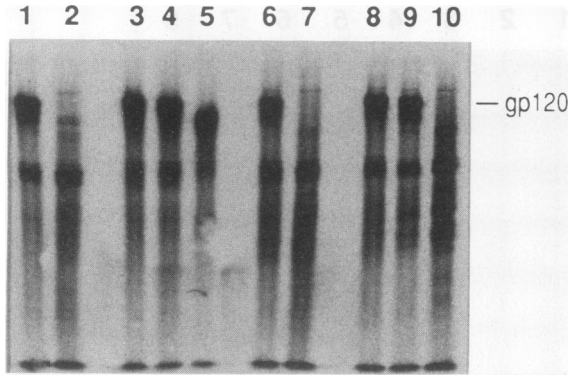

FIG. 5. Radioimmunoprecipitation of mutant envelopes. Cells were transfected with no DNA (lanes 2 and 7), GPG (lanes 1 and 6), ΔGPG (lanes 3 and 8), GFG (lanes 4 and 9), or ΔPND (lanes 5 and 10). The radiolabeled envelope proteins were immunoprecipitated with MAb 1C1 (lanes 1 to 5) or sCD4 and OKT4 (lanes 6 to 10). The location of gp120 is indicated.

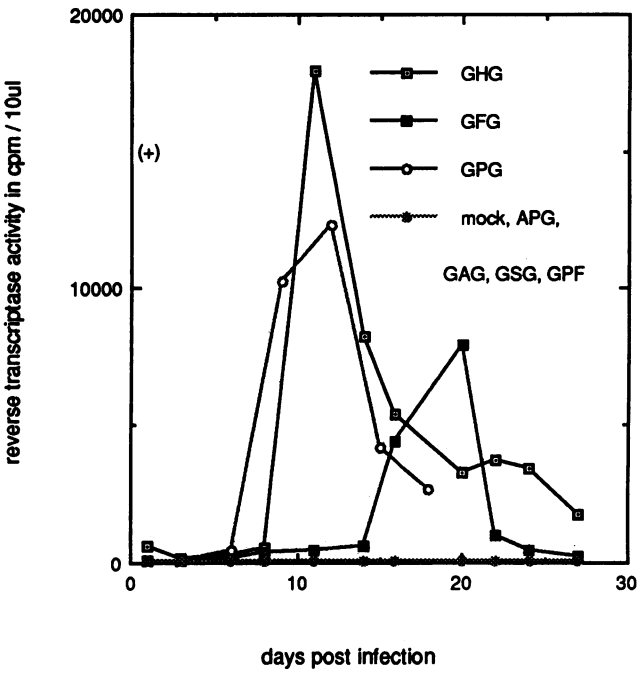

FIG. 6. Growth kinetics of substitution mutant viruses. Virion-associated RT activities in virus expressing the wild-type envelope (GPG) and two substitution mutants (GHG and GFG) were measured. The RT activities of substitution mutant viruses GAG, GSG, APG, and GPF were identical to that of the mock control.

was neutralized. In contrast, although the GHG and GFG mutant viruses grew to a lower titer, neither mutant was neutralized by any of the antibodies. These data show that substitution of the proline at the tip of the PND abolishes the ability of the virus to be neutralized by PND antibodies.

DISCUSSION

The HIV-1 PND is the binding site for antibodies that neutralize virus infectivity and prevent fusion of infected cells with uninfected CD4-bearing cells. It is contained in a 36-amino-acid loop formed by a disulfide cross-bridge (20) between two invariant cysteines. The tip of this loop contains conserved peptide sequences and predicted secondary structures, suggesting that there is selective pressure to preserve these elements (19). Peptides corresponding to the tip of this loop (e.g., IQRGPGRAF [15] and GPGRAF [14]) elicit neutralizing antibody, showing that these amino acids comprise the neutralization determinant.

To determine the effects of PND mutations on virus infectivity, growth kinetics, and susceptibility to neutralization by PND antibodies, we constructed a series of substitution mutants in the highly conserved GPG at the tip of the PND. Several of the GPG variants constructed here are found in natural HIV isolates; however, numerous other differences exist with the IIIB-BH10 sequence and those reported for other envelope genes or the entire envelope sequences have not been determined. Of the 16 substitution and 2 deletion envelope mutants tested, only 2, GFG and GHG, induced syncytia when expressed at low levels. The failure of the remaining envelopes to induce syncytium formation was not due to altered levels of protein expression or envelope processing, because all of the mutant envelopes tested, including the two deletions, were expressed at similar levels, as measured by Western blot analysis and radioimmunoprecipitation with MAb 1C1. The mutations (except ΔPND) also did not significantly alter the abilities of the envelopes tested to bind CD4.

Similarly, the deletion mutant lacking GPG and mutants with a substitution at each position (EPG, GAG, and GPR) were found on the surface of transfected COS cells at levels comparable to that of the wild type, pLOTSA (GPG).

This suggests that the inability of the substitution envelopes to participate in syncytium formation is not due a gross defect in either expression level, processing, or cell surface expression. Freed et al. have recently shown that mutations to the GPG and adjacent amino acids in the PND of the HIV-1 envelope do not alter expression, processing, or cell surface expression in a transient expression assay (10). Freed's limited number of mutations and the larger group reported here demonstrated that single-amino-acid substitutions and deletion of the core GPG residues has little effect on normal envelope expression but has a large effect on envelope function, as seen by the loss of syncytium formation. That COS cells expressing most mutant envelopes can form aggregates with $CD4^+$ cells also suggests surface expression of the envelopes and points to a block in the fusion event.

Several of the substitution mutant envelopes induced syncytium formation when envelope glycoprotein is expressed at 10- to 50-fold higher levels. Mutants GSG, GAG, GQG, GVG, GPF, and APG form syncytia which are comparable in size and number to wild-type GPG. Interestingly, all of the mutations of the proline we tested induced syncytia at higher envelope levels, which is consistent with the results of LaRosa et al. (19) that showed more variability in this position than in the two flanking glycines. The defect in syncytium formation can therefore be overcome, at least for some PND mutants, by elevated expression of the envelope glycoprotein. This suggests that mutations that result in increased envelope expression may be able to compensate for PND mutations that decrease the efficiency of viral infection.

Transient expression allowed screening of 18 envelope mutations for syncytium formation and showed that only two mutants, GHG and GFG, behaved like the wild-type GPG envelope. We tested these envelopes and the GSG,

GAG, APG, and GPF envelopes for effects on viral infectivity, growth kinetics, and antibody neutralization. Only the wild-type GPG virus and the two mutants which formed syncytia in the transient expression assay, GFG and GHG, produced infectious virus. Although the other mutants produced viral particles as judged by the presence of RT in the supernatant after transfection, these viruses were not infectious in CEM-SS cells as measured by either cytopathic effect or virion-associated RT activity. Although the GHG mutant exhibited growth kinetics similar to those of the wild type and the GFG mutant showed delayed growth kinetics, both mutant viruses grew to lower titers. Because the amounts of viral particles (as determined by RT activity) for all three viruses were comparable but the viral titers were either 10 or 100 times lower than that of the wild type for the GHG and GFG mutants, it is likely that the number of infectious viral particles is reduced for the two mutants. Both mutants were resistant to neutralization by PND-specific polyclonal antibodies and MAbs; this was probably due to alteration of the sequence of the binding site for these antibodies.

The mechanism by which PND antibodies neutralize infectivity is unknown. The PND does not directly participate in CD4 binding, because PND antibodies do not block binding of gp120 to CD4 (22, 42), and the results presented herein and those of others show that small deletions, insertions (17), or substitutions (10) do not significantly affect binding of the envelope to CD4. Furthermore, addition of PND antibodies after the virus has bound to CD4 cells completely neutralizes viral infectivity (26). This shows that there is a relatively slow post-CD4-binding step that is blocked by PND antibodies.

The step in the infection pathway blocked by PND antibodies is probably the same as that affected by the PND mutations that abolish the ability of the envelope to induce syncytia or generate infectious virus. The role of the PND in viral infectivity is not known, but it has been suggested that the PND is cleaved by a cellular protease during virus entry and that this cleavage is necessary for syncytium formation. Cleavage of the PND (between the R and A) by thrombin, tryptase (4), or an endogenous protease in Chinese hamster ovary cells (43) has been shown; antibodies to tryptase inhibit HIV-1-induced syncytium formation (12), and proteinase inhibitors block HIV-1 infection (16). It is possible that the mutations in the tip of the PND alter the recognition sequence of the cell surface protease that is required for viral entry, thus making the PND a less efficient substrate for cleavage. This may explain why most mutations do not induce syncytium formation but that some, that may be recognized by the protease, enable the envelope to function. Ivanoff et al. (13) showed that a proviral clone containing a GAG mutation was infectious but that the efficiency of infectivity depends on the cell type. This suggests that different cell types have proteases with different specificities and further suggests that some of our substitution mutants may be infections if other host cells are used.

The origin of PND mutations in vivo is most likely due in part to selective pressure imposed by virus-neutralizing antibody generated during infection. It is possible that, as has been shown for visna virus (29, 30), caprine arthritis-encephalitis lentivirus (31), and equine infectious anemia virus (2, 24, 39), neutralization-resistant variants that are resistant to neutralization by pre-existing antibody are generated during HIV-1 infection. Neutralizing antibody-resistant variants of HIV-1 have been selected both in vitro (25, 35) and in vivo (7, 28) with mutations either in- or outside the PND (25), and our work shows that mutations within the PND that are infectious and resist PND antibody neutralization can be generated. In addition to resistance to neutralizing antibodies, there is evidence that virus isolates obtained from individuals more advanced in AIDS disease progression are more cytopathic, have increased growth kinetics, and display increased cell tropism (3, 9, 38, 45). Our results show that PND mutations affect virus growth kinetics, and recent data (41, 44, 46) implicate the PND in determination of host cell tropism. It will be important to extend the analysis of PND mutants to understand the role of the PND in virus evolution and escape from immunological selective pressure.

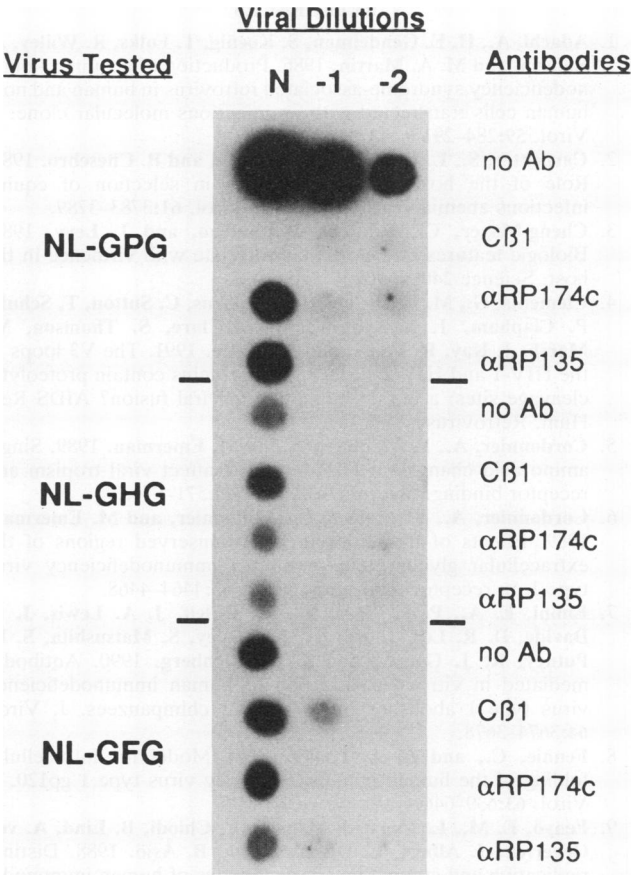

FIG. 7. Neutralization assay. Tenfold dilutions of viruses (N, undiluted; −1, 1:10 dilution; −2, 1:100 dilution) GPG, GHG, and GFG were mixed with no antibody (no Ab), 200 μg of MAb Cβ1 per ml, 1:10 dilutions of polyclonal sera αRP174c and αRP135 and preincubated at 37°C for 30 min before being mixed with 10^6 CEM-SS cells. At day 10 postinfection, supernatants were harvested for RT assay. Labeled products are shown.

ACKNOWLEDGMENTS

We thank Sandra Silver, James Rusche, Walter Herlihy, Al Profy, Greg LaRosa, and Kashi Javaherian for helpful discussions; Michael White for oligonucleotide synthesis and assistance with DNA sequencing; S. Matsushita, S. Tokiyoshi, and Y. Eda for the Cβ1 antibody; and Malcolm Martin for the proviral vector.

This work was supported in part by NIH grant 1 RO1 AI 29825-01 to S.D.P.

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Carpenter, S., L. H. Evans, M. Sevoian, and B. Chesebro.** 1987. Role of the host immune response in selection of equine infections anemia virus variants. J. Virol. **61:**3783–3789.
3. **Cheng-Mayer, C., D. Seto, M. Tateno, and J. Levy.** 1988. Biologic features of HIV-1 that correlate with virulence in the host. Science **240:**80–82.
4. **Clements, G., M. Price-Jones, P. Stephens, C. Sutton, T. Schulz, P. Clapham, J. McKeating, M. McClure, S. Thomson, M. Marsh, J. Kay, R. Weiss, and J. Moore.** 1991. The V3 loops of the HIV-1 and HIV-2 surface glycoproteins contain proteolytic cleavage sites: a possible function in viral fusion? AIDS Res. Hum. Retroviruses **7:**3–16.
5. **Cordonnier, A., L. Montagnier, and M. Emerman.** 1989. Single amino acid changes in HIV envelope affect viral tropism and receptor binding. Nature (London) **340:**571–574.
6. **Cordonnier, A., Y. Rivière, L. Montagnier, and M. Emerman.** 1989. Effects of mutations in hyperconserved regions of the extracellular glycoprotein of human immunodeficiency virus type 1 on receptor binding. J. Virol. **63:**4464–4468.
7. **Emini, E. A., P. L. Nara, W. A. Schleif, J. A. Lewis, J. P. Davide, D. R. Lee, J. Kessler, S. Conley, S. Matsushita, S. D. Putney, R. J. Gerety, and J. W. Eichberg.** 1990. Antibody-mediated in vitro neutralization of human immunodeficiency virus type 1 abolishes infectivity for chimpanzees. J. Virol. **64:**3674–3678.
8. **Fennie, C., and L. A. Lasky.** 1989. Model for intracellular folding of the human immunodeficiency virus type 1 gp120. J. Virol. **63:**639–646.
9. **Fenyö, E. M., L. Morfeldt-Månson, F. Chiodi, B. Lind, A. von Gegerfelt, J. Albert, E. Olausson, and B. Åsjö.** 1988. Distinct replicative and cytopathic characteristics of human immunodeficiency virus isolates. J. Virol. **62:**4414–4419.
10. **Freed, E. O., D. J. Myers, and R. Risser.** 1991. Identification of the principal neutralizing determinant of human immunodeficiency virus type 1 as a fusion domain. J. Virol. **65:**190–194.
11. **Goudsmit, J., C. Debouck, R. H. Meloen, L. Smit, M. Bakker, D. M. Asher, A. V. Wolff, C. J. Gibbs, Jr., and D. C. Gajdusek.** 1988. Human immunodeficiency virus type 1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees. Proc. Natl. Acad. Sci. USA **85:**4478–4482.
12. **Hattori, T., A. Koito, K. Takatsuki, H. Kido, and N. Katunuma.** 1989. Involvement of tryptase-related cellular protease(s) in human immunodeficiency virus type 1 infection. FEBS Lett. **248:**48–52.
13. **Ivanoff, L., D. Looney, C. McDanal, J. Morris, F. Wong-Staal, A. Langlois, S. Petteway, and T. Matthews.** 1991. Alteration of HIV-1 infectivity and neutralization by a single amino acid replacement in the V3 loop domain. AIDS Res. Hum. Retroviruses **7:**595–603.
14. **Javaherian, K., A. Langlois, G. LaRosa, A. Profy, D. Bolognesi, W. Herlihy, S. Putney, and T. Matthews.** 1990. Broadly neutralizing antibodies elicited by the hypervariable neutralizing determinant of HIV-1. Science **250:**590–593.
15. **Javaherian, K., A. J. Langlois, C. McDanal, K. L. Ross, L. I. Eckler, C. L. Jellis, A. T. Profy, J. R. Rusche, D. P. Bolognesi, S. D. Putney, and T. J. Matthews.** 1989. Principal neutralizing domain of the HIV-1 envelope protein. Proc. Natl. Acad. Sci. USA **86:**6768–6772.
16. **Koito, A., T. Hattori, T. Murakami, S. Matsushita, Y. Maeda, T. Yamamoto, and K. Takatsuki.** 1989. A neutralizing epitope of human immunodeficiency virus type 1 has homologous amino acid sequences with the active site of inter-α-trypsin inhibitor. Int. Immunol. **1:**613–618.
17. **Kowalski, M., J. Potz, L. Basiripour, T. Dorfman, W. C. Goh, E. Terwilliger, A. Dayton, C. Rosen, W. Haseltine, and Sodroski.** 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. Science **237:**1351–1355.
18. **Kunkel, T., J. Roberts, and R. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–383.
19. **LaRosa, G. J., J. P. Davide, K. Weinhold, J. A. Waterbury, A. T. Profy, J. A. Lewis, A. J. Langlois, G. R. Dreesman, R. N. Boswell, P. Shadduck, L. H. Holley, M. Karplus, D. P. Bolognesi, T. J. Matthews, E. A. Emini, and S. D. Putney.** 1990. Conserved sequence and structural elements in the HIV-1 principal neutralizing determinant. Science **249:**932–935.
20. **Leonard, C. K., M. W. Spellman, L. Riddle, R. J. Harris, J. N. Thomas, and T. Gregory.** 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. J. Biol. Chem. **265:**10373–10382.
21. **Lewis, N., J. Williams, D. Rekosh, and M.-L. Hammarskjöld.** 1990. Identification of a *cis*-acting element in human immunodeficiency virus type 2 (HIV-2) that is responsive to the HIV-1 *rev* and human T-cell leukemia virus types [sic] I and II *rex* proteins. J. Virol. **64:**1690–1697.
22. **Linsley, P. S., J. A. Ledbetter, E. Kinney-Thomas, and S. L. Hu.** 1988. Effects of anti-gp120 monoclonal antibodies on CD4 receptor binding by the *env* protein of human immunodeficiency virus type 1. J. Virol. **62:**3695–3702.
23. **Matsushita, S., M. Robert-Guroff, J. Rusche, A. Koito, T. Hattori, H. Hosino, K. Javaherian, K. Takatsuki, and S. Putney.** 1988. Characterization of a human immunodeficiency virus neutralizing monoclonal antibody and mapping of the neutralizing epitope. J. Virol. **62:**2107–2114.
24. **McGuire, T., T. Crawford, and J. Henson.** 1972. Equine infectious anemia: detection of infectious virus-antibody complexes in the serum. Immunol. Commun. **1:**545–551.
25. **McKeating, J. A., J. Gow, J. Goudsmit, L. Pearl, C. Mulder, and R. Weiss.** 1989. Characterisation of HIV-1 neutralization escape mutants. AIDS **3:**777–784.
26. **Nara, P.** 1989. HIV-1 neutralization: evidence for rapid binding/postbinding neutralization from infected humans, chimpanzees, and gp120-vaccinated animals. Vaccines **89:**137–144.
27. **Nara, P., W. Hatch, N. Dunlop, W. Robey, L. Arthur, M. Gonda, and P. Fischinger.** 1987. Simple, rapid, quantitative, syncytium-forming microassay for the detection of human immunodeficiency virus neutralizing antibody. AIDS Res. Hum. Retroviruses **3:**283–302.
28. **Nara, P. L., L. Smit, N. Dunlop, W. Hatch, M. Merges, D. Waters, J. Kelliher, R. C. Gallo, P. J. Fischinger, and J. Goudsmit.** 1990. Emergence of viruses resistant to neutralization by V3-specific antibodies in experimental human immunodeficiency virus type IIIB infection of chimpanzees. J. Virol. **64:**3779–3791.
29. **Narayan, O., J. E. Clements, D. E. Griffin, and J. S. Wolinsky.** 1981. Neutralizing antibody spectrum determines the antigenic profiles of emerging mutants of visna virus. Infect. Immun. **32:**1045–1050.
30. **Narayan, O., D. E. Griffin, and J. E. Clements.** 1978. Virus mutation during "slow infection." Temporal development and characterization of mutants of visna virus recovered from sheep. J. Gen. Virol. **41:**343–352.
31. **Narayan, O., D. Sheffer, D. E. Griffin, J. Clements, and J. Hess.** 1984. Lack of neutralizing antibodies to caprine arthritis-encephalitis lentivirus in persistently infected goats can be overcome by immunization with inactivated *Mycobacterium tuberculosis*. J. Virol. **49:**349–355.

31a. **Nelson, M. B., D. Rekosh, and M.-L. Hammarskjöld.** Unpublished data.

32. **Palker, T. J., M. E. Clark, A. J. Langlois, T. J. Matthews, K. J. Weinhold, R. R. Randall, D. P. Bolognesi, and B. F. Haynes.** 1988. Type-specific neutralization of the human immunodeficiency virus with antibodies to *env*-coded sythetic peptides. Proc. Natl. Acad. Sci. USA **85:**1932–1936.
33. **Putney, S. D., T. J. Matthews, W. G. Robey, D. L. Lynn, M. Robert-Guroff, W. T. Mueller, A. J. Langlois, J. Ghrayeb, S. R. Petteway, Jr., K. J. Wienhold, P. J. Fischinger, F. Wong-Staal,**

R. C. Gallo, and D. P. Bolognesi. 1986. HTLV-III/LAV-neutralizing antibodies to an *E. coli* produced fragment of the virus envelope. Science **234:**1392–1395.

34. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, A. N. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature (London) **313:**277–284.
35. **Reitz, M. S., Jr., C. Wilson, C. Naugle, R. C. Gallo, and M. Robert-Guroff.** 1988. Generation of a neutralization-resistant variant of HIV-1 due to selection for a point mutation in the envelope gene. Cell **54:**57–63.
36. **Rekosh, D., A. Nygren, P. Flodby, M.-L. Hammarskjöld, and H. Wigzell.** 1988. Coexpression of human immunodeficiency virus envelope proteins and tat from a single SV40 late replacement vector. Proc. Natl. Acad. Sci. USA **85:**334–338.
37. **Rusche, J. R., K. Javaherian, C. McDanal, J. Petro, D. L. Lynn, R. Grimaila, A. Langlois, R. C. Gallo, L. O. Arthur, P. J. Fischinger, D. P. Bolognesi, S. D. Putney, and T. J. Matthews.** 1988. Antibodies that inhibit fusion of human immunodeficiency virus-infected cells bind a 24-amino acid sequence of the viral envelope, gp120. Proc. Natl. Acad. Sci. USA **85:**3198–3202.
38. **Sakai, K., S. Dewhurst, X. Ma, and D. J. Volsky.** 1988. Differences in cytopathogenicity and host cell range among infectious molecular clones of human immunodeficiency virus type 1 simultaneously isolated from an individual. J. Virol. **62:**4078–4085.
39. **Salinovich, O., S. L. Payne, R. C. Montelaro, K. A. Hussam, C. J. Issel, and K. L. Schnorr.** 1986. Rapid emergence of novel antigenic and genetic variants of equine infectious anemia virus during persistent infection. J. Virol. **57:**71–80.
40. **Schmidt, N., and R. Emmons.** 1989. General principles of laboratory diagnostic procedures for viral, rickettsial, and chlamydial infections, p. 1–35. *In* N. J. Schmidt and R. W. Emmons (ed.), Diagnostic procedures for viral, rickettsial, and chlamydial infections, 6th ed. American Public Health Association Inc., Washington, D.C.
41. **Shioda, T., J. Levy, and C. Cheng-Mayer.** 1991. Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. Nature (London) **349:**167–169.
42. **Skinner, M. A., A. J. Langlois, C. B. McDanal, J. S. McDougal, D. P. Bolognesi, and T. J. Matthews.** 1988. Neutralizing antibodies to an immunodominant envelope sequence do not prevent gp120 binding to CD4. J. Virol. **62:**4195–4200.
43. **Stephens, P. E., G. Clements, G. T. Yarranton, and J. Moore.** 1990. A chink in HIV's armour? Nature (London) **343:**219.
44. **Takeuchi, Y., M. Akutsu, K. Murayama, N. Shimuzu, and H. Hoshino.** 1991. Host range mutant of human immunodeficiency virus type 1: modification of cell tropism by a single point mutation at the neutralization epitope in the *env* gene. J. Virol. **65:**1710–1718.
45. **Tersmette, N., J. M. A. Lange, R. E. Y. DeGoede, F. DeWolf, J. K. M. Eeftink-Schattenkerk, P. T. A. Schellekens, R. A. Coutinho, J. G. Huisman, J. Goudsmit, and F. Miedema.** 1989. Association between biological properties of human immunodeficiency virus variants and risk for AIDS and AIDS mortality. Lancet **i:**983–985.
46. **Westervelt, P., H. Gendelman, and L. Ratner.** 1991. Identification of a determinant within the human immunodeficiency virus 1 surface envelope glycoprotein critical for productive infection of primary monocytes. Proc. Natl. Acad. Sci. USA **88:**3097–3101.
47. **Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. **62:**139–147.