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Human Immunodeficiency Virus Type 1 Protease Inhibitors Irreversibly Block Infectivity of Purified Virions from Chronically Infected Cells

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Synthetic peptide analog inhibitors of human immunodeficiency virus type 1 (HIV-1) protease were used to study the effects of inhibition of polyprotein processing on the assembly, structure, and infectivity of virions released from a T-cell line chronically infected with HIV-1. Inhibition of proteolytic processing of both Pr55*gag* and Pr160*gag-pol* was observed in purified virions from infected T cells after treatment. Protease inhibition was evident by the accumulation of precursors and processing intermediates of Pr55*gag* and by corresponding decreases in mature protein products. Electron microscopy revealed that the majority of the virion particles released from inhibitor-treated cells after a 24-h treatment had an immature or aberrant capsid morphology. This morphological change correlated with the inhibition of polyprotein processing and a loss of infectivity. The infectivity of virion particles purified from these chronically infected cell cultures was assessed following treatment with the inhibitor for 1 to 3 days. Virions purified from cultures treated with inhibitor for 1 or 2 days demonstrated a 95- to 100-fold reduction in virus titers, and treatment for 3 days resulted in complete loss of detectable infectivity. The fact that virions from treated cultures were unable to establish infection over the 7- to 10-day incubation period in the titration experiments strongly suggests that particles produced by inhibitor-treated cells were unable to reactivate to an infectious form when they were purified away from exogenous protease inhibitor. Thus, a block of HIV-1 protease processing of viral polyproteins by specific inhibitors results in a potent antiviral effect characterized by the production of noninfectious virions with altered protein structures and immature morphologies.

Retroviruses, including human immunodeficiency virus type 1 (HIV-1), contain a protease that is responsible for processing the initial translation products of their *gag* and *pol* genes into functional proteins (8). Pr55*gag* and Pr160*gag-pol* contain the nascent structural proteins and enzymes of the virion core in single polyprotein chains (5, 31, 35, 37, 38). Proteolytic processing generates the *gag* and *gag-pol* protein products, each of which has a specific functional role in the fully formed virion (8, 20, 25). Because of the essential roles of *gag* and *gag-pol* cleavage products in structure and enzymatic function, an active protease is required for the production of infectious virions. Indeed, mutations in the protease-coding region of HIV-1 proviruses result in the formation of noninfectious virions which exhibit immature capsid morphologies and reduced reverse transcriptase activities (14, 16, 29). Although proviral mutants demonstrate the importance of the protease for the production of infectious virions, they do not necessarily predict the outcome of protease inhibitor-mediated inactivation of HIV-1 during ongoing chronic infection. It is possible that wild-type immature virions assembled in the presence of a competitive and reversible protease inhibitor might be able to "reactivate" to an infectious form when they are removed from contact with the drug (22).

Rationally designed synthetic peptide analogs containing hydroxyethylene dipeptide isosteres are potent competitive inhibitors of the protease (10) and inhibit processing of cell-associated *gag* and *gag-pol* polyproteins in chronically infected cells (23). In addition, we and others have shown that protease inhibitors block acute infections of T-cell cultures (2, 11, 22, 23, 34). However, none of those studies directly correlated a protease inhibitor block in polyprotein processing with an antiviral effect in the absence of free inhibitor. We used cells that were chronically infected with HIV-1 to investigate the effects of protease inhibitors on the virions that were produced because such cells have been implicated as a reservoir of infectious virus, perhaps contributing to the slow progressive nature of HIV-1-related disease (27, 30). Understanding of the antiviral effects of protease inhibition on the biologic properties of virions will be important in addressing the application of these agents to the therapy of HIV-1 infections and AIDS.

An additional issue addressed in this study is whether virions assembled from chronically infected cell cultures in the presence of protease inhibitors are subsequently capable of infecting cells in the absence of inhibitor. We investigated the effects of cell-associated inhibition of polyprotein processing on the subsequent production, maturation, and infectivity of HIV-1 virions. Results of the studies described here provide important new information on the antiviral effects resulting from an exogenous blockade of viral polyprotein processing.

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TABLE 1. Inhibitors of HIV-1 protease[a]

Compound no.	Structure	K_i (nM)	LEC (μM) of proteolytic processing	IC_{50} (μM)
1	Cbz–Ala-PheΨ($CHOHCH_2$)Gly-Val-Val–OMe	120	5	3
2	Ala-Ala-PheΨ($CHOHCH_2$)Gly-Val-Val–OMe	4	ND	ND
3	Cbz–Ala-Ala-PheΨ($CHOHCH_2$)Gly-Val-Val–OMe	48	2.5	2.1
4	Cbz–Ala-PheΨ($CHOHCH_2$)Ala-Val-Val–OMe	2.2	2.0	0.1
5	Cbz–Ala-Ala-PheΨ($CHOHCH_2$)Ala-Val-Val–OMe	0.7	0.5	0.87

[a] Apparent K_i values and the lowest effective concentration (LEC) and the concentration which inhibited viral infectivity by 50% (IC_{50}) were determined as described in the text. Cbz, benzyloxycarbonyl; Me, methyl. ND, not determinable.

MATERIALS AND METHODS

Virus and cell lines. The H9, Molt-4, and AA5 cells used in this study have been described previously (18, 26, 40). All cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS). H9/IIIB cells, which are chronically infected with the HTLV-IIIB virus isolate (31), were established in our laboratories as described previously (21, 23) and were used for the experiments described here.

Protease inhibitors. Compounds 1 to 5 (see Table 1) were prepared by solution-phase peptide condensation methods, as reported previously (10), by using the protected hydroxyethylene dipeptide isosteres Boc-NHCH(CH_2Ph)CH[$OSiMe_2$ (*tert*-Bu)]$CHRCO_2H$, in which R is H or methyl (Me), Boc is *tert*-butoxycarbonyl, Ph is phenyl, and *tert*-Bu is *tert*-butyl. The synthesis of these hydroxyethylene isosteres will be described elsewhere (9). Inhibitors were characterized by high-pressure liquid chromatography, proton nuclear magnetic resonance, and fast-atom-bombardment mass spectrometry. K_i values for compounds 1 to 3 obtained with recombinant HIV-1 protease were determined as described previously (10, 23), while K_i values for the more potent compounds 4 and 5 were determined by a modification of the method described by Ackermann and Potter (1) as well as other investigators (6, 24). For treatment of virus-infected cells, 10 mM solutions of inhibitors in 100% dimethyl sulfoxide (DMSO) were diluted into culture medium. Control cultures were treated with amounts of DMSO identical to those present in inhibitor-treated cultures. No toxicity, determined by trypan blue staining and viable cell counts, was observed with these inhibitors in the uninfected T-cell lines at concentrations of up to 100 μM compared with that in controls receiving identical amounts of DMSO.

Virion ultracentrifugation. About 6 ml of harvested cell-free culture supernatants was loaded onto a 5% sucrose cushion in phosphate-buffered saline (PBS) and centrifuged for 65 min at 30,000 rpm in an R40 rotor (Beckman). Virus pellets were resuspended in 1.2 ml of PBS, 0.2 ml of which was reserved for biochemical analyses and which represented a fivefold concentration of the starting material. The remaining sample (1 ml) was diluted to 5 ml with medium for virus infectivity assays. Ultracentrifugation did not damage virions, since the infectious titers of control virus following centrifugation were unchanged compared with those of an uncentrifuged aliquot kept on ice following centrifugation.

SDS-PAGE and Western blots (immunoblots). The proteins of virions recovered from the media containing treated cells were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (17). Virion proteins were separated in 12.5% polyacrylamide gels at 40 mA per gel for 4 h and were electroblotted onto nitrocellulose filters (36). Western blot (immunoblot) detection of p24 and p17 *gag* proteins with monoclonal antibodies (Beckman) was carried out as described previously (23). The HIV-1 envelope glycoprotein (gp120) was detected with guinea pig antibodies to strain BH10 gp120 V3 loop peptide (1:100) that were kindly provided by L. Ivanoff (SmithKline Beecham). Anti-guinea pig or anti-mouse immunoglobulin G antibodies conjugated with alkaline phosphatase (Bio-Rad, Richmond, Calif.) were used for the detection of protein-monoclonal antibody complexes.

Virus titration. The primary assessment of virion function was by titration of virus infectivity. Briefly, virion pellets were resuspended to their original volume in RPMI 1640 medium containing 10% FBS. Samples were serially diluted fivefold, and infectious titers were determined on either Molt-4 or AA5 cells in 24-well plates. For this, the medium of cultures was assayed for reverse transcriptase (RT) activity at 7 and 10 days postinfection as described previously (12, 39), and the 50% tissue culture infectious dose ($TCID_{50}$) endpoints were determined as described by Reed and Muench (32).

Electron microscopy. H9/IIIB cells were treated for 24 h with 20 μM compounds 3, 4, and 5 (containing a final concentration of DMSO of 0.2%). Untreated controls received 0.2% DMSO in RPMI 1640 medium containing 10% FBS. Samples were prepared for electron microscopy as described previously (15). Electron micrographs were printed at final magnifications of ×40,000 to ×50,000. Cell-associated virus particles from at least eight cells were evaluated, and the average percentage of virions with mature, immature, and indiscriminate capsid morphologies was calculated. Statistical analysis was performed with the SAS software package (SAS Institute Inc., Cary, N.C.).

RESULTS

Protease inhibitors. The structures of the inhibitors used in this study, along with their K_i values obtained with recombinant HIV-1 protease in vitro (7, 10), the minimum concentrations required to show inhibition of *gag* processing in infected cells, and the concentrations that inhibit viral infectivity by 50% determined in an HIV-1 acute infectivity inhibition assay, are given in Table 1. Compounds 1 to 5 are analogs of peptide substrates in which the scissile dipeptide is replaced by a hydroxyethylene isostere of Phe-Gly [PheΨ($CHOHCH_2$)Gly] or Phe-Ala [PheΨ($CHOHCH_2$)Ala] (10). It should be noted that compounds 4 and 5, which contain the PheΨ[$CHOHCH_2$]Ala isostere, are significantly more potent in vitro against Molt4 cells acutely infected with HIV-1 than are compounds 1 to 3, which contain the PheΨ[$CHOHCH_2$]Gly isostere.

Analysis of virion-associated *gag* proteins. In an initial experiment, accumulated virions in the medium of treated cell cultures were harvested after 72 h of treatment and

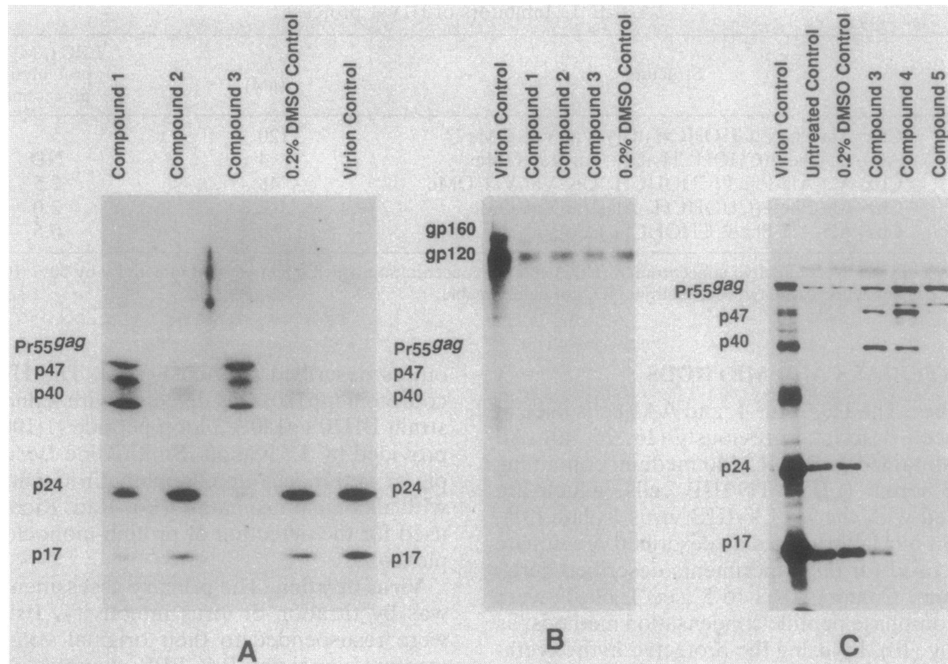

FIG. 1. Effects of HIV-1 protease inhibitor treatment on virion proteins. (A) Virion *gag* proteins. Cultures of H9 cells chronically infected with HTLV-IIIB were treated with the indicated compounds at final concentrations of 20 μM. Fresh compound was added after 24, 36, and 48 h so that accumulated compound concentration on day 3 was 60 μM. At that time, cell-free supernatants were prepared and filtered through a 0.22-μm-pore-size filter and virus was pelleted at 100,000 × *g* for 60 min. The pellet fraction was disrupted in SDS and was analyzed by SDS-PAGE. For detection of viral proteins within the electroblots, p24 and p17 mouse monoclonal antibodies, rabbit anti-mouse antibody, and ^{125}I-labeled protein A were used. Samples include an HTLV-IIIB virion control, virions from H9/IIIB cells treated with compounds 1, 2, or 3 or with 0.2% DMSO. (B) gp120 glycoprotein. Western blot of virion protein samples identical to those shown in panel A were probed with guinea pig antibody made against gp120-hypervariable (V3) loop peptide sequence. Glycoprotein bands were visualized with rabbit anti-guinea pig antibody labeled with alkaline phosphatase (Bio-Rad). (C) Relative inhibition of virion Pr55^{gag} processing by compounds 3, 4, and 5. Cells were treated with 20 μM concentrations of each compound as described above for panel A, except that the cells were pelleted daily and resuspended in medium containing fresh compounds. Virus was pelleted on day 3, and *gag*-related polypeptides were probed by Western blot analysis as described above for panel A. Virion samples included an HTLV-IIIB virus control, untreated H9/IIIB control, 0.2% DMSO-treated control, and virions from H9/IIIB cells treated with compounds 3, 4, and 5, respectively.

pelleted through a 5% sucrose cushion. In virions from untreated controls, virions from DMSO carrier-treated controls, and virions from cultures treated with compound 2, little or no Pr55^{gag} was detected; and most of the *gag* protein within these virions was processed into p24 and p17 subunits (Fig. 1A). However, in virions purified from cultures treated with compounds 1 and 3, unprocessed Pr55^{gag} and two processing intermediates (p47 and p40) were detected. There was also a concomitant reduction of p24 and p17. With compound 3, nearly complete inhibition of processing of Pr55^{gag} and the p47 and p40 intermediates to p24 and p17 (Fig. 1A, lane containing compound 3) was observed. According to earlier studies with cell-associated viral proteins (23), the p47 band probably represents p17-p24-p9 and the p40 band probably represents a mixture of p17-p24 and p24-p9-p6 polyproteins (23, 25).

On the basis of the relative amounts of virion *gag* proteins detected by Western blotting, protease inhibition did not appear to reduce the quantity of virus particles released from these cells. This point was difficult to quantitate more carefully by the p24 antigen capture assays, which only poorly detect p24 in the precursor forms, i.e., Pr55^{gag} (19). We therefore compared the relative levels of a second gene product, the envelope gp120, since the processing of this molecule should be independent of viral protease inhibition. Relative levels of gp120 in the pelleted virus from each of the treatment conditions were similar (Fig. 1B).

In addition to compounds 1 to 3, inhibitors with significantly lower K_i values (Table 1) were also used to treat chronically infected cultures as described above. As seen in Fig. 1C, compounds 4 and 5 appeared to be more potent at inhibiting *gag* processing, with compound 5 being the most potent. Indeed, a new minor band that migrated just above p47 was resolved in virions from cells treated with these compounds and may indicate a unique cleavage event. The inhibition of *gag-pol* processing was also noted in the pelleted virions treated with compounds 3, 4, and 5 and probed with RT-directed monoclonal antibodies (data not shown). Results of these experiments demonstrated that treatment with protease inhibitors results in a blockade of processing of *gag* and *gag-pol* proteins and an accumulation of unprocessed precursors in virion particles. Except for compound 2, the relative level of inhibition of *gag* processing correlated well with the relative potency (K_i) of the individual compounds in the recombinant protease assay (Table 1).

Effects of HIV-1 protease inhibition on virion morphology. The studies described above suggested that viruslike particles are assembled and released from inhibitor-treated cells into the medium. To test that possibility further, we examined these cells and supernatants after 24 h of treatment for retroviruslike particles by electron microscopy. HIV-1 virions with a mature morphology contain a cylindrical condensed nuclear core (Fig. 2A) that is composed primarily of

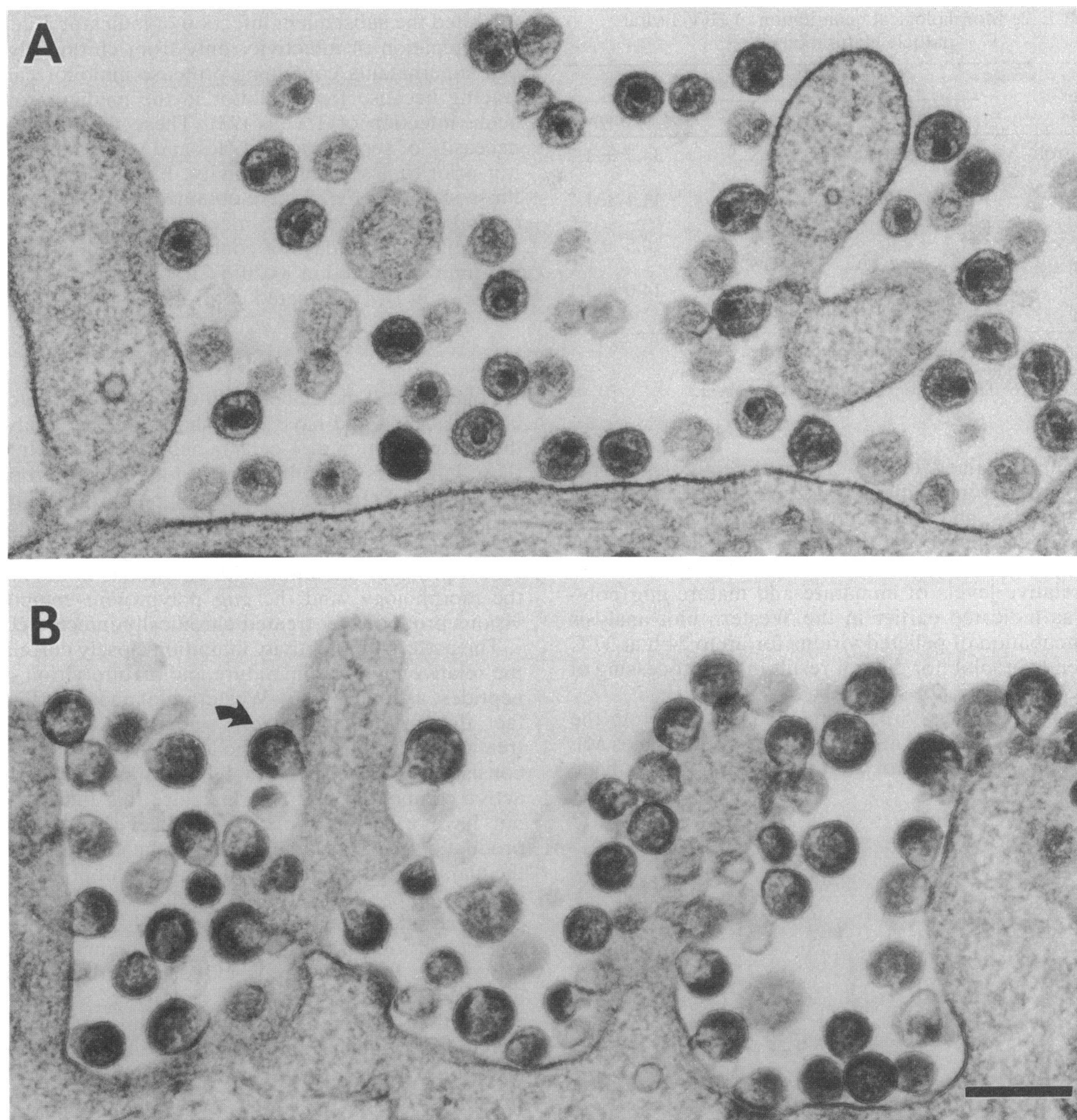

FIG. 2. Electron microscopy. H9 cells infected with HTLV-IIIB treated with the HIV-1 protease inhibitor. (A) Infected cell following 24 h in vehicle (medium plus 0.2% DMSO). Mature virions contain nuclear cores that appear as dark circles or cone-shaped bodies in virions. (B) Cell treated with compound 3 (20 μM in medium–0.2% DMSO) for 24 h. Immature virions are distinguished by the absence of cores and a retention of a dense plaque in a C pattern on the viral membrane. Unprocessed nuclear material remains associated with the viral membrane in a lamellar pattern. Many particles appear somewhat cone shaped (arrow), with the membrane opposite the nuclear plaque bulging out. Bar, 200 nm.

the p24 protein (13). Detached, immature virions, i.e., those that do not contain condensed cores but that possess an electron-dense "annular" plaque similar in appearance to that seen at regions of budding virions associated with the viral membrane, are occasionally seen in control preparations (13). Treatment of chronically infected cells for 24 h with any of the active protease inhibitors (Table 1) led to the appearance of mostly immature particles in the preparations examined (Fig. 2B).

We investigated the relative numbers of mature and immature particles as a function of inhibitor treatment by quantitative analysis of virion morphology after a 24-h treatment with compounds 3, 4, and 5 (Table 2). While a 24-h treatment was not expected to totally eliminate the presence of mature virions, the inhibitors were found to significantly suppress the maturation profile, i.e., the number of mature/number of immature particles observed ultrastructurally. All samples contained a small percentage of particles that could not be clearly identified. Consistent with the *gag* processing inhibition results shown in Fig. 1C, compound 5 also yielded the highest fraction of immature particles in a 24-h treatment.

Infectivity of virus particles derived from inhibitor-treated cultures. Titration of the pelleted inhibitor-treated virions (Fig. 3, day 1, lanes P) revealed that reduced levels of infectious virus were present at day 1 compared with the levels in DMSO-treated and untreated controls. In contrast

TABLE 2. Morphological quantitation of HIV-1 viral particle maturation

Treatment[a]	% Virus particles[b]		
	Mature	Immature	Unknown
DMSO control (n = 188)	73.8 ± 2.8	11.6 ± 2.9	14.7 ± 1.7
Compound 3 (n = 252)	35.6 ± 3.2[c]	53.3 ± 3.3[c]	11.1 ± 1.7
Compound 4 (n = 120)	34.7 ± 7.2[c]	54.9 ± 7.1[c]	10.5 ± 3.8
Compound 5 (n = 134)	21.7 ± 6.4[c]	64.2 ± 5.9[c]	14.0 ± 3.1

[a] Values are means ± standard errors of the means.
[b] n, number of virions counted.
[c] $P < 0.05$; control different from experimental treatment.

to the results obtained on day 1, the infectivities of the virions separated from media on day 2 was detectable only in undiluted samples, and the infectivities of the virions in the day 3 sample was below the threshold of sensitivity of our assay. The pattern of infectivity inhibition closely correlates with the relative levels of immature and mature *gag* polypeptides, as indicated earlier in the Western blot analysis (Fig. 1). Incubation of pelleted virions for up to 24 h at 37°C in the absence of inhibitor did not result in the processing of a *gag* precursor (data not shown).

Titration of infectious virus in medium directly from the treated culture revealed no infectious virus (Fig. 3, lanes M), suggesting that the inhibitor in the medium might have

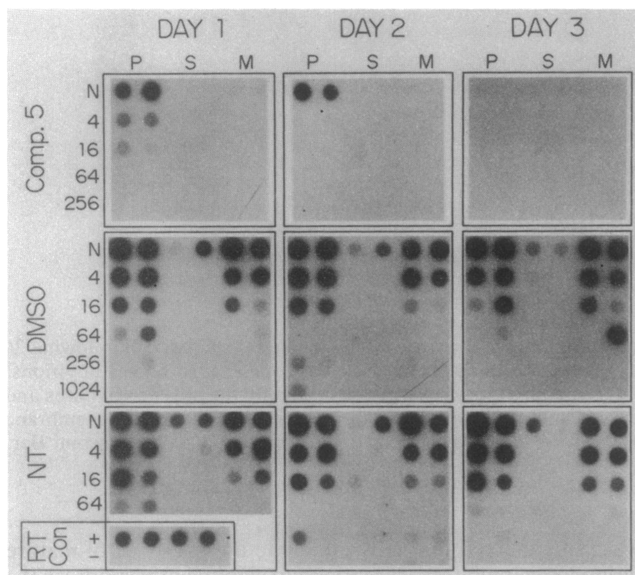

FIG. 3. Infectivity of virions produced by treated H9/IIIB cells. The infectious titers of virus from chronically infected cultures treated with inhibitors after 1, 2, and 3 days were compared. $TCID_{50}$s were determined by micro-RT assay detection of ^{32}P[TTP] incorporation into template DNA (12, 39). Infectious virus in treated culture medium (M), virus pellets (P), or virus pellet supernatants (S) was assayed on Molt4 cells at the indicated dilutions (N [neat], 4, 16, 64, and 256 are inverse of serial fourfold dilutions) at 7 days postinfection. Pelleted virions were resuspended to their original volume in RPMI 1640 medium containing 10% FBS without inhibitor. Virus-containing medium and pelleted virions from 0.2% DMSO carrier-treated and untreated (NT) cultures were run as controls.

inhibited the subsequent infection of indicator T cells. Thus, determination of infectivity only from chronically infected cell supernatants containing protease inhibitor can be misleading because free inhibitor in the medium could block acute infection of T cells (23). These results highlight the necessity of separating particles and virions from the inhibitor prior to assessing infectivity. Finally, results similar to those shown in Fig. 3 were obtained from experiments with compounds 3 and 4. The infectious titers of virus particles assembled after 3 days of continuous treatment were below the limit of detection (<20 $TCID_{50}$s compared with 3,000 $TCID_{50}$s in DMSO-treated controls).

DISCUSSION

Potent, small-molecule inhibitors of HIV-1 protease have been designed and have been shown to effectively prevent the acute infection of T cells in culture (10, 23). In addition, these inhibitors prevented the cell-associated processing of $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ precursor polyproteins in cultures of T cells chronically infected with HIV-1 (23). The purpose of the present study was to correlate the consequence of HIV-1 protease inhibition with alterations in the infectivity, the morphology, and the *gag* polyprotein composition of virions produced by treated chronically infected cells.

The pattern of infectivity inhibition closely correlated with the relative levels of immature and mature virion *gag* polypeptides, as indicated by Western blot analysis (Fig. 1). The fact that gp120 levels were similar in different inhibitor treatments compared with the levels in untreated controls is consistent with the specific inhibition of the viral protease by active compounds and also indicates that the inhibitors did not have a major impact on the level of virus particle production over the time course of the experiment. Rather, the major effects are related to inhibition of processing of the *gag*-related precursors and to a reduction in the infectivities of the particles that were produced. This finding also extends to the virion-associated *gag-pol* precursors (data not shown), as we have reported earlier for cell-associated products (23).

Mutations within the protease-coding region of HIV-1 proviruses result in noninfectious virions which exhibit an immature capsid morphology and reduced RT activity (14, 16, 29). It is possible that wild-type immature virions assembled in the presence of a competitive reversible protease inhibitor might be able to reactivate to an infectious form when they are removed from drug. This has been demonstrated for *gag* processing within pseudovirions derived from vaccinia virus vectors with an inhibitor of moderate potency (22).

Consistent with the results of Ashorn et al. (2), no infectious virus was detectable in the supernatants of cells treated for only 1 day with a protease inhibitor (Fig. 3, day 1, lane M). However, interpretation of this result could be complicated by the existence of free inhibitor in the medium, since HIV-1 protease inhibitors effectively block de novo infection of T-cell cultures by mature infectious virus particles (23). In order to address this issue with virions from chronically infected cells, it was necessary to separate the virus from free inhibitor by gentle sedimentation prior to infectivity analysis. After 24 h of treatment, some mature particles were evident by electron microscopic analysis (Table 2), and although the level was reduced compared with that of DMSO-treated control virions, some infectious virus was detectable at both days 1 and 2 (Fig. 3). However, no infectious virus was detected in virions isolated after 3 days

of treatment (Fig. 3), suggesting that 1 to 2 days of treatment was necessary to completely remove the cell-associated mature virions that were present at the initial time of treatment. Significantly, the virus particles isolated at day 3 were unable to establish detectable infection in indicator T-cell lines after incubation periods of up to 10 days. This result strongly suggests that virus particles produced by inhibitor-treated chronically infected cells are unable to reactivate to an infectious form in the absence of exogenous protease inhibitor.

The results presented here extend the previous finding that protease inhibitors can inhibit the spread of virus in cultures acutely infected at low multiplicities (23). The basis for this inhibitor-induced replication defect in acute infection is not completely understood but may be related to inhibition of early events in the HIV-1 replication cycle in addition to inhibition of maturation of budding particles. Recent studies suggesting that the NF-κB precursor may be processed by HIV-1 protease (4, 28, 33) as well as indications that a protease inhibitor blocked virus replication in early stages of HIV-1 infection (3) offer interesting possibilities for further investigations with this class of compounds. However, results of the studies described here demonstrate that HIV-1 protease inhibitors can also exert a potent antiviral effect on virions produced by chronically infected T-cell cultures, resulting in particles which are defective for infection. We interpret these results to indicate that treatment with an HIV-1 protease inhibitor has the potential to reduce or halt the production of infectious virus particles from both acutely and chronically infected cells. Whereas zidovudine and similar RT inhibitors have little or no effect on the production of infectious virions by chronically infected cells (30), the effects of protease inhibitors on this type of infection suggests a clear operational distinction between their mechanism of action and that of nucleoside analog RT inhibitors. Thus, the unique mechanism of action of HIV-1 protease inhibitors supports the development of these compounds as an alternative or adjunct to current therapies such as zidovudine for the treatment of HIV-1 infection and AIDS.

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