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Antiviral Research 45 (2000) 59–68

Antiviral Research

www.elsevier.com/locate/antiviral

Effect of protease inhibitors on HIV-1 maturation and infectivity

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Received 22 July 1999; accepted 17 November 1999

Abstract

The effects of HIV-1 protease inhibitors on proteolytic processing and infectivity of virions produced from lymphocytes chronically infected with the virus were studied. Protease inhibition was detected by the accumulation of the polyprotein precursors $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ and their cleavage intermediates. Immunoblot analysis demonstrated that while the processing of $Pr55^{gag}$ was largely irreversible, cleavage of $Pr160^{gag\text{-}pol}$ proceeded once the inhibitor was removed, although it was not completed during 96 h of subsequent observation. Virions produced during exposure of cells to protease inhibitors regained some degree of infectivity post-withdrawal of the inhibitor, suggesting that the processing of $Pr160^{gag\text{-}pol}$ following drug withdrawal resulted in the production of those enzymes necessary to enable at least limited viral replication. When cells were exposed to a protease inhibitor for 72 h then the inhibitor withdrawn, a lag phase of up to 24 h occurred before these cells produced virions with equivalent infectivity to virus produced from cells not exposed to drug. These observations may reflect a clinical situation likely to occur as trough plasma concentrations of protease inhibitors fall below the IC_{100} for HIV, highlighting the need for adherence to drug regimens containing these inhibitors.

Keywords: HIV-1 protease inhibitors; HIV-1 infectivity; $Pr160^{gag\text{-}pol}$ processing

1. Introduction

The processing of human immunodeficiency virus type 1 (HIV-1) polyproteins by the viral protease (PR) is crucial to virion maturation (Kohl et al., 1988; Peng et al., 1989). The *gag* gene product ($Pr55^{gag}$) is the precursor for virion capsid proteins (matrix (p17), capsid (p24) and nucleocapsid (p9)). $Pr160^{gag\text{-}pol}$ is the precursor for enzymes essential for viral replication (reverse transcriptase (RT), RNaseH, integrase and the PR itself (Hahn, 1994). During maturation $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ are cleaved to their functional units by the viral-encoded PR (Luciw, 1996). Inhibitors of this enzyme effectively block its proteolytic activity, but have no effect on the formation and function of the viral envelope glycoprotein, or the release of immature viral particles from PR inhibitor (PRI)-treated cells (Schatz et al., 1991; Kaplan et al., 1993; Vacca et al., 1994).

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0166-3542/00/$ - see front matter
PII: S0166-3542(99)00074-1

Previous studies have shown that the exposure of HIV-infected cells to PRIs has profound effects on the processing of HIV structural proteins that normally leads to the maturation and infectivity of virions (Lambert et al., 1992; Rayner et al., 1994; Vacca et al., 1994). An important aspect of the efficacy of PRIs is whether such processing commences and infectivity is gained when the drug is no longer present. Studies investigating this have analysed the processing of $Pr55^{gag}$ to indicate whether virions previously exposed to PRIs undergo maturation subsequent to removal of the drug (McQuade et al., 1990; Rayner et al., 1994; Vacca et al., 1994; Humphrey et al., 1997), and have also used infectivity assays to assess the replication competence of virions post-removal of inhibitor (Kageyama et al., 1994; Humphrey et al., 1997). In general these studies have shown that processing of $Pr55^{gag}$ does not return to normal despite prolonged incubation of virions in the absence of drug (Rayner et al., 1994; Humphrey et al., 1997), and that virions that have been exposed to PRIs are rendered non-infectious (Vacca et al., 1994). However, one study has demonstrated the apparent reversibility of protease inhibition of cleavage within a recombinant virus (McQuade et al., 1990) and others have shown that in some cases infectivity can be restored, at least in a minority of particles (Kageyama et al., 1994; Humphrey et al., 1997). Analysis of the $Pr160^{gag\text{-}pol}$ precursor has not been previously reported. However, because $Pr160^{gag\text{-}pol}$ is the precursor for proteins with enzymatic function in the newly infected cell, its fate post-removal of PRIs is also of considerable importance and has implications for the utilisation of these drugs in a clinical setting.

When assessing the infectivity of virions derived from cells exposed to PRIs, at least two separate scenarios need to be considered; firstly, when virions are produced from cells during exposure to the drug, and secondly, when virions are produced from cells previously exposed to inhibitory concentrations of PRIs but from which the drug has subsequently been removed. Hence, our objectives were to examine the ability of immature virions derived from chronically infected cells exposed to PRIs to mature and become infectious, and to investigate whether cells from which a PRI has been removed subsequently produce HIV with characteristic structural protein profiles and normal infectivity. Considerable emphasis was placed on the processing of $Pr160^{gag\text{-}pol}$ because of its role as a precursor to enzymes required in early events in HIV replication. The mechanism and kinetics of these processes are likely to impact on the efficacy of this class of inhibitor in the clinical setting.

2. Materials and methods

2.1. Cells

H9/HTLV-IIIB and MT2 cells were obtained from the National Institutes of Health AIDS Research and Reference Program. RF-10, an RPMI-1640-based medium supplemented with 10% (v/v) foetal calf serum (Tachedjian et al., 1990) was used for passage and maintenance of these cell lines.

2.2. Drugs

The effect of prior exposure to PRIs on the maturation of HIV was examined using three inhibitors of this class: indinavir (Vacca et al., 1994), saquinavir (Roberts et al., 1990) and DG35 (*t*-butyl-3-isopropyl-3-[(2*S*,3*S*)-2-hydroxy-3-(*N*-quinaldoyl L-asparaginyl)amino-4-phenyl-butyl] carbazate (Grobelny et al., 1997) (Fig. 1). DG35 was supplied by Dr Damien Grobelny (Narhex Operations, Avalon, Australia). Indinavir and saquinavir were supplied by Merck and Co., Inc., and Roche Laboratories, respectively.

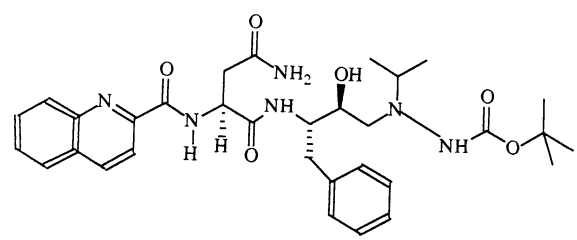

Fig. 1. Structure of the HIV protease inhibitor DG35.

2.3. Proteolytic processing in virions derived from chronically infected (H9/HTLV-IIIB) cells during exposure to PRIs

25×10^6 H9/HTLV-IIIB cells were incubated at 37°C in the presence of 5 μM of each PRI for 72 h, without replacement of the medium. Supernatant fluid was then clarified by centrifugation at $700 \times g$ for 10 min and virions concentrated by ultracentrifugation through 5% (w/v) sucrose in TNE buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA) at $160\,000 \times g$ for 1 h at 4°C. Virion pellets were resuspended in sterile saline, then aliquoted into 20 μl volumes. The aliquots were incubated at 37 and 4°C for various times ranging from h to days. At the appropriate time-points, proteolytic processing was halted by the addition of $5\times$ Laemmli buffer (Laemmli, 1970) and the sample stored at −70°C prior to immunoblot analysis.

2.4. Proteolytic processing in virions derived from chronically infected cells after prior exposure to PRIs

H9/HTLV-IIIB cells were incubated at 37°C in the presence of PRIs for 72 h as described above. The cells were pelleted by low-speed centrifugation, then resuspended in 10 ml of RF-10 and centrifuged as above. The cells were then washed twice more before being resuspended in 40 ml of RF-10, split into 4×10 ml rebound cultures and reincubated at 37°C. At time-points 0, 12 and 24 h, the supernate from one rebound culture was clarified by low-speed centrifugation and the infectivity of the resulting supernatant analysed as described below. The remainder of the supernate was then concentrated by ultracentrifugation as above, and the viral pellet resuspended in $1\times$ Laemmli sample buffer (Laemmli, 1970) for immunoblot analysis.

2.5. Immunoblot analysis

Equal volumes of sample were electrophoresed through a 10% SDS gel and electroblotted onto nitrocellulose. After the transfer, the membrane was blocked using 3% (w/v) casein in TBST (50 mM Tris–HCl [pH 7.5], 50 mM NaCl, 0.5% (v/v) Tween-20) overnight at 4°C. Immunodetection was performed using the HIV-1 $Pr55^{gag}$/p24-specific monoclonal antibody ADP313 (supplied by Drs Ferns and Tedder through the British Medical Research Council AIDS Reagent Project). The primary antibody reaction was carried out for 2 h at room temperature using a 1 in 1000 antibody dilution in 1% (w/v) casein in TBST. After four washes for 5 min each in TBST, the blot was incubated with a 1 in 2000 dilution of anti-mouse IgG conjugated to horseradish peroxidase for 2 h at room temperature. Protein bands were detected using a chemiluminescence procedure performed according to the manufacturers instructions (ECL Western Blot Analysis System, Amersham).

2.6. Infectivity of HIV-1 virions derived from chronically infected (H9/HTLV-IIIB) cells during and after PRI treatment

To examine the infectivity of virions derived from chronically infected cells during exposure to PRIs, H9/HTLV-IIIB cells were incubated at 37°C in the presence or absence of drug for up to 72 h. Following this, supernates were clarified by centrifugation at $700 \times g$ for 10 min and retained. Six serial ten-fold dilutions of the supernates at twice the final required concentration were then prepared in RF-10, and 250 μl of each dilution incubated for 2 h at 37°C with 1.0×10^6 MT2 cells suspended in 250 μl of RF-10. This incubation enabled virions within each dilution to infect the MT2 cells prior to removal of residual PRI. The cells were then resuspended in 10 ml of RF-10 and centrifuged at $700 \times g$ for 10 min at room temperature. The cells were resuspended in 10 ml of RF-10, then washed twice more as above before being resuspended in 2 ml of RF-10. Duplicate one ml volumes of the infected MT2 cells were added to the wells of a 24-well plate (Greiner, Germany) which was then incubated at 37°C in 5% CO_2. Evidence of HIV-specific cytopathic effects (CPE) at each dilution was sought daily for 6 days by light microscopy. The titre of virus derived from cells exposed or not exposed to drug was determined on each day using the Karber formula (Leland and French, 1988). To exam-

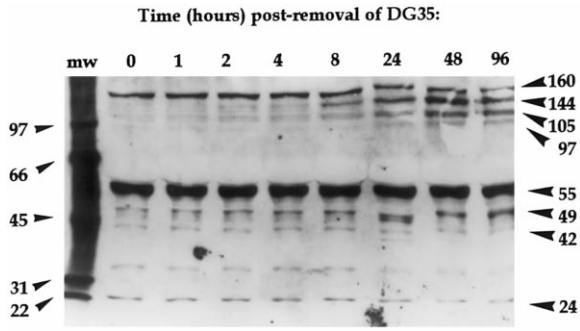

Fig. 2. Proteolytic processing in HIV virions produced from H9/HTLV-IIIB cells in the presence of DG35. Culture supernates were clarified, pelleted through a 5% (w/v) sucrose cushion and then incubated at 37°C in 5% CO_2 for up to 48 h. Proteolytic processing was halted at various times post removal of inhibitor (indicated in h above each lane). HIV-1 structural proteins were examined using a $Pr55^{gag}$/p24-specific MAb. (The molecular weights of HIV proteins are indicated on the right-hand-side; broad range molecular weight markers are on the left-hand side).

ine the infectivity of virions produced from H9/HTLV-IIIB cells previously exposed to a PRI then the inhibitor removed, cell culture supernates obtained at various times post-withdrawal of the PRI were subjected to the same procedure as described above to remove excess inhibitor and follow subsequent HIV replication in MT2 cells.

3. Results

3.1. Proteolytic processing in virions following the removal of PRIs

HIV-1 virions produced from H9/HTLV-IIIB cells incubated in the presence of 5 μM DG35 were concentrated to remove the drug and further incubated at 37 and 4°C for various times up to 96 h. Evidence of cleavage of $Pr160^{gag\text{-}pol}$ and $Pr55^{gag}$ was sought by immunoblot analysis using a $Pr55^{gag}$/p24 specific MAb. Immediately post-removal of inhibitor ($t = 0$), detectable amounts of unprocessed $Pr160^{gag\text{-}pol}$ were present in drug-treated virions (Fig. 2). Following a delay of 4–8 h, processing of $Pr160^{gag\text{-}pol}$ could be observed, with the appearance and subsequent increase in the intensity of cleavage intermediates. Three major species of cleavage intermediates of $Pr160^{gag\text{-}pol}$ were observed, with molecular weights of approximately 144, 105 and 97 kDa. In the absence of further analysis, the composition of these intermediates could not be determined. Some $Pr160^{gag\text{-}pol}$ was still detectable after 96 h, but it was reduced in intensity relative to earlier time points. In samples incubated at 4°C for 96 h, no evidence of cleavage of $Pr160^{gag\text{-}pol}$ or appearance of intermediates could be detected (data not shown).

During the 96-h incubation period there was neither a decrease in the intensity of $Pr55^{gag}$ nor an increase in p24 levels in virions derived from cells exposed to DG35 (Fig. 2). However, some intermediates in the processing of the precursor were present from time zero ($t = 0$), although the level of only one, a 49 kDa intermediate, appeared to increase during the incubation. These results suggest that while some processing of $Pr55^{gag}$ did occur even in the presence of DG35, it was neither efficient nor complete.

HIV-1 virions produced from chronically infected cells in the presence of 5 μM indinavir were investigated for evidence of cleavage of $Pr160^{gag\text{-}pol}$ and $Pr55^{gag}$ for various times up to 96 h (Fig. 3). Virions derived from cells not exposed to indinavir did not contain detectable $Pr160^{gag\text{-}pol}$, indicating that this precursor was rapidly processed in virions not exposed to the drug. In contrast, $Pr160^{gag\text{-}pol}$ was present at the time of immediate drug withdrawal ($t = 0$) in virions derived from indinavir-treated cells, similar to the effect seen with DG35-exposed virions in Fig. 2. Processing of $Pr160^{gag\text{-}pol}$ occurred within 24 h of removal of the drug from virions. By 96 h this precursor had almost disappeared entirely, to be replaced by cleavage intermediates.

Although the immunoblot shown in Fig. 3 is not quantative, the difference in protein bands seen in virions derived from cells not treated with indinavir (lane U) compared to those derived from treated cells at time zero (lane 0) suggests that some processing of $Pr55^{gag}$ did occur in drug-treated cells, since a 49 kDa intermediate was present at time zero in viruses derived from these cells but was absent in virions from untreated cells. Further processing of this intermediate ap-

peared to be inhibited until removal of the drug, following which a 42 kDa intermediate could be seen at 24 h. Beyond 24 h there did not appear to be significant processing of $Pr55^{gag}$ or its intermediates to p24 in virions exposed to indinavir.

A similar pattern of relatively efficient processing of $Pr160^{gag\text{-}pol}$ to its intermediates but less efficient processing of $Pr55^{gag}$ was observed when saquinavir was removed from virions produced from chronically infected cells exposed to this inhibitor (results not shown).

3.2. Infectivity of virions derived from H9/HTLV-IIIB cells exposed to PRIs

Alterations to precursor protein cleavages occurring in virions produced from cells incubated in the presence of DG35, indinavir or saquinavir suggested that the infectivity of these viruses may also be impaired. Consequently, the infectivity of virions from H9/HTLV-IIIB cells exposed to DG35 for 24, 48 and 72 h was investigated and compared to that from untreated cells. Considerable efforts were made to ensure that no residual inhibitor was present during these infectivity experiments (see Section 2). When chronically infected cells were exposed to the drug for 24 h (Fig. 4), virions derived from them were delayed in the time to the first appearance of HIV-1 specific CPE and the time to attainment of maximum titre compared to virions produced from cells not exposed to PRI (3 vs. 4 days, respectively). A similar effect was observed when cells were exposed to DG35 for longer periods (48 and 72 h), although this increased exposure to the drug did not appear to substantially alter the titre of virus derived from these cells compared to that of virus produced from untreated cells. However, irrespective of the duration of exposure, virions produced from DG35-treated cells appeared to be replicatively impaired. This reduced viral fitness was not only demonstrated by delayed time to first appearance of CPE and delayed attainment of maximum titre following removal of the drug, but also by the reduced extent of CPE within each dilution of virus derived from DG35-treated cells compared to that produced from untreated cells (results not shown).

Fig. 3. Proteolytic processing in HIV virions produced from H9/HTLV-IIIB cells in the presence of 5 μM indinavir. Culture supernates were pelleted through a 5% (w/v) sucrose cushion and then incubated at 37°C in 5% CO_2 for up to 96 h. Proteolytic processing was examined at various times post removal of inhibitor using a $Pr55^{gag}$/p24-specific MAb. U (untreated) represents virions derived from cells not exposed to indinavir.

Infectivity experiments were also carried out on virus derived from cells exposed to indinavir and saquinavir (Fig. 5). There was a delay in time to first appearance of CPE in cultures containing virus derived from these PRI-treated cells, and similar to that shown with DG35-exposed virus, virions with previous exposure to either inhibitor did not replicate as efficiently as demonstrated by the extent of CPE within each log dilution (results not shown). However, the titre 6 days after removal of drug did not vary markedly between virus derived from indinavir- or saquinavir-treated cells or untreated cells, irrespective of the duration of exposure (48 or 72 h) to the inhibitor.

3.3. Protein processing and infectivity of virions produced from cells post exposure and removal of indinavir

We compared the protein profiles and infectivity of virions produced from H9/HTLV-IIIB cells for up to 24 h following the removal of indinavir. H9/HTLV-IIIB cells were exposed to 5 μM of indinavir for 72 h, supernate collected and the cells then washed extensively to remove residual

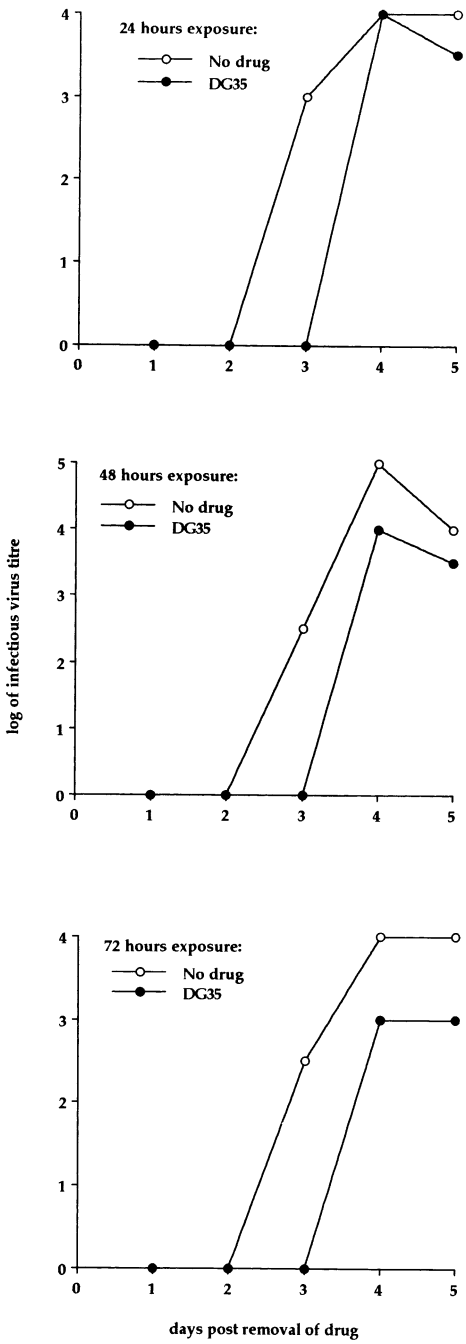

Fig. 4. Infectivity of HIV virions produced from H9/HTLV-IIIB cells previously exposed to 5 μM of DG35 for 24, 48 and 72 h. The infectivity was compared to virions derived from untreated cells.

extracellular drug. Supernate was also collected 12 and 24 h later from cells recultured in the presence of RF-10 medium. Parallel control experiments were performed with cells that had not been exposed to indinavir. Immediately following indinavir treatment ($t = 0$), virions produced from cells exposed to indinavir contained unprocessed $Pr160^{gag\text{-}pol}$ (Fig. 6). In contrast, at this time $Pr160^{gag\text{-}pol}$ was absent in virions produced from cells not exposed to indinavir. At this time,

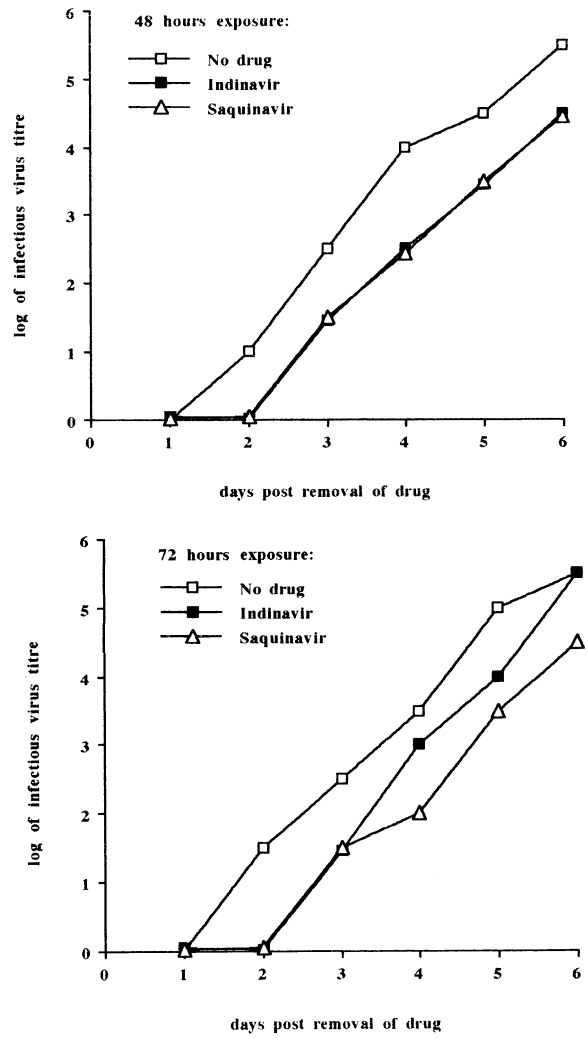

Fig. 5. Infectivity of HIV virions produced from H9/HTLV-IIIB cells previously exposed to 5 μM of indinavir or saquinavir for 48 and 72 h. Comparison to virions produced from untreated cells is shown.

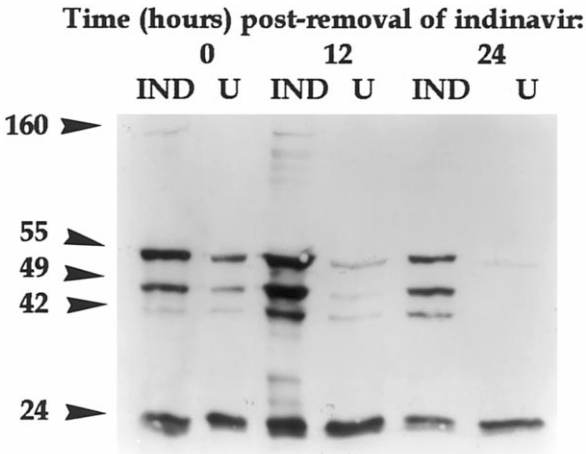

Fig. 6. HIV-1 specific proteins in virions derived from H9/HTLV-IIIB cells not exposed to inhibitor (untreated, (U)) or treated with 5 μM indinavir (IND) for 72 h then the drug removed. Cells were incubated in drug-free medium following either procedure. Immediately on removal of indinavir (0), then 12 and 24 h later, culture supernate was removed and virions concentrated and subjected to immunoblot analysis using a $Pr55^{gag}$/p24 MAb.

$Pr55^{gag}$ levels were considerably greater in virions derived from indinavir treated cells than from cells previously incubated in the absence of drug. After 12 and 24 h exposure to drug-free medium, cells that had never been exposed to indinavir continued to produce virions without detectable $Pr160^{gag\text{-}pol}$ but with evidence of conversion of $Pr55^{gag}$ to p24 through cleavage intermediates. Intermediates resulting from processing of $Pr160^{gag\text{-}pol}$ were present in virions 12 h after removal of indinavir from the cells. These viruses also contained substantially greater amounts of $Pr55^{gag}$ and its intermediates than virions derived from the corresponding previously untreated cells. After 24 h $Pr160^{gag\text{-}pol}$ was no longer detectable in virions derived from indinavir-treated or untreated cells, suggesting that its processing had proceeded to completion. However, compared to virions derived from cells that had not been exposed to indinavir, these viruses still contained elevated levels of $Pr55^{gag}$ and its intermediates, suggesting that inhibition of processing of this precursor by the PR was continuing.

A delay in first appearance of CPE and time to maximum titre in cultures containing virus derived from previously indinavir-treated cells compared to virus derived from untreated cells could be seen when indinavir was first removed from the test cultures (Fig. 7). There were only slight differences between the titres of these two sources of virions 12 h after removal of the drug, and by 24 h their replicative profiles were essentially identical.

4. Discussion

This study shows that virions produced from H9/HTLV-IIIB cells exposed to PRIs have immature protein profiles, and that following the removal of the PRI there may be a delay in protein processing of several hours. The effect was most evident at the level of $Pr55^{gag}$ processing, which rarely returned to normal. Similar effects on $Pr55^{gag}$ cleavage in vaccinia virus recombinants expressing HIV *gag-pol* genes (McQuade et al., 1990) or HIV-1 virions produced in the presence of other PRIs have been observed (Rayner et al., 1994; Vacca et al., 1994; Humphrey et al., 1997), and suggest that PRIs complex with the viral protease and become packaged within newly formed immature particles. The amount of PRI subsequently retained within the virions post-budding is likely to be sufficient to exert a continuing although not indefinite inhibitory effect on the processing of precursor proteins (Rayner et al., 1994). In contrast, cells exposed to PRI then washed clear of inhibitor and reincubated, produced infectious virus within 24 h. This result is in agreement with a previous study which showed that the inhibitory effects of saquinavir and the experimental PRI KNI-272 were reversed within 24 h (Humphrey et al., 1997).

The largely irreversible effect of PRIs on $Pr55^{gag}$ processing within viral particles was not observed to the same degree when processing of the $Pr160^{gag\text{-}pol}$ precursor was examined post-removal of drug. This effect occurred at 37°C but not at 4°C, suggesting that it was mediated by the HIV protease rather than through a general degradative effect. $Pr160^{gag\text{-}pol}$ was processed after

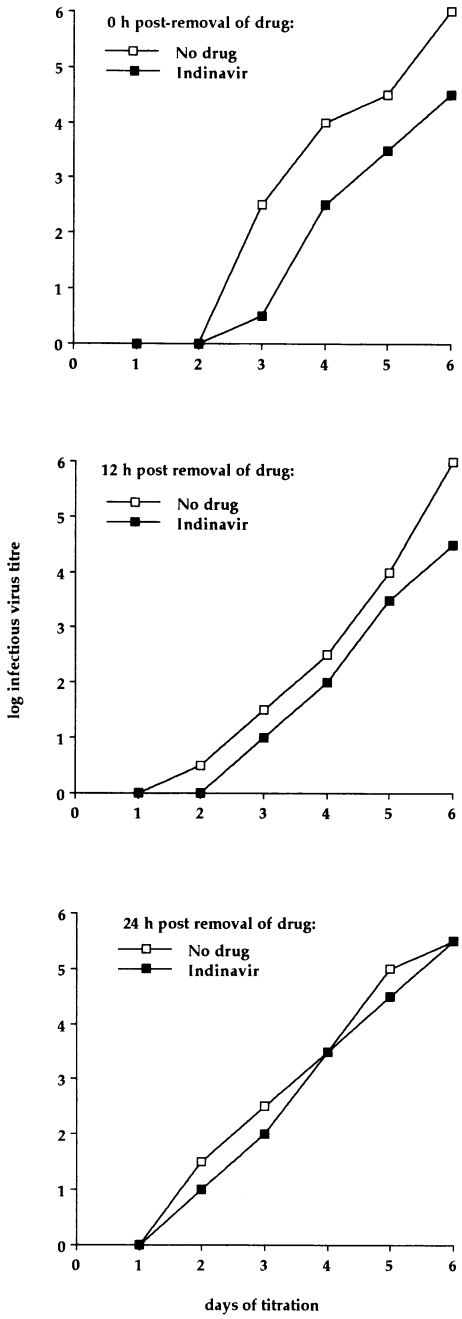

Fig. 7. Infectivity of HIV virions produced from H9/HTLV-IIIB cells exposed to indinavir for 72 h, then the drug removed. Immediately, and at 12 and 24 h post-removal of drug, an aliquot of culture supernate was titrated for infectivity as described.

a delay ranging from 4 to 8 h, an effect most likely due to the reversibility of the protease/inhibitor interaction. Although not specifically examined in this study, it is likely that the hydrophobicity of individual inhibitors is important in determining the time taken for reversal of this interaction with the PR. However, the reason why this reversibility is observed more at the level of $Pr160^{gag\text{-}pol}$ processing and less with $Pr55^{gag}$ processing remains unknown.

We found that virions derived from cells exposed to PRIs were able to regain some degree of infectivity in the absence of drug. However, they were delayed in the time to the first appearance of CPE and the time to attainment of maximum titre by approximately 24 h. In addition, when assessing the titre of virus produced from drug-treated or untreated cells, microscopic observation of cells infected at each dilution of the titration showed that less extensive HIV-specific CPE was present in cells infected by PRI-exposed virus. This suggests that the effect of PRIs on processing also influences the overall replicative fitness of HIV.

Previous studies have suggested that indinavir prevents incorporation of the mature ($Pr160^{gag\text{-}pol}$-derived) RT subunits p66 and p51 into viral particles (Vacca et al., 1994). However, our results suggest that rather than influencing the incorporation of these subunits into the virion, PRIs prevent processing of the precursor to its enzyme subunits within virions. The ability of $Pr160^{gag\text{-}pol}$ processing to recommence following PRI withdrawal may make available the enzymes essential for early events in HIV-1 replication, thereby enabling the virion to regain some degree of infectivity. However, for infectivity to be regained, the limited degree of processing of $Pr55^{gag}$ observed must nevertheless be sufficient to enable nuclear transport of the HIV preintegration complex, a process requiring the matrix protein p17 (Bukrinsky et al., 1993).

Our observations suggest that at the time of their maturation from PRI-treated cells, HIV-1 virions are likely to incorporate sufficient amounts of inhibitor to prevent processing of precursor proteins until reversal of the enzyme/inhibitor interaction. In the clinical situation such reversal is likely to occur when plasma drug con-

centrations decline below that required to completely inhibit replication (the IC_{100}). Pharmacological issues play a vital role in the success or failure of a treatment regimen, and several studies have demonstrated a clear correlation between the pharmacokinetic parameters of PRIs and their antiviral response (Sommadossi, 1998).

Currently, administration of the PRI indinavir requires three times-daily (td) doses. Studies with this drug have shown that twice-daily (bd) dosing (in combination with zidovudine and lamivudine) results in similar reductions in viral load to td dosing for the first 16 weeks. However, beyond this time (out to 24 weeks) td dosing produces a superior antiviral response (Protocol 069, Merck Sharp & Dohme, unpublished data). The early effect of bd versus td dosing may be a result of delayed maturation of virions during the process of enzyme/inhibitor reversibility which occurs in the presence of suboptimal drug concentrations. Failure to achieve a long term antiviral response may be due to unrelated factors, including tolerance and adherence issues and the development of resistance.

Studies on HIV-1 dynamics in vivo have revealed the life-span of HIV virions in blood to be approximately 8 h and that of productively infected cells to be, on average, 2.2 days (Perelson et al., 1996). Given that the maturation of $Pr160^{gag\text{-}pol}$ within virions produced from PRI-exposed cells takes up to 8 h, and that cells previously exposed to a PRI yield infectious virus within 24 h following removal of the drug, the greatest impact of inadequate PRI levels in the blood is likely to come from the ability of previously PRI-exposed cells to recover and produce infectious virus within their relatively short life-span.

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