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Positive and Negative Aspects of the Human Immunodeficiency Virus Protease: Development of Inhibitors versus Its Role in AIDS Pathogenesis

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INTRODUCTION

Proteases (PRs) function critically in a wide variety of cellular and viral processes by exhibiting limited substrate site specificity on their respective precursor proteins. For cellular systems, PRs like chymotrypsin, plasmin, and pepsin are produced after such cleavages, while for viral systems structural proteins as well as enzymes are formed (190, 282).

Further, many cellular PRs are now proving to be essential in abnormal processes related to cancer biology and tumorigenesis, e.g., metastasis and angiogenesis (93). PR inhibitors (PI) which block these processes may eventually prove to be as important as those used in viral diseases such as AIDS. Further, some PRs even help eukaryotic microorganisms such as yeast find mating partners, by secreting a PR that hydrolyzes α factor (14).

In the first part of this review, we focus on retroviral PRs, such as that of human immunodeficiency virus (HIV), and their inhibitors. General examples of viral PR that play a significant role in morphogenesis are hepatitis C virus PR (148), human adenovirus PR (83), and retroviral aspartyl PRs (70, 96, 282). The detailed function of each viral PR is different. For example, although the PR of adenovirus type 2 requires cysteine residues for both activation and catalysis (128), factors required for activation of HIV and other retroviral PRs from their Gag or Gag-Pol precursors are relatively unknown.

Recently, it has been suggested that incorporation and proper folding of the minor (10% of Gag) virion component cyclophilin A is necessary to allow PR dimerization and activation in HIV (341, 342). Alternatively, cyclophilin A may also play a role in viral entry (55). An older model for murine leukemia retroviruses (MLV) suggests that activation of a kinase that phosphorylates Gag or Gag-Pol precursors at specific amino acid residues might be important as well (223). It is also worthwhile to note that since aspartyl PRs function optimally in an acidic (pH 4.5 to 5.0) environment (282), there may be some specific events required for lowering of the pH in retroviral buds.

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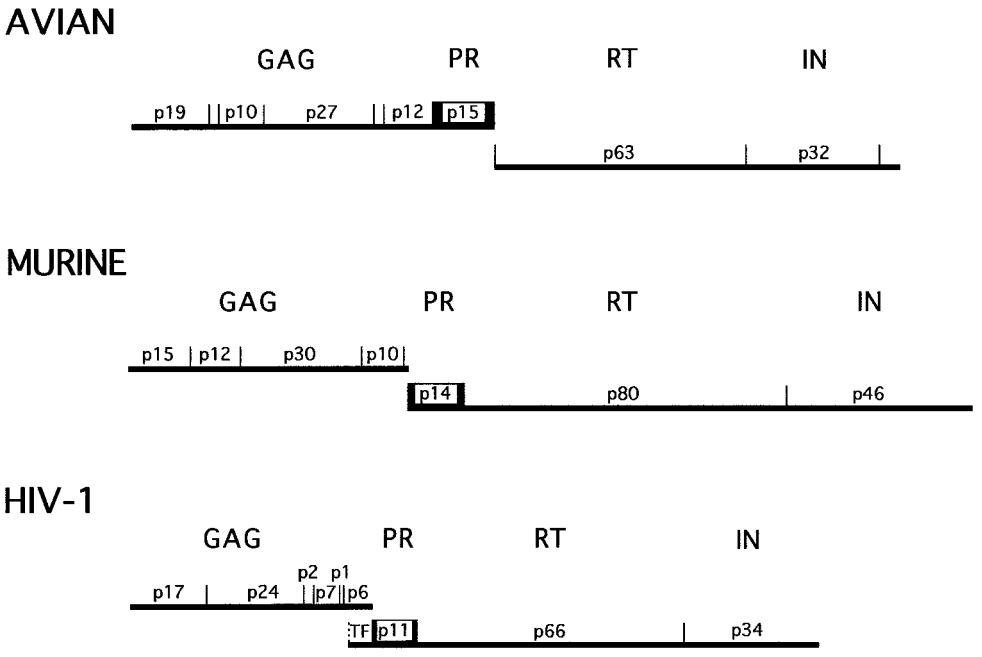

FIG. 1. Comparison of the gene structure of HIV-1 with those of avian and murine retroviruses. The genes for protease (*pro*) are boxed.

Based on predicted models from X-ray crystallization and nuclear magnetic resonance spectroscopy data for the capsid (CA) dimer and matrix (MA) trimer proteins, the assembly of Gag protein precursors (Pr55) of HIV has been suggested to simulate a cocked gun in an unfavorable, precursor conformation, awaiting PR activation before providing realignment of "immature" to "mature" Pr55 Gag cleaved proteins in the virus particles (162). This conformational change is consistent with recent models suggesting that the HIV PR also triggers a "myristyl switch" mechanism that alters exposure of the myristyl moiety from its tight (uncleaved $Pr55^{gag}$) to a loose (cleaved p17 MA) membrane-binding affinity (140, 333). Another aspect of the retroviral morphogenesis process, which is still unknown, involves the role of $Pr55^{gag}$ interactions with the cytoskeleton (224). Recently it was shown that actin molecules can be specifically associated with the nucleocapsid (NC) domain of $Pr55^{gag}$ (308, 366), while other studies have shown that vimentin filaments could be degraded by the HIV PR (149; E. Pichova, personal communication).

In these retroviral assembly models, viral PRs appear to act similarly to cellular aspartyl proenzymes, such as pepsinogen, in that they have to be activated through an autocatalytic mechanism. In cells, pepsin is activated during secretion from gastric cells, while for HIV, PR activation occurs in immature retroviral particles during and/or after budding from the outer plasma membrane. However, both classes of aspartyl PRs differ in a major structural way; HIV and other retroviral PRs are small homodimers of 10 to 14 kDa (282, 293), while pepsin and similar cellular analogs are larger monomers of 25 to 30 kDa that can fold into catalytically active forms (175, 207).

The first retroviral proteolytic enzymes to be studied were those from avian and murine leukosis virus systems. Specifically, von der Helm (55, 358) reported cleavage of avian $Pr76^{gag}$ by a p15 *gag*-encoded gel filtration fraction prepared by 6 M guanidine–HCl treatment of virions, while Yoshinaka and Luftig (371) reported a similar specific cleavage of the murine precursor protein $Pr65^{gag}$ by a non-*gag*-encoded gel filtration fraction obtained from Nonidet P-40 detergent-treated virions. Further, with MLV it was observed that cleavage was accompanied by a morphological transition of "immature" to "mature" particles, which correlated with the production of infectious virus (223, 371).

The MLV PR, like the HIV PR, is found only in small quantities in virions because it is derived from a Gag-Pol precursor located at the 5′ terminus of the *pol* gene (by convention, the PR coding domain of HIV is now named as a separate gene, *pro*, and thus one refers to the precursor Gag-Pro-Pol) (55). In contrast to MLV and HIV PRs, the avian leukosis virus PR is made in large (≥20-fold) excess, since it is located at the 3′ terminus of the *gag* gene (Fig. 1). The latter process appears to be unusual, since not only MLV and HIV but also other retroviral PRs, such as feline leukemia virus, feline immunodeficiency virus, and mouse mammary tumor virus, are produced in small quantities. In addition, as noted previously (256), the preference of MLV and HIV PRs for predominant cleavage between Tyr-Pro and Phe-Pro residues on retroviral precursors contrasts with the inefficient hydrolysis of these sites by cellular PRs, including pepsin (141). This viral specificity accounts in part for the success of PIs in treating AIDS patients.

In this review, we will discuss the role of the retroviral PR in HIV morphogenesis, as well as the potential role of a class of specific PR and accessory gene-mutated particles in enhancing AIDS pathogenesis. As of now, infection of adults who live in most developed countries, including the United States and Europe, occurs predominantly with HIV of the major (M) group, clade B, and leads to AIDS within about 10 to 12 years in the absence of antiretroviral therapy. Specifically, about 20% of such individuals develop AIDS in 5 years, while <2% are long-term (>12 years) nonprogressors (LTNP) (101). For infected individuals, the HIV PR and reverse transcriptase (RT) have been excellent targets for antiviral therapy, since they are both crucial to the formation of infectious, properly assembled particles (69, 70, 256, 331).

It has been well documented over the past several years by several groups that there is a daily HIV-infected versus healthy

$CD4^+$ T-cell replacement war occurring in the patient, so that infected, dying cells continually are replaced (54, 142, 294, 295, 363; D. Havlir, S. Eastman, and D. D. Richman, Abstr. 2nd Natl. Conf. Hum. Retroviruses Relat. Infect., abstr. 299, 1995). In addition, as the disease progresses, there is an increase in the number of latently infected cells, producing defective HIV particles (35, 48, 49, 155). This is due in part to the high error rate of reverse transcription (161), leading eventually to the formation of mutations in critical genes. Although definitive proof of the specific role played by the increased number of defective particles (299) in progression to AIDS is lacking, we believe, based on in vitro experiments with a specific class of defective particles, that similar particles, possibly derived during candidiasis infection, may be involved (164–166; P. Hickman, P. L. Fidel, Jr., J. Leigh, R. Mera, and R. B. Luftig, Abstr. 19th ASV Meet., abstr. 145, 2000).

AIDS pathogenesis can grossly be characterized as a progressive loss of immune cells (100, 211). It can be explained in part by direct killing of host cells due to production and secretion in tissue media of HIV gene products, e.g., gp120 and Tat. Also, defective particles may lead to direct T-cell killing, as well as bystander apoptosis (164, 165). AIDS pathogenesis is further complicated by the HIV life cycle, which varies greatly depending on (i) which HIV strains or host cell genotypes are involved; (ii) which coreceptors are used, leading to non-syncytium-inducing (NSI) or syncytium-inducing (SI), as well as slow/low or rapid/high, viruses; (iii) the temporal production of macrophage- or T-tropic viruses; and, finally, (iv) the physiological state of the host cell, e.g., resting versus activated T cells or naive versus memory T cells.

Thus, there are clearly complex combinations of viruses and host cells interacting in HIV-infected patients, which can cause both rapid syncytium formation and/or cell death in $CD4^+$ T cells. This immediately results in short-lived infected cells that are replaced by healthy T cells. Additionally, infection of a small number of quiescent cells may cause establishment of a persistent or latent HIV infection that apparently results in a small percentage of long-lived infected cells.

Further, a major indirect mechanism that accounts for the loss of immune cells is apoptosis, which can be induced by various secreted products or defective particles resulting from HIV infection. Apoptosis can be induced in immune cells of both the $CD4^+$ and $CD8^+$ T-cell lineage from uninfected patients (105, 127, 138, 164, 260) or as an activation-dependent event after mitogenic stimulation (126, 130).

Another level of complexity in understanding AIDS pathogenesis is that clinical staging of the disease appears to be significantly correlated with virus load (39, 245). Although viral replication occurs in HIV carriers throughout all clinical stages (95, 289, 299), as noted above most of the plasma-derived HIV is produced in short-lived infected cells with a half-life of only a few days (142, 294, 295, 363) and only a small portion of cells carrying the HIV genome can establish an inducible latent infection (35, 95, 292). The importance of latent reservoirs for HIV was recognized by studies with potent antiretroviral drugs that block new rounds of infection and produce rapid dramatic drops in plasma viremia (23, 53, 143).

Currently, combinatorial inhibitors with different protein targets are used in highly active antiretroviral therapy (HAART), e.g., two RT nucleoside inhibitors and one PI. The former inhibits HIV replication in newly infected cells but shows no effect on virus production from persistently or latently infected cells, while the latter inhibits the production of infectious HIV particles from both acutely and persistently or latently infected cells. Possibly due to the high mutation rate of HIV RT, mutations can also arise in other genes, such as the PR, that in turn potentially can generate large amounts of "immature"-appearing defective particles. Using an in vitro assay system, we have not found (with four different PI drugs) that PI-produced "immature" particles are any more SI than is the wild-type virus itself (11). We believe that this is a very positive result, in terms of PI therapy. In contrast, a certain class of HIV defective particles called L-2 particles, which have mutations in the PR gene and three additional genes (*nef*, *gp41*, and *vpr*) (10), are highly SI in vitro when exposed to uninfected T-cells (11, 279).

In addition to the accumulation of defective particles, large amounts of free HIV proteins continue to be generated from both acutely and chronically infected cells. The role of these soluble HIV proteins, as well as of mutant and defective particles and immune products stimulated by them (137), needs to be considered as factors in AIDS pathogenesis (155, 222).

Since this is a rapidly expanding field, we have chosen July 2000 as the cutoff date for references cited in this review.

TABLE 1. Potential use of protease inhibitors in HAART

FDA-approved HIV-1 protease inhibitors[a]		Recommended companion nucleoside inhibitors for triple therapy[b]
Name (synonym)	Company (code)	
Saquinavir (Invirase or Fortovase)	Roche (Ro-31-8959)	AZT + ddI
Ritonavir (Norvir)	Abbott (ABT-538)	d4T + ddI
Indinavir (Crixivan)	Merck (MK-639,L735)	AZT + ddC
Nelfinavir (Viracept)	Agouron (AG-1343)	AZT + 3TC

[a] Some compounds not yet FDA approved but in the pipeline are DMP-450 (analog of DMP-323 but with good oral bioavailability and a low K_i of 0.3 nM), VX-470 (141W94), and KNI-272 (tight-binding transition state analog containing allophenylnorstatine [12]). Recently, amprenavir (Agenerase) was FDA approved, and the information is described in the text.

[b] This table is from a list that was compiled based on recommendations by several pharmaceutical manufacturers at the 1999 ICAAC Meeting in San Francisco. As noted at the July 2000 AIDS World Congress (D. Moodley, personal communication), nevirapine, a non-nucleoside reverse transcriptase inhibitor (NNRTI), is recommended alone in one dose during labor, one at birth, and one after birth as a low-cost replacement for the more complex AZT treatment. Also, there is currently some controversy among clinicians as to whether efavirenz (Sustiva), another NNRTI, should be used first before PIs, due to its simplicity of dosage (one pill a day because of its long half-life [40 to 55 h]) and its prevention of problems with lipodystrophy, saving the PIs for a time when RT inhibitors fail. Thus, there is some flux about which HIV drugs are optimal, and we have provided only a potential snapshot in this table.

HIV PROTEASE INHIBITORS AND THEIR USE IN THERAPY

The recent development of HAART (G. Moyle, Editorial, Curr. Opin. Infect. Dis. **12**:1–4, 1999), also recently called potent antiretroviral therapy (PART) (133), combined with the use of viral load diagnosis led last year for the first time in over a decade to a decrease in the number of AIDS-related deaths in the United States (101). Due to this, certain recently Food and Drug Administration (FDA)-approved drugs developed for treatment of opportunistic infections such as cytomegalovirus retinitis may be less necessary than expected in areas with high AIDS incidence, such as New Orleans (M. Hagensee, personal communication). HAART, as noted above, involves the use of two nucleoside inhibitors and one PI (Table 1), although one recent clinical trial suggests that perhaps a converse treatment, with two PIs plus one RT inhibitor, may be even more effective (101). HAART treatment, however, is not for everyone and can be tolerated or used effectively by only about 50% of AIDS patients. Side effects include

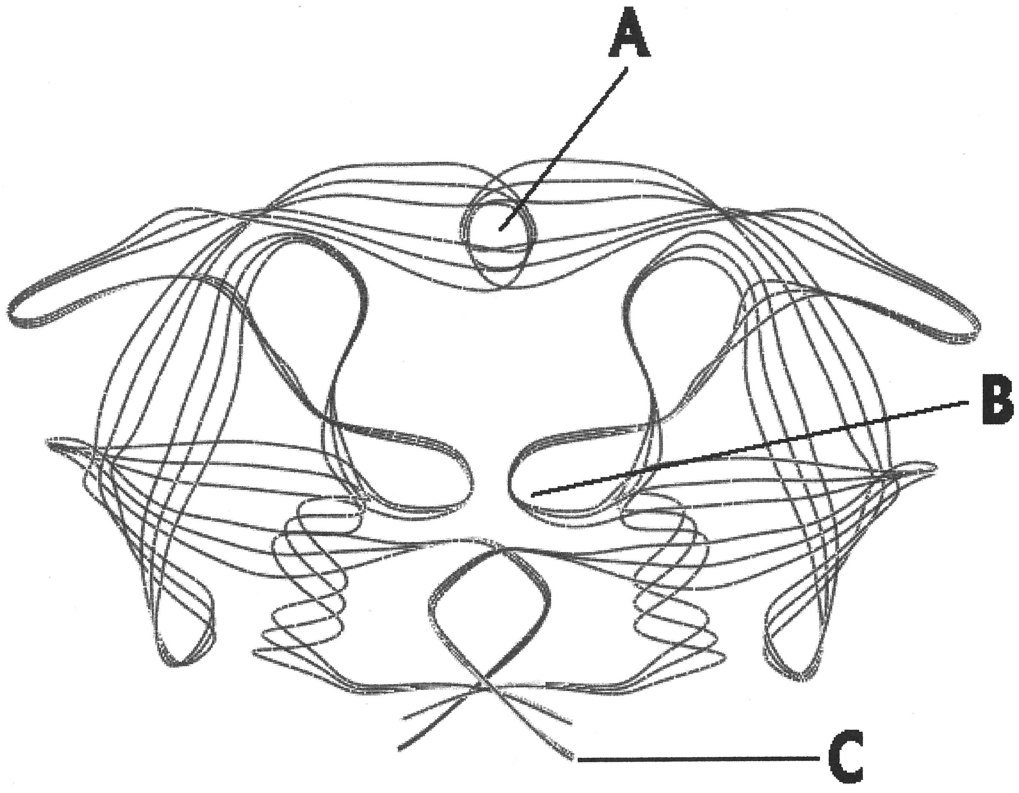

FIG. 2. X-ray crystallographic structure of HIV-1 showing the flap (A), active site (B), and dimer binding (C) regions.

a rise in the level of blood sugar, an increased risk of diabetes, and the formation of unusual body fat deposits (218, 236).

An additional concern about HAART is the eventual selection of nucleoside inhibitor- and PI-resistant HIV strains (17, 19, 28–30, 54, 58, 59, 66, 75, 132, 167; S. Deeks, R. Grant, C. Horton, N. Simmonds, S. Follansbee, and S. Eastman, Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother, abstr. I-205, 1997) which may occur in patients that tolerate and most benefit from the treatment (33). Nevertheless, triple therapy has given a large number of AIDS patients a new lease on life (135), and searches are under way for alternative PI and RT inhibitors, as well as vaccines (144), including unexpected sources such as a novel low-molecular-weight (M_r, 447) cytochalasin isolated in fermentations from a bark-inhabiting fungus (84) or oyster protein peptides (205). A plethora of additional, chemically synthesized potentially new PI have also been recently described (93; Program 12th Int. Conf. Antiviral Res., see abstr. A31–A33 and A40–A46, 1999).

Background of Retroviral Proteases

Historically, pepstatin and its dihydroxystere analogs were first identified as inhibitors of avian and murine retroviral aspartyl PRs, about 12 years ago (26, 172, 282, 309). The genome regions and molecular size of PR for avian (p15, avian sarcoma/leukosis virus), murine (p14, MLV), and human (p11, HIV) viruses all differ (Fig. 1).

Specifically, HIV PR is encoded in the *pol* gene as a homodimer of 99 amino acids, with a molecular mass of about 10 kDa (175, 282). This enzyme is responsible for all of the limited cleavages observed during morphogenesis and permits the separation of Gag, Gag-Pol, and even Nef precursor proteins into their respective, polypeptide components; e.g., Gag is converted to MA, CA, and NC, while Gag-Pol is converted to PR, RT, and integrase (IN). It is even thought that enhanced cleavage of the PR may be provided by NC interactions (326).

The HIV PR aspartyl "fireman's grip" structure (Fig. 2, area B) results in sensitivity to dihydroxystere analog inhibitors, which is similar to but different from those found among inhibitors of cellular and aspartic PRs. Specifically, these compounds block cleavage of the substrate by binding to several residues at the active site, which includes the highly conserved sequence Leu-(Leu/Val)-Asp-Thr-Asp-Thr-Gly-Ala-Asp-Lys (207, 282, 331). Asp, Thr, and Gly are the most essential amino acids for activity.

Another way to look at the HIV PR is to consider how it functions in the timing of viral morphogenesis. In all cases, "immature," uncleaved particles appear (Fig. 3A), and after cleavage by the PR, they appear as "mature" infectious particles (Fig. 3B). In the viral life cycle, the retroviral PR must remain inactive in its precursor form until virus assembly is initiated; otherwise the >1,000 Gag precursors per virion will be abnormally cleaved (199). As noted previously, site-specific mutagenesis has demonstrated that the enzyme is absolutely required for replication of mature, infectious virions; noninfectious particles, containing uncleaved Gag and Gag-Pol polyproteins, are produced if the enzyme is inactivated (191, 219, 231).

Accordingly, an effort was made by many laboratories to use dihdroxystere analogs, such as pepstatin-based compounds, to inhibit avian and murine retroviral assembly as a starting point for the development of HIV-specific PI (256).

Overview of HIV Protease Inhibitors

Currently, although HIV PI appeared among the most effective antiviral agents used to treat HIV infection and AIDS

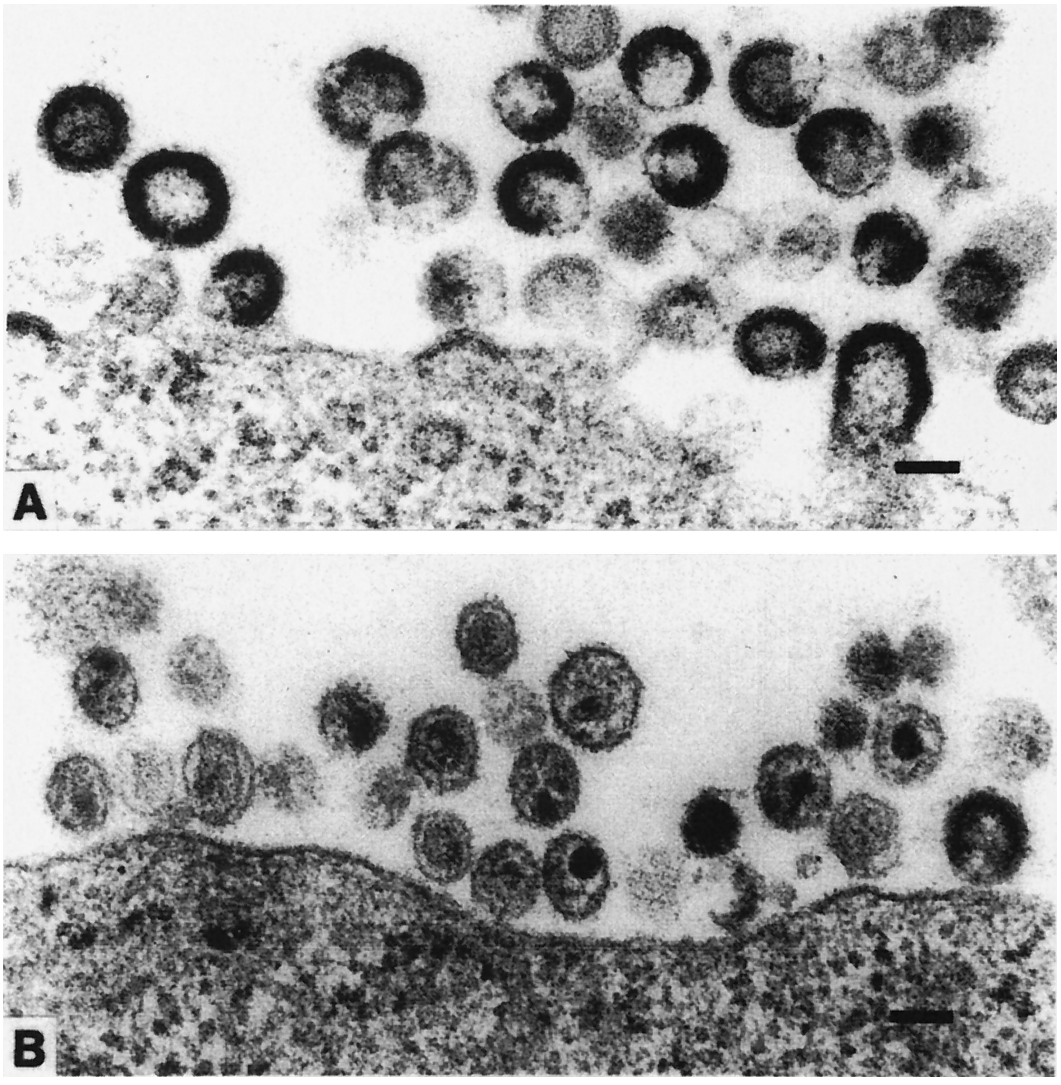

FIG. 3. Electron micrographs of immature (A) and mature (B) HIV-1 particles. Kindly provided by Toshiyuki Goto, Department of Microbiology, Osaka Medical College, Takatsuki, Japan.

when initially used in vitro and in monotherapy (69, 72), PR-resistant mutants arose and specific PI varied in their effectiveness when used alone or in combination (UCSF Website; Deeks et al., 37th ICAAC). Thus, PI were found to be clinically more efficient when used in combination therapy with certain RT inhibitors (Table 1). PI basically differ from the anti-RT drugs, which inhibit virus production only in acutely infected cells and do not affect virus production in persistently infected cells or activated HIV postintegrated quiescently infected cells.

HIV particles produced in the presence of PI can undergo Gag polyprotein cleavage following removal of the PI, but this process does not appear to occur at a high enough level to restore infectivity in the majority of particles (152).

PI-mediated treatment of HIV infection in SCID mice implanted with human fetal thymus and liver tissue is not completely effective, although treated mice have markedly lower viral loads in the region of the implants and spleens (298). Also, a significant correlation has been found between an increase in viral neutralizing-antibody titer and treatment with PI alone or in combination with zidovudine (AZT) but not with AZT alone (322).

Characteristics of Successful HIV Protease Inhibitors

For HIV PI to be successfully used as drugs in patients, they should be effective in vitro at the nanomolar level, have good oral bioavailability, and exhibit minimal side effects against human cellular aspartyl PR. Several excellent reviews have been written to address this area (70, 368). Genetic and X-ray crystallographic studies (27) have been performed for the HIV PR alone (Fig. 2) or with inhibitors in place, and an extensive database is available (368). This has allowed dissection of the inhibition process into three discrete structural features. (i) The first is modification of amino acid residues at the PR substrate binding pocket (Fig. 2B). This involves replacement of substrate scissile bonds by nonscissile bonds in the inhibitor; use of peptidomimetics has permitted this to be the starting point for design of most first-generation PI. (ii) The second is alteration of the PR flap (325) (Fig. 2A). (iii) The final feature is alteration of the stereochemistry of peptide bond replacements in the active site. Thus far, four major PI have been approved by the FDA and are being used in clinical treatments. Many other PI are in clinical trials or in earlier stages of

development. The first four FDA-approved drugs are described in greater detail here.

Saquinavir (RO 31-8959, Invirase), developed by Roche, was the first FDA-approved PI. Since the HIV PR readily cleaves highly hydrophobic scissile, e.g., Tyr(Phe)-Pro, amino acid residues in Gag and Gag-Pol precursors, a strategy was developed to create nonscissile analogs of this dipeptide with five additional binding amino acid residues. Saquinavir has a K_i of 0.12 nM at pH 5.5 against the HIV PR, and it was shown by X-ray crystallography that inhibitors bound in an extended conformation, preserving the homodimeric nature of this enzyme. Recently, Fortivase, a new formulation of saquinavir in soft gelatin capsules, has shown enhanced oral bioavailability. This also provides for higher drug exposure and antiviral activity than the hard-tablet form. Both compounds are recommended in HAART with AZT and dideoxyinosine (ddI), and both exhibit excellent evidence of clinical benefit and sustained suppression of the plasma viral load (123).

In addition to saquinavir, three other PI (ritonavir, indinavir, and nelfinavir) have been developed, and more are in the pipeline (70, 123, 259). As mentioned above, all four drugs are currently recommended in HAART for treatment of established HIV infection (Table 1).

In the development of ritonavir, it is of interest that Abbott originally had developed the synthetic compound A-77003, which, based on in vitro studies, appeared to be an excellent PI; this was related to its mirror-image dimeric structural symmetry, ease of crystallization, and high level of antiviral activity (96). However, A-77003 had low oral bioavailability and resistant HIV strains appeared rapidly in tissue culture. A new direction was thus taken when it appeared that symmetric PR inhibitors could bind the HIV PR in an asymmetric fashion (89, 368, 369). This led to a process where shorter, more orally bioavailable, asymmetric analogs of A-77003, such as ritonavir, were constructed. Ritonavir inhibits the cytochrome P-450 system, making its levels in blood very stable and also stabilizing the levels of other PI, such as saquinavir, in blood (178).

Indinavir (Crixivan) was synthesized by the Merck group, using a similar strategy to that taken by Roche involving nonscissile hydroxyethylene bond analogs. This approach was combined with the use of X-ray crystallography and molecular modeling to test leading compounds. Indinavir, with a K_i of 0.52 nM in vitro against the HIV PR, was synthesized as a first-generation PI. Again, as with other PI, there was high specificity for retroviral versus cellular (e.g., renin) enzymes. Indinavir inhibited the replication of HIV-infected cells at 25 nM, was orally bioavailable, caused a major decrease in plasma viral RNA load, and led to an increase in the number of $CD4^+$ cells (91).

Nelfinavir mesylate (Viracept), an FDA-approved PI, was synthesized by the Agouron group using a totally different approach from that taken with other PI. It contains a DIQ (decahydroisoquinoline) group like saquinavir, which permits tight binding to the water molecule near the D-T-G active site. Nelfinavir mesylate has become a major PI in the United States, in part because it was the first one approved for the treatment of pediatric AIDS (70). An oddity, in some ways, is that none of the above HIV PI inhibit the similarly conformed human T-cell leukemia virus type 1 HTLV-1 aspartyl PR; this may be related to differences in substrate subsite (S_1 and S_1') recognition (297).

Recently, amprenavir (Agenerase, 141-W94, VX-478) was also approved for treatment of HIV infection in the United States. One potential use for amprenavir is as salvage therapy in patients for whom treatment that includes one (or more) of the above four PI has failed. The presence of the N88S mutation and associated amprenavir hypersensitivity may be useful in predicting an improved clinical response to amprenavir salvage therapy (385). Also, treatment with amprenavir plus AZT, and lamivudine, was shown to reduce the levels of HIV RNA significantly more than did amprenavir monotherapy (261).

Some other new approaches to development of HIV PI involve (i) modeling studies with D-amino acids introduced into the PR P1 and P1′ positions of reduced bond inhibitors (70), (ii) C-2 symmetry-based inhibitors targeted against new variants of A-770003 (96), and (iii) placement of a trisubstituted cyclopropane-derived peptidomimetic into a known HIV PI to stabilize the inhibitor extended structure (96).

Emergence of Resistant Mutants

There is evidence of resistance to PI in some patients under HAART after 1 to 2 years, depending in part on location; e.g., in New Orleans, La., this has taken longer to appear (Hagensee, personal communication), while in Costa Rica, although patients are generally doing well on triple therapy, resistance to some PI appeared relatively quickly, attributable in part to earlier monotherapy or noncompliance (K. Visona, personal communication). Resistance, as with tuberculosis, involves selection in the patient of preexisting or newly formed variants containing amino acid replacements at conserved sites such as near the PR active site, the flap, as well as at other sites which decrease binding of the PI (Fig. 2). There is also a certain level of cross-resistance between different groups of PI (86; Deeks et al., 37th ICAAC). Interestingly, as with the RT, one can find second-site mutants that restore enzyme activity (31, 281).

The emergence of HIV variants with a reduced sensitivity to PI also occurs most rapidly when treatment fails to achieve a sufficiently profound suppression of viral replication (D. J. Kempf, R. Rode, Y. Xu, E. Sun, A. Japour, S. Danner, C. Boucher, J. Leonard, and A. Molla, Abstr. Int. Workshop HIV Drug Resist. Treat. Strategies Eradication, abstr. 62, 1997). The evolution of HIV toward high-level resistance to PI is thus the result of a gradual accumulation of those resistance mutations in the PR (58, 59, 96, 253, 258, 311). Most of the HIV PR mutations associated with decreased sensitivity to PI have been identified, e.g., L10I, K20R, M36I, M46I, F53L, L63P, A71V, V82A, D30N, I84V, I54V, L90M, and G48V (58, 59, 143, 167, 234, 237, 290, 291, 312). These mutations are usually not found in HIV isolates which have not been previously exposed to PI (15, 197, 204, 263, 267, 367).

Recently, Rouzine and Coffin (317) proposed a novel mechanism of evolution for the HIV *pro* gene. Variation in *pro* was shown by analyzing a database of 213 sequences restricted to rare variable bases which are highly diverse and differ in location among individuals. The average intrapatient distance per individual variable site is similar for synonymous and nonsynonymous sites, although synonymous sites are twice as abundant. The latter observation excludes selection for diversity as an important, permanently acting factor in the evolution of *pro* and leaves purifying selection as the only kind of selection. Based on this, a model of evolution was proposed in which there are two possible explanations for the high mutant frequency: (i) the frequency of coinfection in the natural host population may be quite low, and/or (ii) a strong variation of the best-adapted sequence between individuals could be caused by a combination of an immune response present in early infection and coselection.

Studies using in vitro cell cultures in the presence of a PI showed that PI-resistant variants have mutations that are often

located close to the enzyme active site (28, 66, 132, 167, 217, 316, 321). In addition, the impairment of HIV replication which results from selection of these resistance mutations can be partially compensated for by secondary mutations that are usually located outside the PR active site (28, 58, 167, 272, 316) or in *trans* (206). Furthermore, for some HIV variants obtained in culture in the presence of a PI, it has been found that adaptive changes that partly correct resistance-associated loss of HIV infectivity can emerge outside the PR coding sequence, e.g., in PR cleavage sites within the Gag precursor protein, such as p7-p1 and p1–p6 (66, 281; A. Carrillo, H. Sham, D. Norbeck, D. Kempf, W. Kohlbrenner, J. Plattner, J. Leonard, and A. Molla, Abstr. Fourth Conf. Retroviruses Opportunistic Infect., p. 462, 1997). Surprisingly, the p1–p6 mutant sequence found in HIV PI resistance variants can promote ribosomal frameshifting both in vitro and in virus-expressing cells (87).

It should be reiterated that although HAART (or PART) is currently the treatment of choice, PI had been initially used in monotherapy trials, where HIV populations in plasma remained genetically constant prior to drug treatment and viremia decreased 10- to 100-fold and during the 1 to 2 weeks following initiation of therapy. Rapid plasma viremia rebounds frequently occurred, and this was associated with either noncompliance or the rapid emergence of drug-resistant virus (75). Thus, as noted above, the switch was made from monotherapy to triple antiviral therapy. Further, this permitted the use of lower PI levels than with monotherapy (23, 312). Another interesting mechanism to explain the positive effectiveness of PI in HAART is that PI seem to be effective at both early and late stages of infection (241, 266); however, there is still controversy about the role of the PR in early virus replication (168).

Secretory Leukocyte Protease Inhibitor

The virtual absence of oral transmission of HIV (114, 188, 254) and reports of antiviral activity in human saliva (6, 21, 63, 111, 119, 229, 313) led to the identification of secretory leukocyte PI (SLPI) as a potent antiviral factor in saliva (239, 240, 358, 360). SLPI is also found in certain other mucous secretions, e.g., cervical and bronchial secretions (198). SLPI does not appear to act on the virus directly (240); instead, its inhibitory activity is most probably due to interaction with the host cell. SLPI most probably inhibits a step of viral infection that occurs after virus binding but before reverse transcription (240). Thus, it differs from the classic PI involved in blocking viral maturation. SLPI, along with other salivary factors with potential HIV-inhibitory activity, has recently been reviewed (328).

A recent new aspect of the role of the HIV PR in the oral cavity has been reported (P. Hickman, P. Fidel, J. Leigh, R. Mera, and R. B. Luftig, Abstr. 19th ASV Meet., abstr. 145, 2000; R. Luftig, P. Hickman, J. Leigh, R. Mera, P. L. Fidel, Jr., A. Mock, and W. Gallaher, Abstr. 99th. Gen. Meet. Am. Soc. Microbiol. 1999, abstr. T-14, p. 630, 1999). In AIDS-infected individuals with oral pharyngeal candidiasis, a common opportunistic infection, one or more yeast aspartyl proteinases are present in the oral cavity. Most of these are secreted by the yeast *Candida albicans* (265) and may play an as yet undefined role in the selection of certain classes of HIV PR mutations (Luftig et al., Abstr. 99th Gen. Meet. Am. Soc. Microbiol.). Further, it has been shown that certain PIs can prevent recurrent oral pharyngeal candidiasis (40, 131), and this may indirectly affect the oral flora, including HIV, as well as the course of viral and/or yeast pathogenesis.

ROLE OF HIV-1 PROTEASE AND OTHER GENES IN ACUTE AND PERSISTENT INFECTIONS

Viral Accessory Genes and Their Role in Overall HIV Production

In the previous section, we have presented in detail the role of the HIV PR in virus production. Even partial inhibition of the PR gene can lead to formation of noninfectious particles, which may be relevant to disease. Further, as we show in the next section, there can also be interactions between mutations in the PR and several accessory genes, such as *nef* and *vpr*, to create defective particles that may play a significant role in AIDS pathogenesis. Toward this end, we briefly review the role of accessory genes in HIV production.

The HIV genome consists of structural (*gag*, *pol*, and *env*), regulatory (*tat* and *rev*), and accessory (*vif*, *vpr*, *vpu*, and *nef*) genes. Both structural and regulatory genes are essential for replication in vitro, while accessory genes are considered nonessential for replication in tissue culture, because each one can be deleted without destroying virus replication in different cell lines (1, 71, 110, 189, 221, 277, 340, 348).

The assignment of molecular functions to accessory genes was initially based on in vitro experiments with cloned viral mutants. *vif* and *vpu* are required for the efficient production of infectious virus particles in certain cell lines (106, 338, 339, 349), while *vpr* has a slight effect on virus replication in cultured T-cell lines (277). Further, Vpr is located in virions (43, 104) as a serine phosphoprotein in stoichiometric amounts with respect to Gag. It is phosphorylated in several HIV-infected cell lines used by different groups, and the serine phosphorylation at position 79 of Vpr appears important for arresting cells in the G_2 phase of the cell cycle (L. Ratner, personal communication; Y. Zhou and L. Ratner, Abstr. 18th Annu. ASV Meet., abstr. 150, 1999). There is still controversy about whether Vif (81) is assembled in particles.

The *nef* gene, located at the 3′ end of the viral genome (partially overlapping the U3 region of the 3′ long terminal repeat [LTR]), encodes a membrane-associated myristylated protein synthesized from early, multiply spliced mRNA transcripts and displays cytotocix activity (67) or can act as a transcriptional repressor of the HIV-1 LTR (1). The Nef protein also acts as a positive factor serving to augment viral replication, most notably in primary lymphocytes and macrophages (2, 79, 134, 182, 247, 334, 348, 350). Further, Nef can be cleaved in vitro between residues 57 and 58 by the HIV PR, generating two Nef polypeptides (115, 120). The myristylated Nef protein itself is incorporated into HIV virions (34, 286, 364), with 60 to 80% of incorporated Nef cleaved by the HIV PR. However, a recent paper suggests that PR cleavage of the Nef protein in virions may not be necessary for its infectivity (249).

Viral Factors Involved in the HIV Life Cycle

HIV is strongly cytopathic for $CD4^+$ T lymphocytes in a variety of tissue culture systems (209). However, biological properties of HIV, such as its replication rate, cell tropism, and cytopathogenicity, can vary during the course of infection (8, 45, 346, 347). In the early asymptomatic phase, preferentially macrophage-tropic, NSI variants of HIV predominate, whereas later in the course of the infection, more T-cell-tropic variants appear; >50% of AIDS cases are associated with the emergence of SI variants (61, 323, 356, 384).

In vitro systems using $CD4^+$ T cells infected with recombinant HIV variants containing single or multiple accessory gene (*vif*, *vpr*, and *vpu*) mutations have shown a loss of cytopatho-

genicity leading to viral persistence (184–187, 273). Also, the same nonsense mutations in *vpr* that can be observed to arise naturally in peripheral blood mononuclear cells (PBMCs) of HIV-seropositive individuals during the early stages of infection increase in number with increasing in vitro serial passage of wild-type virus (268, 274). Further, deletion mutations due to a putative misalignment mechanism have been detected over a region spanning *vif* and *vpr* open reading frames after extensive in vitro serial passage of wild-type virus (269). Similarly, a high percentage of extensively deleted defective HIV genomes generated by such a misalignment mechanism have also been identified in PBMCs from HIV-infected individuals (320).

In summary, a wide spectrum of mutations generating multiple defects in accessory genes during HIV replication appear to correlate in vitro and in vivo with viral persistence and loss of cytopathogenicity. This conclusion is consistent with findings from other laboratories that nondefective Vpr plays a role in cell cycle arrest and apoptosis, as well as in nuclear transport of the preintegration complex (139, 226, 314, 359). It should also be noted that a persistent infection with highly passaged (≥50 passages) *nef* frameshift mutant viruses could not be established, using the cell system described above (117, 274). Thus, the need for wild-type *nef*, in addition to mutations in other accessory genes, appears to be associated with establishment of persistent HIV infection in vitro.

Host Factors Involved in the HIV Life Cycle

Host factors have been known for some time to alter the rate of HIV progression in individuals, including secretion of cytokines; e.g., proinflammatory cytokines like tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and IL-6 upregulate HIV replication, while transforming growth factor β and IL-10 lead to its downregulation (55, 100). Also, about 3 years ago, an extremely important observation was made that HIV tropism is determined predominantly by second-receptor usage (103, 375). As shown below, this has led to further refinements in antiviral therapy.

The study of coreceptors has been a dynamic area. Many groups have shown that as many as 12 coreceptors, CCR2b, CCR3, CCR5, CCR8, CXCR4, CX3CR1, BONZO/STRL33/Tymstr, BOB/GPR15, GPR1, APJ, HCMV-US28, and BLTR, can function in HIV infection or syncytium formation in tissue culture (3, 4, 47, 52, 73, 76, 85, 88, 92, 99, 103, 112, 138, 147, 215, 220, 284, 300, 319, 330). Recent work has shown that the β chemokine receptor 5 (CCR5) and chemokine receptor 4 (CXCR4) are the two major coreceptors for infection by primary isolates (18, 376, 380). This cytokine family of receptors bind conformationally modified surface glycoproteins (SU) from the HIV M group of subtypes A, B, C, D, and E, as well as the outlier (O) group. Other chemokine receptors play a lesser role in facilitating viral entry into stimulated PBMCs (381). Macrophage-tropic NSI variants use CCR5, T-cell line-tropic SI variants use CXCR4, and primary SI variants can use both receptors (4, 25, 47, 76, 85, 88, 103, 329, 377). Based on the chemokine responsiveness of PBMCs, CXCR4 is thought to be abundantly expressed while CCR5 expression on PBMCs is low (24, 275). This result is consistent with the previously observed broader T-cell host range of SI variants (109).

There is no apparent geographical clustering of CCR5 polymorphism in different ethnic populations, suggesting that CCR5 diversity is not the underlying explanation for differences in the spread of different HIV subtypes. While CXCR4 is also widely expressed on resting T lymphocytes, the expression of CCR5 is low on quiescent T cells and is significantly upregulated only after stimulation (24, 275, 353). Moreover, a largely reciprocal expression of CXCR4 and CCR5 among peripheral blood T cells has been reported (24).

Recently, differential patterns of chemokine receptor cofactor usage have been seen in HIV variants from the lungs and blood (330), as well as inhibition of HIV replication by chemokines (Carrillo et al., Abstr. Fourth Conf. Retroviruses Opportunistic Infect.). CXCR4 is expressed predominantly on an unactivated naive $CD26^{low}$ $CD45RA^{+}$ $CD45RO^{-}$ T-lymphocyte subset, while CCR5 is found on $CD26^{high}$ $CD45RA^{low}$ $CD45RO^{+}$ T lymphocytes thought to represent activated/memory cells (25). Thus, since NSI and SI HIV variants may have different cellular tropisms for activated/memory and resting/naive T cells, respectively, specific coreceptor expression coinciding with the presence of a cellular kinase specific for phosphorylation of certain dideoxynucleotides, may contribute to the virus phenotype-dependent effect of different dideoxynucleotide analogs.

This effect led van't Wout et al. (357) to propose a model whereby AZT is preferentially active in activated CCR5-expressing cells that can be infected by NSI HIV variants while ddI preferentially acts in quiescent CXCR4-expressing cells that support SI HIV infection (74). The combination of AZT and ddI then covers both cell types and is used to inhibit both phenotypic variants. In contrast, PI, e.g., ritonavir, which do not require intracellular phosphorylation, are active in both quiescent and activated T cells (327). Thus, combined use of two nucleoside inhibitors and one PR inhibitor for HAART is also justified in terms of covering HIV infection of multiple host cell phenotypes (Table 1).

Primary SI variants are also capable of using both CCR5 and CXCR4 for entry (329, 377). AZT, which is presumed to preferentially inhibit viral infection of CCR5-expressing cells, partially inhibits the SI virus load as well. Primary SI variants do not seem to escape from ddI and are presumed to inhibit infection preferentially in CXCR4-expressing cells by first replicating in CCR5-expressing cells (357). This is in agreement with the finding that NSI variants always remain present and contribute significantly to the viral load even after the emergence of SI variants (195).

Activation from Latency

After initial infection with HIV in most individuals, an asymptomatic carrier (AC) state is established, where there is productive replication of HIV in most $CD4^{+}$ T cells (which are then replaced with uninfected $CD4^{+}$ T cells newly provided from bone marrow) together with a quiescent or latent integrated state of the proviral DNA in a small but increasing number of non-lytically infected cells. This is followed by chronic infection and eventual clinical progression toward AIDS-related complex and then AIDS (238, 303).

The latently integrated state in HIV, although apparently quiescent, can best be described as dynamic, involving interactions between cellular and viral factors, i.e., HIV regulatory and accessory genes on one hand and several internal transcription factors, e.g., NF-κB, and external immune regulatory proteins on the other hand (55, 155). The mechanism of HIV latency has been examined in several cell lines expressing very low levels of viral mRNAs and proteins, and this situation is referred to as postintegration latency (238, 303). Further, different subclones have been established in various laboratories by rescuing and cloning latently or persistently HIV-infected human cell lines such as U937, HL-60, A3.01, Jurkat, and MOLT-4 (37, 51, 107, 116, 118, 296). Under unstimulated conditions, the level of viral transcripts in such subclones is

very low (less than 5% of the total cell population) (302). During isolation of HIV from such model cell lines, it was found that virus latency in the human $CD4^+$ T cell line MOLT-4 could be established only by infection with HIV recovered from acutely infected cells but not with virus from persistently infected cells (118).

Activation leading to virus production from postintegration latency in such model cell lines can then be induced by several reagents such as TNF-α, phorbol esters, or other reagents (20, 116). All of these reagents are presumably involved in induction of cellular transcription factors (122, 264). The mechanism of HIV activation from latency has been further characterized and also shown to exhibit a cell cycle dependence for the G_2/M phase in phorbol ester-treated cells, while it occurs in a cell cycle-independent manner after TNF-α treatment (351).

The accessory proteins Nef and Vpr appear to play an important role in regulating pathogenesis in vivo (181, 201), while extracellular or soluble Nef (116) and Vpr (209, 210) may function to activate HIV from latency. For soluble Nef this may occur through a signal transduction pathway involving *ras*, *raf*, and NF-κB (M. Tobiume and K. Ikuta, unpublished data). The ability of these accessory gene products to activate latent HIV in a manner analogous to that for cellular cytokines could provide a simple and direct means to explain HIV activation.

Further, specific interactions between Nef and the putative Nef binding receptors on T cells (283) and between Vpr and the glucocorticoid receptor type II complex (307) could also be triggers for intracellular signaling pathways that lead to activation of latent HIV proviral DNA, followed by viral antigen expression and particle production.

AIDS PATHOGENESIS

Accessory Gene Products Involved in AIDS Pathogenesis

The *nef* gene was initially shown to be essential for AIDS pathogenicity of simian immunodeficiency virus (SIV)-infected rhesus macaques (181). Efforts have also been made to characterize genetic features of HIV present in long-term survivors (233). Sequence analyses of viral genomes in long-term survivors revealed defects in both regulatory and accessory genes (68, 156, 183, 246). This was observed initially in an Australian cohort of LTNP infected with HIV through a blood transfusion from a single donor; multiple deletions in *nef* and the U3 part of LTR were found in all members of this cohort (68, 203). Another study also revealed a high frequency of defective *nef* alleles in a single LTNP (233). The relative lack of disease in these individuals raised the possibility that one or more specific genetic defects in HIV accounted for the prolonged AC state. This finding appeared similar to those in macaques that remained asymptomatic for a prolonged period after infection with a *nef*-deleted SIV strain (181).

In contrast to the above result, a cohort of LTNP in the United States were infected with HIV in which the *nef* gene was both genetically and functionally intact (150, 151). Detailed sequence analyses of *vif*, *vpr*, and *vpu* in these viruses also revealed the integrity of these genes (379). In the 5′ LTR sequences, all, except for that in one individual which carried G-to-A hypermutations throughout the entire region, shared nearly identical consensus sequences in the binding sites for NF-κB, Sp1, and the viral *trans*-activator Tat (378). Env proteins have also been shown to have functional abnormalities in long-term survivors (60). Taken together, these results suggest that it is unlikely that a single common genetic determinant is responsible for the well-being of LTNP.

When paired isolates obtained by coculture with PBMCs from HIV-infected individuals before and after the panning of $CD8^+$ T cells were compared, it was found that a clear distinction at *nef* occurred between HIV paired isolates from AC but not from LTNP, indicating the importance of wild-type Nef-specific $CD8^+$ T-cell selection pressure for maintaining a stable asymptomatic state (383).

In contrast, the role of *vpr* in development of AIDS is controversial: on one hand, *vpr* gene mutations when combined with *nef* mutations in SIV decreases simian AIDS pathogenesis in monkeys (201); on the other hand, *vpr* deletion mutants of SIV themselves can still induce simian AIDS in infected monkeys (145). Also, molecular analysis of HIV strains derived from the blood and plasma of an HIV-infected long-term-surviving (>13 years) mother-child pair showed the presence of defects (insertions and deletions) and polymorphisms in *vpr* (362).

Examination of the interaction between HIV regulatory gene products and the host immune system is fundamental in understanding the pathogenesis of HIV (225) and could reveal possible targets for AIDS treatment. The HIV *tat* gene is also a potential candidate for this type of strategy. In transgenic mice harboring *tat*, there is an effect on the immune system, i.e., enhanced TNF production (32). Also, the addition of synthetic Tat peptides, but not those from recombinant Nef and Vif proteins, inhibits proliferative responses of $CD4^+$ tetanus antigen-specific, IL-2-dependent T-cell clones in a dose-dependent manner (46).

CD4 Cell Count and HIV Viral Load in Plasma as Markers of AIDS Progression

CD4 cell counts are widely used to predict disease progression in HIV-infected patients (336) and have been employed as a surrogate marker to provide evidence for the effectiveness of therapeutic agents. In addition, viral load or the HIV RNA level determined by PCR is an excellent predictor of the prognosis for patients infected with HIV (121, 242, 276). By investigation of the relationship between these markers, it was shown that the return of the $CD4^+$ cell count to higher levels was significantly related to both the baseline CD4 count and the decline in HIV RNA PCR-determined viral load (101). However, it has also been shown that sole use of a return to high levels of the CD4 cell count can be misleading in monitoring disease progression, since changes in CD4 count after therapy are determined more by the starting CD4 count than by the change in viral load (243), and large increases can occur with minimal antiviral effect (91).

The emergence of SI variants has been shown by many groups to correlate with both accelerated decline in $CD4^+$ T-cell counts and accelerated disease progression in the natural course of HIV infection (171, 192, 197, 310), as well as during treatment (29, 170, 176, 193, 336). Moreover, individuals harboring only NSI HIV variants were shown to benefit more from treatment with AZT than were individuals also harboring SI variants (193). In fact, a preferential inhibition of NSI variants by AZT was observed (355), while ddI treatment resulted in a loss of SI variants in bulk cocultures (74, 382). Also, it has been shown that neither AZT therapy alone nor AZT in combination with alpha interferon or ddI prevents the acquisition of SI strains (352).

On the other hand, it was also known that approximately half of people infected with clade B HIV who develop AIDS never acquire detectable CXCR4 binding (X4) HIV quasispecies (7, 45, 193, 346). It is enigmatic that most studies of primary CCR5 binding (R5) isolates of HIV have detected little if any pathogenesis attributable to these viral isolates in

tissue culture yet many AIDS patients die from infection with exclusively R5 HIV-1 quasispecies. Many R5 isolates of HIV replicate more slowly and to lower titers in stimulated PBMCs than do R5X4 or X4 isolates (61, 354). Nevertheless, some R5 isolates found later in the course of infection, particularly after an AIDS diagnosis has been made, replicate more rapidly and to higher titers in tissue culture than do typical R5 isolates found early in the course of infection (354).

Activation of latently infected or quiescent T cells during HIV infection is becoming another active area of study in AIDS pathogenesis. The levels of $CD4^+$ and/or $CD8^+$ T cells expressing activation markers such as HLA-DR (9, 16, 179, 180, 227), CD25 (146, 227), or CD38 (158, 179, 180, 228, 370) are elevated in HIV infection. However, the mechanism of activation is not yet understood; e.g., it could reflect an antigen-induced T-cell activation in vivo or may be secondary to HIV-induced cytokine production (157, 158).

Treatment with AZT has been shown to reduce activation significantly (17, 194). In addition, treatment with PI, such as ritonavir, reduces the percentage of CD4 and CD8 lymphocytes expressing CD38 (177). Recent cocapping experiments have revealed the gp120-induced association of CD4, the major receptor for HIV, with CD38, suggesting that assembly of abnormal multimolecular complexes could be involved in either gp120-$CD4^+$ T-cell dysfunction or viral entry into cells (33, 82, 102). The association with CD38, but not with chemokine receptors, was observed at increased percentages in both $CD45RA^+$ naive and memory cells (102). Using monoclonal antibody panning and flow cytometry, it was possible to separate PBMCs into $CD4^+$ $CD38^+$ and $CD4^+$ $CD38^-$ subsets, which show susceptibility to HIV with different tropism (146a). This may eventually allow one to examine these populations as potential reservoirs of HIV, even in HAART patients. However, there is still little information about whether the differentially activated $CD4^+$ T cells described above differentially express coreceptors which could contribute to varied stages in the HIV life cycle.

Role of Dendritic Cells

It has been known for some time that the acute phase of HIV infection is followed by an extended period in which humoral and cell-mediated immune responses occur, viremia decreases, and the major virus reservoir is represented by germinal centers in lymph nodes and possibly other peripheral lymphoid tissues (94, 287, 288). Thus, monitoring the status of the viral burden and of trapping and replication of viruses in lymph nodes during combination antiretroviral drug treatment may be critical to an assessment of therapy (56). Plasma cells producing antibodies to HIV have been identified in germinal centers of lymph nodes from asymptomatic subjects (344). Therefore, extracellular immunoglobulin may form immune complexes, as shown by the presence of HIV-specific antibodies, HIV particles, and complement components. In addition, receptor-mediated endocytosis of viral particles by follicular dendritic cells (DC), when virus is abundant in the cytoplasm, leading potentially to the formation of a hidden reservoir, has been observed (144). In this location, HIV may escape recognition by cytotoxic T lymphocytes (CTLs). In contrast, virus budding has seldom been seen, indicating that productive infection of HIV in follicular DC would be rare.

DC play a critical role in the activation of primary immune responses since they are specialized to present antigens to naive T cells in vivo (374). Immature DC capture antigen at peripheral sites such as the skin, airways, mucosa, or gut and then migrate via afferent lymphatics to T-cell-enriched regions in secondary lymphoid organs (57). During this homing process, immature DC lose their capacity to process antigen but upregulate costimulatory molecules (CD86, CD80, and ICAM-1) on their surfaces and display a mature phenotype. These mature DC are then able to select antigen-specific T cells from the circulating pool and stimulate them (42, 374). The yield of DC derived from monocyte-depleted PBMC by culture in human IL-4 and human granulocyte-macrophage colony-stimulating factor was lower in HIV-infected than seronegative subjects, and the lower yield of cells correlates with smaller numbers of peripheral blood $CD4^+$ T cells and higher plasma viral load (98).

Since Langerhans cells express CD4, the major cellular receptor for HIV, their presence in the urogenital mucosa gives rise to the possibility that DC are involved in sexual transmission of HIV, as well as SIV, infection (78, 271, 306, 332). In rhesus monkeys, SIV can be efficiently transferred to lymph nodes within 2 days following mucosal inoculation of the virus (161, 335). Evidence that DC could be directly involved in HIV transmission was deduced from the observation that HIV readily associates with DC and can cause DC and T cells to generate syncytia, which represent sites of vigorous viral replication in vitro and in vivo (38, 304, 305). Both macrophages and DC of mucosal epithelia are among the first cells to be associated with virus following sexual contact involving HIV (113, 248, 250, 374). DC are also capable of transmitting HIV to $CD4^+$ cells of the monocytoid lineage (163).

Recently it was observed that PR-defective, gp120-enriched L-2 particles can bind to DC prepared from PBMC of healthy donors, using IL-4 and granulocyte-macrophage colony-stimulating factor and cause them to acquire an effector function, leading to apoptosis of bystander $CD4^+$ and $CD8^+$ T cells (343).

Accumulation of Infected Cells Producing Defective Particles

According to a deterministic model of HIV population genetics, the frequency of any particular mutation in the differentiated viral quasispecies found a few years after initial primary infection is primarily a function of its production rate and the relative replication fitness it confers on the genome (53, 54). According to this model, any single-nucleotide mutation associated with drug resistance may be found on as many as 0.1 to 1% of the viral genomes in the fully differentiated quasispecies which have evolved following several years of infection. This modeling assumes that 10^8 to 10^9 cells are infected per day (142, 363). Mutation rates are highly dependent on sequence context (17, 161, 234), with an average rate of 3×10^{-5} bp/replication cycle (234).

Interestingly, a novel proposal has been suggested which takes advantage of this high mutation rate by showing that promutagenic nucleosides, such as 5-hydroxydeoxycytidine (5-OH-dC), lead to additional "lethal" mutations and/or loss of viral replication during passage in human CEM cells (219). The fitness cost associated with some drug resistance-associated mutations in the absence of drug selection has been estimated, from their frequency in untreated patients (204, 267) and competition between variants in vivo (125), to be approximately 1% (54). Using such assumptions, a steady-state total of 10^8 cells are expected to carry any particular single-site drug resistance-associated mutation (53, 54). Following the application of drug selection, the continued replication of such a large drug-resistant mutant population would be expected to maintain the genetic composition of the quasispecies at other (nonselected) loci. The emergence of PI-resistant viruses in plasma

therefore reflects a highly complex process. Several explanations for this phenomenon have been proposed, including variable levels of drug efficacy at different anatomical sites of virus production, as well as the selection and amplification of a few drug-resistant lineages, so that the frequency distribution of nonselected gene variants is altered (75).

Apoptosis of Host Immune Cell Populations

Several reports have focused on the possible function of HIV-related proteins such as soluble gp120 and/or Tat to prime signals for induction of apoptosis in bystander cells (13, 213, 285, 365). Although these reports have revealed the ability of these proteins to induce apoptosis by using the Fas/FasL system in $CD4^+$ T cells (169), they mostly examined the effect on T-cell lines after exposure to large amounts of such recombinant HIV proteins. Thus, soluble gp120 was shown to prime apoptosis only in T-cell lines or activated PBMCs but not in freshly prepared resting PBMCs (108, 164, 235). In addition, HIV Tat protein was shown to induce cell death by apoptosis in a T-cell line and in cultured PBMCs from uninfected donors (213). These results thus suggest that the depletion of immune cells by apoptosis in vivo can occur by both direct and indirect mechanisms (5, 244).

Several features have been ascribed to the process of apoptosis in HIV-infected individuals. First, apoptosis can be induced in patient PBMCs as an activation-dependent event after mitogenic stimulation (126, 130). Studies on the surface expression of activation markers on apoptotic and nonapoptotic cells from patient material have shown that in the chronic phase of HIV infection, 50 to 60% of apoptotic cells exhibited activated phenotypes ($HLA\text{-}DR^+$, $CD38^+$, $CD45RO^+$, and Fas^+) and have led to the suggestion that the $CD45RO^+$ T-cell subset was more prone to apoptosis in HIV-infected individuals (127). Second, HIV can induce apoptosis in HIV genome-negative, uninfected cells as a bystander effect (105). In fact, apoptosis has been observed not only in $CD4^+$ T cells but also in $CD8^+$ T cells (105, 127, 165, 212, 260). Third, apoptosis in uninfected cells can be mediated by Fas/Fas ligand (FasL) interactions and/or an imbalance of Th1 and Th2 cytokines (50, 173). Finally, mRNA for FasL is up-regulated in PBMCs from HIV-seropositive individuals and can be expressed in $CD4^+$ T cells (252).

HIV-induced apoptosis has also been correlated with fusion activity of the virus (230). Therefore, the propensity of HIV for apoptosis can in part be estimated by measuring its SI activity. As noted above, SI viruses also predominantly appear in patients who progress most rapidly to AIDS (104, 129). In addition, although continuous virus production is observed even at early infection times as well as during the course of disease (142, 289, 294, 363), a large number of HIV particles in the peripheral blood of infected individuals at late stages of disease are noninfectious (299). Therefore, the possible role of SI-type, PR-defective gp120-containing HIV particles in induction of apoptosis of healthy donor PBMCs, through an activation-dependent mechanism, has also been examined (164, 165).

A subclone (named L-2), which produces noninfectious HIV particles (124, 153, 373) and carries a provirus with a 1-base insertion in the *pol* PR (10), thus is a producer of the doughnut-shaped, RT-negative, and noninfectious HIV particles (similar to those seen in Fig. 3A). These L-2 particles exhibited a higher fusion activity for $CD4^+$ T cells, as shown by their syncytium formation in virus-to-cell fusion, than did the parental wild-type HIV LAI particles (279) and induced apoptosis in PBMC-derived $CD4^+$ and $CD8^+$ T cells in an activation-dependent manner (164). The trigger for apoptosis is related to acquisition of nonspecific killer activity by a subpopulation of $CD4^+$ $CD38^-$ T cells after adsorption with L-2 particles (165). In addition, two unrelated Thai primary isolates of HIV (clade E) exhibited a similar killer activity (165). Also, a $CD4^+$ $CD38^-$ T-cell subset derived from 16 HIV-1 carriers showed significantly higher effector activities than did a subset from HIV-seronegative healthy donors against healthy-donor-derived PBMCs (166). All of these results suggest to us that HIV and its associated disease AIDS is unique (345) and will be around for quite some time in the human population. The epidemic has tended to increase not only among young adult males but also among adolescents (77) and drug users (41). Hopefully, new antiviral approaches, including vaccine development to diminish its virulence, will be developed along with current HAART and immune replacement therapy, but as we learned with multiple cancer therapy, these vaccine methods (see the following section) may not eliminate the virus, as was effectively done 20 years ago by the smallpox vaccine.

FUTURE DIRECTIONS

Multiple-Target Anti-HIV Therapy

HIV inhibitor treatment eventually leads to the selection of HIV variants with resistant mutations, whether they be to nucleoside inhibitors or PI. One advantage of HAART is that for the ≥50% of AIDS patients who tolerate treatment, there is an improvement in life-style, as well as longevity. Generally, mutant formation has been relatively slow to occur for PR, in contrast to nucleoside inhibitors (55, 155; Havlir et al., Abstr. 2nd Natl. Conf. Hum. Retroviruses Relat. Infect.). Thus, one goal is to utilize yet additional protein enzyme targets in the HIV replication cycle for inhibitor design, such as IN, which is involved in integration of proviral DNA, so that by potent quadruple HIV enzyme inhibitor therapy, mutant selection by resistant inhibitors can be further delayed.

Also, one can target sites other than the active site on the PR for drug development, since in bacterial systems, such as that in tuberculosis, it appears that multiple inhibitors are useful for synergistic effects. In a preliminary study, we showed a number of years ago that pepstatin (binds to active site of HIV PR) and cerulenin (binds to an unknown site(s) (perhaps the cysteine-SH group on HIV), when used at 10-fold-lower doses of inhibitor, exhibited a synergistic effect (R. B. Luftig and M. Bu, unpublished data). This approach, however, could have its drawbacks in that binding of inhibitors, such as cerulenin (154, 173), to sites other than the active site either could lead to higher K_is for active-site inhibition and/or may be toxic to cells, so that nontoxic analogs need to be developed (26). On the other hand, such synergy may be beneficial and may imply possible use of smaller amounts of active-site PI, so that there is a longer period before resistance appears in the AIDS patient.

Another strategy would be to select inhibitory compounds, such as loviride (an RT inhibitor) or quinoxiline (a nonnucleoside RT inhibitor) that can be used at high concentrations (0.1 or 2.5 μg/ml, respectively, in CEM cells) to completely suppress HIV replication, so that development of resistance is greatly minimized (70).

Finally, an idea that was propagated several years ago and is winning renewed interest is to transduce cells with DNA encoding single-chain variable-fragment antibodies ("intrabodies"), thus causing intracellular formation of heavy- and light-chain variable domains, joined through a synthetic linker and directed via protein-trafficking signals to specific cellular sites

for HIV proteins, such as gp120, Rev, and PR (315). In the latter case, one can take advantage of the fact that there are different monoclonal antibodies to PR flap epitopes, which undergo dramatic conformational changes on inhibitor binding (65, 251), as well as to NH_2-terminal regions leading to monomer formation (J. Sedlacek and M. Fabry, personal communication).

Vaccine Trials

The development of anti-HIV drugs represents a great effort to reduce the viral load and to prevent the onset of AIDS development. However, most HIV-infected individuals will never benefit from such therapeutic agents. The spread of the AIDS epidemic is concentrated in regions of the world where insufficient financial resources are available to allow access to these drugs.

It is believed by most investigators within the AIDS research community that eventually the only acceptable worldwide treatment for the epidemic will be through formulation of an inexpensive, effective vaccine that induces both humoral and cellular immunity (208). In fact, toward this end the National Institutes of Health (NIH) have created a new Vaccine Research Office, headed by Nobel laureate David Baltimore, the codiscoverer of HIV RT. One may also take some solace in the words of the other RT codiscoverer, Howard Temin, that even if a vaccine or treatment for HIV is not discovered soon, HIV, although different, may not be a unique virus (345). Further, a new NIH Vaccine Research Center has been constructed under the Directorship of Gary Nabel, who recently reported on vaccine strategies for HIV and other emerging viruses (G. Nabel, ASV Satellite Symp. Frontiers HIV Res., 2000).

There is evidence that neutralizing antibodies can protect against HIV infection in certain animal models (22, 36). However, it will not be easy to induce antibodies of the right quality in sufficient quantity by vaccination. Also, there is a wealth of data showing a correlation between the appearance of CTL activity and the containment of viral infection (159, 280, 318). In fact, the early containment of HIV replication in infected individuals coincides temporally with the emergence of a virus-specific CTL response (44, 196, 288). In addition, in individuals chronically infected with HIV, a high-frequency CTL response is correlated with the maintenance of low virus load and a stable clinical status (262, 278). Thus, it is believed that both antibodies and CTL responses will have to be induced for an HIV vaccine to be effective.

Historically, most successful vaccines have been made of attenuated or killed virus. However, both of these approaches appear problematic for HIV. Although attenuated viruses are likely to be quite effective (80), there are genuine safety concerns, as emphasized by the report of morbidity in monkeys after vaccination with attenuated (*nef*-deleted) SIV_{mac} (97).

Recently, as noted above, several HIV vaccines have been made (202, 216, 256, 257), including one from nonenveloped, Gag-containing inactivated HIV particles and shown to give an enhanced benefit in β-chemokine stimulation in HIV-seropositive subjects. It is also hypothesized that the vaccine-increased production of β-chemokines in turn may block the binding of HIV particles to chemokine coreceptors (256, 257).

Other groups are attempting to develop envelope protein vaccines, e.g., gp120 alone or in a poxvirus vaccine vector, or to use other strategies to block the HIV fusion step, e.g., passive immunization with human monoclonal antibodies to gp120 or use of peptides to block gp41 fusion. A particularly novel approach generates "fusion-component" HIV vaccine whole-cell immunogens, which initially capture transient envelope-CD4 coreceptor structures into a complex. Then the complex is used as a formaldehyde-fixed vaccine to elicit antibodies which potentially can neutralize diverse primary isolates (200).

In our view, another possible approach for trials of vaccines against HIV may be the use of PR-defective particles, similar to the multiply mutant L-2 particles described above, for a vaccine immunogen. Subunit envelope vaccines using recombinant forms of the HIV envelope glycoprotein (particularly gp120) are safe but have been disappointing (62, 136). To date, these proteins have not elicited significant neutralizing-antibody responses to representative primary viruses (136), almost certainly because epitope exposure differs between recombinant proteins and the mature oligomer found on the virus (36, 255). Therefore, native forms of the envelope protein in defective particles may play a role as immunogens.

Regulation of Reactivation from Latency

The current guidelines for use of antiretroviral agents in HIV-infected adults and adolescents (101) are very reasonable and suggest that HAART should be introduced in persons acutely infected by primary HIV infection before extensive immune system damage has occurred. It is these patients, who number over 100,000 in the United States, and patients with advanced late-stage disease ($CD4^+$ count < 50 cells/mm) who derive the greatest benefit from HAART even though large increases in $CD4^+$ T-cell counts may not occur. Individuals currently infected with HIV and living in a condition where many proviral DNA copies have already been incorporated into quiescent T cells may present a treatment concern in that such virus can become reactivated and lead to the production of HIV particles, which in turn can enhance AIDS pathogenesis.

To eliminate these possibilities, other approaches must be developed, e.g., using ribozymes or antisense oligonucleotides, which can effectively inhibit reactivation at the DNA level. Thus far, such new approaches have not yet been successful in reaching phase II trials, although there have been positive outcomes in tissue culture, suggesting that these modalities have merit.

Ribozymes are enzymatic RNA molecules that can recognize and cleave other RNA molecules in a specific manner (64). By targeting HIV mRNA, one can block its translation (372). Ribozymes are being considered in both antiviral and anticancer therapy (301, 324). There are three such ribozymes in use, e.g., hammerhead, hairpin, and hepatitis delta ribozymes. Ribozymes are thought to be more advantageous than proteins in gene therapy, since they are less likely to generate an immune response. They are, however, limited in that transcription units on plasmids need to be designed so that larger amounts of the ribozyme are produced intracellularly in order to permit adequate binding and cleavage.

Reducing HIV infection by ribozyme cleavage of target RNA has been a major goal of many laboratories. One example is the development of a hairpin ribozyme (60 to 70 nucleotides) to cleave HIV RNA in the 5′ region of the LTR sequence and then use it to inhibit HIV replication of primary PBMCs, including T cells, or in progenitor stem cells (372). Recently, cleavage of the MLV RT gene by a double hammerhead ribozyme was reported, where the virion content was decreased by >80% in acutely infected cells (J. Tulpinski and B. K. Pal, Abstr. ASV 19th. Annu. Meet., abstr. P29-4, p. 175, 2000).

Finally, in addition to ribozymes, there is a whole class of HIV genetic antiviral strategies, involving RNA decoy and antisense oligonucleotides (phosphothiorates) among others,

which all attempt to eliminate the latent, integrated proviral DNA that cannot be targeted by HAART (90, 214, 270).

One recent drug, called a "molecular tong," has been designed which inhibits homodimerization of the 2 aspartyl PR monomers (Fig. 2). Another possible antiviral drug, methionine enkephalin, has also been described (N. P. Plotnikoff, Abstr. Am. Soc. Biochem. Mol. Biol. and Am. Soc. Pharmacol. Exp. Ther., abstr. A18, 2000). Other approaches suggest that combining several ribozymes and decoys to target multiple sites on HIV RNA may be most effective for treating infection from multiple M-group HIV clades (A to E) (214).

In conclusion, we believe that with the plethora of new PI, as well as other antiviral drugs including small molecular inhibitors against novel targets and, eventually, the development of one or more effective vaccines, the future looks bright for AIDS patients.

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