Highlights of Basic Science Research

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Basic science research is a steadily expanding component of the Retrovirus Conference. There were some surprises from studies that addressed the relation between the host cell cycle and permissiveness to viral infection. Several studies provided recent insights into factors that influence virion morphogenesis and release. New findings were presented on the accessory proteins Vpr and Nef. Studies of viral pathogenesis were highlighted by recent reports on attempts to characterize the reservoirs of viral replication in patients who are being treated with antiretroviral therapy. Studies that detailed the identification and characterization of a novel CCR5 antagonist were one of the highlights of the sessions on tropism and coreceptors. In addition, a number of presentations provided updates on studies aimed at identifying the role of DC-SIGN in HIV replication.

Virology

The activities of many primate lentiviral proteins are mediated through cellular ligands, and these interactions appear to be species-restricted. For example, the activity of the viral transacting protein Tat requires interaction with the cellular protein cyclin T1. However, Tat does not function efficiently in murine cells, because it does not interact efficiently with the murine equivalent of cyclin T1. A single amino acid change in the murine cyclin T1 promotes an interaction with Tat and can support Tat action in murine cells. Several groups have noted that, despite restoration of Tat function in murine cells and efficient expression of viral proteins, there is yet another block to viral replication.

Bieniasz (Abstract S10) and Mariani (Abstract 284) reviewed studies in which the appropriate receptor molecules were expressed, as well as cyclin T1, in mouse, hamster, and rat cell lines. These cells supported efficient reverse transcription, and Tat functioned at almost wild-type levels. These cells produced very little virus, which, nevertheless, was as infectious as human cell–produced virus. The viral precursor polyprotein Pr55 Gag appeared to be poorly processed and secreted from mouse and rat cells. When the investigators produced heterokaryons between these cells and human cells, the assembly and release of viral particles was restored. These studies support the notion that human cells contain a factor that promotes Gag processing and viral assembly and release and that the murine equivalent does not function as a cofactor. The identification of this cellular cofactor for viral assembly and release is important for the development of small-animal models of HIV infection. Furthermore, this cofactor is an attractive drug target, and may lead to the development of agents that interfere with late steps in the viral replication cycle.

Continuing with the theme of cellular processes that influence retroviral Gag functions, Goettlinger (Abstract S11) reviewed studies that point to a role for cellular ubiquitin ligase in promoting the action of Gag during virus release. Goettlinger discussed research from 3 groups that showed that ubiquitin plays an important role in a late step in viral budding. Ubiquitin targets proteins for proteasomal degradation by binding through a C-terminal glycine to lysine residues in the protein. Ubiquitin was previously shown to be contained within viral particles of HIV-1 as well as of murine leukemia virus (MLV). Interestingly, depletion of the intracellular pool of free ubiquitin appears to inhibit the budding step of a number of unrelated RNA-enveloped viruses. By blocking proteasomal function, recycling of ubiquitin is prevented and results in an excess of the unconjugated form of ubiquitin, leading to measurable reductions in the budding of both Rous sarcoma virus and HIV.

It has been suggested that proteasome inhibition leads to an accumulation of defective Gag polyproteins that would normally be subject to proteasomal degradation and that these defective proteins may interfere with budding. Overexpression of truncated forms of the Gag protein of HIV-1 creates a dominant-negative effect on HIV budding. Mice that express a defective Gag product (FV-1 mice) are also highly resistant to viral infection. The ubiquitination of Gag proteins requires an assembly domain in Gag (which is also referred to as a "late" domain) that is required for final detachment of the virion from the plasma membrane. Goettlinger presented evidence that late domains are contained within the Gag proteins of a variety of viruses, including HIV, MLV, and Ebola virus. Goettlinger presented a model in which ubiquitin ligase is recruited to the site of viral budding through interaction with late domains within Gag. The conservation of a ubiquitin-dependent budding mechanism by diverse viruses strongly supports the importance of such an activity in the replication cycle of these viruses and further illustrates how viruses use cellular processes to complete their life cycle.

On infection of the cell, retroviruses initiate reverse transcription of viral complementary DNA within a high-molecular-weight nucleoprotein complex that is commonly referred to as a reverse transcription complex. The composition and nature of the reverse transcription complex remains poorly understood, and McDonald (Abstract 281) presented what may be the first electron microscopy images of the complex. Studies indicate that HIV reverse transcription complexes contain the enzymatic proteins integrase and reverse transcriptase, the structural protein matrix, and the accessory protein Vpr. By conjugating Vpr with green fluorescent protein, incoming reverse transcription complexes could be visualized by fluorescence microscopy. Furthermore, the investigators prepared virions in the presence of fluorescently labeled deoxynucleotides, with the result being that genomic viral RNA within incoming reverse transcription complexes could be further visualized by fluorescence microscopy. Time-lapse observations of incoming reverse transcription complexes suggested that these

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complexes move through cells along microtubules. Using correlative electron microscopy with labeled reverse transcription complexes, the investigators obtained high-resolution images of cytoplasmic reverse transcription complexes. The images suggested a cylindrical structure with a diameter of 100 to 120 nm. Surprisingly, reverse transcription complexes that were derived from cells infected with wild-type HIV-1 or vesicular stomatitis virus–pseudotyped HIV showed that these cylindrical reverse transcription complexes have a globular structure at one end. Since these complexes appear to be larger than the viral core, these results suggest a considerable reorganization of virion components during the formation of the reverse transcription complex. These studies may provide important information on a rather elusive step in the retroviral replication cycle.

The current models of HIV-1 assembly indicate that the major precursor polyproteins Gag and Gag/Pol, from which the structural and enzymatic proteins are derived, respectively, interact at the plasma membrane before virion maturation and assembly. The results of studies presented by Khorchid (Abstract 280) challenged this popular view and indicated that this interaction may occur in the cytoplasm independently of plasma membrane binding. The interaction of Gag and Gag/Pol precursors with the plasma membrane is mediated through a myristic acid moiety at the N-terminal matrix domain of the precursors. Nevertheless, nonmyristylated precursors were able to interact in the cytoplasm. In addition, the interaction of the NC domain of Gag with genomic viral RNA directs the packaging of viral RNA within virions. Khorchid and colleagues showed that the interaction of Pr55 Gag with genomic viral RNA was necessary for interaction between the 2 major precursor polyproteins. These studies further define events in retroviral replication that are required for proper assembly of virions within the infected cell.

The action of reverse transcriptase requires interaction between the p66 and p51 subunits of reverse transcriptase. Previous studies have suggested that mutations within the nonnucleoside reverse transcriptase inhibitor (NNRTI)–binding site within reverse transcriptase can influence the interaction between reverse transcriptase subunits. A study presented by Tachedjian (Abstract 282) found that NNRTIs, which are allosteric inhibitors of HIV reverse transcriptase, affect heterodimerization of the p66 and p51 reverse transcriptase subunits. By fusing p66 and p51 subunits to lexA and gal4AD, respectively, the investigators could examine interactions between p66 and p51 subunits in a 2-hybrid assay in yeast. The investigators found that inhibitors such as efavirenz, nevirapine, and several other NNRTIs markedly promoted p66 and p51 heterodimerization. Delavirdine, which interacts with reverse transcriptase at a site different from that of most other NNRTIs, did not increase heterodimerization. Variants of p66 that contain NNRTI resistance mutations (Y181C) were insensitive to increases in heterodimerization by nevirapine. This study found a previously unsuspected effect of NNRTIs on the heterodimerization of reverse transcriptase. The authors suggest that the interaction of drug with a p66 subunit may create the conformational change that increases its binding to the p51 subunit. This study provides a clear example of how basic research investigations in the structure and function of viral enzymes can point to new strategies to broaden the efficacy of the currently available antiretrovirals.

Accessory Proteins

HIV-1 Vpr is a virion protein that has been shown to promote nuclear targeting of viral preintegration complexes in nondividing cells such as macrophages. Consistent with this activity is the finding that Vpr, when expressed in the absence of other viral proteins, localizes to the nucleus. This creates a paradox in that Vpr must interact with Gag and be transported to the cellular plasma membrane for incorporation into maturing virions. Yet, during viral entry, Vpr must travel in the opposite direction while promoting nuclear localization of the viral reverse transcription complex.

The results of studies presented by Sherman (Abstract 139) and Vodicka (Abstract 140) further suggested that Vpr has the characteristics of a nucleocytoplasmic shuttling protein. To follow the movement of Vpr within cells, the investigators fused Vpr to fluorescent proteins. The wild-type Vpr protein localized predominantly to the nucleus and nuclear envelope, but a truncated form of Vpr (amino acids 1–71) was localized exclusively to the cytoplasm. Furthermore, mutations within a putative nuclear export signal in Vpr also resulted in cytoplasmic localization of the protein. The antibiotic leptomycin B, an inhibitor of the mammalian nuclear export receptor Crm1, caused nuclear accumulation of Vpr_{1-71}. The findings in these studies are consistent with the notion that Vpr continuously shuttles between the nucleus and the cytoplasm and that nuclear export is mediated by Crm1, a major cellular nuclear export receptor.

The functional significance of this nuclear export activity of Vpr remains unexplained. One possibility is that the nuclear export activity of Vpr maintains a sufficient concentration of cytoplasmic Vpr for packaging into assembling virions. Surprisingly, however, the inhibition of Vpr export by leptomycin B did not decrease the amount of Vpr that was contained within virions. Since the virion incorporation of Vpr depends on interaction with the p6 domain of Gag, one possibility is that a previously reported nuclear export activity in Gag (Dupont et al, *Nature*, 1999) may maintain cytoplasmic localization of Vpr even if its nuclear export activity is impaired. An important question that stems from these studies is why the nuclear export activity of Vpr does not interfere with its reported role in promoting nuclear uptake of viral reverse transcription complexes during viral entry. Following viral infection, reverse transcription complexes that localize to the nucleus would be rapidly exported back to the cytoplasm through the action of Vpr. Presumably, therefore, the nuclear export activity of Vpr must somehow be suppressed in the acutely infected cell. One possibility is that the shuttling activities of Vpr are influenced by posttranslational modifications in the protein such as phosphorylation. A study by Zhou and Ratner (Abstract 137) suggested that Vpr is phosphorylated on serine and that this phosphorylation can influence the ability of Vpr to inhibit host cell cycle progression. Thus, phosphorylation of Vpr may impair or promote nuclear export, thereby influencing the steady-state localization of Vpr within the infected cell.

The aforementioned studies suggest that the nuclear export of Vpr is mediated through the cellular nuclear export receptor Crm1. The HIV-1 Rev protein, which regulates the splicing of viral messenger RNA, was the first viral protein to be shown to have nucleocytoplasmic shuttling activity. Nuclear export of Rev also depends on Crm1. Daelemans and colleagues (Abstract 283) described the identification of a low-molecular-weight compound that inhibits Crm1-mediated nucle-

ar export and further inhibits Rev function in human cells. The drug reversibly inhibits the binding of Rev to Crm1. It should be emphasized that such agents are unlikely to be used as antiviral agents. Since Crm1 mediates the nuclear export of a diverse variety of cellular proteins, its inhibition is likely to be accompanied by considerable cellular toxicity. Nevertheless, this drug may prove to be an important reagent in further defining the interplay between viral proteins and the nuclear export apparatus of the cell.

The accessory protein Nef greatly facilitates viral replication and pathogenicity in vivo. A number of activities have been described for Nef. Among these is the facilitation of viral entry in certain cell types. For example, viral infectivity of HeLa cells that express CD4 is increased by Nef, and this has previously been suggested to reflect an increase in the extent of reverse transcription in the target cell. The results of studies presented by Cavrois (Abstract 279) raised the intriguing possibility that Nef may facilitate the cytosolic entry of the virus. When virions bind specifically to receptor and coreceptor molecules on the cell surface the viral membrane fuses with the cellular plasma membrane. In a poorly understood process referred to as "uncoating," the capsid core disassembles and releases genomic viral RNA together with associated virion proteins into the cytosol. The investigators examined the cytosolic association of Gag p24 as a surrogate marker for viral entry. They observed that, while the presence of an intact *nef* gene significantly enhanced cytosolic entry of virions following CD4- and chemokine receptor–dependent entry, the enhancing effect was impaired by mutations in *nef* that had previously been shown to affect the CD4-downregulating activity of *nef* or by mutations in SH3 motifs in *nef* that mediate the interaction with protein tyrosine kinases. Surprisingly, the cytosolic entry of HIV-1 virions pseudotyped with MLV envelopes was also enhanced by Nef. In contrast, virions pseudotyped with vesicular stomatitis virus G envelope were not affected by Nef, suggesting that Nef enhances fusion-mediated entry but not entry that occurs through endocytosis. These studies raise the intriguing possibility that some of the effects of Nef in promoting viral uptake may be mediated through another virion protein. Whether Nef influences the fusion or uncoating steps of viral entry is not yet known.

Nuclear translocation of viral nucleic acids in acutely infected cells has been shown to be promoted by at least 3 virion proteins, including the structural matrix protein, the viral enzyme integrase, and the accessory protein Vpr. The nuclear import activity of these proteins has been shown to be mediated by an interaction with members of the importin family of cellular nuclear import receptors. The findings in studies presented by De Noronha (Abstract 141) point to a novel mechanism through which Vpr may promote nuclear translocation of viral reverse transcription complexes. The investigators showed that the expression of Vpr within cells can influence the subcellular trafficking of cell cycle–regulating proteins fused to GFP. It was found that Vpr induces a loss of nuclear integrity, which leads to the admixing of nuclear and cytoplasmic components. Vpr mutants that did not cause cell cycle arrest did not influence nuclear architecture. The authors showed that the changes in the nuclear envelope resulted from a disruption in the nuclear lamin structure. The authors hypothesize that nuclear localization of large viral reverse transcription complexes in nondividing cells may be facilitated by the disruption of nuclear envelope integrity by Vpr.

Tropism

The coreceptor used most frequently for entry of HIV-1 into macrophages is CCR5. Brain macrophages and microglia are the predominant, if not the exclusive, infected cells in the brain. However, it is not clear whether macrophages and brain microglia share similar coreceptor requirements for viral entry. Studies by Gorry and colleagues (Abstract 5) compared brain-derived viral isolates for their ability to replicate within monocyte-derived macrophages and microglia and for their coreceptor use. Isolates that replicated to high levels in monocyte-derived macrophages also replicated to high levels in microglia. Surprisingly, viral fusogenicity in macrophages, rather than CCR5 use, correlated with infectability for microglia. Furthermore, highly fusogenic X4 isolates were more able to infect microglia than poorly fusogenic R5 variants. The authors suggest that dual-tropic and highly fusogenic viruses may contribute to the neuropathologic manifestations of AIDS.

Dendritic cells have been implicated in viral dissemination. The current models suggest that dendritic cells trap virions and promote infection of T cells in *trans*. A presentation by van Kooyk (Abstract L10) discussed ongoing studies to characterize lectins that promote virion binding to dendritic cells. The investigator had previously identified DC-SIGN as an HIV receptor. Although DC-SIGN does not function independently as a receptor for viral entry, it is able to support *trans* infection of T cells that express the appropriate receptors. The results of studies presented by Lee (Abstract 529) suggest that DC-SIGN, when expressed in *cis*, can mediate more-efficient use of rate-limiting minor coreceptors. When expressed in *cis* and in conditions in which the coreceptor levels were limiting, DC-SIGN significantly increased the use of the coreceptors, including STRL33/BONZO, CCR2, APJ, and, to a lesser extent, the major coreceptors CCR5 and CXCR4. Coexpression of DC-SIGN conferred on some viruses the ability to use STRL33/BONZO to infect cells—they are unable to do so in the absence of DC-SIGN. The requirement for CD4 and a coreceptor was not alleviated by the expression of DC-SIGN. In addition, DC-SIGN was unable to increase infection through coreceptors when it was expressed in *trans*. The enhancing effects of DC-SIGN were further observed when it was expressed in *cis* on 293 fibroblasts. These studies show the importance of DC-SIGN in mediating the ability of dendritic cells to transmit virus to T cells. One feature of dendritic cell–T cell interaction involves the transinfection of resting cells by HIV. It is not clear whether DC-SIGN plays a role in the subsequent activation of T cells that are infected after contact with dendritic cells.

Laboratory-adapted HIV-1 isolates typically have X4 tropism. Despite the presence of CXCR4 on macrophages, these cells do not support efficient infection of laboratory-adapted isolates. Studies summarized by Tokunaga (Abstract 532) found that the expression of elevated levels of CD4 on macrophages increases the infection of primary X4 isolates but not laboratory-adapted isolates, and suggest that the cell surface levels of CD4 may be limiting for infection of primary macrophages by primary HIV-1 isolates.

Cyclophilin A is a cellular target of the immunosuppressive drug cyclosporine. Cyclophilin A is packaged into virions through interaction with the capsid domain of the Gag polyprotein. Virion-associated cyclophilin A significantly increases viral infectivity; however, the mechanism by which infectivity is increased is not well understood. Findings presented by Yurchenko (Abstract 114) and Bukrinsky (Abstract 530) identified a cyclophilin A–binding protein (CD147)

that uses a yeast 2-hybrid screen. The expression of CD147 in CHO cells increased HIV-1 entry. Viruses that contained mutations in Gag that disrupted interaction with cyclophilin A were not enhanced by CD147. Antibodies to CD147 inhibited viral uncoating, as evidenced by a reduced dissociation of HIV-1 core proteins from the membrane. The authors presented a model in which virion-encapsidated cyclophilin A interacts with CD147 of the target cell and this interaction promotes the uncoating step of viral entry.

Arguably, one of the most exciting developments in drug discovery presented at the conference was the identification of a new CCR5 antagonist. Reyes (Abstract L11) described the leading compound SCH C that inhibited both RANTES and MIP-1 □ binding to CCR5. SCH C had subnanomolar antiviral activity against R5 isolates but had no effect on X4 isolates. Surprisingly, SCH C could suppress viral replication even if it had been removed from the cells 25 hours before viral infection. In vitro propagation of R5 virus in the presence of SCH C resulted in the emergence of viral mutants that were greatly resistant to SCH C. Surprisingly, these resistant variants still maintained R5 tropism rather than having switched to an X4 phenotype, and the resistant variants were still sensitive to inhibitors of R5 entry. In addition, viruses passaged in SCID-HU mice in the presence of SCH C acquired resistance, but without having switched from the R5 to the X4 phenotype. A second-generation compound called SCH D, which is approximately 10-fold more potent than SCH C, was also discussed. These compounds are exciting new potential additions to the armamentarium of agents to combat HIV infection.

Pathogenesis

According to the current models of primate lentiviral replication, productive infection of CD4+ T cells requires that they be in cell cycle. Resting (G_0) T cells are refractory to infection because of rate-limiting levels of coreceptor, because of low levels of deoxyribonucleoside triphosphate, which limit the extent of reverse transcription, and because of inefficient nuclear localization of viral nucleic acids. Studies have suggested that T cells must be in the G_{1B} stage of cell cycle or beyond to support productive infection. Several presentations provided surprising findings that, under certain conditions, HIV-1 can apparently infect and replicate within noncycling T cells.

Goldsmith (Abstract S18) presented findings on the characteristics of HIV replication in human lymphoid histocultures. This culture system, which was originally described by Margolis, consists of tonsil and spleen explants that are maintained as "raft" cultures and are highly permissive to HIV infection. Goldsmith compared the replication characteristics of X4 and R5 viruses within the histocultures and showed that, although R5 viruses infect fewer T cells than X4 viruses, the extent of viral replication, as indicated by the amount of secreted p24, is the same with both viruses. Goldsmith also found that viruses that lack Vpr, an accessory protein that has been shown to facilitate the infection of macrophages in vitro, infected far fewer macrophages in these histocultures. Surprisingly, a substantial proportion of the infected T cells in these lymphoid histocultures appeared to be in the G_0/G_{1A} phase of the cell cycle and had a naive cell phenotype. Both X4 and R5 viruses were able to infect naive resting T cells. Furthermore, the presence of p24 antigen in these naive resting T cells suggested that they were permissive for productive viral infection. The investigators ruled out that T cells were infected while in cycle. They further showed that naive cells produce HIV at lower levels than do memory cells. This infection was cytopathic, since these naive resting cells were depleted from these histocultures.

Continuing with this theme, Scales and colleagues (Abstract 80) found that in dendritic cell–T cell cocultures HIV replication was observed in T cells that lack activation markers. Collectively, these studies support the notion that primate lentiviruses can productively infect noncycling T cells. At present, it is not clear whether the productively infected cells are truly quiescent "G_0" lymphocytes or whether these cells are in a very early stage of cell cycle. Since T cells in vitro are not susceptible to HIV infection unless they have been stimulated to enter cell cycle, the microenvironment of the histoculture or the dendritic cell–T cell environment may provide signals such as cytokines that promote the permissiveness of noncycling T cells. Previous studies by the Littman laboratory have found that certain cytokine combinations are sufficient to render noncycling T cells permissive to HIV infection. These studies further extend observations by the Haase group, who found infection of noncycling T cells during SIV and HIV infection. It will be important to determine the extent to which noncycling T cells support HIV replication in vivo, since these cells may have very different turnover characteristics than infected cells that are in cell cycle, and the presence of a reservoir of infected noncycling cells may have considerable implications for viral latency and persistence.

Another presentation indicated that natural killer cells may be susceptible to HIV infection in vivo. Valentin and colleagues (Abstract 505) used 4-color flow cytometry combined with real-time polymerase chain reaction to characterize populations of infected cells in patients who are being treated with highly active antiretroviral therapy (HAART). Using this approach, the investigators identified a subpopulation of natural killer cells that expressed CD4 as well as the coreceptors CCR5 and CXCR4. Proviral DNA was detected in purified natural killer cells from a large percentage of the HIV-1–infected patients, and this proviral DNA appeared to be very stable for up to 18 to 24 months in these patients. Further study is clearly warranted to determine the role of natural killer cells in maintaining viral persistence in the face of HAART.