Developments in Basic Science Research

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As with last year's meeting, perhaps the greatest emphasis in the basic science categories was in the area of cellular cofactors that influence HIV-1 replication either positively or negatively. A number of presentations provided detailed insight into the mechanism by which APOBEC 3G, the cellular target of Vif, effects its antiviral activity. The surprising thing is that this antiviral activity is not restricted to primate lentiviruses but is active against retroviruses and even hepatitis viruses. In the area of positive-acting cellular cofactors, the emphasis was on those cellular proteins that facilitate egress of the virus from the infected cell. It is now apparent that viruses such as HIV-1 can bud into cytoplasmic vesicles in order to establish a unique intracellular reservoir. How viruses move between cells was also the focus of several presentations at the meeting, and there were further surprises about the mechanism by which HIV-1 may establish a latent infection.

Cellular Cofactors That Oppose Viral Replication

When considering mechanisms of host defense against viruses such as HIV-1, one traditionally thinks of the humoral and cellular arms of the immune response. Over the past several years, it has become increasingly apparent that in addition to the immune response, cellular factors exist within cells that counteract HIV-1 replication at various levels. The most dramatic example of a cellular factor that acts as an antiviral defense is the cellular protein APOBEC 3G, which was identified approximately 1to1.5 years ago as the cellular target for the HIV-1 Vif protein (Sheehy et al, *Nature,* 2002). Vif is a viral accessory protein that is essential for the replication of primate lentiviruses in primary cells, and viruses lacking Vif are made in a noninfectious form. Once Sheehy and colleagues identified the cellular target of Vif, a number of groups went on to demonstrate the mechanism by which viral replication is restricted in the absence of Vif (reviewed in Harris et al, *Nat Immunol,* 2003). Several presentations (Abstracts 63, 101, 103, 351) presented evidence that APOBEC 3G induces extensive deamination of deoxycytidine (dC) to deoxyuridine (dU) on minus-strand viral complementary DNA (cDNA) during reverse-transcription. This could negate viral replication by interfering with synthesis of the plus-strand cDNA. In addition, the cDNA could be targeted for destruction by, for example, the uracil DNA glycosolase-dependent pathway. An analogy between this process and the action of another deaminase known as activation-induced cytidine deaminase (AID, which is required for antibody gene diversification) was highlighted in the plenary presentation (Abstract 15). In order to protect themselves from APOBEC 3G, primate lentiviruses have evolved *vif* genes. A number of presentations focused on the mechanism by which Vif inhibits the action of APOBEC 3G (Abstracts 62, 103, 102). The consensus is that in order to induce deamination of minus-strand cDNA during reverse-transcription, APOBEC 3G must be incorporated into viral particles so that it can be present at the site of reverse-transcription after viral entry. Earlier studies in the field demonstrated that the cell that manufactures virions dictates the block to viral replication. Presentations at the conference indicated that in the presence of *vif,* the amount of APOBEC 3G that is incorporated into viral particles is reduced; further, in cells expressing Vif, the stability of APOBEC 3G is greatly reduced. Inhibitors of protease function increased APOBEC 3G stability, suggesting that Vif is somehow promoting degradation of APOBEC 3G in the proteasome. Proteins targeted for proteasome destruction are often ubiquitinated. Presentation 62 presented evidence that Vif directly interacts with E3 ubiquitin ligase, and that this results in mutual destruction of Vif and APOBEC 3G in the proteasome. Although progress in this area is significant, important questions remain. For example, the mechanism by which APOBEC 3G is incorporated into viral particles is not clear. In addition, if interaction between Vif and APOBEC 3G represents a future target for antiretroviral intervention, then it will be important to determine whether APOBEC 3G function is essential to the survival of the cell and to the host. CCR5 is an attractive target for therapeutic agents because individuals lacking functional CCR5 (homozygous Δ-32 deletion) are otherwise immunologically competent. Theoretically, small molecules that prevent interaction of Vif with APOBEC 3G would break down the viruses' defense against APOBEC 3G's antiviral activity.

With the exception of equine infectious anemia virus (EIAV), all primate and nonprimate lentiviruses have a *vif* gene. Therefore, APOBEC 3G or proteins of the same family may exert activity against a variety of lentiviruses. However, one study presented at the conference (Abstract 101) indicated that APOBEC 3G has the ability to inhibit very unrelated viruses. This is exemplified by the demonstration that hepatitis B viral replication is markedly impaired in cells that overexpress APOBEC 3G. It is possible that the levels of APOBEC 3G required to inhibit hepatitis B viral replication are not normally present in cellular reservoirs of hepatitis B viral replication, and that APOBEC 3G under physiologic conditions does not impact hepatitis B viral replication. However, one possibility is that proteins such as APOBEC 3G are regulated by, for example, the interferon response—and this may provide fundamental insight into the mechanism of innate host immunity against viruses. The fact that HIV-1 is a zoonosis was also made apparent by the demonstrations (Abstracts 103, 352, 353) that the species-specific activity of APOBEC 3G is governed by very specific genetic determinants. Thus, human

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APOBEC 3G is efficiently targeted for destruction by HIV-1 Vif, and APOBEC 3G from the African green monkey is not affected by HIV-1 Vif. Exchange of a single amino acid in human APOBEC 3G to the same residue as that in African green monkey APOBEC 3G allows it to be targeted by both HIV-1 and simian immunodeficiency virus (SIV) Vif. This graphically illustrates the fact that cellular defenses that restrict transmission of lentiviruses from monkeys to man can be overcome by subtle polymorphisms within genes such as *vif*.

Although APOBEC 3G acts at the level of reverse-transcription to block virus infection, there are additional cellular defenses that block infection before reverse-transcription, and these appear to target the incoming Gag protein (reviewed in Bieniasz, *Trends Microbiol,* 2003). The existence of cellular restrictions was identified more than 3 decades ago, when mice were found to carry genetic traits that control susceptibility to leukemia induced by the Friend-strain of murine leukemia virus (MLV)—a phenomenon dubbed "Friend virus susceptibility" (Fv). More recently, similar restrictions termed lentivirus susceptibility-1 (Lv1) and restriction factor-1 (REF1) were shown to inhibit a range of retroviruses, including HIV-1. These restrictions appear to target the Gag protein since the block to infection can be saturated by overexpression of capsid proteins from incoming virions. In the case of Lv1, this activity restricts infection of nonhuman primate cells by viruses such as HIV, SIV, and EIAV; REF1, on the other hand, is expressed by human cells and prevents infection by retroviruses such as MLV. Studies presented at the meeting (Abstracts 104, 356, 357) presented evidence that Cyclophilin A confers differential sensitivity to Lv1 and REF1 restrictions. Cyclophilin A is a host cell protein previously shown to be important for efficient uncoating of the viral capsid following viral entry. This rule has now been revised. Owl monkey cells are resistant to HIV-1 infection because of the presence of Lv1. However, if the interaction of Cyclophilin A with capsid is blocked using cyclosporine A, HIV-1 infectivity is increased by 2 logs. Conversely, preventing Cyclophilin A-capsid interactions in human cells restricts HIV-1. How this differential effect is manifested will probably become clearer once the actual proteins that constitute Lv1 and REF1 are identified, and should further provide novel drug targets over therapeutic intervention.

Presentation 105 described the identity of a factor called ZAP that inhibits replication of retroviruses such as MLV, as well as alphaviruses including Sindbis and Semliki Forest. ZAP was not active against HIV-1. In contrast to the previously discussed restriction factors, ZAP does not prevent viral entry and integration but appears to destabilize or inhibit nuclear export of retroviral messenger RNAs (mRNAs). ZAP contains a zinc finger motif that interacts with MLV RNA near the 3'-end. ZAP also contains a protein destabilization motif. It is likely that APOBEC 3G, Lv1, REF1, and ZAP are the first of a number of yet-to-be-identified restriction factors targeted to primate and nonprimate lentiviruses. This is likely to be a fruitful area of research for the next several years.

Positive Cellular Cofactors

Viruses such as HIV-1, despite a limited genetic repertoire, still have to carry out a number of functions. Therefore, it is not surprising that these viruses abscond with cellular proteins and cellular pathways in order to complete various steps in viral replication. Perhaps the best illustration of this is receptor and coreceptor molecules required for viral entry. The transacting factor Rev relies upon the nuclear export factor Crm-1 for its proper functioning, and the transacting protein Tat is simply a recruitment factor for the cellular protein Cyclin T1 that promotes transcriptional activation from the long-terminal repeat (LTR). New cofactors important for detachment of assembling viral particles from the plasma membrane were presented at the conference (Abstracts 6, 64, 65). During HIV-1 assembly, the viral Gag protein binds genomic viral RNA and then interacts with the plasma membrane to assemble into enveloped particles that bud from the infected cell (Pornillos et al, *Trends Cell Biol,* 2002). These enveloped particles must detach from the plasma membrane by a fission event that severs the membrane of the assembling virion from the cellular membrane. Since expression of Gag protein in the absence of other viral proteins is sufficient to recapitulate virus budding and detachment, there must be cellular proteins that interact with Gag that promote this fission event. The first such cellular protein that was recognized to be involved in this step was tumor-suppressed susceptibility gene 101 (Tsg-101). It was identified in a yeast 2 hybrid genetic screen on the basis of its ability to bind to a motif in the p6 region of Gag known as the late domain. This domain is so named because mutations within it lead to a defect at a late step in viral replication (ie, virion detachment). Tsg-101 is a component of the Class E vacuolar sorting (Vps) machinery. As discussed at the meeting, Tsg-101 is one of a family of Class E Vps proteins that participate in the assembly and release of viral particles. Furthermore, the Vps proteins are important for the release of other pathogenic human viruses like Ebola virus and human T-cell leukemia virus Type 1 (HTLV-1). Considering the normal function of Tsg-101, the reason viruses such as HIV-1 have adopted it for their own uses becomes apparent. Tsg-101 normally functions in the cellular vacuolar protein-sorting pathway by selecting cargo for incorporation into vesicles that bud into endosomes, so as to create multivesicular bodies (MVBs). The formation of MVBs, which involves budding of vesicles away from the cytoplasm, contrasts the formation of, for example, endocytic vesicles, which bud into the cytoplasm. Since virus budding to the exterior of the cell parallels vesicular budding into the MVBs, it is expected that these processes depend upon the same cellular machinery. Indeed, more than a decade ago, it was demonstrated that in macrophages, HIV-1 could assemble into cytoplasmic vesicles, and more recently, these vesicles were identified as MVBs (Raposo et al, *Traffic,* 2002). Upon first demonstration, this process was considered an experimental curiosity. However, there is an increasing suspicion that viral particles that enter MVBs in cells such as macrophages may constitute a unique cellular reservoir for viral persistence. Data presented at the conference (Abstract 46) indicated that all the viruses released from infected macrophages originate from MVBs, and other data (Abstract 65) examined how

the processes of plasma membrane assembly at the MVB may be regulated in different cell types. In that study, the ability of Gag to localize to the plasma membrane required interaction with the plasma membrane lipid phosphatidylinositol-(4,5)-biphosphate (PIP_2). Depletion of PIP2 resulted in Gag being targeted to the MVB. One possibility is that in macrophages, where virus budding is predominant, the MVB, PIP_2 may be rate-limiting. These studies illustrate the existence of 2 potential routes for Gag targeting that may define how viruses are sequestered and ultimately transmitted to neighboring cells. MVBs typically fuse with the plasma membrane and release vesicular contents (including viruses) at points of contact with neighboring T cells, and hence may represent an efficient mechanism for cell-to-cell transfer of viral particles. One important question regarding the assembly of HIV-1 virions into the MVB is the fate of those particles. MVBs often undergo acidification that would be predicted to rapidly inactivate vesicularized virions. At present, it is unclear if viruses sequestered within MVBs retain infectivity or if there are specialized MVBs that do not undergo acidification and can retain vesicularized viruses over extended periods. These questions are relevant to the issue of viral persistence in macrophage reservoirs.

A distinguishing feature between lentiviruses such as HIV-1 and oncoretroviruses such as MLV is the former's ability to infect nonmitotic cells. The current consensus is that nucleophilic proteins associated with the incoming viral reverse-transcription complex allow the complex to traverse the nuclear envelope in order to promote contact of viral cDNA with host cell chromatin. Integrase has been suggested as a potential nuclear import factor that allows infection of nondividing cells. Integrase catalyzes the insertion of the viral cDNA into cellular DNA of the infected host cell. Lens epithelium-derived growth factor, p75 (LEDGF) has been identified as a cellular protein that interacts with integrase. Two studies (Abstracts 68, 337B) presented evidence that LEDGF dictates nuclear localization of integrase. Therefore cellular proteins such as LEDGF, through their interaction with integrase, may dictate nuclear import of the viral cDNA. In agreement with this model, both studies presented evidence that mutations in LEDGF that prevent association with integrase result in a redistribution of integrase from the nucleus to the cytoplasm. Both studies then used an RNA-interference strategy to inhibit the expression of LEDGF in cells in order to gauge the effect on viral replication. One study's results (Abstract 337B) found that depletion of LEDGF did not affect viral replication. The other study's results (Abstract 68) found that inhibition of LEDGF expression by RNA-interference blocked viral replication by preventing association of viral cDNA with chromatin, but not nuclear import. The reasons for these differences are unclear, and further studies are required in order to determine whether LEDGF is a cofactor for HIV-1 replication.

Although there is a general consensus that lentiviruses such as HIV-1 infect nondividing cells because they contain nucleophilic functions that promote nuclear entry of the reverse-transcription complex, there is no agreement on the viral factors that promote nuclear uptake of the reverse-transcription complex. One study (Abstract 67) presented surprising findings that MLV/HIV-1 chimeric viruses in which the capsid protein is exchanged altered the viruses' ability to infect nondividing cells. However, there is no strong evidence that the capsid protein of HIV-1 is actually retained within reverse-transcription complexes as it translocates to the host-cell nucleus. Therefore, the authors suggest that capsid acts as a barrier to nuclear import by masking receptors on the reverse-transcription complex that interact with nucleophilic viral or cellular factors. In the case of MLV, which cannot infect nondividing cells, the association of capsid with the reverse-transcription complex would prevent this virus's ability to access the nondividing nucleus. In the case of HIV-1, since capsid is not retained, binding sites on the reverse-transcription complex for nucleophilic factors are exposed for binding nucleophilic factors that promote HIV-1 infection of nondividing cells. The actual viral and cellular factors that mediate the import process itself await validation.

Viral Replication and Transmission

Infectious virion particles serve as conduits for the transmission of genomic viral RNA from the infected cell to new substrate cells. The general consensus is that virions are released into the extracellular space and then randomly encounter new target cells. Studies presented at the conference (Abstracts 44, 45, 46) support the notion that viruses such as HIV-1 may have evolved the ability to exploit the antigen-presenting properties of macrophages and dendritic cells in order to promote efficient cell-to-cell viral dissemination. As discussed earlier, viruses assemble into MVBs of macrophages and are released by exocytosis onto neighboring cells. In dendritic cells, evidence was presented (Abstract 44) that HIV-1 virions are internalized into a late endosomal compartment, as evidenced by expression of tetraspanins such as CD81, a marker for the MVB. This compartment into which virions are internalized lacks early and late endosomal markers, suggesting that viruses may not be rapidly inactivated by acidification. Upon contact with T cells, the compartment harboring virions appeared to localize at the synapse between the dendritic cell and the T cell. Such a synapse would provide a favorable environment for the efficient transfer of internalized particles to target T cells. In addition, since the synapse also involves the display of costimulatory molecules to the T cell, it is likely that T cells involved in the synapse would be brought into a state of readiness to allow viral infection and replication. Continuing on with this theme, abstract 45 presented evidence that synapses formed between infected and uninfected T cells promote recruitment of CD4, CXCR4, and LFA-1 on the target cell, as well as envelope and Gag proteins on the infected cell, and coordinate transfer of viral proteins into the target cell. Receptor and coreceptor recruitment on the target cell were mediated through Actin, suggesting that the effector cell transmits signals, perhaps through envelope engagement of CXCR4 on the target cell, that promote further recruitment of coreceptor and costimulatory molecules that favor viral transmission. Studies of this kind have

important implications regarding the mechanism of viral dissemination and the ability of the immune response to contain it. Cell-free viral particles are rapidly cleared by the reticuloendothelial system of the host and are also subject to neutralization by circulating antibody. These forces may be less able to prevent viral dissemination in the context of a virologic synapse.

The establishment of a reservoir of latently infected cells is considered the single biggest obstacle to HIV-1 eradication by potent antiretroviral therapy. However, the mechanism through which latently infected cells are established is still unclear. Since a number of studies have indicated that truly quiescent (G_0) T cells are refractory to infection, it is generally agreed that latency is established when a cycling cell is infected and then returns to quiescence before viral cytopathic effects or immunologic clearance mechanisms destroy the infected cell. A study presented at the conference (Abstract 123LB) suggests that HIV-1 can infect and integrate within quiescent lymphocytes. This observation is at odds with a number of published studies that suggest the viral life cycle is blocked before integration in quiescent cells. The difference in this study is that the investigators used spin-inoculation to increase the efficiency of infection. One possibility is that the process of spin-inoculation provides a subtle stimulus that brings the cells out of quiescence. Therefore, this observation needs confirmation, but it nevertheless suggests that infected quiescent lymphocytes could potentially represent the immediate precursor to the latently infected T cell.

In the same session, data were presented suggesting that the chronic activation state seen in pathogenic HIV-1 and SIV infections may be partly due to the infection and destruction of cells that suppress T-cell activation. In contrast to pathogenic HIV-1 and SIV infections, SIV in its natural host (for example, the African green monkey) does not cause disease, and this has been correlated with a lack of immune hyperactivation. CD4+ and CD25+ T cells (also known as Treg cells) have the capacity to suppress T-cell activation. In Abstract 124LB, Treg cells were shown to be highly susceptible to HIV-1 infection. This suggests that the disruption of the T-cell regulatory system by HIV-1 infection may remove the controls to hyperactivation of conventional T cells, thereby contributing to the chronic activation state that is characteristic of AIDS progression.

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Conference Abstracts cited in text can be found at www.retroconference.org.

Additional Suggested Reading

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