

Highlights of Basic Science Research

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Three symposia and 6 slide sessions were dedicated to the presentation of basic research at the conference. Unpublished research observations on the activities of the accessory proteins and, in particular, Nef and Vif, were a strong feature of these sessions. In contrast to last year's conference, research on the coreceptors dealt less with coreceptor usage and viral tropism but focused on strategies to block viral entry. Many sessions on accessory protein function dealt with identification of potential cellular intermediaries through which accessory proteins affect viral replication. These studies represent an important first step to the identification of novel targets for the intervention of viral replication. A large number of presentations featuring new data helped make this one of the best showings of basic research at the conference.

Virology

A task in AIDS research is to develop convenient small animal models that may reconstitute some aspects of HIV-1 replication. One of the challenges to developing small animal models for viral replication is the presence of barriers to viral replication in non-primate hosts. For example, HIV-1 infection of a cell requires the presence of CD4 and a coreceptor such as CXCR4 or CCR5. In order to confer permissiveness to HIV-1 infection on mouse cells, investigators have been examining susceptibility of these mouse cells to infection after expression of human CD4 and coreceptor molecules on the cell surface. In addition, efficient Tat function has been shown to require the presence of a cellular protein (cyclin T1). However, the murine equivalent of cyclin T1 is unable to interact with HIV-1 Tat. Substitution of a single amino acid within mouse cyclin T1 confers the ability to efficiently interact with Tat and mediate transactivation of the long terminal repeat (LTR) in mouse cells (Wei et al. 1998).

Landau and colleagues (Abstract 217) presented evidence for yet another block to viral replication in murine cells. The investigators engineered murine 3T3 fibroblasts to express human CD4, CCR5, and cyclin T. These cells supported efficient reverse transcription and integration to equivalent levels following infection of human cells. In addition, Tat functioned efficiently in these cells due to the presence of human cyclin T. The infected cells also manufactured viral proteins. However, the amount of virus production from these cells was significantly reduced relative to human cells. The reduction in virus output was due to a defect in processing of Gag polyproteins. Despite this reduction, the small amount of virus particles that were released from these murine cells were fully infectious for permissive human cells. The investigators then made heterokaryons between HIV-1 infected murine cells and uninfected human cells and found that these heterokaryons released normal quantities of infectious virus. These results suggested that murine cells lack a cofactor that is necessary for efficient Gag processing.

These lines of investigation are important in that they uncover new cellular cofactors that are required by HIV for efficient viral replication. However, they also highlight the problems that may be encountered in trying to adapt small animal models for HIV-1. Another important point that emerges from this research is whether viruses produced from murine cells will exhibit a Vif dependence. As discussed by Gabuzda in the accessory gene symposium (Abstract S27), Vif counteracts the effect of a negative cellular factor that inhibits production of infectious virions. This action appears to be species-restricted, in that simian immunodeficiency virus (SIV) Vif is unable to counteract the activity of the negative cellular factor in human cells (Simon et al. 1998). Thus important questions are whether murine cells express the negative cellular activity and whether HIV-1 Vif is able to counteract it. These questions

will be important in determining whether viral replication can be established in murine models.

The presentation by Hope and colleagues (Abstract S26) provided a fascinating visual insight into how viruses migrate through the cell as they move from the point of cell contact to the host cell nucleus. Hope and colleagues incorporated the marker green fluorescent protein (GFP) into viral particles by fusing it with Vpr, an accessory protein that is packaged within virions. The investigators then pseudotyped the virus with VSV G-glycoprotein to increase the infectivity of the virion and used GFP as a marker to follow early post-entry events in the viral life cycle. Vpr has previously been shown to remain associated with viral nucleic acids within the reverse transcription complex as it translocates to the cell nucleus. Using colocalization analysis and video microscopy, the investigators demonstrated that reverse transcription complexes associate with and move along the microtubule component of the cytoskeleton. The investigators also demonstrated that viral complexes moved along microtubules when entry was mediated through a natural pathway of infection, ie, HIV-1 envelope-mediated fusion. An important question remains as to whether the microtubule component of the cytoskeleton is necessary for viral infectivity, and this will be investigated in the future by examining the influence of microtubule disrupters on viral infectivity.

A well-recognized feature of HIV-1 replication is its cytopathic effect on the host cell during virus replication. Previous studies have suggested that the virus kills cells both by promoting the fusion of infected and uninfected cells (syncytium induction) and by causing the death of individual cells (single-cell killing) by as yet unknown mechanisms. The cytopathic effects of viral replication have been implicated as the underlying cause of lymphocyte depletion in HIV-1 infected individuals. Surprising studies by Bucy and colleagues (Abstract 224) presented

evidence that under certain conditions, HIV-1 is not directly cytopathic for infected primary T cells *in vitro*. Bucy's research group infected primary T cells and then blocked viral spread using antiretroviral agents. When infected cells were cultured at high density, both infected and uninfected cells were lost in the culture, suggesting that direct viral killing and indirect viral killing by a paracrine mechanism were responsible. However, when cells were maintained at a low density, infected T cells were not lost from the culture. The investigators propose that, at a high density of infected cells, there is considerable indirect cytopathicity likely by the effects of released viral products on bystander standard cells. At a low density of infected cells, the level of viral products that are released from the infected cells may be insufficient to induce bystander killing.

These studies have considerable implications *in vivo*, where even in tissue reservoirs the density of infected cells is low. The authors speculate that the *in vivo* death of infected cells may be due to an immune clearance mechanism rather than direct viral cytopathicity. This study may provide an answer to a long-standing question regarding the establishment of latency. Presumably, the establishment of latent proviruses requires that cells be in a cycling state at the point of infection, so as to permit provirus establishment. The issue is how cells, once infected, survive the cytopathic effects of infection long enough to revert to the resting state harboring a latent provirus. According to Bucy's model, the lack of direct cytopathicity would allow productively infected cells to return to a resting state without being cleared by viral cytopathic effects. Presumably, in the presence of antiretroviral agents, there may be insufficient antigen-driven cytotoxic T lymphocyte (CTL) surveillance such that infected cells are not affected by immune clearance mechanisms. Thus it will be important to determine whether highly active antiretroviral therapy (HAART) increases the frequency of latently infected cells.

HIV-1 has been shown to induce cell death by 2 distinct mechanisms, namely syncytium-dependent killing and syncytium-independent, single-cell killing. The mechanism underlying virus induced cell death is still poorly understood. Sheeter and colleagues (Abstract 158)

presented evidence that HIV-1 causes direct cell killing through disruption of mitochondrial function in a process that activates caspase 9-induced apoptosis. HIV infection was accompanied by a release of cytochrome C and apoptosis-inducing factor from the mitochondrial compartment to the cytosol and by a reduction in mitochondrial transmembrane potential. Cells undergoing apoptosis exhibited increased levels of fas-ligand. The investigators suggest that HIV-1 directly kills infected lymphocytes by activating an intrinsic mitochondrial-mediated apoptic pathway.

Following infection of the target cell, reverse transcription of viral RNA leads to the formation of a linear complementary DNA (cDNA) molecule and of circular cDNAs containing either 1 or 2 LTRs. These circular molecules are generally believed to be dead-end products of viral reverse transcription, since HIV-1 lacks factors that stabilize or maintain episomal replication. Since episomes are relatively labile products of reverse transcription, several groups employed these circles as surrogate markers of recent infection events to examine whether replication was ongoing in individuals who were aviremic through antiretroviral therapy (Abstracts 136, 561, and 650). Shaunak and colleagues (Abstract 650) presented evidence that circle forms of viral DNA are unstable *in vitro* under conditions of single cycle infection (See also Sharkey et al. 2000; Pauza et al. 1990). Analysis of patients on long-term antiretroviral therapy who had sustained plasma HIV RNA below detection limits indicated that the majority of these patients had detectable levels of 2-LTR circles suggesting that viral replication is ongoing in these patients. The presence of a reservoir of ongoing viral replication may provide an explanation for the apparent long half-life of the latently infected reservoir, since ongoing replication allows reseeding of latently infected cells.

A number of presentations were concerned with the role of latent viral reservoirs in limiting the effectiveness of antiretroviral therapy. Several groups have demonstrated the existence of resting memory CD4+ T cells that harbor latent integrated HIV and that can release infectious virus following lymphocyte activation. In addition, previous studies (Wong et al. 1997; Finzi et al.

1997) indicated that during antiretroviral therapy, these latent genomes did not acquire drug resistance mutations. Thus, latently infected cells may have been established prior to initiation of therapy and may represent a genetically stable reservoir *in vivo*. Martinez-Picado and colleagues (Abstract 238) examined drug resistance genotype and phenotype of viruses recovered *in vitro* from latently infected lymphocytes from 12 patients with sustained suppression of plasma HIV-1 RNA. Drug resistant mutations were identified in 4 of 5 patients who exhibited periods of transient viremia but not in the 7 patients who did not exhibit transient viremia. These results indicate that transient episodes of viremia are sufficient to promote the acquisition of drug resistance mutations by HIV.

In a further study of viral evolution in the pool of latently infected cells, Persaud and colleagues (Abstract 138) examined viruses that came from latently infected cells for the presence of drug resistance mutations. Resistance mutations observed in virus obtained from the latent pool were likely established by prior partially suppressive regimens, supporting the notion that persistence of latent HIV-1 genomes preserves drug-sensitive viral genotypes.

One approach to eliminating the latent reservoir and improving conditions for viral eradication through HAART is to drive the virus out of latency to where it would be susceptible to antiretrovirals. One approach taken to achieve this is to stimulate latently infected cells with interleukin-2 (IL-2) therapy. Stellbrink and colleagues (Abstract 240) evaluated the extent of productive and latent HIV infection following treatment cessation in patients on and off IL-2 treatment. The investigators did not observe any effect of IL-2 therapy on either virus production, the extent of latent infection, or the immunologic control of HIV-1 infection. One possible complication of "purging" latent reservoirs by IL-2 is the contribution of ongoing viral replication to the pool of virus-infected cells.

The issue of ongoing replication in the face of HAART was further explored by Bucy and colleagues (Abstract 140). They detected the presence of unspliced HIV RNA in peripheral blood lymphocytes in 25 subjects with plasma viral RNA below detection. Limiting dilution analysis indicated that there are approxi-

mately 1000 copies of viral RNA per infected cell. Longitudinal signal analysis to examine the variation in viral RNA-positive cells over time suggested the existence of a reservoir of persistently infected and transcriptionally active cells in patients on HAART with plasma viral RNA below detection limits.

A study by Zhu and colleagues (Abstract 136) examined reservoirs of viral replication in patients on HAART. The investigators detected proviral DNA in resting and activated T lymphocytes and peripheral blood monocytes in patients who had been on HAART for up to 4 years. The investigators also detected the presence of unspliced viral RNA to a higher degree in activated T cells and monocytes than in resting T cells. There was also a higher level of sequence evolution in activated T cells and monocytes relative to that in resting T cells. The authors propose that active replication in monocytes and activated T cells create the conditions for continual reseeding of latent HIV reservoirs. One rather surprising feature of these observations is that monocytes appear to represent a significant reservoir for viral replication in patients on HAART. Several studies demonstrated the presence of HIV-1-infected peripheral blood monocytes *in vivo*. However, freshly isolated blood monocytes are refractory to HIV-1 infection *in vitro* (Sonza et al. 1996). Thus it will be important to determine how circulating monocytes *in vitro* differ from freshly isolated monocytes *in vitro* in terms of their permissiveness to viral infection.

Continuing with the theme of monocyte/macrophage lineage cells as reservoirs for viral replication, Corey and colleagues (Abstract 287) examined the contribution of resident microglia and perivascular macrophages to virus replication in monkeys with terminal AIDS and SIV encephalitis. The investigators demonstrated that perivascular macrophages express CD14, but microglia do not express CD14 even when activated. Using *in situ* hybridization, the investigators demonstrated that perivascular macrophages were the predominant infected cell type, whereas microglia were not infected.

This study has important implications for viral persistence in the central nervous system (CNS) and how it may be impacted by HAART. Perivascular

macrophages are derived from infiltrating monocytes and have a rapid turnover in the tissue whereas microglia are long-lived resident macrophage lineage cells. Thus the viral reservoir in the brain is likely to represent a short-lived reservoir undergoing continual turnover rather than a long-lived reservoir of persistently infected cells, as has previously been suspected. It will therefore be important to determine what distinguishes microglia and perivascular macrophages in terms of their permissiveness to viral infection.

The issue of CNS reservoirs was further investigated by Albright and colleagues (Abstract 435), who like Corey and colleagues observed that monocyte macrophages express CD14 whereas microglia isolated from parenchymal CNS tissue do not. However, they observed that microglia were infectable *in vitro*. The extent of virus production by microglia relied upon their *ex vivo* activation following culture in the presence of serum and cytokines. Thus, it will be important for investigators to determine what distinguishes the permissiveness of microglia to infection *in vitro* and *in vivo*.

Veazey and colleagues (Abstract 447) examined the reservoir of viral replication in the intestine following acute SIV infection. Since the mucosal immune system contains far more lymphocytes than the systemic immune system, the authors speculated that lymphocytes in the mucosal immune system may constitute a major reservoir for viral replication during the acute viremic stage of infection. The investigators presented evidence that effector sites in the lamina propria, which contains a high proportion of activated T cells, are a major site for viral replication. At this site, there was a selective depletion of intestinal memory T cells, which are also highly positive for CCR5 expression. The investigators documented infection of CD4/CD8 double-positive cells expressing high levels of CCR5 and presented evidence that CCR5 plays a central role in determining whether cells are infected, and their activation state dictates whether they are cleared following viral infection. These studies may explain the selective loss of effector CD4+ T cells early in HIV and SIV infection and also underscore the role of the mucosa in viral transmission and replication.

Viral Receptors and Tropism

In general, the expression of the appropriate receptor or receptors on human cells is sufficient to allow entry of primate immunodeficiency viruses. A study presented by McKnight and colleagues (Abstract 648) suggested the presence of additional hurdles to virus entry following HIV-2 infection. Those investigators examined the phenotype of a primary HIV-2 isolate that, although able to exploit a broad range of coreceptors for infection of U87/CD4+ cells, was unable to infect other CD4+ human cell lines such as HOS/CD4 or primary macrophages. The fusion of permissive U87/CD4+ cells with nonpermissive HOS/CD4 cells allowed them to be infected, indicating that HOS cells lack a factor that is necessary for HIV-2 infection and replication. The investigators presented evidence that the defect to HIV-2 entry is at a step between production of full-length proviral DNA and transport to the host cell nucleus.

The studies conducted by Desrosiers and colleagues (Abstract 646) defined sequence changes that promote CD4-independent entry by SIV. The C3 region of HIV and SIV gp120 contains a GGDPE motif where the D residue has been shown to contact CD4. Mutation of the D residue abolished CD4 binding and replication of both HIV and SIV_{mac}. The investigators cloned envelope sequences from the brains of animals infected with SIV_{mac}239 and found that they contained an N residue in place of the D within the GGDPE motif. They demonstrated that there were 4 compensatory mutations in gp120 that confer CD4-independent entry and replication.

A longstanding issue in HIV-1 entry has been whether signaling following receptor engagement is necessary for viral entry. Numerous studies have demonstrated that signaling from both CCR5 and CXCR4 is not required for HIV entry into immortalized cells. Alfano and colleagues (Abstract 422) demonstrated that pertussis toxin (PTX) inhibited entry of R5 but not X4 viruses in primary CD4+ T cells. This effect was shown to be due to the ability of PTX to block CCR5 capping. Thus PTX appears to cross-deactivate CCR5 and capping of coreceptors is necessary for HIV entry into primary T cells.

The issue of coreceptor signaling

events that may affect viral replication was further addressed in a study presented by McManus and colleagues (Abstract 419). Those investigators examined various X4 and RS envelopes for their ability to elicit functional responses from primary human lymphocytes. R5 gp120 up-regulated CD18 and CD69 on peripheral blood mononuclear cells (PBMCs) and this up-regulation was blocked by an antibody to CCR5. The investigators also indicated that the internalization of receptor envelope complexes was mediated through clathrin. The authors propose that the ability of envelope to elicit cellular responses in the absence of infection may influence cellular trafficking and spread of the virus *in vivo*.

In a similar vein, Collman and colleagues (Abstract 410) demonstrated that HIV-1 gp120 promotes ion channel activation by both CCR5 and CXCR4 in macrophages. Using whole-cell patch-clamp studies and single-cell calcium imaging, the investigators demonstrated that recombinant gp120 activated 3 distinct currents in macrophages. The authors propose that envelope interactions with both CCR5 and CXCR4 promote distinct signaling responses in macrophages that may differ functionally from those induced by chemokines and that these responses may be important for macrophage function and permissiveness to infection.

Accessory Proteins

Although a number of activities have been described for the various primate immunodeficiency virus accessory proteins, how these proteins mediate their respective activities is poorly understood. An important area of investigation regards the cellular ligands through which accessory proteins manifest their activities, and a number of presentations at the conference were concerned with the identification of cellular ligands of viral accessory proteins. Nef proteins of HIV-1 and SIV have been shown to interact with cellular serine/threonine and protein tyrosine kinases. Renkema and colleagues (Abstract 220) presented evidence that the serine/threonine kinase that associates with Nef is P21-activated kinase-2 (PAK2). Antibodies to PAK2 were able to immunoprecipitate Nef. Surprisingly, overexpression of PAK2 did not

increase kinase activity associated with Nef but rather displaced endogenous PAK2 from the Nef-kinase complex. These studies indicate that a novel, as yet unidentified cellular factor is rate limiting in the formation of the Nef/kinase complex.

Another Nef interacting protein was identified by Geleziunas and colleagues (Abstract 221). HIV and SIV Nef proteins have previously been shown to activate the expression of fas-ligand on the cell surface. The investigators demonstrated that HIV-1 Nef inhibits the catalytic activity of apoptosis signal-regulating kinase-1 (ASK1), which plays a central role in death signaling through both FAS and tumor necrosis factor (TNF) receptors. The investigators further demonstrated that HIV-1 inhibited FAS- and TNF- α -mediated apoptosis. Mutagenesis of an R106 residue, shown previously to participate in PAK interaction by Nef, impaired the ability of Nef to protect cells from FAS- and TNF- α -mediated apoptosis. The authors propose that virus-infected cells are protected from apoptosis, which ultimately may extend the lifespan of the infected cell and its ability to evade CTL-mediated killing.

Ye and colleagues (Abstract 445) identified 2 novel domains in SIV_{mac} Nef that may participate in the association with and activation of PAK. Residues in the SI-13 domain, shown to mediate the interaction of Nef with protein tyrosine kinases, were shown to be critical for Nef-mediated PAK activation. In addition, analysis of *nef* alleles from monkeys infected with Nef deletion mutants demonstrated the presence of a core domain of Nef which was sufficient for induction of pathogenicity. This core domain was also found to conserve the ability to bind PAK. Thus, it appears that there is a strong *in vivo* selective pressure to maintain the ability of Nef to bind PAK and that this is ultimately linked to SIV pathogenicity.

One of the well-recognized features of HIV and SIV Nef is the ability to down-regulate surface expression of CD4. How this CD4 down-regulation is advantageous to virus replication is unknown. However, it has been suggested that down-regulation of CD4 from the cell surface may prevent reinfection of an already infected cell (superinfection) or alternatively may prevent misincorporation of envelope/CD4 complexes into

virions that may otherwise reduce virion infectivity. Geleziunas and colleagues (Abstract 446) used the SCID-HU thy/liv model to examine the replicative and pathogenic potential of HIV-1 variants that are impaired for CD4 down-regulation. Viruses compromised in the ability to down-regulate CD4 showed marked impairment in virus replication and in pathogenicity as evidenced by an inability to deplete cells within the thymic implants. By comparison, HIV-1 Nef mutants that were impaired for down-regulation of MHC class I (MHC1) or for binding to the kinases Hek or PAK were fully replicative and pathogenic within this model. This provides evidence that the ability to down-regulate CD4 has an important effect on the ability of the virus to replicate and cause lymphocyte depletion within the SCID-HU model.

One issue that is raised by these observations regards the relevance of CD4 down-regulation in the context of SIV infection. The majority of SIV variants do not require CD4 for infection but instead use CXCR4 as a primary receptor. However, the ability of SIV Nef to down-regulate CD4 indicates that such a mechanism has been conserved for some reason other than protection from superinfection resistance, presumably since levels of CD4 on the cell surface do not influence SIV infectivity. Thus, the basis through which CD4 down-modulation by Nef facilitates viral replication and pathogenicity is an important line of investigation.

In addition to CD4, Nef has been shown to influence cell surface expression of MHC1. A number of investigators have focused on the mechanism through which down-regulation of these important immunoregulatory molecules by Nef is elicited. A common theme that is emerging in this research is that Nef promotes the accelerated endocytosis and degradation of Nef and MHC1 by targeting them for lysosomal degradation. Consistent with this, Nef of HIV-1 contains a dileucine-based sorting motif characteristic of signals of cellular proteins that are efficiently endocytosed and targeted to lysosomes. Craig and Guatelli (Abstract 631) investigated the role of the dileucine motif of Nef in mediating interaction with adapter protein complexes. The investigators presented evidence that down-regulation of CD4 and optimization of viral replication, which are

both dileucine-dependent, are mediated through interactions with the AP3-based sorting pathway.

Nef has been shown to influence the infectivity of the virion. The basis of this activity is unclear. It has been suggested that this is related to the ability of Nef to down-regulate CD4. Yu and colleagues (Abstract 632) provided evidence that HIV Nef interacts with the envelope protein via Gag precursors and that this interaction positively influences viral assembly and infectivity. This interaction between Nef was observed for Gag and Gag-Pol precursors but not with proteolytically processed Gag proteins. In addition, the interaction was detected within cells but not within virions. The investigators suggest that this interaction may form the basis for the ability of Nef to influence virion infectivity.

A well-characterized activity of the HIV-1 Vpr protein is its role in nuclear translocation of the viral reverse transcription complex. One of the paradoxes regarding this activity is that Vpr must be transported to the site of virus assembly where it can be incorporated into virions via an interaction with the P6 domain of Gag. Sherman and colleagues (Abstract 627) mapped the nuclear import signal of Vpr to an arginine-rich stretch of amino acids from residue 73 to 96. The authors also demonstrated that Vpr, when fused

with GFP, exhibited a cytoplasmic localization. This cytoplasmic distribution was sensitive to leptomycin-B, which is an inhibitor of CRM1, a major mammalian nuclear export receptor. These observations are reminiscent of the Rev protein and the Gag matrix protein, which have each been shown to shuttle between the nucleus and cytoplasm during virus replication.

The role of Vpr shuttling in the virus life cycle remains an important area of investigation. Within the genomes of HIV-2/SIV, there is an additional open reading frame called Vpx. Previous studies have implicated *vpx* in nuclear targeting of viral reverse transcription complexes in nondividing macrophages. Like HIV-1 *vpr*, HIV-2/SIV Vpx does not contain canonical nuclear localization signals. Mahalingam and Hahn (Abstract 629) mapped the region of *vpx* that was important for its nuclear import. Mutagenesis studies indicated that residues 66 to 75 of *vpx* are important for virion incorporation and residues between amino acids 74 and 112 are important for nuclear localization of Vpx. Thus, it will be important to determine whether this region also mediates interaction with a nuclear import apparatus as has been demonstrated for HIV-1 Vpr.

As was elegantly summarized in the presentation by Dana Gabuzda (Abstract

S27), the HIV-1 accessory protein Vif promotes the infectivity of virions. How Vif manifests this activity on virion infectivity is not understood. Previous studies have suggested that viruses lacking Vif have an abnormal morphology yet otherwise are normal with regard to composition of virion components. Building on previous observations in which Vif-deficient viruses were shown to exhibit defects in endogenous reverse transcription, Dornadula and colleagues (Abstract 635) examined the effects of Vif on endogenous reverse transcription and demonstrated that the effect of Vif is only manifest when virions are treated with mild detergents. The investigators suggest that in the absence of Vif, the reverse transcription complex may become sensitive to mild detergent treatments. These studies are in agreement with studies presented by Gabuzda (Abstract S27), who demonstrated that viral assembly intermediates that are formed in the absence of Vif exhibit an increased detergent sensitivity. These studies are important in identifying the mechanisms through which the Vif protein supports viral infectivity.

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Additional Suggested Reading

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Sonza S, Maerz A, Deacon N, et al. Human immunodeficiency virus type 1 replication is blocked prior to reverse transcription and integration in freshly isolated peripheral blood monocytes. *J Virol*. 1996;70(6):3863-3869.

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