Inactivated HIV-1-Capturing Nanospheres Induce Mucosal Immunity Following Intravaginal Administration in Mice

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The highly active antiretroviral therapy (HAART) has achieved a reduced death rate of AIDS in developed countries. However, considering the high cost and low compliance of long-term HAART, it is obvious that prophylactic vaccine strategy against HIV-1 is the most desirable for the prevention of viral transmission. Since a major route of HIV-1 infection is via sexual intercourse, mucosal immune response may have critical effects on the establishment of the infection.

Concanavalin A (Con A) -immobilized polystyrene nanospheres (Con A-NS) are ultra-fine particles that are able to capture HIV-1 (1). Con A, which possesses a high affinity with the oligosaccharide chains of the HIV-1 envelope, was immobilized on the surface of nanospheres. In our previous study, HIV-1 was efficiently trapped by Con A-NS, resulting in a significant decrease of the infectivity of viral suspensions. Immunostaining and electron microscopic analyses revealed that viral particles and gp120 were densely captured on the surface of Con A-NS (2). Based on these findings, we are now developing Con A-NS as a candidate for the HIV-1 vaccine capable of preventing sexual transmission. Con A-NS could efficiently capture HIV-1 particles irrespective of their cell tropism (R5 or X4). Furthermore, Con A-NS equally captured infectious and heat-inactivated HIV-1. When inactivated HIV-1-capturing Con A-NS (HIV-NS) were intravaginally administered to mice, increased anti-HIV-1 IgA antibody response was identified in the vaginal fluids of immunized mice with HIV-NS. The vaginal fluids showed neutralizing activity against the immunizing HIV-1 strain. A marked difference in vaginal distribution was observed between HIV-NS and other immunogens, and the toxicity of Con A was dramatically reduced by conjugation with nanospheres. Thus, HIV-NS may have great potential as a prophylactic HIV-1 vaccine and should be further examined for its efficacy in monkeys.

Envelope determined properties associated with increased pathogenicity and transmissibility of X4- and R5-tropic SHIVs.

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Background: In vivo adaptation or passage of simian-human immunodeficiency viruses (SHIVs) results in emergence of variants that transmit infection across mucosal barriers more efficiently and cause disease when inoculated in rhesus macaques. Our studies focused on two variants, the X4-tropic SHIVSF33A and R5-tropic SHIVSF162P3. We characterized the properties of Env that could account for increased transmissibility and pathogenicity of these viruses.

Results: Compared to their parental isolates, pathogenic X4-SHIVSF33A and R5-SHIVSF162P3 replicated with greater efficiency and were more resistant to serum and antibody neutralization. Furthermore, the variants were transmitted more efficiently than their wild type counterparts in dendritic cells (DC)/T cell conjugates. An examination of the properties of the envelope glycoprotein gp120 revealed that SHIVSF33A Env mediates better entry and fusion, and confers escape from immune recognition. It also confers increased replicative and cytopathic properties to the virus in vitro as well as in vivo. SHIVSF162P3 Env also mediates better entry and contributes to escape from serum neutralization, but is impaired in its fusogenic ability.

Conclusions: Increased pathogenicity and mucosal transmissibility of SHIVSF33A and SHIVSF162P3 are associated with a common set of envelope-determined properties such as enhanced replicative capacity, neutralization escape and efficient infection and/or capture by dendritic cells. It will be of interest to determine whether increased binding of pathogenic Envs to CD4 and coreceptors underlies the mechanistic basis for changes in these properties.
Functions of HIV-Nef in Cell Activation and Viral Morphogenesis

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The nef gene of the primate lentiviruses is critical for their pathogenesis. Several functions of the Nef protein have been identified in cell culture, however, their relative contribution to the function of the viral protein in vivo remain unknown. Whereas the downmodulation of CD4 and MHC I molecules from the surface of infected cells by Nef could prevent superinfection and lead to immune evasion, respectively, Nef mediated activation of signaling transduction cascades and the enhancement of virion infectivity could directly heighten the replicative potential of HIV. Unraveling the relevant activities of Nef was hampered by the lack of a reliable cell culture system that reflects the positive effect of Nef on viral spread. Our results demonstrate, as previously shown for SIV, that the presence of HIV-Nef represents a prerequisite for viral replication in co-cultures of immature dendritic cells and autologous T-cells. The functional analysis of naturally occurring isoforms of Nef that differ in one amino acid within the otherwise highly conserved PxxP motif allowed us to conclude that the ability of Nef to activate the T cell receptor (TCR) signaling cascade is instrumental to drive viral replication in this system. This function may depend on the recruitment of molecules of the TCR environment into lipid rafts by Nef. These detergent resistant microdomains play also important roles in the morphogenesis of infectious particles as Nef increases virion infectivity by promoting the recruitment of structural viral proteins into rafts. Such a role of Nef during viral morphogenesis is also supported by the existence of a Nef variant that inhibits virus production and virion infectivity. The implications of these findings and current models of their molecular basis will be discussed.
Foamy viruses as live vaccine vectors for HIV.

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Since retroviruses are capable of integrating their genome into host DNA, they provide an efficient means for introducing foreign DNA. However, the use of “integrating” viruses for gene therapy or vaccine approaches to treatment and prevention of human disease raises safety concerns. We evaluated the possibility of using foamy viruses as a vaccine tool to take advantage of the natural history of human and animal infections with these agents, which have shown no pathogenicity. Using the human foamy virus (HFV) infectious molecular clone developed by Rethwilm, we have engineered both HIV-1 env and gag genes into the pFOV vector and analyzed them for gene expression and stability in vitro. The pFOV clone was provided containing GFP in place of the bet 2 gene. Either pFOV 10 (containing an SFFV U3 promoter) or pFOV 7 (containing only an internal promoter) were used to insert HIV-1 env (gp160) or HIV-1 gag (p17-p24), respectively. Results showed that pFOV 10-env produced gp120, as detected by a CD4-binding assay, only transiently after transfection into BHK cells. Further analysis showed the gp160 (approx. 3.0 kb) truncated and abrogated expression prior to foamy virus replication. The pFOV HIV gag construct, however, produced high levels (up to 1 ug/ml) of p24 antigen as detected by p24 antigen capture after transfection. Furthermore, the foamy virus recovered from the transfection could be passaged as infectious virus and retained the HIV gag gene along with p24 expression (>1 ug/ml on day 7 post infection). In addition, we have also developed a pFOVSIVgag construct which stably expresses SIV gag (approx. 300 ng/ml). These data show that stable high-level expression of both HIV and SIV gag can be produced in live, replicating foamy virus vectors.
Potential use of nef-deleted SHIV derived from a nonpathogenic SHIV as a live-attenuated vaccine, that protected macaques against challenge infection of a heterologous pathogenic SHIV

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OBJECTIVE: To evaluate the potential of nonpathogenic SHIVs as anti-HIV-1 live-attenuated vaccines, nonpathogenic gene-deleted SHIVs were examined with the immunogenicity and the protective effect in macaques. And the evidence supporting the safety was collected.

METHODS: SHIV-dn (nef-deleted), SHIV-drn (vpr/nef-deleted) and SHIV-dxrn (vpx/vpr/nef-deleted) were constructed from nonpathogenic SHIV-NM-3rN having \( V_b \) type of HIV-1 Env and vaccinated to macaques. One year later, they were challenged with the homologous parental SHIV-NM-3rN or heterologous pathogenic SHIV-89.6P. The protection was evaluated by measuring plasma RNA, proviral DNA and recovery of infectious viruses in PBMC and lymphnodes.

RESULTS: 1. When the gene-deleted SHIVs were inoculated to each four macaques, neutralizing antibody (Nab) and CTL to HIV-1 Env and SIV Gag were induced most effectively in SHIV-dn-vaccinated macaques and all the macaques were protected completely from challenge infection of the homologous parental SHIV-NM-3rN. In this protection, involvement of not only specific immunity (NAb and CTL) but also nonspecific immunity, such as NK activity, was suggested.

2. When four new macaques vaccinated with SHIV-dn were challenged with heterologous pathogenic SHIV-89.6P, the challenge virus was detected in all the macaques, but the viral loads were about \( 1/10^4 \) of that of unvaccinated macaques. Most importantly, no decrease of CD4\(^+\) cell number was observed in all the vaccinated macaques, while rapid decrease was observed in the unvaccinated macaques. In these vaccinated macaques after the challenge, no sign of the disease was observed clinically and histologically over one year. Interestingly, before the challenge, NAb and CTL to SHIV-NM-3rN were detected, but those to the challenge SHIV-89.6P were not observed. Almost the same but higher protective effect were obtained in intravaginal challenge of SHIV-89.6P in the vaccinated four macaques.

3. The replication of SHIV-dn in macaques was transient and not persistent. Besides, SHIV-dn was considered to be nonpathogenic, because, in contrast to nef-deleted SIV and HIV-1, SHIV-dn was constructed by nef--deletion from nonpathogenic SHIV-NM-3rN, in which no evidence of pathogenicity was observed by clinical and histological examination including apoptosis induction. Nonpathogenicity of the parental SHIV-NM-3rN was also shown in the neonates to which experimentally inoculated or vertically transmitted from carrier mothers. Furthermore, five in vivo passages of SHIV-NM-3rN in macaques did not convert it pathogenic.

CONCLUSIONS: The high protective effect of SHIV-dn against not only homologous but also heterologous pathogenic SHIV challenge was demonstrated. As SHIV-dn can replicate well in human PBMC, the effect observed in macaques can be expected in humans. Nonpathogenicity and no conversion to pathogenic virus was indicated. The most advantage of using SHIVs as live-attenuated vaccines is that the efficacy and the safety can be evaluated in macaques before human trials.
The duodenally absorbable CXCR4 antagonists, KRH, exhibit a potent and selective anti-HIV-1 activity \textit{in vivo} and \textit{in vitro}.

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A series of the novel low molecular weight non-peptide compounds, KRH, efficiently blocked replication of various T-cell line-tropic (X4) HIV-1 in MT-4 cells and PBMCs through the inhibition of viral entry and membrane fusion via the CXCR4 coreceptor, but not via CCR5. They also inhibited binding of the CXC chemokine, SDF-1, to CXCR4 specifically and subsequent signal transduction. One of the compounds, KRH-1636, prevented monoclonal antibodies from binding to CXCR4 without down-modulation of the coreceptor. The inhibitory effect against X4 viral replication by KRH-1636 was clearly reproduced in the hu-PBL-SCID mouse system. Furthermore, this compound was absorbed into the blood after intraduodenal administration as judged by anti-HIV-1 activity and LC/MS in the plasma. Thus, KRH compounds seem to be promising agents for the treatment of HIV-1 infection.
**In vivo apoptosis with HIV-1 infection**

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HIV infection induces gradual loss of CD4⁺ T cells leading to AIDS and neuronal injury in HIV-1-associated dementia (HAD), but the mechanisms of immunodeficiency and HAD remains unclear. Increased destruction of CD4⁺ T cells in secondary lymphoid organs such as LNs and spleen and depletion of neuronal cells in central nervous system have been reported in HIV-infected individuals. To explore the pathological processes occurring in the organs in HIV-infected humans, it is helpful to develop an appropriate animal model. We have previously shown that non-obese diabetic (NOD)-SCID mouse is a useful strain for both successful engraftment of human PBL and massive HIV-1 replication probably due to its severe immunodeficiency including innate immunity (J. Virol. 71:2417-2424, 1997, J. Exp. Med. 193, 651-659, 2001). In the present study, human PBL-transplanted NOD-SCID (hu-PBL-NOD-SCID) mice were used to examine the in vivo apoptosis after HIV-1 infection. Since the NOD-SCID mouse has a low level of phagocyte activity, we were able to analyze apoptosis in human and mouse cells in combination with immunohistological method. As demonstrated by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), massive apoptosis was predominantly observed in virus-uninfected CD4⁺ T cells in the spleens of HIV-1 infected mice. A combination of TUNEL and immunostaining for death-inducing TNF family molecules indicated that the apoptotic cells were frequently found in conjunction with TNF-related apoptosis-inducing ligand (TRAIL)-expressing CD3⁺ CD4⁺ human T cells but not FasL- or TNF-expressing cells. Administration of a neutralizing anti-TRAIL mAb in HIV-1-infected mice markedly inhibited the development of CD4⁺ T cell apoptosis. These results suggest that a large number of HIV-1-uninfected CD4⁺ T cells undergo TRAIL-mediated apoptosis in HIV-infected lymphoid organs. In addition, treatment of lipopolysaccharide in the HIV-1 infected hu-PBL-NOD-SCID mice induced the migration of HIV-1 infected cells into central nervous system and neuronal apoptosis in subcortical region where is mainly damaged in HAD patients. The apoptosis in mouse neuronal cells was only observed in the macrophage-tropic HIV-1 infected mice but not in T cell-tropic HIV-1 or LPS treated mock-infected mice. These results indicated our hu-PBL-NOD-SCID mouse model is an experimental tool to investigate in vivo apoptosis both in the lymphoid organ and central nervous system with HIV-1 infection.
Immunogenicity and Protective Efficacy of Adenovirus-Recombinant Vaccines for AIDS

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Previously, we reported that a combination regimen involving priming with adenovirus (Ad)-HIVenv/rev recombinants and boosting with gp120 envelope protein elicited good immunity in chimpanzees including persistent neutralizing antibodies, cytotoxic T-cell activity, and antibodies in mucosal fluids. Protective efficacy was demonstrated against low and high-dose HIVSF2 challenges and subsequently against the heterologous primary isolate, HIV5016 (Lubeck et al., Nature Med. 3:651, 1997; Robert-Guroff et al., J. Virol. 72:10275, 1998). Similarly, in a rhesus macaque system we showed elicitation of humoral, cellular, and mucosal immunity following immunizations with an Ad5 host range (Ad5hr)-SIVenv/rev recombinant and boosting with SIV gp120 protein. Following intravaginal challenge with the pathogenic SIVmac251 isolate, a diminished viral burden compared to controls was observed during acute infection, and some immunized macaques exhibited slow disease progression (Buge et al., J. Virol. 71:8531, 1997; Buge et al., J. Virol. 73:7430, 1999). To further develop this approach in macaques, we are now examining the value of adding Ad-recombinants containing different SIV genes to the vaccine strategy.

Sixteen rhesus macaques were immunized intranasally and orally with Ad5hr-SIVenv/rev and Ad5hr-SIVgag/pro and then boosted intratracheally at 12 weeks. SIVgp120 boosts were given intramuscularly in QS21 adjuvant at 24 and 36 weeks. Four control animals received Ad5hr vector and adjuvant immunizations, and four macaques were naive. Replication of the Ad5hr recombinant in vivo was demonstrated by assessment of Ad shedding in stool samples and nasal swabs. Cellular immune responses in macaque PBMCs were evaluated by ELISPOT assay using overlapping SIV envelope and Gag peptides and by tetramer staining of CD8 cells from 6 Mamu A*01 macaques. ELISPOT assays using overlapping Rev peptides are ongoing. The strongest cellular immune responses observed were against SIV Gag. Analysis of cells from the jejunal lamina propria, obtained by survival surgery on 4 immunized and 2 control macaques following each immunization, are also being analyzed as representative of cellular immune responses at mucosal sites. The Ad5hr-SIVrecombinants effectively primed antibody responses in serum, which were further increased by 2 gp120 boosts. The strongest recombinant priming effect for secretory IgG was observed in nasal fluids. SIV-specific IgGs were also observed in rectal and vaginal secretions. IgA responses are currently being assessed. These macaques were recently challenged intrarectally with SIVmac251. The outcome of the challenge and any correlations with systemic or mucosal immune responses will be reported. In associated studies, we have immunized 8 additional macaques with an Ad5hr-SIV nef mutant in which 19 N-terminal amino acids, including the myristoylation site, have been deleted. The mutant Nef expressed does not down regulate either CD4 or MHC-1 (Peng and Robert-Guroff, Immunol. Lett., in press). ELISPOT analysis following 2 Ad-recombinant immunizations shows the nef construct is highly immunogenic. Cells of 8 of 8 immunized macaques secreted IFNÎ± in response to overlapping Nef peptides. The contribution of Nef immunity to protective efficacy will be evaluated in future studies.
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Cellular immune responses play a critical role in the control of immunodeficiency virus infections. For efficient induction of the responses, we previously developed a proviral DNA vaccine system using an env- and nef-deleted simian-human immunodeficiency virus (SHIV). In this system, a chimeric SHIV with ecotropic Friend murine leukemia virus (FMLV) env in place of SHIV env, FMSIV, was constructed and used in combination with the FMLV receptor, mCAT1, that is not originally expressed in primate cells. Vaccination of macaques with both the FMSIV proviral DNA and the mCAT1-expression plasmid DNA allowed mCAT1-dependent FMSIV replication and induced resistance against SIV challenge. Further, we examined the potential of a recombinant Sendai virus (SeV) vector as an AIDS vaccine. SeV is a nonsegmented negative strand RNA virus and considered to be nonpathogenic for humans and non-human primates. Vaccination with a recombinant SeV vector expressing SIV Gag (SeV-Gag) conferred resistance against SIV challenge on macaques. In this study, we have combined these two systems to develop a prime/boost vaccine strategy and evaluated its protective efficacy in a macaque AIDS model using a highly pathogenic immunodeficiency virus, SHIV89.6PD.

We used 14 rhesus macaques in total for the evaluation and divided them into four groups; four macaques were naïve control, three received the DNA vaccine only, three SeV-Gag only, and four the DNA-prime/SeV-Gag boost. In case of the prime/boost, DNA vaccinations were performed four times in six weeks, followed by a single SeV-Gag boost on week 12 after the initial DNA prime, and SHIV89.6PD was challenged intravenously on week 26. Frequencies of SHIV-specific CD4+ T cells and CD8+ T cells in peripheral blood mononuclear cells were examined by flow-cytometric analysis of virus-specific intracellular interferon-γ induction. The prime/boost regimen efficiently induced virus-specific T cells, which were maintained for more than three months until challenge. While all the naïve control macaques showed acute CD4+ T cell depletion on week 2 after SHIV89.6PD challenge, all the macaques vaccinated with the prime/boost were protected from the depletion and showed greatly reduced peak viral loads as compared with those in the controls. Vaccination with the DNA alone or SeV-Gag alone was not enough to confer the consistent protection from the depletion, although it lead to efficient secondary CD8+ T cell responses on week 2 after challenge. On week 1, difference in the secondary responses between the protected and the unprotected macaques was clearly shown; rapid augmentation of virus-specific CD8+ T cells was detected in the former but not in the latter. Thus, our results indicate the importance of the rapid secondary responses for reduction in the peak viral loads and protection from the acute CD4+ T cell depletion.
Pivotal Roles of HIV-Coreceptors, CXCR4 and CCR5 in Immune System

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The identification of chemokine receptors as HIV-coreceptors has brought breakthrough in understanding molecular mechanism of preferential infection of HIV to CD4+ macrophages and T lymphocytes, and development of intervention therapy against HIV infection or treatment of AIDS progression targeting these chemokine receptors has been expected. However, the safety of targeting these chemokine receptors has not yet been established except for apparent normal life of Caucasians with CCR5 -/- . Thus, we describe here the phenotypes of irradiated mice reconstituted with hematopoietic progenitor cells expressing SDF-1–intrakine (to specifically down-regulate CXCR4 expression), and CCR5 gene targeted mice. SDF-1 intrakine transduced mice revealed impairment of T lymphopoiesis as well as in B lymphopoiesis and myelopoiesis in adult mice, warning potential serious adverse effect of long term therapy targeting CXCR4. On the other hand, CCR5-/- mice showed no apparent abnormality under regular breeding conditions. But, it turned out that graft versus host reaction (B6 splenocytes to B6XDBA/2 F1 mice) does not occur in these mice. Supporting this in vivo observation, dendritic cells derived from B6XDBA/2 CCR5-/- mice failed in inducing allogeneic response in vitro against T lymphocytes from B6 mice. Furthermore, it was shown that CCR5 moves to lipid rafts after T cell receptor stimulation using confocal microscopy. These data suggest the essential roles of CCR5 mediated signals in eliciting memory CD8+ T lymphocytes as well as in inducing CTL, therefore CCR5 is not necessarily a safe target for the treatment. This finding in mice agrees with the recent report describing complete acceptance of kidney transplant in CCR5 delta 32 -/- persons.
A vaccine is essential for the effective control of the HIV pandemic. However it has proved very difficult to make an anti-HIV vaccine using conventional approaches. In particular it has been impossible to find an HIV envelope construct that stimulates high titre neutralising antibodies. The crystal structure of the gp120 envelope gives some explanation for this – the recessed or hidden receptor binding sites, the trimeric structure and the carbohydrate coat all contribute to the ease with which this virus can escape antibody mediated immunity.

Therefore attention has turned to the T cell responses. There is very good evidence that CD8+ T cells control established infection and in small animals vaccines that stimulate cytotoxic T lymphocyte (CTL) responses can completely protect against a variety of viruses. In the macaques model of HIV infection vaccines that stimulate very strong CTL responses partially protect monkeys from infection with a very aggressive SIV/HIV hybrid virus SHIV89.6. Animals are infected on challenge but do not die and control virus very effectively compared to unvaccinated controls. There are also data from very highly HIV-exposed commercial sex workers in Africa where a few are resistant to infection and these women make anti-HIV CTL responses.

We have therefore initiated a project to make and test a CTL inducing vaccine. The vaccine contains the A clade HIV gag and some epitopes from other virus proteins. The vaccine is given as plasmid DNA and as recombinant MVA (MVA: modified vaccinia virus Ankara, an attenuated version of the old small pox vaccine). In initial phase 1 trials both components stimulate CD8+ T cell responses, MVA particularly effectively.

The vaccine is designed for areas of the world such as Kenya where the A clade of HIV is prevalent. Although there is likely to be some cross-reaction with other virus strains, this is likely to reduce potential effectiveness markedly. Furthermore, the problem of virus escape from the CTL responses will have to be addressed. In order to protect, it is likely therefore that the vaccine will need to stimulate strong CTL responses to five or more epitopes. This requires an understanding, and probably manipulation, of immunodominance. These issues will be discussed.
Natural Killer T cells in HIV infection

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Natural killer T (NKT) cells have received considerable interest recently because of their likely involvement in immunoregulation, autoimmunity and tumor immunity. However, it is not known how these cells are affected by and respond to HIV infection. We have analyzed 57 children with perinatally acquired HIV infection with regard to subsets of Vα24 NKT cells in peripheral blood. The overall numbers of Vα24 NKT cells did not show any apparent correlation with HIV viral load. However, CD4+ and CD4- subsets were differentially affected such that the numbers of CD4+ NKT cells displayed an inverse correlation with viral load, while CD4- NKT cells showed a tendency to increase in frequency at high viral loads. Many pediatric HIV patients retained high overall numbers of CD4+ cells in the face of high viral load, while the CD4+ NKT cells were lost. In contrast to regular T cells a majority of Vα24 NKT cells expressed both CCR5 and CXCR4 HIV co-receptors. Also, CD4+ NKT cells were lost more rapidly than regular CD4+ T cells in an in vitro infection assay, indicating that CD4+ NKT cells are preferential targets for HIV infection. We propose that depletion of the immunoregulatory Vα24 NKT cells in HIV patients may play a role in several AIDS defining illnesses.
“All-in-One Assay”, a novel phenotypic drug resistance assay for anti-HIV-1 combination therapies

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Current guidelines recommend 3-drug combinations for anti-HIV-1 treatment. However, current phenotypic drug resistance assay including ours determine IC_{50-90} of each single drug. Furthermore, clinical effect is influenced by in vivo drug concentration, although the current assays express fold resistance which indicates shift of IC_{90-95} of clinical isolate from that of type strain. In this study, we are developing a novel phenotypic drug resistance assay named “All-in-One Assay”, which can examine viral replication under 3 drugs simultaneously and considers in vivo drug concentrations. In this assay, we first defined the biological cut-off concentration (BCC) of each drug. For NNRTIs and PIs, Cmin is defined 1x BCC and for RTIs, Cmax is defined 1x BCC. 1x BCC of 3 drugs are mixed in one well. Then, the aliquot is diluted from 1x BCC to 0.001x BCC by 10-fold dilutions. Assay procedure is the same as Magic 5 assay as previously reported (Antimicrob Agents Chemother, 45: 495, 2001). As like the inhibitory quotient proposed by Rolf, our assay express BCC/IC95 ratio and fold resistance of a 3-drug combination. Our assay may examine synergistic effect of drug combinations. Our preliminary result showed that BCC/IC95 ratio of NVP-containing regimen was 10-times lower than those of the first line regimens. Cross-sectional study revealed that the first line regimens had higher BCC/IC95 ratio (>100) in HIV-1 from antiviral naïve patients and the lower ratio (<10) in HIV-1 from treatment failure. Retrospective study demonstrated that lower BCC/IC95 ratio was associated with treatment failure by NFV-containing regimen. In future, clinically relevant BCC/IC95 ratios must be determined and a prospective study will prove clinical usefulness of this assay.
Mechanism of HIV transcription and novel therapeutic strategies

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Transcription from HIV proviral DNA in the latently infected cells is the key step of HIV replication. Viral transcription is controlled by host transcription factor, NF-κB, followed by viral transactivator Tat. Upon stimulation of cells by various factors such as proinflammatory cytokines, bacterial mitogen, and oxidative stress, NF-κB is activated through intracellular signal transduction pathways including MEKK3-IKK kinase cascade and redox regulation. We found that Tat interacts with CDK7 (CAK), a catalytic component of a basal transcription factor TFIIH as well as the positive transcription elongation factor b (P-TEFb). Tat was shown to stimulate CDK7. Moreover, we found that CAK is essential in the action of Tat since mC2p, a pseudo-substrate inhibitor peptide for CDK7 blocking CAK but not P-TEFb, inhibited Tat-mediated transactivation and HIV-1 replication. Thus, CAK and P-TEFb appear to act sequentially to maintain the processivity of RNA pol II by phosphorylation of its C-terminal domain (CTD), thus supporting HIV transcription. As TFIIH is released from the pre-initiation complex during “promoter clearance”, recruitment of P-TEFb is required for the progression to efficient elongation.

We previously reported that fluoroquinoline derivatives such as K-37 inhibited HIV replication even from the latently infected cells. Interestingly, K-37 inhibited not only Tat but also other RNA-dependent transactivators. No effect was observed with DNA-dependent transactivators such as NF-κB and Gal4VP16. Since K-37 did not inhibit CTD activities of CAK and P-TEFb, RNA-mediated transactivation may involve a common unidentified factor to which K-37 directly interacts.

Functional loss of antigen-presenting cells (APC) in HIV-1 infection contributes greatly to the pathogenesis of AIDS. We found that Tat inhibits the function of the class II transactivator CIITA, resulting in the suppression of expression of MHC class II genes in APC. As demonstrated by transient transfection assays as well as by flow cytometry using THP-1 cells stably expressing HIV-1 Tat, this action of Tat appeared to be mediated by sequestering the cyclin T1, a component of P-TEFb and a common co-factor for Tat and CIITA. On the other hand, we also found that the overexpression of CIITA could block the Tat activity and subsequently the HIV-1 replication. These findings provide further insights that should provide feasible rationale and practical approaches for novel anti-HIV therapy.

(A part of this study is in collaboration with M. Baba, Kagoshima Univ., Japan and B.M. Peterlin, UCSF, USA)
Host factors in the pathogenesis of HIV-1 disease

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HIV-1 infection is generally characterized by a long-term, chronic disease course gradually progressing to AIDS. However, there are a few but strikingly different scenarios. A small fraction of HIV-1 infections remains normal both clinically and immunologically over 10 years or more after seroconversion. Conversely, another marked fraction is featured by an extremely rapid disease progression taking place even within one year. Determining the host factors of these different disease courses would be extremely helpful for better understanding and control of AIDS. Here we show two examples of single nucleotide polymorphism (SNP), which can affect HIV-1 disease courses.

(1) IL4 -589T.

IL4 is known to down-regulate CCR5 which is a co-receptor for HIV-1 R5 strains. IL4 -589T allele bears a SNP with a C to T exchange at position -589 upstream from the open reading frame of the IL4 gene. This SNP is associated with increased promoter activity for IL4 transcription and influences IgE serum levels. To determine the influence of this allele on HIV-1 disease, we analyzed HIV-1 disease progression and serum viral load according to the IL4 promoter genotype in 427 Caucasian patients with a known date of seroconversion, who were followed in the SEROCO cohort between 1988 and 1996. Serum viral load was 0.20 log lower during the 6-24 month plateau phase after seroconversion in patients with IL4 -589T allele than in those without this allele (p=0.02). Kaplan-Meier survival curves showed a slower progression to clinical AIDS in carriers of IL4 -589T (p=0.04). After adjustment for early serum viral load, the strength of the association was greatly diminished. Adjustment for CCR5delta32, which is the most powerful anti-HIV-1 gene in Caucasians, did not influence the results. These results suggest that IL4 -589T allele protects against HIV-1 disease progression due in part to a reduction in viral load.

(2) CCR5 893(-).

CCR5-893(-) is a single nucleotide deletional mutation which is observed exclusively in Asians. This mutant gene produces a CCR5 which lacks the C-terminal cytoplasmic tail in its entirely. To assess the effect of CCR5-893(-) on HIV-1 infection, we generated a recombinant Sendai virus expressing the mutant CCR5 and compared its HIV-1 co-receptor activity with that of wild-type CCR5. Although the mutant CCR5 has intact extracellular domains, co-receptor activity of this mutant was greatly reduced compared to that of the wild-type CCR5. Flow cytometric analyses and confocal microscopic observation of cells expressing the mutant CCR5 revealed that surface CCR5 levels were greatly reduced in these cells, while cytoplasmic CCR5 levels of the mutant CCR5 were comparable to that of the wild-type. Peripheral blood CD4+ T-cells obtained from individuals heterozygous for this allele expressed very low levels of CCR5. These data suggest that the CCR5-893(-) mutation affects intracellular transport of CCR5 and raise the possibility that this mutation also affects HIV-1 transmission and disease progression.
Variation and Prevalence of non-subtype B infection in Japan and their genotypic patterns related to anti-retrovirus therapy failure.

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Background and Objective: To recognize updated HIV-1 epidemic in Japan, we analyzed subtype variation and frequencies among sexually transmitted HIV-1 cases. We also analyzed drug genotypic patterns of non-B subtypes.

Samples and methods:
Sexually transmitted cases were selected from the samples sent to NIID Japan during November 1996 to September 2000. Drug resistant genotypes were analyzed by sequencing 1.3kb pol fragment, which covers the most of the known drug resistant mutations. HIV-1 subtypes were determined by protease sequence and also from C2V3 region of the envelope protein.

Results:
261 sexually transmitted patients were selected and enrolled in the analyses. We could determine virus subtype for 212 cases and the results were as follows; B:162 cases (70.1%), A/E:45 cases (19.5%), F:3 cases (1.3%), C:1 case, D:1 case. To clarify the mutations related to drug treatment failure, we first analyzed differences in natural polymorphism in treatment naive cases. As for protease region, 57 B and 14 A/E treatment naive cases were analyzed. Significant (Chi-sq test 0.05> p) differences in polymorphism were found in 9 loci between B and A/E, and 3 of these were the secondary drug resistant mutation points. As for RT region, 34 B and 8 A/E treatment naive cases were used. Significant differences were observed on 16 loci, and none of these were involved in known drug resistant mutation. Next, we compared mutation patterns between B and A/E treatment failed cases. As for protease inhibitors, significant differences were found in nelfinavir treatment failed cases. We could not find any D30N (p=0.001), and N88D (p<0.001) in A/E cases. We also found that L63P was less frequent in nelfinavir or indinavir failed A/E cases (p<0.001). No significant differences were found in saquinavir and ritonavir failed cases. Although the background sequence was quit different in RT region, we did not see any differences in mutations between B and A/E nucleoside RT inhibitor (AZT, 3TC, ddL,d4T,ddC) failed cases. For the unique mutation patterns observed in nelfinavir failed cases, recombinant viruses were constructed and drug susceptibilities were analyzed in vitro. Conclusion: non-B subtypes, especially subtype E(A/E), are increasing in Japan. Several significant differences in drug genotypes were found between B and E viruses.
Genetic diversity over time of HIV-1 CRF AE in Thailand: 1993-1999

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Objectives: To characterize genotype and biotype of HIV-1 isolates from HIV-1 infected Thais collected in year 1993-1999 from three risk groups including injecting drug users (IDUs), heterosexual, and perinatal transmission risk groups.

Subjects: HIV-1 nucleotide sequences were analyzed from 250 blood samples collected in year 1993-1999 from Bangkok area of perinatal transmission risk group (n = 30); heterosexual risk group (n = 80); and injecting drug user risk group (n = 30); from Northern area (Chiang Mai, Chiang Rai, Lampang), Northeastern area (Khon Kaen), and Eastern area (Chonburi) of heterosexual risk group (n = 110).

Methods: DNA lysate from peripheral blood mononuclear cells PBMCs was used as template to amplify HIV-1 env (C2-V3), gag (p24), pol (RT), tat, and nef genes for nucleotide sequencing analysis. The nucleotide sequence data was analysed by DNASTAR and phylogenetic analysis was performed using softwares DNASTAR and PAUP.

Result: All 250 HIV-1 isolates were identified as subtype E by C2-V3 nucleotide sequence analysis except for 2 isolates were subtype B’. Glutamic acid (Q) in V3 motif of subtype E (GPGQ) was changed to either “R” or “H” or “K” or “L” about 61.1%, 69.3%, 92.9% and 61.1% (6/15) in HIV-1 isolates from heterosexual from Bangkok, heterosexual from outside Bangkok, perinatal, and IDU transmission risk groups, respectively. While, methionine (M) was found to replace “I” at amino acid position 12 in V3 region about 14.9%, 18.1%, 25%, and 0% in HIV-1 isolates from heterosexual, IDU, and vertical transmission risk groups, respectively. The mean intrasubtype genetic divergence in C2-V3 nucleotide sequence of HIV-1 isolates from heterosexual, vertical, and IDU transmission risk groups lived in Bangkok area were 7%, 12.05%, 16.78% (1993, 1995-1997, 1999); 2.3%, 3.98% (1993, 1999); and 15.18% (1999), respectively. Also, mean genetic distance of C2-V3 sequences from HIV-1 isolates of heterosexual transmission risk group lived in North and Northeastern area were 9.48% and 9.66% in year 1998 and 1999. One extra N-glycosylation site was found mainly in HIV-1 isolates from Bangkok area. While, nucleotide sequence of pol (RT) gene of these HIV-1 strains (n=50) isolates from drug naive heterosexual, IDU, and vertical transmission risk groups in year 1993 and 1998 were quite conserved with mean genetic distance range from 2.2%-2.8%. Nucleotide sequence of gag (p24), nef, and tat genes of these HIV-1 isolates were less diverse than pol gene with mean genetic distance 5.9%, 4.7%, and 8.5%, respectively. Of 250 samples, 200 viruses were isolated by coculture method with macrophage-tropic 19%, T cell tropic 55%, and dual tropic 26%. Macrophage tropic viruses were mainly found in HIV-1 isolates from vertical transmission risk group.

Discussion: Total 250 viruses from HIV-1 infected Thais with three risk groups: heterosexual, vertical, and IDU transmission groups were characterized and collected as reference virus stock. The mean genetic distance in C2-V3 nucleotide sequence of HIV-1 isolates from heterosexual transmission group from Bangkok area collected were more diverged than those found in outside Bangkok with signature glycosylation site in C2-V3 region, and from the other two risk groups that collected in the same year. Hence, genetic characterization of HIV-1 should be routinely surveillance nationwide for HIV-1 vaccine trial and development.
We are investigating the molecular epidemiology of HIV-1 in Myanmar and Southwestern China to elucidate the mechanism of HIV spread and the interrelationship of the epidemic with that in surrounding areas. Myanmar and China became new epicenters of HIV epidemic in Asia, where the high level of HIV prevalence was observed especially among injecting drug users (IDUs): 60-90% in Central and Northeast Myanmar and 40-70% in Yunnan Province of China.

We screened the genetic subtypes based on the nucleotide sequences of various segments in HIV-1 genome, including gag (p17), env (C2/V3) and pol (RT) regions. In Central Myanmar, we have detected HIV-1 subtype C (16%), in addition to the previously identified HIV-1 subtype B' (Thai-B cluster in subtype B) (23%) and CRF01_AE (44%), that were likely to be originated from neighboring Thailand. HIV-1 subtype C in Myanmar belongs to Indo-China cluster, suggesting the close relationship of the epidemic in Myanmar with that in nearby China and India. Interestingly, approximately 18% of the specimens from Central Myanmar showed the discordance between gag (p17) and env (C2/V3) subtypes. The subsequent analyses revealed the emergence of new forms of HIV-1 intersubtype recombinants, including B'/C, E/B' and C/E chimeras.

Unique patterns of distribution of HIV-1 subtypes and CRFs were observed in nearby Yunnan Province of China. A circulating recombinant form (CRF), CRF08_BC, was prevalent (70-90%) in the eastern part of Yunnan. Since the CRF08_BC predominates in Guangxi Province, an east neighbor of Yunnan, this suggests the interrelationship between the epidemic in Guangxi and east Yunnan. In contrast, a variety of unique recombinant forms (URFs) were detected in western prefecture near Myanmar border. Considerable proportions (47%) of specimens appeared to be URFs in west Yunnan. The remainders were subtype B' (33%), C (7%) and CRF08_BC (13%). Most of URFs were comprised of subtype B’ and C with complicated recombination breakpoints. Suggestively, the non-recombinant forms of subtype B’ and C were detected only in the west and not in the east of Yunnan.

Taken together, the multiple occurrences of the HIV-1 intersubtype recombinants with unique chimeric structures both in Myanmar and the western part of Yunnan may suggest the ongoing recombination events between circulating subtypes in these areas. Our finding could provide the insights for the mechanism of HIV spread and for future vaccine strategies for these particular areas in Asia.

(The study was done in collaboration with our colleagues in National Institute of Infectious Diseases, National AIDS Programme in Ministry of Health of Myanmar and Kunming Institute of Zoology in Yunnan, China)