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LETTERS

Adaptation of HIV-1 to human leukocyte antigen class I

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The rapid and extensive spread of the human immunodeficiency virus (HIV) epidemic provides a rare opportunity to witness host–pathogen co-evolution involving humans. A focal point is the interaction between genes encoding human leukocyte antigen (HLA) and those encoding HIV proteins. HLA molecules present fragments (epitopes) of HIV proteins on the surface of infected cells to enable immune recognition and killing by CD8$^+$ T cells; particular HLA molecules, such as HLA-B*57, HLA-B*27 and HLA-B*51, are more likely to mediate successful control of HIV infection[1]. Mutation within these epitopes can allow viral escape from CD8$^+$ T-cell recognition. Here we analysed viral sequences and HLA alleles from >2,800 subjects, drawn from 9 distinct study cohorts spanning 5 continents. Initial analysis of the HLA-B*51-restricted epitope, TAFTIPSI (reverse transcriptase residues 128–135), showed a strong correlation between the frequency of the escape mutation I135X and HLA-B*51 prevalence in the 9 study cohorts (P = 0.0001). Extending these analyses to incorporate other well-defined CD8$^+$ T-cell epitopes, including those restricted by HLA-B*57 and HLA-B*27, showed that the frequency of these epitope variants (n = 14) was consistently correlated with the prevalence of the restricting HLA allele in the different cohorts (together, P < 0.0001), demonstrating strong evidence of HIV adaptation to HLA at a population level. This process of viral adaptation may dismantle the well-established HLA associations with control of HIV infection that are linked to the availability of key epitopes, and highlights the challenge for a vaccine to keep pace with the changing immunological landscape presented by HIV.

The extent to which HIV is evolving at the population level in response to immune selection pressure is under debate[2–6]. Resolving the impact of HLA class I alleles on viral evolution is problematic because it can be obscured by other influences, such as founder effect[6] (polymorphisms present within the early strains establishing the epidemic in a group). In addition, most HLA alleles do not drive significant selection pressure on HIV, a proportion of escape mutations revert to wild type after transmission, and different HLA alleles may drive the identical escape mutation[7].

To test the hypothesis that the frequency of escape mutations in a given population is correlated with the prevalence of the relevant HLA allele in that population, we studied nine distinct cohorts from North America, the Caribbean, Europe, sub-Saharan Africa, Australia and Japan, in which we performed HLA typing, and defined the viral mutations arising within CD8$^+$ T-cell epitopes. We focused initially on a well-characterized mutation, I135X, within the HLA-B*51-restricted epitope, TAFTIPSI (RT 128–135)[8], because it arises in acute infection, non-HLA-B*51 alleles do not also select this mutation[7,9], and it does not revert to Ile 135 after transmission to HLA-B*51-negative subjects[9]. Thus, if highly prevalent HLA alleles drive a high frequency of escape mutations in the population, this would be most obvious in relation to HLA-B*51 and the escape mutant I135X. We then considered an additional 13 well-defined escape mutations, including those known to reduce viral fitness and therefore liable to revert after transmission.

I135X was selected in 205 of 213 (96%) HLA-B*51-positive individuals analysed (Figs 1 and 2, and Supplementary Fig. 1). The I135X variants do not significantly affect viral replicative capacity *in vitro*, other than the rare I135V mutation. This was the only variant observed to revert to wild-type *in vivo* during a 3-year follow-up of 38 HLA-B*51-negative subjects identified during acute HIV infection who carried I135X mutant viruses at transmission (Fig. 1e). The I135X mutants substantially affect HLA binding, and therefore also recognition by CD8$^+$ T cells (Fig. 1f–h). Thus, HIV transmission from HLA-B*51-positive subjects would probably involve transmission of I135X, which would persist in the new host. Newly infected HLA-B*51-positive subjects receiving an I135X mutant would be unable to generate an HLA-B*51-TAFTIPSI-specific response.

To test the hypothesis that the population frequency of I135X is correlated with HLA-B*51 prevalence, HIV sequence and HLA data were collated from the nine study cohorts. One cohort comprised subjects with acute/early HIV infection; the remaining cohorts comprised chronically infected subjects. In all cohorts the odds ratio strongly favoured I135X in the HLA-B*51-positive subjects, even in the acute cohort where I135X was selected sufficiently early to be already over-represented in HLA-B*51-positive subjects (odds ratio 1.65, P = 0.07, Fig. 2a). In Japan, where HLA-B*51 is highly

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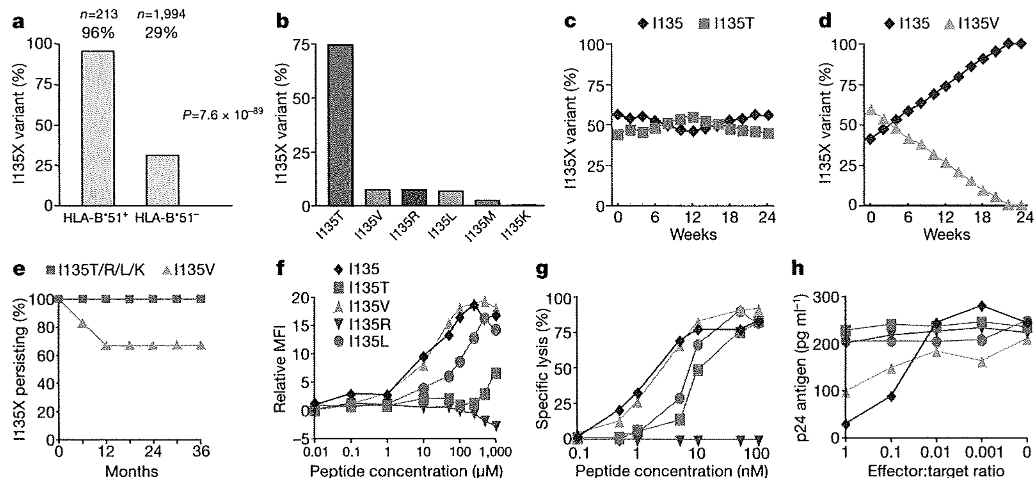

Figure 1 | Selection and fitness cost of I135X escape variants and recognition by the HLA-B*51–TAFTIPSI (RT 128–135)-specific CD8^{+} T cells. **a**, Association between I135X and HLA-B*51 in all study cohorts. **b**, Ile 135 variation in HLA-B*51-positive subjects. **c**, **d**, *In vitro* competition assays between NL4-3 wild-type virus and I135X viral variants (I135T (**c**) and I135V (**d**)). I135R and I135L showed no fitness cost (not shown). **e**, Persistence of I135X mutants in 38 HLA-B*51-negative subjects followed from acute infection. **f**, TAFTIPSI variant binding to HLA-B*51 (see Methods). MFI, mean fluorescence intensity. **g**, **h**, Recognition of peptide-pulsed HLA-B*51-matched targets and viral variants by representative TAFTIPSI-specific CD8^{+} T-cell clones.

prevalent[10] (21.9% of the study cohort), the frequency of I135X was >50%, and overall across all cohorts the I135X frequency was strongly correlated with HLA-B*51 prevalence ($P = 0.0001$, Fig. 2b). To control for the possibility that disproportionately more virus sequences from HLA-B*51-positive subjects were analysed, the same analysis comparing I135X frequency in HLA-B*51-negative subjects only was undertaken, with similar findings (Fig. 2c, $P = 0.0006$). These data suggest that HIV may be adapting to HLA-B*51 with respect to the HLA-B*51–TAFTIPSI response in localities where HLA-B*51 is at high prevalence.

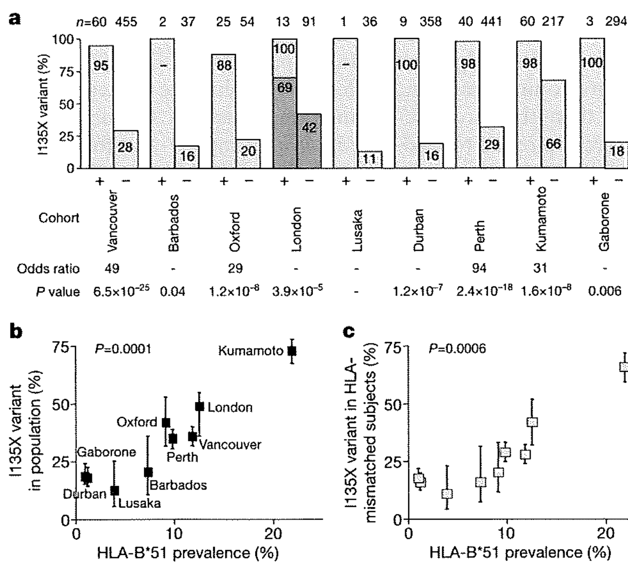

Figure 2 | Correlation between frequency of HLA-B*51-associated escape mutations and HLA-B*51 prevalence in study cohorts. **a**, Frequency of I135X mutations within TAFTIPSI (RT 128–135) in HLA-B*51-positive (+) and -negative (−) subjects within nine study cohorts. In the acute cohort (London) 69% of HLA-B*51-positive subjects expressed I135X mutant at enrolment, 100% within 2 years of baseline (Supplementary Fig. 1). **b**, Correlation between frequency of I135X mutation and HLA-B*51 prevalence in the nine study populations. Logistic regression $P = 0.0001$ (Supplementary Table 1). **c**, Correlation between I135X frequency in HLA-B*51-negative subjects and HLA-B*51 prevalence in nine study populations. Error bars represent 95% confidence limits, obtained using a binomial error distribution.

Additional evidence that I135X is accumulating in Japan comes from the observation that only 3 of 14 (21%) HLA-B*51-negative Japanese haemophiliacs infected in 1983 carried I135X, compared with 30 of 43 (70%) HLA-B*51-negative subjects infected between 1997 and 2008 ($P = 0.002$). Furthermore, HLA-B*51 does not protect against disease progression in Japanese subjects infected between 1997 and 2008, whereas HLA-B*51-positive haemophiliacs infected in 1983 had lower viraemia levels and higher CD4 counts than HLA-B*51-negative haemophiliacs (Supplementary Fig. 2). These data are consistent with fewer HLA-B*51-positive subjects targeting TAFTIPSI during 1997–2008, owing to a population-level increase in the HLA-B*51 I135X escape mutation over this 14–25-year period.

To investigate HIV adaptation to other HLA alleles, we initially examined other escape mutations shown previously to persist stably after transmission[5,7]. We selected the three non-reverting Gag polymorphisms that, from analysis of 673 study subjects in Durban, South Africa[7], were most strongly associated with the relevant restricting allele ($P < 10^{-6}$ after phylogenetic correction), namely, S357X, D260X and D312X within epitopes restricted, respectively, by HLA-B*07 (GPSHKARVL, Gag 355–363), HLA-B*35 (PPIPVGDIY, Gag 254–262) and HLA-B*44 (AEQATQDVKNW, Gag, 306–316). In addition, we analysed a non-reverting I31V variant (LPPIVAKEI, Int 28–36) previously hypothesized to increase in relation to population HLA-B*51 prevalence[5]. These additional polymorphisms show a similar relationship to that between I135X and HLA-B*51, overall showing a strongly significant correlation between variant frequency and prevalence of the restricting HLA allele (Figs 3 and 4a, and Supplementary Fig. 3).

The spectrum of HLA-associated polymorphisms also includes mutations reducing viral fitness[1]. These either revert to wild type after transmission, or persist in the presence of compensatory mutations. We extended these analyses to include epitopes restricted by HLA-B*27 and HLA-B*57, alleles strongly associated with successful immune control of HIV[11,12]. The mutations analysed themselves are associated with precipitating loss of immune control[13–16] and all inflict a documented viral fitness cost, either demonstrated by *in vitro* fitness studies and/or *in vivo* reversion[7,14,17–21] (data not shown for V168I).

Again, a strong correlation between escape mutant frequency and prevalence of the restricting HLA allele was observed (Figs 3c–f and 4b, and Supplementary Fig. 3; overall, for these nine variants affecting viral fitness, $r = 0.69$, $P < 0.0001$). Unexpectedly, this correlation

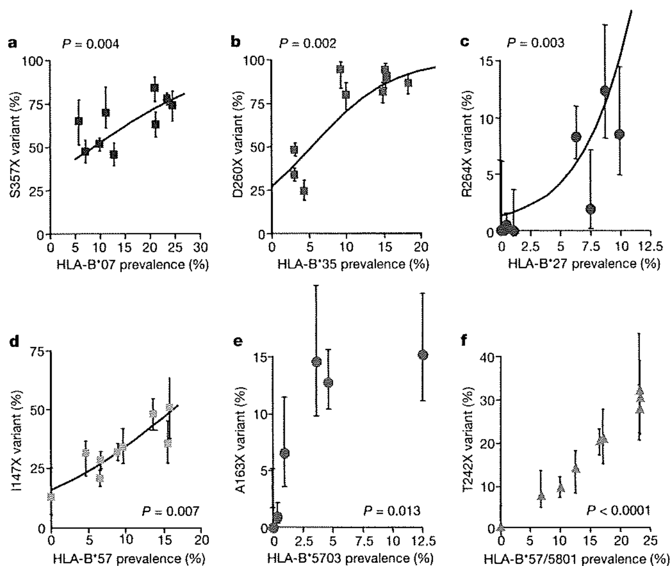

Figure 3 | Correlation between frequency of HIV sequence variant and HLA prevalence for six additional well-characterized epitopes. *P* values calculated after logistic regression analysis as shown (calculations after linear regression analysis are shown in Supplementary Table 1). **a,** Frequency of the S357X mutation within the HLA-B*07-restricted epitope GPSHKARVL (Gag 355–363). **b,** Frequency of the D260X mutation within the HLA-B*35-restricted epitope PPIPVGDIY (Gag 254–262). **c,** Frequency of the R264X mutation within the HLA-B*27-restricted epitope KRWIILGLNK (Gag 263–272). **d,** Frequency of the I147X mutation within the HLA-B*57-restricted epitope ISPRTLNAW (Gag 147–155). **e,** Frequency of the A163X mutation associated with the HLA-B*5703-restricted epitope KAFSPEVIPMF (Gag 162–172). **f,** Frequency of the T242X mutation within the B*57/5801-restricted epitope TSTLQEQIAW (Gag 240–249). Error bars represent 95% confidence limits, obtained using a binomial error distribution.

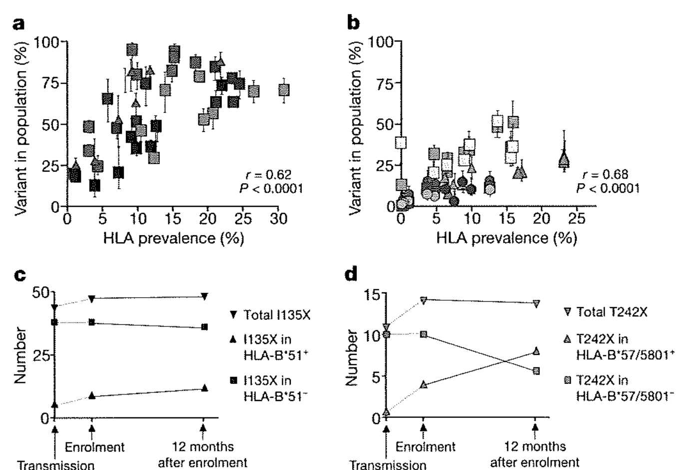

Figure 4 | Correlation between HIV variant frequency and HLA prevalence for all epitopes studied. a, Correlation between HLA prevalence and the five stable, non-reverting variants (symbols in Figs 2 and 3, and Supplementary Fig. 3; grey triangles, I31V; green squares, D312X). **b,** Eight variants demonstrated to reduce viral fitness (see text, Fig. 3 and Supplementary Fig. 3; turquoise triangles, L268X; yellow squares. A146X; sky-blue squares, V168I; yellow circles, I247X). **c, d,** Data from acute London cohort. **c,** Number of HLA-B*51-positive and HLA-B*51-negative subjects carrying the non-reverting I135X variant. The percentage of I135X in HLA-B*51-negative subjects at enrolment (42%) assumed the percentage of I135X in all subjects at transmission (I135X frequency in HLA-B*51-positive subjects at enrolment was 69%, $P = 0.07$). **d,** The reverting HLA-B*57/5801-restricted T242X mutation. T242X frequency in HLA-B*57/5801-negative subjects at enrolment was 7%, versus 33% in HLA-B*57/5801-positive subjects ($P = 0.01$). Error bars represent 95% confidence limits, obtained using a binomial error distribution.

remained significant even when comparing HLA prevalence with variant frequency in the HLA-mismatched population ($r = 0.40$, $P = 0.0004$). As anticipated, non-reverting variants such as I135X accumulate at the population level, but even rapidly reverting[18,20] mutations such as T242N can accumulate, if the selection rate exceeds the reversion rate (Fig. 4c, d).

Although frequency of the analysed HIV polymorphisms and HLA prevalence were strongly correlated overall, some anomalies were observed. For example, despite a 0% prevalence of HLA-B*57 in Japan[10], 38% of the Japanese cohort had the HLA-B*57-associated A146X variant. One potential explanation might be A146X selection by non-HLA-B*57 Japanese alleles. Analysing Gag sequences from Japanese study subjects, we observed a strong association between A146P and HLA-B*4801 ($P = 0.00035$), and then that A146P is indeed selected in HLA-B*4801-positive subjects (Supplementary Fig. 4a, b). We defined a novel HLA-B*4801-restricted epitope (Gag 138–147), showing also that A146P is an escape mutant (Supplementary Fig. 4c–f). These data illustrate that more than one HLA allele can drive the selection of a particular escape mutant (Supplementary Fig. 5). Also, in populations where HIV-specific $CD8^+$ T-cell responses are incompletely characterized, the influences of locally prevalent HLA alleles on HIV sequence variation are unknown.

These data show a strong correlation between HLA-associated HIV sequence variation and HLA prevalence in the population ($r = 0.69$, $P < 0.0001$, Supplementary Fig. 6), suggesting that the frequency of the studied variants is substantially driven by the HLA-restricted $CD8^+$ T-cell responses. Non-reverting variants[5,7], as well as those previously shown to arise at a fitness cost[7,14,16–21], were studied. The latter constitute approximately 55–65% of HLA-associated polymorphisms[7,20]. This current analysis included epitopes whose role in HIV immune control is unknown, as well as those believed to contribute significantly to containment of HIV[4,7,13–19]. Analysis of well-characterized epitopes only also served to limit potential confounding influences of epitope clustering (selection of the same variant by different HLA alleles) and of founder effect. Either would be capable of obscuring a true HLA effect on population variant frequency.

The HLA-B*57-associated A146X mutation illustrates the complexity that may result from epitope clustering. A146X is selected by at least six distinct HLA alleles (Supplementary Fig. 5). A true correlation existing between mutation frequency and individual HLA allele prevalence might thus be obscured by selection of the same mutation by other alleles.

Founder effect also has an undoubted influence on population frequencies of particular polymorphisms[6]. Phylogenetic correction of sequence data excludes founder effect as a confounder[6,7,9], and the highly significant associations between the presence of particular HLA alleles and all 14 HIV polymorphisms studied, persisting after phylogenetic correction (Supplementary Table 3), provide compelling evidence that the effects observed here are substantially HLA-driven. The large numbers of study subjects in these current studies reduce the likelihood of genuine HLA associations with HIV amino acid polymorphisms being obscured by founder effects. The relative impact of HLA and founder effect on variant frequency is harder to quantify, and is likely to differ substantially between particular populations.

The consequence of HIV adapting to certain $CD8^+$ T-cell responses is unknown. For non-reverting polymorphisms such as HLA-B*35-associated D260E, the variant approaches fixation, because even at population frequencies of 90%, D260E is still significantly selected in HLA-B*35-positive subjects (Supplementary Fig. 7b). Important questions relevant to vaccine design include the extent and rate of sequence change in populations. Relevant factors include the selection rate in subjects expressing the HLA allele, the reversion rate in HLA-mismatched subjects, the population HIV

transmission rate, and HLA allele prevalence. Models would need to include factors such as the selection of compensatory mutations to slow reversion rates, and antiretroviral therapy access that would slow transmission rates.

HLA adaptation to certain CD8$^+$ T-cell responses may also alter currently established HLA associations with slow disease progression. Data here suggest that, whereas 25 years ago HLA-B*51 was protective in Japan[11,12], this is no longer the case (Supplementary Fig. 2). The apparent increase in I135X frequency in Japan over this time supports the notion that HLA-B*51 protection against HIV disease progression hinges on availability of the HLA-B*51-restricted TAFTIPSI response. However, whether this is the case remains unknown.

For HLA-B*27 and HLA-B*57, there is more clear-cut evidence that their association with HIV control depends on the Gag-specific epitopes presented and analysed here[4,7,13–15,18,19]. For each of the HLA-B*27- and HLA-B*57-associated Gag mutations studied, an *in vitro* fitness cost or *in vivo* reversion has been observed. A strong correlation between variant frequency and HLA prevalence even for rapidly reverting variants can be explained, either by mutant acquisition exceeding reversion rate (Fig. 4D), or by selection of compensatory mutations slowing or halting reversion altogether. The clearest example of the latter is the HLA-B*27-associated R264K mutation, 'corrected' by S173A[19]. Compensatory mutations are also well described for the HLA-B*57-associated Gag mutations[14,18]. These data suggest that the escape mutations in these HLA-B*27- and HLA-B*57-restricted epitopes are accumulating over time. Several studies have now demonstrated that transmission of viruses encoding escape mutants in the critical Gag epitopes to individuals expressing the relevant MHC class results in failure to control viraemia[2,21,22]. The accumulation at the population level of these escape mutations in HLA-B*27 and HLA-B*57 Gag epitopes is therefore likely to reduce the facility of these alleles to slow HIV disease progression.

The longer-term consequences of this process for immune control of HIV are unknown. Loss of currently immunodominant epitopes would promote subdominant CD8$^+$ T-cell responses, which can be more effective[23,24]. Also, the adapted virus provides new epitopes that can be presented, potentially with beneficial effects. In hepatitis C virus, for example, HLA-A*0301 holds a particular advantage, but only against the specific strain of virus responsible for the Irish outbreak[25]. In HIV, HLA-B*1801 is associated with high viraemia in C clade but not in B clade infection[10,11,26]; the opposite applies to HLA-B*5301.

Thus, the data presented here, showing evidence that the virus is adapting to CD8$^+$ T-cell responses, some of which may mediate the well-established associations (HLA-B*57, HLA-B*27 and HLA-B*51) with immune control of HIV, highlight the dynamic nature of the challenge for an HIV vaccine. Important questions to be addressed include the speed and extent of sequence change, particularly in Gag, the most effective target for CD8$^+$ T-cell responses[1,7,13,21]. The induction of broad Gag-specific CD8$^+$ T-cell responses may be a successful vaccine strategy, but such a vaccine will be most effective if tailored to the viral sequences prevailing, and thus may need to be modified periodically to keep pace with the evolving virus. Moreover, the strong associations between certain HLA class molecules, such as HLA-B*57, HLA-B*27 and HLA-B*51, and slow disease progression may decline as the epidemic continues, particularly where these HLA alleles are highly prevalent, and where HIV transmission rates are high.

METHODS SUMMARY

Overall 2,875 subjects were studied, from 9 previously established study cohorts. These cohorts comprised subjects from North America, the Caribbean, Europe, sub-Saharan Africa, Australasia and Asia. All subjects were antiretroviral-therapy-naive. Apart from the London acute cohort ($n = 142$), all cohorts comprised chronically infected subjects. The 14 variants studied are well-defined escape mutations within well-characterized CD8$^+$ T-cell epitopes, and included those persisting after transmission and likely to have little effect on viral fitness ($n = 5$), as well as those shown previously to reduce viral fitness ($n = 9$). Autologous HIV-1 sequences, and HLA class I types, were determined for all study subjects. The replicative capacity of I135X variants selected within the HLA-B*51-restricted epitope TAFTIPSI (RT 128–135) was assessed via *in vitro* competition assays and also via longitudinal follow-up of HLA-B*51-negative subjects infected acutely with I135X variants. Polymorphism frequency in the study cohorts was compared with prevalence of the relevant HLA molecule in the study cohort using a logistic regression model taking into account the different numbers of study subjects in each cohort. Demonstration of an HLA allele driving escape at Gag 146 in the Japanese cohort was undertaken first by identification of an association between HLA-B*4801 and A146P, subsequent definition of an HLA-B*4801-restricted CD8$^+$ T-cell response to a novel epitope Gag 138–147 (LI10), and finally demonstration that A146P reduced viral recognition by LI10-specific CD8$^+$ T cells.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Information Accession numbers for newly determined viral sequences are included in Supplementary Information. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to P.G. (philip.goulder@paediatrics.ox.ac.uk).

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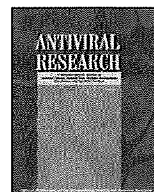

Clinical relevance of substitutions in the connection subdomain and RNase H domain of HIV-1 reverse transcriptase from a cohort of antiretroviral treatment-naïve patients

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ABSTRACT

Some mutations in the connection subdomain of the polymerase domain and in the RNase H domain of HIV-1 reverse transcriptase (RT) have been shown to contribute to resistance to RT inhibitors. However, the clinical relevance of such mutations is not well understood. To address this point we determined the prevalence of such mutations in a cohort of antiretroviral treatment-naïve patients ($n = 123$) and assessed whether these substitutions are associated with drug resistance *in vitro* and *in vivo*. We report here significant differences in the prevalence of substitutions among subtype B, and non-subtype B HIV isolates. Specifically, the E312Q, G333E, G335D, V365I, A371V and A376S substitutions were present in 2–6% of subtype B, whereas the G335D and A371V substitutions were commonly observed in 69% and 75% of non-B HIV-1 isolates. We observed a significant decline in the viral loads of patients that were infected with HIV-1 carrying these substitutions and were subsequently treated with triple drug regimens, even in the case where zidovudine (AZT) was included in such regimens. We show here that, generally, such single substitutions at the connection subdomain or RNase H domain have no influence on drug susceptibility *in vitro* by themselves. Instead, they generally enhance AZT resistance in the presence of excision-enhancing mutations (EEMs, also known as thymidine analogue-associated mutations, TAMs). However, N348I, A376S and Q509L did confer varying amounts of nevirapine resistance by themselves, even in the absence of EEMs. Our studies indicate that several connection subdomain and RNase H domain substitutions typically act as pre-therapy polymorphisms.

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1. Introduction

The zidovudine (AZT)-resistance mutations reside at the DNA polymerase domain of HIV-1 reverse transcriptase (RT). They are associated either with (a) the exclusion mechanism that enhances discrimination at the point of AZT monophosphate (AZT-MP) incorporation through a set of mutations at codons A62, V75, F77, F116 and Q151 of the polymerase domain (Deval et al., 2002; Ueno and Mitsuya, 1997), or with (b) the excision mechanism that involves selective removal of AZT-MP after it has been incorporated by RT into the viral DNA (Boyer et al., 2001; Meyer et al., 1999). The excision mechanism is associated with mutations at the polymerase domain, including M41L, D67N, K70R, L210W, T215F/Y and K219E/Q (excision-containing mutations [EEMs] also known as thymidine analogue-associated mutations [TAMs]).

Certain mutations in the connection subdomain (CD; codons 322–440) of the polymerase domain or in the RNase H domain (codons 441–560) of HIV-1 RT have recently been shown to be associated with resistance to AZT (Brehm et al., 2007; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Ntemgwa et al., 2007; Yap et al., 2007). In some cases it appears that mutations that affect AZT resistance have different phenotypes, depending on the presence or absence of other resistance mutations. For example, the polymorphism G333D/E does not confer drug resistance by itself, but has been reported to contribute significantly to dual AZT-lamivudine (3TC) resistance when combined with EEMs and M184V (Caride et al., 2000; Gallego et al., 2002; Kemp et al., 1998; Zelina

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et al., 2008). Similarly, A371V and Q509L, which were selected in the background of D67N and K70R by high concentrations of AZT *in vitro*, show strong resistance to AZT and weak cross-resistance to 3TC, abacavir (ABC) and tenofovir (TNF/PMPA) in the presence of EEMs (Brehm et al., 2007). Santos et al. (2008) also recently reported that the A360 V and A371V mutations are frequently observed in AZT-treated patients. In contrast, one of the connection subdomain mutations, N348I, is associated with resistance to both nucleoside RT inhibitors (NRTIs) and non-nucleoside RT inhibitors (NNRTIs) and appears to be induced by regimens containing AZT, didanosine (ddI) and/or nevirapine (NVP) (Hachiya et al., 2008; Yap et al., 2007). Recently, it has been shown that the N348I mutation decreases the efficiency of RNase H cleavage and increases excision of AZT from AZT-terminated primer/templates, in the presence of the pyrophosphate donor ATP (Delviks-Frankenberry et al., 2008; Ehteshami et al., 2008; Yap et al., 2007). The decreased degradation of the RNA template by the diminished RNase H cleavage has been proposed to provide additional time for RT to excise AZT-MP and hence result in the observed increased AZT resistance (Delviks-Frankenberry et al., 2008; Ehteshami et al., 2008).

With the exception of N348I, the clinical relevance of these mutations remains to be clarified. A major obstacle to understanding the contribution of connection subdomain mutations to NRTI or NNRTI resistance has been the shortage of relevant sequencing data. This is because, until recently, the majority of commercially available genotypic and phenotypic assays have not been targeting this region of the enzyme. This is now changing, as more attention is being focused on such substitutions, following recent publications from us (Hachiya et al., 2008) and others (Yap et al., 2007) showing that at least one connection subdomain mutation, N348I, contributes to multi-class drug resistance. However, it has not yet been determined if the genotypic substitutions encountered in the connection subdomain of polymerase or in the RNase H domain of RT have any phenotypic impact or any effect on virologic response to subsequent therapies. Another important question is whether resistance testing now performed should include these mutations.

To ascertain whether some mutations at the connection subdomain or at the RNase H domain of RT that appear in the absence of known drug-resistance mutations of the polymerase domain are induced by reverse transcriptase inhibitor (RTI) treatment or are simply pre-existing polymorphisms, we determined the frequency of amino acid substitutions in antiretroviral treatment-naïve patients and assessed whether these substitutions at the reported sites (Brehm et al., 2007; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Yap et al., 2007) can cause drug resistance by themselves. We also explored whether these substitutions may have any effect on the virologic response to subsequent therapies.

2. Materials and methods

2.1. Patients

A total of 123 clinical isolates were obtained from fresh plasma of treatment-naïve HIV-infected patients using MAGIC-5 cells as described previously (Hachiya et al., 2001). Written informed consent was obtained from each patient under approval by the Institutional Review Board of the International Medical Center of Japan (IMCJ-H13-80). The clinical course and antiretroviral therapies used were reviewed retrospectively.

2.2. Recombinant molecular clones

Recombinant molecular clones were generated as described previously (Hachiya et al., 2008). Briefly, the pBS-RT_{WT} contains almost entire RT coding sequence (amino acid position 14–560) containing silent mutations for cloning (restriction enzyme sites, Xma I and Xba I at 5′- and 3′-end of DNA fragment, respectively). After site directed mutagenesis, the mutated RT was ligated into the corresponding restriction enzyme site of the HIV infectious clone pNL101 (Hachiya et al., 2008; Shimura et al., 2008).

2.3. Genotypic and phenotypic assays

For the genotypic assay, viral RNA was extracted from the culture supernatant of clinical isolates, amplified by nested RT-PCR, and then directly sequenced as described previously (Hachiya et al., 2008). For subtype classification, the RT sequences were analyzed using the 'Genotyping' software (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) which uses the BLAST algorithm. HIV-1 sequences in worldwide, treatment-naïve patients were obtained from the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu/index.html, accessed as late as 26 February 2008) and compared with our cohort. Prevalence of mutations at each codon were compared by the χ^2-test, or Fisher's exact test when the number of patients was smaller than 5.

For phenotypic assay, each clinical isolate was directly tested for drug susceptibility in triplicates, using the MAGIC-5 cell-based assay as described previously (Hachiya et al., 2001). Infectious viruses were obtained by transfection of 293T cells with individual HIV molecular clones containing the desired mutations that were introduced by site directed mutagenesis. Cells were subsequently harvested and examined with the MAGIC-5 cell based assay (Hachiya et al., 2001, 2008).

2.4. Measurements of HIV-1 viral load

To assess virologic outcome, HIV-1 viral loads in plasma were measured using the commercially available Amplicor HIV-1 Monitor Test (Version 1.5, Roche Diagnostics K.K., Basel, Switzerland). Mean change from 0 at weeks 4, 8, 12, 16, 20 and 24 were evaluated. The statistical significance of the longitudinal changes of HIV-1 viral load in plasma was assessed by the Mann–Whitney *U*-test.

2.5. Molecular modeling studies

The SYBYL and O programs were used to prepare molecular model of the complexes of HIV-1 RT in complex with RNA/DNA (Protein Data Bank code number 1HYS), and containing mutations A376S, N348I and Q509L that were introduced manually into the original 1HYS structure. After introduction of the mutations, the structure coordinates were optimized through 100 cycles of Coleman energy minimization protocol.

3. Results

3.1. Sequence analysis of RT region

We sequenced nearly the entire RT coding region (amino acid position 9–560) of 123 clinical isolates obtained from treatment-naïve patients. Among these isolates, six contained the known RTI-associated resistant mutations, D67N (n = 2), K238S (n = 2) (http://www.hiv.lanl.gov/content/index), V108I/K238S (n = 1) and V106A/V108I/K238S (n = 1), and thus were excluded from further analysis. The clinical isolates were obtained from six patients within 1 year of the diagnoses for HIV-1 infection. Prevalence of HIV-1 with drug-associated mutations in Japanese treatment-naïve patients is estimated at approximately 4% (Gatanaga et al., 2007) and in American and European patients at 8–27% (Descamps et al., 2005; Little et al., 2002; UK Collaborative Group on Monitoring the Transmission

of HIV Drug Resistance, 2001; Weinstock et al., 2004). Therefore, the prevalence in our cohort (4.8%) seems to be comparable or lower than in previous reports, suggesting that the six patients are treatment-naïve and newly infected from treated patients. The strong majority of the remaining samples in our cohort were of subtype B ($n = 101$ of a total of 117 isolates), while other subtypes were also identified (CRF01_AE, A, C and CRF12_BF, with12, 2, 1 and 1 isolates, respectively).

Substitutions at the connection subdomain and RNase H domain observed in this cohort and in previous reports (Brehm et al., 2007; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Yap et al., 2007) are shown in Table 1. In the treatment-naïve patients of our cohort that were infected with subtype B ($n = 101$), the frequencies of all mutations associated with AZT-resistance (Brehm et al., 2007; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Yap et al., 2007) were comparable to those (treatment-naïve) deposited in the Stanford HIV Drug Resistance Database, except for the A360T mutation. The G335D and A371V substitutions were more prevalent in the non-B, rather than in the B isolates of our cohort. Moreover, the G335D/A371V combination was observed in 9 (56.3%) of the non-B isolates. Other polymorphisms, including E312A/D/N/T, G335E/N/S, A360S/T, A371T, A376T/V and Q509H, were widely observed in all subtypes in our cohort as well as in the Stanford HIV Drug Resistance Database. None of the clinical isolates of our cohort had the G333D, G335C, N348I, A360I/V, and Q509L mutations.

3.2. Phenotypic assay for clinical isolates

Phenotypically, all clinical isolates showed little resistance to tested drugs (Table 1). Isolates with the V365I substitution ($n = 4$ in subtype B) showed slightly reduced susceptibility to 3TC (2.3-fold). However, V365I may not be clinically relevant, since generally at least 3-fold resistance is required for assigning 3TC resistance *in vivo* (Parkin et al., 2004; Rhee et al., 2006). Furthermore, the prevalence of V365I in treated and untreated patients in the Stanford HIV Drug Resistance Database is comparable (3.7% and 3.6%, respectively). Notably, clinical isolates from treatment-naïve patients from our cohort with HIV carrying the E312N, G335E/N or A376V substitutions displayed rather enhanced susceptibility (over five-fold) to

Table 1
Drug susceptibilities of 117 clinical isolates obtained from *treatment-naïve patients.*

Amino acid substitutions	Frequency[a] % (n)		Median fold change in resistance[b]			
	Subtype B (n = 101)	Non-B (n = 16)	AZT	3TC	NVP	EFV
E312	84.1 (85)	18.8 (3)	1.2	1.3	1	1.1
Q[c]	**3 (3)**	**0**	**1.3**	**1.4**	**1.2**	**1.1**
A	6.9 (7)	0	1.1	1.1	1.3	1
D	1 (1)	6.3 (1)	1.7	1.2	1.7	1.1
N	0	6.3 (1)	0.1	1.3	0.2	1.2
T	5 (5)	68.8 (11)[d]	0.8	1	1.1	1.1
G333	94.1 (95)	100 (16)	1.1	1.3	1.1	1.1
D[c]	**0**	**0**	–	–	–	–
E[c]	**5.9 (6)**	**0**	**1.4**	**1.5**	**1**	**1.4**
G335	95 (96)	25 (4)	1.2	1.4	1	1.1
C[c]	**0**	**0**	–	–	–	–
D[c]	**2 (2)**	**68.8 (11)**[d]	**0.7**	**0.9**	**1.1**	**1.1**
E	0	6.3 (1)	0.3	0.06	0.2	0.5
N	1 (1)	0	0.2	0.2	0.8	1.5
S	2 (2)	0	0.6	0.9	1.3	1.4
N348	100 (101)	100 (16)	1.1	1.3	1	1.1
I[c,e]	**0**	**0**	–	–	–	–
A360	79.2 (80)	87.5 (14)	1.1	1.3	1.1	1.1
I[c]	**0**	**0**	–	–	–	–
V[c]	**0**	**0**	–	–	–	–
S	0	6.3 (1)	0.7	1.2	1.5	0.8
T	20.8 (21)[f]	6.3 (1)	0.9	1.4	1	1.2
V365	96 (97)	100 (16)	1.1	1.2	1	1.1
I[c]	**4 (4)**	**0**	**1**	**2.3**	**1.7**	**1.3**
A371	96 (97)	25 (4)	1.2	1.3	1.1	1.1
V[c]	**3 (3)**	**75 (12)**[d]	**0.7**	**0.9**	**0.9**	**1.1**
T	1 (1)	0	0.5	1.3	0.7	0.7
A376	92.1 (93)	75 (12)	1.1	1.3	1	1.1
S[c]	**3 (3)**	**6.3 (1)**	**1.3**	**0.9**	**1**	**0.6**
T	5 (5)	12.5 (2)	1.2	1	1.4	1.2
V	0	6.3 (1)	0.1	1.3	0.2	1.2
Q509	98 (99)	100 (16)	1.1	1.3	1.1	1.1
L[c]	**0**	**0**	–	–	–	–
H	2 (2)	0	0.6	0.7	0.7	0.8

[a] Of 123 clinical isolates, six carried the known RTI-associated mutations and were excluded from this analysis.

[b] The drug susceptibility assay (Hachiya et al., 2001) was clinically accepted in Japan.

[c] Resistant mutations reported previously (Brehm et al., 2007; Delviks-Frankenberry et al., 2007, 2008; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Ntemgwa et al., 2007; Santos et al., 2008; Yap et al., 2007) are indicated in bold. Greater than three-fold increase of EC_{50} compared to that of NL4-3 was defined as resistance.

[d] The prevalence of these substitutions (E312T, G335D and 371V) is significantly difference among treatment-naïve patients between subtype B and non-B isolates ($p < 0.0001$).

[e] N348I confers cross-resistance to NRTIs and NNRTIs (Hachiya et al., 2008; Yap et al., 2007).

[f] The prevalence of A360T is significantly higher in our cohort compared to the Stanford HIV Drug Resistance Database (8.7%, $p = 0.0021$).

Table 2
Drug susceptibilities of molecular HIV-1 clones.

Mutation	EC_{50}, μM (fold increase)[a]				
	NRTI			NNRTI	
	AZT	3TC	TNF[b]	NVP	EFV
WT	0.026 ± 0.009	0.42 ± 0.04	6.2 ± 1.5	0.023 ± 0.01	0.0012 ± 0.0001
E312Q	0.037 ± 0.006 (1.4)	0.36 ± 0.05 (0.9)	4.1 ± 1.4 (0.7)	0.056 ± 0.007 (2.4)	0.0009 ± 0.0002 (0.8)
G333D	0.04 ± 0.01 (1.5)	0.28 ± 0.1 (0.7)	4.5 ± 1.8 (0.7)	0.055 ± 0.01 (2.4)	0.0017 ± 0.0003 (1.4)
G335C	0.04 ± 0.02 (1.5)	0.45 ± 0.1 (1.1)	7.7 ± 1.1 (1.2)	0.065 ± 0.02 (2.8)	0.0007 ± 0.00009 (0.6)
N348I	0.14 ± **0.01 (5.4)**	0.56 ± 0.07 (1.3)	8.8 ± 1.9 (1.4)	0.24 ± **0.04 (10)**	0.0032 ± 0.0005 (2.7)
A360I	0.037 ± 0.01 (1.4)	0.35 ± 0.1 (0.8)	7.1 ± 2.1 (1.1)	0.038 ± 0.01 (1.7)	0.0009 ± 0.00008 (0.8)
A360V	0.03 ± 0.002 (1.2)	0.28 ± 0.09 (0.7)	5.7 ± 2.3 (0.9)	0.051 ± 0.01 (2.2)	0.0016 ± 0.0006 (1.3)
V365I	0.045 ± 0.008 (1.7)	0.27 ± 0.06 (0.6)	6.1 ± 2.0 (1)	0.066 ± 0.02 (2.9)	0.0013 ± 0.0002 (1.1)
A376S	0.053 ± 0.02 (2)	0.3 ± 0.03 (0.7)	5.9 ± 1.6 (1)	0.084 ± **0.02 (3.7)**	0.0022 ± 0.0004 (1.8)
Q509L	0.072 ± 0.02 (2.8)	0.45 ± 0.1 (1.1)	8.1 ± 2.7 (1.3)	0.21 ± **0.06 (9.1)**	0.0032 ± 0.0009 (2.7)

[a] Data means ± standard deviations from at least three independent experiments. The relative increase in the EC_{50} value compared with that in HIV-1_{WT} is given in parentheses. Bold indicates an increase in EC_{50} value greater than three-fold.

[b] TNF (PMPA) [(*R*)-9-(2-phosphonomethoxypropyl) adenine or tenofovir] is the active nucleotide of the clinical prodrug tenofovir disoproxil fumarate.

AZT and NVP, AZT, 3TC and NVP, and AZT and NVP, respectively (Table 1). In our cohort, in the absence of EEM mutations, A371V had no significant effect on drug resistance (Table 1). However, other studies have shown that combined with EEMs, A371V can confer strong resistance to AZT and A371V has also been recently reported to be associated with weak cross-resistance to 3TC, TNF/PMPA and ABC (Brehm et al., 2007). In our cohort, ABC inhibits efficiently the clinical isolates that contain the A371V substitution in the absence of EEMs (n = 13) either in a subtype B, or non-B background (median fold increase was 0.9-fold, data not shown). Further, the combination of A371V and G335D commonly observed in non-B isolates also showed no resistance to AZT, 3TC or ABC (0.7-, 1.0- and 1.1-fold increase in susceptibility as compared to wild-type HIV, respectively). These results demonstrate that none of the above substitutions that were observed in clinical isolates confer any significant resistance to NRTIs or NNRTIs in the absence of EEMs.

3.3. *Phenotypic assay for molecular clones*

To further expand our understanding of the role of substitutions in these RT regions on drug resistance we also prepared HIV-1 recombinant viruses with related mutations that have been reported previously in similar drug resistance studies (Brehm et al., 2007; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Yap et al., 2007). The results shown in Table 2 confirm that in the absence of NRTI or NNRTI resistance mutations, most substitutions in the connection subdomain and RNase H domain (with the exception of N348I, A376S and Q509L) show no significant resistance to AZT, 3TC, TNF/PMPA, NVP or efavirenz (EFV) (less than three-fold), suggesting that these mutations act as secondary mutations and may enhance resistance that is caused by primary mutations and/or may somehow improve replication kinetics impaired by the primary mutations. Q509L, which has been reported to enhance

Table 3
Profiles of patients infected with HIV carrying connection subdomain substitutions, and initial therapies used in patient treatments.

Parameter	Combination for treatment-naïve patients infected HIV-1			
	With substitutions		Without substitutions	
	With AZT (n = 8)	Without AZT (n = 13)	With AZT (n = 16)	Without AZT (n = 24)
Male, n (%)	5(63)	10(77)	15(94)	23(96)
Median age (range)	37 (27–60)	41 (27–54)	36 (24–55)	38 (26–59)
Median baseline viral load, log_{10} copies/ml (range)	5.0 (3.0–6.0)	5.0 (4.2–5.8)	5.0 (4.1–6.4)	5.2 (4.2–6.3)
Median baseline CD4 cell count, cell/μl (range)	217 (3–549)	110 (3–332)	225 (9–613)	170 (4–760)
Substitutions in the connection subdomain, n (%)[a]				
E312Q	–	3 (23)	–	–
G333E	2 (25)	2 (15)	–	–
G335D	3 (38)	6 (46)	–	–
V365I	2 (25)	–	–	–
A371V	2 (25)	5 (38)	–	–
A376S	1 (13)[b]	2 (15)	–	–
Initial therapy, n (%)				
Zidovudine	8 (100)	–	16 (100)	–
Lamivudine	4 (50)	11 (85)	12 (75)	24 (100)
Stavudine	–	11 (85)	–	20 (83)
Didanosine	4 (50)	–	4 (25)	–
Abacavir	1 (13)	1 (8)	1 (6)	3 (13)
Tenofovir	–	1 (8)	–	1 (4)
Emtricitabine	–	1 (8)	–	–
Nevirapine	–	–	–	3 (13)
Efavirenz	2 (25)	3 (23)	9 (56)	9 (38)
One protease inhibitor (PI)	3 (38)	7 (54)	5 (31)	7 (29)
Dual-boosted PI	1(13)	2 (15)	1 (6)	5 (21)

[a] E312Q, G333E, G335D, V365I, A371V and V376S were reported to be AZT-resistant mutations (Brehm et al., 2007; Kemp et al., 1998; Nikolenko et al., 2007).

[b] In this case, the viral load did not fall below the limits of detection.

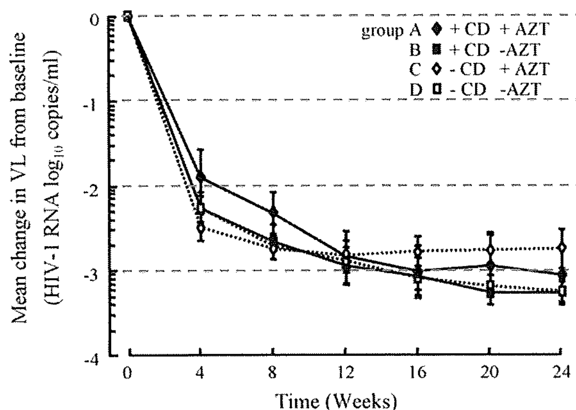

Fig. 1. Virological response up to 24 weeks after initiation of combination therapy. Mean (±standard error of the mean; S.E.M.) changes in plasma viral load (VL) were measured by Amplicor HIV-1 Monitor Test (Version 1.5, Roche Diagnostics K.K., Basel, Switzerland) from 0 to 24 weeks. Treatment-naive patients that were subsequently treated with combination therapy regimens are classified into four groups: patients that were infected with HIV-1 containing connection subdomain (CD) mutations and that subsequently received either combination therapy with AZT ($n=8$, closed diamonds, group A) or without AZT ($n=13$, closed squares, group B) and patients who were infected with HIV-1 with none of connection subdomain substitutions, and who subsequently received combination therapy with either AZT ($n=16$, open diamonds with broken line, group C) or without AZT ($n=24$, open squares with broken line, group D).

cross resistance to NRTIs in the presence of EEMs (Brehm et al., 2007), conferred little resistance to at least AZT, 3TC and TNF/PMPA in this study. Unlike N348I that confers dual resistance to NRTIs and NNRTIs, A376S and Q509L provided only NVP resistance.

3.4. Virological response after initiation of combination therapy

To further assess whether the CD substitutions at baseline are one of predictive factors of virologic outcome, we examined clinical samples from the treatment-naïve patients who subsequently received combination therapy through measuring virus load in plasma from 0 to 24 weeks (Table 3 and Fig. 1). The treatment-naïve patients were classified in four groups: (A) patients who were infected by HIV-1 that carried one or two of the CD substitutions E312Q, G333E, G335D, V365I, A371V or A376S and who subsequently received combination therapy that contained AZT ($n=8$); (B) patients who were infected by HIV-1 that carried the above CD substitutions and who subsequently received combination therapy that did not contain AZT ($n=13$); (C) patients who were infected by HIV-1 that did not carry any of the above CD substitutions and who subsequently received combination therapy containing AZT ($n=16$); and (D) patients who were infected by HIV-1 that did not carry the above CD substitutions and who subsequently received combination therapy that did not contain AZT ($n=24$). The mean change in viral load from baseline (week 0) to week 24 was from −2.76 to −3.28 $\log_{10}$ copies/ml among four groups. There were no significant differences in viral load changes up to 24 weeks among these groups (Fig. 1). Marginal viral suppression was observed in one patient who was infected by HIV-1 carrying A376S and who received combination therapy containing AZT. Any of the drug-associated resistant mutations were detected during the first 5 months of receiving combination therapy. However, HIV-1 protease mutations D30N and M36I that are responsible for resistance to NFV and HIV-1 RT D67N mutation that is responsible for AZT resistance eventually emerged. After switching to a new combination regimen (d4T/3TC/LPV), the viral load was successfully decreased. These results indicate that at least combination of two substitutions in the connection subdomain that are observed in treatment-naïve patients do not affect the virologic response of the ensuing combination therapy. Instead, they merely act as polymorphisms among the treatment-naïve patients.

4. Discussion

According to the crystal structure of HIV-1 RT in complex with RNA/DNA, some amino acids in the connection subdomain may affect binding to the RNA/DNA substrate (Sarafianos et al., 2001). It has been proposed that mutations at the connection subdomain may alter the binding affinity for nucleic acid at the connection subdomain and lead to enhanced resistance to AZT when combined with EEMs. This is thought to happen through a decrease in template RNA degradation which in turn provides additional time for RT to excise AZT-MP from the AZT-terminated template-primer$_{\text{AZT-MP}}$, thus causing resistance to AZT (Delviks-Frankenberry et al., 2007; Nikolenko et al., 2005, 2007). In our cohort, as well as in the Stanford HIV Drug Resistance Database, we observed a considerable number of treatment-naïve clinical samples containing substitutions (E312Q, G333E, G335D, V365I, A371V and A376S) that have been previously associated with AZT resistance. Our phenotypic studies with clinical isolates carrying mutations located in the connection subdomain of the polymerase or in the RNase H domain of RT revealed that in the absence of other known NRTI or NNRTI resistance mutations they do not cause by themselves significant resistance to the tested RTIs. Additionally, results from our cohort establish that the presence of G333E, G335D, V365I or A371V among treatment-naïve patients does not play any significant role in the virologic response after initiation of therapies that may, or may not, include AZT. We identified 25 isolates that have been deposited before 1986, prior to clinical trails for AZT in the Los Alamos HIV Sequence Database (http://www.hiv.lanl.gov/content/index). Some of these isolates also contained E312V, V365I, A376S/T/P, indicating that at least these substitutions are polymorphisms that preceded any antiviral therapy.

None of the isolates in our cohort had the H539N or H549N substitutions which have been proposed to be associated with resistance to NRTIs due to decreasing the frequency of RT template-switching and the level of RNase H activity (Nikolenko et al., 2004; Roquebert and Marcelin, 2008). Furthermore, the G333D, G335C, N348I, A360I/V and Q509L substitutions were not observed in our cohort, and were also rarely observed among treatment-naïve patients (less than 1%) in the Stanford HIV Drug Resistance Database. Their increased incidence among NRTI-treated patients as compared to untreated patients (>3-fold, >40-fold and >12-fold increases for G333C, N348I, and A360V respectively [http://hivdb.stanford.edu/cgi-bin/RTPosMutSummary.cgi]) and in the case of Q509L reported by others (Brehm et al., 2007; Roquebert et al., 2007) suggests that they are associated with AZT resistance. However, site directed mutagenesis studies showed that G333D (Kemp et al., 1998), G335C (Nikolenko et al., 2007), A360I/V (Nikolenko et al., 2007) and Q509L (Brehm et al., 2007) did not confer significant AZT resistance in the absence of other AZT resistance mutations. At present, only N348I has been shown to be involved in resistance to multiple RTIs (Hachiya et al., 2008; Yap et al., 2007). HIV with a serine at codon 376 also exhibits some NVP resistance in the absence of other mutations (Table 2). However, clinical isolates harboring different residues at position 376 exhibited no significant changes in their drug susceptibilities (Table 1). This discrepancy may arise from strain-specific polymorphisms that are present in the clinical isolates or the reference virus used in this study that may influence NVP susceptibility positively or negatively, respectively. In fact, we observe several polymorphisms in the majority of these isolates and it is possible that they somehow affect drug resistance. For instance, V118I has been identified in 2% of treatment-naïve patients as one of strain-specific polymorphisms, but more frequently observed in RTI-treated patients

(Delaugerre et al., 2001). Although this mutation by itself confers no resistance, it has been reported to contribute to hypersusceptibility to NNRTI (Clark et al., 2006) as well as resistance to NRTI in the presence of E44A/D and/or EEMs (Romano et al., 2002). Therefore, it is possible that polymorphisms present in our clinical isolates may also affect drug-susceptibility leading to minor discrepancies with the results obtained with recombinant virus.

In this study, the reference clone has an A376T polymorphism that is observed in a wide range of subtypes. Therefore, it is unlikely that A376T affects NVP susceptibility. Q509L confers moderate (~9-fold) resistance to NVP (Table 2). Although Q509L was not observed in our cohort, this mutation was found in the pretreated patients of another survey (n = 118) (Roquebert et al., 2007). These results indicate that introduction of Q509L may alter virologic responses, especially for NVP, although so far the clinical relevance and virological response of Q509L among the antiretroviral-experienced patients remains to be elucidated by further experiments.

Analysis of the crystal structure of RT bound to RNA/DNA showed that residues 376 (of the p66 subunit) and 509 are located relatively close to the nucleic-acid binding cleft of RT, and residue 348 of the p66 subunit is located close to the hinge region of the thumb subdomain and to the NNRTI-binding pocket (Fig. 2). Recently, Abbondanzieri et al. demonstrated that binding of nevirapine to RT causes conformational changes to the enzyme, allowing it to somehow relax the grip on nucleic-acid substrate (Abbondanzieri et al., 2008; Arnold and Sarafianos, 2008). NVP acts as a rapid-equilibrium inhibitor, not a tight-binding inhibitor as EFV (Maga et al., 2000; Motakis and Parniak, 2002), and it might be more sensitive to changes in the interaction between RT and the nucleic acid substrate. Thus, changes in the interactions of RT with nucleic-acid substrate could also influence the interaction balance between polymerase and RNase H activity and consequently might lead to RTI resistance. Nevertheless, additional biochemical and structural studies are warranted to define the exact mechanisms by which these mutations in the connection subdomain and RNase H domains confer NVP resistance.

Several studies have reported a correlation between two distinct types of EEMs in various HIV subtypes (Kantor et al., 2005; Montes et al., 2004; Novitsky et al., 2007). The Type I EEMs (M41L, L210W, T215Y and occasionally the D67N mutation) appear twice as frequently as Type II EEMs (D67N, K70R, T215F and K219Q mutation) in subtype B (Marcelin et al., 2004), whereas Type II EEMs are mostly observed in non-B isolates (Montes et al., 2004; Novitsky et al., 2007). Type II EEMs confer lower levels of AZT and TNF/PMPA resistance, as compared to Type I EEMs (Cozzi-Lepri et al., 2005; Miller et al., 2004). Addition of A371V to Type II EEM background conferred cross-resistance to AZT and tenofovir (Brehm et al., 2007). A371V was observed in the majority of non-B isolates in our cohort (75%) and the Stanford HIV Drug Resistance Database (96% in CRF01_AE). Therefore, it is possible that in the background of non-B isolates, the majority of which contains drug resistance associated connection subdomain mutations, smaller number of EEMs, especially Type II EEMs, might be preferentially selected for AZT and TNF/PMPA resistance. In the absence of EEMs, mutations at the connection subdomain of non-subtype B HIV, such as E312N, G335E or A376V, appear to act as simple polymorphisms, because they either maintain or enhance drug susceptibility in non-subtypes B HIV (Table 1). However, the A376S polymorphism in samples of treatment-naive patients or in a recombinant virus used in this study conferred mild NVP resistance (Table 2). These mutations were stable even in the absence of any drug treatment, suggesting that viral fitness of these variants is likely to be comparable to wild type non-subtype B HIV.

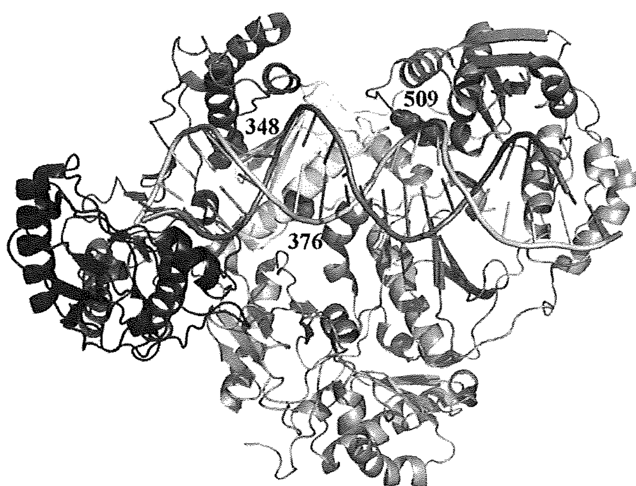

Fig. 2. Structure of HIV-1 RT in complex with RNA/DNA. The fingers, palm, thumb, connection subdomains, and RNase H domain of the p66 subunit colored in blue, red, green, yellow and orange, respectively. The p51 subunit is shown in dark yellow. Residue 348 of the p66 subunit is shown as pink Van der Waals spheres, and located proximally to the hinge region of the thumb subdomain and to the NNRTI binding pocket. Residues 376 and 509 of the p66 subunit are also shown, and are located proximally to the nucleic acid binding cleft.

In this study we report the prevalence of amino acid substitutions in the connection subdomain of the polymerase domain and in the RNase H domain of RT in a cohort of treatment-naïve patients. We also determined the phenotypic susceptibility of these mutants to various RTIs. Our results support the hypothesis that the substitutions observed among treatment-naïve patients have little impact on therapeutic outcome by themselves in the absence of AZT-associated mutations, although certain substitutions, such as N348I, A376S, and Q509L, are involved in drug resistance even by themselves. These results may help improve existing interpretation algorithms and analysis of drug resistance mutations.

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ORIGINAL RESEARCH ARTICLE

High-risk status of HIV-1 infection in the very low epidemic country, Mongolia, 2007

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Summary: Thirty-six HIV-1 cases had been reported by December 2007 in Mongolia. Therefore, Mongolia has been regarded as a very low HIV-1 epidemic country, although the surveillance system is not fully developed. The aim of this study was to evaluate the risk status of HIV-1 infection in Mongolia. A total of 1415 blood samples from high-risk populations including female sex workers, men who have sex with men, mobile men, tuberculosis patients and male sexually transmitted infection (STI) clinic clients and 1050 samples from healthy controls were collected. The seroprevalences of anti-HIV-1/2, anti-*Treponema pallidum*, hepatitis B surface antigen (HBs Ag), anti-hepatitis C virus and hepatitis B surface antibody in the high-risk populations were 0%, 23.1%, 15.5%, 8.0% and 48.2%, and those in the controls were 0%, 3.1%, 14.7%, 4.4% and 44.4%, respectively. HIV-1 prevalence is currently low. However, according to the high prevalence of STIs in the high-risk populations, the risk status for HIV-1 infection is estimated to be high.

Keywords: seroprevalence of HIV, syphilis, HCV and HBV, high-risk population, Mongolia

INTRODUCTION

Mongolia is located in Central Asia bordered by Russia and China. The population of Mongolia is 2635 million, of which 61.0% live in cities and the remaining are nomadic.[1] Geographical conditions and a very low population density make communication, transport and health service provision difficult. Mongolia has witnessed radical changes in its economic and social policies since the democratic revolution of 1990. Along with independence from the former Soviet Union and loss of Soviet support, there has been an increase in unemployment, alcoholism and prostitution and a steady increase in the prevalence of sexually transmitted infections (STIs) and other communicable diseases.[2-8] A recent study demonstrated that syphilis, gonorrhoea and trichomonas were detected in 57 (43%), 18 (14%) and 37 (28%) subjects, respectively, among 132 low-income female commercial sex workers (FSWs) in Mongolia.[9] Mongolia also has a high prevalence of hepatitis B and C viral infection. In a previous study, hepatitis B surface antigen (HBs Ag) and antibodies to hepatitis C virus (anti-HCV) were detected in 24 (10%) and 41 (16%) subjects, respectively, among 249 apparently healthy individuals in Mongolia.[10] However, most of these data were obtained from convenient or non-generalized samples. There is a lack of information regarding exposures and the burden of diseases in the high-risk populations for HIV and STIs. High-risk populations such as FSWs and their sexual contacts with high rates of STIs are important populations contributing to the transmission of HIV and other STIs in developing countries.[11-13]

Since 1992, when data on HIV/AIDS began to be compiled in Mongolia, there had been only five cases reported as of December 2004. Mongolia is considered as an HIV/AIDS low-prevalence country. However, annual new cases of HIV/AIDS have been increasing in recent years. For example, 11, 9 and 11 new cases were detected in 2005, 2006 and 2007, respectively. Among them, 22 (61.1%) cases were men who have sex with men (MSM), seven (19.4%) were heterosexually transmitted and six (16.7%) were FSWs (Mongolian National Center for Communicable Diseases [NCCD], unpublished data). Owing to the lack of a sound surveillance system, the actual situation is uncertain. The primary objective of this study was to evaluate the current risk status of HIV-1 among high-risk populations in Mongolia, examining the seroprevalence of other STIs concomitantly. These data are crucial for taking future preventive measures against HIV-1 infection.

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METHODS

Study design and study population

This study was conducted from September through December 2007. The study protocol was approved by the ethics committees of the International Medical Center of Japan (H19-448) and of the Ministry of Health, Mongolia. After explaining this study and obtaining informed consent, blood samples were

Figure 1 A map of Mongolia. Blood samples were collected from the capital city Ulaanbaatar and four aimags (provinces), such as Darkhan-Uul, Huvsgul, Dornod and Dornogobi. Asterisks indicate the sites where blood samples were obtained

collected anonymously from both high-risk and healthy control populations in Ulaanbaatar (the capital city of Mongolia) and four aimags including Dornod, Huvsgul (borders of Russia), Dornogobi (a border of China) and Darkhan-Uul (Figure 1). A total of 2465 samples were collected: 1415 samples from high-risk populations and 1050 samples from healthy control populations. The high-risk populations included FSWs, MSM, mobile men, tuberculosis (TB) patients and male STI clinic clients. The number of samples in each population and demographic characteristics are listed in Table 1.

Cluster sampling was used for FSWs in locations such as bars, nightclubs, sauna and massage parlours serving as clusters. MSM were sampled only from Ulaanbaatar city, due to the limited data on MSM in other areas of the country. Mobile men were sampled from Ulaanbaatar city and Dornogobi aimag (province), along major road and rail networks and areas such as truck stops and checkpoints at borders. As for TB patients, those who were diagnosed with TB for the first time during the sampling period were enrolled. A male STI clinic client was defined as one who attended public STI clinics during the sampling period. A healthy control group included youth and blood donors. Youth was defined as unmarried, 15–35 years old students in college or university of both sexes. The blood donors were selected in health facilities during the sampling period.

Specimen collection and serology

All sera were stored below −20°C until use. Sera were tested for antibodies to HIV-1/2 (anti-HIV-1/2), *Treponema pallidum* (anti-TP), hepatitis B surface antiboby (HBs Ab), and hepatitis C virus (anti-HCV) and HBs Ag by using the chemiluminescent enzyme immunoassay (CLEIA) (Lumipalus, Fujirebio, Tokyo, Japan) according to the instructions provided by the manufacturer. Seropositive samples for anti-HIV-1/2 by CLEIA were further confirmed by chemiluminescent immunoassay (Architect, Abbott Laboratories, Abbott Park, IL, USA) and a Western blot for the final diagnosis. All laboratory analyses were performed at the AIDS Clinical Center, International Medical Center of Japan.

Statistical analyses

Differences among high-risk and/or healthy control populations were examined by the Fisher's exact test. Univariate logistic analyses were used to determine the odds ratios (OR) with corresponding 95% confidence intervals (CI). All analyses

Table 1 Demographic characteristics of persons who gave blood samples

			Age	
Populations	No.	Sex M:F	Range	Mean ± SD
High risk				
FSWs	410	0:410	17–52	25.3 ± 6.8
MSM	50	50:0	19–48	28.5 ± 6.3
Male STI clients	545	545:0	15–64	29.2 ± 7.9
TB patients	110	41:69	16–70	34.8 ± 12.5
Mobile men	300	300:0	17–57	30.9 ± 8.8
Subtotal	1415	936:479	15–70	28.8 ± 8.7
Healthy controls				
Blood donors	150	101:49	18–49	28.2 ± 9.3
Youth	900	450:450	15–35	19.9 ± 2.6
Subtotal	1050	551:499	17–49	21.0 ± 4.9

FSW = female commercial sex worker; MSM = men who have sex with men; TB = tuberculosis; STI = sexually transmitted infection

Table 2 Seroprevalence between high-risk and healthy controls in Mongolia

	% Positive in each high-risk group					High risk (%)	Healthy controls (%)	OR (95% CI)	*P* value
	FSWs	MSM	Male STI clients	TB patients	Mobile men				
Anti-HIV-1/2	0	0	0	0	0	0	0	–	–
HBs Ag	11.5	18	15.4	16.4	20.7	15.5	14.7	1.1 (0.9–1.3)	0.570
HBs Ab	48.5	42	48.9	50.9	46.7	48.2	44.4	1.2 (1.0–1.4)	0.060
Anti-HCV	6	18	7.5	15.4	7	8	4.4	1.9 (1.3–2.7)	<0.001
Anti-TP	39.5	30	17.2	10.9	14.7	23.1	3.1	9.3 (6.4–13.4)	<0.0001

FSW = female commercial sex worker; MSM = men who have sex with men; TB= tuberculosis; STI = sexually transmitted infection; OR = odds ratio; CI = confidence interval; HBs Ag = hepatitis B surface antigen; HBs Ab = hepatitis B surface antibody; TP = *Treponema pallidum*; HCV = hepatitis C virus

were conducted using the *Stat View* software version 5.0 (SAS Institute, Cary, NC, USA). A *P* value of <0.05 was considered statistically significant.

RESULTS

The seroprevalences of anti-HIV-1/2, HBs Ab and Ag, anti-HCV and anti-TP of each group of the high-risk and healthy populations are presented in Table 2. None of the anti-HIV-1/2-positive samples was detected in this study. The prevalences of HBs Ag and HBs Ab in the high-risk population, including among each high-risk group, were not different compared with those in the healthy control. In contrast, the prevalences of anti-HCV (8%) and anti-TP (23.1%) in the high-risk population were significantly higher than those in the healthy control. The ORs of anti-HCV and anti-TP comparing between the high-risk population and the healthy control were 1.9 (95% CI: 1.3–2.7, $P < 0.001$) and 9.3 (95% CI: 6.4–13.4, $P < 0.0001$), respectively. The prevalences of anti-HCV in MSM and TB patients were higher than those of other risk groups. The prevalences of anti-TP in FSWs (39.5%) and MSM (30%) were surprisingly high.

Geographical differences of seroprevalence are shown in Table 3. Again, there were no significant differences of the prevalence of HBs Ab and Ag in different regions of specific high-risk groups. However, incidences of anti-HCV and anti-TP had some differences in different regions of the specific groups. A striking feature was that the prevalence of anti-TP in Ulaanbaatar FSWs was 54.7%.

The prevalence of HBs Ab was high. However, there were no differences in the prevalence between high-risk and healthy control populations over the country. One reason was that a hepatitis B virus (HBV) vaccination programme in childhood has been implemented 18 years ago. Therefore, we divided the subjects into two age-related groups (below 18 years and over 20 years) and analysed the seroprevalence of HBs Ab (Figure 2). There were no differences between the high-risk and healthy control populations in both age-related groups. However, in both high-risk and healthy control populations, higher age groups had significantly higher prevalence.

DISCUSSION

Since 1992 when the first case of HIV-1 infection was reported in Mongolia, the number of reported cases remained low until 2005. However, the number has been increasing sharply since 2005, and 36 cases have been reported as of February 2008 (Ministry of Health, Mongolia, unpublished data). By the estimated report of the Global Fund for AIDS, Tuberculoses and Malaria ('Impact of AIDS in Mongolia' 2004), without prevention measures, Mongolian HIV/AIDS prevalence will be doubled every two years and 2500 people will die of AIDS by 2014. Our result supports this estimation. A current prevalence of HIV-1 infection is still low but the risk status of HIV-1 infection must be high because of the very high prevalence of syphilis in FSWs (39.5%), especially in Ulaanbaatar (54.7%). Another report also presented similar prevalence among low-income FSWs in Mongolia (43%).[9] Schwebke *et al.* [7] reported the prevalence rate (8.6%) of syphilis among 137 male STI clients in

Table 3 Seroprevalence of HBV, HCV and syphilis among a high-risk population by residence

	No.	% positive for Anti-HIV-1/2	HBs Ag	HBs Ab	Anti-HCV	Anti-TP
Ulaanbaatar						
FSWs	150	0	8.7	48.7	8.7	54.7
MSM	50	0	18	42	18	30
Male STI clients	200	0	10	51.5	5.5	16.5
TB patients	50	0	18	56	12	12
Mobile men	150	0	22	48	5.3	14.7
Subtotal	600	0	14	49.5	7.8	26.3
Darkhan – Uul						
FSWs	200	0	14.5	47	4	31.5
Male STI clients	100	0	28	44	7	26
TP patients	30	0	13.3	46.7	16.7	13.3
Subtotal	330	0	18.5	46	6	28.2
Dornogobi						
FSWs	20	0	5	55	5	30
Male STI clients	45	0	26.7	48.9	13.3	15.6
TB patients	10	0	20	70	10	10
Mobile men	150	0	19.3	45.3	8.7	14.7
Subtotal	225	0	19.6	48	9.3	16
Dornod						
FSWs	10	0	10	40	10	20
Male STI clients	100	0	15	46	5	17
TP patients	10	0	10	40	10	0
Subtotal	120	0	14.2	45	5.8	15.8
Huvsgul						
FSWs	30	0	10	56.7	6.7	30
Male STI clients	100	0	9	51	12	11
TP patients	10	0	20	30	40	10
Subtotal	140	0	10	50.7	12.9	15

HBV= hepatitis B virus; HCV = hepatitis C virus; TP= *Treponema pallidum*; FSW = female commercial sex worker; MSM = men who have sex with men; TB = tuberculosis; STI = sexually transmitted infection; OR = odds ratio; CI = confidence interval; HBs Ag = hepatitis B surface antigen; HBs Ab = hepatitis B surface antibody

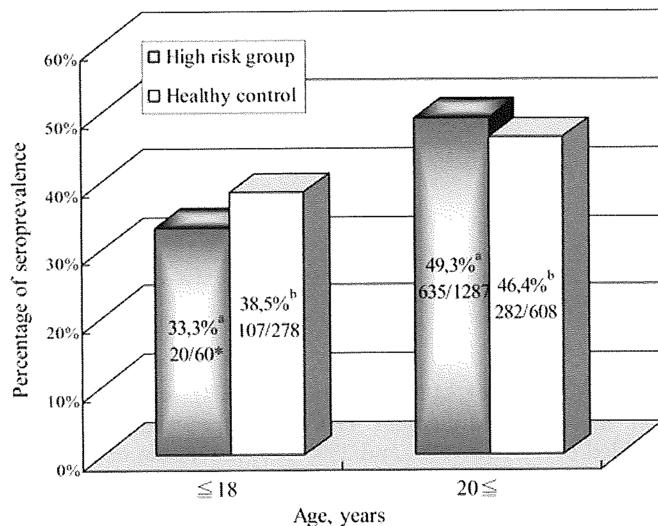

Figure 2 Age-related seroprevalence of hepatitis B surface antibody (HBs Ab). *N-positive for HBs Ab/N-tested. $^{a}P<0.05$. $^{b}P<0.05$

Ulaanbaatar in 1998. In our study conducted in 2007, this rate in Ulaanbaatar was 16.5%, suggesting that the prevalence of syphilis is increasing. It is true that these rates were anti-TP. Therefore, it did not mean active syphilis. However, these rates document that the exposure to syphilis is very high. A 100% condom programme is strongly recommended.

According to the unpublished data by NCCD, another risk factor for HIV-1 acquisition is that the predominant route of HIV-1 transmission in Mongolia is through sexual intercourse in MSM. The present study showed higher rates of anti-TP (30%) and anti-HCV (18%) in MSM than those in neighbouring countries: for example, 7% in Beijing (China) and 10% in St Petersburg (Russia) for syphilis and 0.8% or 5.2% in Beijing (China) for HCV.[14-16] These results indicate active high-risk sexual intercourse in Mongolian MSM. There is strong prejudice and discrimination against MSM in Mongolia. Hence, access to the MSM group was very difficult in this study. This barrier makes the delivery of information to MSM difficult. A quick countermeasure to MSM is crucial and a larger serological survey is necessary to grasp the actual prevalence of HIV-1 in Mongolian MSM.

Compared with other STIs, evaluation of hepatitis B was not simple because of the high-prevalence rate in the general population. A hepatitis B vaccination programme has been conducted 18 years ago. Around 35% of people below 18 years have HBs Ab. In contrast, those over 20 years had a significantly higher rate of HBs Ab in both high-risk and healthy control populations. Analysis of HBc-Ab could make it possible to discriminate between HBV-vaccinated and HBV-exposed individuals, which unfortunately we could not perform in this study. This result also suggests the frequent exposure to hepatitis B in Mongolians. Takahashi *et al.*[10] reported a comparable rate of HBs Ab prevalence, indicating a low selection bias of subjects in this study except for MSM and drug abusers.

The present study demonstrates that HIV prevalence is currently low. However, according to the high prevalence of syphilis and HCV in high-risk populations and the social stigma against MSM, the risk status for HIV-1 infection is estimated to be high. Close monitoring of the HIV epidemic is important in order to take quick measures for the high-risk populations and consequently keep the prevalence of HIV low in Mongolia.

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Favourable use of non-boosted fosamprenavir in patients treated with warfarin

Sir: Warfarin is metabolized by cytochrome P450 2C9 (CYP2C9).[1,2] Due to drug interactions, warfarin dose modification is required when it is combined with other drugs. For example, antiretroviral drugs, nevirapine and lopinavir-ritonavir, reduce the serum concentration of warfarin,[3,4] while efavirenz increases the concentration,[4] probably via CYP2C9 induction and inhibition, respectively. No clinical data are currently available for other antiretrovirals. We describe three HIV-1-infected patients in whom the use of non-boosted fosamprenavir had a favourable outcome. Case 1 was a 60-year-old Japanese man who had been treated with a stable dose of warfarin (mean daily dose, 3 mg) for chronic atrial flutter. The international normalized ratio (INR) was maintained within the optimal range (1.5-2.5). One year later, antiretroviral therapy (ART) was started with abacavir, lamivudine and non-boosted fosamprenavir (1400 mg twice daily). In this patient, dose modification of warfarin was not necessary because INR was controlled within 1.46-3.01. Case 2 was a 33-year-old Japanese man who had been treated with abacavir, lamivudine and lopinavir/ritonavir. He developed deep vein thrombosis followed by pulmonary embolism. Warfarin was given initially at 7 mg/day to maintain INR within the optimal range (2-3). After several months, the control of INR became difficult; at 9 mg of warfarin, INR was 1.38-1.75. Therefore, ART was changed to abacavir, lamivudine and fosamprenavir (1400 mg twice daily), and warfarin dose was decreased to 4.25-5.00 mg/day. The new treatment allowed maintenance of INR within the optimal range. Case 3 was a 49-year-old Japanese man who had been treated with abacavir, lamivudine and fosamprenavir (1400 mg twice daily). He underwent mitral mechanical valve replacement for mitral incompetence and heart failure. After surgery, warfarin was given initially at 2-2.5 mg/day to almost maintain INR within the optimal range (2.5-3.5); at 2.5 mg of warfarin, INR was 2.85-3.65.

Amprenavir is mainly metabolized by CYP3A4 and to a lesser extent by CYP2D6, CYP2C19 and CYP2C9.[5] Interaction between fosamprenavir and warfarin is theoretically rare. Actually in our cases, non-boosted fosamprenavir showed favourable results. Along with the long-term use of ART, cardiovascular events are increasing. When warfarin co-administration is indispensable, non-boosted fosamprenavir can be an antiretroviral agent of choice.

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Retrospective review of *Pneumocystis jirovecii* pneumonia in a French intensive care unit (1994-2000)

Sir: We read with great interest the paper by Travis and colleagues about outcomes of *Pneumocystis jirovecii* pneumonia (PJP) in HIV patients managed in the intensive care unit (ICU).[1,2] Our experience with severe PJP provides both support and nuance to their conclusions.

We also performed a retrospective study, although we included only patients with severe PJP, in 1994-2000, at our 18-bed ICU, to compare outcomes with those in the same ICU in 1989-1993.[3] We identified 76 patients with severe PJP, including 25 (33%) before and 51 during the highly active antiretroviral therapy (HAART) era; only nine (9/51; 18%) were taking HAART at the diagnosis of severe PJP. Median age was 40 years, and 59 (59/76; 78%) patients were men. Only 25 (33%) patients were on PJP prophylaxis, and 34 (45%) patients were not known to be HIV positive before the PJP episode. At ICU admission, median PaO_2 on room air was 49 mmHg (interquartile range [IQR], 43-57). CD4 was 15 cells/L (IQR, 7-51), viral load was 2.1×10^5 copies/mL (IQR, 1.0-4.2), serum lactate dehydrogenase was 1052 IU/L (IQR, 724-1392) and the Simplified Acute Physiology Score II was 32 (IQR, 27-40). Endotracheal mechanical ventilation (MV) was required in 23 (31%) patients, at admission ($n = 12$) or after failure of another method (continuous positive airway pressure, $n = 6$ among 35 who had this treatment; or non-invasive ventilation, $n = 5$ among six who had this treatment). Initial treatment was with cotrimoxazole in 63 (83%) patients, pentamidine in 12 and atovaquone in one. Steroids were used in 66 (87%) patients. Overall ICU mortality was 24% ($n = 18$); 13