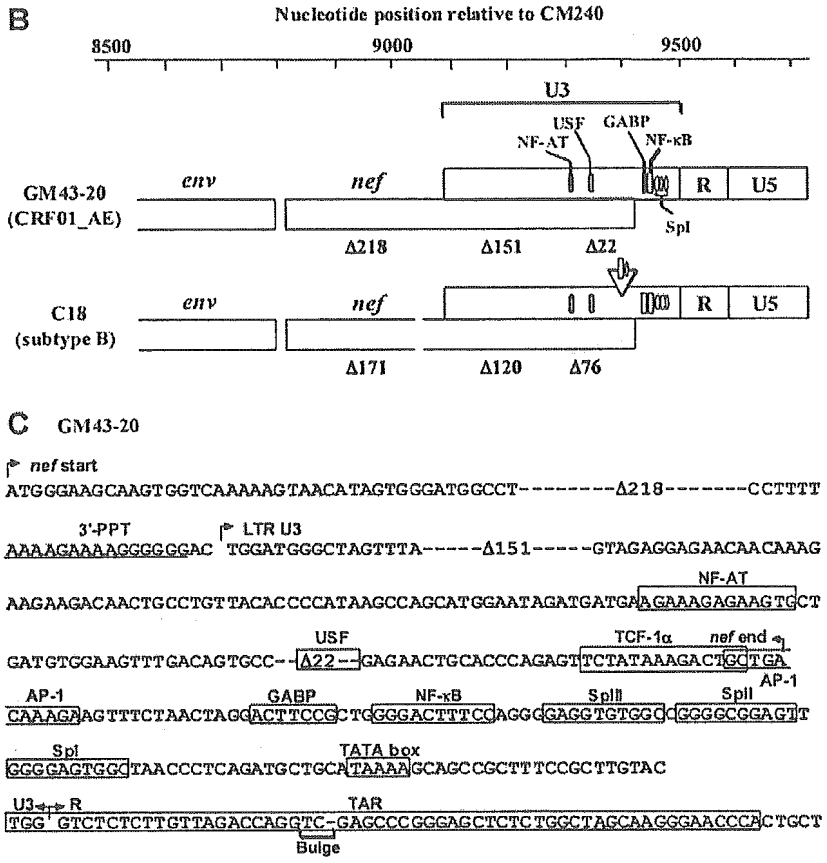

Figure 2. *(Continued.)*

detection of HIV-l RNA from both HIV-1 subtype B and non–subtype B strains, including CRF01_AE [9]. Replication-competent HIV-1 strains were isolated by cocultivation with CD8-depleted peripheral-blood mononuclear cells (PBMCs) from healthy donors. PBMCs were activated by use of anti-CD3 antibody (CLB-CD3; PeliCluster), instead of the standard activation stimuli of phytohemagglutinin and interleukin-2, to improve the efficiency of isolation [10, 11].

The *nef*/LTR regions of HIV-1 provirus genomes were amplified by nested polymerase chain reaction (PCR), with the outer primers Env43F14 (sense; 5′-GAGTTAGGCAGGGATACTCAC-3′; positions 7892–7912 of the genome of CM240, the HIV-1 CRF01_AE reference strain [12]) and 3′LTR43R16 (antisense; 5′-TAAGCACTCAAGGCAAGC-3′; positions 9202–9185 of the genome of CM240) and the inner primers Env43F15 (sense; 5′-AGCCTGTGCCTCTTCAGCTACCA-3′; positions 8052–8083 of the genome of CM240) and MSR5 (antisense; 5′-GCACTCAAGGCAAGCTTTATTGAGGCT-3′; positions 9199–9173 of the genome of CM240). The nucleotide sequences of both strands were determined by the BigDye Terminator cycle sequencing method, using a Prism 310 DNA Sequencer (Applied Biosystems). Nucleotide sequences (GenBank accession numbers AB193797–AB193800) were aligned by use of CLUSTAL W (version 1.4). Phylogenetic trees were constructed by the neighbor-joining method, based on Kimura's 2-parameter distance matrix with 100 bootstrap replicates. Analyses were implemented by use of PHYLIP (version 3.573) [13].

Results. GM43 is a 28-year-old Thai woman who has lived in Japan for the past 6 years. GM43 was infected with HIV-1 via her husband, GM46. GM46 had contracted HIV-1 via heterosexual contact before 1995, presumably in Thailand. Both GM43 and GM46 remain healthy and do not have any clinical symptoms. There is no indication of HIV-2 infection. In January 1996, serologic tests conducted for GM43 at her 18th week of pregnancy (her first visit) showed low marginal HIV-1 seropositivity, with a PA antibody titer of 1:16 (figure 1*A*). Indeterminate WB results (1+ reactivity for gp160 only) persisted for GM43 throughout an 18-month observation period, whereas GM46 was unequivocally positive by WB (figure 1*B*). Empirically, in most HIV-1–infected patients, PA antibody titers

exceed $1:10^3$ within 2 weeks of seroconversion and reach $1:10^4$ one month after seroconversion. However, in GM43, low PA antibody titers ($<1:10^3$) persisted for 1.5 years, and it took >4.5 years until her PA antibody titer reached $1:10^4$ (figure 1*A*).

In parallel with this slow process of seroconversion, GM43's plasma HIV-1 RNA load was persistently below the limit of detection by PCR (<50 copies/mL) during a 4.5-year period (figure 1*A*). HIV-1 proviral DNAs were amplified by nested PCR for the *env* (C2/V3) region only in July 1997 and for both the *gag* (p17) and *env* (C2/V3) regions in December 1998 (figure 1*A*), providing conclusive evidence that GM43 was infected with HIV-1. During the first 3 years after seroconversion, GM43's $CD4^+$ cell count gradually decreased, from 1074 to 600 cells/μL, as her plasma HIV-1 RNA load gradually increased, but her $CD4^+$ cell count remained stable thereafter, at 400–600 cells/μL (figure 1*A*).

To investigate the mechanism of this unusual clinical course, we examined the structural features of the *nef*/LTR region that are known to be associated with slow disease progression [4–6]. The *nef*/LTR regions of HIV-1 proviral genomes were amplified by nested PCR from the PBMC DNAs sampled at 5 different time points between July 1997 and February 2002 (time points I–V, as shown in figure 1). Although the expected size of amplicons of the *nef*/LTR region of the intact HIV-1 genome is 1140 bp, the resulting amplified fragments ranged in size from 500 to 1000 bp, indicating the existence of deletions of ~100–500 bp in the *nef*/LTR region.

To further characterize the genetic alterations in the *nef*/LTR region for GM43, we molecularly cloned (by the TA cloning method) the PCR products and determined the nucleotide sequences of 16–39 independent PCR clones at each time point. Nucleotide-sequence alignment revealed progressive deletions in the *nef*/LTR region over time (figure 2). Plasma HIV-1 RNA load was detectable after the increased deletions in the *nef*/LTR region (time points III–V). A replication-competent HIV-1 strain (GM43-23) was isolated for the first time at time point V. The HIV-1 quasispecies with the 391-bp *nef*/LTR deletion (Δ391) appeared to constitute a major functional (replication-competent) segment of the proviral population in GM43.

We next analyzed the structural characteristics of the HIV-1 genomes for GM46. We attempted to amplify the *nef*/LTR region by PCR at 7 time points (time points 46-1 through 46-7, as shown in figure 2*A*) between 1996 and 1997 (GM46 then dropped out of the follow-up). At all sampling points except the first (46-1), both full-sized and smaller-sized PCR products (containing deletions ranging in size from 12 to 472 bp) were amplified (figure 2*A*) in independent PCR amplification experiments. Although we were not able to establish the exact frequency of defective genomes in GM46, considerable proportions of the HIV-1 quasispecies in GM46 appeared to contain gross genetic alterations in the *nef*/LTR region, especially at later time points. In contrast, no appreciable defects in the *nef*/LTR region were detected among 73 other CRF01_AE–infected individuals (of both Japanese and Thai nationality) in Japan. Phylogenetic-tree analysis based on nucleotide sequences of *env* (C2/V3) and the *nef*/LTR region revealed that HIV-1 sequences from GM43 and GM46 formed a monophyletic cluster within CRF01_AE, with high bootstrap support (100% and 98%, respectively) (data not shown). Furthermore, this GM43/GM46 cluster was distinct from other CRF01_AE local control sequences sampled in the same geographical region. These findings strongly suggest that GM43 was indeed infected via her husband. However, none of the deletions detected in GM46 were identical to those detected in GM43 (figure 2).

The genetic organization of *nef*/LTR deletions detected in GM43 and GM46 are summarized in figure 2. Two large deletions were detected. The first large deletion was located in the amino-terminal half of the *nef* gene that does not overlap with the LTR sequences. The second large deletion was mapped to the *nef*/U3–overlapping region. One or 2 small, additional deletions followed the 2 large deletions. Most of the first large deletion removed the highly conserved acidic (EEEE) domain and $(Pxx)_4$ motif, which are essential for Nef function. However, the downstream deletions located in the *nef*/U3 region left intact the polypurine tract (3′-PPT), NF-κB, and SpI binding sites and the TATA box (figure 2), which are indispensable for HIV-1 replication, as was reported in the previous studies of defective subtype B variants in US and European populations [4–6].

The overall structural configuration of the *nef*/LTR deletions found in the CRF01_AE variant infecting GM43 was remarkably similar to that of the attenuated HIV-1 variant C18 (which belongs to HIV-1 subtype B) detected in the Sydney Blood Bank Cohort [5] (figure 2*A* and 2*B*). Of note, the sequence features unique to CRF01_AE—including the GABP motif (5′-ACTTCCG-3′), a single NF-κB [14], an unusual TATA box (5′-TAAAA-3′), and a 2-nt bulge in TAR stems (figure 2)—were detected in GM43. No appreciable direct repeats that may have caused the deletion in the *nef*/LTR region [6] were detected in GM43.

Discussion. We have identified a unique case of CRF01_AE infection, in which a patient, GM43, experienced an unusually slow increase in HIV-1 antibody titers and had an undetectable viral load over a prolonged period of time. GM43 carried attenuated viral variants with a range of *nef*/LTR deletions that were similar to those found in LTNPs infected with HIV-1 subtype B [4–6]. The present study is the first report demonstrating the association between gross *nef*/LTR deletions and slow disease progression in a patient infected with a non–subtype B strain.

As can be seen in figure 2, striking similarities in the alteration of the *nef*/LTR region between subtype B and CRF01_AE were observed. The genetic alterations observed in the *nef*/LTR region of an attenuated subtype B variant detected in the Sydney

Blood Bank Cohort (isolate C18 [5]) and the CRF01_AE variants found in GM43 in the present study removed most of the sequence elements essential for Nef functions—including the highly conserved acidic (EEEE) domain that is required for the down-regulation of class I MHC molecules and the $(Pxx)_4$ motif mediating the interaction between Nef and signaling molecules—and placed downstream sequences out of frame (figure 2). Although a number of deletions were present in the *nef* region that overlapped U3 in the LTR region, none of these alterations affected *cis*-acting elements known to be critical for viral replication, including the 3′-PPT, the U3 terminal sequences, the TATA box, and the NF-κB and SpI binding sites. This convergent manner of evolution of such genetic alterations in the *nef*/LTR sequences implies the presence of the strong selection pressures that maintain the replication capacities in defective HIV-1 genomes.

Phylogenetic-tree analysis demonstrated that GM43 acquired CRF01_AE from her husband, GM46. Interestingly, GM46 was also found to harbor unique sets of *nef*/LTR deletions, although the profiles of the deletions detected in GM46 were not identical to those detected in GM43 (figure 2). It is tempting to speculate that the defective genomes detected in GM43 may have evolved from a minor viral quasispecies carried by GM46 that was not detected in the present study or that was present only transiently at the time of transmission to GM43. If this is the case, the lack of selection for functional *nef* alleles in GM43 during transmission and/or establishment of infection from GM46 is rather surprising, because functional forms of *nef* alleles are quickly and efficiently selected for in rhesus monkeys infected experimentally with *nef*-defective simian immunodeficiency virus [1]. This suggests that, in certain patients, attenuated viral variants might have a selective advantage over HIV-1 strains with an intact *nef* allele. For instance, an efficient immune response may contribute to the selection of *nef*-defective viruses that could escape the cytotoxic T lymphocyte recognition that is critical to the effective control of viral replication [15]. In light of the identification of this unique case of CRF01_AE infection, a systematic search for the viral and host factors that influence disease progression may be warranted—especially in less-studied regions of the HIV-1 epidemic, such as in developing countries in Asia—with a slow increase in antibody titer used as a convenient marker.

Acknowledgments

We thank the staffs of the participating clinics for their help and Dr. Shingo Kato for his valuable advice on virus isolation.

References

1. Kestler HW 3rd, Ringler DJ, Mori K, et al. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell **1991**; 65:651–62.
2. Du Z, Lang SM, Sasseville VG, et al. Identification of a nef allele that causes lymphocyte activation and acute disease in macaque monkeys. Cell **1995**; 82:665–74.
3. Hanna Z, Kay DG, Rebai N, Guimond A, Jothy S, Jolicoeur P. Nef harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice. Cell **1998**; 95:163–75.
4. Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC. Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection [see comments]. N Engl J Med **1995**; 332:228–32.
5. Deacon NJ, Tsykin A, Solomon A, et al. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients [see comments]. Science **1995**; 270:988–91.
6. Salvi R, Garbuglia AR, Di Caro A, Pulciani S, Montella F, Benedetto A. Grossly defective nef gene sequences in a human immunodeficiency virus type 1-seropositive long-term nonprogressor. J Virol **1998**; 72:3646–57.
7. Casartelli N, Di Matteo G, Argentini C, et al. Structural defects and variations in the HIV-1 nef gene from rapid, slow and non-progressor children. AIDS **2003**; 17:1291–301.
8. Geyer M, Fackler OT, Peterlin BM. Structure-function relationships in HIV-1 Nef. EMBO Rep **2001**; 2:580–5.
9. Triques K, Coste J, Perret JL, et al. Efficiencies of four versions of the AMPLICOR HIV-1 MONITOR test for quantification of different subtypes of human immunodeficiency virus type 1. J Clin Microbiol **1999**; 37:110–6.
10. Spina CA, Prince HE, Richman DD. Preferential replication of HIV-1 in the CD45RO memory cell subset of primary CD4 lymphocytes in vitro. J Clin Invest **1997**; 99:1774–85.
11. Wong JK, Hezareh M, Gunthard HF, et al. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science **1997**; 278:1291–5.
12. Carr JK, Salminen MO, Koch C, et al. Full-length sequence and mosaic structure of a human immunodeficiency virus type 1 isolate from Thailand. J Virol **1996**; 70:5935–43.
13. Felsenstein J. PHYLIP (phylogeny inference package) version 3.5c. Seattle, WA: Department of Genetics, University of Washington, **1993**.
14. Verhoef K, Sanders RW, Fontaine V, Kitajima S, Berkhout B. Evolution of the human immunodeficiency virus type 1 long terminal repeat promoter by conversion of an NF-kappaB enhancer element into a GABP binding site. J Virol **1999**; 73:1331–40.
15. Mariani R, Kirchhoff F, Greenough TC, Sullivan JL, Desrosiers RC, Skowronski J. High frequency of defective nef alleles in a long-term survivor with nonprogressive human immunodeficiency virus type 1 infection. J Virol **1996**; 70:7752–64.

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 21, Number 11, 2005, pp. 977–980

Sequence Note

Molecular Epidemiology of the Heterosexual HIV-1 Transmission in Kunming, Yunnan Province of China Suggests Origin from the Local IDU Epidemic

XIAO-JIE LI,[1,2] SHIGERU KUSAGAWA,[1] XUESHAN XIA,[3] CHAOJUN YANG,[1,4] QIANQIU WANG,[1,2] YUKO YOKOTA,[1] YOSHIMI HOSHINA,[1] TOSHINARI ONOGI,[1] KYOKO NOHTOMI,[1] YUKO IMAMURA,[1] TEIICHIRO SHIINO,[1] RONGGE YANG,[1] NAOKI YAMAMOTO,[1] KUNLONG BEN,[5,6] and YUTAKA TAKEBE[1]

ABSTRACT

Molecular epidemiological investigation was conducted among injecting drug users (IDUs) (n = 11) and heterosexuals (n = 15) in Kunming, Yunnan Province of China. HIV-1 genotypes were determined based on the nucleotide sequences of 2.6-kb *gag*-RT region. The distribution of genotypes among IDUs was as follows: CRF07_BC (5/11) and CRF08_BC (5/11); subtype B′ (1/11). Similarly, a majority of Kunming heterosexuals (14/15) were infected with CRF07_BC (4/15), CRF08_BC (6 /15), or subtype B′ (4/15), known to predominate among IDUs in China. This contrasts with trends in the coastal regions of China and surrounding southeastern Asian countries, where CRF01_AE predominates among heterosexuals. The heterosexual HIV-1 epidemic in Kunming thus appears to derive from the local IDU epidemic. Of note, subtype B′ was the most prevalent strain among heterosexuals before 1997, while CRF07_BC and CRF08_BC became predominant in 2002, indicating a transition of HIV-1 genotype distribution between the early and the more recent samples from Kunming heterosexuals.

THE HIV-1 EPIDEMIC IN CHINA was first detected among injecting drug users (IDUs) in the western part of Yunnan Province in 1989. HIV prevalence among IDUs in initial epidemic sites reached 50–80% by 1993.[1] Yunnan Province accounted for more than 80% of the HIV-1 infections reported in China through 1996[1] and is thought to be an epicenter of the HIV epidemic in China. According to recent HIV-1 sentinel surveys, the HIV-1 prevalence rate among newly tested IDUs in Yunnan has been stable (19.7–24.7% in 1997–1999).[2] However, HIV-1 prevalence rates among female commercial sex workers (CSWs) and wives of heroin users have increased steadily. For example, HIV prevalence among CSWs in Yunnan increased from 1.0% in 1997 to 3.4% in 2001.[2] Figure 1 shows the study site and the geographical distribution of the numbers of HIV reported cases in China as of June 2003 (http://www.aids.net.cn).

HIV-1 strains circulating in Yunnan showed extremely high genetic diversity. Various HIV-1 strains, including subtypes B, B′[3] (Thailand variant of subtype B, also referred to as Thai-B[4]) and C,[5,6] and CRF07_BC and CRF08_BC[7]), have been detected among IDUs. Moreover, in addition to these HIV-1 strains, diverse forms of unique recombinants between subtypes B′ and

[1]Laboratory of Molecular Virology and Epidemiology, AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan.
[2]Institute of Dermatology, Chinese Academy of Medical Sciences, 12 Jiangwangmiao Road, Nanjing 210042, The People's Republic of China.
[3]Kunming University of Science and Technology, Kunming, Yunnan 650224, The People's Republic of China.
[4]Yunnan Provincial Health and Anti-epidemic Center, 158 Dongsi Road, Kunming 650022, The People's Republic of China.
[5]Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, The People's Republic of China.
[6]Kunming Chinaware Biotechnology, Yunnan 650106, The People's Republic of China.

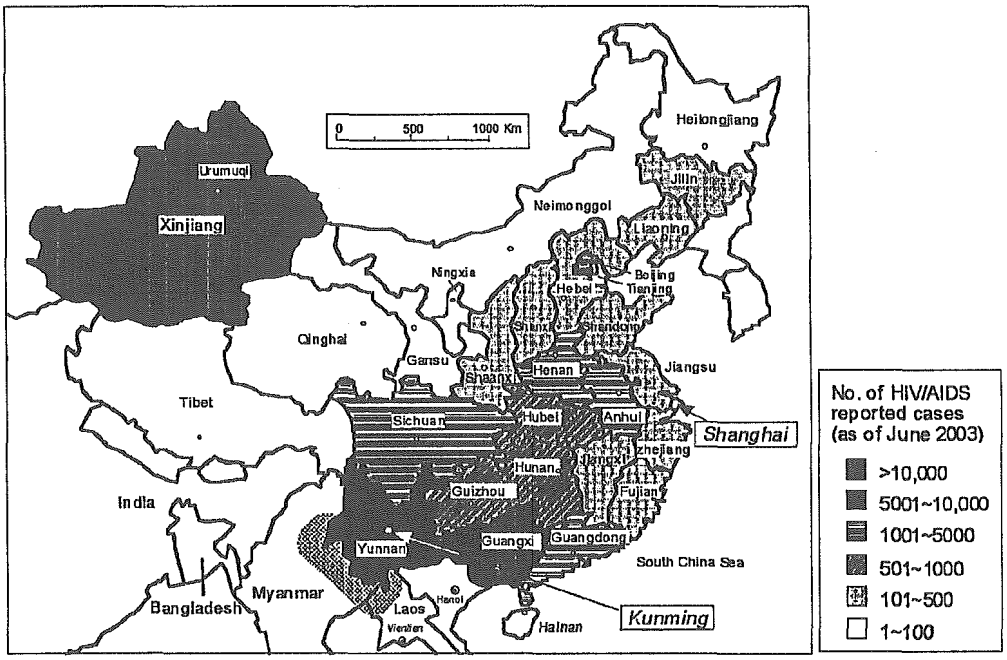

FIG. 1. Map of China. The study site (Kunming, Yunnan Province) and the geographical distribution of the numbers of HIV reported cases (as of June 2003) are shown (http://www.aids.net.cn). The so-called "Golden Triangle," a major heroin production, refining, and trading area, at the borders of Thailand, Myanmar, and Laos, near Yunnan Province, is marked.

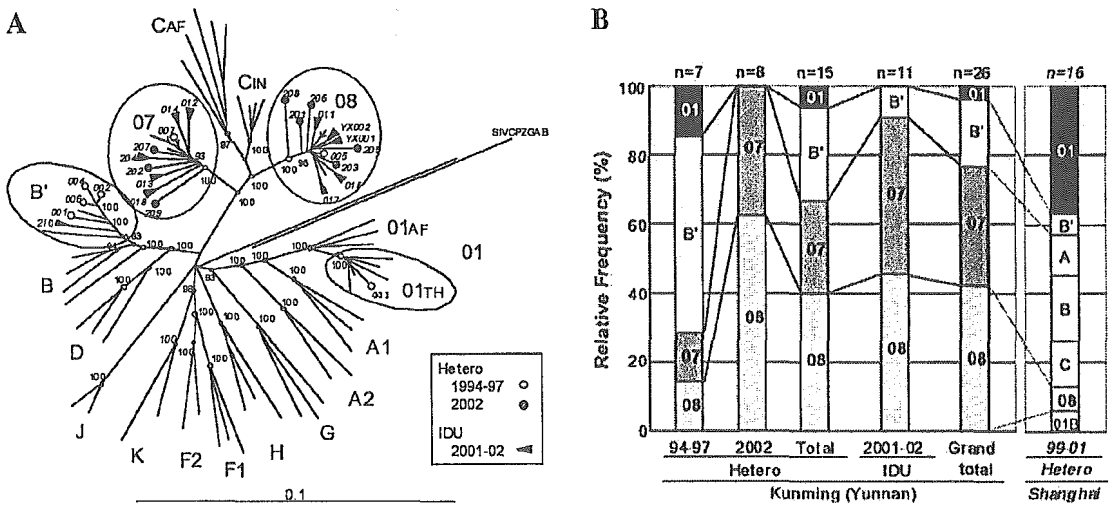

FIG. 2. Phylogenetic tree analysis and the distribution of HIV-1 strains circulating among different risk populations in Kunming, Yunnan. (**A**) Neighbor-joining tree based on the nucleotide sequences of 2.6-kb *gag*-RT regions with HIV-1 group M reference strains (http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REF/Table1.html). $SIV_{CPZ}GAB$ was used as an outgroup. Bootstrap values (>90) are shown at the corresponding nodes. Subtype and CRF designations are shown outside the tree. HIV-1 specimens from IDUs were collected in 2001–2002 (closed arrowheads); HIV-1 specimens from heterosexuals sampled in 1994–1997 (open circles) and in 2002 (striped circles). Three-digit numbers indicate the specimen codes. (**B**) Distribution of HIV-1 genotypes in different risk populations. Bars indicate the relative frequency (%) of the indicated HIV-1 genotype in the respective sample category shown at the bottom. The data on HIV-1 genotype distribution among heterosexuals in Shanghai are adopted from Zhong *et al.*[15] *n* indicates the number of the specimens analyzed in each sample category shown below. B′, HIV-1 subtype B′ (Thailand variant of subtype B); C, subtype C; 01, CRF01_AE; 07, CRF07_BC; 08, CRF08_BC; C_{AF}, African subtype C; C_{IN}, Indian subtype C; 01_{AF}, African CRF01_AE; 01_{TH}, Thailand CRF01_AE.

C,[7] and even the second-generation recombinants comprised of CRF07_BC and CRF08_BC,[8] have been reported among IDUs in Yunnan. In contrast, however, information on the HIV-1 genotypes circulating among heterosexuals in Yunnan is very limited. Although an early study detected CRF01_AE in women who had returned from commercial sex work in Thailand[9] and HIV-1 subtype B/B′, C, CRF01_AE, and CRF08_BC have recently been reported in a small number of heterosexuals in Yunnan,[2] HIV-1 genotypes circulating among persons at heterosexual risk have not been well studied. Ongoing monitoring of the HIV-1 genotype distribution in Yunnan would be important for understanding the evolution of the epidemic as well as for future vaccine strategies in China.

To track the HIV-1 genotype distribution in Yunnan, we collected a total of 26 HIV-1-positive plasma samples from persons in the capital city of Kunming and environs during 1994–2002. Fifteen specimens were from persons who acquired HIV-1 infection through heterosexual contact (7 were sampled in 1994–1997 and 8 were collected in 2002). Eleven specimens were collected from IDUs in 2001–2002. The nucleotide sequences of HIV-1 *gag*-RT regions (2.6 kb) were determined on both strands using BigDye terminator reaction kits on an ABI 373 DNA sequencer as described previously.[7] A multiple alignment with HIV-1 group M references (http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REF/Table1.html) was generated by the Se-Al program.[10] HIV-1 genotypes were screened and determined based on phylogenetic tree (Fig. 2A) and recombination breakpoint analyses of *gag*-RT regions. Phylogenetic trees were constructed by the neighbor-joining method[11] using PHYLIP package version 3.6a3[12] and the reliability of topologies of trees was tested by bootstrap analysis with 100 bootstrap replicates.[13] Bootscanning analyses were performed on neighbor-joining trees for a window of 200 bp moving along the alignment in 30-bp increments, using the Simplot program.[14]

The distribution of HIV-1 genotypes in a total of 26 samples is as follows (Fig. 2): HIV-1 subtype B′ (Thailand variant of subtype B) (5, 19%); CRF01_AE (1, 4%); CRF07_BC (9, 35%); and CRF08_BC (11, 42%). As shown in Fig. 2B, CRF07_BC (5 of 11, 45%) and CRF08_BC (5 of 11, 45%) are predominantly distributed among IDUs. In contrast, HIV-1 subtype B′ (4 of 7, 57%) was the most common strain among specimens from heterosexuals before 1997, while CRF01_AE, CRF07_BC, and CRF08_BC occurred only infrequently (1 of 7, 14% each). Interestingly, however, CRF07_BC (3 of 8, 38%) and CRF08_BC (5 of 8, 63%) were more common among specimens collected from heterosexuals in 2002, indicating a transition of HIV-1 genotype distribution between the early (before 1997) and the more recent samples (in 2002) from Kunming heterosexuals.

It is noted that the specimens, 208 (02CNKM208) and 209 (02CNKM209), are placed slightly outside the clusters of CRF08_BC and CRF07_BC, respectively (Fig. 2A). The raw direct sequencing data of theses specimens contained several ambiguous signals. The clonal sequence analysis by TA cloning revealed that they were coinfected with another lineage of the HIV-1 strain (X.-J. Li, in preparation).

The small proportion of CRF01_AE among heterosexuals in Kunming (Fig. 2) contrasts with the findings in surrounding Southeast Asian countries, where CRF01_AE shows a strong founder effect triggering the explosive epidemic among heterosexuals.[5] As shown in Fig. 2, it appears that CRF01_AE has not accounted for the majority of sexual transmission in Kunming. Although CRF01_AE was detected in the early 1990s among returnees from Thailand,[9] it has not gained the momentum of dissemination through the sexual route in Kunming, as it has in other Southeast Asian countries. In contrast, CRF01_AE constituted a significant proportion of HIV-1 strains among heterosexuals (6 of 16, 38%) in the city of Shanghai in 1999–2001[15] (Fig.2B. right). This suggests a difference in the structure and the genesis of heterosexual epidemics in Kunming and the coastal areas represented by Shanghai. Heterosexual transmission of HIV-1 in Kunming thus appears to be strongly influenced by the local IDU epidemic, while CRF01_AE shows a significant founder effect among heterosexuals in some coastal regions in China.

In conclusion, the apparent predominance of CRF07_BC and CRF08_BC among heterosexuals in Kunming suggests that a large proportion of these infections are related to IDU networks in China. These findings would contribute to our understanding of the HIV-1 epidemic in China.

ACKNOWLEDGMENTS

We would like to thank Tim Mastro for critical reading of our manuscript and Feng Gao for his advice. We also thank Xinming Li for help in the initial phase of our study and Midori Kawasaki for artwork. This study was supported by grants from the Ministry of Health, Labour and Welfare, Ministry of Education, Science and Technology, and Japanese Foundation for AIDS Prevention (JFAP). X.-J. L. is a recipient of a Research Resident Fellowship from JFAP. C.Y. is a research fellow of the Japan–China Medical Association. Q.W. is a Fellow of the Takeda Science Foundation.

The Genbank accession numbers of the nucleotide sequences reported in this article are AB213667–AB213692.

REFERENCES

1. Zheng X, Tian C, Choi KH, *et al.*: Injecting drug use and HIV infection in southwest China. AIDS 1994;8:1141–1147.
2. Yu XF, Wang X, Mao P, *et al.*: Characterization of HIV type 1 heterosexual transmission in Yunnan, China. AIDS Res Hum Retroviruses 2003;19:1051–1055.
3. Kalish ML, Baldwin A, Raktham S, *et al.*: The evolving molecular epidemiology of HIV-1 envelope subtypes in injecting drug users in Bangkok, Thailand: Implications for HIV vaccine trials. AIDS 1995;9:851–857.
4. Ou CY, Takebe Y, Weniger BG, *et al.*: Independent introduction of two major HIV-1 genotypes into distinct high-risk populations in Thailand. Lancet 1993;341:1171–1174.
5. Weniger BG, Takebe Y, Ou CY, and Yamazaki S: The molecular epidemiology of HIV in Asia. AIDS 1994;8(Suppl. 2):S13–28.
6. Luo CC, Tian C, Hu DJ, Kai M, Dondero T, and Zheng X: HIV-1 subtype C in China. Lancet 1995;345:1051–1052.
7. Yang R, Xia X, Kusagawa S, Zhang C, Ben K, and Takebe Y: Ongoing generation of multiple forms of HIV-1 intersubtype recombinants in the Yunnan Province of China. AIDS 2002;16: 1401–1407.

8. Yang R, Kusagawa S, Zhang C, Xia X, Ben K, and Takebe Y: Identification and characterization of a new class of human immunodeficiency virus type 1 recombinants comprised of two circulating recombinant forms, CRF07_BC and CRF08_BC, in China. J Virol 2003;77:685–695.
9. Cheng H, Zhang J, Capizzi J, Young NL, and Mastro TD: HIV-1 subtype E in Yunnan, China. Lancet 1994;344:953–954.
10. Rambaut A: Se-Al (Sequence Alignment Editor) version 1.0 alpha 1. Department of Zoology, University of Oxford, Oxford, 1996.
11. Saitou N and Nei M: The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4: 406–425.
12. Felsenstein J: PHYLIP (Phylogeny Inference Package) version 3.6a3. Department of Genome Sciences, University of Washington, Seattle, 2002.
13. Felsenstein J: Confidence limits on phylogenies: An approach using the bootstrap. Evolution 1985;39:783–791.
14. Ray SC: Simplot for Windows, version 3.2 (distributed by the author via http://www.sray.med.som.jhmi.edu/RaySoft/SimPlot). Johns Hopkins Medical Institutions, Baltimore, MD, 2002.
15. Zhong P, Kang L, Pan Q, *et al.*: Identification and distribution of HIV type 1 genetic diversity and protease inhibitor resistance-associated mutations in Shanghai, P. R. China. J Acquir Immune Defic Syndr 2003;34:91–101.

Address reprint requests to:
Yutaka Takebe
Laboratory of Molecular Virology and Epidemiology
AIDS Research Center
National Institute of Infectious Diseases
Toyama 1-23-1, Shinjuku-ku
Tokyo 162-8640, Japan

E-mail: takebe@nih.go.jp