Molecular Investigation of Transmission of Human Immunodeficiency Virus Type 1 in a Criminal Case

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Very few criminal cases involving human immunodeficiency virus type 1 (HIV-1) transmission have been described. We report on an HIV-1 transmission case with a child being infected by an HIV-1-positive man. The objective was to determine through molecular epidemiology and phylogenetic analyses whether HIV-1 from the HIV-1-positive man could be the source of infection in the HIV-1-positive child, as claimed by the authorities. We conducted genetic analysis of three different parts of the HIV-1 genome (gag, pol, and env) by PCR, direct-sequencing, and phylogenetic analyses. We used maximum likelihood, maximum parsimony, and neighbor-joining methods for the phylogenetic analyses to investigate whether the sequences from the man and the child were related. We found that the viral sequences from the man and the child formed separate clusters in all of the phylogenetic analyses compared to the local controls. A unique amino acid deletion was identified in the C2-V3-C3 region of the env gene in the virus from the man and the child. These results were used in the criminal court to elucidate whether the virus from the man was related to the virus from the child. In summary, the results from the phylogenetic analyses, the sequence distances between the virus from the man and the virus from the child, and the identification of the unique molecular fingerprint in the env gene together indicated that the virus from the man and the virus from the child were epidemiologically linked.

Only a small number of criminal cases involving transmission of human immunodeficiency virus (HIV) have been reported in which the use of molecular epidemiology was used to investigate the possible source of infection. Three previous cases have been described. In the “Florida dentist case,” sequence analysis of the V3-loop of the external envelope glycoprotein gp120 was used in the investigation. However, this case never made it to trial, since the case was settled out of court, but from analysis of the V3-loop, Ou et al. concluded that HIV type 1 (HIV-1) from the dentist was the source of infection in the patients (22).

In The Netherlands, a man was convicted of infecting his ex-girlfriend by injecting blood from an HIV-infected drug user (23). That study investigated the V3-loop of gp120 by nested PCR and showed that the index case and the victim clustered tightly in the phylogenetic analysis. In addition, a mutation was found in codon 215 of the RT gene associated with zidovudine resistance in HIV from the victim, reflecting that the index subject had been in zidovudine therapy and had developed resistance against zidovudine. These sequences, phylogenetic analysis, and the molecular fingerprint strongly supported the allegations concerning the HIV-1 transmission from the drug abuser’s blood to the victim.

The third transmission case used molecular epidemiology to elucidate a rape case with HIV-1 transmission to the victim (1). In this Swedish rape case, different regions of the HIV-1 genome were investigated. Analyses of sequences from the pol and gag genes demonstrated that the HIV-1 strains carried by the male and the female were genetically very closely related.

Analysis of the gag region showed that the two individuals shared an unusual out-of-frame deletion of three nucleotides. From these results, the authors of that study concluded that it was highly likely that the HIV-1 strains from the two individuals were epidemiologically closely linked.

Here we report a possible Danish transmission case, where a sexually abused 12-year-old child contracted infection with HIV-1. The case became known after 10 boys revealed that their recreation center coach (“the man”), who trained them in wrestling, had abused them sexually. The authorities were informed, and the situation was reported to the police. It was later reported that the abuse had been going on since 1994.

All boys were examined at the Institute of Forensic Medicine in Copenhagen in December 1998 and in the beginning of 1999, and one of them tested as HIV positive. It turned out that the man had had sexual intercourse with the boy in question and, moreover, had been sexually abusing him during the period from January 1995 through the summer of 1996, i.e., when the boy was only 12 to 13 years old. The other boys were all about the same age when the victimization took place; however, none of them tested positive for HIV.

The man was therefore charged with violation of the Penal Code, §222, for having performed intercourse with a child under the age of 15 and, since it was assumed that the man was aware of his own HIV-positive status, was also charged with violation of §252 of the Penal Code, for recklessly having caused danger to another person’s life or health.

The man was charged with 22 cases according to the Penal Code, §225 and §252. The man was sentenced to 6 years of imprisonment in March 2000 at the Copenhagen High Court. Genetic analysis of the HIV-1 strains carried by the man and the child was performed to determine whether viral sequences from the child were significantly more closely related to se-
quences from the man than to sequences from relevant controls.

We describe here the molecular epidemiological investigation, the genotypic results, and the phylogenetic analyses related to this case.

**MATERIALS AND METHODS**

**Patient samples.** A sample of whole blood was obtained from both the child (pt845) and the man (pt844). Each sample was prepared separately, and peripheral mononuclear cells (PBMC) and plasma were stored. Furthermore, we had access to frozen PBMC from the man obtained 21 months earlier (prot165). All sample preparations from each individual patient were done separately for DNA extractions (Roche DNA isolation kit for blood) and RNA extractions (Qiagen QiAamp Viral RNA kit). PBMC samples from 14 unrelated HIV-1-infected individuals from the same geographic area as the child and the man (Copenhagen, Denmark) were used as local controls. Because it was possible that the man had been infected during the early 1990s in Thailand with subtype B, we included two Danish samples from individuals infected with subtype B in Thailand. The man had not received antiretroviral treatment prior to the estimated time of sexual abuse leading to the HIV-1 transmission to the child 3 years earlier. Because of low viral load in the sample from the man (pt844), we were not able to perform PCR analysis on the HIV RNA from this sample. Thus, the early sample, prot165, from the man was only analyzed for proviral DNA from the PBMC. Sample pt843 from the child was analyzed for both proviral DNA and HIV RNA.

The local control sequences, which we selected, were from different patient groups with respect to transmission mode. Nine sequence samples were from patients with homosexual transmission, one sample was from an intravenous drug user, five samples were from patients with heterosexual transmission, and one sample was from a patient with hemophilic transmission.

**PCR and direct DNA sequencing.** We performed PCR and subsequent sequencing of three regions in the HIV-1 genome: the C2-V3-C3 region of the env region, the p17\(^{\text{pore}}\) region of the gag gene, and the reverse transcriptase (RT) region of the pol gene.

For amplification of the C2-V3-C3 region of the env gene, we used the primers JA167 (5'-TAT C(C/T)T TTG AGC CAA TAC C(TA) TAC A-3') and JA170 (5'-GTG AGT TAT T(A/G)C ACA AAA ATT C-3') for the first PCR and primers JA168 (5'-ACA ATG C(T/C)AC ACA TCG AAT TA(A/G) GCC A-3') and JA169 (5'-AGA AAA ATT C(T/C)T CTC C(T/C)AC AAT TAA A-3') for the nested PCR, yielding a 410-bp amplification fragment (17).

For the amplification of the p17\(^{\text{pore}}\) region, we used primers JA152 (5'-ATC TCT AGC AGT GGC GCC CGA ACA G3') and JA155 (5'-CTG AGT ATG CTG AAA ACA TGG GTA T-3') for the first PCR and primers JA153 (5'-CTC TCG AGC GAG TAC GCT TGC T-3') and JA154 (5'-CCC ATG CAT TCA AAG TTC TAG GTG A-3') for the nested PCR. A 558-bp fragment was amplified (16).

We amplified the RT region of the pol gene using a nested PCR with primers L3 (5'-GAC CAG ACA CAA CAG C-3') and L4 (5'-ATC ACT AGC CAT TGC TCT CCA-3') for the first PCR and primers A (5'-TTC CCA TTA GTC CTA TT-3') and L3 (5'-CCA GCT GTC TTT TTC TGG CAC CAC TAT-3') for the nested PCR. A 768-bp fragment was amplified (5).

All PCR products were analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining. The PCR products were purified, prior to sequencing, using the Qiagen PCR purification kit (Qiagen GmbH) according to the manufacturer’s instructions. Direct sequencing on the nested PCR products was performed on both DNA strands of all PCR fragments using the Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer) and analyzed on the ABI PRISM 377 sequencer.

All nucleotide sequences obtained were translated and aligned using the software DNASTAR (LaserGene, Madison, Wis.).

Reference sequences of all the known subtypes were obtained from the Los Alamos database. We included 101 complete envelope genes of subtype B obtained from the Los Alamos database used for the phylogenetic analysis shown in Fig. 2.

**Phylogenetic analyses.** Phylogenetic-tree analyses of nucleotide sequences were conducted with three different programs from the PHYLIP package, version 3.57c, provided by Felsenstein (6): DNAML (maximum likelihood [ML]), DNAPARS (maximum parsimony [MP]), and NEIGHBOR (neighbor joining [NJ]). The DNADIST program provided the distance matrix needed for the NJ method. For DNADIST and DNAML, the value for the transition/transversion ratio was set at 1.5 as described by Holmes et al. (13). The trees were constructed using Treeview from the PHYLIP package. Bootstrap analyses were performed on ML and NJ trees with the DNABOOT program of PHYLIP; in each case, 1,000 resamplings were performed.

**RESULTS**

**Analyses of the sequences from the man and the child.** The direct DNA sequencing procedure was performed on both DNA strands to verify all nucleotide positions. Sequences were obtained from both DNA samples (prot165 and pt844) from the man and from HIV RNA and proviral DNA from the child (pt843 and pt844).

The alignment of the sequences from the p17\(^{\text{pore}}\) gene showed that the sequences from the man’s first sample (prot165) diverged by 0.58% from the later sample of proviral DNA (pt844), while the sequences obtained from viral RNA and proviral DNA from the child (pt843) diverged by 0.29%.

The sequence distance between the samples from the man and the child diverged by 2.66 to 2.95%. Sequences obtained from the child (pt843) diverged by 3.87 to 13.00% and sequences from the first sample from the man (prot165) diverged by 3.89 to 12.90% from the local control sequences, respectively. Divergence among the controls was 4.48 to 13.06%.

Results from the nucleotide alignment of the C2-V3-C3 region of the env gene showed that the sequences from HIV RNA and proviral DNA from the pt843 sample from the child diverged by 0.34%, while the sequences from the two samples obtained within a 21-month interval from the man (prot165 and pt844) diverged by 0.69%. Upon comparing the sequences obtained from plasma viral RNA and the proviral DNA in the samples from the child (pt843) and the sample from the man closest to the estimated time of the sexual abuse (prot165), we found that they diverged by 7.18 to 8.33%. A comparison of these sequences with sequences from local controls showed a divergence between the child’s sequences and the controls of 8.71 to 16.63%. The man’s sequences diverged by 8.76 to 15.11% compared to the controls. The divergence between the controls was 7.11 to 19.58%.

When an alignment of 169 sequences, including 101 sequence envelope genes of subtype B from the Los Alamos database and 68 from our own database, was performed, a unique amino acid deletion was observed in codon 269 (strain HIV-IIIb) of the env gene (20). This molecular fingerprint was only found in HIV from the man and the child (alignment not shown). An alignment of the C2-V3-C3 region of the local controls and of the virus of the man and the child was performed and confirmed the previous result concerning the unique deletion in codon 269 (Fig. 1).

Analyses of the amino acid alignment from the pol gene (data not shown) showed that no mutations associated with antiretroviral resistance were detected in either the early (prot165) or the late (pt844) sample from the man or the child (pt843). Sequence analysis of the proviral DNA from the samples prot165 and pt843 showed that sequences of the man and the child diverged by 2.72%. In addition, the child’s sequences (pt843) diverged by 3.56 to 5.53% and the man’s sequences (prot165) diverged by 2.85 to 3.80% compared to the local controls. The divergence between the local control sequences was 3.18 to 4.58%. Furthermore, we did not find any significant amino acid pattern between the two patient samples (pt844-prot165 and pt843) in the pol gene.
Phylogenetic analyses. The genotypic relatedness between HIV-1 from the man and the child was analyzed using different phylogenetic methods, i.e., NJ, ML, and MP. Phylogenetic analyses performed with reference strains of all the known subtypes showed that the viruses from the three samples (pt843, pt844, and prot165) were of subtype B in the env, gag, and pol genes.

We tested the relationship of the local controls by performing phylogenetic analysis (NJ) on the C2-V3-C3 region of these samples, together with 101 subtype B sequences obtained from the Los Alamos database. These analyses demonstrated that the local controls were distributed throughout the phylogenetic tree (Fig. 2).

We used different parts of the HIV-1 genome: the p17gag, env, and pol genes for the analyses of the relationship between HIV-1 from the man and that from the child. The phylogenetic analysis by NJ of the p17gag gene (Fig. 3A) showed that sequences obtained from RNA and DNA from the child clustered together with 100% bootstrap values for the two individuals, and with 79% between their sequences.

For maximum resolution in the phylogenetic analysis, it has been suggested that the sequence data should be combined in different regions of the virus genome. We selected three different genes for our analysis (p17gag, env, gag, pol). By doing so we tried to bypass any randomly phylogenetic relationship in a certain gene that might have disturbed the phylogenetic data obtained by the NJ method.

Finally, we investigated the pol gene and performed phylogenetic analysis on the RT region (Fig. 3D). We selected the prot165 sample from the man and the pt843 sample from the child and analyzed these two sequences, together with eight other control sequences. These results confirmed our previous findings, and the two sequences clustered with a bootstrap value of 83%.

Results from the analyses performed by ML and MP for the three genes are not shown, but these analyses showed that the virus amplified by PCR from samples pt843 (RNA and DNA) clustered together with the samples prot165 and pt844, supporting the phylogenetic data obtained by the NJ method.

DISCUSSION

Use of molecular epidemiology for investigation of transmission cases is often complicated by the fact that the determination always is very difficult, since the molecular analysis is cult and the interpretation cult, since the molecular analysis is difficult, since the molecular analysis is difficult, since the molecular analysis is difficult. The fact that the estimated time of transmission of HIV-1 in this study was more than 4 years prior to the discovery of the HIV-1 infection of the child makes the investigation of the molecular epidemiology more difficult and the interpretation of the phylogenetic analyses harder. Because of these difficulties, we selected three different genes for our analysis (gag, pol, and env). By doing so we tried to bypass any randomly phylogenetic relationship in a certain gene that might have disturbed the investigation. In choosing this approach, establishing a genetic relation was further challenged and demanded a true genetic relationship between the man’s virus and the child’s virus.

FIG. 1. Alignment of the amino acid sequences derived from the C2-V3-C3 region. The child’s sequences correspond to the pt843t and pt843rt sequences, and the sequences from the man correspond to the pt844t and v3prot165 samples. An asterisk indicates the described deletion between the sequences from the two individuals was only 48%.

Because other research groups have reported that the C2-V3-C3 region of the env gene is suitable for analysis of transmission cases (1, 14, 18, 21, 22), we selected this region for investigation of the env gene. The phylogenetic analysis of the C2-V3-C3 region (Fig. 3B) performed by NJ analysis showed that the four sequences from the man and the child clustered together. This is in accordance with the results obtained from the p17gag gene. The bootstrap values for the two individuals’ separate sequences were 100%, while the bootstrap value between the sequences from the two individuals was only 48%.

For maximum resolution in the phylogenetic analysis, it has been suggested that the sequence data should be combined in this case, the p17gag and C2-V3-C3 regions, before performing the phylogenetic analysis (1). In performing these analyses (Fig. 3C), we found that the sequences from the two individuals clustered together with 100% bootstrap values for their individual sequences and with 79% between their sequences.

We tested the relationship of the local controls by performing phylogenetic analysis (NJ) on the C2-V3-C3 region of these samples, together with 101 subtype B sequences obtained from the Los Alamos database. These analyses demonstrated that the local controls were distributed throughout the phylogenetic tree (Fig. 2).

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Results from the analyses performed by ML and MP for the three genes are not shown, but these analyses showed that the virus amplified by PCR from samples pt843 (RNA and DNA) clustered together with the samples prot165 and pt844, supporting the phylogenetic data obtained by the NJ method.
FIG. 2. Phylogenetic tree derived from sequences from the C2-V3-C3 region. A total of 101 subtype B C2-V3-C3 sequences obtained from the Los Alamos database were included in the analyses, together with the 16 local control sequences (in boldface) and the four sequences from the man and the child (in boldface and underlined). The NJ method was used in constructing the tree. An HIV-1 subtype A sequence, U455, was used as an outgroup. The two lines (|) on the outgroup indicate that the branch has been shortened.
virus, which would be elucidated by the phylogenetic analyses. In addition, three different phylogenetic tools were used to analyze the genetic relatedness of HIV-1 from the two subjects: NJ, ML, and MP. However, it is important to note that determination of the transmission direction between the child and the man could not be resolved by these molecular investigations due to the fact that either of these individuals could have been infected by a third party. This molecular investigation should only be used in conjunction with additional evidence.

The data on sequence divergence from the described genes (gag, pol, and env) showed that the divergence differed depending on the gene in question. Other investigations on related transmission cases have shown that the divergence differs from case to case (4, 13, 18, 21). We have previously shown that the DNA sequence of the V3-loop was conserved during the first
24 weeks of infection in a donor-recipient pair (18). In a study by Bobkov et al. it was found that the interpatient variation ranged from 5.9 to 6.6% (4). Belec et al. showed in an investigation of intrafamilial transmission that the divergence in the C2-V3 region was between 1.2 and 5.0% (2).

Goujon et al. investigated a transmission case analyzing the pol gene (9). They found a very high degree of divergence depending on the sequences in question. They further showed that the divergence ranged from 11.33% within group M to 26.37% when group O was included, which shows that reference sequences and control sequences should be selected carefully. Our results for the pol gene show that related sequences can differ substantially.

A previous investigation (14) concerning a nosocomial HIV transmission shows, in comparison with the present study, that the p17^gag^ genes can differ substantially and still be phylogenetically related. Katzenstein et al. found that the p17^gag^ genes of two individuals differed by 0.9% (14), while the man and the child differed by 2.66 to 2.95%. Of note is that the two transmission cases have different time spans from infection to discovery (i.e., the point at which patient samples were obtained). This can explain the difference in divergence between the transmission cases investigated, together with the fact that virus infection evolves in a fashion specific to each infected person, depending on the immunological status of that person and the nature of the virus.

The results from the alignment of the C2-V3-C3 region showed that the divergence was higher in this gene compared
to p17^pol^ and pol. Blanchard et al. found in their investigation of a nosocomial transmission from a surgeon to a patient that the gag sequence divergence was higher compared to the env sequences (3). This was, however, caused by the fact that the individuals were infected by a recombinant HIV-1 A/F subtype.

Because the V3 region has been described as the principal neutralizing epitope, many studies have investigated the variation within the envelope C2-V3-C3 region (7, 8, 19). This has led to speculation on how suitable this region is in resolving transmission cases (11, 12). Leitner et al. investigated which regions were most accurate in the reconstruction of a true transmission case by using phylogenetic methods on sequences derived from the p17^pol^ region or the C2-V3-C3 region and sequences from the two regions in combination (15). These authors found that the most accurate phylogenetic tree compared to the true tree was constructed by using sequences from p17^pol^ and C2-V3-C3 in combination, and they also found that the analysis of the C2-V3-C3 region was more accurate than an analysis of the p17^pol^ region.

When the time spans from the estimated time of infection to the collection of blood samples in this study (21 months and 3 years respectively) were taken into account, the phylogenetic analysis using either p17^pol^ or p17^pol^ plus C2-V3-C3 showed more accurately the time-dependent relationship between the sequences compared to the analysis using the C2-V3-C3 region alone.

The phylogenetic analysis of the pol gene confirmed the relatedness of the two individuals viruses.

The one-amino-acid deletions in the C2-V3-C3 region provided a very strong epidemiological link between the two patients, and we were not able to detect this deletion in any of the sequences from the Los Alamos database that we included in this study.

In conclusion, the results from the phylogenetic analyses, the sequence distances between the virus from the man and the virus from the child, and the identification of the unique molecular fingerprint in the env gene together indicate that the virus from the man and that from the child were epidemiologically linked.

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