High-Rate of Human T Lymphotrophic Virus Type IIa Infection in HIV Type 1-Infected Intravenous Drug Abusers in Ireland

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ABSTRACT
Serological and molecular analyses of a cohort of HIV-1-infected intravenous drug abusers (IVDAs) (n = 103) in Dublin, Ireland have demonstrated that 15 of 103 (14.6%) were infected with HTLV-II, which is the highest infection rate yet recorded for any European country. Restriction fragment length polymorphism (RFLP) analysis of the env region of the provirus demonstrated that the infection involved only the HTLV-IIa subtype; the HTLV-IIb subtype was not detected. Phylogenetic analysis of the nucleotide sequences of the long terminal repeat (LTR) confirmed infection with the HTLV-IIa subtype, and demonstrated that the viruses clustered closely with HTLV-IIa isolates from North American IVDAs. Previous observations that IVDAs in southern Europe, specifically Spain and Italy, appear to be infected predominantly with the HTLV-IIb subtype, along with the present report and evidence that IVDAs in Sweden are infected with the HTLV-IIa subtype, suggest different origins of HTLV-II infection in Europe.

INTRODUCTION
Human T lymphotrophic viruses type I (HTLV-I) and type II (HTLV-II) are closely related retroviruses that have a tropism for T lymphocytes.1,2 HTLV-I infection is endemic in southwestern Japan, the Caribbean basin, and in parts of South America and Africa.3-8 In endemic areas infection has been associated with an aggressive CD4+ T cell malignancy, adult T cell leukemia (ATL)9,10; a chronic neurological disorder, HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP)11,12; and a characteristic uveitis, HTLV-I uveitis (HUV).13,14 HTLV-II infection has been shown to be endemic in a number of native American populations15-17 and high rates of infection have been documented in intravenous drug abusers (IVDAs) in North America, parts of western Europe, and in Southeast Asia.18-21 In contrast to HTLV-I, there are no definite associations between HTLV-II infection and clinical disorders; however, there is growing evidence that infection may be associated with a variety of neurological disorders, some of which closely resemble HAM/TSP.20-27

Molecular characterization of HTLV-II isolates from North America and Europe has demonstrated the existence of at least two major molecular subtypes, designated HTLV-IIa and HTLV-IIb.20 Studies of IVDAs in North America have suggested that HTLV-IIa is the predominant infection in this population.20 While studies of infection in European IVDAs have been somewhat limited, it has been suggested that in southern Europe (specifically Spain and Italy) this involves predominantly, and in many instances exclusively, the HTLV-IIb subtype.18,26-38 In contrast, a single report from Sweden has suggested that HTLV-IIa predominates in that country.39 To appreciate better the extent of HTLV-II infections within Europe, we have now investigated HTLV-II infections in a cohort of HIV-I-infected IVDAs in the Republic of Ireland. The study

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has demonstrated that this population has the highest rate of HTLV-II infection so far documented in Europe, and is equivalent to that seen in urban areas of the United States. Moreover, molecular analysis has indicated that this involves exclusively the HTLV-IIa subtype, suggesting that infections in northern and southern Europe almost certainly have different origins and have probably been introduced at different times.

**MATERIALS AND METHODS**

**Study population and serological studies**

Venous blood samples were obtained from 103 intravenous drug abusers (IVDAs) attending a sexually transmitted disease (STD) clinic and/or a drug rehabilitation unit in central Dublin. The subjects included 71 males (median age. 34.25 years) and 32 females (median age. 32.85 years). All of the subjects were of Irish descent and all were seropositive for HIV-1. Sera were assayed using a commercially available enzyme-linked immunosorbsent assay (ELISA) (HTLV I/II antibody detection assay; Murex, Kent, U.K.) and repeatedly reactive samples were confirmed by Western blot (HTLV blot 2.4; Genelabs Diagnostics, Singapore).

**Polymerase chain reaction and restriction fragment length polymorphism analysis**

DNA for polymerase chain reaction (PCR) studies was extracted from the peripheral blood mononuclear cells (PBMCs), using the Puregene DNA isolation kit (Centra Systems, Minneapolis, MN).

**pX region.** To differentiate HTLV-I and HTLV-II infections, restriction fragment length polymorphism (RFLP) analysis of an amplified product of a region of pX was performed using methods previously reported. Reactions were carried out in a Perkin-Elmer Cetus (Emeryville, CA) 9600 DNA thermal cycler and amplified products were digested with TaqI restriction endonuclease (Stratagene, La Jolla, CA) in accordance with the manufacturer guidelines, and analyzed by agarose gel electrophoresis.

**Envelope region.** To differentiate HTLV-IIa and HTLV-IIb, RFLP analysis of the env region was carried out. Specifically, nested PCR was used to amplify a region encoding the envelope gp21 transmembrane protein. Primers employed in the first round were E2 (5' CTGCAGAAGCTAGCAGTGCTCA 3') and E5 (5' AGCAGGTGTCTCGACTA 3'), corresponding to nucleotides 6661-6641 and 5603-5623 of the HTLV-II isolate Mo-T. The nested primers were E2 and El (5' CTGCAGAAGCTAGCAGTGCTCA 3') corresponding to nucleotides 6031-6051 of HTLV-II Mo-T. Amplification was performed in 100-μl reaction mixtures containing 2 μg of DNA, a 200 μM concentration of each dNTP, 40 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1 mM MgCl₂, and 2.5 U of Taq polymerase. Ten microliters of the first-round PCR product was used as a template for the nested PCR. Thermal cycling conditions used for both the first-round and nested PCRs included an initial denaturation at 94°C for 10 min, and subsequently 35 cycles at 94°C for 40 sec, 57.6°C for 30 sec, and 72°C for 40 sec, both of which were followed by a 10-min extension at 72°C. All PCR products and restriction digestes were analyzed on 2% agarose gels followed by ethidium bromide staining.

**Cloning, nucleotide sequencing, and phylogenetic analysis**

PCR products amplified from the LTR region were excised from agarose gels, purified using the Promega (Madison, WI) Wizard PCR prep system, ligated to plasmid PCR 2.1 (Original TA cloning kit; Invitrogen. San Diego, CA) and used to transform competent Escherichia coli (INVaF') under conditions recommended by the manufacturers. Purified plasmid DNAs from recombinant clones were analyzed using Taq FS dye terminator cycle sequencing on an ABI Prism 373 DNA stretch sequencer (Perkin-Elmer Cetus). Three primers — two universal, M13R (5' GGGAAAGCCCGTGGATTTGCCCCAT 3') and 5'S (5' GGGGGCTTTGGGTATTGGAGTTGGG 3'), and MSW-LTR (5' TGACGATTACCCCCCTGCCCATAAA 3'), corresponding to nucleotides 623-630 and 831-807, respectively, of HTLV-II Mo-T. Amplifications were performed in 100-μl reaction mixtures containing 2 μg of DNA, a 200 μM concentration of each dNTP, 40 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1 mM MgCl₂, and 2.5 U of Taq polymerase. Ten microliters of the first-round PCR product was used as a template for the nested PCR. The same cycling conditions were used in both the initial and nested PCRs and consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 40 sec, 53°C for 30 sec, and 72°C for 40 sec. with a final additional 10-min extension at 72°C The amplified product was then analyzed following digestion with XhoI restriction endonuclease (Stratagene) as described previously.

**Long terminal repeat region.** For nucleotide sequencing and phylogenetic studies a 623-nucleotide region of the long terminal repeat (LTR) region was amplified from five randomly chosen pX and env PCR-positive samples, using nested PCR. First-round PCR primers were P7 (5' GGGAAGCCGTGGATTTGCCCCAT 3') and P2 (5' TGACGATTACCCCCCTGCCCATAAA 3'), corresponding to nucleotides 1-20 of the HTLV-II Mo-T isolate plus a 6-base Nru restriction site at the 5' end; and Long Gag (5' GGGGGCTTTGGGTATTGGAGTTGGG 3'), corresponding to nucleotides 854-831 of HTLV-II Mo-T. Nested PCR primers were Mol6 (5' GCCCTCCAAAGCGACCGAC 3'P and MSW-Gag (5' GGGGAAGCCGTGGATTTGCCCCAT 3'), corresponding to nucleotides 16-31 and 831-807, respectively, of HTLV-II Mo-T. Amplifications were performed in 100-μl reaction mixtures containing 2 μg of DNA, a 200 μM concentration of each dNTP, 40 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1 mM MgCl₂, and 2.5 U of Taq polymerase. Ten microliters of the first-round PCR product was used as a template for the nested PCR. Thermal cycling conditions used for both the first-round and nested PCRs included an initial denaturation at 94°C for 10 min, and subsequently 35 cycles at 94°C for 40 sec, 57.6°C for 30 sec, and 72°C for 40 sec, both of which were followed by a 10-min extension at 72°C. All PCR products and restriction digestes were analyzed on 2% agarose gels followed by ethidium bromide staining.
Nucleotide sequences used in the phylogenetic analyses

Fifty-seven HTLV-IIa and HTLV-IIb LTR sequences were used in the construction of the phylogenetic trees. Isolates and corresponding GenBank accession numbers were as follows. The HTLV-IIa sequences included eight European IVDAs (ATLJ8, U10252; LAS, U10256; SFIU ao 5-5, U73010; SFIU a2 6-2, U73022), four Brazilian Indians (SPWV, Braz.A21, U10253; KAY73, L42509; KAY139, L42508), two North American Indians (PUEB.RB, U10262; NAV.DS, U10257), a Mexican prostitute (Mexy17, L42510), and the prototype HTLV-IIa isolate from the United States (Mo, M1060). HTLV-IIb sequences were from 22 European IVDAs (Gu, X89270; ITA47A, U10254; ITA50A, U10255; SPAN 129, U10256; SPAN 130, U10266; I-AM, Y09149; I-EC Y09150; I-IT, Y09151; I-EA, Y09152; I-GL, Y09153; I-OQ, Y09154; I-OY, Y09149; RC, L77241; 130, L77242; 324, L77243; RVP, L77244), 5 American IVDAs (IJG, L06857; NY185, U10259; PENN7A, U10260; SFIDU bx 4-10, U73016; SFIDU bx 6-4, U73018), 3 Colombian Indians (GI2, L1 1456: WYUI, U12792; WYU2, U12794), and a Cameroon pygmy (PYGCA1M, Z46888). The prototype HTLV-Iib sequence NRA (120734) was also included. An LTR sequence from a primate T cell lymphotropic virus (PTLV) isolated from a pygmy chimpanzee, Pan paniscus, was used as an outgroup in all three tree-construction methods (PP1664, Z46344).22 Also included was an LTR sequence from an Efe pygmy from the Democratic Republic of Congo/Zaire (Efe2, Y14365), which has been designated as a new subtype, HTLV-IId.43

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Sex</th>
<th>Age (years)</th>
<th>ELISA</th>
<th>Western blot</th>
<th>PCR/RFLP</th>
</tr>
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</table>
| DUB095         | M   | 38          | Pos   | HTLV-II      | HTLV-II
| DUB 991        | M   | 33          | Pos   | HTLV-II      | HTLV-II
| DUB408         | M   | 36          | Pos   | HTLV-II      | HTLV-II
| DUB496         | M   | 28          | Pos   | HTLV-II      | HTLV-II
| DUB500         | F   | 34          | Pos   | HTLV-II      | HTLV-II
| DUB501         | M   | 35          | Pos   | HTLV-II      | HTLV-II
| DUB611         | M   | 33          | Pos   | HTLV-U       | HTLV-II
| DUB616         | M   | 36          | Pos   | HTLV-II      | HTLV-II
| DUB717         | M   | 42          | Pos   | HTLV-II      | HTLV-II
| DUB 805        | M   | 38          | Pos   | HTLV-II      | HTLV-II
| DUB812         | F   | 31          | Pos   | HTLV-II      | HTLV-II
| DLB215         | F   | 41          | Pos   | HTLV-II      | —
| DUB376         | M   | 36          | Pos   | HTLV-II      | —
| DUB472         | M   | 42          | Pos   | HTLV-II      | —
| DUB497         | M   | 32          | Pos   | HTLV-I       | —
| DUB3173        | F   | 42          | Pos   | HTLV-II      | —

Abbreviations: Ind, Indeterminate Western blot; —, samples not analyzed.

RESULTS

Serological, PCR, and KI-’LP analysis

All 103 sera were initially analyzed using an enzyme-linked immunosorbent assay (ELISA) and repeatedly reactive samples were confirmed by Western blotting (WB). Sixteen samples (15.5% (4 female, 12 male) were reactive on ELISA. WB confirmed that 12 of these (11.7%) were seropositive with the 4 remaining ELISA-positive samples displaying indeterminate patterns (4 of 103, 3.88%) (Table 1). Eleven of the 12 confirmed samples had WB patterns consistent with HTLV-II infection (10.7%). The remaining sample had a pattern consistent with dual HTLV-I and HTLV-II infection, in that there was reactivity to both HTLV-I and -II recombinant gp46 proteins. The Western blot reactivities of me four individuals with indeterminate patterns are outlined in Table 2, where it can be seen that there was no consistent pattern of reactivity in this group.

DNA was extracted from PBMCs of seven HTLV-II-seropositive individuals and the four with indeterminate patterns, and PCR with RFLP analysis of the tax region was used to confirm and differentiate HTLV-I and HTLV-II infection.40 Amplification produced a product of the expected size (159 bp) and RFLP analysis based on TaqI restriction endonuclease digestion produced three fragments of 85, 53, and 21 nucleotides, confirming that all of the II samples including the indeterminate-nates involved HTLV-II infection.40

RFLP analysis of the envelope region encoding the trans-membrane protein gp21 was used to differentiate the HTLV-IIa and HTLV-IIb subtypes. Amplification of all 11 samples resulted in the expected 63 1-nucleotide product. XhoI digestion resulted in two DNA fragments of 178 and 453 nucleotides, indicating that all samples, also including the WB indeterminate samples, were infected with the HTLV-IIa subtype. The HTLV-Iib subtype was not detected. Unfortunately, whole blood samples could not be obtained from the individual who appeared to be seropositive for HTLV-II infection.
In the present study we have employed serological and molecular methods to characterize human T lymphotropic virus (HTLV) infections in a cohort of intravenous drug abusers (IV-DAs) in Dublin, Ireland. All individuals studied were of Irish descent and were attending a drug rehabilitation and/or a sexually transmitted disease clinic. Initial screening of 103 sera using a commercial ELISA demonstrated immunoreactivity in 16 (15.5%); Western blotting confirmed infection in 12, with the remaining 4 having indeterminate patterns. Of the former, 11 (10.7%) had patterns consistent with HTLV-II infection and I had a pattern suggestive of dual HTLV-I and HTLV-II infection. PCR with RFLP analysis of the tax region of the provirus was used to confirm infection in seven of the HTLV-II-seropositive samples and to analyze further the four WB indeterminate samples. All of the former were confirmed as having HTLV-II infection and, in addition, all of the indeterminate samples were also shown to be HTLV-II infected by this method. The latter observation supports previous reports that certain individuals with HTLV-II infection can be sero-indeterminate or even seronegative.  

**Nucleotide sequence and phylogenetic analysis**

Five samples were chosen randomly for nucleotide sequence analysis of the LTR region. An 831-nucleotide product was amplified from the LTR and sequenced, and of this, 623 nucleotides corresponding to nucleotides 86-707 of the prototype isolate HTLV-IIa Mo-T were used in the phylogenetic analysis. Absolute nucleotide sequence variation between the five Dublin samples and the HTLV-IIa Mo-T isolate ranged from 1.7 to 2.1%. The five Dublin sequences alone displayed divergence of 0.16 to 0.32%. Phylogenetic trees were constructed and evaluated by three different methods: neighbor joining (NJ), parsimony (pars), and maximum likelihood. NJ and pars trees were statistically evaluated using 1000 molecular bootstrap replicates. All of the methods resulted in trees of similar topology with well-documented separation of the HTLV-IIa and HTLV-IIIb subtypes (Fig. 1). The five Dublin samples clearly clustered in the HTLV-IIa group by all three methods and the bootstrap values for the NJ and pars trees clearly supported the inclusion of the Dublin sequences in the HTLV-IIa clade. Using the NJ method with 1000 bootstraps, the calculated value for the HTLV-IIa subtype was 1000/1000 and for the HTLV-IIIb subtype it was 600/1000. In the pars analysis the HTLV-IIa subtype also had a value of 1000/1000 and for the HTLV-IIb subtype, it was 941/1000. For the ML method the clustering of the HTLV-IIa and -IIIb subtypes displayed a p; value of <0.01.

In each of the trees the Dublin samples clustered with the other IVDA strains of the HTLV-IIa subtype, including four isolates from the United States: Los Angeles (LASA), Atlanta (ATL18), and San Francisco (SFI1DU a0 5-5, SFI1DU a2 6-2J44; three isolates from Europe, which included two from the United Kingdom (SMH1 SMH2)45 and one from Norway (NOR2N); and with a Pueblo Indian isolate (PUEB.RB) (Fig. 1). Using the NJ method the bootstrap value for this group was 816/1000, and using the pars analysis it was 549/1000. The ML method showed that this was highly significant, with a p value of <0.01. These values demonstrate that a distinct HTLV-IIa IVDA clade was well supported by all three methods.

**DISCUSSION**

In the present study we have employed serological and molecular methods to characterize human T lymphotropic virus (HTLV) infections in a cohort of intravenous drug abusers (IV-DAs) in Dublin, Ireland. All individuals studied were of Irish descent and were attending a drug rehabilitation and/or a sexually transmitted disease clinic. Initial screening of 103 sera using a commercial ELISA demonstrated immunoreactivity in 16 (15.5%); Western blotting confirmed infection in 12, with the remaining 4 having indeterminate patterns. Of the former, 11 (10.7%) had patterns consistent with HTLV-II infection and I had a pattern suggestive of dual HTLV-I and HTLV-II infection. PCR with RFLP analysis of the tax region of the provirus was used to confirm infection in seven of the HTLV-II-seropositive samples and to analyze further the four WB indeterminate samples. All of the former were confirmed as having HTLV-II infection and, in addition, all of the indeterminate samples were also shown to be HTLV-II infected by this method. The latter observation supports previous reports that certain individuals with HTLV-II infection can be sero-indeterminate or even seronegative.

As noted, one individual appeared to be coinfected with HTLV-I and HTLV-II; however, venous blood samples were not available and PCR analysis could not be used to confirm this. Of the 15 individuals with single HTLV-I infection (11 Western blot positive and 4 Western blot indeterminate), 4 were female (12.5%) and 11 were male (15.5%), and the average age was 36.1 years. Unfortunately, because of unreliable and inconsistent histories we were unable to ascertain if there was a correlation between HTLV-II infection and total years of intravenous drug abuse.

To identify the HTLV-II molecular subtypes and to characterize further the viruses, additional nucleotide sequence analyses were employed. RFLP analysis of the env gene region demonstrated that all samples were infected with the HTLV-IIa subtype. This finding contrasts with a number of studies from southern Europe and specifically in regions of Italy27-30,32 and Spain,31,32 where the HTLV-IIb subtype predominates and in some instances is the exclusive infection. A single report has suggested that, similar to Ireland, the HTLV-IIa subtype appears to predominate in an IVDA population in Stockholm, Sweden.39 In comparison with other studies, where prevalence rates have been reported to range between 0 and 6.5%, the Irish infection rate is the highest yet reported for any European country,18,20,26,39-48 and on the basis of PCR analysis this was 14.6% of the cohort studied. It should be noted that all of the Irish patients tested were coinfected with human immunodeficiency type 1 virus (HIV-1). In other studies it has also been found that the prevalence of HTLV infection, and particularly HTLV-II, is much higher in HIV-positive populations. In one Swedish study it was found that of 1158 IVDA tested for HTLV-I/II, 2.4% were seropositive, whereas if only the HIV-infected IVDA patients in this cohort were examined the prevalence rose to 11.4%.40 In a Spanish HTLV-II-positive cohort of IVDA it was found that 84% of their patients were coinfected with HIV-1.41 It is unclear if the high rate of infection observed in this Irish cohort is representative of all IVDA, and not just those who are HIV-1 infected. Unfortunately, we have up to now been unable to recruit significant numbers of non-HIV-1-infected IVDA in our studies. However, we are currently attempting to establish such a cohort to determine if the rates of infection differ between the two populations. The finding of the two subtypes of HTLV-II in geographically different parts of Europe would indicate that infection probably has been introduced to Europe at different times.

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It is unclear if the introduction of HTLV-IIa infection to Ireland may have occurred recently, as has been suggested in a Swedish study. However, screening of a limited number of archival serological samples from IVDA's in Dublin, which were obtained more than a decade ago, have suggested that few of these were infected with HTLV-II (our unpublished data, 1998) and would support the view that infection has also been recently introduced. A total of 623 nucleotides of the LTR region from five of the Dublin samples and all other available HTLV-II isolates was used to construct phylogenetic trees using neighbor joining (NJ),
Fitch and Wagner parsimony (pars), and maximum likelihood (ML) methods. The grouping of the Dublin isolates in the HTLV-IIa clade could be clearly demonstrated by each method. The bootstrap values for the clade from the NJ and pars analyses (1000/1000 for each) supported this classification, as did the probability values in the ML method (p value < 0.01). The phylogenetic analysis also demonstrated the close relationship of the HTLV-IIa in the Dublin IVDAs with that found in four IVDAs from the United States (Atlanta, Los Angeles, and San Francisco) and three from Europe (two from the United Kingdom and one from Norway). Unfortunately, nucleotide sequence data from the Swedish HTLV-II isolates are not available and the relationship of these isolates to those in Ireland cannot be determined. It has been reported that infection with the HTLV-IIa subtype is the predominant infection among IVDAs in the United States, and it could be speculated that this might be the origin of infection in Dublin. Further studies are now necessary to determine if there has been any transmission of the virus to IVDAs in other parts of Ireland and to other high-risk groups. At present the role of HTLV-II in human disease is poorly understood. Evaluation of the clinical features in our population, which has such a high rate of infection, may allow a better understanding of this and of possible clinical sequelae resulting from interactions of concomitant HIV-I and HTLV-II infections.

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REFERENCES


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