Spread of Hepatitis C Virus among European Injection Drug Users Infected with HIV: A Phylogenetic Analysis

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To describe the spread of hepatitis C virus (HCV) among HCV/human immunodeficiency virus (HIV)–coinfected injection drug users (IDUs), the molecular epidemiology of HCV was studied among 108 IDUs from 7 European countries. Phylogenetic analysis based on the NS5B region showed great sequence variation of HCV within each country and no clear phylogenetic clustering by geographic region. The most prevalent subtypes were 1a and 3a, but the percentage of genotype 4 was also relatively high, ranging from 7% in northern Europe to 24% in southern Europe. Genotype 4 consisted mainly of subtype 4d and has entered the majority of the IDU populations studied. The significantly lower evolutionary distances within subtype 4d suggest that this subtype may have entered the European IDU population relatively recently. In conclusion, HCV exchange between European IDU populations has occurred on a large scale, and, overall, country-specific clustering for HCV was less than that shown for HIV.

Because of the use of contaminated needles and injection equipment, injection drug users (IDUs) are an important risk group for acquiring hepatitis C virus (HCV) infection [1–3]. Although symptoms in the acute phase of infection are usually absent or mild, up to 85% of those infected develop chronic hepatitis C [1, 4–6], and, over several decades, serious symptoms,

such as liver cirrhosis and hepatocellular carcinoma, may develop [7, 8]. IDUs are also a well-known risk group for infection with HIV, which is also transmitted through parenteral exposure, albeit much less efficiently than is HCV [1]. Coinfections with both viruses are common in IDUs [9, 10], with HCV-related liver disease becoming more evident among coinfected individuals in the recent years, since anti-HIV treatment has led to increased life expectancy [11, 12]. As is the case for HIV, the genome of HCV is highly variable because of the lack of proofreading activity during virus replication [13]. HCV is classified into 6 different genotypes, each consisting of different subtypes. The different genotypes are known to differ in their distribution, depending both on geographic region and on mode of transmission [14]. Of the different HCV subtypes, 1a and 3a are the predominant subtypes among IDUs in Europe [15–17]. HCV is one of the few microorganisms for which genotyping, besides providing

epidemiological information, also provides information with regard to treatment [18]. In the case of infection with genotype 2 or 3, treatment is more often successful than in the case of infection with one of the other genotypes [14, 18–20].

From a public health perspective, it is important to know how both viruses spread between IDUs. Very few studies have addressed this issue on a European level. For HCV, one study determined the extent of transmission of subtypes 1a and 3a among several cities, most of which were in the United Kingdom, but the study also included Marseille, France, which is on the mainland of Europe, and Melbourne, Australia. It showed that none of these cities had a virus population that was completely isolated from that circulating in another city. Some regional differences were observed—mainly that the virus strains from London were the most phylogenetically dispersed [21]. For HIV, a study including a greater diversity of European cities showed that the genetic relatedness differs between several European countries [22]. The HIV epidemic was relatively conserved among IDUs in Edinburgh but was relatively diverse among IDUs in The Netherlands, Austria, and Switzerland, most likely due to multiple virus introductions and, perhaps, IDUs with more international contacts. Furthermore, geographical separation between HIV epidemics in northwestern Europe and those in southwestern Europe has faded over time, probably due to virus exchange between IDU populations. For HCV, the genetic relatedness has not yet been studied in such a diverse group of cities across Europe, and that HCV and HIV share the same transmission route does not necessarily mean that the pattern of spread of HCV is similar to that found for HIV.

Since very little is known about the migration of HCV among IDUs across Europe or how it compares with that of HIV, we investigated both the genotype distribution and the genetic links of the HCV strains circulating among different European IDU populations coinfected with HCV and HIV. These are the same IDU study populations for which the molecular epidemiology of HIV has been studied previously, which thereby allows a comparison of the phylogenies of both viruses [22].

SUBJECTS, MATERIALS, AND METHODS

Study subjects and samples. For this study, serum samples from 183 HIV-1–positive European IDUs were available. The samples were collected as part of the European and Italian Seroconverter Studies [22] from sites in Austria (Innsbruck, $n = 24$), Italy (Reggio Emilia and Turin, $n = 15$), The Netherlands (Amsterdam, $n = 31$), Scotland (Edinburgh and Glasgow, $n = 41$), Spain (Alicante and Castellon, $n = 30$), Switzerland (Geneva, $n = 22$), and France (Paris, Marseille, and Nice, $n = 20$). The samples were collected between 1984 and 2001, mostly within 1–3 years after HIV seroconversion (table 1). A study of the phylogeny of HIV was performed previously on these same samples [22].

Serologic testing. Samples were tested for the presence of antibodies against HCV by use of a commercial EIA system (Monolisa anti-HCV plus, version 2; BioRad). The assay was performed in accordance with the manufacturer's instructions. No confirmation testing was performed.

RNA isolation. RNA isolation was performed on all samples containing at least 100 μL of serum. To increase the yield of RNA-positive samples, we used 2 isolation methods. First, isolation was performed by use of the QIAamp viral RNA mini kits (Qiagen; Westburg). If no RNA was detected by polymerase chain reaction (PCR), isolation was performed again, by use of the TriPure method. The pellets were resuspended in a volume of 50 μL containing 10 mmol/L Tris HCl [pH 8.0] and 10 U of RNasin (Roche Diagnostics). Both the QIAamp and the TriPure isolation procedures were performed in accordance with the manufacturer's instructions. All RNA samples were stored at −80°C until amplification.

Reverse-transcriptase (RT) PCR. An RT-PCR targeting the core region of the HCV genome was performed on all the samples. This PCR was used to test for the presence of HCV RNA and to determine the HCV genotype present. On the samples that tested positive by this PCR, a second RT-PCR was performed, targeting the more-variable NS5B region, which was used for phylogenetic analysis. Amplimers were detected and analyzed on 10% acryl-amide gels (BioRad).

Core PCR. The RT-PCR targeting the core region was based on the genotyping system described by Ohno et al. [23]. This PCR was shown to detect all HCV genotypes [23]. For the first-round PCR, the AccessQuick RT-PCR system (Promega Benelux) was used, and 3 μL of sample was added to 22 μL of reaction mixture, resulting in a volume of 25 μL containing 1× avian myeloblastosis virus (AMV)/*Thermus flavus* buffer, 2 mmol/L MgSO$_4$, 200 μmol/L dNTPs, 400 pg/μL each primer, 2.5 U of AMV RT, and 2.5 U of Tfl DNA polymerase. The PCR program consisted of 45 min at 48°C and 2 min at 94°C, followed by 30 cycles for 30 s at 94°C, 30 s at 55°C, and 50 s at 68°C and a final incubation for 7 min at 68°C. For the nested PCR, 2 μL of outer PCR product was added to 23 μL of reaction mixture, resulting in a volume of 25 μL containing 1× PCR buffer, 2 mmol/L MgCl$_2$, 200 μmol/L dNTPs, 800 pg/μL each primer, and 0.5 U of *Taq* polymerase (Eurogentec). The PCR program used for the second-round PCR consisted of 5 min at 94°C, followed by 30 cycles for 30 s at 93°C, 30 s at 55°C, and 1 min at 72°C and a final incubation for 7 min at 72°C. In some samples, we found an additional band of ∼200 bp in mix 1. These bands were not considered, assuming that they were the result of aspecific cross-hybridization of the core primers, in case of a high virus load, as described elsewhere [23].

NS5B PCR. The PCR targeting the NS5B region of HCV

Table 1. Characteristics of the European cohorts of 183 injection drug users (IDUs) infected with HIV.

	The Netherlands	Spain	Austria	France	Italy	Switzerland	Scotland	Total
IDUs	31	30	24	20	15	22	41	183
Age, median, (IQR), years	34 (30–39)	31 (28–33)	28 (25–34)	25 (24–30)	26 (23–29)	29 (26–32)	27 (23–32)	29 (25–34)
Female, no. (%)	11 (35.5)	9 (30.0)	12 (50.0)	8 (40.0)	4 (26.7)	9 (40.9)	12 (29.3)	65 (35.5)
Year of starting injection drug use, median	1979	1986	1982	1986	1989[a]	1982	1981	1985
Sample years, minimum–maximum	1986–1999	1996–1998	1986–1992	1988–1992	1990–1994	1988–1997	1984–2001	1984–2001
Cities	Amsterdam	Alicante, Castellon	Innsbruck	Paris, Nice, Marseille	Reggio Emilia, Turin	Geneva	Edinburgh, Glasgow	
No. of subjects tested for HCV antibody/no. of subjects positive for HCV antibody (%)	30/30 (100)	30/30 (100)	24/22 (91.7)	20/20 (100)	14/14 (100)	21/20 (95.2)	41/34 (82.9)	180/170 (94.4)
No. of subjects tested by core PCR/no. of subjects positive by core PCR (%)	31/21 (67.7)	30/25 (83.3)	24/18 (75.0)	20/19 (95.0)	15/10 (66.7)	22/8 (36.4)	16/7 (43.8)	158/108 (68.4)
No. of subjects for whom NS5B sequences were obtained	20	24	18	17	6	6	5	96

NOTE.   HCV, hepatitis C virus; IQR, interquartile range; PCR, polymerase chain reaction.

[a] Known for only 3 IDUs.

was based on the techniques of Mellor et al. [24] and Cochrane et al. [21]. In addition, primers for genotypes 2 and 4 were selected, since the core PCR, designed to detect all genotypes, indicated the presence of these genotypes. The resulting fragments of the NS5B region consist of 465–494 nt, depending on the genotype. Table 2 shows an overview of the primers used (all primers were obtained from Life technologies). The RT-PCR targeting the NS5B region was performed under the same conditions as those described for the core PCR (see previous paragraph), with the exception that, in the first-round PCR, 1.5 mmol/L $MgSO_4$ was used, and annealing of primers took place at 45°C.

Sequencing. For the sequencing reaction, samples that had tested positive by NS5B PCR were ethanol precipitated. Both the sense and antisense strands were cycle-sequenced by use of the BigDye Terminator (BDT) system (versions 2.0 and 3.0; Perkin Elmer Benelux). For both the BDT reaction mixtures and the PCR program, the manufacturer's instructions were followed, with the adjustment of using 2 μL of reaction reagent (RR) mix combined with 2 μL of RR buffer, instead of 4 μL of RR mix. Subsequently, the products were purified by use of DyeEx spin kits (Qiagen; Westburg), in accordance with the manufacturer's instructions. Analysis of the sequencing products was performed on an Applied Biosystems 310 automated sequencer (Applied Biosystems).

Phylogenetic analysis. The obtained sequences were aligned in the BioEdit sequence alignment editor [25] either by visual inspection or using ClustalW Multiple alignment [26]. Phylogenetic trees for HCV were based on 425 nt of the NS5B region, and, for HIV, trees were based on *env* sequences published elsewhere [22]. Neighbor-joining trees and evolutionary distances were calculated with MEGA software (version 2.1) [27], by use of the Tamura-Nei substitution model with γ distribution ($\alpha = 0.40$ for HCV and $\alpha = 0.38$ for HIV [28, 29]). To analyze the stability of the tree topology, bootstrap values ($n = 1000$) were calculated. The degree to which HCV circulating in one European region (northern, central, or southern) was phylogenetically distinct from HCV circulating in another European region was measured in terms of an association index (AI), as described elsewhere [21, 30]. In brief, the degree of phylogenetic mixing of defined groups (in this case, sequences from different geographic regions) is scored in a phylogenetic tree. This value is then compared with the score from a tree with the same topology, but with the sequences randomly relocated at the tips of the tree; the ratio of the observed score to the control score produces the AI. AI values approaching zero represent almost-complete segregation of sequences, whereas values of ≥1 suggest complete phylogenetic mixing (i.e., no more segregation between the regions than would be expected by chance). Bootstrap resampling of the original sequence data is used to determine the confidence

Table 2. Primers used in polymerase chain reaction (PCR) targeting the NS5B region of hepatitis C virus.

Primer	Sequence (5'→3')	Nucleotide position[a]	Specific for genotype
A1b (O/S)	CTGACRACTAGCTGYGGTAAYAC	8113–8135	1
F1b (O/A)	CCTGGAGAGTAACTRTGGAGTG	8678–8699	1
B1b (I/S)	GCTCCRGGACTGCACSATGCTCGTG	8181–8205	1
E1b (I/A)	AATGCGCTRAGRCCATGGAGTC	8654–8675	1/4
2HCV-OS	GTGTTCACCACYAGCATGGGGA	8110–8131	2
2HCV-OA	WSAGTTCGTGGGGAGWGTATGT	8687–8708	2
2HCV-IS	TGGTRTGTGGMGACGACYTGGT	8201–8222	2
2HCV-IA	GCCCGTGTAGCCTTTCAATTAT	8644–8665	2
A3a (O/S)	ACAATCACTTGTTACATCAAGGCC	8134–8157	3a
F3a (O/A)	TCTACTGGAGAGTAACTGTGGA	8681–8703	3a
B3a (I/S)	GGAACCCGGACTTTCTTGTC	8186–8205	3a
E3a (I/A)	CCATGGAGTCTTTCAATGATTG	8642–8663	3a
4HCV-OS	ACCACCAGCTTYGGRAACAC	8116–8135	4
4HCV-OA	TTCGTGTGGAGAGTATCCRTGCA	8681–8704	4
4HCV-IS	CTGAGAGACTGCACSATGYTGGT	8182–8204	4

NOTE. All primers are expected to show some degree of cross hybridization to targets of other genotypes. A, antisense; I, inner PCR; O, outer PCR; S, sense.

[a] Numbered as in Choo et al. [31].

intervals of the association values obtained. The AI method was also used to compare the HCV and HIV phylogenies. The method can be used for the comparison of different genome regions or different organisms, since it is designed to look at the relative positions of sequences in relation to the nodes and does not consider branch lengths.

Statistical analysis. Differences between groups were tested by either the Student's t test or the χ^2 test, by use of the SPSS statistical software package (version 9.0).

Sequence accession numbers. The obtained sequences were submitted to GenBank and given the following accession numbers: AY131338–AY131435.

RESULTS

Subject characteristics. The median age of the 183 European HIV-positive IDUs was 29 years (interquartile range [IQR], 25–34 years); 65 (36%) of the IDUs were female. The drugs most commonly injected were heroin or heroin plus cocaine. Samples were collected between 1984 and 2001. The median time between the initiation of injection drug use and the date of sampling was 9 years (IQR, 5–13 years). Not all samples contained enough volume to allow both antibody and RNA testing. The presence of HCV antibodies was tested for 180 IDUs, of whom 170 (94.4%) tested positive. For the individual study sites, the percentage that tested positive for antibodies ranged from 83% to 100% (table 1).

HCV RNA prevalence and genotype distribution. Of the 170 samples positive for HCV antibody, core PCR analysis was performed on 150 samples, of which 103 (68.7%) tested positive for HCV RNA. Irrespective of antibody status, the total number of samples that tested positive by core PCR was 108, in which 114 strains were detected, including 6 samples with HCV double infections. Genotyping showed that the European HIV-positive IDUs are most commonly infected with HCV subtypes 1a and 3a and, to a lesser extent, with genotype 4 and subtype 1b. Genotypes 2, 5, and 6 were not found in our study population. The genotype distribution established by the core PCR was the following: 1a, 36.0%; 1b, 11.4%; 3a, 33.3%; 4, 14.0%; and 5.3% could not be typed. Six persons had an HCV double infection: 4 persons were infected with both type 1b and type 4, 1 person with 3a and 4, and 1 with 1b and 3a (table 3). The prevalence of the different genotypes varied by geographic region. Geographic region was categorized as northern Europe (Amsterdam, Edinburgh, and Glasgow), central Europe (Innsbruck, Paris, Geneva, Turin, and Reggio Emilia), and southern Europe (Marseille, Nice, Alicante, and Castellon). In northern and southern Europe, genotype 1a was the most prevalent genotype (44.8% and 41.5%, respectively), whereas, in central Europe, genotype 3a dominated (45.5%). From northern to central to southern Europe, the prevalence of genotype 1b increased (6.9%, 11.4%, and 14.6%, respectively), although not significantly ($P = .32$, χ^2 test). In contrast, the increasing trend from northern to central to southern Europe for genotype 4 was significant (6.9%, 9.1%, and 24.4%, respectively; $P = .03$, χ^2 test). Comparing the genotype distributions of earlier versus later samples for each geographic region separately (the cutoff for earlier vs. that for later, defined as sample dates below or above the median), showed no significant differences. However, it must be noted that, for this subanalysis, numbers were small because of the extra categorization for calendar period.

NS5B PCR. Of all samples that tested positive for HCV RNA by core PCR, 98 sequences were obtained after a second PCR, which targeted the more-variable NS5B region. Comparison of genotyping based on the core region and genotyping based on sequencing of the NS5B region showed a high level of concordance: the results of the 2 methods were in agreement for 88 comparisons (91.7%). The main difference was that only 2 HCV double infections were detected by sequencing of the NS5B region, versus 6 by core PCR. This is to be expected, since core PCR is designed to detect all the genotypes present, whereas, in the case of an HCV double infection, sequencing of the NS5B region will most likely detect the genotype that is present at the highest concentration.

NS5B phylogenetic analysis. In total, 98 sequences of the NS5B region were obtained from samples from 96 IDUs, since 2 HCV double infections were detected by sequencing of the NS5B region. The phylogenetic tree was cut into 3 parts because

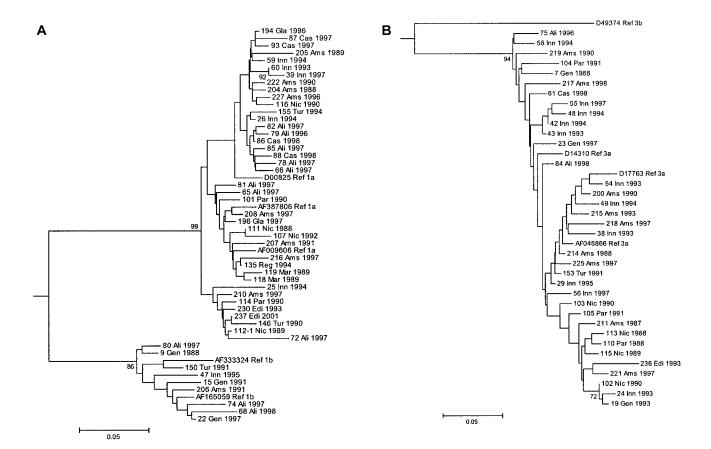**Table 3. Hepatitis C virus genotype distribution by geographic region.**

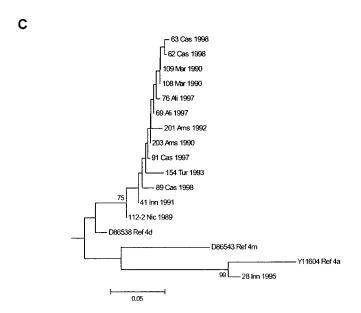| Genotype[a]	Geographic region			Overall
	Northern Europe	Central Europe	Southern Europe	
1a	13 (44.8)	11 (25.0)	17 (41.5)	41 (36.0)
1b	2 (6.9)	5 (11.4)	6 (14.6)	13 (11.4)
3a	10 (34.5)	20 (45.5)	8 (19.5)	38 (33.3)
4	2 (6.9)	4 (9.1)	10 (24.4)	16 (14)
Not typeable	2 (6.9)	4 (9.1)	0	6 (5.3)
Total	29 (100)	44 (100)	41 (100)	114 (100)

NOTE. Data are no. (%) of subjects. Northern Europe: Amsterdam, Edinburgh, and Glasgow; central Europe: Innsbruck, Paris, Geneva, Turin, and Reggio Emilia; southern Europe: Marseille, Nice, Alicante, and Castellon.

[a] The strains found in 6 HCV double infections are included separately in the genotype distribution. These included 1 injection drug user (IDU) with 1b and 4 (northern Europe), 1 IDU with 1b and 3a (central Europe), 3 IDUs with 1b and 4 and 1 IDU with 3a and 4 (southern Europe).

of its large size (figures 1*A*–1*C*). As can be seen in figure 1*A*, all study sites are represented in the tree of genotype 1. Although 2 strains or small groups of strains from the same city can sometimes be found located together in the tree, the overall picture is very mixed. Especially strains from The Netherlands and France are found throughout the tree. In the part of the tree representing subtype 3a, strains from all cities are again highly dispersed (figure 1*B*). For genotype 4, only 1 strain clustered with the 4a reference strain (figure 1*C*). The other strains clustered with the subtype 4d reference strain. Strains from most countries are seen in this part of the tree, although the strains from southern Europe dominate. Strains 108 and 109, which are derived from 2 IDUs from Marseille who were brothers, are identical and were both obtained on the same date. Possibly, these IDUs had infected each other in the period close to sample collection. The 2 subtype 4d strains from Amsterdam originate from IDUs with foreign nationalities (Irish and German). Compared with those of the other subtypes, the distances between the different strains of subtype 4d are clearly smaller. This is supported by the mean evolutionary distances (expressed as proportion of nucleotide difference), which are significantly lower for subtype 4d than for 1a, 1b, or 3a (by use of the Tamura-Nei substitution model; mean, 0.014 vs. 0.067, 0.055, and 0.047, respectively; $P < .001$, Student's t test).

Comparing HIV and HCV sequence diversity among European IDUs. HIV sequences of the V3 region were available for 145 IDUs from the same study population [22], partially overlapping the 96 IDUs for whom we obtained HCV sequences. For the group of IDUs for whom both an HCV and an HIV sequence were obtained ($n = 65$), both the HCV and the HIV phylogeny are shown (figure 2). Comparing the phylogenies of these 2 viruses with the individual level indicates that, in most cases, HCV and HIV do not seem to have been

A

- 194 Gla 1996
- 87 Cas 1997
- 93 Cas 1997
- 205 Ams 1989
- 59 Inn 1994
- 60 Inn 1993
- 39 Inn 1997
- 222 Ams 1990
- 204 Ams 1988
- 227 Ams 1996
- 116 Nic 1990
- 155 Tur 1994
- 26 Inn 1994
- 82 Ali 1997
- 79 Ali 1996
- 86 Cas 1998
- 85 Ali 1997
- 88 Cas 1998
- 78 Ali 1997
- 66 Ali 1997
- D00825 Ref 1a
- 81 Ali 1997
- 65 Ali 1997
- 101 Par 1990
- AF387806 Ref 1a
- 208 Ams 1997
- 196 Gla 1997
- 111 Nic 1988
- 107 Nic 1992
- 207 Ams 1991
- AF009606 Ref 1a
- 216 Ams 1997
- 135 Reg 1994
- 119 Mar 1989
- 118 Mar 1989
- 25 Inn 1994
- 210 Ams 1997
- 114 Par 1990
- 230 Edi 1993
- 237 Edi 2001
- 146 Tur 1990
- 112-1 Nic 1989
- 72 Ali 1997
- 80 Ali 1997
- 9 Gen 1988
- AF333324 Ref 1b
- 150 Tur 1991
- 47 Inn 1995
- 15 Gen 1991
- 206 Ams 1991
- AF165059 Ref 1b
- 74 Ali 1997
- 68 Ali 1998
- 22 Gen 1997

92
99
86

0.05

B

- D49374 Ref 3b
- 75 Ali 1996
- 58 Inn 1994
- 219 Ams 1990
- 104 Par 1991
- 7 Gen 1988
- 217 Ams 1998
- 61 Cas 1998
- 55 Inn 1997
- 48 Inn 1994
- 42 Inn 1994
- 43 Inn 1993
- 23 Gen 1997
- D14310 Ref 3a
- 84 Ali 1998
- D17763 Ref 3a
- 54 Inn 1993
- 200 Ams 1990
- 49 Inn 1994
- 215 Ams 1993
- 218 Ams 1997
- 38 Inn 1993
- AF046866 Ref 3a
- 214 Ams 1988
- 225 Ams 1997
- 153 Tur 1991
- 29 Inn 1995
- 56 Inn 1997
- 103 Nic 1990
- 105 Par 1991
- 211 Ams 1987
- 113 Nic 1988
- 110 Par 1988
- 115 Nic 1989
- 236 Edi 1993
- 221 Ams 1997
- 102 Nic 1990
- 24 Inn 1993
- 19 Gen 1993

94
72

0.05

C

- 63 Cas 1998
- 62 Cas 1998
- 109 Mar 1990
- 108 Mar 1990
- 76 Ali 1997
- 69 Ali 1997
- 201 Ams 1992
- 203 Ams 1990
- 91 Cas 1997
- 154 Tur 1993
- 89 Cas 1998
- 41 Inn 1991
- 112-2 Nic 1989
- D86538 Ref 4d
- D86543 Ref 4m
- Y11604 Ref 4a
- 28 Inn 1995

75
99

0.05

Figure 1. Neighbor-joining tree based on the Tamura-Nei substitution model with a γ distribution ($\alpha = 0.40$). Bootstrap values higher than 70 are shown ($n = 1000$). *A,* Hepatitis C virus (HCV) NS5B genotype 1. *B,* Hepatitis C virus NS5B genotype 3. *C,* Hepatitis C virus NS5B genotype 4.

Figure 2. Hepatitis C virus (HCV)/NS5B and HIV/*env* phylogeny. Neighbor-joining tree based on the Tamura-Nei substitution model with a γ distribution (α = 0.40 for HCV and α = 0.38 for HIV [28, 29]). The HCV type is indicated next to the HIV tree.

transmitted simultaneously or between the same individuals. Concerning the degree of phylogenetic mixing for both viruses, it seems that HIV sequences from the same city were more often located together than were HCV sequences from the same city, although differences between the trees are difficult to assess visually. Therefore, for both HCV and HIV, the degree of phylogenetic mixing of the sequences in the different European regions was measured by calculation of AI values (northern vs. central, northern vs. southern, and central vs. southern Europe) (table 4). This method allows the use of all available sequences for each virus (HIV, 145 IDUs; HCV, 96 IDUs) and the quantification of the degree of phylogenetic mixing. In general, the AI values were somewhat higher for HCV than for HIV (higher values reflecting a higher degree of phylogenetic mixing of virus between 2 regions; table 4). For HCV subtype 3a, there is a modest degree of sequence segregation between the sequences from northern Europe and those from central and southern Europe (AI, 0.56 and 0.60, respectively), whereas sequences from central and southern Europe had an AI of 1.05, which means that they were undifferentiated. Such a difference in the degree of phylogenetic mixing between the sequences from northern Europe and the sequences from central and southern Europe was not found for subtype 1a, which showed a modest degree of segregation of sequences from all the different European regions (AI, 0.65–0.79). HCV subtypes 1b and 4d could not be analyzed, because of the small numbers of sequences.

DISCUSSION

We have shown that transmission of HCV between IDU populations from different European cities has occurred on a very large scale, since strains from all the different European cities are highly dispersed throughout the phylogenetic tree. Also, the HCV epidemic within each European city is diverse, as multiple strains of various subtypes circulate within each country. Overall, country-specific clustering for HCV was somewhat less than that shown previously for HIV in this same study population. Contrary to that for HIV, the HCV phylogeny showed no clear division between epidemics in northwestern Europe and those in southwestern Europe. Furthermore, the HCV-infected populations in most cities were visibly phylogenetically dispersed, whereas, in a study of HIV in this same study population, this finding was shown only for Amsterdam, Innsbruck, and Geneva [22]. Our finding that, for the most part, AI values were higher for HCV than for HIV confirms a higher degree of HCV exchange in Europe. This lower degree of country- and region-specific clustering for HCV could be due to the fact that, compared with HIV, HCV entered the IDU population as soon as injection drug use became popular in the late 1960s, which is almost 2 decades before HIV entered this population [32, 33]. This, together with the fact that, during parenteral exposure,

Table 4. Association indices between different geographic regions, for hepatitis C virus (HCV) subtypes 1a and 3a and HIV.

Region	Association index[a]		
	HCV 1a	HCV 3a	HIV
Northern Europe and central Europe	0.79	0.56	0.72
Northern Europe and southern Europe	0.65	0.60	0.49
Central Europe and southern Europe	0.68	1.05	0.59

NOTE. Northern Europe: Amsterdam, Edinburgh, and Glasgow; Central Europe: Innsbruck, Paris, Geneva, Turin, and Reggio Emilia; southern Europe: Marseille, Nice, Alicante, and Castellon.

[a] Measure of segregation between 2 groups of sequences. Values approaching zero represent almost complete segregation, whereas values of $\geqslant 1$ suggest no more segregation between the regions than would be expected by chance [21, 30].

HCV is transmitted up to 10-fold more efficiently than is HIV [1], probably has allowed HCV to enter the IDU population through more introductions than HIV and to have circulated up to 20 years longer in this specific population. This may also explain why directly comparing HCV phylogenies of IDUs for whom sequences of both viruses were available with HIV phylogenies of the same IDUs does not indicate simultaneous transmission of HIV and HCV in the majority of cases. In most cases, HCV was probably already present before HIV infection was acquired. For all IDUs included in the present study, the most likely route of HIV infection reported was injection drug use. However, we cannot completely rule out the possibility that sexual transmission of HIV could play a role in the differing patterns of HIV and HCV spread, since, unlike HCV, HIV is also sexually transmissible, and the proportion at which these 2 transmission routes contribute to the transmission of HIV in the present study population is not clear [34, 35]. A final explanation could be that it has been well established that an individual can be coinfected with 2 different HCV strains [36–38] or acquire a new infection after clearing the first, whereas, for HIV, this is thought to be less likely.

It has been shown that clearance of HCV occurs in ∼20% of infected persons [39, 40]. This finding explains a major part of the discrepancy between the number of subjects positive for antibody and the number of subjects positive for RNA (68.7% of those positive for antibody) found in the present study. Different storage conditions and different cycles of freezing and thawing, for earlier research purposes, may also have influenced the sample quality, since the percentage of subjects positive for HCV RNA varied largely by site of origin (36%–95%). The overall proportion of samples positive by PCR is similar to that found in the study of HIV conducted in the same study population (71%), with the variation by site being similar as well [22]. Also, RNA-negative samples were stored a median of 3 years longer than were positive samples (sample years 1991 vs.

1994; $P = .04$, Student's t test). It is a unique situation that stored samples from so many different countries were available for the present study. Since it is to be expected that the age of the samples would have some effect on detection of RNA, a 68.7% RNA-positivity rate for the samples positive for HCV antibody can be considered to be a relatively good result.

As is the case for the general IDU population, the present study has shown that, among HIV-infected IDUs, subtypes 1a and 3a are the most prevalent subtypes [15–17]. No IDU in our study was infected with genotype 2, 5, or 6. We did, however, find a relatively high percentage of infections with genotype 4 (overall 14.0%), increasing from 6.9% in northern Europe to 24.2% in southern Europe. Genotype 4 is predominantly found in Africa, where it has been reported in countries such as Cameroon, Gabon, the Central African Republic, and Egypt, a country known for its extremely high prevalence of genotype 4 infections [14, 41]. Although not all Egyptian studies investigated subtype distributions, the majority of the type 4 infections further subtyped are subtype 4a [42], whereas, in Saudi Arabia, subtype 4d is reported to circulate [43]. Recently, genotype 4 has also been reported in low percentages in the general populations, as well as among IDUs, of several European countries [17, 37, 44, 45]. Furthermore, genotype 4 was found in 7.5% of hemodialyzed patients in a Belgian study and in 5.2% of the general population of Vienna, Austria [17, 46], but most prevalences of genotype 4 that are >5% are reported in France and more-southern European countries [47–50]. In the present study, all but 1 of the genotype 4 infections were subtype 4d. Two of the studies reporting genotype 4 in Europe also report subtype 4d [50, 51]. Our study has shown that subtype 4d has entered the majority of the European IDU populations studied. Scotland and Switzerland are the only countries in the present study in which subtype 4d was not detected, but the number of RNA-positive samples from both countries was low. Speculating on the origin of the specific subtype 4d, which is now circulating among European IDUs, is difficult with these data. We do know, however, that subtype 4d was introduced into the IDU population at least 13 years ago, because 1 subtype 4d strain was derived from an IDU in Nice as early as 1989. Also, in Marseille, Amsterdam, and Innsbruck, subtype 4d strains were found in samples obtained early (1990 and 1991). To our knowledge, no other study has reported genotype 4 in European IDUs as early as this. Perhaps, the high prevalence of genotype 4 infections in southern Europe is due to this region's proximity to Africa. However, in the present study, the samples derived from Spain were also the most recent samples obtained (1996–1998), suggesting that, perhaps, the high prevalence of genotype 4 in Spain may reflect an increasing spread of genotype 4 among IDUs in recent years. Such an effect of calendar time on the prevalence of genotype 4 would be in accordance with the findings of 2 previous studies as-sociating genotype 4 in Europe with recent transmission [17, 44]. In general, sequences tend to become more heterogeneous over time, and, therefore, a recent spread of genotype 4 is also supported by the fact that the subtype 4d strains in the present study have significantly shorter evolutionary distances than the other subtypes. Even among subtype 4d strains derived from different countries, the similarity was great. This observation also indicates that subtype 4d most likely did not enter the IDU population by many introductions, since a greater sequence variation should otherwise have been observed. Because of the extent of homogeneity of subtype 4d strains across Europe, we propose that genotype 4d was most likely introduced into the European IDU population relatively recently by only 1 or a few introductions and was thereafter transmitted between IDUs across European borders.

The distribution of HCV genotypes is of particular importance, since genotypes vary in their response to treatment, with genotypes 1 and 4 showing lower success rates than genotypes 2 and 3 [14, 18]. This is of importance for southern Europe, which is the region with the highest proportion of IDUs infected with genotype 1 or 4 in the present study (81% in southern Europe vs. 66% and 56% in northern and central Europe, respectively). Prevention of HCV among IDUs has been shown to be difficult [52, 53], as illustrated by a strong decline in the incidence of HIV infection among IDUs in recent years that has not been matched by a similar decline in the incidence of HCV infection. Prevention campaigns, harm-reduction programs, and a shift to noninjection drug use apparently have had less effect on transmission of HCV than on HIV, despite the fact that, in IDUs, both viruses share the parenteral transmission route [54–56]. Although they have not yet been investigated, differences between the 2 viruses, such as a higher background prevalence of HCV among IDUs and its higher transmission efficiency, may play a role in this phenomenon [57]. In future efforts to prevent HCV infection among IDUs, the high degree of virus exchange between IDUs across European borders is an issue to consider.

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