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ORIGINAL ARTICLE

Serological approaches to subtyping of HIV-1 in injecting drug users in Russia: evidence of subtype homogeneity at the main sites of the epidemic

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Summary: The aim of this study was to develop and evaluate a simple V3 peptide-based enzyme immunoassay (PEIA) for large-scale serotyping of HIV-1 specimens derived from injecting drug users (IDUs) in the Russian Federation. Two synthetic peptides were evaluated, named P1 (RKSIHIGPGRAFYATGD) and P2 (RTSVRIGPGQVFYKTGD), in an PEIA on 63 HIV-1 IDUs sera for which genotypes had been determined by heteroduplex mobility assay (HMA) and sequencing. The sensitivities of P1 (subtype B) and P2 (subtype A) were 87% and 75% respectively. Specificity of the assay was 100% for both peptides, with 100% predictive values of a monoreactive positive test for both peptides. Using the PEIA with peptides P1 and P2, we have serotyped 375 of 477 serum samples derived from IDUs in 4 main sites of the HIV-1 epidemic in Russia. The results demonstrated a high level of subtype homogeneity in all regions studied. In 3 of 4 territories, Tver' (n=345), and Rostov-on-Don (n=61) regions, and Krasnodar Kray (n=27), 100% of typable sera were found to belong to *env* subtype A. On the other hand, all specimens serotyped in the Kaliningrad region (n=38) belonged to *env* subtype B, and there is strong evidence that the recombinant *gag*A*env*B virus which has caused the largest outbreak of HIV-1 in Russia is located in this region. At the present time another parental strain with *gag*B*env*B genotype is of minor importance in the IDUs HIV-1 epidemic in Russia.

Keywords: HIV-1, genetic subtypes, serotyping, synthetic peptides, injecting drug users, Russia

INTRODUCTION

Since the discovery of HIV-1, considerable attention has been focused on the genetic variation of this virus, particulary with regard to the relevance of this variability to vaccine development. HIV-1 isolates from around the world have been divided into 3 groups, M, N, and O[1,2]. The vast majority of isolates fall into group M, which can further be subdivided into at least 9 genetically distinct subtypes (A–H, J)[1]. Moreover, inter-subtype recombination as an important element of the evolution of HIV-1 has been described recently[3].

As soon as HIV-1 viruses began to be subtyped genetically, several laboratory techniques were developed for this purpose, including serological and genetic screening methods. Nucleotide sequencing is the most reliable and comprehensive. However, the HMA[4] based on *env* sequence variation can be performed with less technical equipment and has demonstrated excellent concordance with DNA sequencing in the determination of HIV-1 M group subtypes worldwide[5–7]. However, the HMA requires large amounts of whole blood and such technical procedures as peripheral blood mononuclear cell (PBMC) separation, deoxyribonucleic acid (DNA) extraction, polymerase chain reaction (PCR), and gel electrophoresis for proper analysis. In this regard, for large-scale screening, serological assays seem to be more desirable since only small amounts of sera are

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required and the methods are less technically rigorous.

The great majority of serological assays used for HIV-1 subtyping are based on the detection of host antibodies that are directed against the central part of the V3 loop, an immunodominant epitope within the envelope glycoprotein. At the time the V3-loop enzyme-linked immunosorbent assay (ELISA) was first introduced, it was focused on attempts to determine HIV-1 genetic subtypes using the peptides representing the consensus amino acid sequences of different M group subtypes. It was suggested that the V3 loop could be relatively conserved within different genetic subtypes, and that sera from HIV-1 positive individuals infected with the viruses belonging to the same genetic subtype would react preferentially to V3 peptide derived from the consensus of the appropriate subtype. However, due to highly similar amino acid sequences at the antigenic sites of the V3s throughout the consensus sequences of the M group subtypes, the feasibility of V3 serotyping is limited. Many groups have shown that although the genetic and serological results of HIV-1 subtyping overlap significantly, nevertheless, V3 serotyping does not allow viral genetic subtypes to be determined properly[8–10]. It has been demonstrated that determination of HIV-1 genetic subtypes using the V3-loop ELISA is highly related to the viral diversity within the analysed population[9,10]. V3 serotyping has been successfully used to distinguish subtypes at such geographic regions or within those risk groups where not more than 2 genetic subtypes were recently introduced into a population and where the V3 consensus of the subtypes were distant[11–13]. The best results for determination of the HIV-1 genetic subtypes using the V3-loop ELISA have been reported in Thailand where there are only 2 distinctive subtypes, B and E, commonly circulating[11,12,14].

Since 1995, HIV-1 has been spreading rapidly among populations of IDUs in the former Soviet Union republics. During 1996–1997, the total number of HIV-1 infections has increased 6.5 times, and up to 1 June 1998, 8313 cases have been officially reported[15]. Moreover, in 1997, among 2728 newly registered HIV-1 cases with known risk factors, the proportion of individuals infected through the use of contaminated equipment during drug injection was as great as 90%[15]. More than 10,000 HIV-1-seropositive IDUs have been identified in the Ukraine[16]. In Belarus HIV-1 infection has been diagnosed in more than 1500 IDUs[17]. Cases of HIV-1 infection among IDUs have also been identified in Moldova and Kazakhstan[18]. We have recently demonstrated that 2 strains of HIV-1 belonging to different genetic subtypes, A and B, as well as *gag*A/*env*B recombinants between genomes of these strains, are now circulating simultaneously among IDUs in the former Soviet Union[7,19]. The sequence data show a marked intrasubtype homogeneity of HIV-1, confirming the hypothesis of a point source of virus for each subtype variant. The means of intra-subtype genetic diversity are 0.8% and 2.8% for the p17–24 *gag* and the C2–V3 *env* regions, respectively[7,19]. Moreover, 21 of 23 subjects infected with the A subtype strain had the same amino acid consensus of the V3 loop; 2 other subjects had only one substitution in this region[7]. Similar homogeneity of the V3 regions was found among subtype B and A/B recombinants[19]. A remarkable homogeneity of the nucleotide sequences derived from the IDU samples in the former Soviet Union republics has been recently pointed out by others[20–22]. Here we describe a simple V3 PEIA for large-scale subtyping of HIV-1 specimens derived from IDUs in Russia. This technique was used to determine the proportions of infections due to *env* genetic subtypes A and B or A/B recombinants at the main sites of the epidemic in Russia.

METHODS

Study population, blood collection and specimen processing

Two sets of specimens were used in this study. The first set included samples, lymphocytes and sera, derived from 63 HIV-1 infected IDUs from Russia (n=52), Belarus (n=1), Moldova (n=1) and the Ukraine (n=9). The genetic subtypes of the infecting virus of these patients were determined by HMA (n=63) and DNA sequencing of the p17–p24 *gag* and the C2–V3 *env* regions (n=18). These 63 samples were used to evaluate the sensitivity and specificity of the PEIA. The second set of specimens included sera from 477 HIV-1 infected IDUs collected at the main sites of the HIV-1 epidemic among IDUs in Russia. These samples were taken in the Tver′ region (n=345), Kaliningrad region (n=44), Rostov-on-Don region (n=61), and Krasnodar Kray (n=27). All sera were identified as HIV antibody positive using commercial ELISA tests and Western blot (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France).

Blood specimens of the first set were collected between December 1995 and December 1997. PBMC were separated within 48 h of venepuncture using Ficoll-gradient centrifugation and PBMC DNA was prepared through non-ionic detergent lysis and proteinase K digestion as described previously[6]. DNA lysates and sera were stored at -70° until PCR analysis and PEIA respectively. Serum samples of the second set were collected between July 1997 and June 1998, shipped to the Russian Federal Centre, Moscow and stored at -20°C until PEIA analysis.

Heteroduplex mobility assay and DNA sequencing

Heteroduplex mobility assay was carried out as described previously[4] using *env* gene PCR products generated with primers ED3/ED14 and ES7/ES8

for the first round and for the second round respectively. The nucleotide sequences of the p17–p24 *gag* and the C2–V3 *env* gene regions found in 18 individuals have been described previously, and have been deposited in GenBank under the following accession numbers: U93611–93682, AF051462–051467, AF051474–051507, AF051510–051524.

Serological determination of HIV-1 *env* subtypes by PEIA

Two main peptides were used, named P1 (RKSIHIGPGRAFYATGD) and P2 (RTSVRIGPGQVFYKTGD), each 17 amino acids long, from the principal neutralizing determinant of HIV-1 gp120. These 2 peptides were previously shown to distinguish between infections with HIV-1 of subtypes B (Northern American–European variants) and A (African variants)[23]. The third peptide designated PA-IDU (RTSIRIGPGQTFYATGD) was derived from the consensus sequence found for the Russian/Ukrainian IDUs infected with subtype A and also used in some experiments[7,19]. The peptides were supplied by Proteins and Peptides Research, Exeter, UK, synthesized by a solid-phase method and purified (>80%) by reverse-phase high performance liquid chromatography.

Lyophilized peptides were solubilized in 0.1 M sodium carbonate buffer (pH 9.6) to a final concentration of 2 g/ml. Flat bottomed ELISA plate wells (BIOHIT OY, Helsinki, Finland) were coated with 100 l/well of peptide solution and left for 2 nights at 4°C. The microtitre plate wells were washed 3 times with 0.01 M phosphate buffer saline (PBS, pH 7.4) containing 0.05% Tween 20 (PBS/Tween) and then blocked with blocking buffer (5% dried skimmed milk in PBS/Tween with 10% newborn calf serum) for 2 h at room temperature. Coated plates were washed 3 times with PBS/Tween. Diluted test sera (100 l of 1:100 dilution in blocking buffer) were added to the wells and incubated for 1.5 h at 37°C. Unbound antibodies were removed by 5 washes with PBS/Tween. Mouse monoclonal anti-human immunoglobulin G (IgG) peroxidase conjugate (IMTEK, Moscow, Russia, 100 l/well, 1:1000 dilution in blocking buffer) was added and incubated for one hour at 37°C. After 5 washes with PBS/Tween, antigen-antibody complexes were detected by adding 100 l/well of orthophenylenediamine dihydrochloride, OPD/H_2O_2 substrate. After standing in darkness for 10 min at room temperature, the reaction was stopped by adding 100 l/well of 1 M sulphuric acid. Absorbance was read at 492 nm, and the cut-off value was calculated as the mean of 12 negative controls plus 3 standard deviations. Dual reactions was further clarified as monoreactive if one peptide optical density (OD) was 4 times or more greater than the other peptide OD.

Table 1. Reactivities by enzyme immunoassay of peptides (PEIA) P1 and P2 with genetically confirmed sera from HIV-1 infected injecting drug users

	Subtype by *env* gene sequence *n* (%)		Subtype by HMA *n* (%)	
	A	B	A	B
Sybtype by PEIA				
A (P2)	11 (79)	0	30 (75)	0
B (P1)	0	3 (75)	0	20 (87)
Non-typable*	3 (21)	1 (25)	10 (25)	3 (13)

*Includes specimens which did not react to both P1 and P2 peptides; no dual reactivity was found. HMA=heteroduplex mobility assay

RESULTS

To estimate the sensitivity and specificity of the PEIA for subtyping of HIV-1 in IDUs from the former Soviet Union republics, sera derived from 63 HIV-1 infected individuals from Russia (*n*=52), Belarus (*n*=1), Moldova (*n*=1), and the Ukraine (*n*=9) were analysed. The genetic subtypes of the infecting virus of these patients had been determined previously by HMA (*n*=63) and DNA sequencing (*n*=18). The results of this analysis are summarized in Table 1. Peptide P2 (subtype A) detected 30 out of 40 genotypically-confirmed subtype A specimens, with sensitivity of 75%; peptide P1 (subtype B) detected 20 out of 23 genotypically-confirmed subtype B specimens, with sensitivity of 87%. Specificity of the assay was 100% for both peptides, with 100% predictive values of a monoreactive positive test for both peptides. We did not find any dual-reactive sera among the 63 specimens in the first set. These data demonstrate that the majority of specimens (50/63, 79%) derived from IDUs in the former Soviet Union republics can be correctly subtyped by PEIA with peptides P1/P2.

The lack of reactivity to both P1 and P2 peptides appeared to be not related to the genetic diversity of the V3 loop among the samples studied. All 4 *env* B subtype specimens analysed had the same amino acid consensus sequence of the V3 loop. Similarly, the V3 consensus sequences derived from 12 of 14 *env* subtype A specimens were identical, and 2 others had the only D to E substitution (Table 2). However, peptide P2 used for A subtyping differs from most of subtype A IDUs V3 sequences in 3 positions (Table 2). To determine whether the comparatively low sensitivity of the PEIA with P2 was related to these substitutions, another peptide, PA-IDU, derived from the consensus sequence found for the Russian/Ukrainian IDUs infected with subtype A was studied. We found that the sensitivity of the assay with peptides P1/PA-IDU was the same (78%). Surprisingly, the specificity of PEIA with peptides P1/PA-IDU was significantly less (90%), because 6 of 63 sera were found to be dual-reactive. Non-reactivity of sera thus may be due to weak host immune responses to the V3

*Table 2. Analysis of results for HIV-1 subtypes A and B by peptide-based enzyme immunoassay (PEIA) compared with V3-loop amino-acid sequences**

Specimen	Region	Genetic sequence	Subtype	
			gag/*env* sequence	PEIA (*env*)
P1		RKSIHIGPGRAFYATGD		
RU1226	Kaliningrad	RKGIHIGPGRAFYATGD	A/B	–
UA1216	Ukraine	RKGIHIGPGRAFYATGD	B/B	B
RU1236	Kaliningrad	RKGIHIGPGRAFYATGD	A/B	B
RU1252	Kaliningrad	RKGIHIGPGRAFYATGD	A/B	B
P2		RTSVRIGPGQVFYKTGD		
PA-IDU		RTSIRIGPGQTFYATGD		
UA1127	Ukraine	RTSIRIGPGQTFYATGD	A/A	A
UA1132	Ukraine	RTSIRIGPGQTFYATGD	A/A	–
RU1190	Saratov	RTSIRIGPGQTFYATGD	A/A	A
RU1193	Saratov	RTSIRIGPGQTFYATGD	A/A	A
RU1194	Saratov	RTSIRIGPGQTFYATGD	A/A	A
RU1195	Saratov	RTSIRIGPGQTFYATGD	A/A	A
RU1196	Saratov	RTSIRIGPGQTFYATGD	A/A	A
RU1197	Saratov	RTSIRIGPGQTFYATGD	A/A	A
RU1187	Perm′	RTSIRIGPGQTFYATGD	A/A	–
RU11259	Tver′	RTSIRIGPGQTFYATGD	A/A	A
RU11260	Tver′	RTSIRIGPGQTFYATGD	A/A	–
UA1224	Ukraine	RTSIRIGPGQTFYATGD	A/A	A
RU1176	Krasnodar	RTSIRIGPGQTFYATGE	A/A	A
RU1175	Krasnodar	RTSIRIGPGQTFYATGE	A/A	–
RU1337	Tver′	RTSIRIGPGQTFYATGD	A/A	A,B[†]
RU1340	Tver′	RTSIRIGPGQTFYATGD	A/A	A,B[†]

*The genetic subtypes of infecting virus of 18 HIV-1 infected intravenous drug users from Russia (RU) and the Ukraine (UA) were determined by heteroduplex mobility assay and DNA sequencing. The data were compared with the results of PEIA using peptides P1 (subtype B) and P2 (subtype A). The amino-acid sequences of the crown of the V3-loops derived from patients studied are presented. [†]Dually reacted sample

Table 3. Distribution of env *subtypes A and B among intravenous drug users (IDUs) in Russia by the main geographic sites of the epidemic*

Geographic region	No. of HIV-1 infections officially registered among IDUs*	No. of specimens analysed	Period of specimen collecting	Subtype A *n* (%)	Subtype B *n* (%)[†]	Non-typable *n* (%)
Kaliningrad region	1140	44	12.97–5.98	–	38 (93)	3 (7)
Krasnodar Kray	475	27	4.98–6.98	27 (100)	–	–
Tver′ region	431	345	7.97–6.98	260 (75)	–	85 (25)[§]
Rostov-on-Don region	391	61	8.97–6.98	50 (82)	–	11 (18)

*Up to 1 January 1998. [†]Viruses belonging to genetic subtype B as well as *gag*A*env*B recombinants are included. [§]Includes 2 dually reactive serum samples

epitope or/and to low quality of some sera because of the long shipping time or unsatisfactory storage conditions at some localities where the samples were taken.

Using the PEIA with peptides P1 and P2, we have analysed 477 serum samples derived from IDUs in 4 main sites of the HIV-1 epidemic in Russia (Table 3). We were able to subtype 375 (78%) sera. 100 (22%) sera were not typable because of non-reactivity to both peptides. Two serum samples, RU1337 and RU1340, were found to be dually reactive (Table 3). We have analysed these 2 dually reactive samples from the Tver′ region in detail. HMA was performed on specimens from both individuals, using DNA lysates from patient's PBMCs. HMA results demonstrated that both specimens belonged to subtype A. DNA sequencing of the V3-loop encoded region showed that both specimens contained the same translated amino acid sequences of the V3-loop (Table 2).

Moreover, these sequences were identical to the V3 amino acid sequence found in most IDUs infected with subtype A.

Determination of HIV-1 subtypes at the main sites of the epidemic among IDUs in Russia demonstrated a high level of subtype homogenity in all regions studied. However, there were some geographic differences in the distribution of HIV-1 subtypes among IDUs. In 3 of 4 territories, Tver' and Rostov-on-Don regions, and Krasnodar Kray, 100% of typable sera were found to belong to *env* subtype A. On the other hand, all specimens collected in the Kaliningrad region belonged to *env* subtype B.

DISCUSSION

Previous studies have documented that 2 strains of HIV-1 belonging to different genetic subtypes, A and B, as well as *gag*A/*env*B recombinants between genomes of these strains, are circulating now among IDUs in the former Soviet Union simultaneously[19]. We developed a peptide EIA for serological subtyping of HIV-1 infections among IDUs in Russia and the other former Soviet Union republics that is based on the V3 peptides P1 and P2. These 2 peptides have been previously described as a useful tool for distinguishing between Northern American/European subtype B sera and the African/Asian samples infected with A, C or E subtype viruses[23]. We also observed highly concordant results between serological and genotypic studies when these 2 peptides were used to distinguish between *env* subtypes A and B spreading among IDUs in Russia. In the control experiments the specificity of our assay was equal to 100%, and 2 dually-reactive sera only (0.6%) were found when 477 sera from IDUs in Russia were tested. The sensitivity of the assay was lower, and 3–25% of sera were non-typable. However, we believe that the high percentage of non-reactive sera found in most cases was not related to HIV-1 diversity in the populations studied but resulted from the low quality of some sets of specimens; all V3 loop sequences derived from 5 non-typable specimens (Table 2) were identical to the V3 sequences found in other sera which were readily typed by serology. Moreover, the sensitivity of the assay was higher when freshly collected serum samples were analysed (Table 3). It is interesting to note that both peptides used in the assay differed from the A and B V3 consensus sequences derived from IDUs in Russia, Belarus, and the Ukraine (1 and 3 substitutions in 17-mer peptide sequences respectively). Moreover, the specificity of PEIA with peptides P1 and PA-IDU derived from the IDU subtype A V3 consensus was significantly lower, because 26% (6/23) of positive sera from subtype B infected IDUs reacted with the PA-IDU peptide. It could be suggested that the reactivity of serum antibodies to peptide is most likely to be associated with the conformation of the antigenic site(s) rather than depends on the peptide primary structure alone.

Epidemiological data demonstrate that about 90% of all newly identified cases of HIV-1 infection in Russia as well as in the Ukraine and Belarus are related to the use of contaminated equipment during drug injection[15]. Through use of the PEIA assay we can genotype the absolute majority of newly identified HIV-1 cases in these countries with high specificity. However, it is important to note that the validity of this assay for HIV-1 subtyping in the IDU populations in the former Soviet Union republics is based on (1) current epidemiological situation in these countries where there are only 2 *env* genetic subtypes circulating simultaneously in the IDU population; (2) low level of heterogeneity of HIV-1 within each subtype group. The assay developed is not suitable for genotyping of the specimens derived from HIV-1 seropositive individuals belonging to other risk groups, such as patients infected heterosexually or nosocomially, because of high genomic heterogeneity and multiple genetic subtypes spreading. The introduction of additional HIV-1 subtypes into the population of IDUs in the former Soviet Union or genetic changes in the V3 of prevalent subtypes may limit the effectiveness of our current assay.

All reactive sera from the Tver' and Rostov-on-Don regions, and Krasnodar Kray belonged to *env* subtype A. Taking into account that more than 50% of HIV-1 infections among Russian IDUs have been reported from these 3 regions, we may suggest that this subtype predominates in the Russian Federation nowadays. On the other hand, all samples from the Kaliningrad region were *env* subtype B. However, nucleotide sequencing of the *gag* p17–p24 encoding region showed that all 17 independently obtained specimens had *gag*A genotype (data not shown) and thus, were recombinants. We have described such *gag*A*env*B recombinant in detail previously. The results of sequence analysis of 20 samples from Kaliningrad carried out by another group also revealed that all infecting viruses had *gag*A*env*B genotype[21]. Taken together, these data allow us to suggest that the great majority or even the whole population of HIV-1 positive IDUs in the Kaliningrad region was infected with *gag*A*env*B recombinant virus.

Two isolates with the parental *gag*B*env*B genotype were previously found among IDUs in Russia and the Ukraine[19]. Excepting the Kaliningrad region where recombinant *gag*A*env*B viruses are spreading, we did not observe HIV-1 with *env*B genotype in this study, suggesting that the prevalence of the *gag*B*env*B strain among IDUs in Russia is low, and it was not the cause of the main outbreaks of HIV-1 infection among IDUs.

In summary, this study showed that most of the main local HIV-1 outbreaks among IDUs in the Russian Fderation were caused by *gag*/*env* subtype A virus. Based on these data and considering our previous observations[7,19], the proportion of this

strain in the epidemic among IDUs in Russia may be estimated as 60–70%. The same virus was found to predominate among IDUs in the Ukraine, Belarus, and Moldova[7,19–22]. On the other hand, there is strong evidence that the recombinant *gag*A*env*B virus caused the largest outbreak of HIV-1 in Russia located in the Kaliningrad region[19,21]. Up to 30% of the total number of HIV-1 cases among IDUs have been registered in this region[15]. At the present time another parental strain with *gag*B*env*B genotype is of minor importance in the IDU epidemic in Russia. Future studies will show how the ratio between the recombinant and both parental HIV-1 strains changes in the course of the epidemic.

A simple V3 peptide-based enzyme immunoassay could be useful for tracing the spread of the *env* genetic subtype A and B among IDUs in the former Soviet Union republics and estimating the current proportions between these 2 genetic subtypes in IDU populations. Such monitoring is important both for better understanding of HIV-1 evolution and for future vaccine development.

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