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V3-SEROTYPING PROGRAMME EVALUATED FOR HIV-1 VARIATION IN THE NETHERLANDS AND CURACAO

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INHOUDSOPGAVE

ABSTRACT

To obtain insight into the variation of the HIV-1 V3 neutralization domain of variants circulating in The Netherlands, 126 Dutch, 70 Curacao and 45 African serum samples from HIV-1 infected individuals were screened for antibody reactivity to a set of 16 to 17 mer synthetic peptides, representing the central part of the V3-loop of gp120 of HIV-1 variants circulating in the US, Europe and Africa. These peptides were used in an ELISA and antibody reactivity to the peptides was compared to the actual amino acid sequence of viral RNA circulating in a subset of the same serum samples. For sequencing, RNA was isolated from serum, converted to cDNA and directly sequenced.

84.1% of the Dutch, 80.0% of the Curacao and 73.4% of the Tanzanian sera showed antibodies reactive to one or maximally three of the peptides used. Reactivity to peptides 108, 109 and 110 which differ only one amino acid at position 13 within the V3-loop (H_{108}, N_{109}, P_{110}, respectively), were most frequently found among Dutch and Curacaon samples, whereas reactivity to peptides 169 and 170, representing African V3-loops with a GPGQ sequence at the tip of the loop, was most prevalent among Tanzanian samples. In total 16.6% of the samples were non-reactive and 2.5% showed an non-specific reaction pattern.

Within the Dutch sample set, collected between 1988 and 1990, a division was made according to the provinces. No samples were available from the three northern provinces (Friesland, Groningen and Drenthe). From Limburg and Zeeland only one sample was available in this sample set. Thus, results from the present study are limited to the central part of The Netherlands. Furthermore, Amsterdam was excluded from the present study, as HIV-1 variability among specific Amsterdam cohorts has already been described extensively. Reactivity to peptide 110 appeared to be most prevalent among the samples from Overijssel, whereas reactivity to peptide 108 was most frequently found among the Zuid-Holland samples. Reactivity to peptide 168, representing an African V3 loop was relatively frequent among the samples from Brabant. Reactivity to peptide 110 appeared to be most prevalent among the Curacaon samples and only two samples from Curacao were peak reactive to African peptides.

From 11 Dutch and 7 Curacaon samples HIV-1 V3 RNA was directly sequenced to confirm reactivity patterns found in the synthetic peptide ELISA. In 9 cases the V3-loop

sequence was in accordance to the sequence of the peptide to which reactivity was measured, however broadening of reactivity was frequently seen. All but one of the sequences clustered with the consensus C2-V3 sequence of the genomic subtype B, the remainder appeared to be of subtype A. Analysis of the sequences based on the charge of amino acid substitutions at positions 11 and/or 25 of the V3 loop revealed one syncytium inducing (SI) and 18 non-syncytium inducing (NSI) variants.

In conclusion, we found a relatively high genetic and antigenic homogeneity of the V3 gene of HIV infections in The Netherlands and Curacao during the years 1988-1990. Antibody reactivity to synthetic V3 peptides, as well as sequence analysis confirmed the prevalence of B subtype HIV-1 among the Dutch and Curacaon samples and the prevalence of A/D subtypes among the Tanzanian samples. Screening of HIV-1 positive serum samples for genetic typing by using a set of well defined synthetic V3 peptides appeared to be feasible. In combination with molecular analysis (V3 sequencing and/or hetroduplex mobility assay) of this method can be applied to obtain insight in changes in genetic and antigenic variation of HIV-1 in a population: changes within subtype B HIV-1 variants, as well as introduction of other (new) HIV-1 variants can this be surveyed. This is of importance to obtain insight in the (molecular) epidemiology of HIV-1 as well as with respect to the development and the eventual use of an HIV-1 vaccine.

SAMENVATTING

Doel

Doel van het onderzoek was om inzicht te verkrijgen in de antigene en genetische variatie van het in Nederland en Curacao circulerende humane immunodeficientie virus type 1 (HIV-1).

Achtergrond

De genetische variatie tussen HIV-1 isolaten is aanzienlijk. Dit is niet alleen het geval met HIV-stammen geïsoleerd bij verschillende personen, maar ook bij stammen die geïsoleerd zijn uit één patiënt. De virus populatie is relatief homogeen vlak na besmetting van een individu. Daarna neemt de variatie toe tot aan het einde van de symptoomvrije periode van de infectie, waarna de variatie van HIV-1 weer afneemt, zodat ten tijde van de diagnose AIDS er opnieuw sprake is van een relatief homogene virus populatie. Deze wijkt overigens af van de populatie die wordt gevonden vlak na besmetting. De genetische variatie doet zich vooral voor op een vijftal gebieden van het deel van het virale genoom, dat codeert voor het externe envelop eiwit gp120 van HIV-1.

Van deze vijf gebieden is het derde variabele domein (V3) gelegen tussen aminozuur-positities 269 en 331 van gp120 het meest uitvoerig bestudeerd. Analyse van het V3-gebied en van de afweerreactie tegen epitopen in dit gebied biedt inzicht in het voorkomen van verschillende varianten van HIV-1, hun herkomst en in mogelijke wijzen van verspreiding. Belangrijk is ook, dat het V3 gebied doelwit is van humane antistoffen, die in staat zijn opname van het virus in de cel te blokkeren, anders gezegd: het virus te neutralizeren. Deze neutralizerende eigenschap is echter beperkt en wordt beinvloed door de aminozuur veranderingen, die in datzelfde V3 gebied in de loop van de infectie ontstaan. Het opwekken van neutralizerende antistoffen bij de mens is één van de effecten die met vaccinatie wordt nagestreefd. Bestudering van de variatie in V3 op individueel- en populatieniveau is daarom ook van belang voor enerzijds de ontwikkeling van een anti-HIV/AIDS vaccin en anderzijds de eventuele toepassing ervan.

Materiaal en Methoden

In essentie kan de variatie van HIV-1 op twee manieren worden bestudeerd: een benadering vanuit het virus en een benadering vanuit de antistof-reactie van een geinfecteerd individu op het virus. In deze studie is gekozen voor een studie van de

antistof-reactie op het V3 gebied van het virus. Deze keuze werd vooral ingegeven door het feit dat op populatieniveau naar variatie werd gekeken: serummonsters van een groot aantal individuen werden in de studie betrokken. De laboratoriumtechniek voor het meten van de specifieke antistof respons tegen de V3-loop van het HIV-1 is voor het doen van populatiestudies vooralsnog het meest efficient. Ter bevestiging van verkregen resultaten is in beperkte mate ook gekeken naar de aminozuur-variatie van V3 van het virus zelf.

Onderzoekspopulatie Serummonsters van 126 HIV-1 geinfecteerde personen uit Nederland en 69 uit Curacao werden gebruikt. Deze serummonsters waren ter confirmatie van HIV-1 seropositiviteit toegezonden aan het Virologisch Laboratorium van het RIVM tussen 1988 en 1990. Amsterdam werd van de studie uitgesloten, aangezien variatie in de cohortstudies onder homoseksuele mannen en druggebruikers al uitvoerig bestudeerd was. Ter vergelijking werden serummonsters in de studie betrokken die afgenomen waren in de periode 1988-1991 van 45 HIV-geinfecteerde personen uit Tanzania. Van geen van de serummonsters was bekend op welk moment na besmetting het monster was afgenomen en in welk klinisch stadium van de infectie de persoon waarvan het monster was afgenomen verkeerde.

Synthetische V3 peptiden en ELISA. Er werd gebruik gemaakt van een set van in totaal 15 V3 synthetische peptiden, alle 16 aminozuren lang. De aminozuurvolgorde was afgeleid van sequenties van de V3 loop van verschillende HIV-1 varianten. Antistoffen in serum tegen elk van deze V3 peptiden werden bepaald met behulp van de ELISA techniek.

V3 sequentie onderzoek Uit een aantal serummonsters werd viraal RNA geisoleerd, omgezet in copie DNA en vervolgens geamplificeerd met behulp van de polymerase ketting reactie (PCR). Het PCR product werd gebruikt voor sequentie-onderzoek. Nucleotide sequenties werden na "alignment" vertaald naar aminozuur sequenties.

Resultaten

Van de Nederlandse serummonsters reageerde 54.8% specifiek tegen een van de peptiden p108, p109 of p110, welke representatief zijn voor het genotype B. Dit type wordt het meest frequent gevonden in Europa en de USA. Kruisreactiviteit tussen deze drie peptiden werd frequent gezien. In 5 (4%) Nederlandse monsters werd specifieke reactiviteit gevonden tegen p168, een peptide representatief voor Afrikaanse HIV-1 stammen. Reactiviteit tegen p168 werd met name gevonden in sera afkomstig van HIV-1 geinfecteerde personen uit Brabant en Overijssel. In totaal 17 (13.5%) van de Nederlandse

monsters vertoonden geen specifieke reactie tegen de peptides die in deze studie werden gebruikt.

Voor wat betreft de monsters afkomstig uit Curacao werd een vergelijkbaar resultaat gevonden, met dit verschil dat ten opzichte van de Nederlandse monsters een relatief hoge frequentie van serum reactiviteit tegen p110 werd gevonden. De Tanzaniaanse serummonsters bleken in meerderheid specifieke reactiviteit te vertonen tegen een of twee "Afrikaanse" peptiden. Uit 11 Nederlandse monsters en 7 van Curacao werd viraal RNA geisoleerd voor sequentie-onderzoek. Tien van de 11 Nederlandse en alle Curacaose sequenties bleken afkomstig van subtype B varianten. Eén Nederlandse sequentie behoorde tot subtype A. Opvallend was dat een aanmerkelijke discrepantie werd gevonden tussen de V3 sequentie en de sequentie van het peptide, waartegen specifieke antistof reactiviteit werd gevonden: 5/11 Nederlandse en 3/7 Curacaose samples vertoonden specifieke reactiviteit tegen peptiden waarvan de aminozuurvolgorde identiek was aan de V3 sequentie van het geisoleerde viraal RNA, terwijl de overigen discordantie vertoonden.

Conclusies en aanbevelingen

Op grond van de serologische reactiviteit in het V3 gebied kan worden geconcludeerd dat in de periode 1988 - 1990 in Nederland en Curacao subtype B HIV-1 varianten het meest prevalent waren. De V3-loop reactiviteit bleek vergelijkbaar met die gemeten in de Amsterdamse cohortstudies onder homoseksuele mannen en druggebruikers. Een minderheid van de Nederlandse monsters (mn afkomstig uit Brabant en Overijssel) bleek specifiek te reageren met V3 sequenties die worden gevonden in Afrikaanse HIV varianten. De resultaten van de specifieke antistofreactiviteit werd in het algemeen bevestigd door de resultaten van het V3 sequentie-onderzoek, maar subtiele sequentieverschillen tussen de V3 loop reactiviteit en circulerend viraal V3 werden in een aantal gevallen aangetoond. Dat is vermoedelijk het gevolg van het feit dat de serum monsters die in deze studie werden gebruikt, afkomstig zijn van personen die maanden tot jaren voor de periode 1988-1990 met HIV-1 werden besmet. Bij gebrek aan klinische en epidemiologische gegevens kan dit echter niet nader worden uitgezocht.

Aanbevolen wordt in 1995 een tweede survey uit te voeren, te meer daar inmiddels meer bekend is over verschillende subtypen van HIV-1 en recent het zogeheten subtype O is beschreven. Dit is een cluster van HIV-varianten waarvan het V3 gebied substantieel verschilt van de tot nog toe bekende subtypen A - E en dat vooralsnog vooral een

Afrikaans/Europees probleem lijkt te zijn. Met deze tweede survey kunnen de veranderingen binnen subtype B en de introductie van andere subtypen van HIV-1 ten opzichte van de periode 1988-1990 nader worden gevolgd. Ten opzichte van de in dit rapport beschreven studie worden een aantal veranderingen voorgesteld:

1. Er dienen zo veel mogelijk sera van recent besmette individuen in de studie te worden betrokken; in ieder geval moet het te onderzoeken materiaal klinisch en epidemiologisch beter zijn gedefinieerd.
2. De set van de te gebruiken V3 peptiden moet worden aangepast aan de V3 sequentie data die inmiddels voor de verschillende genetische subtypen bekend zijn: voor elk subtype dient een -beperkte- set van V3 sequenties te worden gekozen, zodanig dat de intra-subtype variatie wordt "gedekt". Een deel van de tot nog toe gebruikte V3 peptiden kan door deze nieuwe peptiden worden vervangen.
3. Het is van belang de methoden voor de screening van sera voor de typering van HIV variatie te koppelen aan de methoden die worden gebruikt in het Europese typeringsprogramma en aan het typeringsprogramma van de WHO. Confirmatie van seroreactiviteit tegen peptiten zou dan met behulp van de heteroduplex mobility assay (HMA) moeten worden gedaan in een random gekozen subset.
4. Sequentie onderzoek van viraal V3 RNA dient in een geselecteerde subset van nieuw te verzamelen samples te gebeuren:
   - in geval van discordante resultaten tussen peptide-ELISA en HMA en in het geval ELISA noch HMA een duidelijk resultaat geven
   - voor het verkrijgen van enig inzicht in het biologisch phenotype van circulerende HIV-1 varianten.

Een dergelijke benadering maakt het mogelijk de resultaten van de eerste in dit rapport beschreven studie te vergelijken met die van de tweede survey. Voorts wordt vergelijking van HIV-1 variatie in Nederland (en Curacao) met andere landen (zowel in Europa als de landen betrokken in het WHO typeringsnetwerk) mogelijk.

INTRODUCTION

Genomic diversity is a well-characterized feature of human immunodeficiency virus type 1 (HIV-1 1-6). It has been described among independent HIV-1 isolates, to a lesser degree among sequential isolates from the same patient, and even within a single patient isolate. Although this genomic heterogeneity is distributed throughout the entire viral genome, most of the heterogeneity is located in the *env* gene (7). Comparison of predicted amino acid sequences from several different isolates revealed that amino acid heterogeneity clusters into five variable regions (V1 through V5) of the surface glycoprotein gp120 (8). When *env* gene nucleotide sequences from sequential isolates were examined, amino acid changes in gp120 were found to occur predominantly in the same highly variable regions, suggesting that sequence variability may play a significant role in generating mutants capable of escaping neutralization (8). From these five variable regions the third (V3) variable region, located between amino acids 296 and 331 of gp120 has been studied extensively. The V3 region is an immunodominant region (9-12) that serves as a major target for isolate-specific neutralizing antibodies (13-21). Antibodies to the V3 region do not prevent binding of gp120 to the CD4 receptor but do prevent virus uptake (22) and are able to block syncytium formation induced by homologous virus (13,15,20,21). Moreover, the V3 loop contains epitopes that elicit cytotoxic T cells responses (23-26) and helper T-cell responses (27-29).

Several studies have shown that V3 has additional functions in virus replication (30) and that determinants of virustropism are located within the V3 region (31-38). Changes flanking the tip of the V3 loop sequence are sufficient to change the cell tropism of the virus (37,39). A minimum of three amino acid substitutions in V3 can confer macrophage tropism and alter T-cell-line tropism (40). A high net charge of V3, due to additional basic amino acids is correlated with the syncytium inducing (SI), fast replicating virus phenotype (39). Comparison of V3 sequences from sets of biological clones isolated sequentially from 12 patients showed a correlation between a basic charge at two positions (aa positions 11 and 25 within the V3 loop) and transition from a NSI to a SI phenotype (41-43).

Zwart et al (44-47) showed that the HIV-1 infected individual produces highly specific IgG antibodies to the V3 domain of the infecting variant, early after infection. For the

measurement of anti-V3 antibodies a set of synthetic peptides, representing circulating V3 variants, was used. Later in the course of infection, V3 sequences obtained from serum often show a discrepancy with the specificity of serum antibodies. In a study among longitudinally followed HIV-1 infected homosexual men in Amsterdam it was shown that most individuals preserved specificity for the V3 variant present at the time of seroconversion in their sera several years after seroconversion, although the initial V3 variants were no longer detected. Some individuals mounted response to newly emerged V3 variants, but most often this response was low. Therefore, the reactivity to V3 sequences usually reflects the HIV-1 subtype, with which the patient was originally infected. The specificity of sera for the infecting variant was also used to probe the antigenic variation of HIV-1 variants circulating among the same cohort of homosexual men. This survey, together with genetic data, showed that HIV-1 variants circulating in this cohort are antigenically similar in the V3 domain and predominantly belong to the genomic subtype B (48,49) cluster of variants. Thus far, serotyping of HIV in the Netherlands was carried out only within the limited situation of the Amsterdam cohort study among homosexual men. The present report summarizes the results of serotyping samples from HIV-1 infected individuals from The Netherlands, excluding Amsterdam, and from Curacao, using an ELISA with the synthetic V3 peptide panel used for the Amsterdam cohort study and including representative European and African V3 variants.

MATERIALS AND METHODS

Study populations

Serum samples of 126 HIV-1 infected individuals from The Netherlands, excluding Amsterdam, were used. Samples were taken between 1988-1990 and had been submitted to the Laboratory of Virology of the RIVM for confirmation of HIV infection. The dates of antibody seroconversion and the clinical status at the moment of sampling were unknown for most of the individuals.

Next to these samples collected in The Netherlands, 69 serum samples of HIV-1 infected individuals from Curaçao were examined. From these individuals, the dates of seroconversion and the clinical status at the moment of sampling were also unknown. Finally, we included 45 samples of HIV-1 infected individuals from Tanzania for comparison. These samples were also taken during 1988-1991 and dates of antibody seroconversion and clinical status at the moment of sampling were unknown.

In all cases, the presence of HIV-1 antibodies was determined by using a commercial EIA (Abbott Laboratories, North Chicago, Illinois, USA) and antibody seropositivity was confirmed by Western Blot (Diagnostic Biotechnology, Singapore).

Peptides

The peptides were 16 amino acid (aa) residues in length and covered the neutralization domain in gp120 V3, aa positions 9 - 24, counting from the amino terminal cysteine of the V3 loop. Sequences were derived from natural HIV-1 variants isolated in the USA, The Netherlands and Tanzania. These variants were selected on the basis of:

* variant frequency, assessed by frequency of occurrence in the V3 sequence data base from Repligen and sequence data from our own laboratory
* physiochemical differences in residues.

The peptides were produced using Merrifield solid-phase synthesis (European Veterinary Laboratory, Amsterdam, The Netherlands). The aa sequences are shown in Table 1.

ELISA

The reactivity of sera with V3-specific peptiden was determined by ELISA as previously described (47). Briefly, peptides were coated overnight at 20°C onto wells of microtitre plates in 100 μl of phosphate buffered saline (PBS). To inhibit non-specific binding wells were incubated with blocking buffer (PBS, 0.1% Tween-20, 2% (weight/vol) bovine serum albumin) for 1h at 37°C. Sera were diluted 1:100 in blocking buffer and 100 μl of diluted serum was incubated in the coated wells for 1h at 37°C. Wells were then incubated with horseradish peroxidase conjugate (goat anti-human IgG; KPL Gaithersburg, MD, USA), diluted 1:500 in blocking buffer, for 1h at 37°C. All incubations were followed by washing with PBS and 0.1% Tween-20. Bound antibodies were visualized using ortho-phenylene diamine and the reaction was stopped with 1 N H_2SO_4. Optical density (OD) was read at 492 nm.

Specificity of sera

All sera were tested on the peptide panel in ELISA at a coating concentration of 10 ng. Assays were performed in duplicate on two separate microtitre plates.

If the reactivity of a serum sample to a peptide tested at 10 ng/well exceeded an OD of 0.7, and was more than 10% higher (in both microtitre plates) than the reactivity of this serum to any of the other peptides from the panel, the serum was considered to react specifically with that peptide.

When a serum reacted to other peptides from the panel showing an OD that was 90% or more of that of the highest reactive peptide, the serum was considered to be reactive to those peptides, also.

If a serum sample did not show reactivity to an OD above 0.7 to any of the peptides at 10 ng/well, serum specificity was judged in the same manner at 100 ng/well peptide coating concentration.

RNA isolation, cDNA amplification and C2-V3 sequencing

RNA was isolated from the supernatant of a subset of serum samples according the procedure of Boom et al. (50). A volume of 10 μl of serum was used for isolation of viral RNA. Conversion of viral RNA to cDNA and amplification conditions were described previously (46). Primers used in the first PCR were 5'-V3-Not (5'GCG CGG CCG GAT

AGT ACA ATG TAC ACA TGG 3') and 3'-V3-Not (5'CGC CGG CCG CCC CCT CTA CAA TTA AAA CTG TG 3'). These primers are complementary to and hybridize to sequences flanking the V3 domain which are well conserved between available HIV-1 sequences (49). 1/25 or 1/5 of the first amplification product, depending on the amount produced, was analyzed in a second round of PCR. Different primer sets were used: 3'EB01 (5'TAA TAC GAC TCA CTA TAG GGT GGG TCC CCT CCT GAG G 3') and SP6-5'-KSI (5'GAT TTA GGT GAC ACT ATA GGC AGT CTA GCA GAA GAA G 3'),or 3'EB02 (5'TAA TAC GAC TCA CTA TAG GGT GGG TCC CCT CCT GAG GI 3') and SP6-5'-KSI. The 5' primers were universally tailed with a SP6 (underlined) oligonucleotide primer and the 3' primers with a T7 oligonucleotide extension. Subsequently, these PCR products were sequenced using ABI taq-dye primercycler sequencing kit SP6/T7 and analyzed automatically with the Perkin Elmer/Applied Biosystems 373 A automatic sequencer.

Sequence analysis

Alignment of the sequences was done using Clustal (51) and corrected by hand to ensure that gaps did not destroy the reading frame. Phylogenetic analysis was done using simple Hamming distance (52).

RESULTS

Reactivity to synthetic peptides

The reactivity of the sera to the various peptides used is summarized in Table 2. From the total of 241 serum samples, 40 (16.6%) showed no reactivity to any of the peptides used in the panel and in 6 (2.5%) samples a non-specific reactivity was found. In these samples OD values > 0.7 were found to > 4 of the peptides used. Among the 195 (80.9%) samples in which a specific or "peak-reactivity" was measured, 136 (69.7%) were "peak-reactive" to one peptide, 43 (22.1%) were "peak-reactive" to two peptides and 16 (8.2%) to three peptides.

Among the Dutch samples reactivity to p108 was the most prevalent, followed by reactivity to p110 and p109, respectively. Combined reactivity to p108, p109 and p110 was frequently found. Next to these samples that were reactive to genomic subtype B representing V3 synthetic peptides, 5 Dutch samples proved to be "peak-reactive" to p168, a V3 peptide representing African strains. The reactivity per Dutch province is depicted in figure 1. The distribution of reactivity to peptides p108, p109 and p110 was more or less the same in Noord-Holland and Utrecht. In the province of Overijssel reactivity to p110 was most prevalent, whereas reactivity to p108 was most frequent among the sera from Zuid-Holland. Remarkably, reactivity to p168 was only found among 3 of the 19 (16%) sera from Brabant and 2 of the 18 (11%) from Overijssel. Comparison of the reactivity to V3 peptides of sera from a group of 129 seroconverters from the Amsterdam cohort studies (45) did not show significant differences between reactivity patterns found in Amsterdam versus other parts of The Netherlands (Table 3).

The "peak-reactivity" pattern among the samples from Curacao was in general similar to that of the Dutch samples (Table 1). Reactivity to peptides p108, p109 and p110 were most prevalent in the Curacao samples, with an equal distribution of reactivity to p108 and p110. Reactivity to p109 was less frequently found than in the Dutch samples. Compared to the Dutch samples, the percentage "not-peak reactive" samples was higher.

Among the samples from Tanzania 19 out of 45 (42.2%) "peak-reacted" to one or two of the peptides representing African V3's. Thirteen of the 45 (28.8%) of the samples were reactive to p108, in 8 of these cases combined with reactivity to an African peptide (Table 1). Ten of the 45 (22.2%) samples were not "peak-reactive" to any of the peptides used in the panel.

Direct sequencing of Dutch and Curacao samples

A subset of samples from The Netherlands (n=11) and Curacao (n=7) was used for direct sequencing of the C2-V3 region of the viral *env* gene. These samples were chosen in order to compare "peak-reactivity" to the various peptides with the V3 sequence circulating in serum. Lack of sufficient material was the reason that only a limited number of samples could be used. Direct sequencing results compared to the "peak-reactivity" found in the synthetic peptide ELISA are given in Table 4A and 4B. Zwart et al. described the importance of the amino acid residue at position 13 of the V3 loop for the specificity and affinity of the binding of antibodies (44). The results of their study were based on genetic subtype B viruses and showed that early in HIV-1 infection the specificity of antibody binding to a particular V3 peptide reflects the V3 sequence circulating, whereas later in infection reactivity does not reflect the V3's circulating at that moment anymore in most cases. In a few cases broadening of the V3 antibody response was shown. In the present study, no data on seroconversion or clinical status was available. From the sequencing performed on the particular subset of samples, it appeared that all sequences from Curacao were of the genetic subtype B and that all but one of the Dutch samples were of subtype B too. The one exception was shown to belong to subtype A. Three of the 7 samples from Curacao showed reactivity to peptides reflecting the V3 sequence found in the same sample, whereas 4 were discordant. Five of the 11 Dutch samples showed V3 peptide reactivity concordant to the V3 sequence found and 6/11 appeared to be discordant. The Dutch genetic subtype A sequence showed reactivity to the in general concordant peptides p116 and p168, which are related to amino acid sequences of V3 loops of African HIV-1 variants. In one sample from Curacao, showing "peak-reactivity" to peptide p111, the V3 loop sequence indeed showed a methionine (M) of position 14, which is frequently found among V3 sequences of subtype B variants from Brazil (49).

Based on basic amino acid substitutions found at the V3 loop positions 11 and 25 (41,42), all variants sequenced were predicted to be of the non-syncytiuminducing (NSI) phenotype, except for one Dutch sample (A910492), which was predicted to be of the syncytiuminducing (SI) phenotype. Except for two, all V3 loop sequences appeared to be 35 amino acids in length.

DISCUSSION

In the present study a serosurveillance was done on a set of sera from HIV-1 infected individuals from The Netherlands and Curacao in order to obtain information on the variability of HIV-1 strains circulating in the Kingdom. Samples from Amsterdam were excluded, since serotyping of these has been described previously (44,45). The samples used were obtained during 1988 - 1990 and were sent to the National Institute for Public Health and Environment (RIVM) for HIV-1 antibody confirmation purposes. No clinical data of these samples were available. Based on the seroreactivity data obtained one can conclude that the majority of HIV-1 variants, circulating during 1988 - 1990 in The Netherlands are of genetic subtype B, showing a V3 loop reactivity comparable to that measured in the Amsterdam cohort studies among homosexual men and drug users (Table 3). However, a minority (5.6%) of the samples showed reactivity to V3 peptides representing African HIV-1 variants. Remarkebly, this African V3 seroreactivity was found only in two parts of The Netherlands: Noord-Brabant and Overijssel. Antibodies to African V3 peptides were not measured in the samples from the Amsterdam cohort studies.

V3 sequencing of a subset of the samples confirmed the serological data in a general way, but showed also the broadening of anti-V3 reactivity as well as the particular differences in V3 loop sequences circulating compared to the anti-V3 reactivity in the same sample. From the Amsterdam cohort studies it was known, that these latter features can be seen in samples obtained from HIV-1 infected individuals in a later stage of infection. Thus, probably most of the samples studied were taken from individuals who seroconverted months to years before 1988/1990. More or less the same holds true for the samples obtained from Curacao. Seroreactivity among the Dutch and Curacaon samples appeared to be significantly different from that of the Tanzanian samples, the majority of which showed reactivity to V3 peptides representing African HIV-1 variants. Seroreactivity to V3 peptides as well as V3 sequences showed a relatively homogeneous virus population among the Dutch and Curacaon samples, whereas among the Tanzanian samples more diverse results were obtained. Finally, based on the V3 sequences obtained within this study, we conclude that the vast majority of the viruses in our samples were of non-syncytium inducing (NSI) capacity.

Interpretation of the results of the present study are limited, because of lacking epidemiological data and of geographical limitations. However, it gives some insight in the genetic and antigenic variation of HIV-1 in the Netherlands and Curacao in the period pre-1988/1990. So far, the results of the present study are in accordance to the results obtained in the Amsterdam cohort studies. It is, however, unclear whether the situation has changed between 1990 and 1994.

At present, six genetic subtypes (A-F) (48,49) are distinguished, based on sequence analysis of the complete *gag* and *env* genes. Next to these subtypes, others (G, H and I) were reported. Recently, an additional HIV-1 type (O) was described (Saragosti et al, personal communication). This subtype O appeared to be of particular interest because of the false-negative test results in some third generation ELISA's for HIV-1/HIV-2 antibodies. Within the Western-European context, HIV-1 subtype B variants are the most prevalent, but one has to take into account that studies on genetic subtypes of HIV are carried out mostly among isolates from homosexual men and drug users. Limited data obtained from Eastern-Europe indicate, that next to subtype B -most prevalent among homosexual men- other subtypes (like subtype A and C) are circulating as well, especially in those individuals who were infected through heterosexual contact or following blood transfusion. Migration of individuals from African and Asian countries to Western Europe consequently leads to migration of genetic HIV-1 subtypes other than B, also.

With these considerations in mind, we suggest to carry out a second HIV-1 antigenic and genetic subtyping survey in 1995. This second survey will give information on possible changes in variability between (pre-)1988/1990 and 1995, as well as in the introduction of genetic subtypes other than B in The Netherlands (and Curacao). Some modifications in the design of the study however are needed in order to obtain more accurate results. These include:

a. serum samples, to be used in this study, have to be accompanied by essential clinical and epidemiological information; mainly samples taken early after primary HIV-1 infection should be used.

b. based on the more recent and accurate V3 sequence data from the various genetic subtypes, the set of synthetic peptides used in the V3 antibody ELISA has to be adapted and completed. For each genetic subtype a set of V3 sequences covering most of the intra-subtype V3 aa variation will be used for synthetic peptide

production. These peptides will replace those in the peptide set used thusfar, to which reactivity was not or less frequently measured.

c. methods for genetic HIV-1 subtype screening of serum samples are to be linked to those used in the European and WHO HIV-1 typing networks: in this second survey the heteroduplex mobility assay (HMA) (53) will be used in order to confirm the results of the synthetic peptide ELISA in an at random chosen subset of samples.

d. sequence analysis of the V3 region should be carried out on a selected subset of samples, namely:
   - those, which give discordant results between the synthetic peptide ELISA and the HMA, as well as other outlayers
   - another subset to be used for the prediction of the biological phenotype.

With this approach it is possible to compare the results of a second survey to those of the first, as described in this report, as well as to compare variation of HIV-1 in The Netherlands (and Curacao) to other countries in Europe and to those countries involved in the WHO Typing Network.

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Table 1

Amino acid sequences of V3 peptides

peptide no	amino acid sequence	region
108	RKSIHIGPGRAFYTTG	Eur/USA
109	----N----------	Eur/USA
110	----P----------	Eur/USA
111	-RR-TM----VL----	Eur/USA
112	SRG-R------ILA-E	Eur/USA
113	-R--YT------H--D	Eur/USA
114	----S-------F---	Eur/USA
115	--R-TM----VY----	Eur/USA
116	-R---V---Q---A--	Africa
117	--G-F-----NI----	Eur/USA
164	RQGTHIGPGRAYYTTR	Africa
168	-KSV-----Q-F-A-G	Africa
169	-ESVR----QTFAY-G	Africa
170	--S-R----Q-L--NK	Africa
172	-KSV-----QTS-A-G	Africa

Table 2

Number of sera reactive to V3 peptides

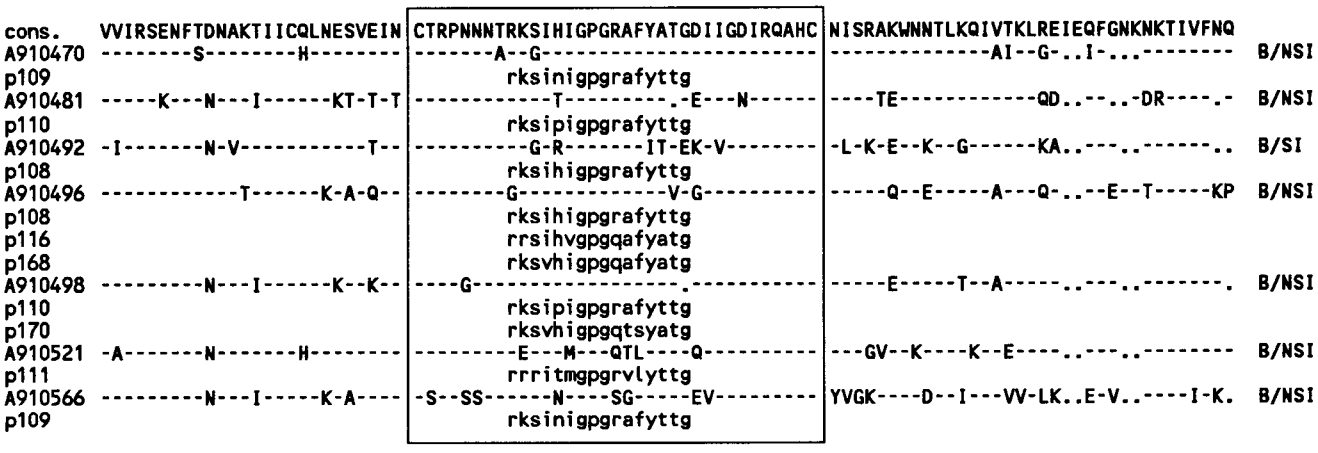
V3 peak reactivity	Netherlands (n = 126)		Curaçao (n = 70)		Tanzania (n = 45)		Total (n = 241)	
	N	%	N	%	N	%	N	%
108	34	27.0	15	21.4	5	11.1	54	22.4
109	14	11.1	4	5.7			18	7.5
110	21	16.7	14	20.0			35	14.5
108/109	7	5.6	4	5.7			11	4.6
108/110	4	3.2	4	5.7			8	3.3
108/164					7	15.6	7	2.9
109/110	5	4.0	4	5.7			9	3.7
108/109/110	6	4.8	4	5.7			10	4.2
108/109/168	1	0.8					1	0.4
108/116/168	2	1.6	1	1.4	1	2.2	4	1.7
109/110/168	1	0.8					1	0.4
109/115	1	0.8					1	0.4
110/170	1	0.8	1	1.4			2	0.8
111			2	2.9			2	0.8
112			1	1.4			1	0.4
114	1	0.8					1	0.4
115	1	0.8					1	0.4
116					1	2.2	1	0.4
164					2	4.4	2	0.8
168	5	4.0	1	1.4	1	2.2	7	2.9
169	1	0.8	1	1.4	4	8.9	6	2.5
170					8	17.8	8	3.3
116/168	1	0.8					1	0.4
168/169					3	6.7	3	1.2
168/172					1	2.2	1	0.4
not peak-reactive	17	13.5	13	18.6	10	22.2	40	16.6
non-specific	3	2.4	1	1.4	2	4.4	6	2.5

Table 3

Comparison of reactivity to V3 peptides between Dutch sera and early sera from HIV-1 seroconverted individuals participating in the Amsterdam cohort studies

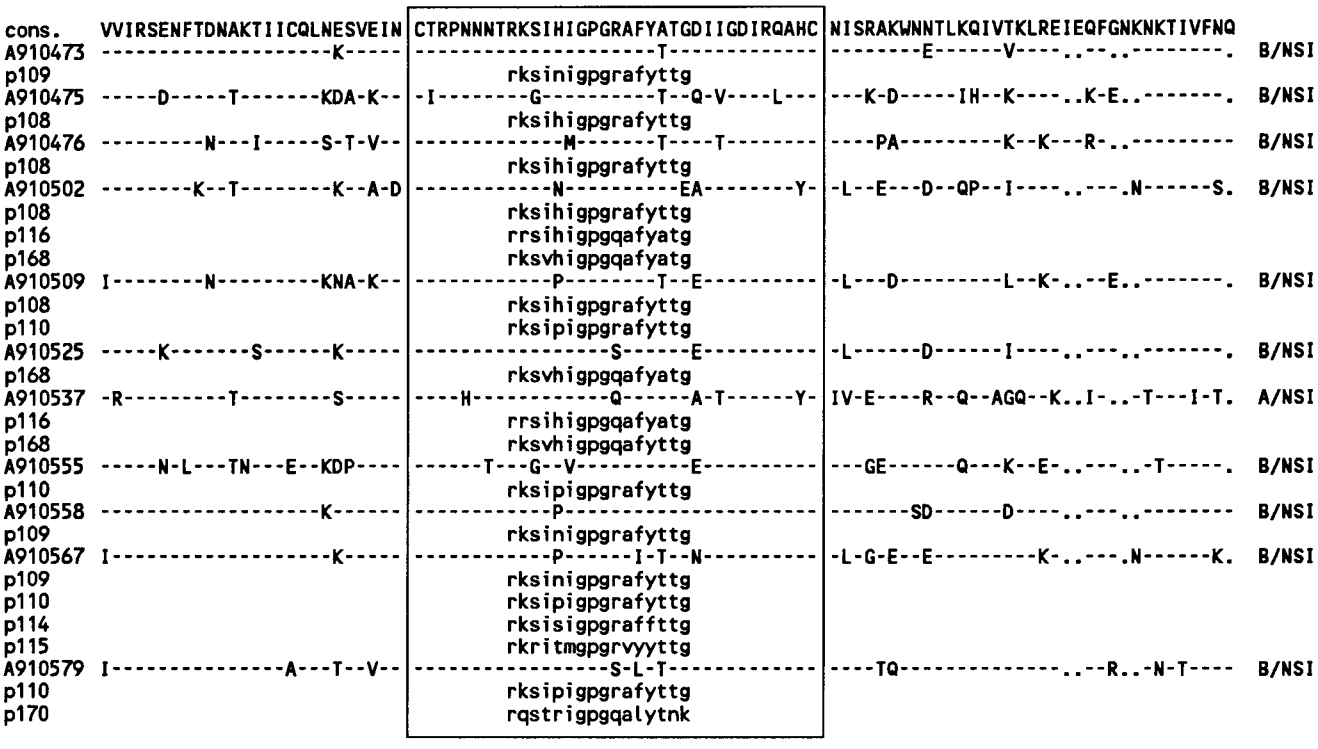
V3 peak reactivity	Netherlands, excluding Amsterdam (n = 126)		seroconverters Amsterdam cohort studies (n=129)	
	N	%	N	%
108	34	27.6	47	36.4
109	14	11.1	15	11.6
110	21	16.7	23	17.8
108/109	7	5.6	14	10.8
108/110	4	3.2	4	3.1
109/110	5	4.0	2	1.6
108/109/110	6	4.8	4	3.1
108/114	0		1	0.8
108/116	0		2	1.6
108/109/168	1	0.8	ND	
108/116/168	2	1.6	ND	
108/109/114	0		1	0.8
108/109/110/114	0		1	0.8
108/109/110/115	0		1	0.8
109/110/168	1	0.8	ND	
109/115	1	0.8	0	
110/170	1	0.8	0	
111	0		0	
112	0		0	
114	1	0.8	0	
115	1	0.8	2	1.6
116	0		2	1.6
'African' V3 peptides	7	5.6	ND	
non-peak reactive	17	13.5	10	7.8
non-spercific	3	2.4	0	

Table 4A C2-V3 sequences of a subset of samples from Curacao versus V3 peptide peak-reactivity.



cons.	VVIRSENFTDNAKTIICQLNESVEIN	CTRPNNNTRKSIHIGPGRAFYATGDIIGDIRQAHC	NISRAKWNNTLKQIVTKLREIEQFGNKNKTIVFNQ	
A910470	--------S--------H--------	-------A--G----------	-------------AI--G-..I-...--------	B/NSI
p109		rksinigpgrafyttg		
A910481	-----K---N---I------KT-T-T	-----------T---------.-E---N------	----TE-----------QD..--..-DR----.-	B/NSI
p110		rksipigpgrafyttg		
A910492	-I-------N-V----------T--	----------G-R-------IT-EK-V--------	-L-K-E--K--G------KA..---..------..	B/SI
p108		rksihigpgrafyttg		
A910496	------------T------K-A-Q--	--------G------------V-G----------	-----Q--E-----A---Q-..--E--T-----KP	B/NSI
p108		rksihigpgrafyttg		
p116		rrsihvgpgqafyatg		
p168		rksvhigpgqafyatg		
A910498	---------N---I------K--K--	----G----------------.----------	-----E-----T--A-----..---..-------.	B/NSI
p110		rksipigpgrafyttg		
p170		rksvhigpgqtsyatg		
A910521	-A-------N-------H--------	---------E---M---QTL----Q----------	---GV--K----K--E----..---..--------	B/NSI
p111		rrritmgpgrvlyttg		
A910566	---------N---I-----K-A----	-S--SS------N----SG-----EV---------	YVGK----D--I---VV-LK..E-V..----I-K.	B/NSI
p109		rksinigpgrafyttg		

Table 4B C2-V3 sequences of a subset of samples from The Netherlands versus V3 peptide peak-reactivity.



cons.	VVIRSENFTDNAKTIICQLNESVEIN	CTRPNNNTRKSIHIGPGRAFYATGDIIGDIRQAHC	NISRAKWNNTLKQIVTKLREIEQFGNKNKTIVFNQ	
A910473	-------------------K-----	---------------------T------------	--------E------V----..--..--------.	B/NSI
p109		rksinigpgrafyttg		
A910475	-----D-----T-------KDA-K--	-I--------G----------T--Q-V----L---	---K-D-----IH--K----..K-E..-------.	B/NSI
p108		rksihigpgrafyttg		
A910476	---------N---I-----S-T-V--	------------M-------T----T--------	----PA---------K--K---R-..---------	B/NSI
p108		rksihigpgrafyttg		
A910502	--------K--T--------K--A-D	-----------N----------EA--------Y-	-L--E---D--QP--I----..----.N------S.	B/NSI
p108		rksihigpgrafyttg		
p116		rrsihigpgqafyatg		
p168		rksvhigpgqafyatg		
A910509	I--------N---------KNA-K--	-----------P--------T--E----------	-L---D---------L--K-..--E..-------.	B/NSI
p108		rksihigpgrafyttg		
p110		rksipigpgrafyttg		
A910525	-----K-------S------K-----	----------------S------E----------	-L------D------I----..---..-------.	B/NSI
p168		rksvhigpgqafyatg		
A910537	-R---------T--------S-----	----H-----------Q------A-T------Y-	IV-E----R--Q--AGQ--K..I-..-T---I-T.	A/NSI
p116		rrsihigpgqafyatg		
p168		rksvhigpgqafyttg		
A910555	-----N-L---TN---E--KDP----	------T---G--V----------E----------	---GE------Q---K--E-..---..-T-----.	B/NSI
p110		rksipigpgrafyttg		
A910558	-------------------K------	------------P---------------------	-------SD------D----..---..--------	B/NSI
p109		rksinigpgrafyttg		
A910567	I-------------------K-----	------------P------I-T--N----------	-L-G-E--E---------K-..----.N------K.	B/NSI
p109		rksinigpgrafyttg		
p110		rksipigpgrafyttg		
p114		rksisigpgraffttg		
p115		rkritmgpgrvyyttg		
A910579	I---------------A---T--V--	----------------S-L-T-------------	----TQ--------------..--R..-N-T----	B/NSI
p110		rksipigpgrafyttg		
p170		rqstrigpgqalytnk		