Performance of V3-based HIV-1 sero subtyping in HIV endemic areas

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INTRODUCTION
Continuous investigation of HIV-1 subtypes’ dynamics in geographical areas with a high incidence of HIV infection and broad virus heterogeneity is important because different HIV-1 subtypes may influence the efficacy of potential vaccines against HIV, the performance of tests to detect HIV infection and the monitoring of HIV-infected patients [1]. A number of techniques, including molecular characterization of PCR-amplified genomic sequences and the HMA (heteroduplex mobility assay), have proved to be highly sensitive in identifying HIV-1 subtypes. However, due to high costs and complexity of these procedures in a poor resource setting, as it is often common in
highly HIV endemic areas, alternative, cost-effective, easy and rapid to perform serological methods, which are based on antibody cross-recognition of synthetic peptides from the V3 variable region of the HIV-1 envelope glycoprotein gp120, have been proposed and used [2]. Nonetheless, antibody cross-recognition can be influenced by the variability of the V3 sequence due to the high heterogeneity of circulating HIV variants [3]. In addition, it is known that avidity of antibodies against HIV antigens is low early after infection, but increases over time as the infection progresses [4, 5]. Thus, V3-based tests could have a weak performance when sera from recently HIV-infected individuals are tested, a common event in areas highly endemic for HIV infection.

In order to evaluate whether V3-based serological testing can be a reliable approach for serotyping in these areas, we investigated the performance of two V3-based serological assays on samples from HIV infected people living in Uganda, a country highly endemic for HIV infection, where several different HIV-1 subtypes and recombinants circulate [6, 7].

**MATERIALS AND METHODS**

**Participants and serum samples**

A total of 148 sera from 118 individuals were collected at the Joint Clinical Research Centre in Kampala, Uganda. Individuals were derived from two independent cohort studies conducted in Uganda (PAVE Study and HC-HIV Study) [8, 9]. These studies were reviewed and approved by local Institutional Review Boards and all participants provided an informed consent. Individuals were 31 male military recruits and 87 women of reproductive age. These individuals seroconverted during the above described studies and seroconversion date was estimated as the midpoint between the dates of the last negative and the first positive HIV test. The interval between the two dates never exceeded 180 days. Specimens were considered HIV-positive when reactive using commercial EIA, confirmed by Western Blotting and/or PCR.

Sera collected within or after 180 days from the estimated seroconversion date were considered as derived from recently or chronically infected individuals, respectively, as previously described [10]. For 30 of the 118 individuals, two serial sera after seroconversion were available, for a total of 148 sera included in the study. Of these 148 sera, 114 (77%) were collected within 180 days from the estimated seroconversion date and 34 were obtained later.

**V3-based assays**

The V3 synthetic peptides were chosen according to previously published data [11] and corresponded to the following HIV-1 clades: A (KSVHIGPGQAYTAF), B (KSIHIGPGAFYTYT), C (KSIHGPGQTFYAT), D (RQTHGPGQALYTT), E (DTSITIGPGQFYRT), F (DKSHLPGQAFYAT), G (CDIQEMRIGPMAWYSMGIGGTAGNS). Peptides were synthesized with a purity > 95% (Biomol International; Matford Court, UK). Two different V3-based indirect EIA assays, Antigen Limiting-EIA (AL-EIA) and Competitive-EIA (C-EIA), were performed [11, 12]. Each sample was tested at least twice with each method.

**Anti-gp120 EIA**

The presence and the titers of anti-HIV-1 gp120 IgG were evaluated by an in-house EIA. Briefly, 96-well microplates were coated with SF162 (clade B)-derived gp120 monomeric protein, at a concentration of 2 ng/µl in Carbonate Buffer, overnight at +4 °C and then washed with PBS containing 0.05% Tween20 and 2% dry milk to the final concentration of 0.05 µg/µl. The subtype was defined according to the V3 peptide showing the strongest competition, as reported in literature [12].

**Avidity Index assay**

The AI assay with sera from both clade B and non-B HIV-1-infected individuals has been previously described and validated in literature. Testing was carried out as previously reported [13]. An optical density of 0.80 has been used as a CO to discriminate recent (<180 days) from established (>180 days) HIV infections, as previously reported [13, 10].

**Statistical analysis**

The Student’s t-test was used to compare the days from seroconversion and the AI values between sample groups with different reactivity to V3-peptides.
RESULTS

The 148 samples were tested using AL-EIA and C-EIA. The samples were classified according to their reactivity to the V3-peptides as following: non-reactive (NR), i.e. samples showing no reactivity to any of the V3-peptides in neither AL-EIA, nor C-EIA assays; cross-reactive (CR), i.e. samples showing reactivity to two or more V3 peptides or with discordant reactivity in the two tests; specific (SP), i.e. samples showing specific reactivity to a single V3 peptide with both methods. Surprisingly, according to the results obtained, a relevant number of 64 out of 148 (43.2%) samples were classified as NR, 16 (10.8%) as CR and only 68 (46.0%) as SP.

We have, therefore, investigated if the lack of reactivity to the V3-peptides of the 64 NR sera was due to the absence or to very low titers of anti-HIV-1 antibodies against the whole Env protein. To this aim, all 148 serum samples were tested for the presence of IgG antibodies against a monomeric, clade B-derived gp120 (Figure 1). Six samples, all classified as NR in the V3 testing, showed no reactivity to the gp120. All the other 142 sera were positive for anti-gp120 IgG antibodies. The median titer of anti-gp120 antibodies in the NR samples was 1600 (range: 0-102 400), as compared to 102 400 (range: 6400-409 600) in the CR samples and 51 200 (range: 100-1 638 400) in the SP samples (Figure 1).

Reactivity of sera against V3-peptides was then analyzed taking into account the estimated seroconversion dates (Figure 2). Results shown in Figure 2 indicate that most of the sera collected within 180 days from the estimated seroconversion date are classified as NR. Only a few of the NR sera were collected after 180 days from the seroconversion date and these sera were all obtained in the period from 181 to 240 days. Conversely, most of the SP and CR sera were obtained later after infection, the majority of them being collected after 180 days from the estimated seroconversion date.

In addition, for 14 individuals a couple of sera collected sequentially were also available: the first specimen was taken within 60 days from estimated seroconversion date and the second one after 60 days. All early collected sera were NR, whereas 43% of the latter specimens were classified as SP (data not shown).
To investigate whether the lack of V3 reactivity of NR sera could be explained by low avidity of antibodies to HIV antigens, all sera were tested for AI (Figure 3). Almost all NR sera showed an lower than 0.80, indicating that these sera had a still low avidity for the HIV antigens. Conversely, SP and CR sera had a higher avidity Index for HIV antigens (AI > 0.80).

As the trend of association between the days from seroconversion and the AI values was comparable in CR and SP samples, we grouped these samples into the reactive cluster (CR + SP). The mean of days from seroconversion of the NR samples was 60.4 (95% CI: 52.2; 68.6), whereas that of one of the CR + SP samples was 150.0 (95% CI: 130.4; 169.3). The difference between the two means was statistically significant (p < 0.0001). Similarly, the mean of AI values of the NR samples was 0.49 (95% CI: 0.44; 0.54), whereas that one of the CR + SP samples was 0.79 (95% CI: 0.74; 0.84). The difference between the two means was, again, statistically significant (p < 0.0001).

**DISCUSSION**

In order to evaluate the performance of V3-based serological subtyping in highly HIV endemic geographical areas, we tested serum samples from Ugandan individuals with estimated date of seroconversion, using two of the most used V3-based serotyping methods, AL-EIA and C-EIA [11, 12]. A relevant aliquot (43.2%) of the tested samples did not react to any V3 peptide (NR sera) with both serotyping approaches. All but six sera had, instead, anti-gp120 IgG antibodies. These data indicate that antibody response maturation could be partially developed in the group of individuals with V3-non-reactive antibodies. This hypothesis is in line with data reported in literature indicating that anti-HIV antibodies, in particular anti-gp120 IgG, can grow up in titers until about 1 year from infection [15, 17].

To further confirm whether a still immature antibody response could influence V3 serotyping performance, we stratified the NR, CR and SP sera according to seroconversion dates and found that almost all NR sera derived from recently seroconverted patients.

Since almost all the sera had antibodies against gp120, the low performance of V3-based assays in the NR sera could be due to the absence of V3-specific antibodies or, if present, to a still low avidity against the V3 epitopes. In fact, it has been described that antibody avidity to HIV antigens is low in the first months after the seroconversion and increases afterwards, as a consequence of the maturation of the humoral immune response [5, 4]. Therefore, still immature antibodies may have a low avidity for the V3 region and hamper V3 serotyping performance. The AI assay confirmed that NR sera had low avidity against HIV antigens. This result is also strengthened by our observation that in individuals for whom more than one sequential serum sample was available, antibodies against V3 peptides were present only in the sample collected later (data not shown). Our data are in agreement with those from Zwart et al., which show that anti-V3 domain antibodies become detectable even up to 13 months after antibodies against other HIV-1 proteins, including gp120, have been developed [18].

Certainly, the high heterogeneity of the circulating HIV variants plays also a role in the low ability of some sera to bind V3 peptides. In fact, our results show that a small portion of cross-reactive (CR) sera is persistently present, in all seroconversion date clusters (Figure 2) and AI clusters (Figure 3). In addition, the fraction of CR sera is proportional to the quota of sera reacting to only one V3 peptide (SP sera).

These results indicate that V3-based assays performance can have, unavoidably, some application limits, due to virus heterogeneity. More importantly, the performance of the assay can be further lowered when sera from recently infected individuals are tested, due to still immature antibody response and low avidity against HIV antigens.

These observations should be taken into account when planning HIV subtype investigations using a serotyping approach in populations with high HIV incidence.
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Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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