Relationship between HIV-1 Env subtypes A and D and disease progression in a rural Ugandan cohort

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Objective: To investigate the role of HIV-1 envelope subtypes on disease progression in a rural cohort of Ugandan adults where two major HIV-1 subtypes (A and D) exist.

Methods: Participants of a clinical cohort seen between December 1995 and December 1998 had blood collected for HIV-1 subtyping. These included prevalent cases (people already infected with HIV at the start of the study in 1990) and incident cases (those who seroconverted between 1990 and December 1998). HIV-1 subtyping was carried out by heteroduplex mobility assay and DNA sequencing in the V3 *env* region. Disease progression was measured by the rate of CD4 lymphocyte count decline, clinical progression for the incident cases as time from seroconversion to AIDS or death, to first CD4 lymphocyte count $< 200 \times 10^6$/l and to the World Health Organization clinical stage 3. All analyses were adjusted for age and sex.

Results: One hundred and sixty-four individuals, including 47 prevalent and 117 incident cases, had V3 *env* subtype data of which 65 (40%) were subtyped as A and 99 as D. In the incident cases, 44 (38%) were subtyped as A and 73 as D. There was a suggestion that for most end-points A had a slower progression than D. The cumulative probability of remaining free from AIDS or death at 6 years post-seroconversion was 0.72 [95% confidence interval (CI), 0.50 to 0.85] for A and 0.58 (95% CI, 0.42 to 0.71) for D, and the adjusted hazard ratio of subtype D compared to A was estimated to be 1.39 (95% CI, 0.66 to 2.94; $P = 0.39$). The estimated difference in rates of decline in square root CD4 lymphocyte counts was −0.41 per year (95% CI, −0.98 to 0.15; $P = 0.15$).

Conclusion: This study suggests that although subtype A may have a slower progression than D, HIV-1 envelope subtype is not a major factor in determining the progression of HIV-1 disease in a rural population in Uganda.

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Introduction

The rate of HIV-1 disease progression varies widely between infected individuals, but the factors responsible for this are not well understood. This variation has been partly explained by the phenotype of the virus, with some reports suggesting a more rapid progression to AIDS in patients with fast-replicating syncytium inducing (SI) viruses [1,2]. A deletion allele of CCR5 co-receptor gene has also been reported to slow disease progression in heterozygotes compared with individuals homozygous for the normal CCR5 gene [3]. Surpris-

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ingly, the levels of chemokine that bind this receptor do not vary significantly between rapid and slow progressors [4]. The age at seroconversion has also been shown to influence disease progression [5]. The role of host HLA genetic variation in relation to disease progression has been controversial, with some studies showing an association and others failing to demonstrate this (for a review see [6]).

So far, 11 different HIV-1 subtypes (A–K) have been identified within the major group M, and more distant or outlier viruses, classified as group O have been reported mainly from Central and West Africa [7]. Another group N composed of highly divergent strains has also been reported [8]. These subtypes have been identified by comparisons of nucleotide sequences in different parts of the HIV-1 genome, especially the *env* and *gag* regions. Recombinant viruses which belong to two distinct subtypes in different regions of the genome are also increasingly being recognized, and it is estimated that around 20% of the current strains in the pandemic are recombinants (for a review see [9]). Some of these recombinant HIV-1 are unique and more prevalent in particular geographical regions, and these have been designated as 'circulating recombinant forms' or CRF [7]. Members of a CRF resemble each other over the entire genome with similar breakpoints reflecting common ancestry. Such CRF include A/E prevalent in Thailand, A/G common in west Africa , A/G/I identified in Cyprus and Greece and recently A/G/J described in patients from Burkina Faso and Mali (for a review see [9]). These CRF have not been reported in East Africa [10] nor in our study area [11].

Very little information exists on the relevance of these subtypes to disease progression. In one study, the rates of disease progression evaluated by the decline in CD4 lymphocyte count were similar between groups of Ethiopian immigrants carrying HIV-1 subtype C and non-Ethiopian Israelis infected with subtype B [12]. However, this study compared two groups on antiretroviral therapy with different demographic composition, mode of transmission, and host genetics. Another study in Senegal reported that individuals infected with HIV subtype A were found to progress clinically more slowly than those infected with non-A subtypes [13]. In their study women infected with a non-A subtype were eight times more likely to develop AIDS than were those infected with subtype A. A study in London reported that differences in disease progression to AIDS and death and CD4 lymphocyte count decline between HIV-1 infected Africans and non-Africans could not be attributed to ethnicity and viral subtypes [14]; however, the Africans were infected with diverse subtypes. A similar rate of disease progression was reported among individuals infected with HIV-1 genetic subtypes A–D [15]. The problem with this study was that the time of seroconversion was not known and it was based on small numbers. Recently, another study in Thailand has reported that HIV-1 subtypes B′ and E were associated with similar degrees of immunosuppresion and opportunistic infection patterns [16] among a group of HIV-1 infected individuals; however, these were AIDS patients in hospitals.

In this study, we have investigated the role of HIV-1 genetic subtype on disease progression as measured by: the rate of CD4 lymphocyte count decline; progression to death; progression to AIDS (stage 4) or death; to the first CD4 count $< 200 \times 10^6$ cells/l; and to stage 3 or 4 or death in an HIV-1 natural history cohort in rural Uganda where two major HIV-1 subtypes (A and D) exist. The majority of the subjects had estimated dates of seroconversion. In this population adult HIV-1 infection is acquired almost exclusively by heterosexual transmission [17] and antiretroviral drugs are not yet available. For this study the envelope subtype was considered based on envelope HMA and V3 sequence data. We decided to determine subtype in the cohort using the *env* (V3 *env*) region because of its association with pathogenesis and host immunity [18].

Subjects and methods

Subjects and sample collection

An HIV-1 natural history cohort (NHC) was established in 1990 in rural SW Uganda [19]; recruits are from a large population study where the dynamics of HIV infection are examined by annual bleeds of the population to test for HIV status. The NHC includes seroprevalent cases of HIV-1 infection detected during the initial survey round of the population study in 1989/1990 [20] and seroincident cases who had an initial negative test followed by a positive test during the annual serosurveys. All participants in the cohort are invited to attend the study clinic every 3 months and provide a detailed medical history, and undergo a full physical examination and laboratory investigation. They also attend for investigations and treatment if ill at other times.

Clinical follow-up

Participants are staged at each routine visit using the proposed World Health Organization (WHO) staging system [21] using a computer algorithm. WHO stage 4 is equivalent to AIDS. CD4 lymphocyte counts were determined every 6 months using FACSCOUNT (Becton Dickinson International, Denderstraat 24, Belgium). Compliance at routine visits is good with $> 90\%$ of patients being seen at least once a year. Deaths in cohort participants are reported by local home visitors, and even if a person has moved and not been seen for several years, information about deaths comes from family residing in the study area. Death

status was unknown for only three participants at the end of 1998, and their follow-up to death was censored at the end of the last year they were known to be alive. For this study, the period of observation is from 1990 to1998.

Subtype analysis

Participants seen between December 1995 and December 1998 had their blood collected in EDTA tubes. These blood samples were transferred to Uganda Virus Research Institute (UVRI), Entebbe within 24 h and frozen at −20°C prior to DNA extraction. DNA was extracted from frozen whole blood using either the commercial Puregene Kit (Gentra Systems Inc., North Carolina, USA) or based on a previously described method [11].

HMA subtyping analysis was carried out at UVRI according to standard procedures [22]. In most cases the primer pairs used were ED5/ED12 for the first round and ED31/ED33 for the second round generating a fragment of 0.5 kb spanning the C2–C3 regions and which includes the V3 *env* region. For samples that were untypable by HMA (n = 36), an aliquot of HMA second round products or unamplified DNA was sent to the Centre for HIV Research, Edinburgh for sequencing as described previously [23].

Statistical methods

CD4 lymphocyte counts

The two-level random effects model is a multiple regression model that recognizes that the data are series of CD4 lymphocyte counts from the same individuals over time, and allows each participant to have their own estimated intercept and rate of decline by introducing participant-specific elements (random effects). We used a square root transformation of absolute CD4 lymphocyte counts which has an approximately linear decline and stable variance over time [24,25]. The statistical package MLn [26] was used to estimate and compare the mean rates of square root CD4 lymphocyte count decline in participants with HIV-1 subtype A and D. The analyses were adjusted for age category (15–24, 25–34, 35–44 and ⩾ 45 years), sex and whether a prevalent or an incident case.

Clinical progression in the incident cases

We calculated the individual survival times from the estimated date of seroconversion. We used Kaplan–Meier survival estimates of the proportion of individuals surviving, or remaining free from death, AIDS (stage 4) or death, the first CD4 lymphocyte count $< 200 \times 10^6$/l, and stage 3 (or 4 or death). The participants' follow-up times were censored at the date last known to be alive for death and staging end points, and to their last CD4 lymphocyte count for CD4 $< 200 \times 10^6$/l. Cox's proportional hazards models were used to compare survival by subtype, adjusting for age category and sex. The analyses were performed using STATA version 6.0.

Results

Up to the end of 1998, 248 HIV-positive participants had been recruited into the NHC of whom 107 were prevalent and 141 were incident cases. The median time from last HIV-negative test to first HIV-positive test for the incident cases was 1.02 years [interquartile range (IQR), 0.92–2.03] and the date of seroconversion was not known for prevalent cases but was before 1989. One hundred and sixty-four HIV-positive patients were successfully subtyped as A or D using the env region (128 by HMA and 36 by sequencing). The distribution of age at enrolment and sex of these participants by subtype is shown in Table 1. The median age at enrolment (Table 2) was higher for those infected with subtype A (31.4 years; IQR, 26.4–43.7 years) compared with those with subtype D (27.7 years; IQR, 23.2–36.3 years; $P = 0.01$).

The ratio of A : D subtypes was 1 : 1.24 [95% confidence interval (CI), 1 : 2.28 to 1 : 0.70)] for prevalent cases and 1 : 1.68 (95% CI, 1 : 2.47 to 1 : 1.55) for incident cases. There was no evidence that these ratios were significantly different (χ^2, 0.70; $P = 0.40$).

The median follow-up time from first to last CD4 lymphocyte count overall was 3.0 years (IQR, 1.7–3.9 years) and the follow-up times were comparable in

Table 1. The age, sex and HIV group of those with subtype A or D.

	Prevalent cases				Incident cases						
	Males Subtypes		Females Subtypes		Males Subtypes		Females Subtypes		All cases		
Age group (years)	A	D	A	D	A	D	A	D	A	D	Total
15–24	3	1	1	5	0	8	9	24	13	38	51
25–34	4	7	2	3	10	13	9	9	25	32	57
35–44	4	3	1	4	7	4	3	7	15	18	33
45 +	5	1	1	2	3	5	3	3	12	11	23
All	16	12	5	14	20	30	24	43	65	99	164

Table 2. Characteristics of the participants by subtype.

	Subtype A (n = 65)	Subtype D (n = 99)	*P*
Age at enrolment (years) [median (IQR)][a]	31.4 (26.4–43.7)	27.7 (23.2–36.3)	0.01
Male (%)[b]	36 (55)	43 (43)	0.13
Incident[b] [n (%)]	44 (68)	73 (74)	0.40
Prevalent [n (%)]	21 (32)	26 (26)	
CD4 count decline			
Participants with CD4 cell counts (n)	64	97	
Total CD4 cell counts (n)	462	652	
Mean (SD) CD4 cell counts[c]	7.2 (2.8)	6.7 (3.2)	0.31
Median (IQR) follow-up (years) first to last CD4 cell count[a]	3.0 (2.0–4.0)	3.0 (1.5–3.8)	0.30
Clinical progression in the incident cases: follow-up from estimated seroconversion date			
Median (IQR) years to death or last known to be alive[a]	4.5 (2.9–6.2)	4.8 (3.6–6.0)	0.49
Median (IQR) years to last CD4 cell count[a]	4.4 (2.9–5.8)	4.6 (3.2–6.0)	0.94
Age at seroconversion[a] [median (IQR)]	30.9 (24.6–41.8)	25.4 (21.5–33.4)	0.02

[a]Mann–Whitney U test. [b]χ^2 test. [c]Two sample t test. IQR, Interquartile range.

those with subtype A and D (Table 2). For the incident cases, the overall median follow-up time from estimated seroconversion date to 31 December 1998 or date of death, if earlier, was 4.7 years (IQR, 3.3–6.1 years) and, again, the follow-up times were comparable in those with subtype A and D (Table 2).

CD4 lymphocyte count decline

There were 47 prevalent and 114 incident cases who had at least one CD4 lymphocyte count during follow-up. These 161 individuals contributed 1098 CD4 counts to the analysis. The estimates from the multivariate analysis are presented in Table 3. Although there was no evidence of an effect of age category on the rate of square root CD4 lymphocyte count decline ($P = 0.18$), the estimates and 95% CI are consistent with more rapid progression with older age. The difference in the rate of square root CD4 lymphocyte count decline per year for those with subtype D compared with subtype A was −0.41 (95%CI, −0.98 to 0.15) adjusting for age category, sex and whether an incident or prevalent case. There was no significant statistical support to suggest that subtype D was associated with faster square root CD4 lymphocyte count decline than subtype A ($P = 0.15$). To place this result in context, we present the estimated rates of decline for a male incident case between 25 and 34 years of age as an example: the estimated mean decline in square root CD4 lymphocyte counts for those with subtype A was −1.58 per year (95% CI, −2.20 to −0.96) and for D −1.99 (95% CI, −2.60 to −1.38).

The results of a subsidiary analysis of 744 CD4 lymphocyte counts from 114 incident cases alone are not dissimilar: the estimated mean difference in decline for incident cases with subtype D compared to A was −0.40 (95% CI, −1.21 to 0.42; $P = 0.34$). An analysis restricted to those with at least three CD4 lymphocyte counts yielded similar results.

Table 3. Analysis of 1098 CD4 lymphocyte counts from 161 individuals: estimated effects on intercept and rate of square root CD4 lymphocyte count decline.

	Estimated intercept ($\sqrt{}$CD4)	95% CI	*P* intercept	Estimated decline ($\sqrt{}$CD4/year)	95% CI	*P* decline
Baseline [a]	26.47	(22.64, 30.30)	–	−1.49	(−2.24, −0.73)	–
Age (years)						
15–24[b]	b			b		
25–34	1.19	(−2.31, 4.69)		−0.09	(−0.78, 0.60)	
35–44	−0.38	(−4.30, 3.53)		−0.71	(−1.51, 0.09)	
45 +	−0.72	(−5.09, 3.65)	0.57	−0.33	(−1.21, 0.56)	0.18
Sex						
Male	b			b		
Female	−0.99	(−3.83, 1.86)	0.50	0.07	(−0.50, 0.63)	0.82
Case						
Incident[b]	b					
Prevalent	0.30	(−2.77, 3.38)	0.85	0.10	(−0.47, 0.68)	0.73
Subtype						
A[b]	b		b			
D	1.47	(−1.29, 4.22)	0.30	−0.41	(−0.98, 0.15)	0.15

[a]Estimates refer to the baseline group of male incident cases between 15 and 24 years. CI, Confidence interval.
[b], Baseline value.

There was no evidence of a significantly modified effect of subtype with age category ($P = 0.60$), although this study has low power to detect such an effect. We have also repeated the analysis using CD4 lymphocyte counts from participants older than 25 years only. The results are similar to those for all ages: the estimated effect of subtype D compared to A on the rate of square root CD4 cell counts was -0.50 per year (95% CI, -1.15 to 0.14; $P = 0.13$).

Clinical progression of incident cases infected with subtypes A and D

The Kaplan–Meier survival curves from the estimated seroconversion date for the end-points death, and AIDS or death, are shown in Fig. 1. The estimated cumulative survival probabilities for each endpoint at 4 years and 6 years from seroconversion are presented by subtype in Table 4. Cumulative probabilities of survival are given because the median survival for the end-points were not reached. There was a suggestion that for all end-points subtype A had a slower progression than subtype D, and this reached statistical significance for the combined stage 3, stage 4 or death end-point.

Discussion

It has been suggested that viral genetics might have a role in HIV-1 disease progression [27]. Very little information exists regarding the relationship between HIV-1 subtypes and the natural history of infection, partly because there are few natural history cohorts in areas with diverse subtypes. The few reported studies have used individuals who were either on antiretroviral therapy, were not homogenous in terms of host genetic composition, mode of transmission or demographic composition, or their date of infection was not known [12,14,15]. The one other longitudinal study that looked at AIDS progression and HIV-1 subtypes compared subtype A with non-A subtypes, which were grouped together due to the limitation of numbers

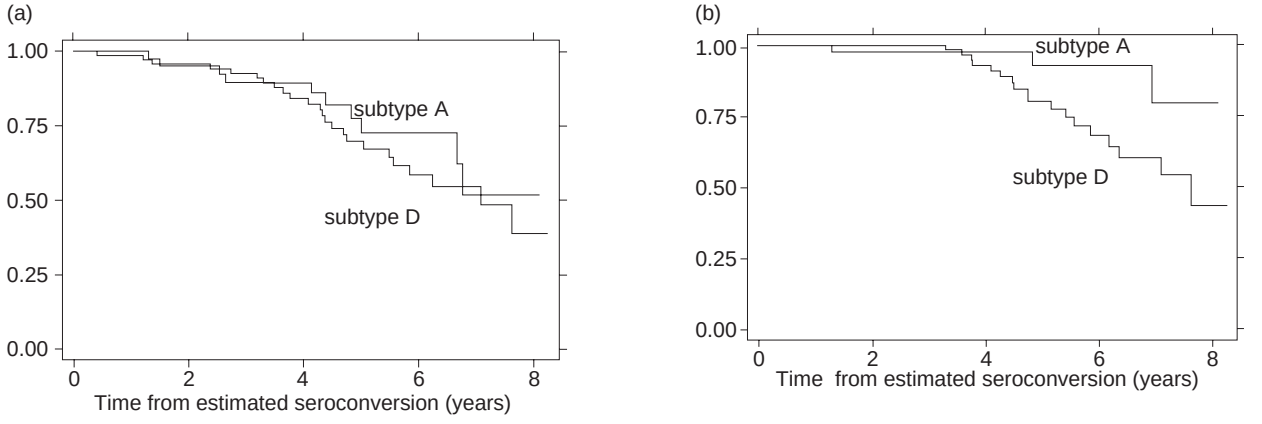

Fig. 1. Kaplan–Meier survival curves for AIDS or death (a) and death (b) in incident cases infected with subtypes A or D.

Table 4. Clinical progression in incident cases from their estimated seroconversion dates by subtype.

Endpoint	Participants	Observed events	Cumulative probability of survival (95% CI) 4 years	6 years	Hazard ratio[a] (95% CI)	P^a
Death						
A	44	3	0.98 (0.84, 0.99)	0.93 (0.72, 0.98)	3.10 (0.89, 10.74)	0.08
D	73	18	0.93 (0.82, 0.97)	0.68 (0.52, 0.80)		
AIDS or death						
A	44	10	0.90 (0.74, 0.96)	0.72 (0.50, 0.85)	1.39 (0.66, 2.94)	0.39
D	73	24	0.84 (0.72, 0.91)	0.58 (0.42, 0.71)		
CD4 cell count $< 200 \times 10^6$/l						
A	43	10	0.85 (0.65, 0.93)	0.66 (0.44, 0.82)	1.28 (0.60, 2.75)	0.53
D	71	22	0.82 (0.69, 0.90)	0.64 (0.48, 0.76)		
Stage 3, or 4, or death						
A	44	21	0.65 (0.47, 0.78)	0.37 (0.19, 0.55)	1.88 (1.11, 3.19)	0.02
D	73	47	0.44 (0.31, 0.56)	0.21 (0.10, 0.37)		

[a]Cox's proportional hazards models were used to compare the hazard rates experienced by those with subtype D compared with subtype A, adjusted for sex and age category at estimated seroconversion date.

[13]. Our study has looked at 164 individuals infected with subtype A or D viruses in a Ugandan population. One hundred and seventeen of these had estimated dates of seroconversion, this being the largest study so far of its kind.

Although disease progression in our participants with subtypes A and D was not significantly different for most end-points, there was a tendency for those with subtype D to progress faster than those with subtype A. For example, there was in excess of three times more deaths in those with subtype D than in those with subtype A. In their study [15], Alaeus *et al.* also did not show a significant difference in progression in individuals infected with subtypes A–D; however, there was a tendency for subtype D to progress faster than A: the mean CD4 lymphocyte count decline in D was twice that in A. Others have also reported slower progression in subtype A infections [13]. As suggested by others it is unlikely that a single characteristic such as subtype can account for significant differences in disease progression [28]. However, an association of subtype with other factors such as phenotype in relation to disease progression needs to be explored.

In this study only patients with blood collected between December 1995 and December 1998 were included in the analysis. The exclusion of those who died before this time could be a potential source of bias; however, of the 141 incident cases enrolled by the end of December 1998, only six (4%) had died before December 1995.

Many studies have tried to establish the significance of the HIV-1 subtype classification in relationship to other properties such as immune recognition, co-receptor usage, transmission and other biological properties. Only a few studies have reported any relation between subtype and these properties. In Thailand, subtype E viruses have been shown to be associated with heterosexual transmission and subtype B with the homosexual mode of transmission [29]. A low frequency of subtype C with SI phenotype has also been reported in Ethiopian AIDS patients [30], and CXCR4 co-receptor usage has been found to be rare among the C subtype isolates [31].

Since CD4 lymphocyte count decline is a good measure of immunodeficiency and a determinant of progression to AIDS [32], mean rates of decline in square root CD4 lymphocyte counts were used in addition to clinical progression. We have not used viral load as a measure of disease progression in this study. Viral load measurement kits are limited by the effect of HIV-1 viral diversity or subtypes on their sensitivity. In addition, most viral load kits amplify either *gag* or *pol* regions; in this study the envelope region was used and the 20% recombination reported in our cohort [11] would cause for substantial misclassification.

It has been reported that the V3 *env* region is important in the pathogenesis of HIV-1 and it also plays a part in both cellular and humoral immunity [18]. This region also determines the viral phenotype and SI viruses are associated with more rapid progression of HIV-1 disease [2]. One report has suggested that subtype D viruses are associated with SI phenotype and are rapidly replicating, 'rapid high' viruses [33]. If HIV-1 exerts its pathogenic effect through direct killing of CD4 lymphocytes, rapidly replicating subtype D viruses would be assumed to be associated with more rapid disease progression. However, this has not been shown convincingly.

Our study further shows that the rate of progression to AIDS in HIV-1 infected individuals is determined by a complex series of interactions between host and virus. Divergent patterns of progression to AIDS after infection from the same source [34] or subtype [35] have been reported and these might be explained by virus evolution and antiviral immune responses. Studies of this nature help with understanding the role of viral subtype in pathogenesis. This may be relevant to the dynamics and evolution of the HIV epidemic and will influence vaccine development.

Currently two major HIV-1 subtype distribution patterns exist globally, the first pattern is characterized by a stable epidemic with the same subtype proportions over time, as shown in our cohort studies and the second pattern as seen in Kalinningrad, in the former Soviet Union [36] is characterized by rapid introduction of new strains spreading the epidemic. Our results showing that *env* subtypes A- and D-infected patients do not differ significantly in disease progression and death might partly contribute to the stable subtype epidemic seen in Uganda.

HIV is a complex virus with different regions that might have different roles in pathogenesis. For example, a number of cytotoxic T lymphocyte epitopes have been identified the *gag* region. In addition, recombinant viruses are common in this and other populations [9,11]. The association between subtypes and disease progression needs to be investigated using other HIV-1 viral regions before meaningful conclusions can be drawn; we are currently investigating this association using the *gag* regions.

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