

Neutralization activity in a geographically diverse East London cohort of human immunodeficiency virus type 1-infected patients: clade C infection results in a stronger and broader humoral immune response than clade B infection

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The array of human immunodeficiency virus (HIV) subtypes encountered in East London, an area long associated with migration, is unusually heterogeneous, reflecting the diverse geographical origins of the population. In this study it was shown that viral subtypes or clades infecting a sample of HIV type 1 (HIV-1)-positive individuals in East London reflect the global pandemic. The authors studied the humoral response in 210 treatment-naïve chronically HIV-1-infected (>1 year) adult subjects against a panel of 12 viruses from six different clades. Plasmas from individuals infected with clade C, but also plasmas from clade A, and to a lesser degree clade CRF02_AG and CRF01_AE, were significantly more potent at neutralizing the tested viruses compared with plasmas from individuals infected with clade B. The difference in humoral robustness between clade C- and B-infected patients was confirmed in titration studies with an extended panel of clade B and C viruses. These results support the approach to develop an HIV-1 vaccine that includes clade C or A envelope protein (Env) immunogens for the induction of a potent neutralizing humoral response.

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INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is a genetically and phenotypically diverse virus, which has challenged the design of a protective vaccine. To rationally construct a vaccine, the immunological parameters that are associated with protection from infection and disease progression to AIDS must be understood. Results of previous studies have implied that both cell-mediated and

humoral responses may be required to prevent infection or disease progression (Nabel, 2002). The Gag and Pol proteins are the predominant targets for the cytotoxic T-cell response (Betts *et al.*, 1997; Cao *et al.*, 1997; Ferrari *et al.*, 1997). These structural antigens are relatively well conserved but nonetheless differ by about 10% between clades (reviewed by Spira *et al.*, 2003). Neutralizing antibodies (NAbs) are directed against the surface gp120 and transmembrane gp41 envelope glycoproteins (Env), which mediate receptor and coreceptor binding and subsequent fusion events for cell entry. Env is the most

A supplementary table is available with the online version of this paper.

divergent of the viral proteins and can differ up to 35% between clades, 20% within the same clade, and 10% within one infected individual (Shankarappa *et al.*, 1999).

HIV-1 is divided into the major (M), the outlying, and the new groups. The M group is the most common and makes up more than 90% of global infections. This group is further divided into clades A, B, C, D, F, G, H, J and K, but also includes circulating recombinant forms, such as CRF02_AG and CRF01_AE. Clade B is mostly prevalent in the Americas, Western Europe, Japan and Australia. Globally, clade C is the most common subtype and is responsible for around half of all infections worldwide. It is found in the southern and eastern parts of Africa, China, India and Nepal. Clades A and D are widespread in eastern parts of Africa. CRF02_AG is endemic in the western parts of Africa and CRF01_AE, although originating in Central Africa, can also be found in South-east Asia.

Clade C, A, D and CRF02_AG infections are widespread in sub-Saharan Africa, where HIV-1 and AIDS are most prominent. Hence, research in recent years has focused on these non-clade B viruses. Attempts have been made to understand if infections by certain clades mount humoral responses that differ in neutralization breadth and potency. However, reports so far fail to agree and indeed are conflicting. A number of early studies reported that neutralization activities are sporadic and independent of genetic clade (Kostrikis *et al.*, 1996; Louisirothchanakul *et al.*, 1998; Moore *et al.*, 1996; Nyambi *et al.*, 1996; Weber *et al.*, 1996). Bures *et al.* (2002) demonstrated that clade C viruses from South African subjects were generally more susceptible to neutralization by heterologous subtype C plasma than to clade B plasma. Li *et al.* (2006a) showed that Zambian clade C plasmas could neutralize autologous viruses significantly better than plasma from American clade B-infected patients. Cavacini *et al.* (2005) showed that antibodies from clade C-infected patients from Zambia could inhibit infection of heterologous clade C virus, whereas antibodies from clade B plasma could not. However, plasmas from clade B and C subjects were equally efficient at inhibiting infection by clade B virus. Li *et al.* (2006b) showed that subtype C, A and D plasma pools were better at neutralizing clade C virions than the B pool. Finally, Brown *et al.* (2008) showed that a clade C plasma pool was exceptionally potent and broad at neutralizing a panel of viruses from six clades compared with five other pools of plasma. Discrepancies can be due to structural differences between viruses of different clades (Li *et al.*, 2006a; Lynch *et al.*, 2009). Patel *et al.* (2008) proposed subtype-specific (B versus C) V3 loops may limit cross-neutralization.

To date most studies have been limited by both patient sample size and a lack of geographical diversity. Also in the process of pooling sera, stronger or weaker activities of individual patient samples will be missed and may mask general patterns of neutralization potency. For vaccine design, a clear idea of how viral genotypes may influence

neutralization is important and we aimed to contribute to this debate.

The array of HIV subtypes encountered in East London, an area long associated with migration, is unusually heterogeneous, reflecting the diverse geographical origins of the population. Surveillance data indicate that approximately 53% of the HIV infections diagnosed in North East London between 2000 and 2009 have been acquired outside the UK, of which 82% were acquired in sub-Saharan Africa (Health Protection Agency, 2010). This unique population provides a particular opportunity to examine clade neutralization specificity independently of regional differences. First, we investigated the neutralization profile against a panel of 12 viruses from six different clades and showed that plasmas from individuals infected with either clade C, A and, to a lesser degree, CRF02_AG and CRF01_AE, were better at neutralizing a number of the viruses across clades compared with plasmas from clade B-infected individuals. We expanded the study of C and B plasmas to include 17 clade C viruses and 16 clade B viruses and confirmed the difference in potency.

RESULTS

Patients

We set out to characterize the neutralization profile in a sample of HIV-infected individuals who are receiving treatment and care in East London. So far, we have enrolled 396 patients (see Methods) who had been infected for at least 1 year at the time of enrolment. Of these, 210 were antiretroviral treatment-naïve at the time of recruitment. The sample of 396 subjects is made up of 61% men and 39% women. The country of birth is depicted (Table 1) showing that 50% of the sample comes from sub-Saharan Africa. Of these, Nigeria (14%), Uganda (17%) and

Table 1. The East London HIV-1 cohort: region of birth

The number of patients born in different regions is shown in the second column (percentages of total in parentheses). In the third column, the number of clade B patients from these areas (in parentheses: percentage of clade B-infected patients from specific region) are given.

Region	n (%)	Clade B; n (%)
Africa	198 (50)	2 (1)
UK	117 (29)	50 (43)
Europe	37 (9)	22 (60)
Asia	12 (3)	2 (17)
Americas	8 (2)	3 (38)
West Indies	6 (1)	1 (17)
Australia/New Zealand	7 (2)	2 (29)
Middle East	2 (0.5)	0
Unknown	7 (2)	0

Zimbabwe (16%) make up almost half of the African cohort. Twenty-nine per cent of the subjects are from the UK and the majority of the 21% non-African, non-UK recruits come from Europe (9%), although a number of patients from Asia, the Middle East, the Americas and Australia/New Zealand are included. As expected (Spira *et al.*, 2003), patients infected with clade B make up 43% of the UK-born sample and 60% of those from mainland Europe, whereas only 1% of the sub-Saharan African subjects are infected with clade B (Table 1, column 3). Where information is available, the majority of the viruses appear to have been acquired in the county of birth (Health Protection Agency, 2010). This geographical distribution corresponds to the clade profile, as clades A, C, D, F, G, H, CRF02_AG and CRF06_cpx, which are the most common viruses in sub-Saharan Africa, make up 50% of the infections (Table 2).

The African London cohort

Fig. 1 shows the map of Africa, where each patient is represented by a symbol and the clades are symbolised with different shapes. As expected from previous reports patients from the southern parts of Africa (South Africa, Zambia and Zimbabwe) predominantly harbour clade C viruses. Patients infected with clade CRF02_AG originate largely from the western part of Africa, although patients from here also carry HIV-1 of subtypes C, F, G and H. The

virus profile in patients from eastern Africa is not as clear, although, in addition to clade C, this is predominantly where clade D and A viruses are found (reviewed by Spira *et al.*, 2003). In summary, the clade profile correlates with the origin of the subjects and expected geographical clade adherence. By studying the clade and geographical correlations, at least for the African cohort, virus is generally likely to have been transmitted in the country of origin. We confirm that our cohort indeed is representative of the African pandemic in terms of clade identity.

Neutralization by patient plasma

HIV-1-infected individuals develop antibodies against the gp120 and gp41 proteins in the first few weeks following infection (Moore *et al.*, 1994; Tomaras *et al.*, 2008), and NAb to the autologous virus emerge a few weeks later (Li *et al.*, 2006a; Wei *et al.*, 2003). With time, a broader humoral reactivity can develop in some individuals (Aasa-Chapman *et al.*, 2004; Doria-Rose *et al.*, 2009; McKnight *et al.*, 1992; Richman *et al.*, 2003; Sather *et al.*, 2009; Wei *et al.*, 2003) targeting conserved sites in the Env. As part of an international consortium within the Bill and Melinda Gates Foundation, we have set out to identify patients with strong neutralizing activity to heterologous virus, with the ultimate goal to develop neutralizing mAbs (Corti *et al.*, 2010) as tools for vaccine design. We studied the humoral response in treatment-naïve, chronically infected individuals (>1 year, $n=210$) and compared neutralization activity between groups of individuals infected with different clades. Similar studies have been done in the past, suggesting that neutralization activities are relatively independent of genetic subtype (Kostrikis *et al.*, 1996; Moore *et al.*, 1996; Nyambi *et al.*, 1996; Weber *et al.*, 1996), but contrasting findings have also been reported (Braibant *et al.*, 2006; Bures *et al.*, 2002; Cavacini *et al.*, 2005; Li *et al.*, 2006a; Mascola *et al.*, 1996; Moore *et al.*, 1996; Samleerat *et al.*, 2009). The discrepancies between such reports could be due to differences in geographical rather than clade origin of infected patients. Our cohort is geographically diverse, where for example clade C-infected individuals come from 20 different countries and those infected with CRF02_AG are born in nine countries. This variety can potentially exclude regionally associated factors in clade specificity of neutralization.

We looked at the neutralization capacity in plasma of 210 treatment-naïve patients against a panel of 11 replication-competent viruses and one pseudotyped virion (CA018) from six different clades (see Methods). We selected the viruses to cover coreceptor usage (six CCR5, four CXCR4 and two dual-tropic) and a range of neutralization sensitivity. As the gp160 of CRF02_AG is predominantly made up from clade A (Howard & Rasheed, 1996) we used CRF02_AG to represent both subtypes. Patients infected with an unknown subtype, dually infected, HIV-2, or belonging to groups of less than five patients (clade F, H,

Table 2. The East London HIV-1 cohort: clades

The number of individuals infected by different clades is shown in the second column. In the third column, the number of treatment-naïve patients whose plasmas were tested in the initial neutralization screen is shown. Only groups with more than five tested samples are further discussed, and results from patients infected with an unknown clade, or recombinants ($n<5$) are excluded from further analysis.

Clade	Patient (n)	Off therapy (n)
A	39	9
B	82	75
C	89	34
D	17	6
F	3	2
G	8	5
H	1	1
CRF01_AE	19	15
CRF02_AG	31	16
CRF06_cpx	6	1
CRF11_cpx	1	1
CRF12_BF	1	1
CRF30_0206	1	1
CX	2	1
BG	3	2
Others recombined	11	9
Unknown	78	28
HIV-2	4	3



Fig. 1. The clade diversity of the African East London HIV-1 cohort. Numbers in parentheses are the numbers of patients sampled who were born in that country. Each symbol represents one patient.

cpx and some recombinants) are not discussed in detail. Only 160 infections (clades A, B, C, D, G, CRF01_AE and CRF02_AG were significantly represented; Table 2) were included for analysis.

Neutralization of clade C viruses

In the first instance, plasmas were screened at a dilution of 1/40. The level of neutralization was calculated as percentage of inhibition of infection where 100% means a complete block of infection. Comparing the distribution of neutralization capacity in plasmas from patients infected with viruses from different clades indicates that there are some interesting trends in our cohort (Fig. 2). We had chosen the two clade C viruses 38G1 (also known as 28g) and 27B based on their genetic diversity, which are described in detail in Koh *et al.* (2010). A third clade C virus [K530(6)] was included, as it is a highly neutralization-sensitive virus, obtained from an asymptomatic patient (infected >5 years). Plasmas from C-infected individuals ($n=34$) showed a particularly strong neutralization of all the three clade C viruses (Fig. 2). For example, the median of clade C plasmas for the 38G1 virus is 72.8 [interquartile range (IQR) 51.1–87.7], whereas the corresponding values for plasma from clade B-infected subjects ($n=75$) is 44.5 (IQR 28.8–59.7), a difference that is statistically significant (non-parametric, Mann–Whitney test: $P(38G1) < 1 \times 10^{-4}$). Similarly, neutralization by clade C-infected plasma of the very neutralization-sensitive virus

K530 (81.8; IQR 66.4–90.0) was higher [$P(K530) < 1 \times 10^{-4}$] than the clade B plasma values (48.4; IQR 34.9–59.7). The clade C plasmas neutralized virus 27B significantly better than clade B plasmas [$P(27B)=1 \times 10^{-4}$]. Similarly, clade A plasmas ($n=9$) neutralized two out of the three C viruses more efficiently than the B plasmas [$P(K530) < 1 \times 10^{-4}$; $p(27B)=0.05$], as did the D plasmas [$n=6$; $P(K530)=0.037$, $P(27B)=0.005$]. The clade CRF02_AG plasmas ($n=16$) neutralized the C viruses 27B and K530 better than clade B plasmas [$P(27B)=0.029$, $P(K530)=0.004$], whereas CRF01_AE ($n=15$) and the G ($n=5$) plasmas neutralized the K530 virus better than the B samples ($P=0.005$ and $P=0.04$, respectively).

In HIV-1 infection, neutralization breadth and potency develop with time (Aasa-Chapman *et al.*, 2004; Doria-Rose *et al.*, 2009; McKnight *et al.*, 1992; Richman *et al.*, 2003; Sather *et al.*, 2009; Wei *et al.*, 2003). However, the time of infection is unknown for the majority of patients in this cohort, hence we used age as a surrogate marker. Here, the mean age of the clade B-infected individuals was 38.4 (median 38; IQR 33–43) whereas that in the clade C group was 40.8 (median 40; IQR 35.5–47), a difference that is not statistically significant (Student's *t*-test). Additionally none of the other clade groups (A, B, D, G, CRF01_AE and CRF02_AG) were older than the clade B patients. Gender differences cannot explain the greater potency of neutralization as there was no difference in neutralization potency between plasmas from clade C-infected women and men.

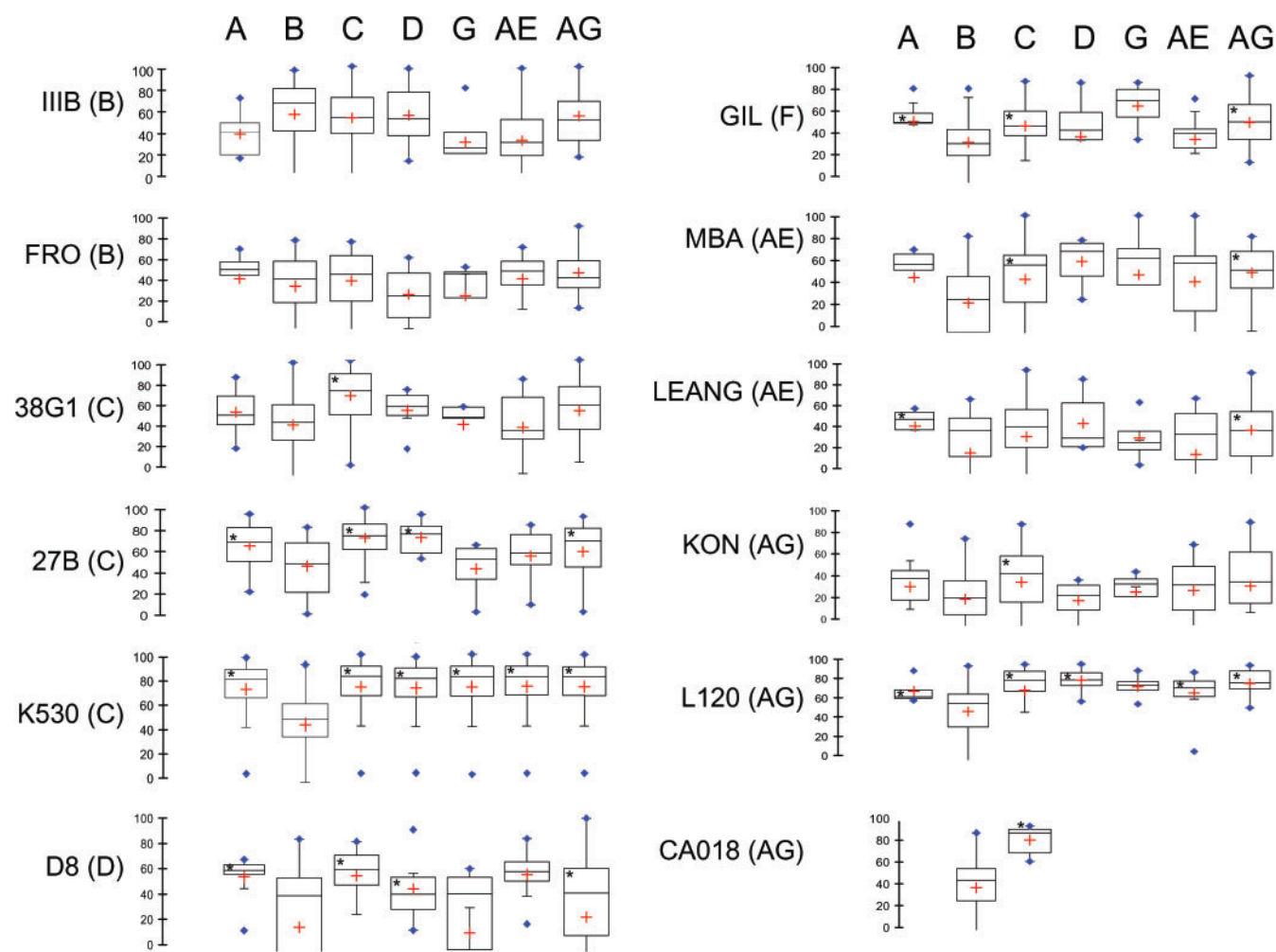


Fig. 2. Neutralization assays were performed on TZM.bl cells with 12 different viruses. Patients were grouped by clades (upper rows). Box plots show the distribution of neutralization (0–100%, y-axis), where the horizontal lines represent the lower adjacent, 25th, median, 75th and upper adjacent percentiles. The mean score is indicated by a red plus sign and far out values are shown by blue diamonds. Stars (*) show significant differences between clade B and the other groups (Mann–Whitney non-parametric test: $P < 0.05$).

Other factors such as CD4 counts and viral loads have been previously excluded as contributing to neutralization potency (Wei *et al.*, 2003).

Neutralization of CRF02_AG viruses

The clade CRF02_AG virus L120 was relatively sensitive to neutralization by most plasmas (Fig. 2), although antibodies from clade B-infected patients were significantly less potent at neutralizing this virus, compared with plasmas from clade A- ($P < 0.0001$), C- ($P = 0.003$), D- ($P = 0.007$), CRF01_AE- ($P = 0.044$) and CRF02_AG- ($P = 0.001$) infected individuals. KON was a more neutralization-resistant CRF02_AG virus, but, again, clade C plasmas were more effective at neutralizing this virus than clade B samples ($P = 0.006$). The third clade CRF02_AG virus CA018 virion was added to the panel at a later time point, and only clades C and B plasma

results are shown. Again, this virus was significantly better neutralized by clade C plasmas ($P = 0.002$).

Neutralization of one D and one F virus

The clade D virus D8 was a relatively neutralization-resistant virus, but clade C ($P < 0.0001$), A ($P = 0.008$), D ($P = 0.05$) and CRF01_AE ($P = 1 \times 10^{-5}$) plasmas neutralized D8 significantly better than the B plasmas. GIL (clade F) was neutralized to a larger extent by antibodies from C- ($P = 0.006$), A- ($P = 0.05$) and CRF02_AG- ($P = 0.009$) infected subjects than patients infected with clade B.

Neutralization of CRF01_AE viruses

Following the report from the HIV-1 vaccine trial in Thailand (Rerks-Ngarm *et al.*, 2009) it is interesting to look

at viruses derived from this region, as well as focusing on the immune response of people infected with CRF01_AE virus. LEANG and MBA were both more sensitive to neutralization by plasmas from CRF02_AG-infected individuals [$P(\text{LEANG})=0.03$, $P(\text{MBA})=0.006$] than from clade B-infected patients. Plasmas from clade C-infected patients neutralized MBA better than the B plasmas ($P=0.005$), whereas A plasmas were better at neutralizing the virus LEANG ($P=0.03$). Interestingly, the CRF01_AE plasmas did not neutralize the CRF01_AE viruses any better than the clade B plasma antibodies.

Neutralization of clade B viruses

Plasmas from clade B-infected individuals were relatively poor at neutralizing many of the non-clade B viruses, compared with those infected with other clades. However, there did not seem to be a general weakness to neutralize, as the T-cell line-adapted IIB was equally sensitive to antibodies from clade B-, C-, D-, CRF01_AE- and CRF02_AG-infected patients. The potency of plasmas from clade A and G seem somewhat less; however, a significant difference was not obtained. Similarly, the relatively neutralization-resistant virus FRO (Barin *et al.*, 2004) was equally sensitive to neutralization from all subtypes of plasma.

Inhibitory dose (ID₅₀) of C and B plasmas on a separate virus panel

IIB is a well-characterized T-cell line-adapted tier 1B virus (Seaman *et al.*, 2010). Interestingly, it was not more sensitive to neutralization than some of the primary isolates (Fig. 2) used in this study, notably K530(6), but also 27B (clade C) and L120 (CRF02_AG). To ensure that the inclusion of such neutralization-sensitive viruses had not skewed the interpretation of the results, we expanded the panel of C and B viruses (17 and 16, respectively) to include tier 2-matched viruses (Seaman *et al.*, 2010) (see Supplementary Table S1, available in JGV Online). The titres of nine randomly chosen clade C and 37 clade B plasmas were determined. Again, the geometric mean ID₅₀ for the C and B plasmas (Fig. 3) imply a general trend of stronger neutralization of clade C viruses by plasmas from clade C-infected individuals than by the clade B plasmas (Fig. 3a), and significance ($P \leq 0.05$) was achieved with 16 of the 17 viruses. This confirms the results in Fig. 2 and supports previous reports (Bures *et al.*, 2002; Cavacini *et al.*, 2005; Li *et al.*, 2006a, b). The tendency of more potent neutralization in clade C plasmas was confirmed using clade B viruses. Significance was reached for eight out of 16 viruses (Fig. 3b).

DISCUSSION

We show that infection with HIV-1 clade C, A and to a lesser degree D, CRF02_AG and CRF01_AE induce stronger and broader NABs against a wide range of virus clades compared with patients infected with clade B viruses.

Clade C is endemic in eastern and southern Africa. Moreover, transmissions in both India and China are continuing to rise, suggesting a further clade C subepidemic in Asia (Arora *et al.*, 2008). For this reason, attention to HIV-1 clade C has surged in recent years, with vaccine attempts high on the agenda. One major question is whether clade-specific viral proteins need to be included in a vaccine to protect against certain subclasses of virus. Hence, understanding the details of the immune response to the virus would guide the rational design of vaccines. Previous reports have suggested that clade C patients generate more potent NABs against heterologous or autologous clade C viruses than B-infected patients (Brown *et al.*, 2008; Bures *et al.*, 2002; Cavacini *et al.*, 2005; Li *et al.*, 2006a). It could be argued that such neutralization potencies are due to the genetics of specific ethnic groups rather than immunogenicity of the virus.

Since our cohort is more geographically diverse than many previously reported patient groups (we have recruited clade C-infected subjects from 20 countries), we wanted to see if patient plasmas would preferentially neutralize heterologous viruses of the same clade or those of different clades.

Indeed, we demonstrated that antibodies from the clade C-infected group were better at neutralizing the three clade C viruses in our panel, compared with the clade B plasmas. This was further strengthened by comparing ID₅₀ values from a smaller set of patient plasmas on a larger viral panel. Again, the C plasmas neutralized 16 out of 17 viruses better than the B plasmas. Interestingly, the ID₅₀ values implied that the C plasmas were even more potent than the B plasmas in neutralizing the clade B panel, although significance was only reached for eight out of 16 viruses. This somewhat contrasts with the findings of Cavacini *et al.* (2005) who showed that C and B plasma neutralized B viruses equally well. This disparity may be a result of the different viral panels used, as well as the different cohorts, ours being more geographically diverse (see above).

Similar to the potency observed in the clade C plasmas from the initial screen with 12 viruses, clade A-infected subjects, and to a lesser degree plasmas from D-, CRF02_AG- and CRF01_AE-infected subjects had more potent NABs than clade B-infected individuals. This is very interesting as most studies have focused on comparing B- and C-infected individuals. Brown *et al.* (2008) included clades A, B, C, D, CRF01_AE and CRF02_AG in their study, and like us, demonstrated the exceptional breadth of potency of clade C and the relatively weak activity of clade B plasma. In contrast, they did not observe the potency of the clade A group. We conclude that clade B-exposed individuals are less efficient at generating NABs against heterologous viruses from other clades, compared with individuals infected with clades A, C and to a lesser extent D, CRF01_AE and CRF02_AG, who appear to develop a more robust and broader neutralization profile. This may be due to structural differences, where clade B viruses do not expose epitopes that trigger breadth of neutralizing response. Interestingly these differences still leave the clade

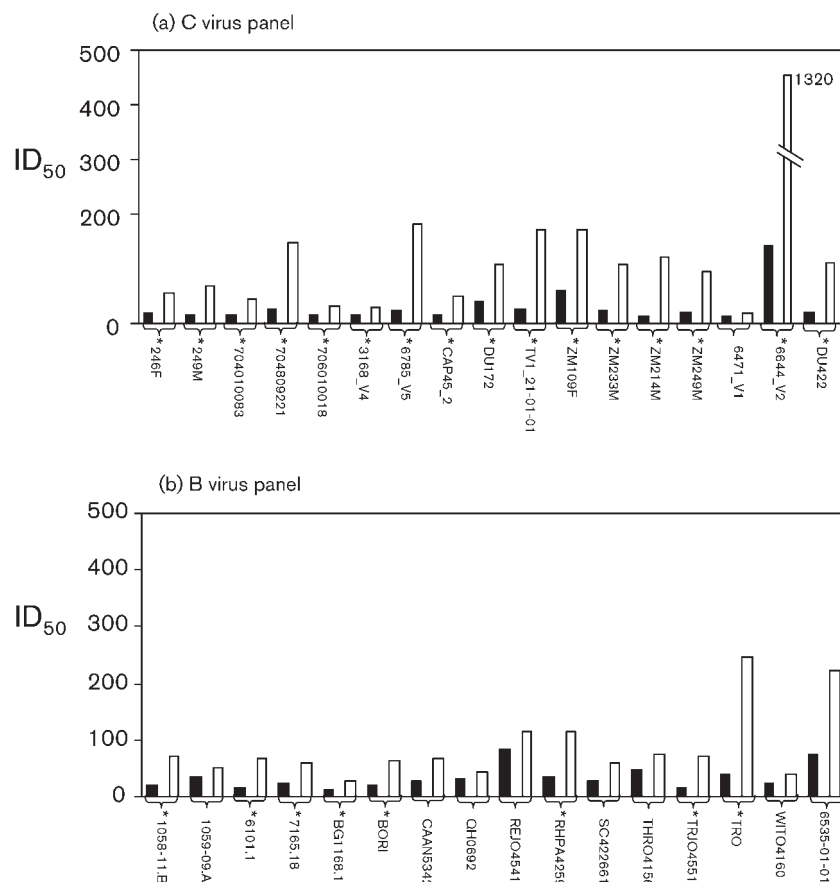


Fig. 3. Geometric mean of the ID₅₀ values of nine clade C plasmas (white bars) and 37 clade B plasmas (black bars) were determined (y-axis) for (a) 17 clade C viruses and (b) 16 clade B viruses. Stars (*) indicate viruses where there is a significant difference between the clade C and clade B plasmas (Mann–Whitney non-parametric test: $P < 0.05$).

B viruses vulnerable to neutralization by plasma from people infected with clade C (Fig. 3b).

A previous study (Wei *et al.*, 2003) excluded a role for clinical factors such as CD4 cell counts or viral loads in the potency of NAbs. In our study all the tested plasmas came from asymptomatic individuals who were off therapy at the time of recruitment; there was only one LTNP (long-term non-progressor; clade B, asymptomatic >10 years with normal CD4 counts). We found no connection between NAb potency and age (a surrogate for time since infection) or gender. We cannot, however, exclude the possibility that the mode of infection (heterosexual versus homosexual) could influence the potency of the humoral immune response. This warrants further investigation.

Taken together, we suggest that the differences we see are more likely to be due to the genetic subtype of the infected virus rather than the factors discussed above. Hence, in the era of cautious vaccine enthusiasm, these findings support the case for immunization with clade C- or A-derived Env antigens, at least for the induction of broad NABs.

METHODS

Cell lines. The TZM.bl (JC53-bl) cell line was obtained through the NIH AIDS Research and Reference Reagent Program, as contributed by

J. Kappes and X. Wu. This is a genetically engineered HeLa cell clone that expresses CD4, CXCR4 and CCR5 and contains Tat-responsive reporter genes for firefly luciferase (Luc) and *Escherichia coli* β -galactosidase under regulatory control of an HIV-1 LTR. HEK 293T cells originated from the ATCC. The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated FBS (Biosera). Cells were passaged with trypsin/EDTA solution (Gibco/Invitrogen) and grown at 37 °C in humidified air containing 10% (TZM.bl) or 5% (HEK 293T) CO₂.

Plasma samples. Informed consent was obtained from all study participants (ethical approval 06/Q0603/59) who were recruited at the Grahame Hayton Unit, Barts and The London Hospital; Centre for the Study of Sexual Health and HIV, Homerton University Hospital; and Andrewes Unit at St Bartholomew's Hospital. Clade data were available as part of the in-house antiretroviral resistance assay, in which sequences were obtained for all of protease and for aa 1–335 of reverse transcriptase. The plasma samples were heat inactivated at 56 °C for 1 h followed by 10 min centrifugation at 10 000 r.p.m. (Thermo Scientific IEC MicroCL 17).

Primary and tissue culture adapted HIV-1 stocks. LEANG (Cambodia, CRF01_AE, CCR5/CXCR4; Samleerat *et al.*, 2008), MBA (Cambodia, CRF01_AE, CCR5/CXCR4), GIL (clade F, CCR5), FRO (clade B, CXCR4) and KON (CRF02_AG, CXCR4), described in Barin *et al.* (2004), were kindly provided by Professor Francis Barin at Université François Rabelais, Tour, France. Viruses were passaged once or twice in phytohaemagglutinin-stimulated PBMC. IIIIB (T-cell line-adapted, clade B, CXCR4) has been described previously (Matthews *et al.*, 1986; Weiss *et al.*, 1986) and was propagated in H9 cells (Centralised Facility for AIDS Reagents).

Construction of patient gp160-derived infectious chimaeric molecular clones. K530(6) (Zimbabwe, clade C, CCR5) and L120 (Congo, CRF02_AG, CCR5) are molecular clones where the gp160 genes were amplified from genomic DNA from patient blood (infected for >1 year) using nested PCR, as described previously (Zheng & Daniels, 2001), with some modifications (inner forward 5'-AGC-TCGAGCAGAAGACAGTGGCAATGA-3' and inner reverse 5'-CTA-TGGAATTCTTCGACCACTTGCCCCATTT-3'). The PCR products were subcloned into TopoTA vector (Invitrogen) and transferred into the C2/NL43 vector (Zheng & Daniels, 2001) between the *XhoI* and *EcoRI* sites. The molecular clones 27B (Malawi, clade C, CCR5, GenBank accession no. FJ977091), 38G1 (Scotland, clade C, CCR5, GenBank accession no. FJ977093) (Koh *et al.*, 2010) and 92UG001.D8 (Uganda, clade D, CXCR4) were generous gifts from Dr Willie Koh and Dr Anna Forsman at University College London.

Virus stocks. Virus stocks from the replication-competent HIV-1 molecular clones were produced by polyethylenimine (PEI; Polysciences) transfection of HEK 293T cells. Briefly, 10 cm-tissue culture dishes were seeded with HEK 293T cells 24 h prior to transfection. The full-length molecular clone (6 µg) was mixed with 150 µg PEI in 1 ml serum-free DMEM, incubated for 10 min at room temperature and added dropwise to the cells. After 4–6 h at 37 °C, the medium was replaced with 7 ml DMEM with 10% FCS (Biosera). The viral supernatants were harvested after 48 h.

Pseudovirion stocks. The envelope gene in pcDNA3.1 from patient CA018 (Netherlands, CRF02_AG, CCR5) was provided by Dr Sunita Balla-Jhaghoorsingh from the Institute of Tropical Medicine, Antwerp. Virions were produced in HEK 293T cells with the packaging-reporter vector pNL4-3.Luc.R-E-, obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), NIH from Dr Nathaniel Landau. Briefly, 4 µg CA018 vector and 4 µg pNL4-3.Luc.R-E- were mixed and processed as described above.

Neutralization assay. Virus neutralization was measured using a Luc-based assay in TZM.bl cells as previously described (Li *et al.*, 2005). For the large throughput screen, all plasma samples from drug-naïve patients were diluted 1/20 in 50 µl DMEM with 10% FCS in a 96-well flat-bottom opaque Nunc plate. To this was added 50 µl of virus (200 TCID₅₀; yielding 10⁴–1.5 × 10⁵ light units) and the plate was left for 1 h at 37 °C. Then 10⁴ TZM.bl cells in 100 µl DMEM with 10% FCS, containing 50 µg DEAE-dextran (Sigma) ml⁻¹ was added. Assay controls included TZM.bl cells alone (cell alone control), plasma from HIV-1 negative control subjects and wells with virus and cells only (positive control). After 48 h of incubation at 37 °C, 150 µl of the medium was removed from each well, and 50 µl Bright-Glo Luc reagent (Promega) was added. After 2 min, luminescence was measured using a FLUOstar Optima (BMG Labtech). Percentage of neutralization was calculated as 100 × [1 – (sample luminescence/positive control luminescence)] after the cell alone control luminescence was subtracted.

The ID₅₀ neutralization assays were performed in the laboratories of Michael Seaman, David Montefiori and the NIAID Vaccine Immunogenicity T-cell and Antibody laboratory (NVITAL) for the Collaboration of AIDS Vaccine Discovery (CAVD).

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