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# Escape of human immunodeficiency virus type 1 from broadly neutralizing antibodies is not associated with a reduction of viral replicative capacity *in vitro*

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**Abstract**

Although the majority of primary HIV-1 variants can be neutralized by broadly neutralizing antibodies such as b12, 2G12, 2F5 and 4E10, resistance to these antibodies has been reported as well. The ability of the broadly neutralizing antibodies to inhibit a variety of viruses suggests that their epitopes are conserved and escape from these antibodies may thus come at a cost to viral fitness. Here we demonstrate that resistance to broadly neutralizing antibodies was in general not associated with a reduced replicative capacity of the virus *in vitro*. This indicates that loss of replicative capacity due to escape from broadly neutralizing antibodies may be limited.


*Keywords:* HIV-1; Escape; Broadly neutralizing antibodies; Replicative capacity

## Introduction

The envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) is exposed at the viral surface and is the target for neutralizing antibodies (Parren et al., 1999; Poignard et al., 2001). Primary virus isolates are relatively resistant to neutralization by antibodies directed to the envelope glycoprotein. Most primary HIV-1 isolates, however, can be neutralized by the four broadly neutralizing monoclonal antibodies (mAbs), b12, 2G12, 2F5 and 4E10 (Binley et al., 2004; Burton et al., 1994; Conley et al., 1994b; Moore et al., 1995; Parren et al., 1998; Trkola et al., 1995; Zwick et al., 2001), although additional mAbs of somewhat more limited neutralization breadth have also been described (e.g. 447-52D (Conley et al., 1994a), IgG1 X5 (Moulard et al., 2002), D5 (Miller et al., 2005)).

Neutralizing antibodies can be detected early in HIV-1 infection within 2 months after seroconversion (Albert et al., 1990; Montefiori et al., 2001; Moog et al., 1997; Parren et al., 1999; Pilgrim et al., 1997; Richman et al., 2003; Wei et al., 2003). Neutralizing activity of autologous serum against contemporary viruses is often low, suggesting that these viruses have escaped from humoral immune pressure and that neutralizing antibodies are functional in infected individuals (Albert et al., 1990; Arendrup et al., 1992; Bradney et al., 1999; Montefiori et al., 2003; Trachtenberg and Sande, 2002; Tremblay and Wainberg, 1990; Wei et al., 2003).

In newly HIV-1 infected individuals, a relatively neutralization sensitive virus population is assumed to be responsible for the primary infection (Derdeyn et al., 2004) although the enhanced neutralization sensitivity of recently transmitted HIV-1 was not confirmed by others (Frost et al., 2005). After development of a neutralizing antibody response in the infected patient, viruses with escape mutations will be rapidly selected, resulting in a relatively neutralization resistant virus population (Richman et al., 2003; Wei et al., 2003). This would imply that, in the absence of neutralizing antibodies, neutralization sensitive

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HIV-1 variants have a selective advantage over neutralization resistant viruses, which might be caused by an increased replicative capacity of the neutralization sensitive virus variants as compared to the resistant variants. Primary HIV-1 variants display differential neutralization sensitivity for broadly neutralizing antibodies (Quakkelaar et al., submitted for publication). As broadly neutralizing antibodies are considered to be directed at conserved epitopes, escape mutations in these epitopes may be associated with even larger changes in replicative capacity.

Here we studied the replication kinetics of HIV-1 variants isolated at different time points during infection from 6 patients and subsequently analyzed the potential relationship between the replication rate of these viruses and their sensitivity to broadly neutralizing antibodies.

## Results

### *Replication kinetics*

We previously showed differential sensitivity of primary HIV-1 variants ($n=55$) obtained at different time points in the course of infection from 4 recipients of homosexual transmission and 2 recipients of parenteral transmission to neutralization by the 4 broadly neutralizing antibodies IgG1b12, 2G12, 2F5 and 4E10 (Quakkelaar et al., submitted for publication). The differential neutralization sensitivity was even observed between coexisting clonal virus variants for a single antibody. Given the high level of genetic homology between these coexisting HIV-1 variants, they provide a unique tool to study the relationship between viral replicative capacity and neutralization sensitivity. Replication kinetics were determined during a period of 18 days after inoculation of PHA-stimulated PBMC with equal viral inocula (100 $TCID_{50}$ per culture). Replication rates varied between viruses obtained from a single individual, and even between viruses obtained from the same time point. As we reported previously (Blaak et al., 1998a), viruses isolated early in infection after homosexual transmission tended to have slower replication kinetics compared to viruses isolated later in infection, which was significant in patient ACH658 at multiple time points in the viral replication assay ($P=0.04$; Fig. 1A). HIV-1 variants isolated at sequential time points after parenteral transmission showed similar replication kinetics (data not shown). However, the time between the moments of virus isolation in these parenteral recipients may be too short to observe differences in replication rates (<8 months of follow-up for parenteral compared to >4 years for homosexual transmission cases).

ACH9012 was infected with both R5 and X4 viruses. P24 production of R5 and X4 viruses is shown in Fig. 1B. R5 viruses showed lower replication kinetics than coexisting X4 viruses relatively early in infection ($P=0.03$). This difference was less pronounced for viruses that were isolated 8 months later ($P=0.05$).

The lower replication rate of viruses isolated early in infection as compared to viruses isolated late in infection seems

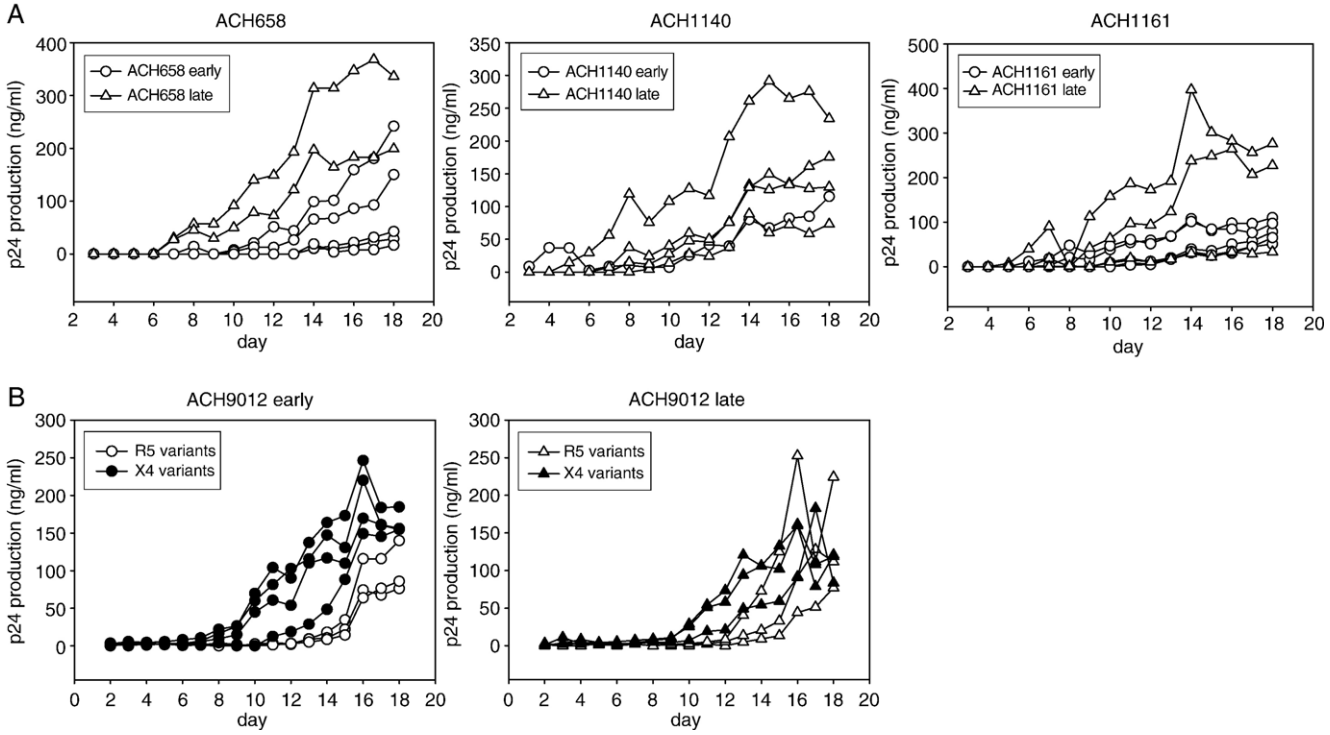


Fig. 1. Replicative capacity of viruses isolated at sequential time points after homosexual transmission (A) and parenteral transmission (B). P24 production of early variants (circles) and late variants (triangles) is plotted against the day of culture. Open symbols represent R5 variants, closed symbols represent X4 variants. For determination of replicative capacity $2*10^6$ PHA-stimulated PBMC were inoculated with 100 $TCID_{50}$ of a given HIV-1 variant in a total volume of 1 ml for 2 h at 37 °C in a shaking water bath. P24 antigen production in culture supernatant that was harvested each day was determined in one in-house p24 antigen capture ELISA.

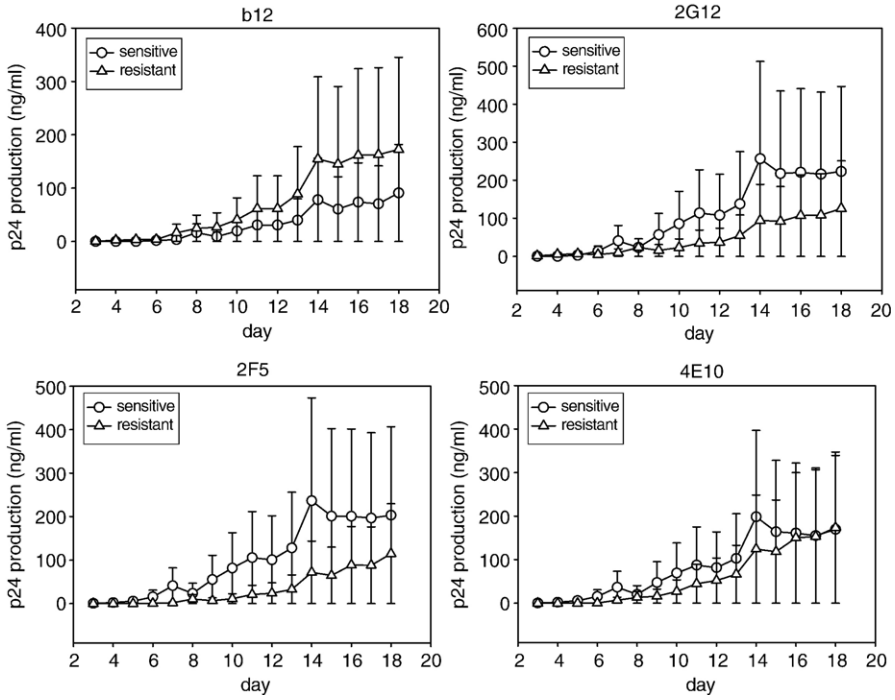


Fig. 2. Replication kinetics of viruses that were sensitive (circles) or resistant (triangles) to neutralization by b12, 2G12, 2F5 and 4E10. Mean p24 production of average values per patient values and standard deviations are indicated.

to contrast the current hypothesis that neutralization sensitive virus variants with high replication kinetics would be responsible for the primary infection (Derdeyn et al., 2004). In our study, the earliest viruses available after homosexual transmission were isolated 3 weeks after seroconversion which makes it unlikely that these viruses were slowly replicating due to adaptation to host immune responses since autologous neutralizing antibodies are absent this early in infection (Richman et al., 2003; Wei et al., 2003). To further address this issue, we determined the dynamics of viral replication early in infection using virus clones isolated at several time points within the first 2 months after seroconversion upon homosexual transmission from an individual with acute infection. Replication rates of these viruses were low and did not change within the first 2 months after seroconversion (data not shown). In summary, viruses isolated early in infection from recipients of homosexual transmission tended to have slow replication rates which increased during long-term infection.

*Viral replication kinetics in relation to sensitivity to broadly neutralizing antibody neutralization*

As stated above, we previously determined the neutralization sensitivity of the viruses in our study for the broadly neutralizing antibodies b12, 2G12, 2F5 and 4E10 (Quakkelaar et al., submitted for publication). We next determined whether HIV-1 replication kinetics and neutralization sensitivity for the broadly neutralizing antibodies are indeed correlated. Replication kinetics of neutralization sensitive and resistant viruses obtained from recipients of homosexual transmission are shown in Fig. 2. Viruses were considered neutralization sensitive when the $IC_{50}$ value for the broadly neutralizing antibody was lower than 12.5 μg/ml. Replicative capacity was not different between viruses that were resistant or sensitive to neutralization by 4E10. Viruses resistant to b12 neutralization tended to have higher replication kinetics than viruses that were sensitive to b12 neutralization ($P=0.08$). In contrast, viruses that were sensitive to 2G12 and 2F5 neutralization replicated faster than resistant viruses ($P=0.01$ and $P=0.04$, respectively). Replication kinetics of neutralization resistant and sensitive viruses obtained from recipients of parenteral transmission were not different (data not shown).

We subsequently studied whether the level of virus replication was correlated with the level of viral neutralization sensitivity for individual virus variants from each patient. We calculated Pearson correlations between $IC_{50}$ values of IgG1b12, 2G12, 2F5 and 4E10 for a given virus variant and the gag p24 production in culture medium on the average day of maximum virus production (day 15) of the replication assay performed with that same virus variant. Pearson correlations are indicated in Table 1. When only 2 virus variants were studied,

Table 1
Correlation between replicative capacity and neutralization sensitivity (Pearson correlations are indicated)

| Individual | | $IC_{50}$ b12 | $IC_{50}$ 2G12 | $IC_{50}$ 2F5 | $IC_{50}$ 4E10 |
|---|---|---|---|---|---|
| ACH1140 | $n=5$ | 0.472 | 0.396 | 0.911[¶] | 0.039 |
| ACH658 | $n=7$ | 0.884* | −0.872[§] | −0.480 | 0.734 |
| ACH1161 | $n=8$ | 0.043 | −0.263 | NA | −0.730[$] |
| F | $n=17$ | −0.026 | NA | −0.448 | −0.108 |
| p127 | $n=11$ | −0.323 | 0.820[#] | 0.163 | NA |
| ACH9012 | $n=6–7$ | −0.084 | −0.693 | −0.586 | −0.833[$] |

NA, not applicable. $IC_{50}$ value is constant. *$P=0.008$; [§]$P=0.01$; [¶]$P=0.03$; [$]$P=0.04$; [#]$P=0.001$.

results were excluded from the analysis. HIV-1 variants from 3 of 6 patients showed a negative correlation between replication rate and neutralization sensitivity for one of the antibodies (ACH658, ACH1161 and ACH9012 for antibodies 2G12, 4E10 and 4E10, respectively). However, 3 of 6 patients showed a positive correlation between replication and neutralization for one of the antibodies (patients ACH658, ACH1140 and p127 for antibodies b12, 2F5 and 2G12 respectively).

In conclusion, reduced replicative capacity was not a general feature of neutralization resistant virus variants. However, negative correlations were observed for some viruses from some patients.

## Discussion

It is generally accepted that a vaccine against HIV-1 should elicit broadly neutralizing antibodies to provide sterilizing immunity. To date, only 4 such antibodies (b12, 2G12, 2F5 and 4E10) have been identified although additional mAbs of somewhat more limited neutralization breadth have also been described (Binley et al., 2004; Burton et al., 1994; Conley et al., 1994a,1994b; Miller et al., 2005; Moore et al., 1995; Moulard et al., 2002; Parren et al., 1998; Trkola et al., 1995; Zwick et al., 2001). Considering the breadth and reactivity of mAbs b12, 2G12, 2F5 and 4E10, their epitopes are expected to be conserved between HIV-1 variants (Binley et al., 2004). The conserved nature of these epitopes suggests that mutations in HIV-1 that allow escape from these antibodies may come at a severe cost to viral fitness. In the present study, we determined for the first time the replication kinetics of viruses from which we previously established the sensitivity to the broadly neutralizing antibodies b12, 2G12, 2F5 and 4E10 (Quakkelaar et al., submitted for publication). Viruses were isolated at sequential time points in the course of infection from 6 patients. We observed that replication rates were different between viruses from different patients as well as between viruses from the same patient, even between viruses that were isolated from the same blood sample. Overall, virus variants isolated early in infection tended to have slower replication kinetics than viruses isolated later in infection, in agreement with previous observations by us and others (Blaak et al., 1998a; Kwa et al., 2003; Repits et al., 2005; Troyer et al., 2005; Van 't Wout et al., 1998). Apparently, the virus phenotype best adapted to be transmitted or to replicate and persist in the early asymptomatic phase of infection coincides with a reduced replicative capacity *in vitro*. Probably, during infection, viruses with the highest replication kinetics are selected which may relate to target cell availability and (co)receptor expression levels on these target cells.

Overall, we did not observe a correlation between viral replication kinetics and neutralization sensitivity for the broadly neutralizing antibodies. In other words, resistance to broadly neutralizing antibodies did not generally coincide with a reduction in replicative capacity. However, resistance to 2G12 or 4E10 neutralization of HIV-1 variants from 3 patients was associated with reduced replicative capacity, whereas resistance to neutralization by b12, 2G12 or 2F5 was associated with increased replicative capacity for viruses from 3 patients.

In our previous study we noticed that resistance to broadly neutralizing antibodies was not necessarily associated with mutations in the respective epitopes (Quakkelaar et al., submitted for publication) suggesting a role for mutations outside the targeted epitopes that influence for instance the conformation of the epitope. We therefore next compared the replication kinetics of neutralization sensitive and resistant viruses that had or did not have substitutions of residues involved in the epitopes of the broadly neutralizing antibodies under study (Calarese et al., 2003; Sanders et al., 2002; Scanlan et al., 2002; Zwick et al., 2005). Mutations in the epitopes for 2F5 and 4E10 did not affect viral replication *in vitro*. Viruses that lacked at least one of the five potential N-linked glycosylation sites (PNGS) involved in 2G12 binding had higher replication kinetics than viruses with all 5 PNGS present ($P<0.01$, Mann–Whitney $U$ test, data not shown). Although the absence of 1 or more of the 5 PNGS involved in 2G12 binding does not reflect a lower overall number of PNGS in the viral envelope, it suggests that fewer PNGS at the specific positions that contribute to the 2G12 epitope may favor higher replication rates, for instance by facilitating binding of the virus to the cellular entry complex. However, a correlation between replicative capacity and number of PNGS in V1-V5 was not observed (data not shown).

The fact that from all patients at least some virus variants could be neutralized by the broadly neutralizing antibodies supports the idea that these antibodies are directed against conserved epitopes in the envelope glycoprotein. Alternatively, these epitopes may be only conserved due to the absence of b12, 2G12, 2F5 and 4E10 like antibodies in most HIV-1 infected individuals and thus the lack of selection pressure on these epitopes. Some virus variants studied here were, however, resistant to neutralization by the broadly neutralizing antibodies, which may imply that neutralizing antibodies directed against these epitopes are present in these individuals and that selection against these conserved epitopes does exist *in vivo*. Viral replication rate and sensitivity to broadly neutralizing antibodies may not be associated for the majority of HIV-1 variants. Alternatively, a reduction in viral fitness associated with escape mutations in this region might be too small to be detected in the replication assay as we performed here. In agreement, subtle differences in viral fitness associated with escape from cytotoxic T lymphocytes (CTL) were also not detected when replication kinetics were compared and only observed in direct competition experiments between wild type and mutant viruses (Martinez-Picado et al., 2006). On the other hand, fitness differences that are relevant *in vivo* may be absent *in vitro* due to for instance different target cell availability. Finally, as suggested for CTL escape variants from HIV-1, neutralization resistant viruses may develop compensatory mutations by which replicative capacity is ultimately restored. The reduced replicative capacity of virus variants that were resistant to some antibodies in 3 patients might be due to the absence of compensatory mutations.

In this study, we have focused on the association between replicative capacity and the neutralization resistance of primary HIV-1 variants to 4 broadly neutralizing antibodies. One could

argue that it would have been more relevant had we tested the sensitivity to neutralization by broadly reactive polyclonal sera as resistance to a mix of neutralizing antibodies directed against different epitopes may indeed have more impact on viral fitness. Some of the viruses in our study, however, were resistant to all 4 broadly neutralizing antibodies tested, yet had replication kinetics similar to their coexisting neutralization sensitive HIV-1 variants (data not shown). These data imply that a vaccine eliciting broadly neutralizing antibodies of this kind may, when not providing complete sterilizing immunity, select for HIV-1 escape variants that will maintain optimal replication capacity, thus not slowing down the pandemic. This observation supports the need for additional neutralizing antibody specificities to be elicited by a successful vaccine.

## Material and methods

### *Patients and viruses*

Clonal virus variants isolated at sequential time points in the course of infection from 4 recipients of homosexual transmission who participated in the Amsterdam Cohort Studies on HIV infection and AIDS, and from two recipients of accidental or deliberate parenteral transmission (Lange et al., 1990; Veenstra et al., 1995) were used in this study (Blaak et al., 1998b; Van 't Wout et al., 1994). In addition, clonal virus variants isolated from an individual with primary HIV-1 infection were studied.

Clonal virus variants were previously obtained in multiple micro co-cultures of limiting amounts of HIV-infected patient peripheral blood mononuclear cells (PBMC) and phytohaemagglutinin (PHA)-stimulated PBMC from healthy seronegative blood donors, as described before (Schuitemaker et al., 1992; Van 't Wout et al., 1994). Virus stocks were prepared on PHA-stimulated PBMC. The total number of passages was kept to a minimum as this may change the primary phenotype of the viruses (Beaumont et al., 2004). Virus production was monitored using an in-house p24 ELISA (Tersmette et al., 1989). Upon sufficient virus production, virus stocks were frozen and the infectious titers of the stocks were quantified by determination of the 50% tissue culture infectious dose ($TCID_{50}$) in PHA-stimulated PBMC.

### *Cells*

PBMC were isolated from buffy coats by Ficoll density centrifugation. For stimulation, $5*10^6$ cells/ml were cultured for 2 days in Iscoves Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS, Perbio, Logan, Utah, USA), penicillin (100 U/ml, Gibco, Paisly, Scotland, UK), streptomycin (100 μg/ml, Gibco), cyproxin (5 μg/ml, Bayer, Mijdrecht, The Netherlands) and PHA (5 μg/ml, Remel Europe, Dartford, England, UK). Subsequently, PBMC ($10^6$/ml) were grown in the absence of PHA, in medium supplemented with 10 U/ml recombinant interleukin 2 (rIL2, Cetus Corporation, Emeryville, CA, USA) and polybrene (5 μg/ml; Hexadimethrine Bromide, Sigma, Zwijndrecht, The Netherlands).

### *Characterization of HIV-1 replication kinetics*

For determination of HIV-1 replication kinetics, $2*10^6$ PHA-stimulated PBMC were inoculated with 100 $TCID_{50}$ of a given HIV-1 variant in a total volume of 1 ml for 2 h at 37 °C in a shaking water bath. Subsequently, cells were washed with 8 ml IMDM containing 10% FBS (Perbio), penicillin (100 U/ml, Gibco) and streptomycin (100 μg/ml, Gibco) and resuspended at a concentration of $10^6$ cells/ml for culture. Fresh PHA-stimulated PBMC ($10^6$) in a volume of 0.75 ml were added at days 5, 8, 11 and 14. Cultures were maintained for 18 days. Samples (50 μl) for determination of p24 antigen production in culture supernatant were harvested each day. P24 production in all samples was determined at the same time using an in-house p24 antigen capture ELISA. P24 production per ml supernatant was determined and corrected for the differences in volume of culture supernatant.

### *Statistical analysis*

Statistical analyses were performed using the Mann–Whitney *U* test and Pearson correlations were determined using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance of differences in replication rate between viruses isolated early or late in infection or between neutralization sensitive and resistant viruses was calculated at each time point of the virus replication assay and considered significant when the *P* value was lower than 0.05 at multiple time points. Only the *P* value from the comparison of the last time point is given. Overall a result was considered statistically significant when a *P* value was lower than 0.05.

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