HIV-1 sensitivity to neutralization: biological and molecular studies

Beaumont, T.
Human immunodeficiency virus envelope V1V2 and alanine 370 determine CD4-binding site dependent neutralization

Tim Beaumont, Ad van Nuenen, and Hanneke Schuitemaker

Department of Clinical Viro-Immunology, Sanquin Research at CLB, and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, The Netherlands

submitted for publication
HIV-IIIB resistance to CD4-bs directed agents

Human immunodeficiency virus envelope V1V2 and alanine 370 determine CD4-binding site dependent neutralization

We previously reported the reversal of the neutralization sensitive human immunodeficiency virus type-1 (HIV-1) IIIB virus towards a neutralization resistant phenotype in an accidentally infected laboratory worker. This increased resistance coincided with a number of mutations, among which a change of the highly conserved glutamate (Glu) to an alanine (Ala) at position 370. Here we report that such an in vivo reversed isolate became sensitive again to neutralization by soluble CD4 (sCD4) and the CD4-binding site specific monoclonal antibody (MAb) IgG1b12 after propagation on primary peripheral blood leukocytes. Limiting dilution culture provided two viral clones with different neutralization sensitivity. The in vitro reversal to a neutralization sensitive phenotype was associated with 10 amino acid substitutions in gp120, including a back mutation Ala to Glu at 370. Chimeric molecular clones revealed that the Glu370 and mutations in V1V2, contributed to the in vitro increased neutralization sensitivity for CD4-binding site directed agents. Sensitivity to neutralization by chemokine antagonists or other envelope directed MAbs, except for the 48d MAb in the presence of sCD4, did not differ between the biological clones. Ala370 was not associated with reduced replication competence of the virus. Our data suggest that the Ala370 variant was best adapted to growth in vivo, whereas in the absence of neutralizing antibodies in vitro the Glu370 variant was most favorable in the HIV-IIIB like envelope structure.

Introduction

Human immunodeficiency virus type 1 (HIV-1) is able to persist in the human host by replication in CD4 T-helper-cells and macrophages, ultimately causing AIDS. For sterilizing immunity or even control of virus replication, a vaccine should induce significantly high levels of protective humoral and cellular immunity. Recent vaccine studies in macaques demonstrated that partial protection could be achieved, however control of virus replication was mainly achieved by CD8+ T-cell responses (1-4). One of the major problems in vaccine development is the neutralization resistance of primary HIV-1. Only upon passage and adaptation to T cell lines, HIV-1 becomes neutralization sensitive (5-9). The molecular conditions associated with this phenotypic change are still not fully understood (10;11), reviewed in (12). Insight into these conditions may provide ways to circumvent neutralization resistance and to increase the efficacy of humoral immunity. Sequence variation in HIV-1 envelope gp120/gp41 determines the molecular conformation of the complex, which can explain differences in chemokine receptor usage and cell tropism (13-15). Previously we studied viruses isolated from a laboratory worker (LW-F) who was accidentally infected with the HXB2d molecular clone in 1985 (16;17). Over time a broad cellular and humoral immune response developed and the patient experienced a typical clinical course, with the first AIDS defining event in 1993 (18-21). We showed that disease progression coincided with the in vivo escape from humoral immunity of the T cell line adapted (TCLA) HXB2d. Isolate FF3346, obtained 7 years after the assumed moment of infection, was resistant to neutralization by pooled patient sera and CD4 binding site (CD4-bs) directed agents like recombinant soluble CD4 protein (sCD4) and the IgG1b12 monoclonal antibody (MAb) (16). A total of 38 amino acids changes between HXB2d and the LW-F isolate were observed. According to the crystal structure and mutagenic studies of gp120, three mutations in FF3346, namely Ala281 to Val, Glu370 to Ala and Lys429 to Glu are directly involved in CD4 and coreceptor binding (numbers according to HXB2d sequence) (22-26). Of these three
residues, the Glu370 together with residues Asp368 and Trp427, have been described to be essential for CD4 binding (27) and to contribute 57% of the interatomic contacts between gp120 and CD4 (22;28). In the present study, we focussed on the role of amino acid 370 in the differential neutralization sensitivity of two biological cloned HIV variants that were obtained by limiting dilution of the bulk FF3346 isolate.

Materials and Methods

Cells
Experiments were performed on phytohemagglutinin (PHA) stimulated pooled peripheral blood mononuclear cells (PBMC) of at least five healthy blood donors by Ficoll density gradient centrifugation. For stimulation, 5 x 10⁶ cells/ml were cultured for 3 days in Iscoves modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), cyproxin (5 μg/ml) and PHA (5 μg/ml). Subsequently, cells (1 x 10⁶/ml) were grown in the absence of PHA, in medium supplemented with 10 U/ml recombinant interleukin 2 (Chiron Benelux BV).

Virus isolation
An HIV-1IIIB variant (FF3346) was re-isolated from an accidentally infected laboratory worker (LW-F) in 1992, approximately seven years after the assumed moment of infection (before 1986) (17). Isolate FF3346 was obtained by cocultivation of patient PBMC with healthy donor PBMC. Biological clones of FF3346 were obtained by cocultures of 50 FF3346 infected PBMC with 3 days PHA-stimulated healthy donor PBMC (10⁶ cells/well) in 96-well microtiter plates (29). Each week 1/3 of the culture supernatant was tested for the presence of p24 by an in-house p24 antigen capture ELISA. At the same time half of the cells were transferred to new 96-well plates, and 10⁵ fresh PHA stimulated PBMC were added to propagate the culture. When less than 1/3 of the microcultures showed evidence of virus production, clonality of a p24 positive culture was assumed. From p24 positive cultures virus stocks were grown and 50% tissue infectious dose (TCID₅₀) was determined by endpoint dilution.

Molecular cloning and expression of replication-competent viruses

Genomic DNA was isolated from PBMC infected with two biological clones (LW_G9 and LW_H8). Complete envelope was amplified by Taq and Pwo DNA polymerases (Expand High Fidelity; Boehringer Mannheim) using primer combinations and PCR conditions as described previously (16;31). PCR products were purified using GFX Purification Kit (Amersham Pharmacia) and inserted in the pGEM-T easy vector (Promega). Colonies were sequenced to check for proper inserts using BigDye Terminator Cycle Sequencing reaction containing AmpliTaq DNA polymerase, FS (ABI Prism, Applied Biosystems, Warrington, UK), according to the manufacturer's protocol on an ABI 373S automated sequencer. SalI/BamHI-digested plAI-2 vector (Figure 6a) (32). A glutamate was introduced in the LW_H8 region of LW_G9 was obtained by digestion with SalI and SstI (5786 to 6833) and ligated into the pH8 and pH8/Glu vectors, creating two additional vectors (pG9/118 and pG9/118/Glu).

To obtain recombinant viruses, 10 μg of SalI and BamH1 (5786 to 8480), numbers relative to HXB2d digested envelope fragments from LW_G9 and LW_H8 that were expressed in the pGEM-T vector were ligated into the plAI-2 plasmid containing the different envelope constructs were used to transfect 70% confluent 293T cells, using lipofectamine (Gibco BRL) as describe by the manufacturer. Two days after transfection, supernatant of 293T cells was used to infect PHA-stimulated PBMC.

Neutralizing agents and neutralization sensitivity of HIV-1 variants

Biological and molecular virus clones were tested for their neutralization sensitivity against increasing concentrations of scD4 and the IgGb12 MAb. The biological clones were also tested for sensitivity to neutralization by HIV1g, a preparation of purified polyclonal Ig derived from HIV-infected donors and AIDS, pooled sera of 34 patients from the Amsterdam Cohort. In addition we tested MAb gp13, gp68, F105, which recognize epitopes surrounding the CD4-bs of gp120 (33;34), MAb 902 which binds to the CD4-bs of gp120 (35), and IgGb12 which recognizes the CD4-bs of gp120 (36). We also tested sensitivity for MAbs 17b and 48d which both recognize the coreceptor binding site of gp120. This area is more exposed after binding to CD4, therefore neutralization by both antibodies was tested in the absence or presence of 0.5 μg/ml sCD4 (26;37).

Furthermore, we tested sensitivity of the viruses to the CXCR4 antagonists bicyclam AMD3100 (38) and polyphenasmin II-derived peptide T22 (Bachem AG, Bubendorf) (39). Both antagonists specifically bind to CXCR4 and inhibit entry of CXCR4-using but not CCR5-using HIV variants.
HIV-IIIB resistance to CD4-bs directed agents

From each virus isolate, an inoculum of 100 TCID₅₀/ml in a 100 μl final volume was incubated for 1 hour at 37°C with increasing concentrations of the neutralizing agents. Subsequently, the mixtures of virus with sCD4, sera or antibodies were added to 10° 3-day PHA stimulated human PBMC in 96 wells microtiter plates. For testing sensitivity to the CXCR4 antagonists, PHA stimulated PBMC were incubated with increasing concentrations of AMD3100 or T22 for 1 hour before viruses were added. To avoid interference of anti-p24 Abs in patient sera in our p24 ELISA, plates incubated with HIV-Ig or Amphi were washed the following day. On days 7 and 14 virus production in supernatants was analyzed in an in-house p24 antigen capture ELISA. Percent neutralization was calculated as the mean reduction in p24 production of triplicate cultures in the presence of the neutralizing agent compared to cultures with virus only (100%). If possible, 50% inhibitory concentrations (IC₅₀) were determined by linear regression.

Replication characteristics of HIV-1 variants

Analysis of replication kinetics was performed as described previously (31). In brief, PHA-stimulated PBMC (5.0 × 10⁶ cells) were incubated with 100 TCID₅₀ in a total volume of 1.5 ml for two hours at 37°C. Virus supernatant was removed and cells were incubated at a concentration of 1.0 × 10⁶ cells/ml. Every day 50 μl of supernatant was collected to measure p24 production. Fresh PHA stimulated PBMC (3 × 10⁶) were added on day 5, 8 and 11.

Results

Isolation and sequence analysis of biological LW-F clones

An HIV-IIIB isolate (FF3346) was obtained 7 years after accidental infection, by cocultivation of patient cells with stimulated donor PBMC (16;17). The homogenous nucleotide sequence obtained from this bulk FF3346 virus, suggested a clonal virus population. Relative to the original HXB2d virus, 38 amino acid substitutions were present, among which a glutamate (Glu) to alanine (Ala) at position 370 (16). After prolonged in vitro culture of this FF3346 isolate on PHA-stimulated PBMC, a variant with Glu at this position became evident (Figure 11). To study whether the FF3346 Glu370 variant was already present in a small minority in the earliest virus isolate, we performed a limiting dilution of the oldest available FF3346 PBMC coculture sample (29). Envelope sequence analysis of 15 biological clones that were obtained in this way indeed revealed the presence of a mixed virus population. Two virus clones with either Ala (clone LW_H8) or Glu (clone LW_G9) at position 370 were selected for further experiments. In addition to the substitution at position 370, nine mutations were present in the

Figure 1

Sequence analysis of two biological HIV-IIIB clones that were isolated by limiting dilution from PBMC infected with an isolate obtained seven years after accidental infection of LW-F (isolate FF3346). The predicted amino acid sequence of the envelope glycoproteins was derived from the consensus sequence of the envelope fragment V1 to V5 which was amplified and subsequently sequenced from DNA isolated from infected PBMC cultures. LW_H8 and LW_G9 are the two isolates obtained by biological cloning that either contain an alanine (H8) or a glutamate (G9) at position 370. Variable and constant domains are indicated and numbering of amino acid positions is relative to HXB2; dots indicate identical residues to reference sequence and - indicate insert or deletions compared to HXB2.

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Figure 2
Neutralization sensitivity of the biological clones LW_H8\textsuperscript{A} and LW_G9\textsuperscript{E}. (A) Virus (100 TCID\textsubscript{50}/ml) LW_H8\textsuperscript{A} and LW_G9\textsuperscript{E} were incubated with increasing concentrations of sCD4 and the IgGl12 MAb, or (B) with non-CD4-bs directed MAbsp gp13, gp68, 902 and F105 and polyclonal serum of HIV-1 infected patients (HIVIg and Ams\textsuperscript{hps}), for 1 hour at 37\textdegree C before PHA-stimulated PBMC were added. P24 production in supernatant was measured, and mean OD values were calculated from triplicate cultures. The percent neutralization was calculated by determining the reduction in supernatant p24 production in the presence of the neutralizing agent relative to control cultures lacking these agents. Experiments were performed at least twice.

Sensitivity to neutralization of LW_H8\textsuperscript{A} and LW_G9\textsuperscript{E}.

Since the 370 residue has been shown to be critical for CD4 binding \textit{in vitro} (24,25,40), we tested the sensitivity of LW_H8\textsuperscript{A} and LW_G9\textsuperscript{E} for neutralization by sCD4 and IgGl12 (Figure 2). Virus stock preparation, determination of virus titer and neutralization experiments were all performed on the same pool of human CCR5\textsuperscript{+/+} PHA-stimulated PBMC obtained from at least five healthy donors. In contrast to the LW_H8\textsuperscript{A} clone, the LW_G9\textsuperscript{E} variant was highly sensitive to neutralization by sCD4 and IgGl12 (Figure 2a). Fifty percent inhibitory concentrations (IC\textsubscript{50}) for sCD4 and IgGl12 were 12 \textmu g/ml and >100 \textmu g/ml for the LW_H8\textsuperscript{A} virus variant compared to 0.8 \textmu g/ml and 1.6 \textmu g/ml for the LW_G9\textsuperscript{E} variant. To exclude the development and selection of variants containing a Glu at position 370 in the background of the LW_H8\textsuperscript{A} isolate during the neutralization experiment, DNA was isolated from p24 positive cultures and sequence analysis was performed. In all experiments, only input virus was detected (data not shown).

In addition, we also tested whether the isolates differed in their sensitivity to neutralization by agents directed against other epitopes. This was determined by measuring the sensitivity of LW_H8\textsuperscript{A} and LW_G9\textsuperscript{E} to neutralization by the MAbsp gp13, gp68, F105 and 902 (Figure 2b).
No differences in neutralization sensitivity were found. Similarly no variation in sensitivity to polyclonal sera AMSp and HIV Ig was observed (Figure 2b).

Replication kinetics of LW_H8^A and LW_G9^E
The direct interaction of the Glu370 residue with CD4 is assumed to be structurally necessary for virus binding, entry and subsequent replication (24;41). Although we isolated a replication competent and genetically stable LW_G9^E and LW_H8^A variant by limiting dilution, the predominant outgrowth of the LW_G9^E isolate during bulk culture, prompted us to measure differences in short term replication capacity. As compared to the FF3346 isolate containing the conserved Glu amino acid at position 370, the LW_H8^A virus replicated with identical kinetics to comparable maximum levels (Figure 3).

Sensitivity to neutralizing agents directed against CD4-induced epitopes
As the 370 residue is located in the central part of gp120 and directly interacts with CD4, the presence of Ala or Glu at this position may have consequences for neutralization directed at structures that are only exposed after CD4 binding (26;37). Therefore the LW_H8^A and LW_G9^E isolate were tested for their sensitivity to neutralization after CD4 binding. Virus supernatant (100 TCID50/ml) was incubated with MAbs 17b or 48d in the absence or presence of 0.5 fig/ml of sCD4. The mix of virus, sCD4 and MAbs was incubated at 37°C for 1 hour before PHA stimulated PBMC were added. Both the LW_H8^A and LW_G9^E variant were resistant to 17b and 48d neutralization in the absence or presence of sCD4 (Figure 4). LW_G9^E, however, was more sensitive to 48d MAb induced neutralization when cultured in the presence of sCD4, compared to LW_H8^A. No neutralizing effect of MAb17b in the absence of presence of sCD4 was found for both viruses.

Sensitivity to CXCR4 antagonists
Both the LW_H8^A and LW_G9^E variants still showed a CXCR4 restricted coreceptor usage (data not shown). However, as the 370 residue could influence CXCR4 coreceptor binding affinity or possibly induce a changed binding site usage of CXCR4, we studied the sensitivity of the LW_H8^A and LW_G9^E variants for inhibition by the CXCR4 antagonists bicyclam AMD3100 (38) and the T22 peptide (39), which both specifically bind to the CXCR4 coreceptor. Replication of both variants was inhibited to the same extent by AMD3100 and the T22 peptide (Figure 5).
CD4-bs directed neutralization is determined by the V1V2 domain and residue 370

To gain insight in the minimal genetic changes responsible for the observed changes in neutralization sensitivity, chimeric viruses were generated. By using site-directed mutagenesis at position 370, a Glu residue was introduced in the PCR amplified envelope fragment of LW_H8\(^A\) and the complete envelope was cloned in the background of LAI. Individually or in combination with the Glu370, a Sall-StuI fragment containing the V1V2 region of the envelope gpl20, was exchanged (Figure 6a). Exposure of the chimeric viruses to sCD4 and IgG1b12 revealed that exchange of only the V1V2 domain (pG9/H8) or the presence of only the 370 Glu residue (pH8/Glu) in the background of LW_H8\(^A\) did not result in virus with a neutralization sensitive phenotype (Figure 6b). However, exchange of the V1V2 region of the LW_G9\(^E\) clone together with a Glu at position 370 in the background of the neutralization resistant envelope of the LW_H8\(^A\) virus (pG9/H8/Glu), resulted in a neutralization sensitive variant, analogous to the biological clone LW_G9\(^E\).

Discussion

We previously reported the reversal of the neutralization sensitive HIV-1 HXB2 variant to a neutralization resistant phenotype in an accidentally infected laboratory worker. The LW-F isolate FF3346, which was obtained at the time of AIDS diagnosis, was resistant to neutralization by CD4-bs directed agents (16). This increased resistance, as compared to the inoculum and the LW-F isolate fe0233 which was obtained earlier in the course of infection, was accompanied by a number of mutations in gp120, among which a mutation of the highly conserved residue 370 (Glu to Ala). The α3 sheet of gp120, which contains the 370 residue, is assumed to be important in folding of the bridging sheet during CD4 binding and in establishing the conformational changes between the inner and outer domains of gp120 after CD4 binding (23;42). As concluded from crystal structure studies, the CD4 binding pocket does not allow major amino acid changes, especially when the Phe43 of CD4 or the Trp100 of IgG1b12 is inserted (22;43). Therefore we hypothesized that the resistance to sCD4 and IgG1b12 could be simply due to the absence of the glutamate residue in the CD4-bs. Surprisingly, during long term passage on PHA-stimulated human PBMC in vitro, the coexistence of variants with either Ala or Glu at position 370 became evident. This Glu370 variant (LW_G9\(^E\)) was indeed sensitive to sCD4 and IgG1b12 MAb neutralization. Sensitivity to neutralization by polyclonal sera or MAbs recognizing residues surrounding the CD4-bs, the V3 loop or other conformational epitopes did not differ, a phenomenon that has been observed before (44). By construction of chimeric viruses in the background of the Ala370 variant (LW_H8\(^A\)) variant, we could demonstrate that in addition to the residue at position 370, mutation of Ser141 to Asn, Ser144 to Gly and Asn164 to Ser in the V1V2 domain contributed to this increased sensitivity to CD4-bs directed agents.

Neutralization by sCD4 and IgG1b12 but lack of neutralization by the pooled serum samples

![Figure 5](image-url)
HIV-IIIB resistance to CD4-bs directed agents

HIV-Ig and Ambs suggested that in these samples no IgG1b12 like monoclonal antibodies are present, or at least not in sufficient concentrations to neutralize the sensitive LW_G9E virus. Since these pools contain sera of many individuals this would implicate that in most patients this type of antibodies are not present.

It is tempting to speculate that in the patient CD4-bs directed antibodies were generated that may have selected for the escape mutant LW_H8A, as was also described by Mo et al. (45). Alternatively, the selection of Ala370 may be due to a more optimal receptor interaction, as compared to the Glu370 variant. However, prolonged in vitro culture did select for a variant containing a Glu at position 370, suggesting that Glu370 in the HXB2 envelope conformation was more beneficial to the virus, which is supported by the high level of conservation of this residue among different HIV-subtypes and clades.

Others have shown with site-directed mutagenesis that substitution of Glu370 in either the HXB2 (X4 dependent) or YU2 (R5 dependent) background resulted in loss of gp120 binding to both sCD4, MAbs that recognize the CD4-binding domain and to the MAbs 17b and 48d (23-26;40). Variation of this residue in the background of the LW-F IIIB variant influenced the CD4-bs directed neutralization sensitivity but was not associated with changes in short term replication capacity or coreceptor affinity. This suggests that during the evolution in vivo of HXB2, an envelope conformation was selected that allowed or even required the 370 Glu to Ala substitution. As previously reported (45), preservation of fitness of LW_H8A may be established by compensatory mutations elsewhere in gp120, such as the substitutions Arg146Gly, Arg166Lys, Lys171Glu and Lys192Thr in V1V2 (Figure |1) (16). Alternatively or in addition, compensation may

Figure|6
Schematic overview of chimeric LW-F clones (A) and sensitivity of these chimeras to CD4-bs directed neutralization (B). Four chimeric clones as described in the materials and methods section were constructed. Envelope constructs were cloned in the background of the pLAI-2 vector, using the unique Sall and BamHI restriction sites. The white bar in pH8 represent the envelope part derived from the neutralization resistant LW_H8A clone. The black bars, in pG9/H8 and pG9/H8/Glu represent the V1V2 domain of the neutralization sensitive LW_G9E clone. Site-directed mutagenesis of Ala370 to Glu in the background of pH8 is denoted pH8/Glu, or pG9/H8/Glu when combined with the V1V2 domain of LW_G9E. Neutralization experiments were performed as described in figure|1.
have been achieved by loss of a proline at 369, together with the Ile424Val, Lys429Glu, which are in close proximity of 370 and possibly are solvent accessible before CD4 binding. Furthermore these residues, like Ala281Val may be directly involved in CD4 binding (22).

Based on the differential sensitivity of LW_G9E and LW_H8A to neutralization by MAb 48d in the presence of sCD4 it is tempting to speculate that a Glu or Ala at 370 differentially affects the conformation of distal structures within gp120. Especially since no mutations in the coreceptor binding domain of the two isolates were observed (sequences were compared with studies of Rizzuto (23) and Thali (26)). Distal effects of mutations on antibody recognition in different epitopes, in different regions of the protein have been described before, and are indicative for the flexibility of the gp120/gp41 oligomeric complex (42;46-50). Alternatively, due to the resistance of LW_H8A for sCD4, the epitope for 48d may not have been exposed on this variant. This observation at least excludes a CD4-independent phenotype of LW_H8A since CD4-independent isolates are highly sensitive to neutralization by 48d and 17b in the absence of sCD4 (51;52).

Based on the in vivo reversal of the TCLA HXB2 variant to a neutralization resistant variant, we previously concluded that the neutralization resistance of primary HIV can indeed be considered as an escape mechanism. This was supported by the observation that despite cellular and humoral immune responses elicited in LW-F, virus replication could not be suppressed (18-21). In our present study, we have identified the residues in gp120 that seem to be crucial for this phenotypic change in the background of HXB2. It demonstrates that possibly deleterious mutations can be reversed in vivo, resulting in a phenotype generally observed for pathogenic primary HIV-1.

Acknowledgements
The authors wish to thank Ray Sweet (Smithkline Beecham) for kindly providing recombinant soluble CD4 and Alfred Prince for HIV1g, Jaap Goudsmid for Ams<sup>®</sup>, and Dominique Schols for the bicyclam AMD3100. The human monoclonal antibodies gp13 and gp68 were a kind gift of Martin Schutten, IgG1b12 was kindly provided by Paul Parren and Dennis Burton and we thank James Robinson for providing us with the 17b. The MAb 48d was obtained through the AIDS Research and Reference Reagent Program, NIH, contributed by James Robinson, respectively. For providing us with the full length sequence of pLAI-2 we thank Ben Berkhout. For critically reading the manuscript and helpful discussion we thank Ronald van Rij and Frank Miedema.

This work was supported by The Dutch AIDS Foundation grant number 1304.

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