HIV-1 sensitivity to neutralization: biological and molecular studies

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chapter|3

Reversal of human immunodeficiency virus type 1 IIIB to a neutralization-resistant phenotype in an accidentally infected laboratory worker with a progressive clinical course

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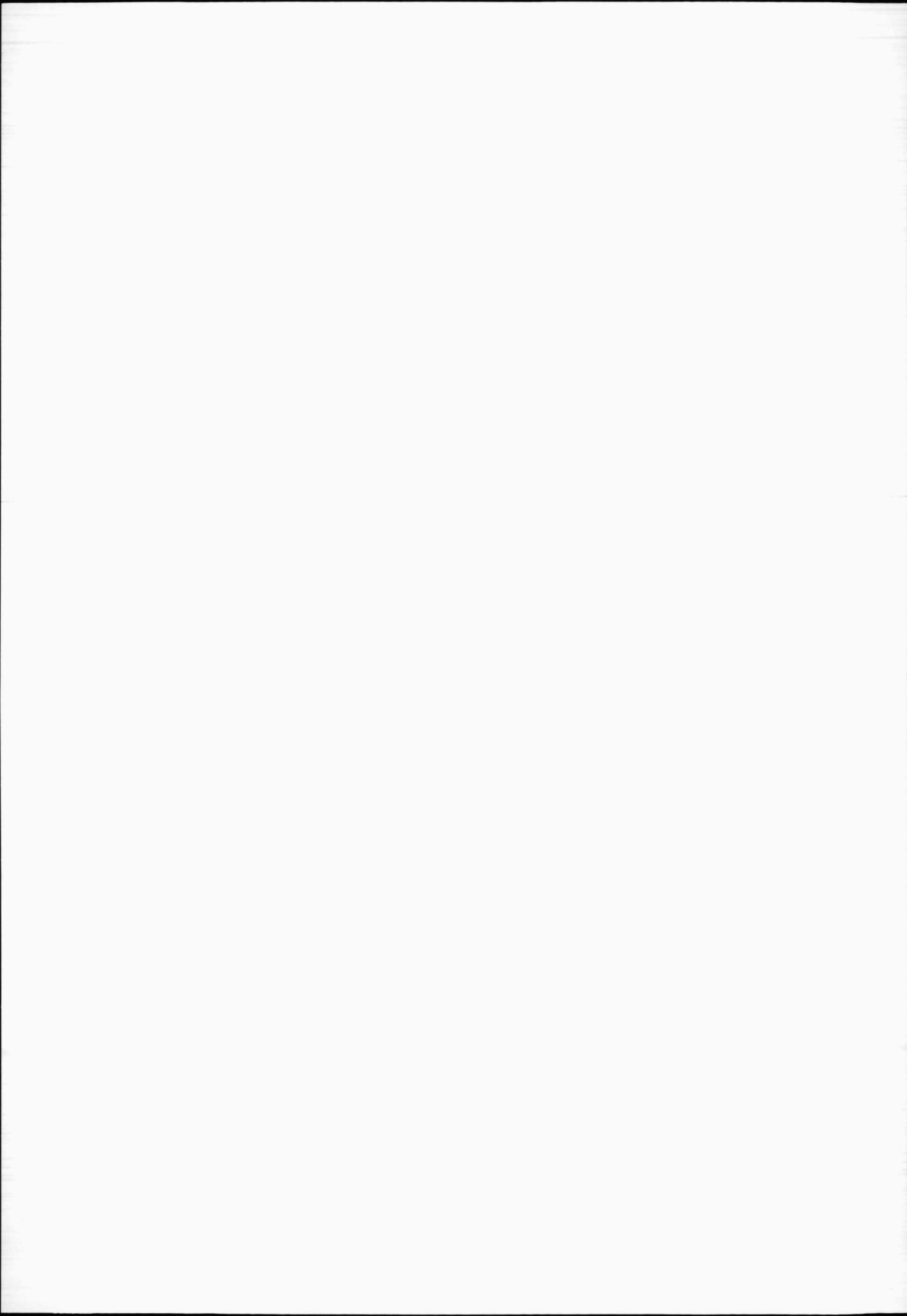
Reversal of human immunodeficiency virus type 1 IIIB to a neutralization-resistant phenotype in an accidentally infected laboratory worker with a progressive clinical course

The role of humoral immunity in controlling human immunodeficiency virus type 1 (HIV-1) is still controversial. The resistance of primary HIV-1 variants to neutralization by antibodies, sera from HIV-1-infected patients, and soluble CD4 protein has been suggested to be a prerequisite for the virus to establish persistence *in vivo*. To further test this hypothesis, we studied the neutralization sensitivity of two IIIB/LAV variants that were isolated from a laboratory worker who accidentally was infected with the T-cell-line-adapted neutralization-sensitive IIIB isolate. Compared to the original virus in the inoculum, the reisolated viruses showed an increased resistance to neutralization over time. The ratio of nonsynonymous to synonymous nucleotide substitutions in the envelope gene pointed to strong positive selection. The emergence of neutralization-resistant HIV preceded disease development in this laboratory worker. Our results imply that the neutralization resistance of primary HIV may indeed be considered an escape mechanism from humoral immune control.

Introduction

The length of the asymptomatic period between the moment of infection with human immunodeficiency virus type 1 (HIV-1) and the development of AIDS-like symptoms differs between patients. This may be interrelated with variables such as the level of immune control, the biological properties of the virus, and host susceptibility. High frequencies of cytotoxic T lymphocytes have indeed been correlated with the clearance of viremia during primary infection and prolonged asymptomatic survival (1-3). Neutralizing antibodies emerge only relatively late in the course of infection (4-6) and may contribute to the control of virus replication. Indeed, passive immunization in animal models provided partial protection (7-9), although this was not confirmed by all studies (10). In addition, titers of neutralizing antibody correlated with a lack of disease progression in long-term survivors of HIV-1 infection (11-15). Finally, the emergence of neutralization escape mutants has pointed to the presence of humoral immunity (5;16-18). The efficiency of antibody neutralization *in vivo* may be limited by the neutralization resistance as generally observed for primary HIV-1 variants (4;16;19;20). This resistance is observed *in vitro* for immune sera from HIV-infected patients and from vaccinees, for monoclonal antibodies, and for soluble CD4. With adaptation to replication in immortalized cell lines, HIV-1 but also other lentiviruses, such as equine infectious anemia virus and simian and feline immunodeficiency virus variants, become highly neutralization sensitive (21-26). It is at present still unclear whether neutralization resistance of primary HIV-1 should be considered an escape mechanism from humoral immunity. Neutralization resistance *in vivo* might be a prerequisite for pathogenicity of HIV because it will allow the virus to persist in the presence of neutralizing antibodies.

To further study the clinical significance of primary HIV-1 neutralization resistance, we analyzed HIV-1 variants that were isolated longitudinally from a laboratory worker (LW-F) who progressed to AIDS within 8 years after accidental infection with the T-cell-line-adapted (TCLA) neutralization-sensitive IIIB strain (27).

Materials and Methods

Cells

Virus isolation and virus stock preparation were performed with human phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC)

according to standard procedures (28). PBMC were isolated from buffy coats from healthy blood donor volunteers by Ficoll-Isopaque density gradient centrifugation. For stimulation, 5×10^6 cells/ml were cultured for 3 days in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and PHA (5 μg/ml). Subsequently, cells (10^6/ml) were grown in the absence of PHA, in medium supplemented with 10 U of recombinant interleukin-2 (Chiron Benelux, BV, Amsterdam, The Netherlands)/ml. The T-cell line H9 was cultured in IMDM supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Viruses and neutralizing agents
The IIIB isolate was a kind gift of R. Gallo. IIIB variants were reisolated from an accidentally infected laboratory worker (LW-F) at approximately three (4 May 1988; isolate fe0233) and seven (7 May 1992; isolate FF3346) years after the assumed moment of infection (before 1986) (27). All viruses, including the originally H9-cell-line-adapted IIIB virus, were propagated on PHA-stimulated PBMC. Each week, virus production in the supernatant was monitored by an in-house p24 antigen capture ELISA (29). If sufficient p24 antigen production could be demonstrated, the titer of the virus stock was quantified by determination of the 50% tissue infectious dose ($TCID_{50}$) on PHA-stimulated healthy donor PBMC.

Viruses were tested for their relative neutralization sensitivity against increasing concentrations of recombinant soluble CD4 (sCD4), HIV-1 immune globulin (HIVIg), human sera Ams^{hps}, and the monoclonal antibodies (MAb) gp13, gp68, IgG1b12, F105, 902, 1577, and 2F5. In short, HIVIg is a preparation of purified polyclonal Ig derived from HIV-infected donors; Ams^{hps} consists of pooled plasma of 34 patients from the Amsterdam Cohort studies on AIDS and HIV-1 infection. The gp13, gp68 and F105 antibodies recognize epitopes surrounding the CD4bs of gp120 (30;31), IgG1b12 recognizes the CD4bs of gp120 (32), and the antibody 902 reacts with the immunodominant hypervariable loop of gp120 (33) and the gp41-directed antibodies CHO 2F5 (epitope ELDKWA; amino acids 662 to 667 of BH10 gp160) and 1577 (region 735 to 752 of IIIB) (34;35).

Neutralization sensitivity of HIV-1 variants
From each virus isolate, an inoculum of 100 $TCID_{50}$/ml in a 100-μl final volume was incubated for 1 to 2 h at 37°C with increasing concentrations of the neutralizing agents. Subsequently, the mixtures of virus with sCD4, sera, or antibodies were added to 10^5 3-day-stimulated human PBMC in 96-well microtiter plates. The same mixtures of human PHA-stimulated PBMC were used to grow virus stocks and to determine virus titers. The following day,

plates incubated with HIVIg or Ams^{hps} were washed extensively. On days 7 and 14, virus production in supernatants was analyzed in an in-house p24 antigen capture ELISA. Means of quadruplicate experiments of each agent, tested at least twice, were plotted. Percent neutralization was calculated by determining the reduction in p24 production in the presence of the agent compared to that for the cultures with virus only. When possible, 50% (IC_{50}) and 90% (IC_{90}) inhibitory concentrations were determined by linear regression.

Determination of virus coreceptor usage and cell tropism
U87 cells stably expressing CD4 alone or in combination with CCR5 or CXCR4 (a kind gift of D. Littman), were seeded at 10^4 cells per well in 96-well plates in IMDM supplemented with 5 μg of Polybrene/ml and 1 μg of puromycin/ml. Occasionally 200 μg of G418/ml was added to select for CD4-expressing cells. The next day, cells were washed with phosphate-buffered saline, and 10^2 to 10^4 $TCID_{50}$ of virus/ml was added in a 100-μl final volume. After 24 h, cells were washed twice with phosphate-buffered saline and 200 μl of fresh medium was added. Supernatants were harvested on days 7, 14 and 21 and tested for the presence of p24 antigen in an in-house ELISA.

Replication in macrophages was determined as described previously (36). In brief, PBMC were obtained from heparinized venous blood by Percoll density gradient. The monocyte fraction was purified by centrifugal elutriation. Monocytes were cultured for 5 days at a cell concentration of 10^6 monocytes/ml in IMDM supplemented with 10% human pooled serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in 24-well plates.

DNA isolation, PCR, and sequence analysis
Total DNA from PBMC infected with the HIV-1 variants under study was isolated as described previously (37). PCR amplification of the complete envelope gene was performed using primer TB/3 (5'-GGCCTTATTAGGACACATAGTTAGCC-3') (positions relative to the HXB2D proviral genome: nucleotides 5405 to 5430) and TB/C (5'-GCTGCCTTGTAAGTCATTGGTCTTAAAGG-3') (nucleotides 9018 to 9046) using a mixture of *Taq* DNA and *Pwo* DNA polymerases (Expand High Fidelity; Boehringer Mannheim). All reactions were performed in the presence of 15 mM $MgCl_2$ and 5mM deoxynucleoside triphosphate (dNTP). Thermo-cycling conditions were the following: 2 min 30 s at 94°C once, 15 s at 94°C, 45 s at 50°C (2°C/sec slope), 6 min at 68°C repeated 10 times, followed by 30 times of the same program at 53°C and subsequently an additional 10-min extension at 68°C. PCR products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany). The gp120-gp41 region of the HIV envelope gene was completely sequenced with

Dye terminator cycle sequencing with Ampli *Taq* DNA polymerase, FS (Perkin-Elmer, Applied Biosystems Division, Foster City, Calif.), performed according to the manufacturer's protocol on an ABI 373S automated sequencer.

Phylogenetic analysis
From each isolate unambiguous DNA sequences were obtained, and within the two sequence sets nucleotide sequences were aligned manually. Phylogenetic analysis was performed by using the PHYLIP DNADIST and NEIGHBOR software (38), based on Kimura two-parameter distance estimation method with the HIV-1 MN sequence as an out-group. For both sequence sets, the bootstrap analysis was performed using SEQBOOT and CONSENSE (100 replicates). Synonymous (D_s) and nonsynonymous (D_n) distances were calculated by using the MEGA software (39) as previously described.

Results
Sensitivity to neutralization of IIIB and the LW-F isolates
From a laboratory worker who accidentally was infected in 1985 with IIIB (27), HIV variants that were isolated in 1988 (fe0233) and 1992 (FF3346) were used in this study. Viruses were isolated by coculture with PHA-stimulated PBMC. These HIV isolates have not been passaged through T-cell lines unless specifically indicated.ly. A 10- to 15-fold-lower level of resistance to neutralization by sCD4 was observed for IIIB and isolate fe0233, which showed IC_{50} and IC_{90} values ranging from 2 to 8 µg of sCD4/ml for IIIB and 2 to 13 µg of sCD4/ml for fe0233 (Figure | 1a). The sensitivity of IIIB to sCD4 neutralization after adaptation to replicate on human PBMC was not different from that of the original H9-adapted IIIB virus (data not shown). Passage of FF3346 through the H9 T-cell line resulted in a neutralization sensitive T-cell-line-adapted phenotype with an IC_{50} and IC_{90} of sCD4 comparable to those observed for IIIB and fe0233 (data not shown). Subsequently the sensitivity to neutralization by two polyclonal anti-HIV-1 sera, HIVIg and Ams[hps], was tested (Figure | 1b). Again, FF3346 was relatively neutralization resistant, with 50 and 90% inhibition at serum dilutions of 1:110 and 1:68 for HIVIg and 1:85 and 1:65 for Ams[hps], respectively. These dilutions where about fivefold lower than those required for neutralization of IIIB (50 and 90% neutralization at serum dilutions of 1:600 and 1:115 for HIVIg and 1:600 and 1:275 for Ams[hps], respectively). Compared to IIIB and FF3346, isolate fe0233 showed an intermediate neutralization-sensitive phenotype, with 50 and 90% neutralization at serum dilutions of 1:175 and 1:70 for HIVIg and 1:200 and 1:100 for Ams[hps].

Next, a panel of monoclonal antibodies was used for further characterization of the neutralization sensitivity of IIIB, fe0233, and FF3346. The IgG1b12 antibody, which recognizes a conserved epitope in the CD4 binding site, indeed, like sCD4, neutralized IIIB at an IC_{50} and IC_{90} of 0.7 and 0.9 µg/ml, respectively, and neutralized fe0233at an IC_{50} of 1.5 and an IC_{90} of 1.7 µg/ml (Figure | 1c). Although IgG1b12 has been reported to be capable of neutralizing many primary viruses (32;40), FF3346 was relatively resistant (IC_{50} and IC_{90} of 15 and 31 µg/ml, respectively). The gp68, gp13, and F105 monoclonal antibodies, which all recognize amino acid residues surrounding the CD4 binding cavity, could neutralize IIIB only with IC_{50} values between 1.0 and 7.0 µg/ml and IC_{90} values between 22 and 65 µg/ml. The same phenomenon was seen for the monoclonal antibody 902, which recognizes the hypervariable immunodominant loop of HIV-1 (Figure | 1c). Neutralization with antibodies against gp41 (1577 and 2F5) (Figure | 1d) was not different among the three HIV-1 isolates. Although there was a tendency towards a more neutralization-resistant phenotype for the two LW-F isolates, IIIB was also relatively resistant at the concentrations tested. These results suggest ongoing evolutionary progression towards an increased resistance to neutralization within the CD4 binding region of the gp120 envelope protein.
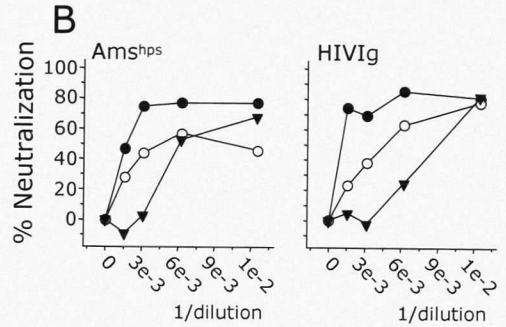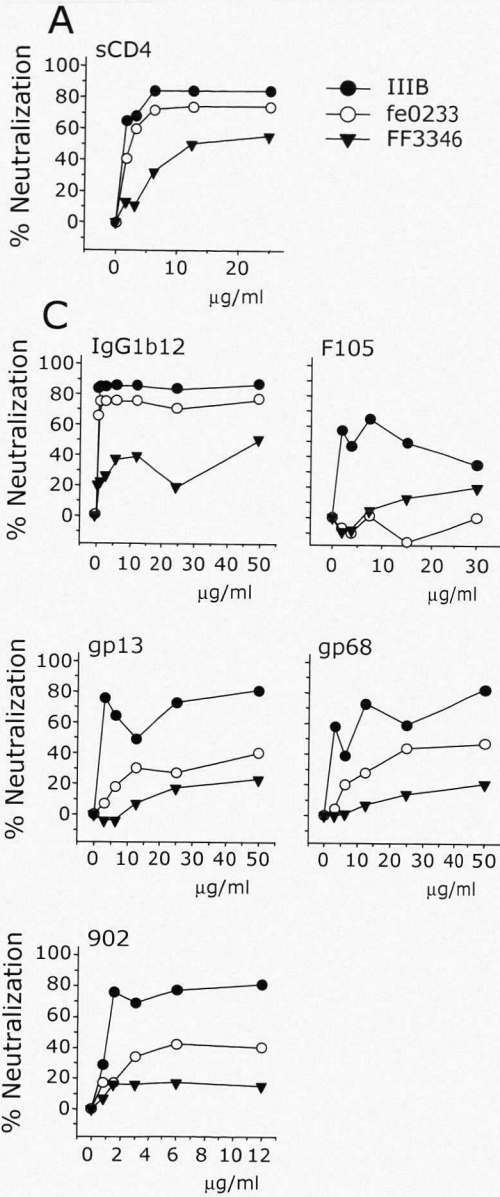
Figure|1

Neutralization sensitivity of HIV-IIIB and LW-F isolates as determined on human PHA-stimulated PBMC. Of the tested viruses, HIV-IIIB (●), fe0233 (O) and FF3346 (▼), 100 TCID$_{50}$/ml was incubated with increasing concentrations of recombinant sCD4 (a), polyclonal pooled serum of HIV-1 infected patients (HIVIg and Ams^{hps}) (b), anti-CD4 binding-site directed MAbs (IgG1b12, 902, gp13, gp68 and F105) (c), and anti-gp41 MAbs 2F5 and 1577 (d). In the neutralization assay, p24 was measured, and mean optical densities (OD) were calculated from quadruplicate cultures from at least duplicate experiments. The percent neutralization was calculated by determining the reduction in supernatant p24 production in the presence of the neutralizing agent relative to that for control cultures lacking these agents.
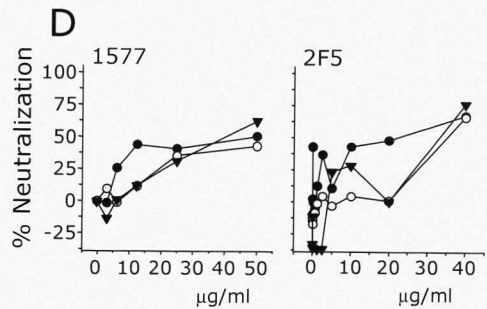
Phylogenetic and sequence analysis

Sequence analysis of the nucleotides spanning the gp120 envelope region and subsequent phylogenetic analysis showed that fe0233 and FF3346 clustered together with HXB2D, BH10, PV22, and LW90-2 (GenBank accession no. K03455, M15654, K02083, and U12053, respectively) (Figure|2). BH10 and PV22 are H9/HTLV-III proviral DNA clones, and LW90-2 is another isolate from LW-F (isolated in 1990) from which the complete envelope genome was sequenced (41). Genetic distances between the IIIB isolate and the two LW-F isolates never exceeded 3%, indicative of their close relatedness (data not shown). We next analyzed changes in the deduced amino acid sequence of gp120 that
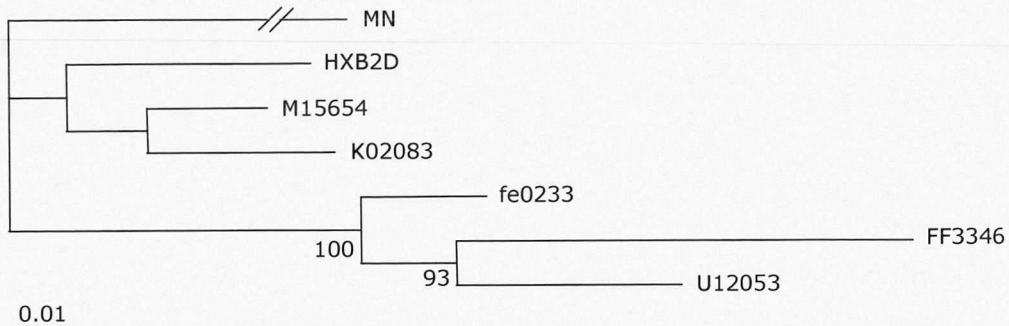
Figure|2
Phylogenetic analysis of complete HIV envelope sequences of the HXB2D and LW-F isolates. Included are two other IIIB-related sequences, BH10 (M15654) and PV22 (K02083), respectively, and a LW-F clone named LW90-2 (U12053) from which the envelope sequence was determined previously (50). The phylogenetic tree was constructed with the neighbor-joining, Kimura two-parameter distance estimation method. The MN branch is truncated to facilitate presentation and was used as an outgroup.

could be associated with the observed phenotypic changes in the viruses isolated from the laboratory worker (Figure|3). Amino acid substitutions in fe0233 and FF3346 were observed mainly in the variable regions of gp120 and in the loop region of gp41. Both of V3 and potential N-linked glycosylation sites in gp120 have been implicated in biological properties of HIV-1 (42;43). Despite the high number of 5 amino acid substitutions in V3, only one (Arg-Gly 304) resulted in a change of the overall charge (-1) of the V3 loop in fe0233 and FF3346. However, the fe0233 and FF3346 V3 loops still had a charge of +8, compatible with their restricted CXCR4 co-receptor use (44). In agreement with the preserved syncytium-inducing (SI) phenotype, there was a positively charged amino acid residue at position 311 in all three viruses. We were able to confirm a mutation in the highly antigenic GPGRAF sequence in the top of the V3 loop, which was already found 1 year after infection (45). The overall charge of the V2 loop was also reduced (-2) in isolates fe0233 and FF3346 compared to that in isolate IIIB. In isolate FF3346, two potential N-linked glycosylation sites were lost (positions 289 and 674). In addition, the two amino acid substitutions Val-Ala 275 and Ala-Val

281 may redirect the glycoprotein moiety on the Asp at position 276, which could influence the accessibility of the underlying CD4 binding cavity. The importance of this specific N-linked glycosylation site in neutralization sensitivity has been described previously (42).

A relatively high number of amino acid substitutions in the LW-F isolates compared to the IIIB isolate were present in the so-called anti-hotspots (43;46). These surface-accessible residues are located adjacent to amino acids that are directly involved in CD4 binding and are positioned mainly in the constant regions C2, C3, and C4 but also in V5. Two amino acid residues that are directly involved in CD4 binding were also changed (Ala-Val 281 in fe0233 and FF3346; Glu-Ala 370 in FF3346). Amino acid residues in C1, C4, and C5 of gp120 that are involved in the non-covalent association between gp120 and gp41 were preserved in all three isolates. In addition, we observed a recovery of the VPR open reading frame, which underscores the importance of VPR for viral replication in primary cells (data not shown).

Finally we analyzed whether the sequence changes were due to selective immune pressure or random mutations. This was analyzed by calculating the ratio of synonymous versus

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        gp120     .     .   20    .    C1    .   40    .     .     .   60    .     .     .   80    .     .   .   100
HXB2d  : MRVKEKYQHLWRWGWRWGTMLLGMLMICSATEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLVNVTENFNMWKNDM
fe0233 : .........R..........................................................................................
FF3346 : .........R..........................................................................................

            .     .     .  120    .     V1    .   140 -----  .     .     V2    .     .   180    .     .   .
HXB2d  : VEQMHEDIISLWDQSLKPCVKLTPLCVSLKCTDLKNDTNTNSS-----SGRMIMEKGEIKNCSFNISTSIRGKVQKEYAFFYKLDIIPIDNDTTSYKLTS
fe0233 : ..........................................-----..G.................................................M...
FF3346 : ..........................................-----G.G...................K....E.......................T...

        C2  200    .     .     .  220    .     .     .  240    .     .     .  260    .     .     .  280    .     .
HXB2d  : CNTSVITQACPKVSFEPIPIHYCAPAGFAILKCNNKTFNGTGPCTNVSTVQCTHGIRPVVSTQLLLNGSLAEEEVVIRSVNFTDNAKTIIVQLNTSVEIN
fe0233 : ...............................................................................A....V.......Q....
FF3346 : ...............................................................................A....V.......QP....

        V3  300    .     .     .  320    .     .     .    C3  .  340    .     .     .  360    .     .  380 V4  .     .
HXB2d  : CTRPNNNTRKRIRIQRGPGRAFVTIGKIGNMRQAHCNISRAKWNNTLKQIASKLREQFGNNKTIIFKQSSGGDPEIVTHSFNCGGEFFYCNSTQLFNSTW
fe0233 : ...K.....G.........T...................................Y..............L..........................
FF3346 : ..K.....G....H....T.........V..........V.....Y......R....LA..................N..

            .  400    .     .     C4  .     .     .  440    .     .     V5    .   C5  .  480    .     .   .
HXB2d  : FNSTWSTEGSNNTEGSDTITLPCRIKQIINMWQKVGKAMYAPPISGQIRCSSNITGLLLTRDGGNSNNESEIFRPGGGDMRDNWRSELYKYKVVKIEPLG
fe0233 : ....----.....................E.......................N.G.......................
FF3346 : ....----................V....E.......................N.G.......................

            .  500    .     .     .  520    .    gp41    .   540    .     .     .  560    .     .     .  580    .     .   .
HXB2d  : VAPTKAKRRVVQREKRAVGIGALFLGFLGAAGSTMGAASMTLTVQARQLLSGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQQLLGI
fe0233 : ...................................................................................................
FF3346 : ...................................................................................................

            .  600    .     .     .  620    .     .     .  640    .     .     .  660    .     .     .  680    .     .   .
HXB2d  : WGCSGKLICTTAVPWNASWSNKSLEQIWNHTTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWFNITNWLWYIKLFIMIVGGLVGL
fe0233 : ........................R..................................................................V.I.......
FF3346 : ..................T.....T.E...Q...Q.....S.....................................D.......I..........

            .  700    .     .     .  720    .     .     .  740    .     .     .  760    .     .     .  780    .     .   .
HXB2d  : RIVFAVLSIVNRVRQGYSPLSFQTHLPTPRGPDRPEGIEEEGGERDRDRSIRLVNGSLALIWDDLRSLCLFSYHRLRDLLLIVTRIVELLGRRGWEALKY
fe0233 : ...................................G.......K.......................................................
FF3346 : ..I................................G...............................................................

            .  800    .     .     .  820    .     .     .  840    .     .   .
HXB2d  : WWNLLQYWSQELKNSAVSLLNATAIAVAEGTDRVIEVVQGACRAIRHIPRRIRQGLERILL
fe0233 : ............................................................
FF3346 : ..................................R....V....................
```

Figure|3

Sequence of the parental HXB2D and the two reisolated LW-F isolates. The predicted amino acid sequences of the envelope glycoproteins of the fe0233 and FF3346 viruses, based on the consensus sequence of the Env fragment amplified from infected human PBMC, are aligned with the HXB2D sequence. Differences in amino acid residues are indicated. The start sites of gp120 and gp41 are shown, as are the locations of the different gp120 regions.

nonsynonymous mutations for gp120, gp41 and the total envelope sequence (Table|1). A relatively low D_s/D_n ratio (approximately 0.35) suggests positive selection of favorable amino acid substitutions, whereas a low level of immune pressure is represented by a high ratio (approximately 0.65) (47). The D_s/D_n ratio of 0.57 between IIIB and fe0233 for gp120 was somewhat higher than the ratio found between fe0233 and FF3346 (D_s/D_n = 0.42). In gp41, D_s/D_n ratios between fe0233 and FF3346 were also markedly lower than between IIIB and fe0233, 0.33 and 1.0, respectively. D_s/D_n ratios between parental HXB2D and isolate FF3346 showed a relatively normal ratio for gp120 and the complete envelope and a low mean D_s/D_n ratio for gp41. The lower ratios for fe0233 versus

FF3346 are indicative of positive selection pressure on the virus in the period from 3 to 7 years after infection, which is in agreement with the observed changes in neutralization sensitivity of the viruses.

Coreceptor usage and cell tropism.
In recently infected individuals, the HIV-1 quasispecies in general is very homogeneous, with a non-syncytium inducing (NSI) macrophage-tropic phenotype and a CCR5 restricted coreceptor usage (48). This is also observed in recipients who were infected by individuals with SI CXCR4 using viruses. In these cases, selective transmission or outgrowth of NSI HIV-1 occurred, although at least low-level SI virus replication persisted (49). Cell-free

Table|1. Intrahost evolution of HIV-IIIB in the laboratory worker as determined by the D_s/D_n ratio based on synonymous (D_s) and nonsynonymous (D_n) mutations in different regions of the envelope gene[a]

| Region | Results of comparison | | | | | | | | | | | |
| | HXB2D vs fe0233 | | | | fe0233 vs FF3346 | | | | HXB2D vs FF3346 | | | |
	No. of nuc. mutations	D_n	D_s	D_s/D_n	No. of nuc. mutations	D_n	D_s	D_s/D_n	No. of nuc. mutations	D_n	D_s	D_s/D_n
gp41	10	5	5	1.0	16	12	4	0.33	20	12	8	0.67
gp120	23	14	8	0.57	17	12	5	0.42	36	25	10	0.40
Env	33	19	13	0.68	33	24	9	0.38	56	37	18	0.49

[a]Insertions and deletions were not taken into account. Per time frame the D_s was determined by subtracting the number of mutations encoding amino acids (D_n) from the total number of nucleotide (nuc.) mutations. Gp41 and gp120 are the transmembrane and extracellular part of the envelope protein (Env), respectively.

inoculation of 5-day-cultured monocyte-derived macrophages revealed that fe0233 and FF3346 indeed both had the capacity to replicate in macrophages (Table|2). However, as determined by infection of the MT2 cell line and microscopic analysis for the appearance of syncytia, isolates fe0233 and FF3346 were also both classified as T-cell line tropic and SI. The macrophage tropism of the LW-F viruses prompted us to analyze their coreceptor usage. U87 cell lines stably expressing CD4 and CCR5 or CXCR4, the two coreceptors that are considered most relevant for *in vivo* HIV infection (50), were inoculated with fe0233 or FF3346. Both isolates established a productive infection only in the U87/CD4/CXCR4 cell line and lacked the capacity to use coreceptor CCR5 (Table|2).

Table|2 Coreceptor usage and macrophage tropism of isolate IIIB and HIV variants isolated from LW-F[a]

| virus | Replication in CD4-expressing U87 cells (OD)[b] | | Replication in macrophages (OD) | |
	CXCR4	CCR5	Donor 1	Donor 2
IIIB	0.88	Neg.[c]	Neg.	Neg.
fe0233	0.99	Neg.	0.09	0.10
FF3346	0.78	Neg.	0.07	0.09

[a] Viral replication in U87 cell lines or macrophages is expressed as the maximum OD measured in a p24 antigen capture ELISA reduced by background. Only samples with an OD value of more than two times the background were considered positive.
[b] U87 cells were cotransfected with either CXCR4 or CCR5 coreceptor.
[c] Neg., negative.

Although we cannot exclude that NSI CCR5 viruses may have been present very early in infection or coexist as a minor population, our data at least confirm that CCR5 usage is no prerequisite for macrophage tropism or neutralization resistance (51-54).

Discussion

The neutralization resistance of primary HIV-1 variants is considered instrumental for HIV-1 persistence in the presence of neutralizing antibodies and HIV-1 pathogenicity *in vivo* (15;17;18;20;42;55). Since all infections are established by primary neutralization-resistant HIV-1, it has been impossible to conclude whether neutralization resistance should indeed be considered an escape mechanism. The unfortunate accidental infection of a laboratory worker (LW-F) with the TCLA neutralization-sensitive IIIB variant provided the opportunity to study directly the relevance of HIV-1 neutralization resistance *in vivo*. LW-F had a typical clinical course, developing AIDS within 8 years after infection (27). Comparison of viruses that were isolated from the laboratory worker 4 and 7 years after infection showed a gradual loss of HIV neutralization sensitivity, preceding clinical progression to AIDS. Based on this observation, we conclude that the neutralization resistance of HIV may be considered an escape mechanism from humoral immunity. The clinical relevance of HIV-1 neutralization resistance is in line with our finding that a IIIB variant re-

isolated from an experimentally infected chimpanzee after 10 years of asymptomatic HIV infection was still sensitive to neutralization by CD4-binding-site-directed antibodies and sCD4 (56). Symptom-free follow-up of this animal has now extended to more than 18 years. It is remarkable that the neutralization sensitive IIIB virus could persist in LW-F, since steadily increasing and broadening antibody responses against the gp160 and IIIB-derived V3 peptides were demonstrated even 5 years after infection (57). Moreover, a strong antibody response was already measured in serum one year after infection (6),(58) and neutralizing activity in serum against TCLA viruses was demonstrated between 3 and 5 years after infection (41;57;59). However, since binding to monomeric gp120 in a CD4 binding inhibition assay or neutralization against TCLA isolates is not a relevant quantification for neutralizing activity (6;25;58), it may be possible that titers of neutralizing antibody were absent or at least too low to fully suppress viral replication.

Other mechanisms to escape humoral immunity have been hypothesized. HIV-1 macrophage tropism may be critical for viral replication in the presence of neutralizing antibodies *in vivo* (60). Spreading of virus during close cell-cell contact, which frequently occurs between macrophages and T cells, would prevent a cell-free state during which HIV-1 otherwise would be vulnerable to neutralizing antibodies, and would select for macrophage-tropic HIV-1. In support of this is the macrophage tropism of the LW-F isolates which, however, did not coincide with the capacity to use coreceptor CCR5.

Although not sufficient to suppress virus replication, even a modest autologous neutralizing antibody response may have been sufficient to drive evolution of the IIIB variants in LW-F towards neutralization resistance. Comparison of the synonymous versus nonsynonymous mutations between HXB2D and fe0233 and between fe0233 and FF3346 indeed pointed to an increasing selection pressure on the virus, which may be humoral immunity (57). The impact of the increasing selection pressure was most pronounced in gp41, as can be concluded from the low D_s/D_n ratio of 0.33 for this region (47;61). We did not observe a change in neutralization sensitivity for two gp41 directed antibodies, and in agreement there were no mutations in their respective epitopes. We cannot exclude the possibility that antibodies directed against the region in gp41 that shows nonsilent mutations may have been present *in vivo*, although the level of gp41 antibodies is generally considered to be very low. In addition, only part of the region in gp41 with the high number of positively selected mutations may be accessible for antibodies, which makes antibody-mediated selection unlikely. Therefore, an alternative explanation for the positive selection of the gp41 mutations could be that the positively selected gp41 mutations contribute to a favorable configuration of the gp41-gp120 complex (62-65).

A relationship between the presence of HIV-specific humoral immunity and delayed or even absent disease progression has been suggested by several studies (11;12;14;19;66). A progressive disease course in the presence of neutralizing antibodies was in most studies attributed to the emergence of viral escape mutants (4-6;15-18;55). The LW-F viruses had gained a broad neutralization resistance against immune sera from HIV-infected patients, sCD4, and different antibodies. The molecular basis for neutralization resistance of primary HIV-1 is still unknown. With knowledge of the mechanism of neutralization resistance, we may be able to circumvent it, opening up new therapeutic strategies.

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