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In Vivo and In Vitro Escape from Neutralizing Antibodies 2G12, 2F5, and 4E10[▽]

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Recently, passive immunization of human immunodeficiency virus (HIV)-infected individuals with monoclonal antibodies (MAbs) 2G12, 2F5, and 4E10 provided evidence of the in vivo activity of 2G12 but raised concerns about the function of the two membrane-proximal external region (MPER)-specific MAbs (A. Trkola, H. Kuster, P. Rusert, B. Joos, M. Fischer, C. Leemann, A. Manrique, M. Huber, M. Rehr, A. Oxenius, R. Weber, G. Stiegler, B. Vcelar, H. Katinger, L. Aceto, and H. F. Gunthard, Nat. Med. 11:615–622, 2005). In the light of MPER-targeting vaccines under development, we performed an in-depth analysis of the emergence of mutations conferring resistance to these three MAbs to further elucidate their activity. Clonal analysis of the MPER of plasma virus samples derived during antibody treatment confirmed that no changes in this region had occurred in vivo. Sequence analysis of the 2G12 epitope relevant N-glycosylation sites of viruses derived from 13 patients during the trial supported the phenotypic evaluation, demonstrating that mutations in these sites are associated with resistance. In vitro selection experiments with isolates of four of these individuals corroborated the in vivo finding that virus strains rapidly escape 2G12 pressure. Notably, in vitro resistance mutations differed, in most cases, from those found in vivo. Importantly, in vitro selection with 2F5 and 4E10 demonstrated that resistance to these MAbs can be difficult to achieve and can lead to selection of variants with impaired infectivity. This remarkable vulnerability of the virus to interference within the MPER calls for a further evaluation of the safety and efficacy of MPER-targeting therapeutic and vaccination strategies.

Neutralizing antibodies are considered a key component of protective vaccines against human immunodeficiency virus type 1 (HIV-1), but despite tremendous efforts, the vaccination approaches tested so far have failed to induce broad neutralization activity (4, 5, 14, 18, 20, 34). Challenges for vaccine design have proven manifold. Most notably, the high genetic variability of the HIV envelope proteins (27), their high degree of glycosylation (58), and their complex conformation as a membrane-embedded trimer of two subunits (15, 59, 60, 62) represent considerable obstacles to vaccine development. The most promising leads in vaccine design remain the few potent and broadly neutralizing antibodies known to date; foremost are the four intensively characterized antibodies IgG1b12, 2G12, 2F5, and 4E10 (2, 5–7, 38, 45, 51, 54, 65). The epitope characteristics, modes of action, and potency, as well as biochemical properties and structures, of these antibodies have been unraveled and provide the basis for the design of vaccines aiming to elicit like responses. Both antibodies recognizing gp120, i.e., IgG1b12, which binds to a distinct epitope overlapping the CD4 binding site (6, 45), and 2G12, which recognizes a unique mannose-dependent epitope within gp120 (49, 54), target fairly complex, nonlinear epitopes and are, in general, more potent against subtype B than against other subtypes of HIV (1, 54). The binding sites of 2F5 and 4E10, however, lie within a well-defined region of the membrane-proximal external region (MPER) of gp41 (2, 38, 51, 65). MPER-based vaccine approaches have gained particular interest because of the high conservation of this region and its role in the fusion process (36, 48, 63). In a comprehensive screening of 90 HIV isolates of diverse subtypes, 4E10 was found to be active against 100% of the viruses and 2F5 still neutralized 67%, whereas IgG1b12 and 2G12 neutralized only 50% and 41% of the isolates in this survey, respectively (1). Nevertheless, thus far attempts to elicit 2F5- and 4E10-like responses have failed to yield potent neutralization activity (10, 11, 16, 22, 26, 28, 29, 32, 37, 61, 63). Recent biophysical analysis of the MPER revealed that this region adopts a helical conformation which may be required for proper recognition by neutralizing antibodies and likely needs to be considered in vaccine development (8, 9, 41, 50). On the basis of these findings, efforts to redefine immunogen design are under way and raise hopes that it may be possible to elicit neutralizing MPER-reactive antibodies (63). However, whether or not MPER-specific antibodies will be able to effectively control infection in vivo will not be known for years. To investigate the in vivo activity of these antibodies to preassess their potential in vaccine-mediated protection, we performed a clinical trial consisting of a passive-immunization study with a cocktail of monoclonal antibodies (MAbs) 2G12, 2F5, and 4E10 which was designed to mimic conditions of therapeutic vaccination (52). The study provided formal proof that neutralizing antibodies are active in vivo, as a delay of viral rebound occurred in several trial participants. Strikingly, in all of the patients who initially harbored 2G12-

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sensitive virus strains, resistance to 2G12 but not to the MPER antibodies developed and loss of sensitivity to 2G12 coincided with viral rebound in plasma (52). In sum, from all 14 of the patients enrolled in this passive-immunization trial, 14 isolates obtained before immunization and 40 isolates obtained during the trial were assessed for sensitivity to the three MAbs. None of the strains revealed phenotypic changes in sensitivity to the MPER MAbs upon in vitro exposure to the antibodies (52). The fact that viral rebound was associated with resistance to 2G12 but to neither of the MPER antibodies raised questions about whether the latter antibodies had been active in vivo. In view of the MPER- and carbohydrate-targeting vaccines under development, these findings necessitated an in-depth analysis of the underlying events.

MATERIALS AND METHODS

Clinical specimens. The patient plasma and isolates utilized in this study were derived during a recently conducted passive-immunization trial as previously described (52). Written informed consent was obtained from all individuals according to the guidelines of the ethics committee of the University Hospital Zurich.

Stimulated primary CD8-depleted PBMC. Buffy coats obtained from three healthy blood donors were depleted of $CD8^+$ T cells with Rosette Sep cocktail (StemCell Technologies Inc.), and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque centrifugation. Cells were adjusted to 4×10^6/ml in culture medium (RPMI 1640 medium, 10% fetal calf serum, 10 U/ml interleukin-2, glutamine, and antibiotics), divided into three parts, and stimulated with 5 μg/ml phytohemagglutinin, 0.5 μg/ml phytohemagglutinin, or anti-CD3 MAb OKT3 as previously described (46). After 72 h, cells from all three stimulations were combined (referred to as three-way-stimulated PBMC) and used as a source of stimulated $CD4^+$ T cells for infection and virus isolation experiments.

Virus stock preparation. Autologous virus was isolated from patient PBMC by coculturing patient $CD4^+$ T cells with stimulated, CD8-depleted PBMC as previously described (52). Virus was isolated in the absence of the neutralizing antibodies to ensure that escape from antibody treatment occurred in vivo and not in vitro. Only early-passage virus was used for further studies (passages 1 to 3). The 50% tissue culture infective doses ($TCID_{50}$) and coreceptor usage of the virus stocks obtained were determined as previously described (46).

Generation of in vitro escape mutants. Three-way-stimulated primary CD8-depleted PBMC (1×10^6/ml) were infected with 1,000 $TCID_{50}$ of the respective HIV-1 isolates in a total volume of 6 ml. Cultures were maintained either in medium alone or in the presence of 2G12, 2F5, 4E10, or a cocktail of all three antibodies. Initial MAb concentrations for the selection experiments were adjusted to the 70% inhibitory doses of the MAbs for the respective virus. Viral growth was monitored weekly by measuring p24 antigen levels. Continuous viral replication was maintained by passaging 1/10 of the infected culture (supernatant and cells) to freshly stimulated PBMC weekly. During this passage, the appropriate antibody concentration adjustments were also made. Testing for sensitivity changes to determine the required antibody concentration changes is not possible in this regimen, as the readout of the inhibition assay is only available with a 7-day delay. Growth kinetics were thus compared on the basis of p24 antigen determination. By comparing the viral antigen production of a specific antibody selection culture to the growth in the same culture in preceding weeks and that of the untreated control culture of the respective isolate, it was then decided whether or not antibody concentration increases or decreases were necessary in order to maintain significant selection pressure while still allowing sufficient viral production. The readout based on p24 antigen production provides only rough estimates of the effect of the antibodies, as viral replication in these long-term cultures can vary substantially from week to week because of differences in PBMC donor infectibility. The selected viral cultures were therefore continuously assessed in parallel for sensitivity to the MAbs. Virus supernatant and cells were collected weekly and stored at −80°C. Virus supernatants where then used to characterize escape variants in neutralization assays for sequencing and as a source for virus stock generation.

Neutralization assay. The neutralization activity of MAbs against sequential virus isolates was evaluated on CD8-depleted PBMC as previously described (53). Briefly, a virus inoculum (100 $TCID_{50}$) was incubated with serial dilutions of antibodies for 1 h at 37°C. Stimulated PBMC were then infected with aliquots of this preincubation mixture. The total infection volume was 200 μl. Cultures were incubated in 96-well culture plates and assayed for p24 antigen at multiple time points between days 4 and 14, depending on the viral growth kinetic. The antibody concentrations (micrograms per milliliter) causing 50%, 70%, and 90% reductions in p24 antigen production were determined by linear-regression analysis. If the appropriate degree of inhibition was not achieved at the highest or lowest drug concentration, a greater-than or less-than value was recorded.

Viral cDNA synthesis and amplification. Viral RNA was extracted by an automated extraction robot (MagNA Pure instrument; Roche) according to the manufacturer's instructions. cDNA synthesis of the full-length envelope was done with SuperScript III reverse transcriptase (Invitrogen) with primer envM (17), envN (17), PR5 (5′-AGCTGGATCCGTCTCGAGATACTGCTCCCACC C-3′), or PR10 (5′-CGAGCTGGATCCTTTTGACCACTTGCCACCCATCTT ATAGC-3′), depending on the virus isolate. Subsequent amplification was performed in 50-μl reaction volumes (94°C for 2 min; 5 cycles of 94°C for 10 s, 58°C for 30 s, and 68°C for 180 s; 45 cycles of 94°C for 10 s, 60°C for 20 s, and 68°C for 150 s; and 68°C for 10 min) with Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen). The primer pair env1A (31)-PR5 was used for the majority of the isolates. When PR5 did not match the isolate's sequence and failed to amplify *env*, the alternative primer set env1A-envM, env1A-envN, or env1A-PR10 was used. PCR amplicons were loaded onto a 1% agarose gel and purified with the QIAquick gel extraction kit (QIAGEN, Basel, Switzerland) before sequencing and cloning were performed. Clones were sequenced after high-purity plasmid miniprep (QIAprep 8 Turbo Miniprep kit; QIAGEN, Basel, Switzerland).

Sequence analysis. Bidirectional sequencing was performed by dye terminator cycle sequencing (ABI Prism BigDye version 2.0; Applied Biosystems, Rotkreuz, Switzerland) with an automated capillary sequencer (ABI 3100). Full-length sequencing of 10 overlapping regions spanning the genes *rev* through *nef* was performed with specific primers including 5allspl (5′-AAGAAGCGGAGACAG CGACGAAGA-3′), MF178 (5′-ATGGTAGAACAGATGCATGAGGATATA AT-3′), V3Fin2 (24), MF181 (5′-TGCAGAATAAAACAATTTATAAACATG TGGC-3′), MF159 (47), PR10, MF182 (5′-TGTATTAAGCTTGTGTAATTGT TAATTTCTCT-3′), MF155 (52), MF169 (5′-TGATGGGAGGGGCATACAT TG-3′), MF180 (5′-TGAGTTGATACTACTGGCCTAATTCCATGTG-3′), and MF179 (5′-CACATGGCTTTAGGCTTTGATCCCAT-3′).

Precipitation with ethanol-sodium acetate was used for purification of the extension products. Where specified, sequencing was confined to the C2-to-V4 region of gp120 or the MPER-spanning region in gp41, respectively. Primers V3Fin2 (24) and MF169 were used to sequence the C2-to-V4 region. MPER sequencing gp41 was done with primers MF158 (52) and MF155 (52).

Sequence heterogeneity was detected in some instances as a consequence of direct sequencing of bulk PCR products derived from samples containing mixed virus populations. Only the major variants represented in bulk sequences, i.e., those exhibiting the strongest signal in the chromatograms, are depicted, except where mixed populations are indicated. Mixed populations in bulk sequences were identified by visual examination of chromatograms for various sets of data (see Fig. 1 and 4; data not shown). Where specified, clonal analysis was performed.

Clonal analysis of the MPER in gp41. Extraction of viral RNA from week 12 plasma samples from patients NAB01, NAB02, and NAB08; cDNA synthesis; and hot-start PCR were performed in duplicate as previously described (47, 52). Alternatively, MPER cDNA synthesis was performed with primers MF155 and MF158 and amplification for 1 cycle of 50°C for 30 min, 95°C for 15 min and 50 cycles of 95°C for 10 s, 55°C for 15 s, and 72°C for 120 s, followed by 72°C for 7 min. Cloning (pCR 4 TOPO TA or pcDNA3.1/V5-His TOPO TA; Invitrogen, Groningen, The Netherlands), amplification (HotStarTaq Master Mix; QIAGEN, Basel, Switzerland), and sequencing (BigDye, Applied Biosystems, Rotkreuz, Switzerland) of individual clones were performed as previously described (24), with the primer pair MF155-MF158 under otherwise identical conditions. This procedure has previously been demonstrated to result in low error rates (24).

Full-length envelope cloning and generation of envelope-pseudotyped HIV particles. Full-length envelope genes amplified for sequencing were also used for cloning of envelope genes into expression vectors. To this end, the PCR amplicons were introduced into the Expression Kit (Invitrogen, Groningen, The Netherlands), followed by transformation into One Shot TOP10 or STBL3 competent bacteria (Invitrogen, Groningen, The Netherlands). Since ligation of inserts by AT cloning can occur bidirectionally, the orientation of the envelope inserts in derived clones was verified by PCR with two different primer pairs, PR12 (5′-G GCTAACTAGAGAACCCACTGCTTA-3′)-PR13 (5′-ACTACTTACTGCTTT GATAGAGAAACTTGA-3′) and PR14 (5′-GCCCGAAGGAATCGAAGAA GAAGGTGG-3′)-PR15 (5′-AGGCTTACCTTCGAACCGCGGGCCCTCTA-

3′), spanning small regions at both ends of the cloning site, resulting in the amplification of different-size products, depending on the orientation of the insert. PCR was performed with the HotStarTaq Master Mix (QIAGEN, Basel, Switzerland) in 22-μl reaction volumes (94°C for 15 min; 33 cycles of 94°C for 10 s, 55°C for 15 s, and 72°C for 60 s; and 72°C for 7 min). Subsequent gel electrophoresis of PCR amplicons allowed us to select clones which carry correctly oriented envelope inserts. Pseudotyped reporter HIV strains were generated as previously described (31, 56). Briefly, 293T cells were transfected in 24-well plates with 6 μl of the Effectene transfection reagent (QIAGEN, Basel, Switzerland); 3 μl of the plasmid carrying the reporter gene expressing the virus backbone, pNLluc-AM (43; a kind gift from A. Marozsan and J. P. Moore); and 2 μl of the purified respective *env*-carrying plasmid (QIAprep 8 Turbo Miniprep kit; QIAGEN, Basel, Switzerland). Viral supernatants were harvested at 2 days posttransfection and screened for infectivity. To this end, TZM-bl cells (National Institutes of Health AIDS Research and Reference Reagent Program) (13, 42, 55) were infected with viral supernatants in Dulbecco's modified Eagle medium–10% heat-inactivated fetal calf serum–1% penicillin-streptomycin (BioWhittaker) containing 10 μg/ml DEAE-dextran (Amersham Biosciences). Luciferase activity was measured 3 days after infection as previously described (23, 31). Functional envelope clones were used to produce large-scale virus stocks for further analysis in infection assays and for sequence verification.

Neutralization assays with Env-pseudotyped reporter gene viruses. Large-scale virus stocks of Env-pseudotyped virus were prepared by transfecting 10-cm dishes seeded with 293T cells with 15 μg of the backbone plasmid (pNLluc-AM), 5 μg of the functional envelope clone, and 40 μg of polyethylenimine (linear, 25 kDa; Polysciences, Inc.). Virus stocks were titrated as previously described (23). Neutralization activity of MAbs against pseudotyped virus carrying the patient-derived and in vitro-selected envelope genes was evaluated on TZM-bl cells essentially as previously described (35). Briefly, 200 $TCID_{50}$ of the virus was preincubated with serial dilutions of the antibody. The antibody concentrations causing 50% (50% inhibitory concentration [IC_{50}]), 70% (IC_{70}), and 90% (IC_{90}) reductions in luciferase reporter gene production were determined by regression analysis.

Infection experiments with replication-competent TN6 NL constructs. The TN6 NL vector, a replication-competent viral backbone engineered from strain NL4-3 (40), was kindly provided by M. Dittmar. The NL4-3 envelope was deleted from the TN6 NL vector by digestion with the restriction enzymes BstEII and NcoI (New England BioLabs). *env*-depleted vectors were purified through separation on agarose gel. The NAB01 envelope genes carried by pcDNA3.1 vectors were amplified by a primer pair introducing a BstEII restriction site (PR20 [5′-AATTGTGGGTCACCGTCTATTATGGGGTACCTGT-3′]) at the 5′ end and an NcoI restriction site (PR21 [5′-GACTGCGTGCCATGGCTTATAGCA AAGCTC-3′]) at the 3′ end of *env* under PCR conditions identical to those described for *env* amplification above. *env* PCR products were digested with BstEII and NcoI, purified, and ligated into the linearized TN6 NL vectors with T4 DNA ligase (New England BioLabs) according to the manufacturer's instructions. Virus stocks were generated by transfecting 293T cells with the various TN6 constructs. The p24 antigen contents of stocks and the $TCID_{50}$ of the stocks on three-way-stimulated PBMC were determined to evaluate the infectivity of the various virus mutants.

Nucleotide sequence accession numbers. The clonal envelope sequences reported here have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html). The assigned accession codes are EF643652 to EF643695.

RESULTS

Characteristics of in vivo escape from 2G12. Resistance to 2G12 has been commonly reported to result from mutations at one or several of the N-linked glycosylation sites composing the antibody's epitope. Five N-linked glycosylation sites have been defined to form the canonical sites of the 2G12 epitope and are located at amino acid positions 295, 332, 339, 386, and 392 within gp120 of reference strain HXB2 (49). Of note, a recent study suggested that escape from 2G12 may also occur without the involvement of these five sites (39). Since 2G12 is considered a prototypic antibody to model vaccines that elicit anticarbohydrate responses blocking HIV, we performed an in-depth analysis of the 2G12 escape mutants that evolved in our in vivo study to derive further insight into the composition of the antibody's epitope and in vivo escape pathways. To this end, we sequenced for each patient envelope (*env*) genes spanning the C2-to-V4 region from isolates derived before passive immunization (preisolates) and directly from plasma at relevant time points during the trial when viral rebound and phenotypic resistance had been observed (Fig. 1). By comparing the 2G12-sensitive pretreatment sequences with those of the matching escape variants, we analyzed whether resistance-conferring mutations mapped to those glycosylation sites previously defined as the 2G12 epitope or whether alternate glycosylation sites or amino acids may also be involved in forming the epitope and were preferentially changed during in vivo escape. Pretreatment sequences were derived from the cultured virus isolates used as patient-relevant reference strains throughout the study (52). Thirteen of the 14 patients enrolled in the clinical trial were included in this analysis. Samples from patient NAB14 were not available for this analysis, as this patient suppressed viremia throughout the entire observation period (24 months). Sequences of the escape variants that evolved in vivo in the remaining 13 patients were derived directly from bulk viral RNA isolated from plasma. This strategy allowed us to determine the dominant mutations in the viral quasispecies which evolved during the in vivo treatment with the MAbs, excluding potential influences of in vitro culturing (Fig. 1). In the majority of patients, escape mutants emerged that had sequence changes in at least one of the five previously defined glycosylation sites composing the 2G12 epitope. Notably different were the results obtained initially for bulk sequences from patient NAB05, which despite phenotypic resistance to 2G12, portrayed no apparent change in the canonical sites of the epitope in this analysis (data not shown). To verify whether resistance in this case was indeed conferred by mutations in other regions, as previously suggested (39), we performed a clonal analysis of the envelope genes derived from viral RNA in the plasma of this patient (Fig. 1). Data obtained in this survey revealed that multiple different mutations within the 2G12 epitope had occurred. The latter could not be detected in a bulk analysis, as none of them by itself was forming a majority among viral quasispecies. However, in sum, these mutant sequences formed the majority, clearly outnumbering wild-type sequences, and thus were in concordance with the observed phenotypic resistance profile (Fig. 1 and data not shown). Of note, virus from patient NAB09 was already relatively resistant to 2G12 at the pretreatment time point (IC_{90}, >25 μg/ml; IC_{70}, 8.71 μg/ml). Although sensitivity to 2G12 did not further decrease in the phenotypic assay, we nevertheless observed new mutations in the 2G12 epitope, suggesting that the antibody even in this case exerted some selection pressure. Several patients already lacked one of the 2G12 canonical sites before treatment. Two patients (NAB02, NAB10) lacked the glycosylation site at position 332, three individuals (NAB01, NAB05, NAB13) lacked the site at position 339, and patient NAB07 lacked the glycan at site 386. Interestingly, isolates from these patients were phenotypically sensitive to 2G12 in the PBMC-based neutralization assay. Thus, depending on the viral strain, 2G12 reactivity appears not always to require all five glycosylation sites (Fig. 1). Nevertheless, higher antibody concentrations were needed to achieve inhibition of several of these isolates, supporting the notion that these sites are relevant for 2G12 binding and activity (Fig. 1 and reference 52).

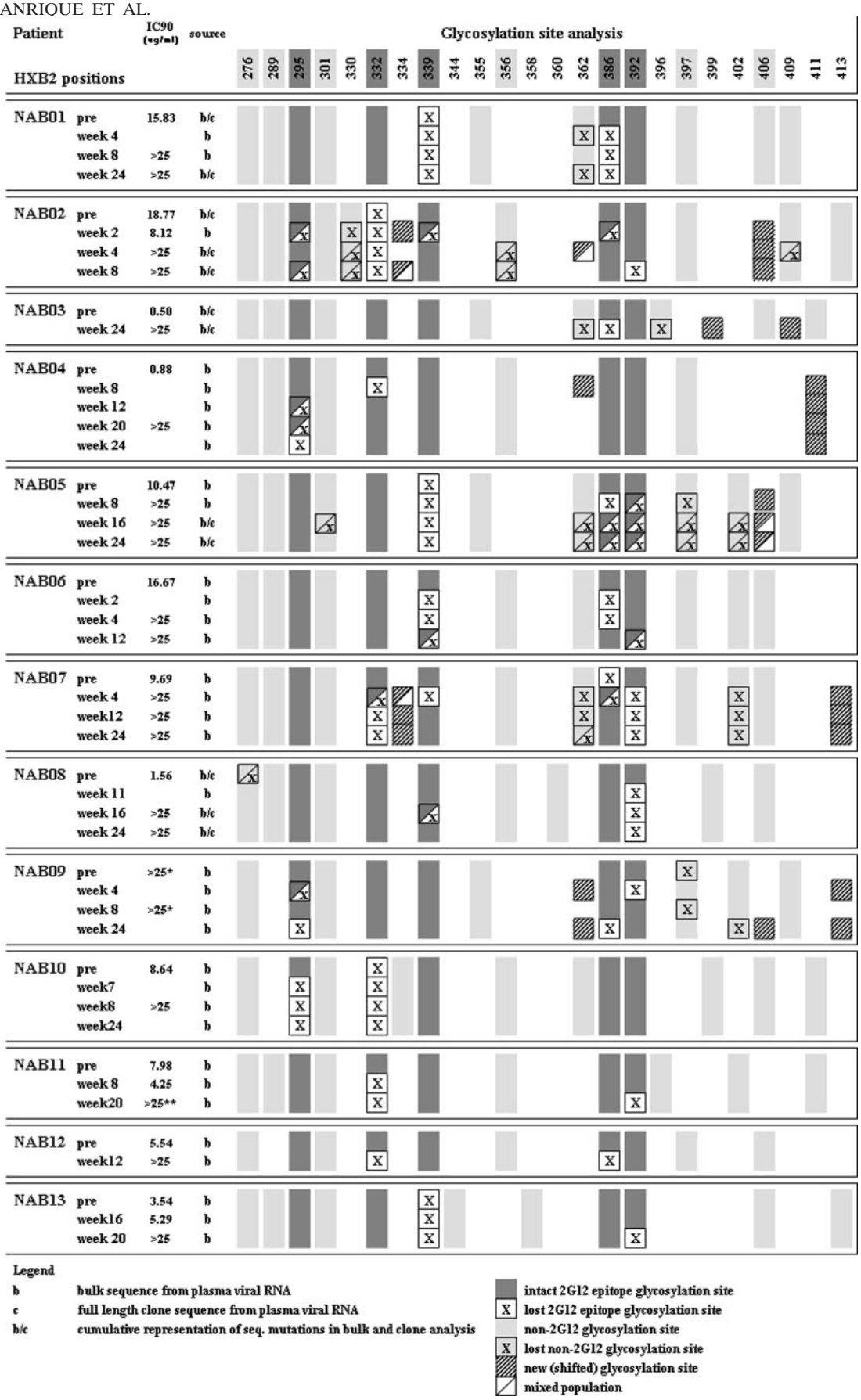

TABLE 1. Clonal analysis of the 4E10 epitope in plasma virus isolates obtained during in vivo treatment with MAbs[a]

Patient and virus	Amino acid at position:												
	668	669	670	671	672	673	674	675	676	677	678	679	680
NAB01													
Pretreatment isolate	S	L	W	S	W	F	D	I	T	Q	W	L	W
Wk 12 clone 05	S	L	W	S	W	F	**G**	**M**	T	Q	W	L	W
Wk 12 clone 13	S	L	W	S	W	F	D	**M**	T	Q	W	L	W
Wk 12 clone 27	S	L	W	**N**	W	F	D	I	T	Q	W	L	W
NAB02													
Pretreatment isolate	S	L	W	N	W	F	D	I	T	K	W	L	W
Wk 12 clone 35	S	L	W	N	W	—	—	—	—	—	—	L	W

[a] Shown are sequence changes in the 4E10 epitope retrieved from the analysis of 51 and 37 clones derived from patients NAB01 and NAB02, respectively, at week 12 of the in vivo passive-immunization study. The 4E10 epitope is in boldface. The amino acid numbering corresponds to that of the HXB2 reference strain. —, amino acid deletion in the sequence.

Reactivity in at least some of these cases may have been preserved through the introduction of adjacent glycosylation sites at positions 330 and 334 (NAB02), position 334 (NAB10), and position 344 (NAB13). Upon in vivo exposure to 2G12, we observed different patterns of escape mutations among individuals. Patients NAB01, NAB03, NAB10, and NAB13 lost only one glycosylation site, patients NAB04, NAB05, NAB08, NAB11, and NAB12 lost two sites, patients NAB06, NAB07, and NAB09 lost three sites, and patient NAB02 lost four sites at different time points. Mutations at sites 295, 332, and 339 each occurred in four individuals, whereas mutations at sites 386 and 392 were detected in seven and eight individuals, respectively (Fig. 1 and data not shown). As expected, the number of 2G12 core epitope glycosylation sites was significantly decreased after passive immunization ($P = 0.0002$; Wilcoxon two-tailed signed-rank test), whereas no influence on other N-linked glycosylation sites within amino acids (aa) 264 and 415 was apparent ($P = 1.00$; Wilcoxon two-tailed signed-rank test).

Characteristics of viral evolution during in vivo treatment with 2F5 and 4E10. We previously reported that, in contrast to the effects seen for 2G12 upon prolonged in vivo exposure, we failed to detect escape from the MPER-reactive antibodies 2F5 and 4E10 (52). Neither changes in phenotypic sensitivity to the MAbs nor mutations in the core epitopes of 2F5 and 4E10 were detected. To exclude the possibility that mutations had been lost during the culturing of virus isolates, we had previously sequenced both the derived cultured isolates which emerged during the trial and viral RNA derived directly from plasma (52). The two analyses provided identical results and gave no evidence of mutational changes (52). The epitopes of 2F5 and 4E10 are prime candidates for the development of vaccines because of the exceptionally broad reactivity of these MAbs to divergent viral strains. It was thus of particular interest to define why escape from these antibodies had not occurred in vivo and whether this reflects an overall inability of the antibodies to function under in vivo conditions. As a first step to address this, we performed an in-depth clonal analysis of the MPER of three representative patient isolates to define whether escape mutants had developed at low frequency. In total, we analyzed 150 clonal sequences of virus from patients NAB01 (51 clones), NAB02 (37 clones), and NAB08 (62 clones). Sequences were derived directly from viral RNA isolated from patient plasma at the peak of antibody exposure (week 12), which is when, we assumed, the strongest selection pressure was induced. Within the core epitope of 2F5, no amino acid mutation was detected among the clones analyzed for patients NAB01 and NAB08. One clone of patient NAB02 had a synonymous substitution in the epitope at position 664D. Within the 4E10 epitope, patients NAB01 and NAB02 had nonsynonymous substitutions in three clones and one clone, respectively (Table 1). Changes of S to N at position 671 and D to G at position 674 that occurred in clones of NAB01 are known not to interfere with 4E10 activity (1, 47, 64). The I675M substitution detected in two clones is highly uncommon. Only 8 of the 187,347 entries currently listed in the Los Alamos HIV sequence database (http://www.hiv.lanl.gov/content/hiv-db/mainpage.html) contain the respective residues. Importantly, we found that introduction of this mutation into the wild-type *env* sequence of virus NAB01 does not affect infectivity or sensitivity to 4E10 (P. Rusert and A. Trkola, data not shown). The deletion spanning the 4E10 epitope observed in one clone of patient NAB02 has not been previously described and likely represents a defective viral sequence. Taken together, the results of this clonal analysis confirmed our previous observations that no escape mu-

FIG. 1. Changes in potential glycosylation sites within C2 to V4 induced during in vivo treatment with 2G12. Samples derived at different time points from plasma bulk cultures and single clones from 13 patients (NAB01 to NAB13) following passive immunization were sequenced, and changes are summarized. The five glycosylation sites defining the 2G12 epitope are shaded in dark gray. Non-2G12-related potential glycosylation sites are shaded in light gray. X denotes a sequence mutation in the glycosylation site that lead to loss of that specific site. Glycosylation sites created through mutations during passive immunization are indicated by dashed boxes. The presence of mixed populations is indicated by boxes with diagonal divisions. The sensitivity to 2G12 (IC_{90}) of available replication-competent viruses derived during our passive-immunization study was measured in a PBMC-based assay (52). An asterisk indicates that the isolate was sensitive to 2G12 at the IC_{70}. Double asterisks indicate the measured IC_{90} for the isolate derived at week 24.

tations in the epitopes of 2F5 and 4E10 occurred in vivo, despite prolonged exposure to high concentrations of the MAbs.

In vitro escape from 2G12, 2F5, and 4E10. The central question thus remained why escape solely to 2G12 and not to the MPER MAbs had occurred in vivo. Our previous studies suggested that effective doses of MPER MAbs reached by passive immunization may have been too low to reach in vivo efficacy (52). While the emergence of escape mutations is evidence that a MAb induced selection pressure, we can nevertheless not conclude with certainty that absence of escape proves the MAbs' inactivity. Clearly, the MPER antibodies by themselves had had no effect in our in vivo study, as otherwise lower viremia should also have been observed after escape from 2G12. However, it has to be considered that the three MAbs can act in synergy (30, 33, 66). While the impact of the MPER MAbs by themselves must have been too low for in vivo efficacy, it cannot be ruled out that as long as all three antibodies were present and active, 2F5 and 4E10 might, in combination, have increased the 2G12 selection pressure. Once resistance to 2G12 was established, the pressure of the MPER MAbs alone may have been too low to affect selection. If this scenario is correct, it would imply that 2G12 resistance can be obtained more easily without loss of fitness, whereas MPER mutations are selected against. Nevertheless, virus isolates that are phenotypically resistant to both MPER MAbs that occur naturally or emerge in vitro or in vivo have been previously described and appear, at least in the case of 2F5, not to be uncommon (3, 33, 44, 51, 57, 65).

To determine if restrictions to the generation of HIV mutants able to escape MPER antibodies exist, we performed in vitro escape selection experiments with viral isolates derived from four patients, NAB01, NAB02, NAB03, and NAB08, before the passive-immunization study. These 4 isolates were chosen from among the 13 patient isolates because of their high sensitivity to all three MAbs at prescreening, which allowed escape selections at comparable antibody concentrations. By exposing these virus isolates in vitro to the three MAbs separately, we investigated if isolates from our patient cohort were particularly refractory to changes within the MPER.

All four of the patients selected had experienced a rebound of viremia during the trial that coincided with the emergence of 2G12-resistant viral strains (52). Virus resistant to 2G12 evolved rapidly in patients NAB01 and NAB02 (after 8 and 4 weeks, respectively). NAB03 rebounded only when passive immunization had stopped and antibody levels had declined. NAB08, a patient who initiated antiretroviral therapy during acute infection, showed delayed viral rebound compared to a control group of untreated, acutely HIV-1-infected patients. Importantly, loss of sensitivity to 2G12 was associated with loss of viremia control in all of these patients.

To explore the patterns of escape from the three antibodies in vitro, we cultivated preimmunization virus isolates on stimulated PBMC in the presence of the respective antibody. Antibody concentrations were adjusted in order to attain high selection pressure while still allowing residual viral replication (for a detailed description, see Materials and Methods). To closely mimic in vivo conditions, both supernatant and infected cells were passaged weekly onto fresh stimulated PBMC, allowing free virus infection, as well as cell-cell transmission, to occur. Viral replication was monitored weekly by assessing p24 content in cultures, and antibody concentrations were increased according to the extent of viral replication to guarantee sufficient selection pressure. Figure 2 depicts a representative profile of the selection experiments performed with each individual antibody to the isolate from patient NAB01. After a series of long-term selection experiments with all four isolates, we performed additional experiments with isolates from patients NAB01 and NAB03 to obtain information on the reproducibility of our observations (Fig. 3). Viral supernatant was collected weekly and assayed periodically for sensitivity to all three MAbs. Resistance increased gradually under elevated antibody pressure in those cases where successful selection of escape variants occurred (Fig. 2 and 3). Irrespective of the isolate used, we observed a relatively rapid escape from the 2G12 antibody in all nine selection experiments. The timing of the phenotypic detection of escape varied, to some extent, between patient isolates but also in repeat experiments with the same isolates and ranged from 3 to 11 weeks. This is not unexpected, as viral replication in these experiments depends on PBMC infectivity, which fluctuates from donor to donor.

With respect to 2G12, the in vitro experiments confirmed our in vivo observation. While the MAb is capable of exerting pressure on viral replication, the virus nevertheless fairly easily evades 2G12. However, in contrast to the in vivo situation, virus able to escape both MPER antibodies could be generated in vitro. Viruses from all four patients developed resistance to 2F5 during immune selection, albeit escape selection was not successful in all attempts (Fig. 3). The fastest resistance evolved after 4 weeks of culture (NAB02), and the slowest evolved after 8 weeks (NAB01). In comparison, 4E10 escape mutants were even more difficult to induce. No 4E10 escape mutants could be generated in virus cultures derived from patients NAB03 and NAB08. Virus of patient NAB01 developed 4E10 resistance in all three selection experiments after 9 to 16 weeks of culture, and 4E10 resistance of virus NAB02 only developed after 17 weeks. We noted that, compared to 2G12, infected cultures were highly sensitive to 4E10 concentration increases and, to a lesser extent, also sensitive to 2F5 concentration increases. Elevation of MPER antibody levels repeatedly resulted in abortive infection (Fig. 3). The latter confirmed that, at least in vitro, the antibodies are highly effective and that at the same time resistance is not easily achievable by the virus. Notably, we did not succeed in generating virus populations that were stable and fully resistant to 4E10 in these experiments. While the virus strains that emerged in vitro clearly had lost substantial sensitivity to 4E10, they remained partially sensitive to the MAb (IC_{90}, <25 μg/ml) (Fig. 3).

Genotypic analysis of in vitro escape variants. We next used genotypic analysis to confirm the introduction of resistance-conferring mutations into the viral variants generated. The in vitro-selected virus cultures are expected to contain a mixture of wild-type subspecies that will progress to a population with increasing contents of escape variants as antibody pressure is elevated. Sequencing of bulk virus cultures therefore has limitations in defining escape mutants at early stages of mutant selection, as only the most frequent population is represented in this analysis. Since clonal analysis at all time points in our extensive mutant selection would not have been feasible, we chose a two-geared approach. We initially performed bulk

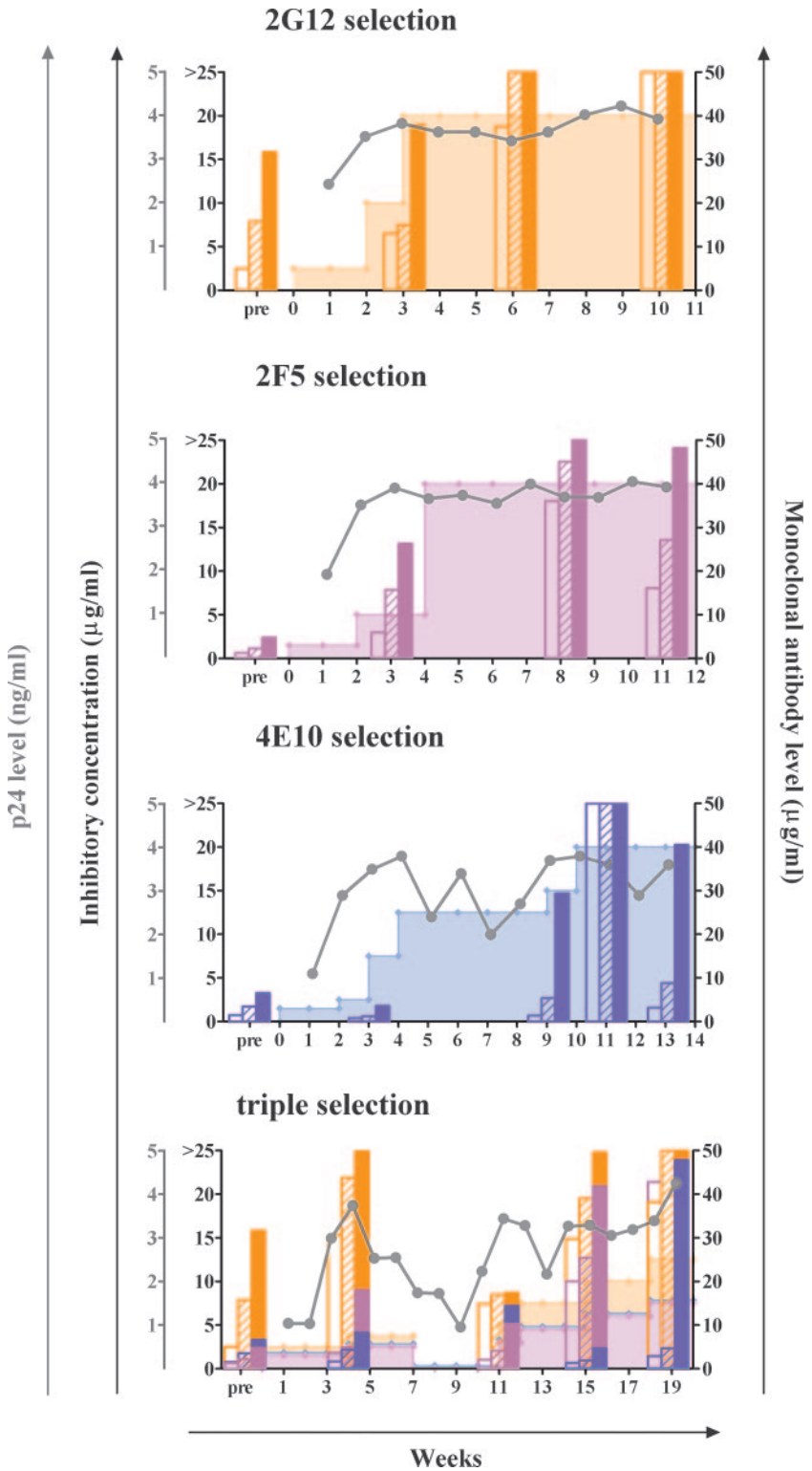

FIG. 2. Representative profile of in vitro selection experiments performed with MAbs 2G12, 2F5, and 4E10. Individual panels depict the evolution of the resistant viral variants of isolate NAB01 for each of the three MAbs (2G12, 2F5, and 4E10) tested and all three MAbs in combination. Gray lines and symbols (gray axis) signify p24 antigen production in cultures. Shaded areas represent the antibody level present in the culture during selection (orange for 2G12, magenta for 2F5, and blue for 4E10). The same colors are used to illustrate the neutralization sensitivities (IC_{50}, clear bars; IC_{70}, dashed bars; IC_{90}, filled bars) of the viruses that emerged to the respective antibodies at the indicated time points.

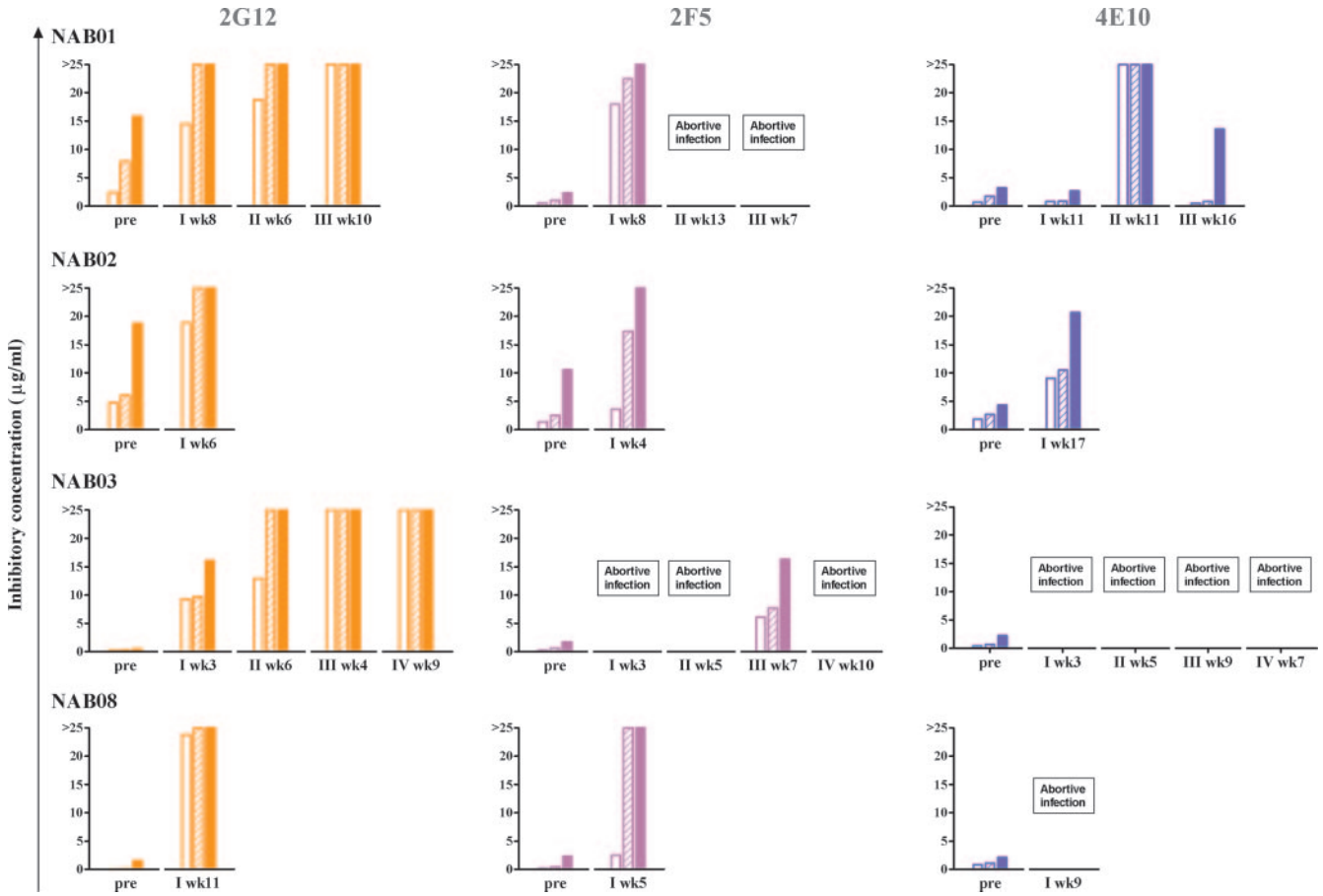

FIG. 3. Overview of in vitro selection experiments with 2G12, 2F5, and 4E10. Selection experiments were conducted one to four times (indicated by roman numerals) with isolates from patients NAB01, NAB02, NAB03, and NAB08. The neutralization sensitivity (IC_{50}, clear bars; IC_{70}, dashed bars; IC_{90}, filled bars) attained during each selection series is depicted. Genotypic resistance was further characterized by sequencing (see Fig. 6 and 7). When selection resulted in abortive infection, the timing of the termination of an experiment is indicated.

sequencing of virus cultures at all of the time points when phenotypic resistance was detected, by analyzing the respective epitope regions (C2 to V4 for 2G12; aa 620 to 720, spanning the MPER, for 2F5 and 4E10). Selected time points of interest were analyzed more thoroughly by single-clone sequence analysis of the full-length gene for gp160. In all of our selection experiments, untreated controls were cultured in parallel to provide reference viral strains that were subjected to identical culturing conditions. This allowed us to specifically discriminate between changes introduced upon long-term in vitro culturing and those induced directly by MAb pressure.

Genotypic analysis of in vitro-selected 2G12 escape variants. In total, we analyzed the 2G12 epitopes of nine viral cultures with phenotypic resistance to the MAb and the matching control cultures. While in vivo-selected 2G12-resistant strains harbored multiple amino acid substitutions that were not associated with potential N-glycosylation sites, the pattern observed in vitro was markedly different (Fig. 1 and 4 and data not shown). Mutations that did not affect glycosylation sites were only found occasionally. The majority of substitutions occurred in N-glycosylation-relevant sites. As expected, mock-treated controls displayed no sequence changes within the 2G12-relevant N-glycosylation sites, with the exception of the mock-treated culture strain from patient NAB02, where a glycosylation site at position 332 was reintroduced. Accordingly, all control culture isolates remained fully sensitive to 2G12 (Fig. 4).

Interestingly, 2G12 selection of the virus from patient NAB01 gave rise to the same single mutation (T394I), which resulted in loss of the glycosylation site at position 392 in all three of the long-term selection experiments performed with this virus. These escape mutants thus differed from those obtained in vivo, where only mutants that lost the N-glycan at position 386 had evolved (Fig. 1). Phenotypic resistance to 2G12 was already detected for the patient NAB02-derived virus isolate at week 6. However, at this early time point of the selection, wild-type virus was apparently still present, as detected by bulk sequencing (Fig. 4). Further clonal analysis revealed that resistant species with a T341I mutation (loss of N-glycosylation site at position 339) had emerged. The same mutation also occurred in vivo, although there it was not the dominant variant as this patient developed multiple different escape mutants over time (Fig. 1). In vitro cultivation of all four selection experiments performed with the virus from patient NAB03 yielded virus with phenotypic resistance to 2G12. Three escape mutants had lost the glycosylation site at position

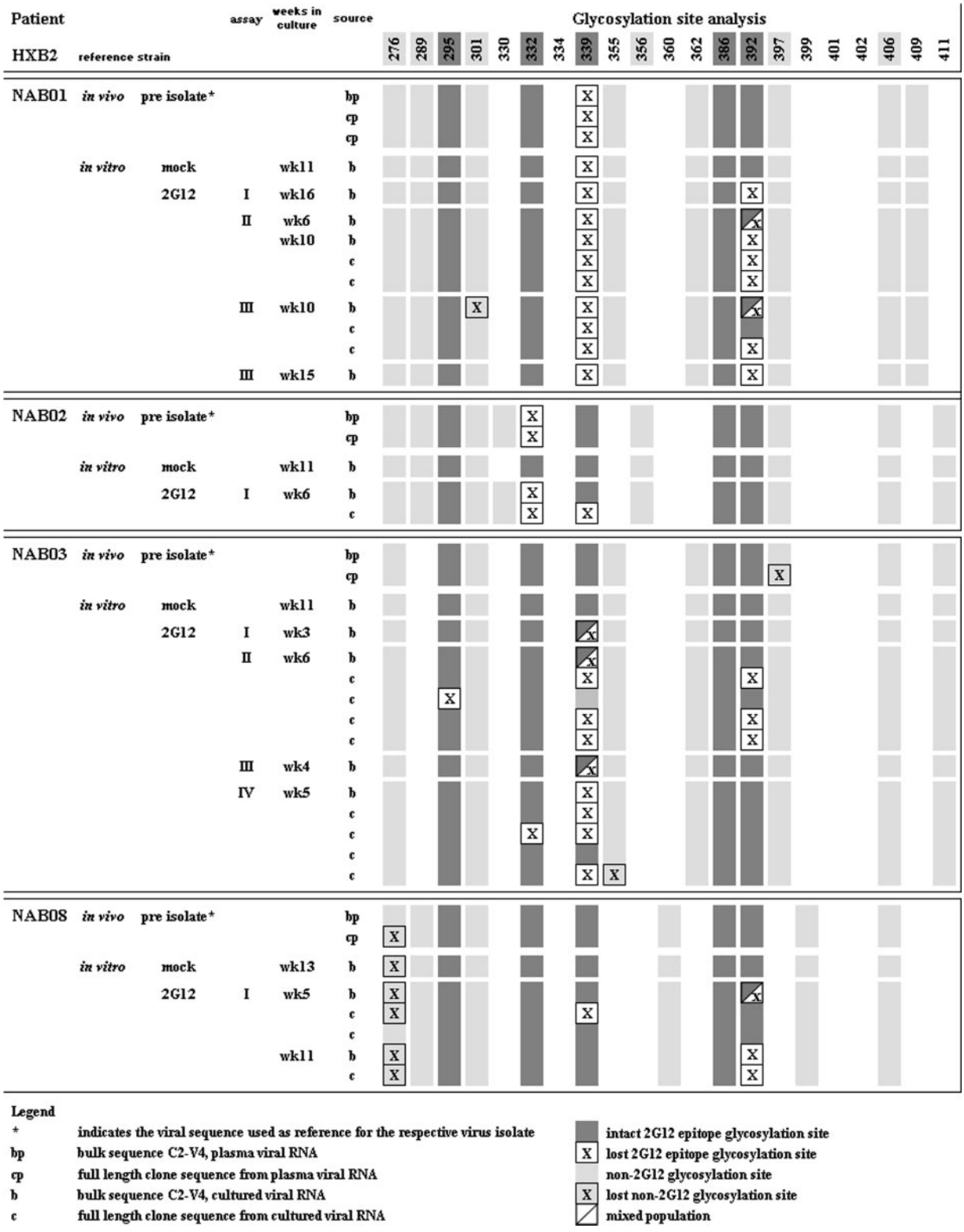

FIG. 4. Overview of sequence changes within the C2-to-V4 region of isolates selected with 2G12 in vitro. The C2-to-V4 region of the 2G12-selected isolates derived from patients NAB01, NAB02, NAB03, and NAB08 are shown together with the respective mock-treated isolates and the sequence changes found in vivo during passive immunization. 2G12-relevant glycosylation sites are shaded in dark gray. Non-2G12-related potential glycosylation sites are shaded in light gray. X denotes a sequence mutation in the glycosylation site that lead to loss of that specific site. The presence of mixed populations is indicated by boxes with diagonal divisions.

339 (N339K), whereas in vivo again a different mutation had evolved (N386D). Clonal analysis of the virus obtained from patient NAB03 in in vitro selection experiments revealed that while loss of site 339 was predominant, mutations of N-glycosylation sites at positions 295 and 332 also occurred. Clones of the escape virus derived from patient NAB08 after 5 weeks of selection harbored a mutation at aa 339 which affects N-glycosylation. Interestingly, sequence analysis of the same long-term culture at week 11 revealed that, over time, a different mutant had become dominant which lacked the glycosylation site in position 392 (T394I). Of note, both sites had been lost during in vivo escape.

Genotypic analysis of the MPER upon in vitro exposure to 2F5 and 4E10. In view of the fact that during in vivo treatment with the MPER antibodies no loss of neutralization sensitivity or mutations in the antibody epitopes were detected, it was of particular interest to phenotypically and genotypically analyze the in vitro-generated mutants able to escape 2F5 and 4E10 (Fig. 2, 3, and 5).

In one of three experiments with virus derived from patient NAB01, we succeeded in generating a 2F5 escape variant at week 8 of culture which harbored the mutation D664N within the core epitope of 2F5. All three attempts to generate 4E10 escape mutants of the virus from patient NAB01 resulted in the evolution of viral variants with mutations within the 4E10 epitope. Two selection series gave rise to mutation F673L, whereas the third selected strain had introduced a W672G change (Fig. 5). However, phenotypically, these virus isolates had gained only a relatively modest decrease in sensitivity to 4E10 (Fig. 3), which could have been caused by a mixed population of wild-type virus and escape mutants. We thus cloned full-length viral envelopes from these cultures and performed inhibition experiments with Env-pseudotyped virions and TZM-bl cells (Fig. 6). Both, *env* mutants carrying W672G and F673L were fully resistant to 4E10 in this analysis.

Virus from patient NAB02 developed a 2F5 (K665T) and a 4E10 (F673V) escape mutation after culturing for 4 and 17 weeks with the respective antibody (Fig. 3 and 5). Only one of the four attempts to generate a 2F5 escape mutant virus from patient NAB03 was successful. Bulk sequencing at week 7 revealed that the majority of quasispecies carried mutation K665E. However, clonal analysis detected quasispecies carrying a K665N mutation which became prevalent by week 12 of culture. All four attempts to generate 4E10 escape mutants of the virus from patient NAB03 failed and resulted in abortive infection at relatively early time points (Fig. 3). The virus from patient NAB08 also introduced a mutation at position D664N which appeared after 5 weeks of culture with 2F5. However, in the same series, 4E10 escape selection of the virus from patient NAB08 was unsuccessful. To more closely mimic the in vivo experiment, we also performed selection experiments with the three MAbs in combination (Fig. 2). Briefly, we found that triple-selection experiments very much followed the profile seen for the MPER MAb selection in that frequent abortive infection was observed (data not shown). By applying relatively low antibody pressure, we nevertheless succeeded in maintaining three triple-selection cultures derived from the isolate from patient NAB01 (Fig. 2 and data not shown). Resistance was evaluated phenotypically by inhibition assays, and resistance-conferring mutations were located by sequencing of the respective epitopes (data not shown). Two triple-selected strains developed resistance to both MAbs 2F5 and 4E10 (mutations D664N and F673L, respectively), while the third isolate only developed resistance to 2F5 (D664N). However, likely as a result of the lower 2G12 concentration used in the combination, no resistance to 2G12 developed (data not shown).

In summary, all of the isolates tested were capable of tolerating mutations in the MPER. Importantly, the resistance-conferring mutations observed were all located at the canonical sites of the respective antibody epitopes defined in previous studies (12, 38, 44, 64, 65).

Infectivity and stability of in vitro-selected escape mutant viruses. Our observations during the in vitro selection of MAb escape mutants strongly suggested that 2G12 escape mutations are tolerated well while 4E10 and, to some extent, also 2F5 resistance-conferring mutations appear to impose constraints on viral replication. To address this more directly, we compared the functionality and infectivity of *env* genes of the isolates derived from patient NAB01 during selection in vivo and in vitro (Fig. 7). To exclude influences on viral infectivity outside of *env*, we inserted the respective envelope genes of the mock-treated control virus and the respective MAb escape mutants into the TN6 vector, a replication-competent viral backbone engineered from strain NL4-3 (40). Infectivity was determined by measuring the $TCID_{50}$ of the viruses on stimulated PBMC. Figure 7 depicts $TCID_{50}$ normalized to the p24 content of the respective stock ($TCID_{50}$/pg p24). The infectivity of the mock-treated control from patient NAB01 at week 11 of culture was the highest in this analysis. 2G12 and 2F5 escape variants displayed lower but still relatively high infectivity. In contrast to this, 4E10-resistant virus had markedly decreased infectivity, substantiating the evidence that the resistance-conferring mutations may potentially interfere with viral fitness.

To further investigate the phenotypic features of MPER MAb- and 2G12-resistant viruses, we probed to what extent these mutations restrict viral replication by analyzing the stability of the mutations in selected clones from patient NAB01 during long-term culturing in the absence of antibody pressure (Fig. 8). Cultures were propagated in analogy to the escape selection on PBMC with weekly passaging onto fresh donor cells. Viral supernatants were periodically analyzed for sensitivity to the three MAbs to assess if and how rapidly resistance-conferring mutations revert to wild-type sequences. As anticipated, 2G12 escape virus showed no apparent changes in replication pattern in the long-term culture and remained fully resistant to the MAb until the termination of the experiment at week 16. Considering the observed replication impairment of the MPER-resistant clones and the comparative difficulties in selecting these mutants, we hypothesized that a rapid reversion to wild-type sequences upon the removal of antibody pressure could occur. Surprisingly, the 2F5 escape mutant replicated for several weeks in the absence of the antibody without regaining sensitivity. Only by week 11 did sensitivity to 2F5 begin to moderately increase (Fig. 8). However, compared to the wild-type strain, the mutants retained reduced sensitivity to the MAb. These data were supported by clonal analysis of virus collected at the beginning of the long-term culturing and at weeks 11, 13, and 16, which revealed that the majority of the clones still harbored the resistance-conferring mutation

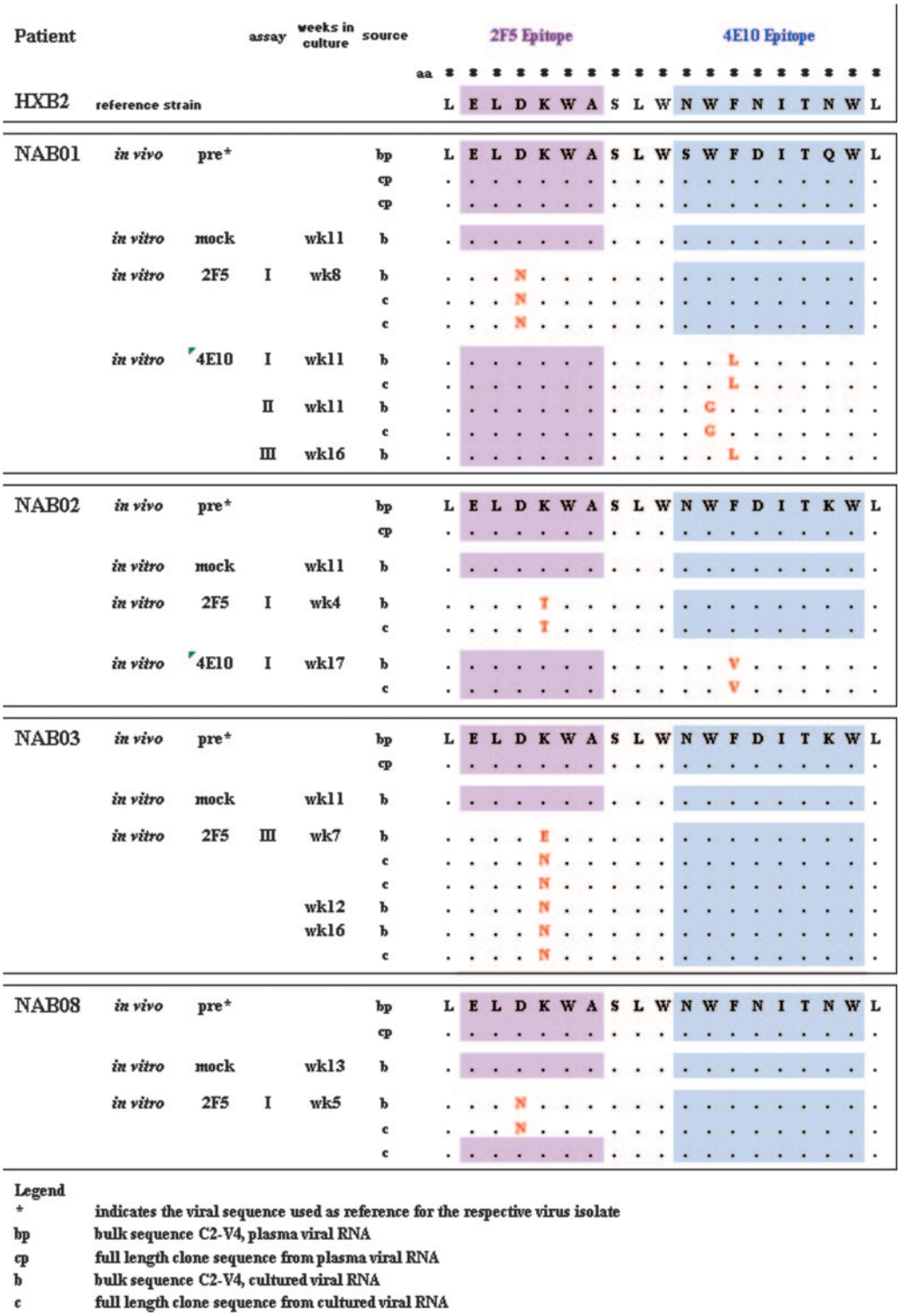

FIG. 5. Overview of sequence changes in isolates selected for resistance to 2F5 and 4E10. The epitopes of the selected isolates derived from patients NAB01, NAB02, NAB03 and, NAB08 are shown together with the respective pretreatment and mock-treated isolates. Intact epitopes for 2F5 and 4E10 are shaded in magenta and blue, respectively; mutations in the epitopes conferring resistance are in red. aa corresponds to the amino acid numbering in the HXB2 reference strain.

D664N (Fig. 9 left panel). Notably, though, at week 11 one clone carrying the 2F5-sensitive wild-type sequence was detected while different minor variants were detected at weeks 13 and 16.

In contrast to 2F5, upon phenotypic evaluation of 4E10 escape isolates during long-term culture, a gradually increased sensitivity to 4E10 from week 7 onward was detected. Surprisingly, clonal analysis revealed that this gain in neutralization

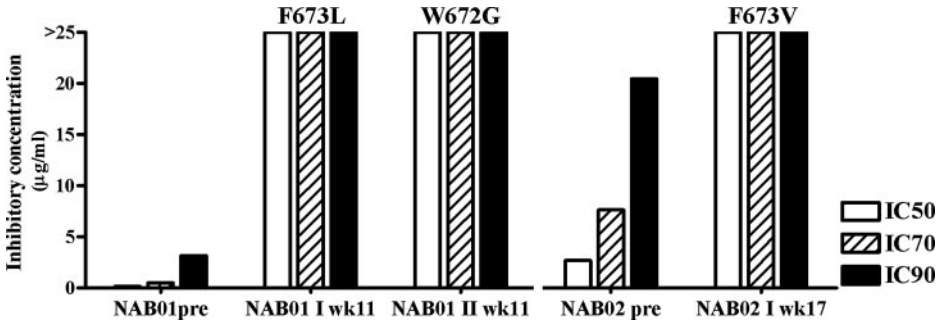

FIG. 6. Neutralization sensitivities of 4E10 escape mutants. Neutralization sensitivities of Env-pseudotyped viruses expressing gp160 derived from 4E10 escape virus strains from patients NAB01 and NAB02 were measured on TZM-bl cells. ICs (IC_{50}, clear bars; IC_{70}, dashed bars; IC_{90}, filled bars) of the wild-type virus (pre) and 4E10 escape mutants are depicted. The respective mutations in the core epitopes of the envelope clones are indicated.

sensitivity was not linked to a reappearance of quasispecies carrying the wild-type 4E10 epitope (Fig. 9). Only at one time point (week 13) had 2 of the 36 clones tested mutated to wild-type residue W672. Determination of which of the changes introduced rendered the 4E10 escape viruses more sensitive to neutralization upon long-term culture requires further analysis. Similar to the 2F5 escape viruses, other fluctuations in the epitope were apparent but none of these mutants increased in frequency. In sum, these analyses suggest that, in the absence of competition with fitter viral strains and selection pressure, MPER mutants are relatively stable.

DISCUSSION

We recently conducted a passive-immunization study to evaluate the in vivo potency of MPER MAbs 2F5 and 4E10 together with that of carbohydrate-specific MAb 2G12. The outcome of this trial was remarkable. A cocktail of these three neutralizing antibodies was capable of suppressing or delaying viremia rebound in several individuals. However, activity was lost as soon as a 2G12-resistant virus emerged. The fact that no resistance to the MPER MAbs occurred raised concerns that these MAbs were not active in vivo.

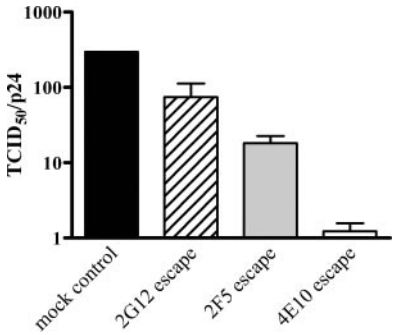

FIG. 7. Infectivity of neutralization escape viruses. The influence of 2G12, 2F5, and 4E10 resistance-conferring mutations in the envelope on the infectivity of the virus from patient NAB01 was studied. The *env* genes of the mock-treated control (black) and 2G12 escape (NAB01 2G12 II wk 10, gray), 2F5 escape (NAB01 2F5 I wk 8, dashed), and 4E10 escape (NAB01 4E10 II wk 11, white) mutants of the isolate from patient NAB01 were introduced into the replication-competent NL4-3 backbone TN6 NL and compared for infectivity on three-way-stimulated PBMC. The infectivity ($TCID_{50}$/pg p24) of the viral stocks is displayed. Means of two independent experiments, each performed with pools of three independent donor PBMC, are shown.

To elucidate the mechanisms behind the selection and resistance patterns in vivo and to gain insights into potential effects of MPER vaccines, we set out in the present study to investigate the pathways used by patient isolates to escape the three antibodies both in vivo and in vitro.

In sum, our analysis confirmed that 2G12 activity depends on the presence of glycosylation sites at positions 295, 332, 339, 386, and 392, as previously determined (49). However, the relevance of the individual sites appears to vary, depending on the viral strain and in the context of some viral *env* genes, 2G12 also remains active when one of the sites is lacking. It may be possible that adjacent N-glycans that were introduced in some cases can compensate for the loss of the 2G12 core epitope sites (Fig. 1). Most frequently, we observed amino acid changes at positions 386 and 392 (7 of 12 and 8 of 13 patients, respectively) among the escape variants that emerged in vivo. 2G12 escape variants that emerged in vivo included mutations not only at the epitope core sites but also at other potentially glycosylated and nonglycosylated amino acids positions. Whether or not the latter changes compensate for 2G12 escape mutations, support resistance to 2G12, or reflect selection processes of the autologous immune responses cannot be dissected with certainty. Our present analysis nevertheless allows us to conclude that in vivo 2G12 escape requires a mutation in one or more of the five canonical sites. Ensuing introduction of novel glycosylation sites may occur to secure the viral glycan shield. Thus, carbohydrate-targeting vaccines could have the potential to be effective if they succeed in recognizing a variety of epitope compositions. When we induced escape from 2G12 in vitro with prestudy isolates from four patients who had participated in the passive-immunization trial, we found different escape patterns. Mutations were almost exclusively restricted to the N-glycosylation sites, and the mutations within the 2G12 core epitope differed from those found in vivo in three of the patients. Escape was, in general, rapid, confirming that the virus can quite easily evade the MAb pressure applied.

Our detailed analysis of in vivo and in vitro escape pathways of 2F5 and 4E10, however, gave strikingly different results. To verify our previous observations that no resistance-conferring mutations had occurred in vivo, we performed a detailed clonal analysis of the MPER in plasma viral RNAs derived from three

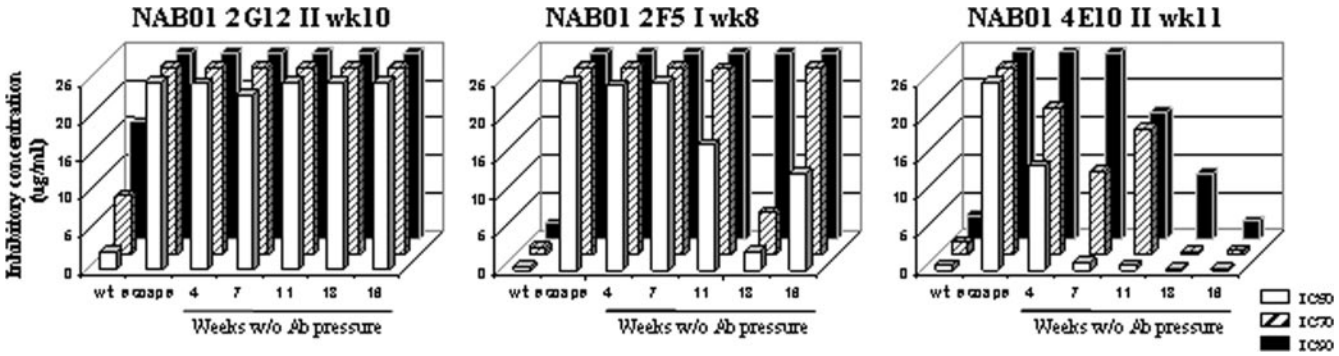

FIG. 8. Stability of escape mutations without (w/o) antibody (Ab) pressure. Patient NAB01 isolates selected to escape 2G12 (NAB01 2G12 II wk 10) (left panel), 2F5 (NAB01 2F5 I wk 8) (middle panel), and 4E10 (NAB01 4E10 II wk 11) (right panel) were subjected to long-term in vitro culture in the absence of antibody pressure. Inhibitory doses of the respective antibodies for virus strains derived during these long-term cultures are depicted (IC_{50}, clear bars; IC_{70}, dashed bars; IC_{90}, filled bars). wt, wild type.

patients at week 12 of antibody treatment, which is the latest time point under high antibody pressure in the study. The results confirmed our previous analyses. Among the 150 clones tested, none with a mutation conferring resistance to 2F5 or 4E10 was detected.

We conducted in vitro selection experiments with the MPER MAbs to verify whether or not these isolates from study participants can tolerate MPER mutations needed to gain resistance to the MAbs. The outcome of these in vitro selection experiments was notably different from that of experiments with 2G12, as frequent abortion of infection occurred when antibody levels were increased. The latter observations confirm that the MPER antibodies are highly active against these isolates and that escape from the MAbs is difficult to achieve. We nevertheless succeeded in generating 2F5-resistant strains of all four of the isolates tested and 4E10-resistant strains of two of the four isolates tested. Phenotypic and genotypic analyses demonstrated that resistance was, in all cases, associated with changes in the known core epitopes of the MAbs.

The often slow process of evasion of the MPER MAbs and the observed fragility of the emerging viruses prompted us to investigate whether the MPER mutations required for resistance to 2F5 and 4E10 may, to some extent, impair viral replication. Indeed, the infectivity of the 4E10 escape mutant virus tested was markedly reduced. Surprisingly, though, long-term culture in the absence of antibody pressure did not lead to rapid reversion of the epitope to the wild-type sequence for any of the three MAbs. Nevertheless, variation in both MPER epitope regions occurred, suggesting that a certain drift was induced.

Several reasons for a potential inactivity of the MPER antibodies in our previous trial have been considered. As MPER antibody concentrations in plasma were significantly lower than those of 2G12 during passive immunization, the concentration required for in vivo efficacy may not have been reached (25, 52). Lower MPER antibody concentrations were the consequence of considerably shorter elimination half-lives, the cause of which remains unknown (25). Of note, no endogenous

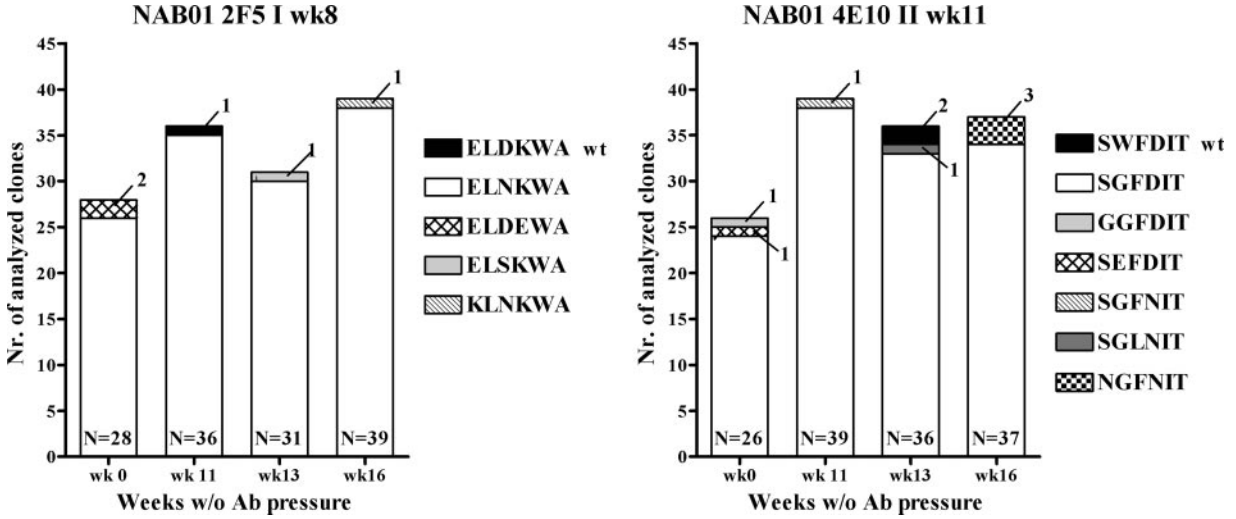

FIG. 9. Clonal analysis of the MPER after long-term culture. MPERs of 2F5-resistant (NAB01 2F5 I wk 8) and 4E10-resistant (NAB01 4E10 II wk 11) virus strains obtained after long-term culture without (w/o) antibody (Ab) pressure. Sequences of the respective antibody epitopes were analyzed to detect whether reversion to the respective wild-type (wt) sequences (ELDKWA and SWFDIT for the epitopes of 2F5 and 4E10, respectively) occurs.

antibody response to either MAb developed (25, 52). Antibody distribution to various tissues was not measured directly, but pharmacokinetic analyses showed no differences in distribution half-lives or central and steady-state distribution volumes, suggesting that the antibody distributions may generally not be very different. However, whether or not antibody distributions to the relevant sites of infection are comparable remains unknown (25). Concerns that the activity of 4E10 and 2F5 was blocked in vivo because of cross-reactivity with host antigens such as phospholipids (19) could thus far not be corroborated, as in ex vivo studies all three passively administered antibodies maintained their neutralization activity in plasma (Trkola et al., unpublished data). Determination of which, if any of these effects could account for inactivity in vivo requires further investigation. Despite the fact that the MPER MAbs lacked in vivo activity by themselves, as no effect on viral load upon escape from 2G12 or selection of mutants able to escape from the MPER MAbs was seen, it cannot be ruled out with certainty that the MPER antibodies were active in vivo (52). While difficult to assess, it is possible that the in vivo activity of the antibody cocktail depended on a synergistic effect of all three MAbs. Synergy, as previously described for these MAbs (30, 33, 66) in combination with 2G12, could potentially have augmented the selection pressure induced by the latter. We have recently investigated these synergy effects in detail and found evidence that the antibodies acted in combination (Trkola et al., unpublished).

Here we provide further information on the nature of the evolution of escape from the three MAbs. Whereas escape from 2G12 is also rapid in vitro, selection of resistance to MPER antibodies is notably difficult and can result in abortive infection or—as in the case of the 4E10-resistant variant analyzed—in virus strains with low infectivity. Both a high threshold of resistance evolution and impaired infectivity of escape mutants that emerge would be highly desirable features of vaccine-elicited immunity and highlight once more the potential of MPER-targeting immunogens or drugs. A further detailed survey of the potential, but also the suggested risks (21), of MPER-specific strategies for therapy and prevention is therefore crucial.

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