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The Membrane-Proximal External Region of the Human Immunodeficiency Virus Type 1 Envelope: Dominant Site of Antibody Neutralization and Target for Vaccine Design

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INTRODUCTION

In spite of 25 years of effort, a vaccine against human immunodeficiency virus type 1 (HIV-1) is not available. However, there are several appropriate vaccine targets that are exposed on the viral surface and play an important role in infection. Infection of the target cell by HIV-1 is mediated by two envelope proteins (Env): surface gp120 and membrane-anchored gp41. Initially, Env is produced as a highly glycosylated gp160 precursor, which is processed by a host protease into the two subunits (96, 250). These two proteins remain associated by noncovalent interactions and form heterotrimeric spikes on the viral surface (65, 132); Env is the only viral protein expressed in the viral membrane as well as in the membranes of infected cells. The infection process is initiated when gp120 binds to the primary CD4 receptor on the target cell (52). This interaction induces conformational changes to gp120 that expose and/or form the coreceptor binding site that is specific for the chemokine receptors CCR5 and CXCR4 (14, 226). Coreceptor binding triggers several conformational changes in gp41, which leads to the fusion of viral and host membranes, pore formation, and, ultimately, the release of the viral nucleocapsid core into the cell (39, 73, 244).

A vaccine that targets Env could potentially block the infection process by eliciting neutralizing (Nt) antibodies (Abs) against HIV-1. However, producing a vaccine that targets an immune response against HIV-1 has been extremely challenging for several reasons. First, HIV-1 mutates easily, thus creating a large number of quasispecies that act as a decoy for the immune response. In addition, the virus evades immune recognition by expressing a small number of viral spikes, shedding gp120, and masking Nt epitopes. The immunogenicity of Env is decreased by the trimeric structure of the spike, which occludes important epitopes, and by surface glycosylation. Moreover, HIV-1 infects T cells, which are critical for orchestrating the immune response to viral infection. Taken together, these viral characteristics complicate approaches to HIV-1-targeting vaccines.

Due to their exposure on the viral surface, both gp120 and gp41 are the targets of Ab-mediated viral neutralization (178, 185). These proteins are immunogenic, as shown by the large amount of Ab that is produced during the course of natural infection; however, Nt Ab levels are generally low and/or isolate specific (156). This low level of Nt Ab is perpetuated by the persistent nature of HIV-1 infection since high viral replication and mutation rates lead to the appearance of viral escape mutants against which the immune system responds with new Abs. This constant interplay between the virus and the host immune response results in the inability of the immune system to clear an established infection (192). Nevertheless, broadly Nt (bNt) Abs are sometimes generated. So far, only six bNt monoclonal Abs (MAbs) have been isolated from HIV-1-infected donors; they are all directed against Env. bNt MAbs b12, 447-52D, and 2G12 bind to gp120 (32, 48, 228, 230), whereas, 2F5, Z13, and 4E10 recognize the membrane-proximal external region (MPER) of gp41 (162, 275). These bNt MAbs inhibit infection by multiple genetic HIV-1 clades in vitro and prevent experimental infections in animal models with viruses bearing the envelope proteins from primary HIV-1 isolates (18, 141, 142).

The discovery of bNt MAbs and their characterization have introduced the possibility of targeting their production in vivo by active immunization. This has proven difficult due to a number of factors including the structural complexities of the sites targeted by these Abs. For example, MAb b12 is directed against a complex discontinuous epitope that overlaps the CD4 binding site of gp120, whereas 2G12 recognizes the termini of several oligosaccharide chains on the highly glycosylated face of gp120. MAbs 2F5 and 4E10 bind adjacent linear epitopes located on the MPER; however, it is thought that those linear regions do not completely represent their full immunogenic epitopes. In addition to the complexities of their epitopes, bNt MAbs share several uncommon structural features, including a long hypervariable loop comprising the third complementarity-determining region of the heavy chain (CDR-H3) (b12, 2F5, 447-52D, Z13, and 4E10) (38, 48, 170, 203, 216) as well as VH domain swapping (2G12) (35). In spite of these complex features, the goal of eliciting bNt Abs continues to be crucial for the development of an HIV-1 vaccine.

Of the six known bNt MAbs, three are directed to the MPER, thus defining this region as major target for vaccine efforts. This review focuses on the MPER as a target for HIV-1 vaccine design and describes (i) the current understanding of the structure and function of gp41 and in particular the MPER, (ii) the structure and function of bNt MAbs against the MPER, (iii) challenges in producing bNt Abs against the MPER, and (iv) potential approaches that could be used to make an MPER-targeting vaccine. It is generally accepted that an efficient anti-HIV-1 vaccine should involve both the cellular and humoral arms of the host immune response; however, our discussion will be limited to approaches that target the production of Nt Abs.

STRUCTURE AND FUNCTION OF gp41

Env protein gp41 anchors the infectious spike to the viral membrane and plays an important role in cell entry. It consists of ~345 amino acids (aa) with a molecular mass of 41 kDa, does not contain clearly defined variable regions, and is more conserved than gp120. As shown in Fig. 1, it is divided into three major domains (74): the extracellular region, also called the ectodomain (aa 512 to 683; numbering is based on HIV-1 HXB2 [120] unless otherwise specified), the transmembrane (TM) domain (aa 684 to 705), and the cytoplasmic tail (CT) (aa 705 to 856). The ectodomain contains several distinct functional determinants involved in the fusion of viral and host cell membranes: (i) an N-terminal hydrophobic region that func-

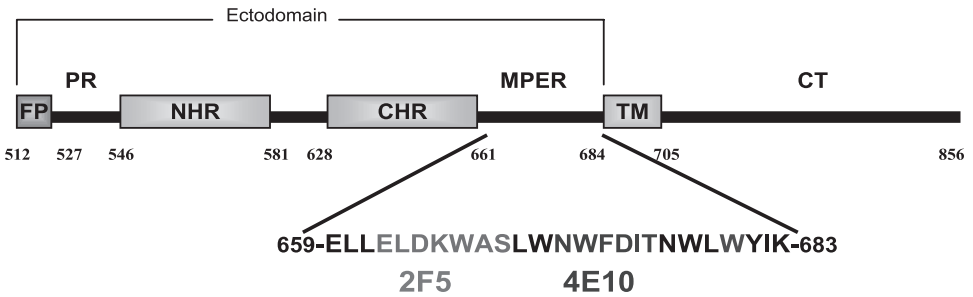

FIG. 1. Schematic representation of the gp41 domains.

tions as a fusion peptide (FP) (aa 512 to 527); (ii) a polar region (PR) (aa 525 to 543); (iii) two α-helix repeat regions referred as the N-terminal heptad repeat (NHR) (aa 546 to 581) and the C-terminal heptad repeat (CHR) (aa 628 to 661) (39); (iv) a disulfide-bridged hydrophilic loop that connects the two heptad repeats, also known as the connecting loop, the cluster I epitope, or the immunodominant (ID) loop (aa 598 to 604); (v) a Trp-rich region known as the MPER (aa 660 to 683); and (vi) a membrane-spanning domain, also called TM, and the CT. Thus, gp41 is comprised of several distinct regions that each contribute unique functions.

The structure of whole gp41 is not clearly defined, since the available crystal structures of the HIV-1 gp41 ectodomain core do not contain the FP and the ID loop and have deletions of the MPER (39, 223, 244). These studies revealed a six-helix bundle (6HB), which is considered to be the postfusion structure of the ectodomain and different from the native metastable structure of gp41 in the viral spike. Recently, two different structural models for the viral spike of the whole virion have been solved using cryoelectron tomography (263, 268). Roux and colleagues proposed that the MPER and TM "stalk" of each trimer are composed of three separate legs that project obliquely from the head of the trimer as a tripod-like structure. In contrast, the structural model proposed by Zanetti et al. shows the TM domain of simian immunodeficiency virus (SIV) gp41 as a stem in the viral surface; the tripod-like structure model correlates with the current view of the possible membrane involvement of the gp41 MPER (for more details on the spike structure, see the recent review by Roux and Taylor [195]). This technique is still being developed, and these conflicting structures could be attributed to the methods used to collect the data and/or the computational approaches used to determine the structures (222). Thus, the structure of the native gp41 protein in the viral spike remains an open question to be revealed by future studies. The lack of agreement between those two studies demonstrates one of the main challenges involved in understanding the complexities of this virus.

How many Env proteins are required for infection? This question has intrigued HIV-1 researchers for years. A study reported by Yang et al. supports the notion that only a single viral spike could be required for fusion (256); however, newer structural studies indicate a different picture. Recently, the structure of the HIV-1–T-cell interaction at the time of infection has been solved using microscopic tomography. Sougrat et al. observed the arrangement of about six to seven dense rod-like structures at the contact site between the cell and viral membrane (212). These rods were hypothesized to comprise a single Env spike. This structurally unique contact zone comprises an arrangement of closely packed rods (presumed to be Env) extending from the virus into the membrane of the target cell; this structure was named the "entry claw." However, at this atomic level of resolution (~ 20 Å), structural information is not refined enough to reveal how the fusion process occurs. Those authors proposed two different models describing the last step of fusion between virus and the host cell membranes. In the first model, the entire width of the "entry claw" is required for the fusion of the viral and the host membranes. This results in the merging of the contents of the two membranes. In the second model, a rod at the center of the "entry claw" creates a pore in the center of the contact zone, which permits the release of the viral core into the cell (212). Thus, gp41 appears to change its structure repeatedly and to change its location on the surface of the virion during infection. This structural flexibility may be yet another reason behind the difficulties in designing immunogens that elicit bNt Abs.

The FP

As shown in Fig. 1, the FP corresponds to the first 15 aa of gp41 and is followed by the PR. It is believed that the nonfusogenic state of the FP is buried in the gp120/gp41 quaternary complex and is exposed only transiently for interactions with the host cell membrane after gp120 binding to the CD4 receptor. The involvement of the FP in triggering virus-cell membrane fusion has been confirmed by mutational studies (21, 71, 76, 206). It has been proposed that the FP causes membrane destabilization by oblique insertion into the cell membrane, thus resulting in membrane fusion (23).

There is some controversy over the functional structure of the FP. For example, an α-helical structure has been proposed by Martin et al., who used Fourier transform infrared spectroscopy to determine the structure of a 16-mer synthetic peptide interacting with a phospholipid bilayer. However, the peptide adopts mainly a β-sheet conformation in the presence of lipids (137–139). Other studies with a 23-mer peptide suggested that the FP is a β-pleated structure (167, 168, 180–183, 220, 255). These conflicting results could be explained by the differences in peptide lengths, lipid compositions of the membrane model, and/or methods used. Thus, although the role of the FP in the fusion process is well established, the structure of the region in the native conformation of gp41 or in the fusogenic state is not known.

To solve this controversy, Buzon et al. studied the structure

and membrane interaction of FP using peptides comprised of 16 and 23 aa (33). Membranes comprised phosphatidylcholine and phosphatidylethanolamine with variations in the cholesterol and 6-ketocholestanol content. They found that when the peptide was in solution, it formed a mixed aggregate of α-helical and unordered structures; however, peptide interactions with the membrane resulted in an aggregated β-structure (33). More recently, the structure of the FP was evaluated using solid-state nuclear magnetic resonance (NMR) spectroscopy (266). That study revealed that the structure adopted by the FP in the context of the membrane could be influenced by the concentration of cholesterol used in the model vesicles. At low cholesterol concentrations, the FP adopts an α-helical conformation, whereas at higher cholesterol concentrations, it adopts a β-strand conformation. The FP was shown to be fusogenic under both conditions, suggesting that this region has a plastic nature. Thus, those authors speculated that structural flexibility is important, conferring the ability to infect cells with different cholesterol concentrations and/or to regulate the rate of fusion on HIV-1.

In another example, Li and Tam reported a helical structure for FP in dodecylphosphocholine (DPC) micelles using solution NMR. They proposed that the α-helix is the most likely physiologically active conformation of the FP during HIV-1 infection (125). Taken together, the studies described here indicate that the active structure of the FP remains a debatable topic; future studies should clarify this issue and reveal the events that occur upon interactions of the virus with receptors and coreceptors.

NHR and CHR

The presence of the NHR and the CHR in the HIV-1 gp41 ectodomain is a feature that is shared with TM envelope proteins of other retroviruses (for reviews, see references 211 and 245). It is thought that these regions play a key role in virus-host membrane fusion with gp41 by undergoing conformational changes that result in the formation of 6HBs. This is a stable structure (a trimer of hairpins) in which three NHRs form a core bundle in parallel, with three CHRs associated antiparallel to the NHR bundle; the latter bind in the hydrophobic grooves formed between the strands of the NHR trimer core. The formation of 6HBs was originally accepted as being the process that brings the viral and the cellular membranes together and allows the aggregation of several activated Env complexes to form a pore, leading to the entry of the nucleocapsid into the cell (73, 149, 244; for a review of gp41 structure and fusion, see reference 242). This hypothesis is supported by the fact that NHR and CHR synthetic peptides can inhibit HIV-1 infection and cell-cell fusion at nanomolar concentrations (39, 109, 247, 248) and that a CHR peptide can bind gp41 after receptor activation (73).

Nevertheless, there is no consensus on a model explaining the events leading to viral entry; two entirely different propositions have been made concerning the timing of 6HB formation. Some studies (73, 85) indicated that the transition of gp41 into 6HBs probably drives membrane fusion by bringing the two membranes into close proximity. Other studies (136, 149) proposed that although 6HB formation is required for the fusion process, some 6HBs, if not all, are formed after pore formation. It has also been suggested that two parallel pathways of gp41 conformational rearrangement may coexist, one leading to 6HB formation and the other leading to the generation of hairpin monomers (34). A couple of studies have supported this model (60, 119); thus, fusion is considered to be a very dynamic process during which different conformations of gp41 may coexist at the same time.

The parameters governing fusion are not understood; however, agreement on the mechanism of infection is very important for HIV-1 vaccine research. A clear definition of the structural intermediates (extended or folded conformations) could define targets for vaccines that elicit the production of Nt Abs that halt fusion. Current structural data on Env are very limited and thus do not establish a clear picture of how the whole fusion process of HIV-1 infection actually occurs. In addition, many of the currently used models are deduced from mutagenesis and biochemical studies, which have led to controversial results. Thus, this a critical area to which future studies could contribute results that clarify the fusion process.

The TM Region

The TM is composed of 22 aa and anchors Env into the lipid bilayer. It is highly conserved among different HIV-1 isolates and is thought to play a direct role in viral fusion, since substitution of the TM region for a covalently linked lipid anchor abrogates fusion (198, 243). In contrast, Wilk et al. (249) reported that infectivity was not affected by the substitution of the gp41 TM with the TM from the cellular protein CD22, suggesting that the TM is not important for fusion. Other studies support that fusion is impaired by replacing the gp41 TM with the TM domain from glycoporin A, vesicular stomatitis virus (VSV) G (155), and, more recently, the TM domain from influenza virus hemagglutinin (HA) (128). This is further supported by observations that TM plays a role in fusion in other viral proteins (128, 224).

The length and amino acid composition of the TM-spanning region determine the localization of the protein to specific regions of the plasma membrane; this is called the "hydrophobic matching principle" (for a review, see reference 108). Thus, a modification of those parameters may influence TM activity (for a review, see reference 169). In fact, the gp41 TM has been proposed to have a direct involvement in the formation of the fusion-competent Env protein (246). The full structure of gp41 including the TM region remains to be solved; TM structures are difficult to elucidate due to problems associated with the expression and purification of such hydrophobic proteins. However, early computer modeling suggested that the TM region adopts an α-helix (77).

The CT

The gp41 cytoplasmic domain is unusually long for a TM protein (~150 aa); its exact functions are not clearly understood, although it is thought to have important functions in vivo. Mutagenesis studies of CT suggest that it is involved in Env incorporation into the virus (184, 260), decreased virus infectivity (251), and structural perturbations of gp120 leading to increased sensitivity to Ab-mediated neutralization (67). Other functions have been described for CT, including interaction with the viral matrix protein (48, 62, 72, 160, 260), targeting to vesicles (56, 129, 194), as well as interaction with other proteins (15, 102, 152, 171, 252). The presence of an

TABLE 1. The MPER of gp41 is critical for HIV-1 infection

Evidence	Reference(s)
The region is very conserved	162, 189, 199, 273
Important contribution to the activity of the viral inhibitor T-20	113, 123
Epitopes of bNt MAbs 2F5, 4E10, and Z13 are located in this region	162, 273
Deletion or substitution of sequence within this region affects fusion	13, 159, 186, 199, 271
Peptides from this region associate with lipid membrane and cause membrane leakage	157, 220, 221
Possible involvement in gp41 oligomerization and location in the membrane	196
Binding of MPER to galactosyl ceramide is important for mucosal infection mediated by transcytosis	4, 5, 259

endocytic motif, YXXΦ, in the CT (aa 712 to 715) has been reported (56, 129, 130), and substitution of Tyr_{712} for Ser increased the expression of the Env proteins on the cell surface by reducing the endocytosis of Env (27). Taken together, the CT domain is associated with a wide variety of functions.

The structure of the CT region is not known, but a computer model predicts the presence of three aliphatic regions called lentivirus lytic peptides: LLP-3 (aa 789 to 815) (117), LLP-2 (aa 768 to 788), and LLP-1 (aa 828 to 855) (68, 93, 94, 153). Several studies with these peptides have shown that they bind to membranes (118, 214, 231), perturb membranes (9, 40, 47), and bind to calmodulin (213, 225). Structural modeling has described gp41 as a type I single-pass membrane protein (77); however, the work of Cleveland et al., who used a MAb that targets intracellular CT, suggests that the protein could have other membrane-spanning domains (45). Thus, those authors proposed a model for a possible structure that shows CT doubling back through the membrane surface of the virus and back inside, possibly exposing the Kennedy epitope on the surface of the virion (45). Those studies indicate that the CT domain is another example of the confounding structural and functional characteristics of HIV-1 Env.

THE MPER

This section provides a detailed review of MPER structure and function and the impact of these features on vaccine design; these features are summarized in Table 1. The MPER comprises the last 24 C-terminal amino acids of the gp41 ectodomain, LLELD KWASLWNWF(N/D)ITNWLWYIK (aa 660 to 683) (271), and contains at least two attributes that make it very attractive as a vaccine target: it is highly conserved (162, 189, 199, 273), and the MPER contains epitopes that are recognized by three HIV-1 bNt MAbs (162, 273).

Diverse Structures of the MPER

The actual structure of the MPER is in dispute; for example, the region has been described as an α-helix in some studies and as an extended β-turn in others. It was originally predicted that the MPER has an α-helical structure (77), and this conformation is supported by crystallographic structures of this region determined by three independent studies. Those studies showed that the region immediately upstream of and partially overlapping the 2F5 epitope exists as an α-helix, at least during the fusogenic state of the virus (39, 223, 244). In support of those studies, an NMR structure of the19-mer peptide from HIV-1 gp160 (KWASLW NWFNITNWLWYIK) (aa 665 to 683) shows that the MPER adopts an α-helical conformation in DPC micelles in which the aromatic and polar residues are distributed around a helical axis (Fig. 2A). The peptide was shown to interact with the H_2O-DPC interface of the micelles, and it was observed that the aromatic groups of Trp and Tyr residues in the MPER were positioned in the same plane (209). Accordingly, other structural studies showed that the 2F5 epitope adopts a helical conformation (19). In this case, the structure of a 42-residue peptide, NN-T-20-NITN (aa 638 to 673), was analyzed using heteronuclear two- and three-dimensional NMR. The secondary structure at near-physiological conditions showed that the NN-T-20-NITN peptide is mostly unstructured in the N-terminal region but contains a helical region beginning at the center of T-20 (YT SLIHSLIEESQNQQEKNEQELLELDKWASLWNWF) that extends toward the C terminus. Taken together, these studies support the generally accepted idea that the functional structure of the MPER is a helical conformation.

More recently, other structural studies have raised the possibility of alternative MPER structure models. Contrary to the studies that showed a helical MPER, structures of the 2F5 Fab bound to a peptide revealed a different conformation. As shown in Fig. 2B, the structure of 2F5 with the 7-mer peptide ELD KWAS (174) and a second structure with a 17-mer peptide, EK NEQELLELDKWASLW (aa 654 to 670) (170), revealed that this region of the MPER forms an extended conformation with a distinct β-turn at the DKW in the core of the peptide epitope. As the DKW core is buried in the Fab interface, these residues are most likely exposed on the Env trimer prior to fusion. In contrast, the structure of the 4E10 Fab bound to its epitope (aa 670 to 678) (Fig. 2C), which is adjacent to the 2F5 epitope, adopts a helical structure (38). It is thought that these structural differences in the MPER could be related to the overall gp41 structural changes that occur during fusion. This raises the possibility that the MPER undergoes a structural transition during fusion from an extended conformation to a helical structure (11). Intriguingly, the structure of the MPER in DPC micelles shows the residues that directly contact MAb 4E10 pointing into the micelle (Fig. 2A and C). This supports the possibility that these residues interact with the membrane due to their proximity to the membrane and the hydrophobic nature of the 4E10 paratope. Similarly, it was observed that 4E10 and Z13, which bind overlapping epitopes, interact with two different faces of the MPER helix (164). This was recently reported by Nelson et al., who mapped the Z13 epitope in Ala substitution studies (164). Taken together, these observations support the flexible nature of the region and suggest the existence of more than one structure relevant to neutralization.

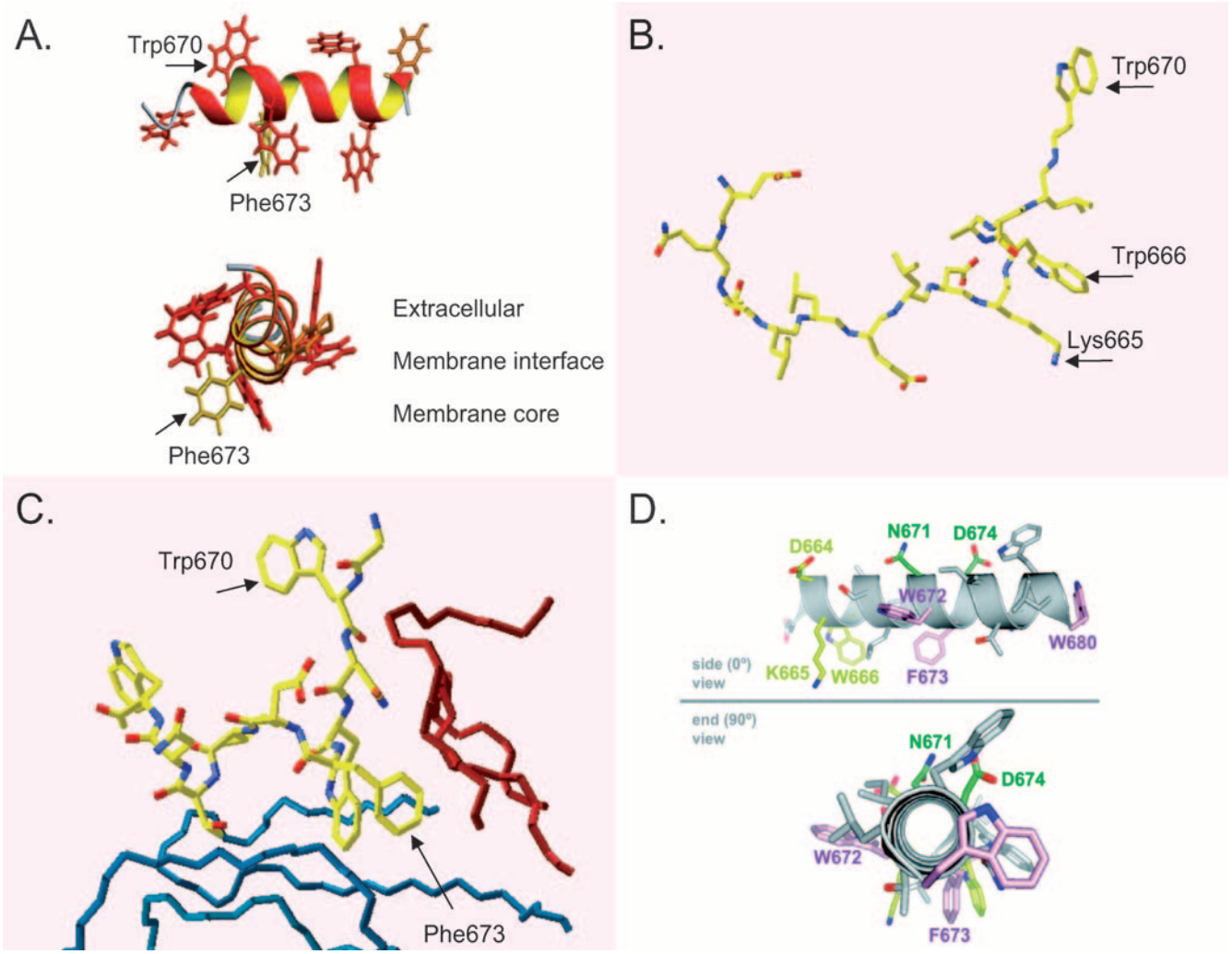

FIG. 2. The flexibility of the MPER is revealed by the differences in its structure. (A) NMR spectroscopy structure of the MPER peptide (KWASLWNWFNITNWYIK) in DPC micelles. (Top) Side view with the exposed face oriented toward the top of the figure. (Bottom) End view. (Reprinted with permission from reference 209. Copyright © 2001 American Chemical Society.) (B) Crystal structure of the 2F5 epitope peptide (EKNEQELLELDKWASLW) (yellow) in complex with the 2F5 Fab (not shown). This figure was made using DeepView Swiss-PDB Viewer from PDB coordinates (PDB accession number 1TJI) described previously (170). (C) Crystal structure of Fab 4E10 in complex with its epitope peptide (GWNWFDITNWGK) (yellow). The light chain is shown in red, and the heavy chain is shown in blue. This figure was made using DeepView Swiss-PDB Viewer from PDB coordinates (PDB accession number 1TZG) described previously (38). (D) Hypothetical model of the MPER (DKWASLWNWFDITNWLW) as described by Nelson et al. (164). (Reprinted from reference 164 with permission.)

This implies that several distinct immunogens with different orientations of the MPER helix, or even with extended structures, might be useful for the generation of Nt Abs. However, it also raises the concern that the structural flexibility of the MPER is responsible for its poor immunogenicity in neutralization studies (Tables 2 and 3).

The idea of MPER flexibility is supported by a study described previously by Granseth et al. (87), who showed that the membrane-proximal region for membrane-inserted proteins is unusually enriched in irregular structure and in interfacial helices that run roughly parallel with the membrane surface; β-strands are extremely rare. In this region, hydrophobic and aromatic residues tend to point into the membrane, and charged/polar residues tend to point away from it. This analysis is very interesting considering our current knowledge of the gp41 MPER, for which the existing data do not support a single static structure. Whether the α-helical structure of the MPER is present in the native prefusion state of gp41 or is induced upon binding of the virus to its receptor(s) has yet to be determined.

Role of the MPER in Fusion

It has been well established that the MPER plays a role in the fusion of the viral and cell membranes; however, the exact nature of that role is yet to be confirmed. This region contains numerous hydrophobic residues and is unusually rich in Trp (Fig. 1). The substitution of some of these residues dramatically affects the efficiency with which gp41 is incorporated into virions, and (possibly as a consequence) inhibits viral entry into target cells (199). Salzwedel et al. (199) showed that a deletion of 17 aa from the

TABLE 2. Vaccines targeting the production of MAb 2F5

Target Ab (reference[s])	Immunogen	Epitope supported by membrane	Animal	Immunization schedule[a]	ELISA antigen[b]	Ab titer[c]	50% Nt titer[d]	Strain(s) tested *and assay method*
2F5 (161)	Chimeric influenza virus with ELDKWA epitope inserted into site B of HA	No, virus is enveloped, but B loop is not next to membrane	OF-1 mice	Mice were immunized with chimeric influenza virus or WT control; 10^2 PFU i.n. (1st), 5×10^5 PFU i.n. (2nd), 20 μg inactivated virus in incomplete Freund's adjuvant (3rd); 21-day intervals between immunizations	Glutathione S-transferase–E LDKWA fusion protein	IgG, medium; high IgA, low	40–320	**MN**, **RF**, and **IIIB**, by syncytium inhibition
2F5 (140)	Chimeric potato virus X with ELDKWA at N terminus of coat protein	No	C57BL/10 mice	Mice received chimeric virus or WT control without adjuvant; 50 μg i.p. 6 times every 14 days or 50 μg i.n. 11 times every 7 days	QTQQEKNAQELLELDK WASL	i.p. IgG, medium; i.n. IgG, medium; medium-high i.n. IgA, low to medium (endpoint titers)	~180–200	**IIIB**, by syncytium inhibition
2F5 (264)	Chimeric bovine papillomavirus VLPs expressing ELDKWA in N terminus of L1 protein	No	BALB/c mice	Mice received chimeric virus or WT control without adjuvant; 10 μg i.m. 2 times at 14-day intervals or 10 μg, orally 2 times at 14-day intervals	ELDKWA	Low	1/20 dilution of sera decreased p24 by threefold	**MN**, **Bal**, and Ada, by detection of p24 by ELISA
2F5 (121)	Chimeric hepatitis A virus in which 2F5 epitope is inserted into domain 2A at C terminus of structural protein VP1	No	Guinea pigs and marmoset	For guinea pigs, 1.7 μg of 70S procapsids in complete Freund's adjuvant s.c. and i.m. 3 times at 2-wk intervals; for marmosets, 100 μg of 160S particles 1 time i.o. and i.v.; no adjuvant	GGGLELDKWASLW	Guinea pigs, low; marmoset, low	1/40 serum dilution was tested	**LAV_{LAI}**, by detection of reverse transcriptase activity
2F5 (126)	DNA expressing LLEL DKWASL inserted into HIV-1 gp140 V1, V2, V3, or V4 loop	No	BALB/c mice, guinea pigs	Animal groups were inoculated with DNA expressing the 2F5 epitope in gp120 V1, V2, V3, or V4 loops; 100 μg DNA (mice) and 800 μg DNA (guinea pig) i.m. 2 times at 28-day intervals	ELLELDKWASLWN and soluble gp120	Anti-2F5 titers, low to medium Anti-gp120 titers, medium to high (mice immunized with V2 insert); anti-2F5 titers, medium to medium-high; anti-gp120 titers, medium-high to high (Guinea pigs immunized with V2 insert)	Not Nt	**IIIB**, by syncytium inhibition
2F5 (46)	ELDKWA inserted at various sites of MBP of *E. coli*	No	BALB/c mice	Mice were immunized with MBP bearing ELDKWA epitope or WT MBP; 10 μg protein adsorped to alum i.p. 3 times at 21-day intervals	LLELDKWASL	For BALB/c mice immunized with MBP with 4 tandem repeats of 2F5 epitope, low to medium-high	Not Nt	MN, RF, and primary isolate, by syncytium inhibition

2F5 (100)	ELDKWAS sequence fused into framework regions of anti-HLA-DR MAb	No	New Zealand White rabbits	Rabbits were immunized with anti-HLA DR MAb bearing ELDKWAS sequence at different locations or WT or control Abs; 200 μg protein in complete Freund's adjuvant or saline boosted two more times at 4-wk intervals, first with 200 μg protein and then with 100 μg protein	gp160	Low to medium	Not Nt	R2, by single-cycle HIV infectivity assay
2F5 (112)	Synthetic peptides containing 2F5 epitope constrained to enhance α-helix structure and conjugated to KLH	No	Guinea pigs	40 μg conjugate administered 3 times at 4-wk intervals with QS21 adjuvant	Synthetic peptides containing 2F5 epitope and constrained in α-helix	High to very high	Not Nt	HXB2, by single-cycle HIV infectivity assay
2F5 (145, 146)	Cyclic β-turn peptides containing ELLELDK WASLW conjugated to KLH or outer membrane protein of N. meningitidis	No	Guinea pigs	40 μg conjugate administered 3 times at 4-wk intervals with QS21 adjuvant	ELLELDKWASLW, disordered and constrained into β-turn	Very high	Not Nt	HXB2, by single-cycle HIV infectivity assay
2F5 (57)	DNA expressing gp160 and CCR5 and peptides with variations on the EL DKWAS sequence, gp41 coil peptide (QLQAR VL), and CCR5 second-loop peptide	DNA, yes; peptide, no	C57BL/6 mice	Prime i.n. with 25 μg gp160/CCR5 DNA and boost with 10-μg mix of ELDKWAS, gp41 coil, and CCR5 second-loop peptides 8 wk after prime (group 1); 2 i.n. immunizations with 10-μg mix of ELDK WAS, gp41 coil, and CCR5 second loop (group 2); 2 i.n. immunizations of PBS	ELDKWAS, coiled-coil peptide, V3 loop peptide, CCR5 second-loop peptide, gp160	Group1 and 2 anti-ELD KWAS IgG, medium; groups 1 and 2 anti-coiled-coil peptide, V3 loop peptide, CCR5 second-loop peptide, and gp160 IgG, low; group 5, no Ab response detected; all IgA responses were low	Group 1, ~100–240; groups 2 and 3 did not reach 50% neutralization at serum dilutions of 1/20	**SF2** and **primary isolate**, by p24 production

[a] Immunization routes are abbreviated as follows: intranasal, i.n.; intraperitoneal, i.p.; subcutaneous, s.c.; intramuscular, i.m.; intraosseous, i.o.; intravenous, i.v.
[b] Amino acid sequences describe synthetic peptides used to determine Ab titers.
[c] Titers are described as follows: low, <1,000; medium, 1,000 to 15,000; medium-high, 15,000 to 50,000; high, 50,000 to 1,000,000; very high, >1,000,000. The difference between endpoint and midpoint titers was not taken into account.
[d] Strains that were neutralized are in boldface type.

TABLE 3. Vaccines that target the production of Abs to gp41 or the MPER

Target region (reference)	Immunogen	Epitope supported by membrane	Animal	Immunization schedule[a]	ELISA antigen[b]	Ab titer[c]	50% Nt titer	Strain(s) tested,[d] assay method
gp41 (134)	gp41 fused to C terminus of p15E from PERV and expressed on VSV	Yes	New Zealand White rabbits	2×10^6 PFU VSV recombinant viruses (1st) and boost with purified protein emulsified in incomplete Freund's adjuvant at 3-wk intervals (2nd, 3rd, and 4th); injection route and dose were not specified	gp41-$MPER_{655–682}$ expressed on the surface of cells	Titer not tested; presence of Abs shown in Western blot by binding of antisera to cell lysate and using flow cytometry	Only 1/20 dilution tested	**HXB2** and **JRFL**, by single-cycle HIV infectivity assay
gp41 (258)	DNA vaccine comprising gp41 fused to the C terminus of influenza virus HA	Yes	BALB/c mice	Mice were immunized with fusion protein alone or displayed on proteoliposomes; 100 μg DNA i.m. 3 times at 4-wk intervals	gp41 fused to N terminus of the SV5 HN protein	Medium	40% neutralization at a 1/40 dilution of pooled sera	**SF162**, by single-cycle HIV infectivity assay
MPER and CHR (124)	Trimeric gp41 C-terminal domain in proteoliposome or without liposome	Yes	BALB/c mice	Two i.p. immunizations at a 3-wk interval; dose was not specified	C terminus of gp41	C terminus of gp41 in proteoliposome, medium; C terminus of gp41 without proteoliposome; low	Not Nt	RF, by syncytium inhibition
MPER (143)	$MPER_{649–684}$ fused to cholera toxin B subunit (MPER/CTB)	No	BALB/c mice	Prime with 42 μg i.n. 5 times every wk and then 2 more either i.p. or i.n. with cholera toxin at wk 10 and 18 or prime with 3.5 μg i.p. adsorped to alum weekly 4 times, followed by i.p. or i.n. administration at wk 10 and 18	Synthetic $MPER_{649–684}$	Medium-high	Not tested	NA
MPER (115)	Three gp41-based prefusion constructs expressed on VLPs or on Sf9 insect cells; gp41 fused to C1 and C5 regions of gp120 with other gp120 segments replaced by the SH3 domain of CD2BP1, a 24-aa sequence linking gp120 to gp41 NHR, CHR, MPER, and the TM domain (C1); gp41 NHR, CHR, the MPER, and the TM (C2); trimeric BAFF fused to CHR, MPER, and TM (C3)	Yes	Outbred guinea pigs	All groups received 3 immunizations at 2-wk intervals of 5×10^6 cells i.d. with or without *E. coli* LT or 2 or 10 μg i.d. or i.m. with or without LT (VLPs)	VLPs, soluble C1 that does not contain the MPER and TM, and synthetic peptides bearing the MPER sequence from HIV MN and ADA	Titers to VLP were high; titers to fusion protein were low to medium; binding to MPER peptide was so low that titers were not measurable	Not Nt	MN, SFI2.LS, ADA, and NL-ADArs, by single-cycle HIV infectivity assay

MPER (122)	DNA prime followed by recombinant protein boost with MPER or 4E10 epitope expressed in place of gp120 V1/2	No	BALB/c mice and New Zealand White rabbits	4 i.m. DNA immunizations (40 μg) at wk 0, 3, 6, and 18 and boost two times with 10 μg protein (s.c.) in Ribi adjuvant at wk 28 and 32 or wk 35 and 39 (mice); prime-boost with 4 i.m. DNA doses (0.5, 1, or 2 mg) at wk 0, 4, 8, and 12 and 3 s.c. protein boosts (50 μg) with Ribi adjuvant plus cell wall skeleton were given at wk 20, 25, and 30 (rabbits); for protein, 5 (100 μg) s.c. immunizations administered at wk 0, 5, 10, 20, and 30 (rabbit)	WT JR-FL gp120 and a panel of peptides bearing overlapping sequences from V3 and MPER	High titers to WT JRFL gp120; no detectable Abs to MPER peptides produced	Mice, 10–40; rabbits, 10–800	JR-FL and **SF162** (mice) and **JRFL, SF162, MN**, HxB2, ADA, and JR-CSF (rabbits), by single-cycle HIV infectivity assay

[a] Immunization routes are abbreviated as follows: intranasal, i.n.; intraperitoneal, i.p.; subcutaneous, s.c.; intramuscular, i.m.; intradermal, i.d.
[b] Amino acid sequences describe synthetic peptides used to determine Ab titers.
[c] Titers are described as follows: low, <1,000; medium, 1,000 to 15,000; medium-high, 15,000 to 50,000; high, 50,000 to 1,000,000; very high, >1,000,000. The difference between endpoint and midpoint titers was not taken into account.
[d] Strains that were neutralized are in boldface type. NA, not applicable.

MPER (aa 666 to 682) completely abrogated the ability of Env to mediate both cell-cell transmission and viral entry; however, Env maturation and binding to CD4 were not affected. That study concluded that the MPER is essential for fusion activity and for the incorporation of Env into virions (199). In addition, deletion of the sequence LLELDKWASLW (aa 660 to 670) has shown that it is important for fusion, since the absence of this region produced impaired syncytium formation (186). An additional study by Muñoz-Barroso (159) et al., who used a cell-cell fusion assay, allowed the classification of MPER mutants into three different phenotypes: those showing reduced activity, defective variants unable to mediate fusion, and mutants able to assemble nonexpanding fusion pores. These results once again support the role of the MPER in the fusion process (159). Besides those studies, there is no model explaining the mechanism by which the MPER mediates fusion, even though a 36-aa synthetic peptide (YTSLIHSLIEESQNQQEKNEQEL LELDKWASLWNWF) (aa 638 to 673) derived from the CHR (also called DP-178, T-20, enfuvirtide, and Fuzeon) (248) is currently used as a new class of antiviral drug and works by inhibiting fusion. This peptide sequence overlaps with the sequence recognized by MAb 2F5 (113, 123). The role of the MPER in fusion is further supported by the ability of three bNt MAbs (2F5, 4E10, and Z13) to recognize epitopes in this region; thus binding to the MPER by these MAbs blocks infection, presumably by interfering with one of the critical steps required for viral entry.

A recent study evaluated the crucial role of the FP-proximal PR and the MPER during HIV-1 infection using two different modes of viral transmission: cell-to-cell and virus-to-cell fusion (13). Interestingly, Ala substitution of Trp_{666}, Trp_{672}, Phe_{673}, and Ile_{675} in the MPER reduced viral entry potential by ~120-fold without affecting cell-to-cell fusion. However, a single Ala substitution in the MPER in combination with one of Leu_{537} in the polar region inhibited cell-to-cell fusion as well as viral entry. Therefore, those authors proposed that the MPER might have distinct roles during the different steps of the fusion process.

To test the contribution of individual MPER residues to Nt activity, Zwick et al. generated Env mutants by substituting MPER residues with Ala (271). The mutant Envs were used to make pseudoviruses, which were tested in neutralization assays with 2F5 and 4E10. The results of that study are in agreement with data on the critical binding residues required for the interaction of the MPER with 2F5 (188, 227). However, the situation is slightly different for 4E10, for which the critical binding residues and Nt activity did not coincide. 4E10 critical binding residues were Trp_{672}, Phe_{673}, and Thr_{676}, whereas residues critical for Nt activity were Trp_{672}, Phe_{673}, and Trp_{680} (26, 38). It was suggested that these differences may be related to the use of MPER in two different contexts (synthetic peptide versus membrane-attached region). Taken together, those studies confirm the role of the MPER during viral transmission and support current efforts to target this region for neutralization of HIV-1 infection.

HIV-1 infection occurs mainly via mucosal transmission; thus, it must be understood how Abs neutralize the virus at the epithelial surface. Bomsel et al. sought to understand the process of transmission and Nt activity. They showed that HIV-1-infected cells that are in direct contact with the apical surface of an epi-

thelial cell can generate virus that crosses the tight epithelial barrier by transcytosis. This event could be blocked by immunoglobulin M (IgM) or dimeric IgA generated against Env (20). That study suggested that the induction of mucosal Abs against Env could block HIV-1 mucosal transmission. In addition, that group showed that HIV-1 binds to epithelial cells using an alternative receptor, glycosphingolipid galactosyl ceramide. This virus-receptor interaction is mediated by the conserved ELDKWA epitope on gp41, since secretory IgA from the colostrum and cervicovaginal secretions from several HIV-1-positive patients were capable of Nt intracellular HIV-1 transcytosis through epithelial cells by binding to the ELDKWA epitope. This finding further supports the potential of an MPER HIV-1 vaccine, since it could be used to elicit protective Abs at mucosal sites (5).

MPER and Membrane Interaction

The HIV-1 membrane is considered to be highly ordered and contains a very high ratio of cholesterol to phospholipid, >1.00 (191); it is thought that lipids play a role in infection by affecting Env structure and function. This is supported by the observation that viral infectivity is impaired when the lipid content is modified by increasing the temperature or treating the virus with 50% ethanol (6). In addition, cholesterol plays an important role in viral infection since its depletion ablates infection (204, 207). Moreover, a cholesterol-binding motif has been found in the MPER (aa 679 to 683) (238). Saez-Cirion et al. studied the ability of the interfacial sequence preceding the TM, DKWASLWNW FNITNWLWYIK (aa 664 to 683), to interact with membrane and showed that it forms lytic pores in liposomes composed of the main lipids occurring in the HIV-1 envelope: 1-palmitoyl-2-oleoylphosphatidylcholine, sphingomyelin, and cholesterol (1:1:1 molar ratio) (197). These results suggest that the MPER sequence may participate in the clustering of gp41 monomers within the HIV-1 envelope and may destabilize the bilayer architecture at the locus of fusion. Those authors hypothesized that the interfacial MPER sequence behaves as a signal for lipid domain targeting and proposed several mechanisms by which the presence of lipid domains could influence the fusion process, for example, by supporting clustering of the MPER, and further activation of fusion activity. Another mechanism could be that the MPER targets the lipid domain, causing the surface aggregation of gp41 trimers, which assist in the formation of the oligomeric complexes that function in fusion pore opening. Last, it was proposed that the MPER is involved in the creation of membrane projections (nipples) enriched in cholesterol/sphingomyelin, thus favoring viral fusion. This step represents the main energetic barrier for initial bilayer merging and subsequent fusion pore formation. The interaction of the MPER with the membrane was also demonstrated by studies in which the peptide DKWASLWNWFNITNWLWYIK (aa 664 to 683) mediated membrane partitioning, fusion, and permeability (220, 221). The role of the MPER in membrane partitioning was also confirmed in a recent study by Veiga and Castanho (236). Here, we have reviewed the structure of the MPER and described the different studies that support its role in infection. Table 1 summarizes the studies that support the critical role of the MPER during infection by HIV-1.

Besides the exceptional Nt activity of the anti-MPER bNt MAbs isolated to date, it has not been possible to recapitulate the gp41 structure and/or structures required for eliciting such Nt Abs. Thus, HIV-1 vaccine research faces the challenge of developing a vaccine to a structurally unknown immunogen. Without a clear understanding of gp41's structural changes and the events that lead to virus infection, it will be difficult and a matter of trial and error to generate an MPER-targeting vaccine. Also, if we consider that gp41 undergoes drastic structural changes, then the immune system could be constantly exposed to diverse structures of the MPER during natural infection. Such structures may or may not be Nt, thus making the overall response to the different epitopes located in the MPER very low, which could explain the low immunogenic nature attributed to the MPER. However, it has been shown that some, albeit weak, activity is directed to the MPER in the Ab response to HIV-1. This is described in detail below.

Ab RESPONSE AGAINST gp41

Although gp41 is mostly occluded by gp120 on native viral spikes, gp120 shedding can expose gp41 to the immune system. In addition, gp41 may be exposed in different transient structures during the stages of the fusion process. Reflecting this, the Ab response to HIV-1 during natural infection typically includes strong reactivity against gp41 (101). There are several immunogenic regions on gp41, including the PR, the NHR, the ID loop, the MPER, and, to a lesser extent, the CT (Fig. 1). Here, we describe Ab reactivity against the regions on the gp41 ectodomain (for a more extensive review on Ab responses against gp120 and gp41, see reference 86).

Ab Reactivity to the PR, the NHR, the ID Loop, the CHR, and the CT

PR reactivity. A few human Nt MAbs have been isolated against the PR AAGSTMGAASMTLTVQARQ (aa 525 to 543) (28).

NHR reactivity. Cross-reactive Abs to the NHR have been detected in HIV-1-positive sera, indicating its immunogenic characteristics (187). Moreover, an Nt Ab, D5, was generated from a native scFv library; it has been shown to bind to the NHR trimer and, like the T-20 peptide, inhibits the assembly of the fusion intermediate in vitro (154). In addition, new Nt MAbs with limited potency against the NHR trimer of HIV-1 have been isolated by screening a phage-displayed immune Ab library (M. Zwick, personal communication). Those studies indicate the potential of the inner NHR trimer as a target for vaccine design.

ID loop reactivity. The ID loop (also known as epitope cluster I) was identified as the primary ID region on gp41 by early "serum-mapping" studies (43, 82, 83). Although most of the Abs against this region are not Nt, one MAb (termed clone 3) has been shown to neutralize both diverse T-cell laboratory-adapted (TCLA) viruses from clade B and three primary isolates from group O (50, 70). Interestingly, some MAbs generated against this region show an infection-enhancing activity (193).

CHR reactivity. It has been reported that epitopes in the CHR (aa 628 to 661) are exposed only after the interaction of gp120 with CD4; these regions are probably masked by gp120 (205).

However, a relatively ID region, located near the N-terminal end of the 2F5 epitope (ELDKWA), has been described and named epitope cluster II (aa 644 to 663) (254).

CT reactivity. Although it is thought that the CT of gp41 is contained by the viral membrane, there are several MAbs that recognize a hydrophilic region in the so-called "Kennedy epitope" (PRGPDRPEGIEEEGGERDRDRS) at the N terminus of the CT (aa 724 to 745) (29, 44). Two possible structures have been proposed for the CT, with each being adopted as part of drastic conformational changes produced by gp41 during viral infection (41, 61). Thus, regions within the CT could interact with the membrane, and perhaps cross it, to be exposed and form epitopes (e.g., the Kennedy epitope) on the viral surface.

Ab Reactivity to the MPER

In contrast to the NHR and ID loop, the MPER is not strongly immunogenic, yet three bNt MAbs against this region, 2F5, 4E10, and Z13, have been discovered (28, 162, 273). Both 2F5 and 4E10 neutralize a broad range of both laboratory-adapted and primate isolates of HIV-1 (49, 218). Both MAbs were originally identified as IgG3s and were subsequently changed to IgG1; these are commonly used in the latter form. Of the two MAbs, 2F5 is the most potent Nt Ab, whereas 4E10 neutralizes a broader range of HIV-1 isolates, as shown by pseudovirus studies using an extensive panel of Envs derived from primary isolates (18, 148). There have been some reports on the Ab response against the MPER in natural infection (see below); unfortunately, most serum Ab responses to this region are not as bNt as 2F5 or 4E10 in neutralization assays (22, 24, 36, 79, 101, 158).

A number of studies have reported results from serum-mapping studies using synthetic linear peptides covering the Env sequence to detect epitope-specific Ab reactivity in serum samples (101). Using this approach, some studies have identified Ab responses against the MPER, indicating that it is immunogenic during natural infection. Broliden and colleagues (24) revealed the presence of cross-reactive Abs with the peptide QQEKNEQ ELLELDKW (aa 652 to 666) in sera that neutralized HIV-1 strains IIIB, SF2, and RF. The frequency of seroreactivity to this region was 56% among samples from 50 HIV-1-positive subjects. Furthermore, individual serum Nt activity was inhibited when the peptide was preincubated with the sera (24), indicating that the specificity of the Nt response was against the region bearing the same sequence as the peptide. Interestingly, this peptide partially overlaps with the epitope of the bNt MAb 2F5, but as the full 2F5 epitope (ELDKWAS) is not present, it is likely that the Abs are specific for the region just preceding the 2F5 epitope.

There are a number of studies that have mapped Abs to the region at the N-terminal end of the 2F5 epitope. For example, Ugen et al. (234) investigated HIV-1 vertical transmission with 20 maternal sera from HIV-1-positive individuals by screening against the peptide QNQQEKNEQELLEL (aa 650 to 663); they found that 70% of the maternal serum samples had cross-reactivity with this peptide. Surprisingly, reactivity against this peptide seemed to be inversely related to the HIV-1 transmission status (234). In another study, a set of overlapping MPER peptides was used to map sera; the region adjacent to the C terminus of the ELDKWA sequence was observed to be ID in most of the sera studied. In addition, more than 30% of the sera were reactive to the ELDKWA peptide (36). In another study, the peptide ELLE LDKWAS (aa 659 to 668) was used to affinity purify and quantify epitope-specific Abs from HIV-1-positive sera (235). It was observed that a reduction in reactive Abs correlated with disease progression. This is supported by the work of Geffin et al. (79), who showed that there is an association between Ab reactivity to the ELDKWA epitope peptide and disease progression in 29 children who had been perinatally infected with HIV-1. Approximately 50% of infected individuals displayed a detectable Ab response against the peptide, and the Ab levels measured over time were inversely associated with the levels of p24 antigen in the plasma (79). Furthermore, Muhlbacher et al. found that serum reactivity against an MPER peptide (aa 642 to 673) corresponded directly to the recognition of infected T cells and to CD4 cell counts (158). More recently, Srisurapanon and coworkers studied the serum Ab reactivity against the peptide ELDKWA in HIV-1-infected subjects and showed that its frequency was low (15 to 35%) (215). Moreover, Ab titers to this epitope in sera from AIDS patients were significantly lower than those in sera from asymptomatic subjects that were collected the same year (215). Those studies suggest that Ab reactivity to the region containing the 2F5 epitope is produced during natural infection and suggest that the immunogenicity of this region may be enhanced in vaccination strategies.

Following the peptide-mapping idea, Opalka et al. reported the use of a fluorescence-based, multiplexed Ab binding and mapping assay to characterize the specificity, breadth, and magnitude of the Ab response to gp41 (172). Using this assay, Ab responses to the 2F5-4E10 regions were detected in HIV-1-positive serum, but there was no direct correlation between Ab binding and serum neutralization potency (172). This is not surprising, as the Nt activities in many sera are restricted to gp120 (58).

Other methods have also been used to detect Nt Abs specific to the MPER. Braibant and colleagues used competition assays to determine the presence of 2F5- and 4E10-like Abs in HIV-1-positive sera. Sera were used to compete with biotinylated MAbs 2F5 and 4E10 for binding to Env captured on microplates (22). They detected 2F5 blocking Abs in all the sera from HIV-1-positive long-term nonprogressors, although the levels were low. They also analyzed the binding of Abs to the MPER peptide by direct enzyme-linked immunosorbent assay (ELISA) and showed that 60% of sera were positive for Ab against this region. This study demonstrates that almost all long-term nonprogressors develop MPER binding Abs, albeit at very low levels, but does not address the question of whether these reactivities are Nt. There is a possibility that Nt Abs against the MPER are at such a low titers or low affinities in serum that they are not sufficient to inhibit HIV-1 infection and are therefore undetectable in neutralization assays.

Recently, a new approach has been developed for detecting the prevalence of epitope-specific Nt Abs in HIV-1-positive sera, especially against the 2F5 and 4E10 epitopes. The HIV-1 epitopes for these two MAbs were grafted into the MPER of SIV (262) or HIV-2 (16), replacing homologous SIV and HIV-2 sequences with that of HIV-1. The chimeric pseudovi-

ruses were neutralized by Abs 2F5 and 4E10, indicating that they should be useful for detecting 2F5- and 4E10-like Nt activity in patient sera. Thus, the chimeric pseudoviruses were used to determine the prevalence of 2F5- and 4E10-like Nt Abs during natural infection. The chimeric SIV constructs were used to screen 96 HIV-1-positive samples for Nt Abs, but none of the sera neutralized chimeric SIV bearing the 2F5 epitope, and only one was able to neutralize the SIV chimeric virus bearing the 4E10 epitope (262). Similarly, when these SIV and HIV-2 chimeric viruses (displaying 4E10 and 2F5 epitopes) were used in a separate study to further characterize the nature of the bNt sera from three asymptomatic patients (two from clade B and the other from clade C), these sera also failed to neutralize the viruses (58). In another study, the neutralization activity of 14 sera from HIV-1 clade C were evaluated for the presence of 2F5- and 4E10-like Nt Abs using the HIV-2 chimeric pseudoviruses. MPER-specific Nt Abs were detected in ~28% of the subjects within the first year of infection (89). However, clade C viruses have been reported to be insensitive to 2F5 Nt activity (18, 30, 88).

From these studies, it could be concluded that MPER Abs have been frequently detected in HIV-1-positive sera. However, recent studies using chimeric pseudoviruses with epitopes in a context more closely related to the MPER structure suggest that MPER-specific Nt Abs are rare or absent during natural infection. One reason for why these studies may show no neutralization activity is that the levels of 4E10- and/or 2F5-like Abs are at levels too low to detect in the neutralization assay. Moreover, the fact that new Nt Abs are mapping to a region in the MPER different from those of 2F5 and 4E10 provides hope by suggesting that vaccine-induced Nt Abs are achievable (16).

It is not known if the lack of bNt activity shown by MPER binding sera is due to low titers of Nt Ab or if the Nt Abs are completely absent. This issue of quantity versus quality has not been fully resolved but has significant implications for vaccine design. In the following sections of this review, we focus on the structural features of bNt MAbs against the MPER, 2F5, Z13, and 4E10, together with their epitopes, and the challenges in eliciting bNt Abs in vivo.

bNt MAbs 2F5, 4E10, AND Z13

MAb 2F5 and Its Epitope

The 2F5 epitope was first mapped to a linear sequence (ELDKWA) (aa 662 to 667) on the MPER (162). This epitope has since been confirmed by others and has been expanded from the original 6-mer to a longer 17-mer linear epitope (aa 655 to 671) (11, 49, 151, 177, 227). A site-directed mutagenesis study has demonstrated that the critical binding residues on the peptide are the DKW residues (271). Consistent with this, peptides bearing the DKW motif are repeatedly selected from phage-displayed peptide libraries (49, 151, 233) as well as a phage-displayed gp160 gene fragment library (273).

However, Neurath et al. (165) showed that 2F5 reacts with sequences from two partially overlapping peptides from CT in addition to the ELDKWA epitope (PTPRGPDRPEGIEEEGG ERDRDRSIRLV [aa 722 to 749] and GGERDRDRSIRLVNG SLALIWDDLRSLC [aa 737 to 764]). These two peptides do not show any homology with aa 638 to 675 and did not block the reactivity of 2F5 with peptides from that region; however, the peptide at aa 722 to 749 inhibited 2F5 binding to HIV-1 by 63.1%, while a different peptide (aa 737 to 764) did not have a measurable effect. Those authors suggested that the epitope for 2F5 is discontinuous and that the ELDKWA (aa 662 to 667) segment represents only a portion of the epitope, providing a major contribution to the gp41-binding capacity of the MAb. This is an isolated result that requires further investigation. Since this region of gp41 (aa 722 to 764) is located in the CT of the protein and presumably is not exposed, it seems unlikely that this domain could play any role in the binding of the Ab to the virus; however, this notion has been challenged by Cleveland and coworkers (45) (for a review, see reference 61).

The crystal structure of the 2F5 Fab in complex with a series of short synthetic peptides or an elongated 17-mer peptide, EKNE QELLELDKWASLW (aa 654 to 670), has been resolved by two different laboratories (170, 174). Both studies showed an extended β-turn conformation of the peptide in the region DKW. One interesting characteristic of the longer peptide is that only 41% of it (the charged face) is bound by Ab 2F5, while the other hydrophobic face is unbound. This suggests that the non-2F5-bound face may be occluded by other portions of the envelope protein or may be buried in the plasma membrane.

Another striking feature of the 2F5 Fab-peptide complex is its unusually long (22-aa) CDR-H3. Curiously, of 22 aa, only 10 aa at the base interact with the peptide, whereas the apex remains largely unbound. However, mutagenesis studies showed that the substitution of the Trp at the hydrophobic tip of 2F5's long CDR-H3 significantly decreases the binding affinity of 2F5 to both gp41 and its epitope peptide and also decreases neutralization activity (272). This suggests that the tip of long CDR-H3 is involved in further interactions and/or that the hydrophobic tip is required for the Ab to maintain its overall structure and thus its MPER binding paratope. Given the hydrophobicity of the CDR-H3 and the proximity of the 2F5 epitope to the viral membrane, the long CDR-H3 may also interact directly with the membrane and facilitate MPER binding (170).

A study reported recently by J. P. Julien, J., S. Bryson, and E. F. Pai (presented at the Keystone Symposium, HIV Vaccines: From Basic Research to Clinical Trials [X7], Whistler, British Columbia, Canada) revealed a new X-ray structure for the 2F5 Fab bound to an elongated peptide. It supported previous studies in that it showed that the structure at the N terminus of the DKW core and the β-turn motif of the core remain unchanged, while the residues at the C terminus of the epitope did not adopt any specific conformation. The new structures also revealed that the CDR-H3 loop does not adopt a single conformation, indicating that the interaction between Ab and peptide does not lock the H3 loop into a single conformation. This newly found result sheds light on the controversial issue of the 2F5 epitope and supports a dynamic role for CDR-H3 in neutralization.

A more recent report from Lorizate et al. provides evidence that the 2F5 epitope may be supported by the N-terminal region of gp41 (131). Those authors hypothesized that, in the native or prefusion structure of the spike, the FP and the MPER are located at the same end of the ectodomain and that this arrange-

ment helps to maintain the gp41 structure recognized by MAb 2F5 (131). In this conformation, the MPER and the FP are adjacent and may form the entire epitope. In addition, the natural epitope of Ab 2F5 may include more than the core epitope (ELDKWA). In support of this, Ala substitutions in ELDKWA indicate that the motif "DKW" is a determinant for 2F5 recognition (271); however, different viruses bearing the DKW core have shown resistance to 2F5 neutralization (30), suggesting that other regions of the protein could be involved in the formation of the entire epitope. Structural requirements or membrane participation could not be ruled out. The possibility that the 2F5 epitope extends beyond ELDKWAS is also supported by the observation that Ab 2F5 binds to a variety of sequences C terminal to the DKW core when used to screen random phage-displayed peptide libraries (151).

A study reported by Haynes et al. suggests that bNt MAbs 2F5 and 4E10 are polyspecific, as they bind self-antigens (whole cells and cardiolipin [CL]) (88), and could be considered to be auto-Abs. However, CL is not a predominant lipid in the HIV-1 membrane (7) and is not found in high levels in the plasma membrane. A recent study from the Haynes group compared the CL reactivities of 4E10 and 2F5 with those of two human thrombogenic anti-CL MAbs using surface plasmon resonance. They showed that the bNt Abs bind to CL with similar affinities; however, they observed a difference in the kinetics of binding of 2F5 and 4E10 to their protein epitopes conjugated to liposomes compared to those of binding to their protein epitopes alone. The binding of 2F5 and 4E10 to their respective epitope peptides conjugated to lipid, was better defined by a two-step binding model, whereas the interaction of the MAbs with linear peptide followed a Langmuir binding model. Those authors concluded from this finding that 4E10 and 2F5 interact with the membrane and MPER in a two-step model involving an initial encounter followed by conformational docking. In further support of the hypothesis reported by Haynes et al., a study reported by Sanchez-Martinez et al. (200) demonstrated that CL liposomes can inhibit the neutralization activity of MAb 2F5. However, the interaction of 2F5 with liposomes does not necessarily mean that the liposomes mimic the 2F5 MPER epitope. Alternately, liposomes may interact with 2F5 by a different mechanism, as noted for cross-reactivity identified for MAb b12 by a peptide ligand (B2.1) and gp120 (202, 270) (see below). Given these results, it would be interesting to clarify whether mutations in MAb 2F5 that abrogate binding to the MPER also affect binding to CL.

The biological consequences of the CL reactivity of 4E10 and 2F5 is unclear. Interestingly, when HIV-infected individuals were passively immunized with 2F5 and 4E10, no immunopathological side effects were reported (111, 229). Previous studies reported by Trkola and colleagues evaluated the passive transfer of 2F5 and 4E10 in combination with bNt Ab 2G12 in HIV-1-infected individuals. Those studies raised concerns about the in vivo activities of 2F5 and 4E10, since the viral rebound observed in several patients occurred along with the emergence of 2G12-resistant virus, with no resistance to the MPER MAbs emerging. However, a more recent study by Manrique et al. showed a different picture when they performed a more in-depth analysis of the virus in vivo and in vitro (135). No mutations in the MPER were found in virus that was obtained from patients at different time points. In contrast, the Env sequences of viruses isolated from the same patients but cultured under high levels of Nt MAbs revealed that neutralization resistance viral variants did emerge, indicating that they comprised a subdominant subset of the total virus in vivo. Interestingly, the mutations that supported escape from 2F5 and 4E10 were identical to residues in the MPER previously defined as being critical for neutralization of the virus (mutations D664N and F673L). Moreover, it was noted that viral escape mutants have reduced infectivity, which could account for the reduced frequency of these viruses in vivo. This newly reported study also supports the potential of the MPER as a target for HIV-1 vaccine development.

MAb 4E10 and Its Epitope

The 4E10 epitope was first mapped to AEGTDRV (aa 823 to 829) on gp160 (28). Later, Zwick et al. showed the 4E10 epitope comprises the linear sequence NWFDIT (aa 671 to 676), which is just C terminal to the 2F5 epitope in the MPER (273). As with 2F5, there is also conflicting information regarding the "full" 4E10 epitope, as a recent study suggested that 4E10 binds both FP at the N terminus of gp41 and the MPER epitope (95). Those authors hypothesized that the full 4E10 epitope was missing in previously reported studies because soluble gp41 does not include the FP. Recent mutagenesis of the 4E10 peptide showed that Trp_{672}, Phe_{673}, and Thr_{676} are essential for 4E10 binding (26), whereas Trp_{672}, Phe_{673}, and Trp_{680} located C terminal to the core epitope (7 aa away) are important for 4E10-mediated neutralization (271). Thus, similar to the epitope of 2F5, the 4E10 epitope seems to involve residues other than those originally mapped in the core epitope.

The crystal structure of 4E10 in complex with the MPER peptide WNWFDITNW (aa 670 to 678) reveals several interesting features (38). First, the 4E10 epitope peptide adopts an unusual helical conformation when bound; yet typically, β-turns are the predominant secondary structure of Ab-bound peptide (217). The importance of the helical epitope conformation for 4E10 binding was supported by a study reported Zwick et al., which showed that Ab binding was reduced by the denaturation of recombinant gp41 (273). Second, the crystal structure of the Fab 4E10-peptide complex illustrates that the core sequence WFXIT makes the greatest number of selective contacts with 4E10, with Trp_{672} being the most highly contacted residue. The WFXI(T/S) motif, in which the X residue does not play a major role in 4E10 binding, appears to be highly conserved among all HIV-1 viruses, thus explaining 4E10's ability to neutralize a huge range of HIV-1.

Previously, Ala substitution studies of the MPER supported the crucial role of the conserved Trp_{672} in virus infectivity (199). Consistent with this, more sequence variation occurs on the opposite side of the helical epitope flanking the conserved WFXIT where there are fewer contacts with Ab. The Ab-combining site of 4E10 is remarkably hydrophobic due in part to the CDR-H3 loop as well as an unusually hydrophobic CDR-H2 loop. This makes it considerably more hydrophobic than those of most Abs. The CDR-H3 of 4E10 is also relatively long (18 aa). Only two residues at the base of the CDR-H3 contact the C-terminal region of the peptide epitope, with the apex of the loop bending away from the peptide. The tip of the

CDR-H3 loop of 4E10 is composed mainly of nonpolar residues that form a very hydrophobic flat surface, which has been suggested to interact with the adjacent viral membrane (see details below). Since it does not directly bind to the peptide epitope, the importance of this long CDR-H3 in neutralization is still unknown; mutagenic analysis of the 4E10 Ab is not yet available.

In a recent study by Cardoso et al. (37), a series of peptides were developed to further characterize the epitope recognized by MAb 4E10. The crystal structures of 4E10 in complex with peptides containing residues that increased helical character or length were shown to positively influence 4E10 binding to its epitope. An extended and modified core epitope for 4E10 with a sequence motif of WFX(I/L)(T/S)XX(L/I)W was identified. This type of study is of significance since vaccines in which residues that are not critical for 4E10 binding (e.g., X) are replaced with those that impose helix-promoting constraints could be designed. Such vaccines would promote a 4E10 binding structure and may be more likely to produce Nt Abs.

MAb Z13 and Its Epitope

The Z13 Fab was isolated by screening a Fab phage-displayed library made from bone marrow cells of an HIV-1-positive individual, referred to as FDA-2, whose serum is bNt (69, 179, 239). The Fab libraries were screened against the synthetic peptide LLELDKWASLWNWFDITNWLW (aa 660 to 680) from HIV-1 MN. Positive Fab clones were selected by ELISA with the same peptide used in the screening and then analyzed by affinity studies; the Z13 Fab showed the highest reactivity with the peptide. Importantly, the entire HIV-1_{MN} sequence was also used for the library screening, and an Ab similar to Z13 was selected, indicating that the epitope recognized by the Z13 Fab is actually exposed in the viral surface structure.

Using competition ELISA, it was determined that the epitope recognized by Z13 overlapped the region recognized by Abs 2F5 and 4E10; however, the majority of the overlap was with the 4E10 epitope, since 4E10 inhibited the binding of Z13 to the peptide more strongly than 2F5 (>90% and >70%, respectively). Using different lengths of peptides covering the 4E10 and 2F5 epitopes from different HIV-1 isolates, it was determined that the core region of the MPER was recognized by both Z13 and 4E10. Both Abs recognize a peptide as short as the one comprising the sequence NWFDITK (aa 671 to 677). Interestingly, Z13 did not bind to gp41 from HIV-1 isolate IIIB; comparison of the sequences of JR-FL and IIIB showed the presence of a different amino acid composition for IIIB (NWFNIT); thus, the importance of D_{674} for Z13 interaction was revealed, further supporting that the core epitope recognized by Z13 overlaps the one recognized by 4E10.

To further characterize this new MPER-specific Ab, the Z13 Fab was tested in neutralization assays against TCLA isolates as well as a primary isolates. The monovalent nature of the Z13 Fab was taken into account for the comparison of the Z13 Fab to 4E10 and 2F5 IgGs in a neutralization assay. However, that study showed that the Nt capacity of Z13 was weaker than those of 2F5 and 4E10 even though it was able to neutralize primary isolates as well as TCLA ones (273). Nevertheless, the discovery of the Z13 Fab has further encouraged researchers to continue efforts to produce an HIV-1 vaccine based on MPER targeting.

Recently, an improved version of Ab Z13 was developed by Nelson and colleagues (164). A random mutation procedure on the CDR-L3 of the Z13 coding sequence was used to generate a phage-displayed Fab library. A Fab with a higher MPER affinity was selected (Z13e1), with affinity studies showing that Z13e1 binds gp41 and the MPER peptide at levels ~35-fold greater than that for Z13. Interestingly, the Z13e1 Ab binds better to a peptide that is elongated at the N terminus (LLELDKWASLWNWFDI TNWLWYIKKKK) (aa 660 to 683); thus, those authors proposed that the optimized Z13e1 Ab has a core epitope located N terminal to the core epitope recognized by Ab 4E10 and C terminal to the 2F5 epitope. The newly optimized Z13e1 Ab showed an increase in affinity for the MPER and an increase in its neutralization potency.

The critical residues recognized by the Z13 Fab were mapped using Ala substitution mutants on the MPER, and it was revealed that the most important residues for Z13 binding were Asn_{671} and Asp_{674}. (Fig. 2D). Although the structure of Z13e1 with its cognate epitope is not available, a hypothetical model of the MPER sequence (DKWASLWNWFDITNWLW) (aa 664 to 680) as an ideal α-helix was created (Fig. 2D). According to that model, the critical MPER-contacting residues for Abs 4E10 and Z13e1 are located on opposing faces of the helix, placing the Z13e1 epitope on the "non-Nt" face of the helix (26, 38). This could explain the lower neutralization potency of Z13e1 than those of Nt Abs 4E10 and 2F5, since the Z13e1 epitope may be less exposed than the 4E10 epitope. Figure 2D shows that the critical interacting residues for the two MPER Nt Abs (4E10 and Z13e1) are located on opposite faces of the MPER helix (164). This reflects the fact that the MPER is flexible and probably changes its conformations to bind 4E10, Z13, and 2F5 during infection.

2F5 and 4E10 Epitopes and the Viral Membrane

The design of vaccine against the MPER is limited by the lack of complete immunogenic, antigenic, and structural data on 2F5 and 4E10 epitopes. It has been proposed that the viral membrane may be a key component of the full epitopes recognized by these MAbs by supporting a particular MPER structure and/or by direct membrane contact with the Ab paratope. In support of these possibilities, one low-resolution structure of the HIV-1 spike shows the MPER as a foot embedded in the viral membrane (268). In addition, a cholesterol-binding motif has been identified in the MPER (LWYIK) (aa 679 to 683) (238), suggesting the possibility that the MPER anchors gp41 into cholesterol-rich regions within the viral membrane (6, 7). Furthermore, NMR studies of an MPER peptide embedded in lipid micelles (Fig. 2A) (209) show that this region adopts a helical conformation, with most of its Trp residues being on one face of the helix and oriented toward the membrane. Again, this indicates that the MPER may be in close association with the viral membrane, since Trp residues tend to localize at membrane interfaces (257). The interaction of the MPER with liposomes comprising HIV-1-specific lipids was studied by Saez-Cirion et al., who found that MPER peptides established specific interactions with cholesterol-containing membranes (197). Collectively, these data suggest that the

membrane plays an important role in defining the structure of the MPER and, therefore, the formation of the 2F5 and 4E10 epitopes.

The proximity of the MPER to the TM and its probable association with the membrane suggest that both 2F5 and 4E10 interact directly with the viral membrane during viral neutralization (i.e., that the viral membrane is an integral component of the 2F5 and 4E10 neutralization-sensitive epitopes). In support of this, crystal structures of the 2F5 and 4E10 Fabs in complex with their peptide epitopes have revealed that the peptides interact with the base of the long CDR-H3, whereas the distal parts of the CDR-H3s do not (38, 170, 174). The CDR-H3 region of an Ab is normally crucial to its interaction with antigen (253); thus, it is reasonable to assume that if the H3 region is not engaged in interactions with the peptide epitopes, it may contact some other component(s) of the viral surface. One appealing candidate is the viral membrane (38, 170).

The notion that the lipid membrane is important for the formation of the 2F5 and 4E10 epitopes in the MPER is supported by functional data. Grundner et al. used proteoliposomes and flow cytometry to characterize the interaction of 2F5 and 4E10 Abs with their epitopes (in the context of gp160 with CT deleted) presented in a lipid environment and observed that both 2F5 and 4E10 bound better to their epitopes in this context (91). They also showed that treatment of the proteoliposomes with detergent, which reduces the lipid content, decreased the binding of the Abs to their epitopes (91). In addition, Ofek et al. showed that MAbs 2F5 and 4E10 bound better to MPER in the context of liposomes than to the MPER without lipid (170). In contrast to these results, Veiga and Castanho did not observe any significant interaction of 2F5 with model viral membranes (237), and Ou et al. showed that 2F5 neutralizes chimeric murine leukemia virus with the same efficiency when the 2F5 peptide is inserted into the Env surface protein or at its natural position near the TM region (173). Those studies suggest that membrane proximity is not required for neutralization by 2F5. Interestingly, others have shown that 2F5 and 4E10 bind to phospholipids and to CL, a characteristic found in auto-Abs produced during antiphospholipid syndrome (2, 25, 97, 200, 201). Although a lipid binding function of these Abs may at first support the concept of membrane binding, the implications of these observations in the recognition of the MPER by 2F5 and 4E10 are not clear.

In summary, there is conflicting evidence concerning the involvement of the membrane in the cognate 2F5 and 4E10 epitopes and its role in structuring protein epitopes within the MPER and/or contributing directly to Ab binding. Clarification of this question is critical for designing MPER-based immunogens that best resemble the cognate MPER structure so as to elicit 2F5/4E10-like bNt Abs.

2F5 and 4E10 Neutralization Mechanisms

The exact mechanisms of neutralization by 2F5 and 4E10 are not fully understood. In general, Nt Abs can act at different stages during the viral infection process (for reviews, see references 31 and 269). Most likely, the mechanisms of neutralization by 2F5 and 4E10 do not involve blocking virus attachment to cellular receptors but appear to interfere with the subsequent fusion of virus with the target cell membrane, during which the MPER is assumed to be exposed (17).

2F5 and 4E10 bind to both native and fusion-activated structures; however, it has been pointed out that the reactivities of 2F5 and 4E10 decrease after the engagement of gp120 with the CD4 receptor. In that study, flow cytometry was used to evaluate the binding of several anti-gp41 MAbs to cells infected with HIV-1 and to cells expressing Env. 2F5 showed constitutive binding to gp41, but this binding was reduced after the cells were incubated with soluble CD4 molecules (205); this was similar to results reported by de Rosny et al. (55). Those authors suggested that the addition of soluble CD4 may reduce 2F5 binding to gp41 by initiating a conformational modification of the epitope induced directly by the interaction of soluble CD4 with gp120 and/or with gp41. Alternately, this may be due to epitope masking caused by the interaction of gp41 with other molecules in the infected cells or viral membrane. Interestingly, Zwick et al. reported the weak binding of 2F5 and 4E10 to their epitopes when the Env protein was expressed on the cell surface, but no substantial differences in epitope binding were observed after treatment with soluble CD4 (273).

In an elegant study, Binley et al. monitored the interaction of 2F5 and 4E10 with fusion intermediates by using a mutated gp160 in which gp120 and gp41 were tethered together by a disulfide bridge. Pseudoviruses bearing this mutant Env can bind to CD4 and the coreceptor, but fusion and cellular infection cannot proceed unless the disulfide tether is broken by a reducing agent such as dithiothreitol (17). Using those experimental conditions, the phase during which Nt MAbs blocked cellular infection was identified using three different assays: (i) a standard assay in which the MAb can bind to virus at any point before viral entry, (ii) a preattachment neutralization assay in which the MAb can bind to the virus only before it binds to the target cell, and (iii) a postattachment assay in which the MAb can bind to the virus only after it has bound to CD4 on the cell surface. MAb b12 prevented infection in the standard assay but not the postattachment assay; this is probably due to the CD4 binding site being occupied by the target cell. In contrast, 2F5 and 4E10 neutralized effectively in both the standard and postattachment assays. Furthermore, 2F5 performed relatively poorly in the preattachment assay. Those authors concluded that both MAbs 2F5 and 4E10 are able to neutralize virus in a standard and postattachment assay but not in a preattachment one. Those data suggest that the 2F5 epitope is not as exposed in free virus. Using the same model, Crooks et al. confirmed the results from Binley et al. showing that 2F5 and 4E10 are able to neutralize the virus in different stages of infection and proposed that neutralization may occur after receptor binding (51). This notion for 2F5 neutralization was also supported by the study reported by de Rosny et al., who tested the binding of 2F5 to native and fusion intermediates and suggested that neutralization by 2F5 is accomplished by the inhibition of a late step of the fusion process (55).

Recent efforts to understand the neutralization mechanism of bNt MAbs 2F5 and 4E10 indicate that Ab-mediated fusion inhibition occurs at the same time as inhibition by peptide C34 (corresponding to a region of the CHR, like T-20), but they do not appear to use the same mechanisms (59). This conclusion is based on the biochemical analysis of the interaction of gp41 with C34 and Abs; however, it is very difficult to interpret the

data in the absence of a consensus model for HIV-1 membrane fusion with target cells.

Taken together, we have described, in detail, the structural and functional complexity of the MPER and the bNt MAbs that bind it. We will examine below how these characteristics impact the design of vaccines aimed at targeting this very important region.

THE MPER FOR TARGETED VACCINE DEVELOPMENT

Current FDA-approved vaccines use whole pathogens, conjugated polysaccharides, or purified protein subunits to elicit protective immunity. However, these classical approaches have not succeeded in producing a protective HIV-1 vaccine. Immunization with whole Env or its subunits did not elicit bNt Abs; instead, nonprotective epitopes dominate the immune response and divert the Ab response from protective epitopes. Thus, new approaches that target the production of Abs against conserved sites such as the MPER need to be developed. An MPER-based vaccine would direct Ab responses against this region, avoiding the problem of Abs against other sites on Env. This "targeted vaccine design" is a new challenge for vaccinologists. The bNt MAbs 2F5, Z13, and 4E10 each bind a unique epitope on the MPER and in so doing have delineated a region to which a targeted vaccine could be developed. Targeted vaccines are distinguished by whether a vaccine elicits Abs to a specific epitope or produces a preselected Ab (i.e., one that resembles one of the bNt Abs). Thus, for the sake of clarity, we refer to vaccines that reproduce a particular Ab as "Ab-targeting vaccines," and we refer to vaccines that produce Abs against a particular epitope as "epitope-targeting" vaccines. A potential advantage of targeted vaccine design is that the Ab response is focused against protective epitopes and away from nonprotective ID epitopes (78). Initial attempts to elicit bNt Abs against the 2F5 epitope (46, 66, 112, 126, 140, 161) and the 4E10 epitope (115, 122) have failed, indicating some of the many challenges associated with producing a targeted vaccine against the MPER.

Challenges for Producing an MPER-Targeting Vaccine

In order to make an MPER-targeting vaccine, this region should be presented in a form so that when used as an immunogen, it will elicit Abs with Nt activity and thereby prevent infection. Thus, there should be a clear understanding of the structural requirements of the region and how the structure supports the biological function of the virus. This information is critical for designing an MPER vaccine. There is a limited understanding of the cognate MPER structure in the context of the Env spike in prefusion, fusion-intermediate, or postfusion conformations, and the available structural data show conflicting information (see above). Another question is the role of the plasma membrane in the epitopes for MAbs 4E10 and 2F5. It is not known if the membrane supports the cognate structure of the antigen and/or if it also contacts Ab. The current approach to vaccine design favors the presentation of the MPER in the context of the membrane, but further structural studies should be done to resolve some of these questions and conflicting data.

Serum studies from HIV-1-infected people and immunization studies have shown that the MPER is weakly immunogenic. Thus, a second challenge is identifying methods that produce high titers of Abs to the MPER. As described below, an approach that combines the exposure of Nt sites on the immunogen in the correct structure, masking of ID epitopes (78, 175, 232), new adjuvants, and the use of novel immunization strategies (e.g., prime-boost) (12, 170, 270) may be effective.

A third challenge is determining whether or not an MPER-targeting vaccine should elicit Abs with long CDR-H3 loops, such as those found on 4E10, 2F5, and other bNt MAbs against HIV-1. The production of this type of Ab may require a special Ab-targeting approach, yet it is not known how long H3 Abs are elicited. Some researchers have suggested that tolerance may have to be broken to do so (98) (see below), which brings up further problems, since such an approach could lead to autoimmunity. However, it is not known if the long H3 loops are absolutely required for broad neutralization, so additional data are needed to resolve this issue.

We have described some of the major challenges that should be considered in the design of an MPER-based vaccine. The sections below discuss (i) previous vaccines attempting to target the 2F5 epitope, (ii) strategies for designing immunogens that resemble the cognate MPER structure, (iii) methods to enhance MPER immunogenicity, and (iv) ways in which the success of an MPER-targeting vaccine could be assessed. Clinical trials will not be discussed (for more reviews on HIV-1 vaccines, see references 64, 81, and 274).

Vaccines To Target Abs against the 2F5 and 4E10 Epitopes

There have been a number of attempts to produce Nt Abs against the 2F5 epitope. So far, these approaches, described below and summarized in Table 2, have not produced Nt Abs.

Chimeric viruses. Due to the strong immunogenicity of influenza virus HA, Muster et al. introduced the ELDKWAS (aa 662 to 668) sequence into a loop on antigenic site B of HA and produced a virus bearing the chimeric protein (161). Mice immunized with the chimeric virus produced strong anti-ELD KWAS peptide IgG titers, and sera showed 50% neutralization of strains MN, RF, and IIIB at titers ranging from 40 to 320 (161). Similarly, Marusic et al. fused the ELDKWA sequence into the N terminus of the potato virus X coat protein (140). Once again, mice immunized with chimeric virus particles produced strong anti-2F5 epitope IgG titers as well as low levels of IgA, as shown by binding to a synthetic peptide containing the ELDKWA sequence in an ELISA. Sera from two mice out of six that were immunized neutralized HIV-1 strain IIIB at low serum dilutions (140). Last, Zhang et al. gave mice both oral and intramuscular (i.m.) immunizations with chimeric virus-like particles (VLPs) bearing the 2F5 ELDKWA epitope at the N terminus of the viral capsid protein L1 of the nonenveloped bovine papillomavirus (264). Anti-ELDKWA Ab titers were very low, likely because the mice were immunized only twice. Sera at a 1/20 dilution from four of eight mice neutralized HIV-1 Bal and MN weakly but not HIV-1 Ada (264). Although there is evidence of weak neutralization in these examples, further progress has not been achieved with

these approaches.

More recently, Kusov et al. produced a live chimeric virus in which the 2F5 epitope replaced the 2A C-terminal extension of the major viral structural protein, VP1, of hepatitis A virus (121). This virus was shown to be infectious in the feces of marmosets 20 days after inoculation, and the serum from one of two marmosets showed weak binding to synthetic 2F5 peptide at a serum dilution of 1/50. Guinea pigs immunized three times with the chimeric virus emulsified in complete Freund's adjuvant for the first immunization also produced weakly detectable serum Ab responses to the 2F5 peptide (titers were not reported). However, these responses were not tested for neutralization, so it is unclear if this approach produced 2F5-like Abs or other Nt Abs. In addition, the immunization protocols could be optimized to increase anti-2F5 epitope titers since the Ab responses were very low. Taken together, while some approaches that display 2F5 in the context of a chimeric virus have elicited strong peptide reactivities, they have not been very successful in eliciting HIV-1 Nt Abs (for another example using chimeric viruses, see reference 66).

Fusion proteins. Liang et al. replaced HIV-1 gp140 V1, V2, V3, or V4 loops with the LLELDKWASL sequence (126). Mice and guinea pigs immunized with plasmid DNA encoding the 2F5 epitope in an optimized position within the V2 loop produced the strongest anti-2F5 epitope Ab titers ranging from 100 to 10,500 for mice and 3,200 to 51,200 for guinea pigs. However, no neutralization of HIV-1 IIIB was observed when sera were tested in a syncytium inhibition assay (126). On the other hand, Coeffier et al. incorporated the 2F5 epitope into maltose binding protein (MBP) of *Escherichia coli* using ELD KWAS (aa 662 to 668), LLELDKWASL (aa 660 to 669), or tandem repeats of the sequences (46). Murine immune sera elicited by the fusion proteins showed binding to the LLELDK WASLK peptide; however, neutralization was not observed for HIV-1 strains MN and RF and a primary isolate (46). Ho et al. designed immunogens that reflected the 2F5 epitope structure (100). They generated fusion proteins that stabilized the 2F5 structure as a β-turn or α-helix by incorporating the ELDKWAS sequence into different locations in the framework regions of an anti-HLA-DR MAb, with the rationale that the MAb would bind to major histocompatibility complex class II and aid antigen presentation. Rabbits whose major histocompatibility complex class II cross-reacts with HLA-DR were immunized with the different constructs three times with or without complete Freund's adjuvant in the priming immunization. Endpoint Ab titers to the anti-DR immunogen were high after the third immunization, reaching over 1,000,000 in the Freund's adjuvant-primed groups and up to 100,000 in the groups that did not receive Freund's complete adjuvant in the prime. In contrast, anti-gp160 titers were lower, with the strongest titer being 10,000 and the lowest being around 20. Protein G affinity-purified rabbit IgG did not show neutralization in a pseudovirus-type assay (100). These examples have shown that fusion proteins bearing the 2F5 epitope have failed to produce immune sera that could neutralize HIV-1.

Peptide-based vaccines. Joyce et al. developed an α-helical peptide bearing the 2F5 epitope and conjugated it to keyhole limpet hemocyanin (KLH) to produce an immunogen (112). Similarly, McGaughey et al. immunized guinea pigs with β-turn-constrained peptides conjugated to KLH or the outer membrane protein of *Neisseria meningitides* without producing Nt Abs (146; for a review, see reference 145). Thus, peptide conjugates also have not produced Nt Abs. In addition, Abs can be directed against the carrier protein and away from the peptide, as was observed by Ho et al., in which anti-HLA-DR reactivity exceeded reactivity to gp120 (100). There are several other examples describing immunization with 2F5 peptides, including 2F5 peptides conjugated to a carrier such as tetanus toxoid (53), bovine serum albumin (127), a CD4 helper T-cell epitope (54), and multivalent peptides on a carbohydrate scaffold (166). The studies described here show the trend that immunization with the 2F5 epitope peptide elicits antipeptide antibodies but does not produce Nt Abs. This is in spite of efforts by scientists to optimize the structure of the peptides so as to provide the epitope in its cognate state. Thus, the results from those studies revealed that new approaches are required for producing Nt Abs that go beyond using the 2F5 linear epitope. This is further supported by Fab-peptide structural data, which indicate that the complete 2F5 epitope (170) may not be fully defined and may include nonprotein components such as membrane.

More recently, immunogen and immunization studies that target 4E10 (see below) have been proposed. For example, Brunel et al. designed immunogens that optimize the 4E10 MPER epitope peptide to keep it extended and constrained to stabilize its helical conformation (26). They proposed to first immunize with the constrained peptide and then give a boosting immunization with an immunogen that would have the "non-Nt face" obscured by a "nonimmunogenic bulk" in order to amplify Abs with a specificity for the Nt epitope in the cognate configuration (26). This approach involves a prime-boost immunization strategy as well as removing unwanted epitopes (see below for a further description of these concepts). Immunizations with these constructs have not yet been reported, so it is unknown whether or not this approach will yield 4E10-like Abs.

The example of targeting the 2F5 epitope has illustrated the technical and immunological challenges involved in producing an epitope-targeting vaccine. Given the similarities between 2F5 and 4E10 (i.e., their unusual Ab structures and the fact that both bind a membrane-stabilized weakly immunogenic region), it is possible that such challenges will transfer to vaccines that target the 4E10 epitope. Recently, Law et al. designed a 4E10 epitope-targeting vaccine using a DNA prime, protein boost strategy with a monomeric gp120-based immunogen in which the V1/V2 variable loops were replaced with the MPER sequence (122). This is similar to the approach that Liang et al. used for the 2F5 epitope (see above). Eight gp120/MPER variants were made: four incorporated variations on the 2F5 and 4E10 epitopes with sequentially removed residues between the two epitopes (LWN) and another four included the 4E10 epitope with sequentially removed residues (LWN) at the N-terminal end of the epitope. These deletions were intended to rotate the α-helix and thus expose different residues of the 4E10 epitope for binding to Ab (neutralizing face). Soluble monomeric gp120-bearing MPER variations all showed binding to Ab 4E10 within a twofold range compared to gp41; however, binding by 2F5 was significantly decreased, perhaps due to the occlusion of the epitope caused by protein fold-

ing. The immunogenicity of all eight constructs was first tested in mice, who were given four priming immunizations with DNA encoding the gp120/MPER variants; these were followed by two boosting immunizations with the corresponding soluble proteins (for doses and injection routes, see Table 3). The DNA immunizations elicited a strong anti-wild-type (WT) JR-FL gp120 Ab response that reached a plateau after the third DNA immunization (endpoint titers of ~10,000), and boosting with protein further enhanced the anti-gp120 Ab response by two- to threefold. However, epitope mapping of mouse sera (at a 1/50 dilution) after the second protein boost showed no binding to MPER-specific peptides but did show binding to V3 loop-specific peptides. In addition, only weak neutralization (50% neutralization at a 1/10 to 1/40 dilution) was observed for HIV-1 strain SF162 but not JR-FL. Those authors speculated that the lack of Nt Ab responses could be due to the inability of mice to produce Abs with long CDR-H3 regions. Thus, they tested the Ab response produced by rabbits (that do produce such Abs) to a construct containing only the 4E10 epitope, which showed the best binding to 4E10 IgG, using a prime-boost regimen that was similar to the one used with the mice. In addition, protein immunization without DNA was also tested. As was observed in the mice, rabbit Ab responses to WT JR-FL gp120 plateaued after the third DNA immunization and were further enhanced by boosting with protein; no binding to MPER peptides was observed. Unlike the mice, rabbit sera from the prime-boost regimen (but not protein-only immunizations) were able to neutralize HIV-1 strains JR-FL, SF162, MN, and HxB2 with 50% neutralization observed at serum dilutions ranging from 1/10 to 1/800. However, the neutralization activity could not be attributed to anti-MPER Abs and was shown to be V3 loop specific since V3 loop peptides blocked neutralization activity (122). This example illustrates that the MPER is weakly immunogenic even in the context of the V1/V2 loops, which typically produce strong Ab responses. However, those authors did not display the MPER in the context of the membrane, and the Ab response was directed to ID epitopes on the V3 loop. Importantly, those authors dissected the Ab response and showed to which epitopes the neutralization activity was directed. This type of analysis is instructive for all vaccinologists attempting to produce anti-MPER vaccines.

To date, approaches to target Abs against the 2F5 epitope (summarized in Table 2) have not succeeded at producing Nt Ab (i.e., chimeric viruses, fusion proteins, and peptides), and those studies have demonstrated that novel approaches are required to produce an MPER-targeting vaccine. Thus, scientists targeting the 4E10 epitope and/or the whole MPER will learn from these examples and should apply new approaches to vaccine design. In addition, new structural information provides a more accurate picture of how immunogens can be made to represent the Env spike on the viral surface. Some of these novel approaches are described below.

Using "mimotopes" to target Nt Abs. In the introduction to this section, we described two approaches for vaccine design, Ab-targeted vaccines and epitope-targeted vaccines. The examples described above show vaccines that target epitopes; that is, immunogens are made to resemble the cognate epitope and are evaluated by the binding ability of a known bNt MAb (e.g., 2F5 linear epitope peptides are constrained so that 2F5 binding is optimized). The goal of such a vaccine is to produce Nt Abs against the target epitope. Conversely, the other approach is to recapitulate the production of the bNt MAb itself (e.g., b12, 2G12, 2F5, and 4E10). Ab targeting starts by optimizing an antigen for binding to one specific MAb and thus may not resemble the cognate immunogen. For example, Pantophlet et al. mutated gp120 to produce an immunogen that was optimized for binding to MAb b12 but not to other Abs against the CD4 binding site (see below) (175). Another approach to Ab targeting is to identify MAb-specific ligands from phage-displayed random peptide libraries; these have the advantage of being a source of peptides for MAbs that bind to linear and discontinuous epitopes. Once selected, peptides (often referred to as "mimotopes") can be further optimized for binding and tested as vaccines (for a review, see reference 104). Here, we describe Ab targeting using mimotopes for HIV-1 bNt Abs b12, 2G12, 2F5, and 4E10.

(i) Mimotopes for MAbs that bind discontinuous epitopes and carbohydrates. Zwick et al. (270) isolated and optimized a MAb b12-specific peptide (B2.1) that competed for binding with gp120. However, when used as an immunogen, the B2.1 peptide does not elicit Abs that cross-react with gp120 in spite of producing high antipeptide Ab titers (202). This lack of immunogenic mimicry is clearly explained by the crystal structure of a complex of b12 and the B2.1 peptide that shows that the peptide occupies a different binding site on the Ab paratope (202) than gp120 (267). Furthermore, B2.1 immune sera showed different reactivity patterns to a peptide Ala scan than b12. In contrast, Dorgham et al. described a peptide selected by b12 that shared common residues with the B2.1 peptide, and immunization of mice with whole phage bearing the peptide elicited some weak gp120 cross-reactivity (63). However, the elicited Abs did not show HIV-1 neutralization activity; moreover, the antipeptide antibodies in the antiphage sera were neither measured nor tested for competition with the b12 Ab (63). Therefore, it is unclear whether or not the gp120 cross-reactivity was due to "b12-like" Abs. Other examples show that the mimotope approach is unlikely to work for targeting the production of Abs that bind to discontinuous epitopes, as we have tested several peptides that bind to MAbs with discontinuous epitopes on hen egg lysozyme and cytochrome *c*, and none of these peptides were able to produce cross-reactive Abs to the cognate antigen (103). These results may be explained by the possibility that Abs select peptides that are optimized for binding to Ab and do not necessarily resemble the cognate structure of the antigen.

Peptides specific for MAbs that bind to carbohydrate antigens (such as 2G12) have also been used to target the production of a specific Ab. For example, Menendez et al. isolated peptides specific for MAb 2G12. The crystal structure of MAb 2G12 bound to a "mimotope" peptide shows that the peptide occupies a different site on the Ab than the cognate carbohydrate (150). Unsurprisingly, immunization of rabbits with recombinant phage-bearing 2G12 peptides did not elicit Abs that showed cross-reactivity with gp120, although antipeptide Ab reactivity reached half-maximal titers of ~800 to 1,600 after four immunizations. Similarly, Vyas et al. reported that peptide makes different contacts with an anticarbohydrate Ab

paratope than the cognate oligosaccharide (240). These differences in structural interactions may explain our immunization results with the 2G12 binding peptide. However, others have reported some success by immunization with peptides that bind to carbohydrate-specific MAbs using prime-boost strategies (12, 90). Taken together, peptides that bind to MAbs against discontinuous epitopes and carbohydrates are not likely to elicit cross-reactive Abs on their own. However, MAbs that bind to linear epitopes are more likely to select peptides that resemble their cognate antigen and thus may be better for producing Ab-targeting vaccines.

(ii) Mimotopes for MAbs that bind to linear epitopes. We used MAb 4E10 to select peptides from phage libraries and then further optimized the peptide for binding to 4E10 by building sublibraries that contained fixed consensus residues (10). Seven immunizations of rabbits with a recombinant phage clone produced low reactivity with the immunizing peptide and low cross-reactivity with gp41; however, sera did not show binding to a peptide bearing the cognate 4E10 epitope. Furthermore, immune sera showed no neutralization activity at a 1/20 dilution (data not shown); however, if Nt Abs were present, they would likely be at too low of a titer to produce detectable Nt activity. Thus, further studies are aimed at determining the nature of the cross-reactivity that was observed with gp41, and antigen-specific Abs will be concentrated by affinity purification before retesting them for neutralization.

Peptides specific for MAb 2F5 have also been reported (151), and sera from rabbits immunized with phage bearing the recombinant peptide (DXR4.22EL) showed reactivity with gp41 with titers of ~1/2,000. Further studies will test these Abs for Nt activity; however, others that have used a similar approach did not report the presence of Nt Ab activity in anti-peptide immune sera (233). Thus, peptides that are selected by MAbs that bind to linear epitopes are more likely to produce cross-reactive Abs, although the nature of the cross-reactivity observed in the examples described above still needs to be explored.

Such peptides could play a role in producing an MPER-targeting vaccine; however, due to their small size, a peptide immunogen may not fill the paratope of a targeted Ab in the same way that a larger protein would; thus, a peptide may not represent a complete epitope. One approach to overcome this limitation is to use anti-idiotype Abs whose paratopes are complementary to the targeted Ab and, when used as immunogens, are occasionally able replicate an Ab response that is very close to that of the targeted Ab (84). This approach was used by Gach et al., who immunized mice with an anti-2F5 idiotype MAb and produced Abs that reacted with a peptide bearing the 2F5 epitope sequence; purified Abs were able to compete with 2F5 for binding to the same peptide (75). However, it is not clear whether these Abs demonstrate Nt activity, since in vitro neutralization experiments were not reported.

These examples illustrate two problems. The first challenge is that a vaccine should produce the correct Ab (i.e., one that shows Nt activity), and the second challenge is that the correct Ab needs to be produced at high titers. Although a few of the examples described here were tested for Nt activity, it should be distinguished whether the lack of Nt activity is due to low specific Ab titers or a lack of the correct Ab. Thus, experiments where Abs are concentrated by affinity purification followed by neutralization studies should demonstrate the challenges that vaccinologists are facing.

IMMUNOGENS AND IMMUNIZATION STRATEGIES FOR MPER-TARGETED VACCINES

As described above, Abs Z13, 2F5, and 4E10 are rare and unusual in structure. Thus, it is unlikely that the Ab-targeting approach will be successful. It may be that targeting the MPER as a region could be more effective by producing neutralizing Abs that bind to the MPER. However, the requirements that make those Abs able to neutralize and the structural characteristics of the MPER for those Abs to be produced are not known. To date, there are no targeted vaccines. This new approach requires molecular-level design based on the known structure of the MPER and an in-depth understanding of how the Ab response to certain epitopes develops. Using current technology, an MPER-based vaccine could include (i) an immunogen built in the context of membrane, (ii) reduced ID epitopes so as to focus the Ab response to Nt epitopes, and (iii) novel immunization strategies to enhance immunogenicity. These concepts and their limitations are discussed below.

Should MPER-Targeting Vaccines Be Presented in the Context of Membrane?

Current region-targeting vaccine design favors the presentation of the MPER in the context of the membrane so as to present the putative 2F5 and 4E10 epitopes in a state that resembles that of the natural Env epitope; recombinant enveloped viruses are one approach to this. For example, Luo et al. made recombinant VSV particles displaying the HIV-1 MPER as a fusion to the C-terminal end of the p15E protein from porcine endogenous retrovirus (PERV); p15E has structural similarities to gp41 (134). The gp41 MPER either replaced PERV Nt epitope E2 or was placed C terminal to it. 2F5 was shown to bind to cells expressing these constructs by flow cytometry; however, 4E10 binding was not shown. Sera from rabbits that were immunized with recombinant VSV and soluble protein produced anti-gp41 MPER Abs, as shown by binding to cell lysates expressing gp41 that contained MPER sequences (aa 628 to 705) in a Western blot. These Abs neutralized HBX2 and JRFL HIV-1 strains at 1/20 serum dilutions; however, sera from control rabbits also showed weak neutralization (134). As the serum Ab response was not mapped to specific epitopes, further analysis could better reveal whether this approach was successful at targeting Abs against the MPER and if the Nt activity observed was authentic.

DNA vaccines are another way to present the MPER in the context of membrane provided that the protein to be expressed is fused to a TM domain. Ye et al. made a DNA vaccine in which the HA2 domain of the influenza virus HA was replaced with gp41, reasoning that HA1, a slightly smaller protein than gp120, would conserve the prefusogenic structure of gp41 while exposing epitopes that are normally obscured by gp120 (258). When this construct was expressed on cells, the fusion proteins formed trimers that bound Abs 2F5 and 4E10 twofold better than did the native Env trimer, as shown by binding to

cells in a flow cytometry assay; this suggested increased exposure of the 2F5 and 4E10 epitopes, although it was not shown that similar amounts of HA/gp41 and native Env trimers were present on the cell surface. Sera from mice immunized with plasmid DNA encoding the HA/gp41 fusion bound to whole gp41, and pooled sera neutralized SF162 at a 1/40 dilution compared to empty-vector-immunized control sera. As with the above-described example, no epitope-mapping experiments were performed, so it is unclear to what epitope specificity the Nt fraction was directed (258). Furthermore, it is possible that the use of whole gp41 directed the Ab response away from Nt epitopes on the MPER and that the Ab response could be further focused to 2F5 and 4E10 epitopes by making constructs that express the MPER and subunits of gp41 rather than the whole protein. If this is true, then perhaps altering some of the ID epitopes on the immunogen could enhance MPER targeting.

Liposomes that stabilize membrane-dependent structures for targeting 2F5-like Abs have been proposed (170). In one example, Lenz et al. produced proteoliposomes with trimeric truncated gp41 comprising the C-terminal heptad repeat, the MPER, and the TM region of gp41 (124). Mice were immunized with truncated gp41, truncated gp41 in a liposome, or empty control liposomes. IgG1 and IgG2a Ab titers to truncated gp41 reached 2,500 in the mouse group immunized with truncated gp41 in a liposome but not when the immunogen was presented without liposome. Serum Abs did not neutralize HIV-1, although the immunogens were shown to inhibit viral entry, suggesting that this proteoliposome may have potential therapeutic qualities (124). This example shows that liposomes may present the MPER in the context of membrane; however, it may be important to optimize lipid content, as bilayer thickness can alter the tilt of a TM helix, potentially affecting antigen display (114, 219). This approach will require further optimization to confirm whether or not liposomes are an effective carrier for the MPER.

Taken together, the evidence indicates that the presentation of gp41 in the context of membrane can produce low-titer Nt Abs. However, the MPER is weakly immunogenic and may not produce a strong Ab response when other gp41 epitopes that could divert the Ab response away from Nt epitopes on the MPER are present. Matoba et al. designed a vaccine to target the MPER in which they fused the MPER (aa 649 to 684) to the cholera toxin B subunit (143). They immunized mice seven times using immunization protocols that included intraperitoneal (i.p.) and intranasal (i.n.) immunizations, or a combination thereof, and produced anti-MPER Ab endpoint titers as high as 100,000. While sera were not tested for neutralization, fecal and vaginal mucosal Abs were shown to decrease HIV-1 transcytosis in a human tight epithelial model compared to control Ab (143). Although this approach does not present the MPER in the context of the membrane, those authors recognized the importance of producing mucosal immunity in a vaccine. Thus, it would be interesting to map the serum Abs to epitopes on the MPER and test for neutralization in a standard assay (156, 192).

Another interesting example of MPER targeting was reported by Lorizate et al., who showed how subtle changes in structure can affect immunogenicity (131). Those authors postulated that the prefusion structure of the MPER would be stabilized by the FP by forming a structurally defined complex with hydrophobic sequences of the FP. To test this hypothesis, those authors combined peptides bearing 2F5 epitope sequences and gp41 fusion peptide to make a complex that could resemble the prefusion structure of gp41. A complex of the two peptides showed better binding by MAb 2F5 by ELISA than did the 2F5 epitope alone. Circular dichroism was used to show that the 2F5 epitope peptide and a soluble version of the fusion peptide formed ordered structures in solution, indicating that the enhanced binding by 2F5 observed in the ELISA was not due to nonspecific hydrophobic interactions of the peptides with the ELISA plate. Structures were further enhanced when complexes were made between the FP and a peptide with the more complete MPER sequence bearing both 2F5 and 4E10 epitopes, and these complexes showed moderately improved binding (compared to MPER alone) to 2F5 when used in a competition assay for binding gp41. To test the immunogenicities of these complexes, rabbits were immunized with linear MPER peptide or a complex of the fusion and MPER peptides in alhydrogel or Freund's adjuvant; details such as immunization route and timing were not reported. Immune IgGs were then affinity purified on protein G columns. Interestingly, IgG from rabbits immunized with the peptide complex in alhydrogel inhibited the binding of 2F5 to its epitope peptide in an ELISA. Although these Abs competed with 2F5, they are not likely to be Nt, since preincubation of the cells with both the immune IgG and 2F5 in a syncytium formation assay blocked the capacity of MAb 2F5 to inhibit fusion. These results indicate that immune IgGs are blocking the binding of 2F5 to its epitope but are not capable of preventing syncytium formation. 2F5 alone prevented syncytium formation, whereas syncytium inhibition by immune IgGs alone was not reported. IgG from rabbits immunized with the MPER peptide alone in alhydrogel or the complex in Freund's adjuvant did not compete with 2F5. This may be because Freund's adjuvant is an oil-based emulsion that could affect the structure of the peptide complex. Although the 4E10 epitope was part of the immunogen, the production of 4E10-like Abs was not explored (131). This novel example clearly illustrates that subtle changes in the structure of the immunogen and even the adjuvant can affect the outcome of the Ab response.

It is unclear from those studies whether supporting the MPER by membrane and/or other gp41 regions results in the production of bNt Abs. Furthermore, this approach may result in unintended consequences, as was observed for a lipidated peptide immunogen bearing the MPER sequence from feline immunodeficiency virus. When this peptide was displayed in multilamellar lipid vesicles and used as an immunogen in cats, the resulting Abs enhanced infection in an in vitro neutralization assay (80). In addition, studies described previously (92, 124, 131, 134, 144) did not map Ab specificities or correlate neutralization activity to specific epitopes. While peptide mapping may not detect all relevant Abs, it is important to determine if epitope targeting has taken place and to show which epitopes on the immunogen play a dominant role. If so, then there may be a need to overcome the influence of irrelevant ID epitopes that may be contained in those structures. The section below discusses approaches on how this could be accomplished.

Engineering of gp41 Epitopes on an MPER-Targeting Immunogen Could Focus an Nt Ab Response to the MPER

As mentioned above, an MPER-based vaccine should mimic its cognate structure on infectious virus. It is possible that other portions of gp41 as well as the viral membrane are required to stabilize the MPER. However, in an MPER-targeting vaccine, ID epitopes (such as the ID loop) could divert the Ab response from poorly immunogenic MPER epitopes. Fortunately, there is consistent evidence indicating that altering an ID epitope can divert the Ab response to other epitopes on a protein. For example, serum IgG responses to a multiepitope peptide were focused on a single 4-aa epitope even though the IgM response focused on multiple epitopes. Point mutations in the epitope redirected the IgG response to other epitopes in the peptide (1) (for more examples, see references 42, 44, and 208). Thus, this approach may be an effective way to make the MPER more immunogenic while supporting its cognate structure.

Modification of ID epitopes by glycosylation was proposed by Garrity et al. (78). They engineered N-linked glycosylation sites into the V3 loop on gp120 at single sites along the V3 loop or in combinations of four sites (i.e., at site 1:3, 1:4, 2:4, 1:2:4, or 1:2:3:4); that is, there are four sites on the V3 loop where minimal mutagenesis was required to insert a glycosylation site. These sites were labeled sequentially 1 through 4, orienting from N terminus to C terminus. Single glycosylations are referred to as 1 or 2, etc., whereas multiple glycosylations are referred to as a combination of numbers describing the different sites. For example, 1:2:4 would contain glycosylation at sites 1, 2, and 4. Guinea pigs were immunized with recombinant vaccinia virus expressing WT or glycosylated gp120 and boosted with the respective recombinant proteins. WT gp120 immune serum mapped predominantly to the V1 and V3 peptides. In contrast, immune sera from animals immunized with gp120s glycosylated at positions 2, 4, 2:4, and 1:2:4 showed increased reactivity to the C1 peptides and a relative decrease in binding to a V3 loop peptide. Neutralization studies that included either V1 or V3 loop-competing peptides showed that when gp120 was glycosylated at all four sites (1:2:3:4), it elicited Abs that neutralized HIV-1 via the V1 loop, whereas WT immune sera neutralized via the V3 loop (78). This example shows that the effect of ID epitopes can be diluted to the extent that correlates of neutralization can be redirected to other epitopes (also see reference 232).

Others have applied this approach to focus the Ab response to the CD4 binding site on gp120. Pantophlet et al. produced two immunogens. One was comprised of a gp120, glycosylated at seven different sites, that bound to the bNt Ab b12 but showed reduced binding by V2 and V3 loop-specific Abs (176). The second immunogen was comprised of a gp120 mutant with four Ala replacements that enhanced the binding of b12 to gp120 but reduced binding by non-Nt CD4 binding-site-specific MAbs (GDMR gp120, where GDMR indicates residues replaced by Ala) (175). Rabbit immune sera to the two glycosylated gp120s and WT gp120s showed similar anti-WT gp120 Ab titers. Serum from only one rabbit immunized with the glycosylated gp120 was able to neutralize SF162, whereas GDMR gp120 immune sera neutralized HIV-1 strains SF162, JR-FL, JR-CSF, and ADA. The predominant neutralization activity for both GDMT and WT gp120 immune sera was mapped to the V3 loop, and serum-mapping studies showed a strong binding of all sera to two V3 loop peptides (210); thus, the Ab response, while diluted, did not target the b12 epitope on gp120.

Another method for redirecting Ab responses away from ID epitopes is to remove them. This has been moderately successful for diverting Ab responses but has not yet improved broad Nt activity (107, 116, 133). One caveat of this approach is that the removal of ID epitopes can change the structure of the protein, risking an alteration of conformational epitopes.

Although the studies discussed above do not describe MPER targeting, they provide several approaches that could be used to divert Ab responses away from ID epitopes on gp41 and possibly to the MPER. Ultimately, this information could be used to design an MPER-targeting vaccine in which structural support is provided by other gp41 "domains" and/or the cell membrane, with ID epitopes modified on the supporting regions to decrease their immunogenicity. This could be done by glycosylation (78, 175, 232), the removal of the ID region (if not required for structure) (107, 116, 133), or the replacement of charged or bulky residues with less immunogenic amino acids (such as Ala, Gly, or Ser).

More recently, Kim et al. incorporated these concepts into the design of three membrane-presented, MPER-targeting vaccines that did not display ID epitopes such as the ID loop (115). Constructs that expressed fusion proteins included portions of gp120, gp41, MPER, and gp41 TM domains; these were displayed in the context of membrane on enveloped VLPs and on the surface of Sf9 insect cells. Specifically, the first construct (C1) comprised the gp120 C1 and C5 regions, with the other segments of gp120 (including ID variable regions) replaced by the smaller SH3 domain from CD2BP1, an adaptor protein that binds to the cytoplasmic tail of CD2 (LFA-2); gp120 C5 was fused to a soluble 24-aa linker region that replaced the hydrophobic gp41 fusion peptide, followed by gp41 N- and C-terminal heptad repeats, the MPER, and the gp41 TM domain (115). Previous studies used cross-linking followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to show that a similar construct lacking the MPER and the TM domain formed trimers (190), and the binding of Abs specific for pre- and postfusion conformations showed that this construct did not form the postfusion 6HB. A second construct (C2) comprised the gp41 N- and C-terminal heptad repeats in which four residues were mutated to Asp to prevent the formation of the 6HB; this was followed by the MPER and the TM domain. The third construct (C3) comprised the homotrimeric extracellular domain fragment of human B-cell-activating factor (BAFF) fused to the CHR, followed by the MPER and the TM region. Although BAFF is implicated in B-cell development, maturation, and maintenance of follicular B cells (105), it is unclear whether BAFF was used exclusively to maintain the gp41 trimeric structure and/or to play a role as a molecular adjuvant. However, this was human BAFF, which may not be active in guinea pigs. 4E10 and 2F5 were shown by flow cytometry to bind to insect cells expressing all three constructs compared to insect cells alone. To test the immunogenicities of these constructs, guinea pigs were inoculated three times with 5×10^6 cells (expressing C1) or 2- or 10-μg doses of VLPs (bearing C1, C2, or C3) using intradermal (i.d.) injection routes with or without *E. coli* heat-labile enterotoxin

(LT) as an adjuvant; in some cases, the i.m. route was used. Antisera were tested by ELISA for binding to VLPs or soluble C1 lacking the MPER and TM domains (C1-MPER/TM). In all immunization groups, significant anti-VLP Ab titers were observed (except for mock-transfected cells and phosphate-buffered saline [PBS] immunogens), with endpoint titers ranging from 10,000 to 15,000; however, anti-C1-MPER/TM Ab responses were substantially lower, with endpoint titers ranging from ~100 to 1,000. These results indicate that while there were significant Ab responses to the Sf9 membrane proteins, this was not the case for the expressed fusion protein. Furthermore, Western blots that used pooled sera to determine the epitope specificity of the Ab response showed that immune Abs were directed to the CHR but not to peptides that bear the MPER linear sequence; no neutralization was observed. Taken together, these results indicate that the display of the MPER along with other ID domains does not appear to enhance Ab responses against the weakly immunogenic MPER (115).

Interestingly, and in agreement with results reported by Kim et al. (115), a recent paper by Alam et al. suggested that the presence of the ID epitope in the region corresponding to the CHR (previously defined as the cluster II epitope) can mask the Ab response elicited against the MPER (3). After immunizing mice with gp140, two MAbs (5A9 and 13H11) that bind to CHR epitopes were isolated and partially cross-blocked 2F5 Ab binding to Env. Using competition ELISA, they also showed the presence of Ab with CHR reactivity in 83% of sera from HIV-1-positive donors. They hypothesized that B cells make ID non-Nt Abs to the CHR that block less prevalent B cell clones from recognizing the 2F5 epitope region (3).

Although those approaches did not produce high anti-MPER Ab titers, further modifications (e.g., enhancement of MPER copy number on VLPs or the use of a less immunogenic cell type), possibly combined with an immunization strategy designed to enhance the Ab response to a restricted site (i.e., prime-boost), could elicit high titers against the MPER. In the next section, we discuss immunization strategies that could be used to enhance an Ab response to the MPER.

Prime-Boost Immunization Strategy for Targeting and Enhancing Specific Ab Subsets

An MPER-targeting immunogen may require novel immunization strategies to enhance its immunogenicity. We proposed prime-boost immunization as a method for targeting the bNt MAb b12 (270) based on the work by Beenhouwer et al. (12), whereas Ofek et al. proposed the reverse of this model for targeting 2F5 (see below) (170). Such a strategy to target the MPER could comprise a priming immunization with gp41 that activates and expands a variety of B-cell clones that includes clones producing the targeted anti-MPER Ab, with a boosting immunogen comprising 4E10- and/or 2F5-specific peptides; the reverse of this may also be effective. The boost with a peptide may amplify memory B cells that produce the targeted Ab. Ab production could be further enhanced by incorporating the same T-cell epitope into the priming and boosting immunogens. Thus, the prime-boost method could enhance a specific subset of B cells in the Ab response and divert a polyclonal Ab response in the boost by amplifying restricted Ab specificities.

This approach has worked for eliciting Abs against the *Cryptococcus neoformans* capsular polysaccharide, glucuronoxylomannan. Mice were primed with small doses of a glucuronoxylomannan-tetanus toxoid conjugate, and no antiglucuronoxylomannan Abs were detected. A peptide (P206.1) was optimized for binding to a neutralizing antiglucuronoxylomannan MAb and used as a boosting immunogen in the form of free peptide, in the form of a multiple antigenic peptide (P206.1-MAP), or conjugated to tetanus toxoid. Only mice that were boosted with the P206.1-tetanus toxoid conjugate produced antiglucuronoxylomannan titers that were significantly higher than those of the tetanus toxoid-boosted control group, indicating the importance of including the same T-cell epitopes in the priming and boosting immunogens. The antiglucuronoxylomannan serum Abs cross-reacted with both glucuronoxylomannan and peptide, as shown by competition ELISA, and did not bind to de-O-acetylated glucuronoxylomannan, which is preferentially bound by nonprotective Abs. Immune mice were not challenged with *C. neoformans* to assess protection (12). This elegant work clearly shows that prime-boost immunizations can significantly augment the production of peptide-targeted Abs.

Other groups have attempted prime-boost immunizations for targeting the 2F5 epitope with ambiguous results. Devito et al. tried several immunization conditions, which included priming mice with DNA plasmids encoding gp160 and CCR5 and boosting with a mixture of peptides, including (i) 2F5 epitope peptides from clades A through D, (ii) gp41 coiled-coil peptide, and (iii) CCR5 second-loop peptide (57). A second mouse group received the gp41 2F5 peptides, the coiled-coil peptide, and the CCR5 peptide in both the priming and boosting immunizations; a third group received PBS for priming and boosting. Sera from groups 1 and 2 showed statistically similar anti-gp41 2F5 peptide IgG titers; however, group 1 showed increased fecal IgA levels to the 2F5 epitope peptide, whereas this was not observed for group 2 or 3. Moreover, the increase in IgA titers was very small, so this prime-boost protocol was not very effective; no increase in IgG production was observed. In addition, sera from group 1 showed the best neutralization of SF2 HIV-1, and an HIV-1 primary isolate with 50% neutralization was shown at dilutions ranging from ~200 to 300 and ~150 to 250, respectively (57). This approach showed that the DNA immunization followed by a peptide boost was more effective than immunization with peptide alone, as demonstrated by the improved IgA response and Nt activity. In addition, those authors showed that the immunity lasted for 12 months corresponding to 50% of the life span of the mouse.

Ofek et al. proposed that prime-boost immunizations that target 2F5-like Abs should comprise a prime with 2F5 epitope peptide presented in the context of membrane (VLP or liposome) and a boost comprising native Env trimers, also in the context of membrane, to select Abs that can bind to the native envelope spike (170). The "peptide-first" approach has been applied to meningococcal group A polysaccharide (MGAPS). Grothaus et al. primed mice with a peptide specific for an Nt MAb in a proteoliposome and boosted them (90) with MGAPS. The prime-boost appeared to elicit similar to higher anti-MGAPS Ab titers compared to those with three immunizations with the peptide-proteasome complex or MGAPS alone. Bactericidal assays showed that the sera from mice that were primed with peptide-proteasome groups and boosted with MGAPS were sixfold better at killing bacteria than serum

from mice immunized twice with the peptide-proteasome (90). These data suggest that the "reverse" approach can be successful, but as with the example described by Beenhouwer et al., this study was designed to target an anticarbohydrate MAb; these techniques have yet to be shown to be successful in targeting antiprotein Abs.

Prime-boost for targeting carbohydrates has shown some success; however, our work on a prime-boost model for targeting antiprotein Abs has shown several limitations. First, the targeted Ab must be produced in the priming immunization, and complex Abs such as 2F5 and 4E10 may be difficult to elicit. Second, the targeted Ab should be produced at a high frequency across an animal population. This may not be likely for Abs such as 2F5 and 4E10 but may true for other anti-MPER Abs. Third, the boosting immunogen can stimulate a de novo response, producing Abs to new epitopes rather than targeted ones. Last, peptide immunogens may represent only portions of the cognate epitope, thereby acting as poor immunogenic mimics. In spite of these problems, optimization of the prime-boost approach could make this a valuable immunization strategy.

OTHER CONSIDERATIONS IN VACCINE DESIGN

Targeting the MPER and Breaking B-Cell Tolerance

The sections above describe general approaches that are currently used for targeted vaccine design. These have included examples of incorporating membrane into an MPER-based immunogen (134, 170, 258, 264), removing or altering ID epitopes to enhance the Ab response to weakly immunogenic epitopes (44, 78, 107, 210), and various prime-boost strategies to enhance immunogenicity (12, 57, 90). It is unlikely that a single approach will produce a successful MPER-targeting vaccine; a combination of these approaches may be required.

In producing an MPER-targeting vaccine, some important questions are as follows. Do Abs need to have long CDR-H3s to neutralize HIV-1 via the MPER, and if so, how could they be produced by a vaccine? There is limited evidence that long CDR-H3s are required for neutralization via the MPER; for example, it is known that a Trp residue at the tip of the 2F5 CDR-H3 is required for neutralization, but this does not suggest that the CDR-H3 should be long (272). Furthermore, it is not understood how putative interactions between long CDR-H3s and the viral membrane might lead to neutralization. If future studies show that long CDR-H3s are crucial for neutralization, then the issue of producing them by a vaccine should be addressed. Currently, there is limited understanding as to how to elicit Abs with long CDR-H3s, and there are several hypotheses regarding how they could be produced. Our unpublished analysis of several hundred human MAbs indicates that Abs associated with chronic viral infection have long CDR-H3s in general (F. Breden, C. Lepik, J. K. Scott, and M. Montero, unpublished data). Thus, HIV-1 causes a chronic infection, and long CDR-H3 Abs may arise as a result of persistent exposure to antigen. Under these circumstances, a vaccine may need to be administered many times or be delivered by time release preparations to be effective.

Others have proposed that 4E10 and 2F5 are auto-Abs (163) and suggested that producing long CDR-H3 Nt anti-MPER Abs would require breaking B-cell tolerance. This is based on the observations that pre-B cells in adult bone marrow have longer CDR-H3s than mature B cells in the periphery and are autoreactive (147, 241). As B cells mature, they undergo several developmental checkpoints to produce tolerance to self-antigens. The first checkpoint occurs in the bone marrow, where a significant percentage of polyreactive and autoreactive B cells are removed through mechanisms such as deletion, anergy, and receptor editing (241). A second checkpoint occurs in the periphery in newly migrated B cells before maturing into naïve B cells that are ready for activation by antigen and T-cell help (241). Thus, if the auto-Ab hypothesis is true, both checkpoints would likely have to be broken in order to produce long CDR-H3 Abs such as those found in the early stage of development in the bone marrow. This approach may produce unintended outcomes, as the loss of these developmental checkpoints should be associated with increased levels of anti-self-Abs and polyreactive Abs, such as those produced in autoimmune disorders (e.g., systemic lupus erythematosus) (261). It has been proposed that strong adjuvants could be used to break tolerance (98), which brings up additional questions concerning the immune responses elicited by such an action. Thus, further work is required to ascertain whether the autoreactive Ab precursors are needed to develop bNt Abs against HIV-1.

Identifying Correlates of Neutralization Activity

How should the success of a targeted vaccine be measured? Epitope-mapping studies in which immune serum is tested for binding to overlapping synthetic peptides (see, e.g., references 78 and 210) are limited to identifying linear epitopes, whereas the majority of Abs are produced against discontinuous epitopes (110). In addition, there has been an assumption that linear epitope peptides cross-react with membrane-associated peptides, but this may not be true. Serum Abs that bind to discontinuous epitopes can be identified using competition assays with MAbs that bind known epitopes. However, this approach does not show specificity but shows only that Ab binding sites overlap. In addition, affinity, steric hindrance, and conformational changes induced by Ab binding can all affect how a competing Ab binds (210). Testing Abs for epitope-specific neutralization can be done by including inhibitory peptides in neutralization assays (for example, see references 78 and 210) or producing chimeric viruses that display the epitope of interest and using them in neutralization assays (262). Again, these approaches are limited to linear epitope peptides that may not fully resemble the cognate antigen structure.

These limitations are especially troubling when targeting a region such as the MPER in which the epitopes for 2F5 and 4E10 are not clearly defined and may have the added complexity of involving membrane. Thus, the nature of Nt epitopes should be clarified. It is well established that the binding of immune sera to the ELDKWAS peptide is not an indication of neutralization (see above). Therefore, the correlates of neutralization for 2F5 and 4E10 should be determined at the molecular level so that vaccine-produced Abs could be assayed for the same characteristics. This should include an understanding of the MAb effector mechanisms, since viral clearance may be enhanced by mechanisms other that neutralization; for

example, MAb b12 can initiate Ab-dependent cellular cytotoxicity (99). What is urgently required is an assay that defines the correlates of neutralization at a molecular level. Specifically, such an assay should present the MPER in a "neutralization-competent" conformation. This structure could be defined by the binding of Nt Abs but not non-Nt Abs. This distinction is important since non-Nt Abs do not bind Nt competent structures on the virus; if they did, they would show Nt activity. Such an assay could be used to answer questions regarding the binding mechanisms of bNt Abs (such as 2F5, 4E10, and Z13) and would help determine the structure of the MPER that is required for neutralization. Answers to these questions could be used to develop immunization approaches for eliciting Abs that bind this neutralization-competent structure with high affinity. Furthermore, such an assay could be used to probe Abs from vaccinated animals and infected individuals to identify methods that successfully produce Nt Abs to the MPER.

CONCLUSIONS

The identification of three bNt Abs against the MPER have defined this region as an important vaccine target. In this review, we have described the current state of understanding regarding the structure and function of the HIV-1 MPER as well as progress toward making an MPER-targeting vaccine. This is a challenging task since the MPER is a structurally and functionally complex region. Structurally, it has been shown that the MPER, or peptides containing MPER sequences, adopts different conformations, including α-helix and β-turn. Functionally, the MPER plays a role in the fusion of the viral membrane to its target cell and viral entry; however, the exact mechanism of its involvement is not understood and requires further study. Fortunately, new structural information and biochemical analyses continue to reveal the true nature of the epitopes of these Abs; this information could lead to the development of assays that determine the neutralization-competent structure of the MPER and would allow vaccine designers to critically analyze their immunogens as well as define the outcome of immunization studies. In addition, future studies that investigate the role of the long CDR-H3 in neutralization and the biological origins of these Abs would reveal the B-cell subsets that should be targeted by immunization strategies. Taken together, it is clear that many challenges remain in producing an MPER-targeting vaccine; however, approaches that recapitulate the structural features of the MPER Nt epitopes, together with the use of new immunization strategies, should support the future development of an MPER-based vaccine for HIV-1.

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ADDENDUM IN PROOF

Since this article was accepted for publication, Z. J. Sun, K. J. Oh, M. Kim, J. Yu, V. Brusic, L. Song, Z. Qiao, J. Wang, G. Wagner, and E. L. Reinherz (Immunity **28:**52–63, 2008) used NMR and electron paramagnetic resonance to solve the structure of an MPER peptide in the context of the membrane; several different lipid compositions were tested. Sun and colleagues observed that the MPER adopts a helical structure that is kinked and somewhat L-shaped and that the region that contains the 4E10 epitope is embedded within the membrane. Furthermore, analysis of NMR structures of the MPER in the context of DPC micelles in the presence and absence of 4E10 Fab fragments suggests that 4E10 initiates a conformational change in the MPER, perhaps by lifting its critical binding residues out of the membrane and into the paratope of the Ab (Z. J. Sun et al., Immunity **28:**52–63, 2008; see also B. F. Haynes and S. M. Alam, Immunity **28:**10–12, 2008). The MPER structure described in this study conflicts with previously solved structures (see Fig. 2). In addition, it is unclear if this or other structures resemble the cognate structure of the MPER in the context of the trimeric protein on the viral membrane. For example, it may be that the MPER associates with protein in the context of the trimer on the viral surface and/or that the structure of the MPER is affected by the TM region, which is C terminal to the MPER and was not included in the analysis of Sun et al. Thus, this study adds to our understanding of the structure of the MPER in the context of lipids, but the question of its native structure(s) on the cell surface in the context of gp41 remains open.

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