Immunization with Hybrid Proteins Containing the Membrane Proximal External Region of HIV-1

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Abstract

The transmembrane envelope (TM) protein gp41 of HIV-1 is an attractive target when designing a vaccine to induce neutralizing antibodies. A few broadly neutralizing antibodies (2F5, 4E10, and 10E8) that target conserved epitopes in the membrane proximal external region (MPER) of gp41 have been isolated from infected individuals. However, attempts to induce such antibodies by immunizations with gp41 and Env derivatives containing the MPER were successful only to some extent. In contrast, immunizations with the ectodomain of the TM protein p15E of different gamma retroviruses resulted in the induction of neutralizing antibodies. These sera recognized epitopes located in the MPER and in the fusion peptide proximal region (FPPR) of p15E. Based on these results, both regions of p15E were substituted with the corresponding sequences derived from gp41 of HIV-1. Thus, four different hybrid antigens were produced. One of the inserted sequences contained the epitopes of 2F5 and 4E10 in the MPER; the other corresponded to the FPPR. Vaccination of rats, guinea pigs, and a goat induced binding antibodies directed against the FPPR of gp41 and the 2F5 epitope (ELDKWA) located in the MPER. Despite the exact recognition of the 2F5 epitope, no or very weak neutralization of HIV-1_{NL4-3} by the immune sera was demonstrated. Nonetheless, using the strategy of hybrid proteins, antibodies targeting the desired epitope were successfully induced.

Introduction

THE DESIGN OF ANTIGENS that are able to induce broadly neutralizing antibodies (bnAbs) against the human immunodeficiency virus 1 (HIV-1) is one of the major challenges in vaccine development. Although sera with broad neutralizing capacity are observed in only about 2% of infected patients, 10–30% of HIV-1-infected individuals develop neutralizing antibodies over time.^{1–3} However, the design of antigens capable of inducing neutralizing antibodies against HIV-1 is hampered by dense glycosylation and variable conformations of the envelope proteins.⁴ After the interaction of gp120 with the CD4 receptor, conformational changes enable binding of gp120 to one of the coreceptors with subsequent insertion of the fusion peptide of the transmembrane envelope (TM) protein gp41 into the cell membrane and generation of a prehairpin conformation of gp41. This conformation is due to an interaction of the C-terminal helical region (CHR) with the N-terminal helical region (NHR) in an antiparallel manner, forming a six-helix bundle.^{5–7} The conformation of gp41 required to induce bnAbs is still unknown, although either prehairpin or six-helix bundle formation is most likely to be targeted.

Several bnAbs targeting gp41 have been isolated from infected individuals and some of them are directed against the tryptophan-rich membrane proximal external region (MPER). The bnAbs 2F5 and 4E10, binding to juxtaposed epitopes [amino acid sequences ELDKWA for 2F5 and WNWF(N/D)IT for 4E10] are the most extensively investigated. It has been shown that 2F5-like antibodies were found in about 0.3%⁸ and 4E10-like antibodies in 3% of HIV-1-infected individuals and that these antibodies appear years after infection.² Furthermore, the recently identified bnAb 10E8 was found in about 8% of infected individuals, indicating a relative high prevalence in infected individuals.⁹ Immunization studies with virosomes,¹⁰ recombinant proteins,¹¹ chimeric viruses,¹² DNA or virus-like particles,¹³ all comprising the MPER sequence, induced HIV-1 neutralizing MPER-specific antibodies, but not with broad neutralizing capacity. Possible explanations for the failure to induce such broadly neutralizing antibodies have been discussed,^{14,15} but most likely, the antigens possessing MPER epitopes were presented in a nonoptimal conformation.^{16–18}

In contrast, neutralizing antibodies have been easily induced in animals immunized with the TM protein p15E of gamma retroviruses. This was reported for the porcine

endogenous retroviruses (PERVs),^{19,20} the koala retrovirus (KoRV),²¹ and the feline leukemia virus (FeLV).^{22–24} The neutralizing potential of these antibodies has also been demonstrated *in vivo*, in immunized cats challenged with FeLV.²⁵ Epitope mapping of these neutralizing sera identified epitopes in the FPPR and in the MPER of p15E of all three gamma retroviruses. Of note, one epitope in the MPER (FEGWFN) showed partial homology to the epitope of the bnAb 4E10 (NWFNIT, identical amino acids in bold).^{19,22,26} Although a binding of 4E10 to the FPPR was previously reported,²⁷ our epitope mappings of 4E10 and 2F5 demonstrated only epitopes located in the MPER.²⁶ Structural interaction of the MPER and the FPPR has been suggested as a reason for an increased binding of 2F5 to its epitope in the presence of peptides corresponding to the FPPR.²⁶ In addition, a direct interaction of the FPPR and the MPER sequences during infection²⁸ and a stabilization of the 6-helix-bundle formation by peptides corresponding to the MPER and FPPR have been demonstrated.²⁹ These results allow speculations that an interaction of the FPPR with the MPER may be required to induce neutralizing 2F5-like and 4E10-like antibodies.

On the basis of the results mentioned above, a set of hybrid antigens was designed to direct immune responses toward HIV-1. Epitopes in p15E recognized by PERV neutralizing sera were substituted with the FPPR and MPER derived from gp41 of HIV-1, assuming that the intramolecular interaction of the p15E scaffold may induce a conformation essential for the induction of neutralizing antibodies.

The hybrid antigens were produced, purified, characterized, and used for immunization. Improvement of the binding of the induced antibodies to the 2F5 epitope was achieved by

step-by-step changes in the structure of the hybrid antigens. Despite good recognition of the epitope only two of the 21 immune sera or purified IgG tested neutralized HIV-1 weakly.

Materials and Methods

Cloning, expression, and purification of recombinant proteins

Recombinant hybrid proteins were designed by combining fragments of gp41 of HIV-1 (GenBank: K03455) and p15E of PERV (GenBank: AY953542) (Fig. 1). Codon-optimized DNA constructs of N1 were synthesized by Geneart (Regensburg, Germany) and cloned into a pQE30Xa expression vector (Qiagen, Hilden, Germany) using the restriction sites *StuI*/*Bam*HI. N2 was generated by PCR amplification using primers 5'-GAACCGATTAGCCTGACCCTGGCA-3' and 5'-TCGACTACTTACTTAAACGGTCAGTAACAG-3'. The amplicon was inserted blunt end into the pQE30Xa expression vector via the *StuI* restriction site. Primers 5'-GCAGCCGGTAGCACCATG-3' and 5'-ATACCACAGCCAATTGGTAA-3' were used to generate the amplicon for the construct N3 using pQE30Xa-N1 as the template. The amplicon was also inserted blunt end via *StuI* into the multiple cloning site. The construct pQE30Xa-N4 was cloned from pQE30Xa-N3 by truncation of 26 amino acids via polymerase chain reaction (PCR) using the primers 5'-CTGCTGCTG AACAATACCAGA-3' and 5'-AGCCTGAGCGAAGTTGTTCTG-3'.

All constructs contained N-terminally located hexahistidine tags for purification procedures and were transformed into the *Escherichia coli* expression strain SCS1/pSE111.³⁰ Transformants were grown in 2YT medium at

FIG. 1. (A) Schematic presentation of p15E, gp41, and the hybrid antigens N1, N2, N3, and N4 containing sequences of gp41 of HIV-1 (GenBank: K03455) and p15E of porcine endogenous retrovirus (PERV)-A (GenBank: AY953542). White squares indicate sequences of gp41 of HIV-1; light gray squares represent sequences of p15E of PERV-A. The fusion peptide proximal region (FPPR) and membrane proximal external region (MPER) domains are derived from gp41, substituting analogue domains in p15E. E1 and E2 located in the FPPR or MPER, respectively, of p15E correspond to epitopes recognized by neutralizing sera after immunization with recombinant p15E. N1 and N2 contain the fusion peptide (FP), the membrane spanning domain (MSD), and a short intracytoplasmic tail from gp41 (N1) or p15E (N2). Construct N3 was designed by removing the FPPR and MPER flanking regions to prevent potential masking of antibody binding sites. N4 contained a 26 aa truncated p15E linker between FPPR and MPER. (B) Sequences of the gp41-derived domains from the FPPR and MPER regions introduced into the p15E backbone.

37°C containing 100 µg/ml ampicillin and protein expression was induced with 1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) for 3 h. Bacteria expressing recombinant proteins were subsequently harvested by centrifugation at $13,000 \times g/4^\circ\text{C}$ for 20 min.

The recombinant proteins N1 and N2 were insoluble and thus were not purified by affinity chromatography. Cell pellets from expression cultures were resuspended in 6 M guanidine hydrochloride buffer (6 M guanidine hydrochloride, 100 mM NaH_2PO_4 , 10 mM Tris-Cl, pH 8.0) and subsequently sonicated (three pulses of 60 s, break 60 s) using a Branson Sonifier II 250 (Danbury, CT), followed by centrifugation ($0.5 \text{ h}/13,000 \times g$ at room temperature). This procedure was repeated five times: the supernatants were collected and analyzed by western blot. The fourth and fifth supernatant fractions containing purified proteins N1 or N2 were dialyzed against double distilled water (ddH_2O) and used for immunization. N3 and N4 were purified using affinity chromatography.

Pelleted bacteria expressing N3 or N4 were resuspended in 8 M urea buffer (8 M urea, 100 mM NaH_2PO_4 , 10 mM Tris-HCl, pH 8.0) and disrupted by sonication as described above. Crude lysates were centrifuged ($1 \text{ h}/13,000 \times g$ at room temperature), supernatants were added to Ni-NTA agarose (Qiagen, Hilden, Germany), and eluates containing purified protein were dialyzed against ddH_2O and used for immunization. The histidine-tagged recombinant ectodomain of gp41 (aa 533–681, GenBank: AF324493) was expressed and purified under denaturing conditions as described for N3 and used for the analysis of anti-gp41 responses in the immune sera. The recombinant protein p15E (rp15E; GenBank: HQ688786; aa 488–596) was expressed and purified as previously described.²⁰ The recombinant CHR of gp41 (gp41-CHR), which was used for the isolation of CHR-specific antibodies (HIV-1; GenBank: K03455, aa 605–681) was produced as a GST fusion protein. Pelleted bacteria of 1 liter BL21 (DE3) expression culture expressing the recombinant CHR of gp41 were resuspended in 50 ml phosphate buffered saline (PBS)/1% glycerine/10 mM dithiothreitol, sonicated as described above, and centrifuged for 20 min at $25,000 \times g/4^\circ\text{C}$. Then, the supernatant was applied to the glutathion Sepharose 4B (GE Healthcare, Uppsala, Sweden) and incubated on a rotating shaker at 4°C for 2 h. After five subsequent washing steps with PBS/1% glycerine, the recombinant protein was eluted in 2 ml elution buffer fractions (50 mM Tris-HCl, 10 mM reduced glutathione, pH=8). One liter expression culture yielded 5 mg of purified protein, which was dialyzed against PBS.

PAGE and western blot analysis

Recombinant hybrid proteins N1, N2, N3, and N4 were characterized by SDS-PAGE and western blot analysis using the human monoclonal antibodies (mAbs) 2F5 and 4E10 for detection. Binding properties of antibodies in the immune sera were analyzed by western blot as described in the Supplementary Information (Supplementary Information S1; Supplementary Data are available online at www.liebertpub.com/aid) using 0.5 µg/lane of rgp41 or rp15E as antigens.

Surface plasmon resonance analysis

Apparent K_d values of 50% binding equilibrium of mAbs 2F5 and 4E10 binding to the N3 and N4 antigen were esti-

mated using a BIAcore X100 (GE Healthcare, Waukesha, WI). A sensor chip CM5 containing a carboxymethylated dextran matrix was coated with 4,000 RU of N3 on flow cell 2 (Fc2) and flow cell 1 (Fc1) was left uncoated as control. Affinity determination by steady-state analysis was carried out in $1 \times \text{HBS-EP}^+$ running buffer containing 10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.05% surfactant P20, pH 7.4 at a constant temperature of 25°C . To determine apparent K_d values, mAbs 2F5 and 4E10 were diluted in running buffer and tested by injecting 50 µl (600 s, flow rate 5 µl/min) of diluted protein solution at concentrations ranging from 113 ng/ml to 1.13 ng/ml for 2F5 and 119.4 µg/ml to 0.1194 µg/ml for 4E10. The apparent affinity of 2F5 to the hybrid protein N4 was measured at concentrations ranging from 22.6 µg/ml to 0.226 µg/ml. 4E10 was tested at equivalent concentrations as used for affinity testing to N3. The chips were regenerated for 60 s with 50 mM glycine/HCl, pH 1.5, and results were evaluated with the Biacore X100 software 1.0.

Animals, immunization schedule, and antibody isolation

Guinea pigs (Dunkin-Harley strain), Wistar rats (both Charles River, Sulzfeld, Germany), and one goat were immunized using a 3 week immunization schedule and pre-immune sera were collected before immunization (Fig. 1A). Guinea pigs and rats were immunized subcutaneously (sc) with 250 µg of recombinant proteins mixed 1:1 with complete Freund's adjuvant (Thermo Scientific, Bonn, Germany). Three booster immunizations of rats and guinea pigs were performed with 250 µg of antigen mixed 1:1 with incomplete Freund's adjuvant (Thermo Scientific, Bonn, Germany). The goat received 500 µg N4 with complete Freund's adjuvant as initial immunization, injected intramuscularly (im) and sc, followed by two booster immunizations with 500 µg N4 in incomplete Freund's adjuvant.

Total IgG was isolated from serum using Protein G HP Spin Trap (GE Healthcare, Munich, Germany) according to the manufacturer's protocol. Briefly, decomplexed serum was diluted 1:4 with resin binding buffer (20 mM sodium phosphate, pH 7.0) and incubated with Protein G Sepharose for 15 min. Four washing steps with binding buffer were followed by two elution steps using 200 µl elution buffer (0.1 M glycine-HCl, pH 2.7). Elution fractions were immediately neutralized with 1 M Tris-HCl, pH 9.0, pooled and dialyzed against phosphate-buffered saline (PBS) for 48 h at 4°C to allow refolding of the purified antibodies. Isolated IgG (2–5 mg/ml) was diluted to reach the IgG concentration in serum and tested in an ELISA, using rgp41 as antigen.

The gp41-CHR recombinant protein was coupled to CNBr Sepharose in order to isolate and concentrate CHR/MPER-specific antibodies from goat serum. Freeze-dried CNBr-activated 4B Sepharose 4B (0.5 g; 1.5 g gel matrix) was washed with 100 ml of 1 mM HCl and subsequently equilibrated with freshly prepared matrix coupling buffer (0.1 M NaHCO_3 , 0.5 M NaCl). Gp41-CHR protein was dialyzed against coupling buffer and concentrated by vacuum evaporation to 0.5 µg/ml. Then 20 ml of protein solution was incubated with the Sepharose overnight at 4°C on a rotation shaker to couple the protein. Unbound ligand was washed away with 20 ml of coupling buffer and unspecific binding

sites were blocked with blocking buffer (0.1 M Tris-HCl buffer, pH=8) for 2 h. The protein-coupled Sepharose was then washed three times with alternating pH (0.1 M NaHCO₃, 0.5 M NaCl, pH=8, and 0.1 M NaAc, 0.5 M NaCl, pH=4), applied to the column and subsequently washed with PBS. Goat serum obtained 3 and 8 weeks after the third immunization was diluted 1:3 with PBS and applied to the column, circulating overnight at 4°C. The next day, the column was washed with PBS and CHR/MPER binding antibodies were eluted in 1.5-ml fractions with elution buffer (0.1 M glycine-HCl, pH 2.7) and immediately neutralized with 1 M Tris-HCl, pH 9.0. Eluted fractions were analyzed by SDS-PAGE and fractions containing high antibody concentration were pooled and dialyzed against PBS for 48 h. Antibody concentration was determined by a NanoDrop 1000 spectrophotometer at 280 nm (Thermo Scientific, Bonn, Germany). CHR/MPER-specific antibodies were concentrated 2-fold compared to serum concentrations, which was used for neutralization assays.

ELISA and epitope mapping

ELISAs using rgp41, peptides corresponding to FPPR or MPER, cardiolipin, or sphingomyelin were performed as described in the Supplementary Information. Two methods were used to map epitopes of the antibodies induced by immunization, utilizing peptides corresponding to the ectodomain of gp41, spotted on microarray glass slides or linked to nitrocellulose membrane as described in the Supplementary Information.

Virus neutralization assays

Neutralizing activity of the sera or the purified IgG antibodies was measured in a 96-well format using reporter TZM-bl cells³¹ or lymphocyte [peripheral blood mononuclear cells (PBMCs) or C8166 cell]-based assays and HIV-1NL4-3 (clade B, GenBank: AF324493), measuring relative luciferase units (RLU) or estimating the inhibition of provirus integration using a duplex real-time PCR.

In the TZM-bl cell-based assay 2×10^4 cells were seeded per well and grown for 24 h. Sera or purified IgG were used at concentrations comparable to those in serum, diluted 1:20 in Dulbecco's modified Eagle's media, followed by four steps of 2-fold serial dilution. Then 25 μ l of virus (infectious titer 5×10^5 /ml) and 25 μ l serum/IgG dilution were preincubated for 30 min at 37°C and subsequently transferred to the cells. After incubation for 48 h at 37°C, neutralization activity was estimated using the Bright-Glo Luciferase Assay System (Promega) and assessed with a GloMax-96 luminometer (Promega, Mannheim, Germany). The cut-off for neutralization was set at 50% relative luminescence units (RLU) of the value measured using virus without serum as control. Sera that recognized the 2F5 epitope as shown by the epitope mappings were also tested in a neutralization assay based on the measurement of the reduction of provirus integration taken as a marker for neutralization.^{26,32}

Briefly, 10 μ l serum dilution was mixed with 90 μ l virus (MOI=1) and incubated for 30 min/37°C. C8166 cells or phytohemagglutinin (PHA) (5 μ g/ml)-stimulated PBMCs (5×10^4 cells in 100 μ l) were added and incubated for 65 h. Cells were pelleted and lysed as previously described³³ and provirus integration was assessed by duplex real-time PCR using an HIV-1_{NL4-3}-specific probe and primers (Table 1).

TABLE 1. PRIMERS AND PROBES USED FOR THE DUPLEX REAL-TIME POLYMERASE CHAIN REACTION

Primers/probes	Sequence (5'-3')
HIV-1 probe	FAM-tgacgctgacggtacaggccagac-BHQI
68i-spec-fwd	ggagcagcaggaagcactatgg
69i-spec-rev	ccccagactgtgagttgaaca
GAPDH probe	HEX-ttcaccaccatggagaaggctggg-BHQI
hGAPDH-fwd	ggcgatctggcgctgagtag
hGAPDH-rev	tggtccacacccatgacga

Equal load and quality of DNA were monitored by amplification of human GAPDH as a housekeeping gene (Table 1). Duplex real-time PCR was performed using MX3000P (Stratagene, La Jolla, CA) with the following cycle conditions: 95°C/10 min, 95°C/1 min, 52°C/1 min, 72°C/30 s. Delta-CT values of preimmune serum plus three times the standard deviation were taken as cut-off values and an inhibition of infection more than 50% was defined as positive neutralization.

Results

Design and characterization of the antigens

The TM protein p15E of PERV was used as scaffold to expose the MPER of gp41 of HIV-1. The epitope domains in p15E were substituted by the FPPR and MPER of gp41 of HIV-1 (Fig. 1). When the amino acid sequences of the introduced FPPR and MPER of HIV-1 were compared with the substituted PERV sequences, a sequence homology around 20% identical amino acids in the entire sequence and 50% within the 4E10 epitope was observed (Table 2). Prediction of the conformation and antigenicity of the recombinant proteins was performed using the programs Protean (Largene version 10 software, DNASTar, Madison, WI) and Phyre2 (Protein Homology/Analogy Recognition Engine V2.0). It revealed conformational and overall structural similarity with gp41 by homology modeling (Supplementary Fig. S1). Four different recombinant hybrid proteins were designed, all containing the MPER and FPPR of gp41 (Fig. 1). Hybrid antigen N1 contained the membrane spanning domain (MSD) and the fusion peptide (FP) of gp41 and N2 comprised the MSD and FP domains of PERV. N3 did not contain an MSD or FP and N4 is characterized by a truncated p15E linker in between the MPER and FPPR of gp41 of HIV-1.

The fusion proteins were expressed and purified to 95% purity under denaturing conditions (Fig. 2). Further analysis of the proteins showed dimerization in a modified SDS-PAGE (using sample buffer without SDS or reducing agent) for all antigens (N1, N2, N3, and N4), as well as trimers and tetramers for N3 and N4 (Fig. 2A–C). All purified proteins were recognized by the mAbs 2F5 and 4E10 in western blot analyses (Fig. 2).

2F5 has a higher affinity to N3 and N4 than 4E10

To characterize binding of the bnAbs 2F5 and 4E10 to the recombinant hybrid proteins an SPR analysis was performed. 2F5 bound to N3 with an apparent K_d value of 0.3448 nM, showing a 100-fold higher affinity value compared to that

TABLE 2. HOMOLGY OF HIV-1 AND PORCINE ENDOGENOUS RETROVIRUS SEQUENCES SUBSTITUTED IN THE HYBRID PROTEINS

Env	Accession no.	Sequence FPPR	Identity
HIV-1 HXB2	K03455	AAGSTMGAASMTLTVQARQLLSGIVQQQ	
PERV-A	AY953542	AAGVGTGTAALITGPQQLEKGLSNLHRI	21.4%
PERV-A (E1)	AY953542	GPQQLEK	14.3%
Env	Accession no.	Sequence MPER	Identity
HIV-1 HXB2	K03455	QQEKNEQELLELDKWAS LWNWFNITNWLWYIK	
PERV-A	AY953542	LRERLERRRREREADQGWFEFEGWFNRSPWATLL	24.1%
PERV-A (E2)	AY953542	FEGWFN	50%

Identical amino acids are indicated in bold and sequences introduced in the hybrid proteins are underlined. FPPR, fusion peptide proximal region; MPER, membrane proximal external region.

FIG. 2. Characterization of the purified recombinant proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie brilliant blue G 250. Protein samples were treated with sample buffer containing SDS and 2-mercaptoethanol (red) or sample buffer without SDS or reducing agent (non-red). Arrows show recombinant proteins as monomers (MM), dimers (DM), trimers (TM), and tetramers (TTM). Western blot analyses were performed with purified recombinant proteins using the monoclonal antibodies 4E10 and 2F5. (A) Hybrid proteins N1 and N2, (B) hybrid protein N3, (C) hybrid protein N4.

measured for 4E10 (32.0 nM). N4 was bound by 2F5 with a 20-fold lower apparent K_d value of 18.34 nM compared to the affinity for 4E10 (344.8 nM). These results revealed a significantly stronger binding of 2F5 to both hybrid antigens compared to that observed for 4E10.

Immune responses induced by immunizations with hybrid antigens

Since preimmune sera of rats may contain preformed antibodies against the p15E backbone as previously observed,²⁰ which may influence the immunization, they were tested by western blot analysis using recombinant p15E of PERV. All sera were negative throughout the immunization study (Supplementary Fig. S2), indicating lack of interference. Rats and guinea pigs were immunized with N1, N2, and N3, and rats and one goat were immunized with N4 applying the immunization schedule shown in Fig. 3A. The immune sera and preimmune sera were tested by western blot analyses using recombinant gp41. All immune sera recognized gp41, whereas the pre-immune sera did not (Fig. 3B). Sera collected after the third boost were tested in addition in an ELISA using gp41 (Fig. 3C) or peptides corresponding to the FPPR or the MPER (Fig. 4). Moderate titers (between 10^4 and 10^6) of antibodies against gp41 were detected in all immune sera, which supported the western blot data with recombinant gp41.

Reactivity against an FPPR-derived peptide was observed in all antisera from animals immunized with antigens N1, N2, N3, or N4 with the exception of a single guinea pig (N3g#4) immunized with N3. In contrast, responses against the MPER were either weak (1:250) or undetectable in guinea pigs or rats immunized with N1 (Fig. 4). All sera from rats immunized with N2 reacted with the MPER peptides. Sera from rats or guinea pigs immunized with N3 showed significantly higher titers of anti-MPER antibodies compared with rats and guinea pigs immunized with N1 or N2. Importantly, immunization with N4 resulted in the highest titers of anti-MPER antibodies compared with the other hybrid antigens (Fig. 4).

Antisera from animals immunized with N3 and N4 recognized the 2F5 epitope

Epitopes recognized by the immune sera were evaluated by epitope mappings using overlapping peptides corresponding to the entire TM protein (Fig. 5A). Immunization with

FIG. 3. (A) Immunization schedule for guinea pigs and rats vaccinated with 250 μ g of recombinant hybrid proteins N1, N2, N3, and N4 mixed with complete Freund's adjuvant for initial and incomplete Freund's adjuvant for booster immunization. (B) Western blot analysis of rat immune sera derived after the third immunization boost, using recombinant gp41 as antigen in a reducing SDS-PAGE. Preimmune sera were used as negative control (–) and 2F5 as positive control (+). (C) Titers of antibodies specific for rgp41 in the sera of animals immunized four times with the hybrid proteins N1, N2, N3, and N4 as determined in an anti-gp41 ELISA.

N1 induced preferentially antibodies binding to the FPPR (Fig. 5B), confirming the ELISA result. Sera from rats immunized with N2, N3, and N4 also recognized epitopes in the N-terminal region of gp41, mainly the sequence TLTVQARQL. Surprisingly, peptides corresponding to the MPER were not recognized by sera from animals immunized with N2, although these sera reacted in an ELISA with a longer peptide comprising the complete MPER sequence (aa 657–681), suggesting an influence of the peptide length, conformation, or both on antibody binding.

The goat immunized with N4 produced antibodies that recognized recombinant gp41 in western blot analysis already after the first boost (Fig. 6A). Antisera from guinea pigs, rats, and the goat immunized with N3 or N4 reacted specifically with the epitope of 2F5 (ELDKWA) (Fig. 5). Of note, the serum from guinea pig N3g#4 immunized with N3 recognized only the ELDKWA epitope in the MPER, representing binding specificities similar to the broadly neutralizing antibody 2F5. CHR/MPER-specific antibodies isolated by affinity chromatography from the goat immune serum after the third booster immunization with N4 also bound exactly to the 2F5 epitope (Fig. 6C).

Despite recognition of the epitope sera neutralized only weakly

Immune sera were tested for neutralizing activity using a TZM-bl reporter cell assay and HIV-1_{NL4-3}. None of the sera

from the animals immunized with N1, N2, or N3 inhibited HIV-1 infection despite the fact that some of these sera bound to the same epitope as the bnAb 2F5. To minimize the influence of cytokines or endotoxins³⁴ on the outcome of the neutralization assays, IgG were isolated from the serum of immunized animals and normalized to the concentration in the serum as controlled by ELISA using recombinant gp41 as antigen. TZM-bl cell-based neutralization assays were performed with isolated IgG, but again no neutralizing activity was observed. Sera and IgG from rats and guinea pigs immunized with N3 that reacted with the 2F5 epitope were further analyzed in a neutralization test based on C8166 cells or stimulated PBMCs (Supplementary Fig. S3A and B). As in the TZM-bl cell-based neutralization assay, neither sera in different dilutions (1:20 or 1:50) nor isolated IgG were able to inhibit provirus integration to a greater extent than the corresponding preimmune serum.

Immune serum from one rat (N4r#2) collected after the third immunization with N4 (bleeding S3) inhibited HIV-1_{NL4-3} infection in the TZM-bl cell-based neutralization assay at a 1:20 dilution, which was supported by the weakly neutralizing activity of isolated IgG from these immune sera (Fig. 7A) at the same dilution. The immune serum from rat N4r#2 after the third immunization (bleeding S3, diluted 1:20) also inhibited HIV-1_{NL4-3} infection when the C8166-based neutralization assay was used, although this neutralizing activity was lost after the fourth immunization (bleeding S4) (Supplementary Fig. S3C). Furthermore, immune serum

FIG. 4. Titration of rat immune sera obtained after immunization with N1 (A, E), N2 (B, F), N3 (C, G), and N4 (D, H) in an ELISA using a peptide corresponding to the MPER region (aa 657–681) (A–D) and a peptide corresponding to the FPPR region (aa 525–552) (E–H) as antigen. PI indicates preimmune sera. STD are indicated, $n=3$. Titrations A–D and E–G were normalized using positive control sera on the ELISA plates; H was performed independently.

of the goat immunized with N4 inhibited HIV-1_{NL4-3} infection at a dilution of 1:20 (Fig. 7B). However, isolated IgG from this serum or MPER-specific antibodies showed no inhibitory effect on virus infection in the TZM-bl cell-based neutralization assay (Fig. 7B).

Antisera did not bind cardiolipin or sphingomyelin

Since MPER-specific antibodies were previously reported to exhibit lipid-binding properties,³⁵ cardiolipin and sphingomyelin were used as antigens to analyze binding of 2F5 and 4E10 in an ELISA. 4E10 showed binding to cardiolipin and to a lesser extent to sphingomyelin, but 2F5 did not bind to either of the lipids. Although only one serum was weakly neutralizing HIV-1, the immune sera of immunized animals were also tested for lipid binding. None of the immune sera derived from the immunization experiments reacted with sphingomyelin or cardiolipin, which is consistent with the fact that only binding to the 2F5 epitope was observed (Supplementary Fig. S4).

Discussion

Hybrid antigens containing the MPER and the FPPR of gp41 of HIV-1 and sequences of the TM protein p15E of PERV were used for immunization of different animal species. All antigens (N1, N2, N3, and N4) contained the MPER of gp41 and were recognized by 2F5 and 4E10. Whereas antisera of animals immunized with N1 predominantly recognized the FPPR, immunization with N2 induced responses against the FPPR and the MPER, indicating accessibility of the MPER *in vivo*. Immune sera of animals immunized with N3 and N4 reacted with the MPER-derived peptide as demonstrated by ELISA and the epitope mapping showed an exact recognition of the 2F5 epitope. Although immunization of antigens N1, N2, and N3 did not induce antibodies capable of neutralizing HIV-1_{NL4-3}, immune sera from one rat and one goat immunized with N4 weakly inhibited virus infection. Whereas neutralization by the serum of rat N4r#2 was mediated by IgG, this was surprisingly not observed with IgG isolated from the serum of rat N4r#4. The immune serum of

FIG. 5. Epitope mappings of the antisera from animals immunized with the recombinant hybrid proteins. **(A)** Representative epitope mapping on a nitrocellulose membrane of the serum from guinea pig N3g#3 immunized with N3, showing one epitope in the FPPR and the exact 2F5 epitope within the MPER (the epitopes were boxed). The reactivity of the serum with two additional peptides (numbers 17 and 18) is unspecific since this sequence was not present in the recombinant hybrid protein. **(B)** Summary of epitope mappings of guinea pig and rat immune sera. Bars represent the recognized epitopes. Sequences corresponding to the FPPR and the MPER of gp41 in the hybrid constructs are underlined. Boxed sequences indicate the epitopes of bnAbs 2F5 (ELDKWA) and 4E10 (NWNFNIT).

the goat immunized with N4 inhibited virus infection to a minor extent, but this could not be shown with purified CHR/MPER-specific antibodies. It was also not due to toxicity as shown in a cytotoxicity assay.

One explanation could be that inhibition of virus infection was due to conformational antibodies that did not bind the recombinant gp41-CHR protein used for isolation of CHR/MPER-specific antibodies. The neutralizing effect of the immune serum 3 weeks after the third immunization might also have been due to FPPR-binding antibodies or antibodies not isolated by CHR-specific antibody purification or inhibiting virus infection by mechanisms other than neutralization.

The conformation required to induce potent and broad neutralizing antibody responses at sufficient serum concentrations is still largely unknown. Since it was shown that (1) the FPPR and MPER of HIV-1 interact,^{27–29} and (2) this interaction enhances binding of 2F5 to its epitope,²⁶ we suggested that a complex conformation involving FPPR and MPER might be required for the induction of neutralizing antibodies such as 2F5. A weak neutralization was observed by the immune sera from animals immunized with N4 at serum dilutions of 1:20. It remains unclear whether such a weak neutralizing activity might be sufficient to inhibit virus infection effectively *in vivo*. We assume that a gradual improvement of the structure resulted in better recognition by the bnAb 2F5 and obviously in weak neutralization. The detectable neutralizing activity observed after the third immunization may be attributed to maturation of the antibodies, but other explanations are also possible.

A number of studies using passive immunization with high titer broadly neutralizing antibodies showed protection in macaques from intravenous^{36,37} or mucosal^{38–40} challenge with SHIV. In contrast, a study simulating a more natural route of infection using multiple vaginal low-dose exposures⁴¹ demonstrated that lower concentrations of the passively administered neutralizing antibody b12 were required to reduce virus infection compared with an intravenous challenge model. In addition, we could not exclude that the concentration of 2F5-like neutralizing antibodies in our studies was too low to be detected in our neutralization assays despite detection levels of 3.125 μ g 2F5/ml. Neutralization assays with increased sensitivity as well as concentration of the MPER-specific antibodies by affinity chromatography using suitable ligands may solve this problem.

Moreover, no antibodies binding to the 4E10 epitope were induced, although the antigens used for immunization reacted with 4E10. It may be speculated that the 4E10 binding site was not accessible *in vivo*, maybe by shielding of the epitope by the fusion peptide, at least in the constructs N1 and N2. A similar explanation was proposed previously in a DNA immunization regimen using the ectodomain of gp41 in a membrane bound context.⁴² Alignments of p15E and gp41 showed a similar length of the NHR, but the CHR comprised the double amount of amino acids in gp41 compared to p15E. There are also differences in length and amino acid composition of the cytoplasmic tail of the TM proteins that may have an impact on folding of the TM protein and contribute to the accessibility of the neutralizing epitopes.

FIG. 6. Immunological characterization of the immune sera from a goat immunized with N4. (A) Western blot analysis of goat immune sera obtained 3 weeks after each immunization (S1–S3) and 6 weeks after the third immunization (S4) and of CHR/MPER-specific antibodies from sera S3 and S4 (CHR/MPER-Ab) isolated by affinity chromatography. Sera and antibodies were diluted 1:400. (+) 2F5 diluted 1:100,000 and (–) represents preimmune serum; STD are indicated, $n=3$. (B) ELISA results of the sera diluted 1:1,000, binding to N4, rgp41, FPPR, or MPER-derived peptides after each immunization. (C) Epitope mapping of rgp41-CHR/MPER-specific antibodies purified from the serum of a goat immunized with N4 by affinity chromatography using peptide arrays. The sequence ELDKWA was identified as the core epitope. Only peptides corresponding to the N-terminal and C-terminal sequences are shown.

FIG. 7. Neutralizing activity of the immune sera. (A) TZM-bl cell-based neutralization assay of sera, isolated immunoglobulins (IgG), or MPER-specific antibodies purified by affinity chromatography (CHR/MPER-Ab) from sera from rats (A) and a goat (B) immunized with N4. The sera were taken after the third (S3) and fourth (S4) immunization of the rats. Immune serum of the goat was taken 3 (S3) and 8 (S4) weeks after the second boost. Sera were used in 2-fold dilutions from 1:20 to 1:160. IgG were used in 2-fold dilutions (corresponding to the concentration in the original serum) from 1:20. MPER-Ab were applied in 2-fold concentrations, starting from 1:20. 2F5 was used as control in concentrations of 50/12.5/3.125/0.78 µg/ml. Luciferase reporter activity is presented as RLU (relative luminescence units) and the dotted line indicates the cut-off defined as 50% of untreated virus infectivity control; STD are indicated, $n=2$.

The broadly neutralizing antibodies 2F5 and 4E10 had been reported to exhibit polyreactivity and to react with self-antigens.⁴³ Although the induction of such polyreactive antibodies during HIV-1 infection demonstrates that such antibodies can develop,¹⁷ we cannot exclude that B cells reacting with our antigens were depleted. However, the findings concerning the interaction of 2F5 with lipids were controversial.^{44–46} In this regard the antisera were tested for reactivity to lipids such as cardiolipin or sphingomyelin, also shown to be bound by polyreactive autoantibodies in HIV-1 patients.⁴⁷ None of our sera reacted with cardiolipin or with sphingomyelin. Given the fact that these immune sera recognized only the 2F5 epitope and 2F5 also did not bind both lipids tested, this result was not surprising.

In summary, the approach described here was successful in inducing antibodies recognizing the core epitope of 2F5. Immune sera derived after immunization with N4 showed a weak neutralization capacity. Further modifications of the hybrid antigens or prolonged antigen presentation may improve the presentation of the MPER conformation required to induce increased titers of potent neutralizing antibodies.

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Author Disclosure Statement

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