Interaction of the HIV-1 gp120 Viral Protein V3 Loop with Bacterial Lipopolysaccharide

A PATTERN RECOGNITION INHIBITION

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HIV-1 represents an elusive target for therapeutic compounds due to its high rate of mutation. Targeting structural patterns instead of a constantly changing specific three-dimensional structure may represent an approach that is less sensitive to viral mutations. The V3 loop of gp120 of HIV-1, which is responsible for binding of viral gp120 to CCR5 or CXCR4 coreceptors, has already been identified as an effective target for the inhibition of viral entry. The peptide derived from the V3 loop of gp120 specifically interacts with the lipid A moiety of LPS, as does the full gp120 protein. NMR analysis of V3 in complex with LPS shows formation of an amphipathic turn. The interaction between LPS and V3 relies on the structural pattern, comprising a combination of hydrophobic and charge interactions, similar to the interaction between antimicrobial peptides and LPS. LPS inhibition of viral entry involves binding of gp120 to the surface of target T cells. Non-endotoxic LPS antagonists inhibited viral infection, demonstrating the possibility for the development of an inhibitor of HIV-1 attachment to T cells based on the recognition of a conserved structural pattern.
immature response to Gram-negative bacteria. It is recognized by a complex cascade of extracellular “pattern recognition receptors,” which recognize the conserved motif of lipid A and present LPS to the lipid A-binding protein, MD-2, associated with the transmembrane receptor TLR4 (23). Recognition of various forms of LPS from different strains of Gram-negative bacteria is accomplished through the lipid A moiety, which represents the pathogen-associated molecular pattern, triggering the signaling cascade, which results in a release of pro-inflammatory mediators, such as cytokines, chemokines, and others (24). The conserved lipid A moiety is the minimal structural fragment of LPS that triggers the cellular response (25). Although there are structural differences among key extracellular LPS-sensing and -relaying receptors, lipopolysaccharide-binding protein, CD14, and MD-2, the lipid A recognition motif can be reduced to the arrangement of negatively charged groups at the defined distance with a large adjacent hydrophobic patch (24). There are many studies on the role of LPS in HIV-1 replication or inhibition. Contradictory studies may be a consequence of the activation immune system receptors, which may result in the transcriptional activation or repression of gp120 coreceptors. For example, although it has been shown that LPS inhibits HIV-1 replication in macrophages (26), several studies have also shown that LPS enhances HIV-1 replication in monocytoid cell lines (27). As we show in this study, it may also impart its effect through direct binding to gp120.

Our research was initiated by the finding that a peptide from the V3 loop of gp120 exerted antimicrobial activity against Gram-negative bacteria (28). We demonstrate that gp120 and the 15-residue V3 peptide (29) directly and specifically interact with different chemotypes of LPS. We determined the conformation of the V3 peptide in complex with LPS using high resolution NMR and docking methods. The specific binding of V3 to LPS was confirmed by fluorescence spectroscopy, by a binding assay, and by the neutralization of activation of a monocytoid cell line Mono Mac 6. We demonstrated that LPS inhibited binding of gp120 to the receptors on a target T cell line H9. Nonendotoxic LPS antagonists (tetraacylated MLK986 LPS and lipid IVa) inhibited infection by an HIV-1 pseudovirus in the U87.CD4.CCR5 cell line, indicating that nonendotoxic compounds could be potentially used in an anti-HIV therapy.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Peptide V3 (RKRHIGPGRAFYTT) was synthesized at the Keck Biotechnology Resource Laboratory, Yale University (New Haven, CT) (purity >95%). Recombinant HIV-1 envelope glycoprotein gp120 was obtained from Protein Sciences Corp. (Meriden, CT). BODIPY TR cadaverine (BC) and Alexa Fluor 488 dye were purchased from Molecular Probes (Eugene, OR). LPS from *Escherichia coli*, serotype 055: B5, was obtained from Fluka (St. Louis, MO), and synthetic lipid IVa was from Peptide Institute Inc. (Osaka, Japan). Smooth-form LPS (S-LPS) from *Salmonella abortus equi* (strain HL83), rough-form LPS (Ra-LPS) from *Salmonella enterica* (serovar Minnesota, strain R60), and deep rough mutant Re-LPS from *S. enterica* (serovar Minnesota, strain R595) were kindly provided by Dr. Klaus Brandenburg (Forschungszentrum, Borstel, Germany). LPS from *E. coli* MLK986; msbB-, htrB1—double mutant; rough-form LPS (MS analysis confirmed that this LPS contains tetraacylated LPS) was a kind gift from Dr. Ignacio Moriyon (University of Navarra, Pamplona, Spain). LPS were solubilized in water (except lipid IVa, which was solubilized in 50% dimethyl sulfoxide (DMSO) or in 2 mM HEPES, pH 7.4) by vortexing and sonication in a water bath. S-LPS, Ra-LPS, and Re-LPS were labeled with biotin as described previously (30). LPS or biotinylated LPS molecules were then subjected to three cycles of heating to 56 °C for 15 min and cooling down to 4 °C for 5 min. After that, the LPS samples were stored at least 12 h at 4 °C before use. Other chemicals and reagents were from Sigma, Invitrogen (Lofer, Austria), BioWhittaker (Walkersville, MD), or Promega (Madison, WI).

**Cell Lines**—The HEK 293T cell line was cultured in DMEM, 10% fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (The Mono Mac 6 human monocytoid cell line was a kind gift from Dr. Kathy Triantafillou (University of Sussex, Brighton, UK) and was cultured in a complete culture medium made of RPMI 1640, 10% FBS, OPI media supplement (1 ml/100 ml), nonessential amino acids (2 ml/100 ml), and penicillin/streptomycin. The Human T lymphoblastoid cell line H9 (31) was cultured in RPMI 1640, 10% FBS, and penicillin/streptomycin. The U87.CD4.CCR5 cell line (32) was cultured in DMEM, 15% FBS, 2 μg/ml puromycin, 0.3 mg/ml G418, and penicillin/streptomycin. All cells were grown at 37 °C in a 5% CO₂ environment.

**ELISA Assay for V3 and gp120 Binding to LPS**—Wells in a Nunc 96-microwell plate MaxiSorp (Langenselbold, Germany) were coated with increasing concentrations of V3 or 25 nM gp120 in 100 μl of a coating buffer (50 mM sodium carbonate buffer, pH 9.6) at 4 °C overnight. After blocking with 3% (w/v) bovine serum albumin in phosphate-buffered saline with 0.05% Tween 20 (PBST) for 1 h at room temperature and washing five times with PBST, biotinylated S-LPS (3 μM) in the case of coating with V3 or at increasing concentrations in the case of coating with gp120 was added to the wells and incubated for 2 h at 37 °C followed by washing five times with PBST and incubation with a streptavidin/peroxidase-conjugated polymer, diluted 1:1000 in PBST, for 2 h at 37 °C. Finally, after washing five times with PBST, 50 μl of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid liquid substrate (ABTS) was applied to each well, and the absorbance at 390 nm was read in a Mithras LB 940 reader (Berthold Technologies, Bad Wildbad, Germany).

**ELISA Assay for V3 Binding to LPS**—Wells in microtiter plates were coated with V3 (10 μM) or gp120 (25 nM) in 100 μl of the coating buffer at 4 °C overnight. During blocking with 3% (w/v) bovine serum albumin in PBST for 1 h at room temperature and washing five times with PBS, V3 or polymyxin B at increasing concentrations was preincubated with 3 μM biotinylated S-LPS, Ra-LPS or Re-LPS in PBST for 30 min at 37 °C. These mixtures were then added to the wells after blocking and incubated for an additional 2 h at 37 °C followed by washing and incubation with a streptavidin/peroxidase-conjugated polymer, dilution to 1:1000 in PBST for 2 h at 37 °C, and detection...
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using ABTS liquid substrate. For competitive inhibition of binding of biotinylated S-LPS to V3 peptide, the peptide (2.5 μM) was first preincubated with increasing concentrations of lipid IVa or a control lipid molecule (L-α-phosphatidylinositol) for 30 min at 37 °C in the coating buffer, and then mixtures were added into the wells in microtiter plates, and coating was performed at 4 °C overnight. After blocking with 3% (w/v) bovine serum albumin in PBST for 1 h at room temperature and washing five times with PBST, 0.75 μM biotinylated S-LPS in PBST was added to the wells and incubated for 2 h at 37 °C. After washing, the streptavidin/peroxidase-conjugated polymer, diluted 1:1500 in PBST, was added and incubated for 1 h at room temperature. Lastly, ABTS liquid substrate was used for detection, and the absorbance at 405 nm was read in a PowerWave XS reader (BioTek Instruments Inc., Winooski, VT).

Fluorescence Spectroscopy—A fluorescence displacement assay was performed on a multimode Mithras LB 940 reader. 100 μl of mixtures of S-LPS (0.5 μM) with V3 or polymyxin B at increasing concentrations in 50 mM Tris, pH 7.4, was added to the wells in microtiter plates. Fluorescence reading was performed immediately after the addition of 5 μM BC into each well. The BC excitation wavelength was 560 nm. Displacement of BC from LPS was monitored by the fluorescence emission at 611 nm at room temperature. An estimate of the percentage of free BC was calculated according to the formula \((F - F_{\text{min}}) / (F_0 - F_{\text{min}}) \times 100\), where \(F_0\) is the fluorescence intensity of BC alone, \(F_{\text{min}}\) is the intensity in the presence of S-LPS alone, and \(F\) is the intensity of the S-LPS:BC mixture at varying concentrations of V3 or polymyxin B.

Inhibition of Stimulation of Human Monocytic Cells by LPS—Mono Mac 6 cells were resuspended in the culture medium without antibiotics, and their number was adjusted to \(1 \times 10^6\) cells/ml. For stimulation, \(1 \times 10^5\) cells were transferred into each well of a 96-well culture plate (TPP Techno Plastic Products AG, Trasadingen, Switzerland). Phorbol 12-myristate 13-acetate at 50 ng/ml and V3 or polymyxin B at increasing concentrations were added to the cells and incubated for 1 h at 37 °C. Afterward S-LPS at 20 ng/ml was added to the wells. The cultures were incubated for 16–18 h at 37 °C under 5% CO₂. Supernatants were collected after centrifugation of the culture plate for 10 min at 1200 rpm and used for the immunological determination of TNFα in a sandwich ELISA using a human TNFα ELISA kit (ImmunoTools, Friesoythe, Germany).

NMR Spectroscopy—NMR spectra were obtained in 95% H₂O, 5% D₂O solution at 298 K and peptide concentrations of 1–2 mM. All experiments were performed at pH 4–5. The concentrations of LPS from E. coli, serotype O55:B5, are given in mass per volume because of the heterogeneity of the polysaccharide outer core. The assignment of 1H resonances was performed using standard total correlation spectroscopy (mixing times 10 and 70 ms) and two-dimensional NOE experiments (NOESY and rotating frame Overhauser effect spectroscopy (ROESY), mixing times 80–200 ms) on a Varian Inova 600 spectrometer (Palo Alto, CA) and a Bruker DMX 600 spectrometer (Billerica, MA). Water signal suppression was achieved using presaturation or WATERGATE. Two-dimensional NOESY experiments with mixing times of 80–150 ms were carried out using mixtures of V3 and LPS that correspond to 10:1 w/w ratios of both components. A NOESY experiment with a mixing time of 80 ms was used for structure determination. The spin systems in the total correlation spectroscopy NMR spectra of free V3 in aqueous solution are fairly well resolved at 25 °C and allow complete assignment of proton resonances using \(d_{\alpha}(i, i+1)\) and \(d_{\beta}(i, i+1)\) connectivities in the NOESY spectra (33).

Computational Methods—100 different structures of V3 were calculated using the torsion angle dynamics program DYANA (34); 10 structures selected according to lowest target function were refined in a solvent box with explicit water molecules using the program CNS (35, 36). The coordinates of the lipid A portion of LPS from (37) (including two 3-deoxy-d-manno-2-octulosonate residues) and the V3 coordinates of five structures with the lowest target function from NMR refinement were used for molecular docking calculations using the program AutoDock (38). The peptide backbone was kept rigid using the data from the transferred NOE experiments, whereas all side chains were defined as flexible using the deftors module; lipid A was treated as the macromolecule part of the docking calculation and was kept rigid. The AutoGrid calculation was run with 100 points (separated by 0.325 Å) in each spatial dimension, with the grid centered at the H2 atom of the GlcN II residue of the lipid A moiety. 20 structures were produced for each starting structure using the AutoDock module (for details, see Ref. 39); the top 10 generated docked structures were evaluated in the analysis step.

Flow Cytometric Binding Assay—The inhibition of binding of gp120 to the receptors on the T cell line H9 by LPS was measured by flow cytometry. First, a recombinant gp120 was labeled with Alexa Fluor 488 dye using an Alexa Fluor® 488 protein labeling kit following the kit instructions. A sample containing gp120-Alexa Fluor 488 (10 nM) was preincubated in PBS with Re-LPS at a concentration of 1 μg/ml in a total volume of 20 μl for 30 min at 37 °C. H9 cells (5 × 10⁶) were resuspended in 180 μl of RPMI and then incubated together with preincubated gp120:LPS mixtures for 30 min at 37 °C in the dark. Afterward unbound gp120 was removed by two washes in PBS. Afterward the cells were resuspended in 500 μl of PBS followed by flow cytometric analysis using a Coulter EPICS Altra flow cytometer (Beckman-Coulter Electronics, Luton, UK). Alexa Fluor 488 was excited using a 488 argon ion laser and detected using a 525-nm emission filter. 10,000 events were acquired for each sample. Dead cells were excluded by appropriate gating.

HIV-1 Pseudovirus Preparation and Neutralization of Pseudovirus Infection—Pseudoviruses were prepared by transfecting HEK 293T with 10 μg of env-deficient HIV-1 backbone vector pNL4-3.Luc.R-E- (40) and 10 μg of AC10.0, clone 29 (SVPB13), an env/rev expression vector (41), using Lipofectamine 2000, as recommended by the manufacturer. Pseudovirus-containing medium was harvested 3 days after the transfection and was filtered through a 0.45-μm filter (Millipore, Billerica, MA), concentrated using a 100-kDa Vivaspin20 (Sartorius Vivascience, Aubagne, France) and stored at −80 °C. The titer of pseudotyped viruses was measured using an ELISA assay kit for the p24 antigen (PerkinElmer Life Sciences). A day before infection, U87.CD4.CCR5 cells (1 × 10⁵/well) were seeded in a 96-well culture plate. 80 pg/well of p24 gag equiv-
lent pseudoviruses were preincubated with MLK986 LPS (2 μg/ml), lipid IVa (0.1 μg/ml), or medium for 90 min at 37 °C. The cells were incubated with pseudoviruses for an additional 36 h. Cells were lysed with a passive lysis buffer, and the luciferase activity was measured on a Mithras LB940 luminometer. The results are represented as mean values, and the statistical significance was calculated using the Student’s t test.

RESULTS

V3 Peptide Binds to LPS—Based on the study on the antimicrobial activity of V3-based peptide 6286 (28), we decided to investigate whether this peptide could bind to bacterial LPS as this could be the basis of its antibacterial activity. Indeed we demonstrated a concentration-dependent binding of S-LPS to the immobilized V3 peptide (Fig. 1A). The V3 peptide was able to displace different chemotypes of LPS differing in the size of their inner core or O-antigen from the immobilized V3 (Fig. 1C). Lipid A is the central and essential moiety responsible for the biological activity of LPS; therefore we tested whether V3 binds to LPS through its lipid A moiety. For this purpose, we used polymyxin B, a cyclic polycationic lipopeptide antibiotic, which binds to lipid A with high affinity and is considered as the “gold standard” for LPS-sequestering agents (42). Polymyxin B displaced different chemotypes of LPS from the immobilized V3 (Fig. 1D), which confirmed the specific binding of V3 to lipid A of LPS. The strongest binding was observed with Re-LPS, which is the smallest LPS molecule (contains only lipid A and the core without any O-antigen), and the weakest was observed with the S-LPS, which contains the largest carbohydrate chain. Specific binding of V3 to lipid A was also demonstrated using a fluorescent displacement assay with BC. BC binds to lipid A and can be displaced by compounds displaying an affinity for lipid A (42). We showed similar concentration-dependent competition with BC for S-LPS binding by V3 or polymyxin B (Fig. 1B). The addition of V3 also neutralized the biological activity of LPS by sequestering it from the cellular receptors as the V3 peptide inhibited LPS stimulation of the signaling pathway in Mono Mac 6 cells, as determined by the amount of secreted cytokine TNFα (Fig. 1E). Inhibition was nevertheless weaker than inhibition by polymyxin B, which is a high affinity LPS sequestering agent. The V3 peptide also inhibited biological activity of the LPS in the limulus amebocyte lysate assay (data not shown).

Conformation of the V3 Peptide in Complex with LPS—The V3 peptide has no intrinsic tertiary structure and adopts a defined conformation only in complex with antibodies or in the presence of the structure-promoting agent trifluoroethanol (for a review, see Ref. 43). The NOE (Fig. 2A, left) and ROE (data not shown) patterns of the free peptide contain only intraresidual and sequential connectivities and are indicative of a highly flexible molecule. The addition of LPS at one-tenth of the peptide amount (w/w) leads to a large increase in the number and intensity of the signals in the NOESY spectra due to the
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transferred NOE effect (44) (Fig. 2B, right). This type of experiment detects the conformation of a small ligand (the V3 peptide, in this case) bound to a larger receptor (LPS or LPS aggregates, in this case), where the ligand is in exchange between the free and bound state. As can be seen in Fig. 2A (right), the addition of LPS results in structuring of the peptide as it forms a complex with LPS. Long range NOE connectivities involving the peptide residues Ile$^4$ and Phe$^{12}$ appeared upon the addition of LPS (from Ile$^{4}$-H$^{61}$ to Phe$^{12}$-H$^{6}$ and Phe$^{12}$-H$^{5}$) that were not observed in the spectra of the free peptide (see supplemental Figs. S1 and S2). The increased number of NOE correlations was used for NMR structure refinement using, in total, 65 meaningful distance restraints. The observation of the transferred NOE is proof that the ligand-receptor system is in the kinetic regime of fast averaging of NOEs that occurs in conditions of relatively low affinity (dissociation constant $K_D = >100$ mM) and submillisecond lifetimes of the free and bound state of the ligand. Only one averaged set of NOE signals is observed for the ligand, with the contributions from the free state being almost negligible because of the proximity of the molecular orientation correlation times $t_c$ to the inverse of the $^1$H NMR resonance frequency, i.e. the zero-NOE region. For this reason, a 10–100-fold excess of ligand can be used.

The 10 best structures of V3 with the lowest target function after the energy minimization protocol after the energy minimization protocol displayed a family of well defined conformations comprising residues 4–12 (root mean square deviation superposition at the polypeptide backbone atoms 2.1 ± 0.7 Å; Fig. 2B). None of the residues was found in the forbidden regions of the Ramachandran plot (see supplemental Fig. S3); however, the proportion of residues in the most favored regions of the Ramachandran plot was low when compared with conventional protein structures; thus the structures from the NMR ensemble were never used to draw detailed conclusions on local bond geometries. A representative structure is shown in Fig. 2C. The backbone forms an almost planar hairpin-like structure surrounding a hydrophobic core consisting of Ile$^4$, Pro$^8$, Phe$^{12}$, and Tyr$^{13}$, making the structure amphipathic. The structure of V3 induced by the interaction with LPS does not form a regular β-turn at Gly-Pro residues; a bend, however, does occur in these residues, forming a loop that is indicated by NOE contacts between the side chains of Ile$^4$ and Phe$^{12}$.

In molecular docking calculations, five peptide structures were docked to LPS, each producing 20 docked structures of which 10 with a low final docked energy were analyzed. Not all structures converged to the same binding mode; however, for all five peptide structures, the ones with the lowest final docked energy showed the same binding mode (Fig. 3). Residues Arg$^1$, Arg$^9$ and Arg$^{10}$ (corresponding to Arg$^{309}$–Arg$^{311}$ and Arg$^{318}$, respectively, in the full gp120 protein of HIV-1 MN isolate (29)) of V3 are involved in electrostatic contacts with the phosphate groups of GlcN II and GlcN I of LPS, respectively, whereas Ile$^4$, Pro$^8$, Phe$^{12}$, and Tyr$^{13}$ (corresponding to Ile$^{312}$, Pro$^{316}$, Phe$^{320}$, and Tyr$^{321}$ of gp120) are involved in hydrophobic contacts with the acyl chains of LPS. The two C-terminal Thr residues do not show a consistent pattern of interaction with LPS. The V3 docked structure is significantly different from the structure of the V3 loop in the gp120 core in complex with CD4 and antibodies (for both, backbone root mean square deviation was 4.04 Å) (4, 11) and also from the structure of the V3 peptide when complexed to the human monoclonal antibody 447-52D (12) (backbone root mean square deviation 3.69 Å).

gp120 Binds to LPS—Based on the results of the binding studies with the V3 peptide, we anticipated that LPS would bind to the integral gp120 protein. The binding assay demonstrated a concentration-dependent binding of LPS to gp120 (Fig. 4A). V3 displaced S-LPS, as well as the Ra- and Re-LPS, from the immobilized gp120, which indicates that V3 comprises the LPS binding site of gp120 (Fig. 4B). Re-LPS exhibited the strongest competition for binding to gp120, whereas S- and Ra-LPS have approximately the same potency. As polymyxin B also displaced LPS from the immobilized gp120 (Fig. 4C), we concluded that not only V3 but also gp120 binds LPS through its lipid A portion. Polymyxin B outcompeted Ra-LPS from gp120 by...
better than S-LPS, indicating some differences in the binding specificity between the V3 peptide and polymyxin B.

Inhibition of Binding and Viral Attachment to Cells by LPS—Binding of LPS to gp120 would be expected to interfere with the biological role of gp120 in the process of attachment of virus to cells. gp120 is essential for binding to the cellular surface receptors. We investigated whether LPS interferes with gp120 binding to CD4+ T cells using flow cytometry. We used the T cell line H9, selected for its high yield permissive growth with HIV-1 (31). We first preincubated gp120 and LPS and afterward added them to the H9 cells to achieve masking of the V3 loop in gp120 from coreceptors. We demonstrated that Re-LPS inhibited binding of fluorescently labeled gp120 to the surface of the H9 cells (Fig. 5A) similarly to anti-gp120 monoclonal antibodies 257-D IV or 268-D IV (45), which react with the HIV-1MN V3 epitope KRIH or HIGPGR (data not shown).

As LPS was able to inhibit binding of gp120 to the surface of the H9 cells, the next step was to show whether binding of LPS to gp120 expressed on the surface of HIV-1 pseudoviruses could prevent the infection. Using binding assays and NMR, we clearly demonstrated that LPS specifically interacts with the V3 peptide based on 15 amino acid residues of the V3 loop in X4 strain MN (Table 1) and with the recombinant HIV-1MN gp120. To demonstrate that the observed LPS binding pattern is present not only in the V3 region in X4 strains but also in the V3 region in R5 strains, we prepared macrophage-tropic HIV-1 pseudovirus for pseudoviral neutralization experiments. It was prepared by transfecting exponentially dividing 293T cells with env/rev expression vector AC10.0, clone 29 (SVPB13), and env-deficient HIV-1 backbone vector pNL4-3.Luc.R-E-. The deduced amino acid sequence in the V3 region from AC10.0.29 differs from the sequence in our MN V3 peptide by only one amino acid (Gly instead of Arg3, corresponding to Arg311 in the full gp120 protein). The amino acid sequence of the whole V3 loop in R5 strain AC10.0.29 is otherwise very similar to the sequence of the V3 loop in X4 strain MN (Table 1). LPS has strong immunostimulating activity, activating cells at picomolar concentrations, and is, as such, not appropriate as a thera-

FIGURE 3. Stereoview of the calculated complex of V3 peptide (thin sticks, in front) and LPS (thick sticks, in the back).

FIGURE 4. Binding of gp120 to LPS. A, concentration-dependent binding of biotinylated S-LPS to immobilized 25 nM gp120 in wells in the microtiter plate. B and C, competitive inhibition of binding of biotinylated LPS to immobilized gp120 by V3 and polymyxin B. V3 or polymyxin B was preincubated with 3 μM of different biotinylated LPS molecules and transferred to each well, coated by 25 nM gp120. Inhibition of gp120 binding was performed with biotinylated S-LPS (■), Ra-LPS (○), and Re-LPS (▼). The average results of three independent experiments are shown.
peutic neutralizer of viral attachment to cells or in the organization. Lipid A antagonists, structurally similar to the endotoxic molecule but differing in the acylation of the diglucosamine moiety of lipid A, could still retain the binding affinity without activating an immune response. Therefore we first determined whether the tetraacylated lipid A (lipid IVa) was able to bind to V3. Tetraacyl lipid A is endotoxically inactive in humans and antagonizes active LPS at multiple sites in the LPS recognition pathway (46). We confirmed that lipid IVa binds to the V3 peptide as it inhibited binding of biotinylated S-LPS to the immobilized V3 peptide. On the contrary, L-\(\text{H9251}\)-phosphatidylinositol, a control lipid molecule, showed no inhibitory activity, demonstrating a specific effect of lipid IVa (Fig. 5B). For demonstration of inhibitory effect of LPS antagonists on HIV-1 pseudoviral infection, we used two nonendotoxic LPS antagonists: synthetic lipid IVa and MLK986 LPS, an LPS from \(\text{E. coli}\) MLK986 containing tetraacylated LPS (47). The MLK986 LPS did not activate human monocytes (data not shown). Pseudoviruses were preincubated with medium, MLK986 LPS, or lipid IVa (0.1 \(\mu\text{g/ml}\)), or medium. Complexes were added to the U87.CD4.CCR5 cells and incubated for 36 h. Cells were lysed, and luciferase activity was measured. R.L.U., relative luciferase units; n.c., noninfected cells. The significance of differences was assessed by the Student’s \(t\) test; **, \(p < 0.05\), ****, \(p < 0.005\).

TABLE 1

| HIV-1 strain | Coreceptor use | Amino acid sequence alignment* |
|--------------|----------------|--------------------------------|
| MN           | X4             | CTRPHYNKMKIKHGGRAFTTVKIIGTIRQAHC |
| AC10.0.29    | R5             | C1RPHYNKMKIKHGGRAFTTVKIIGTIRQAHC |

* Amino acid residues corresponding to the region of V3 peptide from HIV-1 MN isolate are in bold.

FIGURE 5. Binding of the V3 peptide to lipid IVa and inhibition of gp120 and HIV-1 pseudovirus binding to target cells. A, inhibition of gp120 binding to H9 cells with Re-LPS. H9 cells were exposed to 10 \(\mu\text{M}\) gp120-Alexa Fluor 488, preincubated in PBS with Re-LPS at a concentration of 1 \(\mu\text{g/ml}\). The mean fluorescence intensity reflecting gp120-Alexa Fluor 488 binding to receptors on the surface of H9 cells was measured. The gp120-treated cells alone (positive control) are represented by a straight line; the LPS-treated group is represented by a dashed line; and the PBS-treated cells (negative control) are represented by a dotted line. These are the data from one of three representative experiments. B, competitive inhibition of the binding of biotinylated S-LPS to immobilized V3 by lipid IVa. V3 or polymyxin B (PMB) (2.5 \(\mu\text{M}\)) were first preincubated with increasing concentrations of lipid IVa or \(\text{L-\alpha-phosphatidylinositol (PI)}\); afterward mixtures were added into the wells in microtiter plates, and 0.75 \(\mu\text{M}\) biotinylated S-LPS was added. The average results of three independent experiments are shown. C, inhibition of HIV-1 pseudovirus infection. 80 pg of p24 gag equivalent of pseudotyped viruses was preincubated with MLK986 LPS (2 \(\mu\text{g/ml}\)), lipid IVa (0.1 \(\mu\text{g/ml}\)), or medium. Complexes were added to the U87.CD4.CCR5 cells and incubated for 36 h. Cells were lysed, and luciferase activity was measured. R.L.U., relative luciferase units; n.c., noninfected cells. The significance of differences was assessed by the Student’s \(t\) test; **, \(p < 0.05\), ****, \(p < 0.005\).
IVa) decreased the expression of luciferase, showing the inhibition of pseudoviral infection (Fig. 5C).

**DISCUSSION**

One of the strategies for the treatment of AIDS is the prevention of the interaction of HIV-1 with target cells and its entry into these cells. The HIV-1 envelope protein gp120 protein is weakly immunogenic, and only a few antibodies are able to afford protection. Antibodies against gp120 targeting the V3 loop are effective against individual HIV-1 strains, but fast viral mutations can attenuate their affinity and therapeutic efficiency. Despite variability, certain structural features of the V3 peptide have to be conserved to maintain viral function. Recognition of this structural pattern comprising the conserved amphipathic arrangement of residues in the V3 loop and the tip of the V3 loop, containing the GPG sequence (48), could be used to avoid the mutational variability.

LPS, or lipid A as its active component, might therefore be viewed as a type of inhibitor that recognizes conserved structural features, regardless of the changes that might otherwise decrease binding of antibodies. To identify the candidates for the inhibition of gp120 binding to T cells, we tested different chemotypes of LPS and nonendotoxic LPS antagonists and determined specific interactions of the V3 peptide with LPS (Fig. 1, A–D) as well with LPS antagonist lipid IVa (Fig. 5B). ELISA assays demonstrated that integral gp120 protein also interacts with LPS (Fig. 4A), where the V3 loop represents the lipid A binding site (Fig. 4C). Among the different LPS chemotypes, only rough Re-LPS exhibited full inhibition of LPS binding to V3 peptide. It has been reported previously that the V3 loop of different HIV-1 isolates interacts with different polyanionic compounds, such as sulfated polysaccharides (dextran sulfate, heparin, heparin sulfate, heparan sulfate, and others) (49–51) or ceramide and related glycolipids (52). Concentrations of these polyanions in experiments were in the similar concentration range as LPS in our binding assays.

As determined by NMR spectroscopy, the conformation of the positively charged V3 peptide adapts to the amphipathic LPS molecule and displays two separated clusters of positively charged residues Arg1-Lys2-Arg3 and Arg10 that interact with the LPS molecule and displays two separated clusters of positively charged V3 peptide adapts to the amphipathic LPS in our binding assays. In the past studies on the V3 loop structure in complex with anti-gp120 antibodies, authors have focused on the specific conformation of the loop rather than on the structural signature that is required for binding to the chemokine coreceptor. Sharon et al. (17) report that the N-terminal strand and four residues from the C-terminal strand contribute to almost all the interactions between the V3 loop and the 447 Fv antibody, whereas in Stanfield et al. (12), sequence specificity is conferred through interaction of the type-II turn at the apex of the V3 hairpin. Another study using an 18-residue HIV-1 V3 peptide in complex with a fragment of an anti-gp120 antibody revealed a β-turn comprising residues RGPG at the center of the β-hairpin (18). The central glycine and proline residues of this turn were found to be linked by a cis-peptide bond; however, we found no evidence for a cis-peptide bond in the present study. In the same study, the residues of the turn interact extensively with the antibody (18); the same is also true for the docked structure of the V3 peptide with LPS. The flexibility of the V3 peptide framework seems to be essential for the induced fit of the peptide tertiary structure observed with the different binding partners, as was also observed with the V3 peptide complexed to antibody 447-52D (12). The structure of V3 in the complex with LPS is different from the structures of V3 peptides when bound to antibodies. The latter bind the V3 peptides from three sides (see e.g. Ref. 20), engaging hydrophobic as well as hydrophilic residues; LPS binds the V3 peptide from one side only, inducing a structure with a large hydrophobic core flanked by two strongly basic regions.

The structural model of the complex between V3 and LPS, where the lipid A moiety is the main contact site (Fig. 3), confirmed the results of our binding assays. This model is important for the identification of intramolecular interactions that could be applied for the selection or design of new compounds based on the lipid A structural motif that binds to the V3 loop of gp120 with high potency. The less conserved residue in our V3 peptide that interacts with LPS is Arg3, whereas all other residues in V3 that interact with LPS belong to highly conserved residues of the V3 loop. The Pro8 in the V3 peptide (residue from GPG sequence at the top of the loop, which is the most conserved part of the V3 loop (48)) also interacts with LPS. Apart from removal of both separated clusters of cationic residues (Arg1-Arg2 and Arg10) that would probably be deleterious to LPS binding, some point mutations in the conserved hydrophobic core of the molecule (e.g. Phe12 or Tyr13 → Trp, Ala11 → Val or Leu, etc.) could favorably modulate the hydrophobic intermolecular interactions. It is important to note, however, that mutations that significantly affect binding of V3 to lipid A would probably also affect binding to the coreceptors and viral entry.

The NMR experiments were carried out at pH 4–5. In the LPS-bound structure of V3, the His5 residue does not participate in the interaction with LPS and is clearly exposed to the solvent; its protonation state is thus unlikely to have any effect on the LPS-bound structure as His5 does not compete with Lys2 and Arg1 for interaction with the phosphate group of LPS. We thus believe that the bound structure at lower pH accurately represents the conformation under the physiological pH.
Interaction between LPS and gp120 inhibited the interaction of gp120 with receptors on the surface of target T cells. Similarly to anti-gp120 monoclonal antibodies, which react with V3 epitope KRIH or HIGPGR, Re-LPS (and S-LPS, data not shown) also inhibited binding of gp120 to the receptors at the surface of T cell line H9. We preincubated gp120 and LPS before adding to the cells. gp120 otherwise first interacts with CD4 on the cell surface, and the V3 loop interacts with a coreceptor (3–5). A shift in the flow cytometric analysis of gp120 binding to the surface of H9 cells (Fig. 5A) demonstrated that LPS hinders binding of gp120 to the cells. Therefore we propose that LPS masks the docking site (i.e. the V3 loop) of gp120 for the coreceptor. Similarly, another negatively charged molecule sulfated polymannuroguluronate (SPMG), a sulfated polysaccharide from marine algae, inhibited gp120 binding to CD4+ T lymphocytes at a similar concentration as LPS in our experiment (55), and ceramide analogs inhibited HIV-1 entry into CD4+ and CD4− cells through masking of the V3 loop (52).

As we used V3 peptide based on the sequence of X4 strain MN, we examined the biological activity of HIV-1 isolates with different and especially less charged V3 loops, which typically characterize the R5 HIV-1 strains. There are two separate clusters of positively charged residues in our V3 peptide involved in the electrostatic contacts with lipid A (Figs. 2C and 3). According to our model, we estimated that the absence of one arginine residue in the first cationic cluster, which consists of three basic residues, in the V3 loop of macrophage-tropic AC10.0.29 pseudovirus (Table 1) will still enable contacts with the first phosphate group of LPS, whereas the electrostatic contacts with the second phosphate group of LPS and hydrophobic contacts with acyl chains of lipid A will remain the same. In fact, some of the second phosphate group of LPS and hydrophobic contacts with phosphate group of LPS, whereas the electrostatic contacts with the residues, in the V3 loop of macrophage-tropic AC10.0.29 pseudovirus, are significantly inhibited R5 viral infection (Fig. 5C), confirming that LPS can inhibit the viral entry. Our other results suggest that LPS binding to the V3 loop of gp120 on the surface of HIV-1 pseudovirus. Using R5 HIV-1 pseudovirus, we confirmed that not only amino acids in the V3 loop of X4 strains but also amino acids in the V3 loop of R5 strains enable such geometric arrangement of charged and hydrophobic interactions, which is actually the LPS binding pattern.

It has been shown that a Gram-negative bacterial infection can drive HIV-1 LTR regulation by increasing the amount of free NF-kB stimulated by bacterial LPS (27). LPS affects HIV-1 replication through the up-regulation of type I interferons (26), whereas it also up-regulates the transcription of chemokines MIP-1 and RANTES (regulated on activation, normal T cell expressed and secreted), which can compete for binding with chemokine coreceptors, protecting macrophages from infection by HIV-1 (58, 59). Down-regulation of CCR5 via LPS also contributes to the protection from viral infection (60). With respect to the effect of bacterial and HIV-1 infection, we propose that the effects of LPS in HIV-1 patients could be modified by nonendotox LPS antagonists as wide-range inhibitors of HIV-1 entry, which may reduce the HIV-1 load and the inflammation caused by opportunistic Gram-negative bacteria. Alternatively known polyanionic HIV-1 inhibitors, such as dextran sulfate or heparin sulfate, could be modified by the introduction of a hydrophobic moiety to increase the affinity to the V3 and HIV-1 neutralization potency.

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