Resistance to Neutralization by Antibodies Targeting the Coiled Coil of Fusion-active Envelope Is a Common Feature of Retroviruses*

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The human T-cell leukemia virus transmembrane glycoprotein (TM) is a typical class 1 membrane fusion protein and a subunit of the viral envelope glycoprotein complex. Following activation, the TM undergoes conformational transitions from a native nonfusogenic state to a fusion-active pre-hairpin intermediate that subsequently resolves to a compact trimer-of-hairpins or six-helix bundle. Disruption of these structural transitions inhibits membrane fusion and viral entry and validates TM as an anti-viral and vaccine target. To investigate the immunological properties of fusion-active TM, we have generated a panel of monoclonal antibodies that recognize the coiled-coil domain of the pre-hairpin intermediate. Antibody reactivity is highly sensitive to the conformation of the coiled coil as binding is dramatically reduced or lost on denatured antigen. Moreover, a unique group of antibodies are 100–1000-fold more reactive with the coiled coil than the trimer-of-hairpins form of TM. The antibodies recognize virally expressed envelope, and significantly, some selectively bind to envelope only under conditions that promote membrane fusion. Most importantly, many of the antibodies potently block six-helix bundle formation in vitro.

Nevertheless, viral envelope was remarkably resistant to neutralization by antibodies directed to the coiled coil. The data imply that the coiled coil of viral envelope is poorly exposed to antibody during membrane fusion. We suggest that resistance to neutralization by antibodies directed to fusion-associated structures is a common property of retroviral TM and perhaps of other viral class I fusion proteins. These observations have significant implications for vaccine design.

Enveloped viruses gain entry into cells by fusion of the viral and cell membranes. For many viruses membrane fusion is carried out by viral class I fusion proteins (1). In response to an activation trigger, the fusion protein inserts an N-terminal hydrophobic peptide into the target cell membrane, which results in the formation of a rod-like fusion-active pre-hairpin intermediate (1, 2). The pre-hairpin intermediate is stabilized by a triple-stranded coiled-coil that is assembled from the N-terminal α-helices (N-helices) of adjacent fusion protein monomers. Subsequently, a C-terminal α-helical (C-helix) segment of each monomer docks into grooves on the surface of the coiled coil to yield a six-helix bundle or trimer-of-hairpins structure. Resolution to the trimer-of-hairpins brings the viral and cellular membranes into close proximity, destabilizes the lipid bilayers, and ultimately promotes membrane fusion (1–5).

The retroviral envelope glycoprotein complex consists of a trimer of surface glycoproteins (SU) 2 held on a trimer of a class I fusion proteins referred to as the transmembrane glycoprotein (TM) (1, 6). The fusogenic properties of TM are activated following binding of SU to the relevant cellular receptor. For human T-cell leukemia virus type 1 (HTLV-1), the structure of the TM-derived trimer-of-hairpins has been particularly well resolved (7). For each monomer of TM, an N-terminal hydrophobic fusion peptide is connected via a glycine-rich linker to an α-helical motif that interacts with the equivalent helix of adjacent TM monomers to form a central triple-stranded coiled coil. At the base of the core coiled-coil, the peptide backbone folds back in a disulfide-bonded 180° loop referred to as the chain reversal region. The C-terminal segment, which includes a short α-helix, runs in an anti-parallel manner along the grooves formed on the surface of the core coiled-coil.

Important, synthetic peptides that mimic the C-terminal α-helix of the trimer-of-hairpins motif of human immunodeficiency virus type 1 (HIV-1) (2, 8–10) and HTLV-1 (11, 12) are potent and highly specific inhibitors of viral entry. These peptides most likely dock into the grooves of the pre-hairpin coiled coil and act in a trans-dominant negative manner to prevent engagement of the groove by the C-terminal α-helical segment of the trimer-of-hairpins (1, 2, 12–15), thereby blocking six-helix bundle formation and consequently preventing membrane fusion and viral entry into cells. The ability of C-helix-based mimetic peptides to inhibit viral infection of cells has prompted speculation that antibodies targeting the coiled-coil motif of fusion-active envelope may also display inhibitory activity. Furthermore, immunogens that elicit neutralizing antibodies targeting the coiled coil are likely to be of value as antiviral vaccine candidates.

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2 The abbreviations used are: SU, surface glycoprotein; TM, transmembrane glycoprotein; MBP, maltose-binding protein; MAb, monoclonal antibody; HIV, of human immunodeficiency virus; HIV-1, HIV type 1; HTLV-1, human T-cell leukemia virus type 1; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; FBS, fetal bovine serum; eGFP, enhanced green fluorescent protein; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay.
Despite the use of a variety of immunogens, and immunization strategies, potently neutralizing antibodies targeting the fusion-active coiled-coil or six-helix bundle structures of HIV envelope have remained elusive (16–20). In contrast, Kaumaya and co-workers (21) have reported that a peptide antigen incorporating a fragment of the N-helical region of HTLV-1 TM is a potent immunogen, which stimulates production of polyclonal antibodies that recognize the coiled coil of HTLV-1 TM and antagonize envelope activity. These observations suggest that neutralizing antibodies to pre-hairpin structures can be obtained and that resistance to neutralization by such antibodies may be an attribute that is peculiar to HIV-1.

The conserved nature of the viral fusion process implies that immunization strategies that elicit neutralizing antibody responses to the coiled coil of viral fusion proteins will be of general utility to diverse viral pathogens. Given the potential importance of the pre-hairpin intermediate to vaccine design, we sought to generate antibodies with reactivity to a recombinant trimeric form of the HTLV-1 coiled coil and to determine the specificity and neutralizing properties of such antibodies. We demonstrate that monoclonal antibodies (MAbs) targeting the trimeric α-helical coiled coil of the HTLV-1 envelope can be generated. Many of the MAbs are highly conformation-specific and recognize the coiled coil but fail to recognize denatured antigen. Significantly some of the MAbs exhibit a marked specificity for the exposed coiled coil but recognize poorly the tri-m-of-hairpins form of TM. Nevertheless, such antibodies fail to block HTLV-1 envelope-mediated membrane fusion and viral entry. The significance of our observations for vaccine design and viral pathogenesis is discussed.

**EXPERIMENTAL PROCEDURES**

**Animal Immunization, Hybridoma Production, and Cell Culture**—Six- to eight-week-old CD1 mice were immunized with the recombinant antigen MBP-fishhook emulsified in adjuvant (TiterMax® Gold; CytRx Corp.) following the manufacturer’s instructions. Briefly, equal volumes of adjuvant and antigen were mixed to a final antigen concentration of 1.25 mg/ml. Mice were immunized subcutaneously with 50 μg of antigen, and immunization was repeated at 3-week intervals (three times). Seven days after the last boost, samples of serum from each animal were collected for determination of antibody titer. The animals were given a final boost by intravenous injection of soluble antigen (10 μg) without adjuvant 5 days prior to fusion. The mice were euthanized and the spleens removed, washed, minced, and gently agitated to release splenocytes. The splenocytes were fused to myeloma cells (SP2/0-Ag14, European Collection of Cell Cultures) using polyethylene glycol (PEG 4000, Sigma) and plated out in 96-well plates in RPMI medium supplemented with 20% FBS and hypoxanthine/aminopterin/thymidine (Sigma) to select for hybridomas. Two weeks later, hybridomas were transferred to HT-supplemented media, and the culture supernatants were tested by ELISA for antibodies with reactivity to the immunogen. Antibody isotype and IgG subclass was determined using the mouse monoclonal antibody isotyping reagents kit (Sigma) as recommended by the manufacturer.

HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. Myeloma SP2/0 cells, the HTLV-1 infected cell line MT-2, and uninfected Sup-T1 cells were maintained in RPMI 1640 medium supplemented with 10% FBS.

**Plasmids**—The plasmids pHTE-1 (22), pMAL-gp21hairpin (MBP-hairpin), pMAL-gp21fishhook (MBP-fishhook) (12), pMAL-N-helix (MBP-N-helix), and pMAL-STOP (MBP-stop) have been described previously (23, 24).

**ELISA**—Microtiter 96-well plates (NUNC, MAXI-Sorp) were coated overnight at 4 °C with the chimeric MBP-TM fusion proteins, (MBP-Fishhook, MBP-Hairpin, MBP-N-helix, or control MBP; all at 10 μg/ml) in PBS, pH 7.2. Plates were blocked (5% Marvel, PBS, 0.2% Tween 20) for 1 h at room temperature and washed (five times), and antibodies at the concentrations indicated were added and incubated with the immobilized target antigen for 2 h at room temperature. Peroxidase-conjugated anti-mouse IgG (1:10,000 dilution) (Sigma) was added, and the bound antibody was detected using fresh 2,2’-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate at 15 μg/ml (in 0.1 M citric acid, 0.06% H₂O₂). After 10–20 min, color development was stopped and absorbance read at 415 nm.

For assays comparing antibody reactivity with native versus denatured antigen (23, 25), microtiter 96-well plates were coated overnight at 4 °C with an anti-MBP sheep polyclonal antibody at 10 μg/ml in PBS, pH 7.2. Plates were washed twice with Wash buffer (PBS, 0.05% Tween 20) and blocked with Blocking buffer (1% bovine serum albumin in PBS) for 2 h at room temperature. After further washing, native MBP-Hairpin (10 μg/ml) in Tris-buffered saline (TBS) containing 10% FBS was added to the wells (100 μl). For comparison, denatured MBP-Hairpin was prepared as above but also mixed with 50 mM dithiothreitol with 1% SDS and boiled for 5 min. Nine volumes of TBS/FBS containing 1% Nonidet P-40 (TBS/Nonidet P-40) were then added, and the solution was cooled to room temperature before addition to the plates. Monoclonal antibodies at the concentrations indicated were added and incubated with the native or denatured target antigen for 2 h at room temperature. Plates were washed five times to remove unbound antibody, and peroxidase-conjugated anti-mouse IgG (1:10,000 dilution) was added and incubated for 1 h, and the bound antibody detected as described above.

**Peptide Binding in the Presence of Anti-coiled Coil Antibodies**—The peptide Bio-P²⁴-400 (24) was prepared as a stock solution in Wash buffer containing 5 mM dithiothreitol. Bio-P²⁴-400 (2.0 μg/ml) was incubated with immobilized MBP-fishhook in the presence or absence of the test antibody at the indicated concentrations, at room temperature, for 1 h. Preliminary binding assays indicated that a peptide concentration of 2.0 μg/ml is within the upper linear range of the concentration curve for binding of the peptide to the coiled coil (24). Subsequently, plates were washed (five times) to remove unbound peptide, and bound complexes were incubated for 1 h at room temperature with 100 μl of streptavidin/horseradish peroxidase (Sigma) (1:10,000 dilution). Plates were washed five times to remove unbound streptavidin/horseradish peroxidase and two times with PBS to remove residual detergent. Finally, bound
Expression and Purification of MBP-TM Proteins—The expression of recombinant MBP-TM proteins in *Escherichia coli* and purification by affinity chromatography on amylose-Sepharose were carried out as described (12). The concentration of the eluted fusion proteins was estimated by the Bradford assay (26, 27). HeLa cells (13.7 kDa; Amersham Biosciences). Gel filtration analyses were calibrated with ferritin (440 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), and RNase A (13.7 kDa; Amersham Biosciences).

Syncytium Interference Assay—Syncytium interference assays were performed by standard methods (26, 27). HeLa cells (3 x 10^5) transfected with the envelope expression vector pHTE-1 were added to untransfected HeLa target cells (7.0 x 10^5). The effector and target cells were co-cultured in the absence or presence of the MAbs at the concentrations specified. The cells were incubated for 12-15 h at 37 °C, washed twice with PBS, and fixed in PBS, 3% paraformaldehyde. Assays were performed in triplicate, and the number of syncytia from five low power fields per replicate was scored by light microscopy.

Pseudotyping Assay—Pseudotyped virus was prepared as described (27). Briefly, 293T cells were co-transfected using FuGENE 6 with 10 μg of the Env-defective, luciferase-encoding HIV proviral clone pNL4-3.LUC.R.E-. Sodium butyrate (20 mm) was added after 18 h, and 48–72 h later the viral supernatants were harvested by centrifugation at 400 x g and filtration through a 0.45-μm micropore filter. For transduction of HeLa or HOS cells, triplicate samples of 7 x 10^5 target cells in 1 ml were incubated with 1 ml of undiluted or serially diluted virus stock and plated into wells of a 6-well tissue culture dish. Following overnight incubation at 37 °C, 5% CO_2, cell lysates were harvested, assayed for luciferase activity following the manufacturer’s instructions using the luciferase assay system (Promega, Madison, WI) and a Turner Designs TD-20/20 luminometer (Sunnyvale, CA). For antibody inhibition experiments, 1 μl of HTLV-1 Env-pseudotyped virus was incubated with 1 μl of unlabeled HeLa or HOS cells (4 x 10^5 cells) in the presence or absence of serial dilutions of purified MAbs. After overnight culture, the cells were lysed and assayed for luciferase activity.

FACS Analysis—HTLV-1-infected MT-2 and noninfected (control) Sup-T1 cells at a density of 10^6 cells per ml were mixed with 0.1 ml of hybridoma culture supernatant or 1 μg/ml purified MAbs and incubated at room temperature for 1 h with rotation. The cells were pelleted (1000 rpm for 5 min, in an Eppendorf C5415C microcentrifuge), washed, and incubated with anti-mouse FITC antibody (1:1000 dilution) in RPMI medium at room temperature for 30 min in the dark. The cells were washed (PBS, 0.1% sodium azide) and fixed (0.5% paraformaldehyde in PBS, pH 7.4), and the cells were kept in the dark at 4 °C until subjected to FACScan (BD Biosciences) analysis as directed by the manufacturer.

Microscopy—HTLV-1-infected MT2 cells were labeled by transduction with a lentiviral vector expressing enhanced green fluorescent protein (eGFP) allowing the identification of the virally infected cells. The eGFP-MT2 cells (0.5 x 10^6) were co-cultured with the unlabeled Sup-T1 (0.5 x 10^6) in the presence of 1 μg/ml of the purified MAbs. The cell population was incubated at 37 °C, 5% CO_2 for 30 min and plated onto collagen (5 mg/cm^2)-coated glass coverslips (Sigma). The cells were incubated at 37 °C, 5% CO_2 on the coverslips for 60 min. Cells were washed with PBS, fixed with ice-cold methanol for 5 min at room temperature, and then blocked (PBS, containing 1% FBS and 0.5% bovine serum albumin). The cell-bearing coverslips were carefully washed twice in Blocking buffer and incubated in the dark with anti-mouse IgG/TRITC (1:500) (Sigma) at room temperature for 1 h. Coverslips were mounted onto glass slides with Vectashield (Vector Laboratories), sealed with nail varnish, and left to dry in the dark before the examination by fluorescence microscopy and differential interference contrast imaging using a Zeiss Axioplan 2 microscope with Openlab (Improvision) data acquisition software.

RESULTS

Production of Anti-TM MAbs—We have expressed a fragment of HTLV-1 TM that spans the N-terminal α-helical leucine/isoleucine heptad repeat as a fusion to maltose-binding protein (MBP) (12, 23) (Fig. 1). This recombinant TM-derived fusion protein, referred to as MBP-fishhook, is trimeric as revealed by size exclusion chromatography and by native PAGE (12), and specifically binds to synthetic peptides that mimic the C-helical region of the HTLV-1 trimer of hairpins (12, 24). Thus the recombinant TM fragment faithfully mimics the critical features of the exposed core coiled coil of fusion-active TM.

In an effort to obtain monoclonal antibodies that specifically target the HTLV-1 coiled coil, the recombinant trimeric antigen was used to immunize CD1 mice. Booster immunizations were given at 4-week intervals; and the mice were subsequently euthanized, the spleens recovered, and the splenocytes fused with the myeloma cell line SP2/0-Ag14. Thereafter, hybridomas secreting monoclonal antibodies were selected using standard methods. From a bank of 600 hybridomas, a panel of nine independent clones secreting MAbs reactive with the immunogen were selected for detailed analysis. Each of the MAbs demonstrated strong binding to the immobilized coiled-coil fusion protein, MBP-fishhook (Fig. 2), but exhibited little or no reactivity with the carrier protein MBP. Thus, each of the selected MAbs exhibits specific reactivity with the coiled-coil fragment of HTLV-1 TM. Analysis of antibody subclass and isotype indicated that the panel of MAbs consist of six IgG1, two IgG2a, and one IgG2b antibodies (Table 1). Moreover, non-linear regression analysis (GraphPad, Prism) of the dose-dependent binding curves for each antibody indicated that there is a 35-fold range in relative binding affinity of the MAbs against the coiled coil of HTLV-1 TM (Fig. 3; Table 1).

MAbs Directed at the Coiled Coil Are Highly Conformation-dependent—Our approach to immunization and hybridoma screening employed a trimeric antigen (Fig. 1B) that faithfully...
mimics the exposed coiled coil of fusion-active envelope (12, 23, 24). We therefore anticipated that some antibodies would exhibit a degree of conformational specificity to the epitopes recognized. Indeed, we have found previously that MAbs reactive with the post-fusion trimer-of-hairpins structure of HTLV-1 TM are highly conformation-specific (23). We therefore examined the panel of MAbs for reactivity to native and denatured forms of the coiled coil.

An established ELISA methodology (23, 25) was employed to examine MAb binding to native or denatured antigen. Briefly, the antigen MBP-fishhook was denatured by heating in SDS-Tris buffer containing irrelevant carrier protein, and thereafter the antigen was diluted 10-fold in SDS-free buffer. Denatured or native antigen was subsequently captured on the surface of microtiter plates pre-coated with anti-MBP polyclonal rabbit sera. Murine MAbs were then examined for the ability to bind to the immobilized native or denatured antigen. Importantly, the MAB M20-1 known to bind to a linear epitope of the carrier protein MBP was used as a control to monitor antigen capture (Fig. 4). The control MAB, M20-1, displayed highly efficient binding to both native and denatured MBP-fishhook. By contrast, whereas the anti-coiled-coil MAbs bound efficiently to native antigen, there was a dramatic loss in binding to dena-

### TABLE 1

| Hybridoma | Isotype and subclass | MAb at 50% binding µg/ml |
|-----------|---------------------|-------------------------|
| N4-4      | IgG1                | 0.104                   |
| N7-2      | IgG1                | 0.715                   |
| N9-7      | IgG1                | >2.50*                  |
| N9-8      | IgG1                | 0.096                   |
| N15-4     | IgG1                | 0.055                   |
| N18-1     | IgG1                | 2.283                   |
| N10-4     | IgG2a               | 0.041                   |
| N16-1     | IgG2a               | 0.149                   |
| N13-3     | IgG2b               | 0.061                   |

* Saturation was not achieved for N9-7.
HTLV-1 Coiled-coil-specific MAbs

FIGURE 4. Epitopes recognized by anti-TM MAbs are lost on denatured antigen. Each of the purified monoclonal antibodies at the concentrations indicated was examined for the ability to bind to native (closed squares) or denatured (closed triangles) MBP-fishhook. Native or denatured antigen (boiled in SDS and dithiothreitol and diluted in the presence of carrier protein) was captured onto the solid phase using polyclonal anti-MBP rabbit sera; subsequently, the anti-TM MAbs were incubated with the immobilized antigen; following extensive washing bound MAB was detected (mean ± S.D. of triplicate assays). As a control for antigen capture, the native and denatured antigen was also examined for reactivity with the monoclonal antibody M20-1, which binds to a linear epitope of MBP (graph top left). Absorbance was measured at λ 415 nm.

tured coiled coil (Fig. 4). Moreover, these MAbs failed to bind to a set of overlapping but monomeric 27–35 amino acid synthetic peptides (23, 24) that cover the entire core coiled coil of envelope (data not shown). Thus binding of each of the MAbs to the coiled coil is highly dependent on appropriate presentation of epitopes that are sensitive to the conformational status of the coiled coil.

The MAbs Discriminate between TM Structures—The observation that the epitopes recognized by the MAbs are highly dependent on the conformation of the antigen prompted us to test the ability of the MAbs to discriminate between trimeric and monomeric fragments of recombinant TM (Fig. 1). This analysis demonstrated that the MAbs fall into two groups with overlapping reactivity profiles (Fig. 5). As expected, all of the MAbs bound efficiently to the trimeric recombinant core coiled-coil protein MBP-fishhook but failed to bind to the control carrier protein MBP (Fig. 5). Notably, the MAbs also failed to recognize a TM fragment, MBP-CX6CC, representing the disulfide-bonded chain reversal region of the trimer-of-hairpins, a construct that has been shown to be monomeric (23). Moreover, the MBP-fishhook immunogen lacks the C-terminal helical region of the trimer-of-hairpins, and consistent with this the MAbs failed to bind to monomeric MBP-C-helix (23, 24). These data are consistent with the view that the MAbs recognize epitopes contained within the trimeric region of the exposed coiled coil.

Most significantly, a group of five MAbs efficiently recognized both the trimeric coiled coil and trimer-of-hairpins structure; these MAbs are N10-4, N15-4, N9-7, N18-1, and N8 (Fig. 5). By contrast, a second group of four MAbs, N4-4, N13-3, N16-1 and N7-2, demonstrated considerably better binding to the coiled-coil structure relative to the trimer-of-hairpins. This indicates that although both groups of MAb recognize epitopes within the
N-helical segment of TM, they recognize this region in functionally different ways. Furthermore, the ability of the TM fragments to form stable trimers appears to be a prerequisite for recognition by the MAbs, as both groups of MAb failed to bind to a recombinant fragment of TM that emulates the N-helical region of the core-coiled coil. Importantly, we have previously demonstrated that the isolated N-helical region, which spans amino acids Met338–Trp387 of envelope (Fig. 1), fails to form trimers under the conditions used in these assays (23, 24).

MAbs Specific to the Coiled Coil Exhibit Poor Reactivity with the Trimer-of-Hairpins—The epitope mapping data indicate that the MAbs raised against the core coiled coil fall into two reactivity groups. Group I MAbs are reactive with both the trimer-of-hairpins and the trimeric coiled coil. These MAbs appear to have similar reactivity profiles to a previously described subset of antibodies raised against the intact trimer-of-hairpins of HTLV-1 TM (23). By contrast, group II MAbs, represented by N-4-4, N-13-3, N-16-1, and N-7-2, have properties that set them apart from the group I MAbs and distinguish them from the previously described six-helix bundle-specific MAbs. To further investigate the reactivity profiles of the MAbs, we compared each of the group I and group II MAbs and a representative anti-hairpin MAb, M17-21 (23), for dose-dependent binding to both the coiled coil and trimer-of-hairpins. Examination of the binding curves (Fig. 6) and estimation of the half-maximal binding concentration of the antibody against each of the TM-derived structures indicated that group I MAbs, such as N-18-1, N-9-8, N-9-7, and N-10-4 and the anti-hairpin antibody M17-21, recognized the trimer-of-hairpins and coiled coil equally well. By contrast, the relative affinity of group II MAbs, including N-4-4, N-13-3, N-16-1, and N-7-2, was 100–1000-fold greater for the exposed coiled coil than the trimer-of-hairpins (Fig. 6). Thus, the accumulating data indicate that both group I and II MAbs recognize the coiled coil and that the epitopes recognized by the antibodies are highly conformation-specific and are available for binding only on trimeric forms of TM. Most importantly, the epitopes recognized by group II MAbs are presented appropriately on the core coiled coil but are poorly accessible to antibody on the trimer-of-hairpins form of TM.

Recognition of Viral Envelope on Infected Cells—Having demonstrated that the MAbs bind to recombinant forms of TM, we wished to determine whether the antibodies are capable of recognizing virally expressed envelope and whether such antibodies can bind to the fusion-active structures of TM. Previously, we have demonstrated that antibodies directed to the trimer-of-hairpins of HTLV-1 envelope efficiently recognize TM on the surface of infected T-cells even in the absence of overt membrane fusion (23). We therefore examined the panel of anti-coiled-coil MAbs for reactivity with envelope expressed on the surface of the chronically infected T-cell line MT2. Importantly, MT2 cells abundantly express viral envelope glycoproteins but exhibit little spontaneous envelope-mediated membrane fusion and syncytium formation, most likely because of low surface expression of HTLV-1 receptors such as Glut-1 (28). Flow cytometry (Fig. 7) revealed that most of the group I MAbs, including N-9-8, N-10-4, N-15-4, and N-18-1, efficiently recognized envelope on infected cells. In contrast, three of the group II MAbs, N-4-4, N-16-1, and N-13-3, and also the group I MAb, N-9-7, failed to bind to native envelope on infected cells; typical negative data for N-4-4 and N-16-1 are shown (Fig. 7). In the absence of obvious envelope-mediated membrane fusion, N-7-2 was the only group II MAb that efficiently bound to envelope on infected cells. Therefore, group I and II MAbs display distinct binding properties with respect to recombinant antigen and virally expressed envelope (Fig. 8).

The coiled coil is a structure thought to be associated with envelope-mediated membrane fusion. Therefore, it was anticipated that the epitopes recognized by highly coiled-coil-specific MAbs might become available only during the fusion process. To test this view, the ability of the MAbs to bind to viral envelope on MT2 cells that had been co-cultured with the uninfected T-cell line Sup-T1 was examined (Fig. 9). Our group and others (26, 29, 30) have demonstrated that Sup-T1 cells express functional receptors for HTLV-1, and that these cells are permissive for envelope-mediated membrane fusion and HTLV-1 entry. No envelope staining was observed with any of the group II MAbs on MT2 cells cultured in the absence of Sup-T1 cells, and no staining was observed on control Sup-T1 cells cultured in the absence of HTLV-1-infected cells (Fig. 9). However, upon co-culture a weak but highly reproducible staining of the cell population was observed (Fig. 9).

The weak antibody-dependent staining of co-cultured cells was examined further. The HTLV-1-infected cell line MT2 was first labeled by transduction with a lentiviral vector expressing enhanced green fluorescent protein (eGFP), thereby allowing unambiguous identification of the virally infected cells. Subsequently, following co-culture, the eGFP-MT2 and unlabeled Sup-T1 cells were probed with either MAb N-13-3 or N-16-1 and examined by fluorescence microscopy. Significantly, pronounced fluorescent staining because of the bound coiled-coil-specific MAb was localized discretely to the junction of interacting MT2 and Sup-T1 cells (Fig. 10). Fluorescent staining was not observed on either infected MT2 cells or Sup-T1 cells grown in monoculture (data not shown). Most importantly, the
coiled-coil-specific staining on co-cultured cells was highly evocative of the polarized pattern of envelope localization found at sites of cell-to-cell contact and viral transfer that have been referred to as the virological synapse (31). Taken together, the accumulating data are consistent with the fusion-dependent exposure of a novel epitope on viral TM that is recognized by the coiled-coil-specific MAbs.

Lack of Neutralizing Activity—A critical issue for envelope-based vaccine design is the ability of the immunogen to elicit antibody responses that target viral envelope and neutralize viral entry. The immunogen used in these studies was constructed specifically to generate antibodies against a functionally important structure of fusion-active envelope. We therefore used two complementary assays to examine the ability of the purified MAbs to block envelope-mediated membrane fusion and neutralize viral entry. In the first assay, each MAb was tested for the ability to inhibit syncytium formation between HeLa cells transfected with the envelope expression vector HTE-1 and untransfected HeLa target cells. The second assay assessed the ability of the MAbs to block infection of HOS target cells with HIV particles pseudotyped with HTLV-1 envelope. Even at very high antibody concentrations (20 μg/ml), or following release from fusion arrest by returning the cells to 37 °C after culture at suboptimal temperatures for membrane fusion (18, 31), no neutralizing activity could be detected for any of the group I or group II MAbs in either of the neutralization assays. Thus, despite the ability of many of the MAbs to bind to virally expressed envelope, and despite the ability of group II MAbs to bind preferentially to the coiled coil, the MAbs invariably failed to block envelope-mediated membrane fusion or neutralize viral entry.

Antibodies Directed at the Coiled Coil Prevent Six-helix Bundle Formation—One plausible explanation for the inability of MAbs targeted to the core coiled coil to neutralize viral entry is that despite the ability of the antibodies to bind to the exposed coiled coil, they are unable to prevent docking of the C-terminal α-helical segment of the TM ectodomain into the hydrophobic grooves of the
coiled coil. If this were the case, the MAbs would be unable to prevent six-helix bundle formation and therefore fail to inhibit envelope-mediated membrane fusion. We therefore examined MAbs known to bind specifically to the recombinant coiled coil, and we compared them to control antibodies and MAb M17-21, a non-neutralizing antibody known to bind both the trimer-of-hairpins and the coiled coil, for the capacity to block six-helix bundle formation in vitro (Fig. 11).

Using a robust and established assay (24), we monitored binding of a synthetic N-terminally biotinylated peptide, representing the C-helix region of the HTLV-1 trimer-of-hairpins, to immobilized recombinant core coiled coil in the presence or absence of the test antibodies. Importantly, we have demonstrated that C-helix-based synthetic peptides, such as Pcr-400, are potent inhibitors of HTLV-1 envelope-mediated membrane fusion (12). These peptides bind directly to the hydrophobic grooves of the coiled coil that are normally occupied by the anti-parallel C-terminal segment of the trimer-of-hairpins (12, 24). Therefore, binding of the synthetic peptide to the coiled coil mimics six-helix bundle formation. Notably, the control antibodies failed to inhibit six-helix bundle formation in vitro (Fig. 11A). By contrast, and most significantly, the anti-coiled-coil MAbs N4-4, N7-2, N16-1, and N13-3 demonstrated potent and dose-dependent inhibition of peptide binding to the coiled coil, whereas N9-7 was a poor inhibitor of six-helix bundle formation (Fig. 11A). Furthermore, the profile of the dose-response curves indicated that the inhibition of six-helix bundle formation by the MAbs broadly reflects the relative binding affinities of the MAbs for the coiled coil. Therefore, antibodies of high relative affinity such as N13-3 and N4-4 displayed considerably better inhibition of peptide binding than the relatively low affinity N9-7. Taken together, the results reveal that, at least in vitro, MAbs specifically targeted to the coiled coil are capable of blocking six-helix bundle formation.

As group II MAbs are capable of blocking six-helix bundle formation, we also assessed the activity of selected MAbs that recognize both the coiled coil and hairpin structures. Surprisingly the ability to block six-helix formation was not limited to the group II MAbs that bind specifically only to the core coiled coil. A number of group I MAbs that recognize both the coiled coil and the trimer-of-hairpins (Fig. 11B) and some anti-hairpin MAbs (Fig. 11C) were also able to block assembly of the six-helix bundle. However, the capacity to block bundle formation was not a typical feature of all anti-TM MAbs as M17-21 and M16-28 failed to block bundle formation or were poor inhibitors of bundle assembly (Fig. 11C).

**DISCUSSION**

Immunogens and immunization strategies that elicit neutralizing antibodies against the fusion-active structures of viral fusion proteins are likely to have broad application against a range of clinically important viruses, including HIV, HTLV-1, paramyxoviruses, and emerging viruses such as Ebola. Despite considerable effort, conventional immunization approaches employing peptide immunogens, which mimic fusion protein structures, have failed to produce neutralizing antibody activity (16, 23, 24), or the derived antibodies have antagonized membrane fusion and viral entry only under highly selective conditions (18). Interestingly, Kaumaya and co-workers (21) recently reported that a peptide immunogen, which emulates a fragment of the N-helical region of the HTLV-1 TM coupled to a lysine scaffold, induced HTLV-1-neutralizing antibodies in coiled coil. If this were the case, the MAbs would be unable to prevent six-helix bundle formation and therefore fail to inhibit envelope-mediated membrane fusion. We therefore examined MAbs known to bind specifically to the recombinant coiled coil, and we compared them to control antibodies and MAb M17-21, a non-neutralizing antibody known to bind both the trimer-of-hairpins and the coiled coil, for the capacity to block six-helix bundle formation in vitro (Fig. 11).

Using a robust and established assay (24), we monitored binding of a synthetic N-terminally biotinylated peptide, representing the C-helix region of the HTLV-1 trimer-of-hairpins, to immobilized recombinant core coiled coil in the presence or absence of the test antibodies. Importantly, we have demonstrated that C-helix-based synthetic peptides, such as Pcr-400, are potent inhibitors of HTLV-1 envelope-mediated membrane fusion (12). These peptides bind directly to the hydrophobic grooves of the coiled coil that are normally occupied by the anti-parallel C-terminal segment of the trimer-of-hairpins (12, 24). Therefore, binding of the synthetic peptide to the coiled coil mimics six-helix bundle formation. Notably, the control antibodies failed to inhibit six-helix bundle formation in vitro (Fig. 11A). By contrast, and most significantly, the anti-coiled-coil MAbs N4-4, N7-2, N16-1, and N13-3 demonstrated potent and dose-dependent inhibition of peptide binding to the coiled coil, whereas N9-7 was a poor inhibitor of six-helix bundle formation (Fig. 11A). Furthermore, the profile of the dose-response curves indicated that the inhibition of six-helix bundle formation by the MAbs broadly reflects the relative binding affinities of the MAbs for the coiled coil. Therefore, antibodies of high relative affinity such as N13-3 and N4-4 displayed considerably better inhibition of peptide binding than the relatively low affinity N9-7. Taken together, the results reveal that, at least in vitro, MAbs specifically targeted to the coiled coil are capable of blocking six-helix bundle formation.

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**DISCUSSION**

Immunogens and immunization strategies that elicit neutralizing antibodies against the fusion-active structures of viral fusion proteins are likely to have broad application against a range of clinically important viruses, including HIV, HTLV-1, paramyxoviruses, and emerging viruses such as Ebola. Despite considerable effort, conventional immunization approaches employing peptide immunogens, which mimic fusion protein structures, have failed to produce neutralizing antibody activity (16, 23, 24), or the derived antibodies have antagonized membrane fusion and viral entry only under highly selective conditions (18). Interestingly, Kaumaya and co-workers (21) recently reported that a peptide immunogen, which emulates a fragment of the N-helical region of the HTLV-1 TM coupled to a lysine scaffold, induced HTLV-1-neutralizing antibodies in
HTLV-1 Coiled-coil-specific MAbs

Vaccinated mice. Such observations suggest that immunization strategies that target the fusion-active structures of viral fusion proteins are a viable objective of prophylactic vaccination.

We therefore set out to generate a panel of monoclonal antibodies that specifically target the fusion-associated trimeric coiled-coil region of HTLV-1 TM. A recombinant mimetic of the coiled coil (7, 12, 23, 24) was used as an immunogen to generate a panel of hybridomas producing antibodies that are reactive with the HTLV-1 TM. The approach is validated by the demonstration that binding of the derived antibodies to the assembled trimer of hairpins is highly dependent on appropriate folding of the antigen and that epitope recognition is severely impaired on denatured protein. Therefore, effective epitope recognition by the antibodies is highly dependent on both the tertiary and quaternary structure of the coiled coil.

Based on antigen recognition and reactivity with viral envelope, the antibodies fell into two groups. Group I MAbs recognize both the coiled coil and the trimer-of-hairpins structure, and most of these antibodies also recognize envelope on HTLV-1-infected cells. Anti-hairpin antibodies (20), group I MAbs bind to virally expressed envelope even under nonfusogenic conditions. These data imply that there are multiple forms of envelope on infected cells and that some of the envelope complexes exist in post-fusion conformations that include the trimer-of-hairpins. Why do MAbs raised against the core coiled coil also recognize the trimer-of-hairpins? The TM crystal structure (Fig. 1) (7) provides an explanation. The trimer-of-hairpins presents a large surface area to solvent, and within this structure much of the core coiled coil is exposed and potentially available for antibody binding. Of course, much of the exposed surface is not directly involved in docking with the C-helical region of TM. Consequently, antibody binding to these surfaces may have little impact on fusion, which may, in part, explain the failure of group I antibodies to neutralize envelope function and prevent viral entry. However, in our view, it is difficult to reconcile binding of a large antibody even to a nonfunctional surface of envelope with maintenance of membrane fusion activity. We therefore favor alternative scenarios for the failure of antibodies to neutralize (discussed below).

Interestingly, group II MAbs are notable for their superior binding to the coiled coil relative to the trimer-of-hairpins. For some antibodies binding to the core coiled coil was 100–1000-fold greater than binding of the same antibody to the trimer-of-hairpins. Therefore, the epitopes recognized by group II antibodies are exposed on the core coiled coil but are not exposed, or only poorly exposed, on the trimer-of-hairpins form of TM. It is likely that the epitopes recognized by these antibodies include amino acid residues that lie within or overlap with, the hydrophobic groove of the coiled coil. In the context of the trimer-of-hairpins (7) the C-terminal α-helical region and extended nonhelical leash residues occupy the groove on the coiled coil (Fig. 1). Thus, docking of the C-terminal helical region of TM with the coiled coil occludes the epitopes recognized by group II antibodies. Importantly, binding to the groove of the coiled coil is a property that is predicted for neutralizing antibodies.

Current models indicate that the coiled coil is assembled during fusion but is not present on the pre-fusogenic form of native envelope (1, 2, 5). Therefore, it was anticipated that antibodies directed at the coiled coil would recognize envelope only during active membrane fusion. Significantly, most of the group II MAbs fail to bind envelope on infected cells under nonfusogenic conditions, but following co-culture with uninfected T-cells, conditions that are known to promote fusion (12, 29), some MAbs (N4-4, N13-3, N16-1 and N7-2) demonstrated weak but reproducible binding to cells. Therefore, membrane fusion induces exposure of novel epitopes on envelopes that are recognized by group II MAbs, and this is consistent with recognition of the fusion-active coiled-coil structure.

In vitro, many group I and group II anti-coiled-coil MAbs and, as we show here, some anti-hairpin MAbs (23) are capable of blocking assembly of the six-helix bundle (Fig. 11). Therefore, it is surprising that none of the antibodies are capable of
inhibiting envelope-mediated membrane fusion or neutralizing entry of retroviral particles pseudotyped with HTLV-1 envelope. The results are consistent with findings made for the related but highly divergent retrovirus HIV-1 (16, 18, 19), but they are in stark contrast to a previous study in which animals immunized with an HTLV-1 N-helix-derived peptide immunogen were found to have neutralizing antibody activity (21).

Why is there such a dramatic difference between the results presented here and the study (21) by the Kaumaya group? One possible, but in our view unlikely, explanation is the very divergent retrovirus HIV-1 (16, 18, 19), but they are in stark contrast to a previous study in which animals immunized with an HTLV-1 N-helix-derived peptide immunogen were found to have neutralizing antibody activity (21).

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It is difficult to reconcile the experimental data from these studies. However, given that the MAbs produced in this study bind preferentially to trimeric TM structures, including the coiled coil, that many of the MAbs recognize envelope expressed on virally infected cells, and that many of the MAbs block six-helix bundle formation in vitro, it is surprising that none of the MAbs display neutralizing activity. Current models of envelope-mediated membrane fusion (1) and the data presented here suggest that if the coiled coil of fusion-active envelope or, alternatively, envelope complexes that have stalled at a pre-hairpin stage of the fusion pathway. On the other hand, group II MAbs may, in fact, bind to fusion-competent pre-hairpin forms of TM, but the kinetics of binding are insufficient to neutralize all of the abundant envelope complexes that exist on the surface of infected or transfected cells.

We have now characterized a variety of independently selected MAbs targeted to HTLV-1 TM. This study examined nine MAbs targeted to the core coiled coil, and in a previous
study 32 MAbS were raised against the trimer-of-hairpins of which 16 were characterized in detail (23). In addition, we have identified a single MAb reactive with the C-helix region of the six-helix bundle (24). Despite the ability of many of these MAbs to block six-helix bundle formation in vitro, the MAbs are unanimously unable to block membrane fusion and viral entry into cells. The simplest explanation for the inability of so many MAbs to neutralize envelope-mediated fusion is that the coiled coil and pre-hairpin structures are not accessible, or only poorly accessible, to antibody. Therefore, HTLV-1 appears to have evolved mechanisms to exclude potentially neutralizing antibody from the functionally important epitopes of envelope. One mechanism may be direct structural masking or occlusion of the functionally important surfaces. A second strategy may rely on the preferential cell-to-cell transfer of viral particles at sites of intimate cell-to-cell contact, such as the virological synapse (33), which may act as a privileged site that provides a shield from immunological surveillance.

Significantly, HIV-1 is also resistant to neutralization by antibodies targeted to the fusion-associated structures of TM (16, 18–20). Again, one mechanism of resistance appears to be limited access to antibody (16, 18–20, 34–36). The coiled coil of HIV and HTLV-1 are of comparable size (70 and 75 A, respectively) and, as illustrated by Zwick et al. (37) for HIV, the attached SU and proximity of the viral and possibly the cellular membranes act to restrict free access of antibody to TM. Therefore, resistance to neutralization, by antibodies directed to the coiled coil and fusion-associated structures, is a common feature of retroviral TM and perhaps of other viral class 1 fusion proteins. This resistance to neutralization is likely achieved by restricting the access of antibody to the fusion-active structures.

Generation of neutralizing antibodies against viral fusion proteins is a major objective of current anti-viral vaccine programs. Nevertheless, the accumulating evidence indicates that successful strategies may be difficult to achieve using conventional immunogens and immunization methods. Reassuringly, an elegant and novel approach reveals that neutralizing antibodies targeted to the pre-hairpin structures of retroviral TM are achievable (38). Using phage display technology, Miller et al. (38) identified an HIV-1 coiled-coil-specific single chain antibody that exhibits potent neutralizing activity. When engineered into a full antibody framework, the resulting MAb retained neutralizing potency and blocked viral entry across viral clades. Thus, the restricted access to antibody, which provides intrinsic resistance to neutralization, can be successfully penetrated. However, it remains to be determined whether immunization schemes can be developed that surmount the limited accessibility of the fusion-active pre-hairpin structures and thereby elicit potently neutralizing antibodies.

In contrast to SU-specific antibody (39–43), we have shown that HTLV-1 displays remarkable resistance to neutralization by antibodies targeted to the pre-hairpin and fusion-associated structures of TM. We suggest that, because HIV also exhibits resistance to many TM-directed antibodies (16, 18–20), such resistance is likely to be a common feature of retroviral TM. The accumulating evidence illustrates that it will be difficult to develop immunization strategies that elicit robust neutralizing antibody activity against viral class 1 fusion proteins. Nevertheless, providing that the restrictions to antibody binding can be overcome, it is likely that neutralizing antibodies targeted to pre-hairpin forms of class 1 fusion proteins will be of value in the dissection of fusion protein function and ultimately in the therapeutic treatment of viral infections.

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