Antibodies to the Envelope Glycoprotein of Human T Cell Leukemia Virus Type 1 Robustly Activate Cell-Mediated Cytotoxic Responses and Directly Neutralize Viral Infectivity at Multiple Steps of the Entry Process

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Antibodies to the Envelope Glycoprotein of Human T Cell Leukemia Virus Type 1 Robustly Activate Cell-Mediated Cytotoxic Responses and Directly Neutralize Viral Infectivity at Multiple Steps of the Entry Process

Chien-Wen S. Kuo, Antonis Mirsaliotis, and David W. Brighty

Infection of human cells by human T cell leukemia virus type 1 (HTLV-1) is mediated by the viral envelope glycoproteins. The gp46 surface glycoprotein binds to cell surface receptors, including heparan sulfate proteoglycans, neuropilin 1, and glucose transporter 1, allowing the transmembrane glycoprotein to initiate fusion of the viral and cellular membranes. The envelope glycoproteins are recognized by neutralizing Abs and CTL following a protective immune response, and therefore, represent attractive components for a HTLV-1 vaccine. To begin to explore the immunological properties of potential envelope-based subunit vaccine candidates, we have used a soluble recombinant surface glycoprotein (gp46, SU) fused to the Fc region of human IgG (sRgp46-Fc) as an immunogen to vaccinate mice. The recombinant SU protein is highly immunogenic and induces high titer Ab responses, facilitating selection of hybridomas that secrete mAbs targeting SU. Many of these mAbs recognize envelope displayed on the surface of HTLV-1-infected cells and virions and several of the mAbs robustly antagonize envelope-mediated membrane fusion and neutralize pseudovirus infectivity. The most potently neutralizing mAbs recognize the N-terminal receptor-binding domain of SU, though there is considerable variation in neutralizing proficiency of the receptor-binding domain-targeted mAbs. By contrast, Abs targeting the C-terminal domain of SU tend to lack robust neutralizing activity. Importantly, we find that both neutralizing and poorly neutralizing Abs strongly stimulate neutrophil-mediated cytotoxic responses to HTLV-1–infected cells. Our data demonstrate that recombinant forms of SU possess immunological features that are of significant utility to subunit vaccine design. The Journal of Immunology, 2011, 187: 361–371.
geometry of native viral envelope and the mode of interaction with Abs have a profound impact on the ability of Abs to neutralize virus infectivity. Understanding the immunogenic properties of HTLV-1 envelope is therefore a critical issue for the development of effective vaccines and immunological treatments to combat HTLV-1 infection.

Although the crystal structure for HTLV-1 SU has not yet been determined, rigorous examination suggests that the SU is comprised of two autonomously folding domains separated by a proline-rich linker peptide (31–36). The N-terminal region, also known as the receptor-binding domain (RBD), is necessary and sufficient for binding to Glut-1 on target T cells (21, 33). Moreover, transfer of the RBD into a heterologous viral envelope confers upon the recipient virus the cellular tropism that is typical of HTLV-1 (32, 37). By contrast, the C-terminal domain (CTD) is less well characterized, but accumulating evidence suggests that the CTD not only provides an important structural function in the envelope trimer, but is also involved in recognition of additional entry factors or coreceptors (38, 39), in determining the differing tropisms of HTLV-1 and HTLV-2 (40), and in transducing the envelope activation signal upon receptor binding (23, 24, 41). To date, the contribution of these domains to recognition by Ab and to the sensitivity of HTLV-1 to Ab-mediated neutralization is incompletely understood.

In this study, we investigate whether a recombinant form of SU that is competent for receptor binding can induce Ab responses and whether such Abs are capable of inhibiting envelope-mediated membrane fusion and viral entry. We demonstrate that many anti-SU Abs exhibit some neutralizing activity, whereas some are potently neutralizing. The most robustly neutralizing Abs tend to recognize the RBD of SU, but, although the relative binding efficiency of mAbs may be similar, there are marked differences in the inhibitory properties of RBD-targeted Abs. Moreover, we demonstrate that even poorly neutralizing Abs have the ability to recognize HTLV-1 viruses and to recruit cytoxic responses to HTLV-1–infected cells. Finally, we provide evidence that neutralizing Abs block HTLV-1 infectivity at distinct steps of the viral entry process. We discuss the implications of our findings for HTLV-1 immunotherapy.

Materials and Methods

**Plasmids and cell lines**

The plasmids pHTE-1, pMgp46-Fc have been described (42, 43). HeLa and human osteosarcoma (HOS) cells were maintained in DMEM supplemented with 10% FBS. Sup-T1 cells and primary lymphocytes were maintained in RPMI 1640 supplemented with 10% FBS. Insect cells were maintained in Shelds and Sang medium supplemented with 10% heat-inactivated FBS.

**Expression and purification of sRgp46-Fc proteins**

The expression of recombinant sRgp46-Fc protein in insect cells and purification by affinity chromatography on Con A-Sepharose and protein A-Sepharose were carried out, as described (43), with the exception that sRgp46-Fc was eluted from protein A-Sepharose using Ab Gentle Elution buffer (Pierce), as recommended by the manufacturer. The concentration of the eluted protein was estimated by Bradford assay, and the recombinant protein was stored at −80°C in column buffer (20 mM Tris [pH 7.5], 500 mM NaCl) supplemented with 20% glycerol. Purified sRgp46-Fc was shown to bind to Glut-1–expressing cells, as determined by flow cytometry (43).

**Western blotting**

Western blotting was carried out using standard methods. HTLV-1 envelope expression was probed using supernatant from murine hybridoma cell lines producing mAbs raised against sRgp46-Fc, and detected with anti-mouse HRP-conjugated secondary Ab at a dilution of 1:2500 in PBS containing 0.2% (w/v) Marvel and 0.025% Triton X-100.

ELISA

Microtitre 96-well plates (Nunc; MAXI-Sorp) were coated with anti-human IgG (Fc-specific) goat antiserum (Sigma-Aldrich) for 1 h at 37°C. The plates were blocked (5% Marvel/PBS/0.2% Tween 20) for 1 h at room temperature; sRgp46-Fc–containing Drosophila medium supernatant was added and incubated for 1 h at room temperature; and unbound SU was removed by washing (5×). Subsequently, murine envelope-specific and control Abs in hybridoma supernatants or purified mAb at the concentrations indicated were added and incubated with the immobilized target Ag for 2 h at room temperature. Plates were washed extensively, and peroxidase-conjugated anti-mouse IgG (1:10,000 dilution; Sigma-Aldrich) was added and the bound Ab detected using fresh ABTS substrate at 15 μg/ml (0.1 M citric acid, 0.06% H2O2). After 10–20 min, the absorbance was read at 415 nm. Dose-dependent Ab binding was compared by nonlinear regression using GraphPad by Prism, and minima and maxima for each data set were constrained to 0 and 100%, respectively.

**Syncytium interference assays**

Syncytium interference assays were performed by standard methods (28, 29, 43, 44). HeLa cells (2 × 105) transfected with the envelope expression vector pHTE-1 were added to untransfected HeLa target cells (8 × 105). The effector and target cells were cocultured in the absence or presence of the mAbs at the concentrations specified. The cells were incubated for 12–15 h at 30°C and returned to 37°C for 1–2 h, washed twice with PBS, and fixed in PBS containing 3% paraformaldehyde. Syncytia were stained with Giemsa’s solution (BDH). Assays were performed in triplicate, and the number of syncytia from five fields (×200) per replicate was scored by light microscopy.

**Pseudotyping assay**

Pseudotyped virus was prepared, as described (26, 45). Briefly, 293T cells were cotransfected using Genejuice (Novagen) with 10 μg envelope, luciferase-encoding, HIV proviral clones, pNL4-3.LCUR.E- (46) obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, and donated by N. Landau, New York University School of Medicine) in the presence or absence of 10 μg pHTE-1. Sodium butyrate medium was changed to normal growth medium after 24 h, and the viral supernatants were harvested after an additional 24-h incubation by centrifugation at 2500 × g and filtration through a 0.20-μm microfilter. For transduction of HeLa or HOS cells, triplicate samples of 7 × 105 target cells in 1 ml were incubated with 1 ml undiluted or serially diluted virus stock and plated into wells of a 6-well tissue culture dish. Following overnight incubation at 37°C/5% CO2, cells were harvested in PBS, assayed for luciferase activity following the manufacturer’s instructions using the Luciferase Assay System (Promega) and a Turner Designs TD-20/20 luminometer (Sunnyvale, CA). For Ab inhibition experiments, 1 ml HTLV-1 Env-pseudotyped virus was incubated with 1 ml HeLa or HOS cells (4 × 105) in the presence or absence of serial dilutions of purified mAb. After overnight culture, the cells were lysed and assayed for luciferase activity using the Promega Luciferase Assay System, as directed by the manufacturer.

**Flow cytometry**

HTLV-1–infected MT2 and noninfected (control) Sup-T1 cells at a density of 105 cells/ml were mixed with 0.1 ml cell-free hybridoma culture supernatant or 1 μg/ml purified mAbs and incubated at room temperature for 1 h with rotation. The cells were pelleted at 1600 rpm for 5 min, in an Eppendorf C5415C microfuge, washed, and incubated with anti-mouse FITC–labeled Ab (1:1000 dilution) in RPMI 1640 medium at room temperature for 1 h in the dark. The cells were washed (PBS/0.1% sodium azide), fixed (0.5% paraformaldehyde in PBS [pH 7.4]), and kept in the dark at 4°C until analyzed by flow cytometry (FACScan; BD Biosciences).

**Microscopy**

MT2 cells (2 × 105 in 2 ml) were incubated with 250 μl SU MAb hybridoma culture supernatant at 37°C/5% CO2 for 20 min and plated onto poly-l-lysine–coated glass coverslips (Sigma-Aldrich). The cells were incubated at 37°C/5% CO2 on the coverslips for 60 min. Cells were washed with PBS, fixed with 3% paraformaldehyde for 15 min at room temperature, and then blocked (PBS, containing 10% FBS and 0.25% BSA). The cell-bearing coverslips were carefully washed three times in blocking buffer and incubated in the dark with anti-mouse IgG-FITC (1:750; Sigma-
Aldrich) at room temperature for 1 h. Coverslips were mounted on glass slides with Mowiol (Mowiol 4-88; Calbiochem) mounting medium or with Vectashield (Vector Laboratories) for differential interference contrast microscopy. Slides were sealed with nail varnish and left to dry in the dark before examination by fluorescence microscopy and/or differential interference contrast imaging using a Zeiss Axioplan 2 microscope with Openlab (Improvement) data acquisition software.

Isolation of peripheral blood polymorphonuclear neutrophils

Buffy coat fractions from healthy donors were obtained from the Scottish Blood Transfusion Service. Polymorphonuclear neutrophils (PMN) were obtained by standard procedures including dextran sedimentation to remove erythrocytes and platelets, hypotonic lysis to remove remaining erythrocytes and platelets, and Ficol sedimentation to separate mononuclear cells from neutrophils. In brief, buffy coat fractions from donors (50 ml each) were layered onto citric acid, sodium citrate, and dextran (ACD) solution with 1 ml ACD for every 5 ml blood. Dextran solution (6% dextran 0.9% NaCl) was then added to the ACD/blood mixture. The ACD/dextran blood mixture was incubated at room temperature for 45 min, or until sedimentation was complete. The separated supernatant was centrifuged at 1000 rpm (Heraeus; Labofuge 400) for 12 min at 4°C without break. The cell pellet was resuspended in ice-cold ddH2O, and 0.6 M KCl was added to the cell suspension with 1 ml KCl for every 3 ml cell/H2O suspension. Cells were recovered by centrifugation at 1000 rpm for 6 min at 4°C with break, and the process was repeated until no RBCs remained. The cell suspension was layered onto Ficoll-Hypaque (density 1.077; Sigma-Aldrich) and centrifuged at 1200 × g for 30 min at 4°C, and mononuclear cells were collected at the Ficoll interface, whereas the neutrophils sedimented to the bottom of the Ficoll, where they were recovered. Puriﬁed neutrophils were ready. The MT2 cells were washed once with PBS, PBS, and HBSS/BSA, in turn, and 100 μl luminol (4-amino- phenylhydrazide [Sigma-Aldrich]; 25 × 10−3 M in HBSS/BSA) was added per well. Subsequently, 2 × 106 neutrophils were added to each well. The cells, in the presence of luminol, were incubated for 10 min at 37°C/5% CO2 and centrifuged at 1000 rpm for 3 min to precipitate cells. The plate was immediately transferred to the luminometer, and the luminescence of each sample was determined at 1-min intervals over a period of 30 min. Chemiluminescence was recorded as relative light units. The maximal relative light units were determined for each Ab-sonopied sample and control. Maximal relative light values were routinely obtained within 5 min of initiating the assay.

Immunoprecipitation of HTLV-I virions with SU-specific mAbs

SU mAb (1 μg) in PBS was incubated with 50 μl μMACS protein A microbeads (Miltenyi Biotec) for 30 min on ice. MT2 cell-free supernatant (1 ml) was subsequently incubated with the Ab-conjugated microbeads for 20 min at room temperature with mixing. The microbeads were captured on μ columns (Miltenyi Biotec) and washed with five-column volumes of wash buffer. Intact virions were eluted from the magnetic microbeads following the protocol of the manufacturer. The RNA was extracted from captured virions using TRizol (Invitrogen) and used for RT-PCR using published RT-PCR primers (49); the remaining eluate was prepared for analysis of viral protein by Western blotting.

Results

To investigate the immunological properties of HTLV-I envelope, a functional soluble form of recombinant SU (sSU), sRgp46-Fc, which includes amino acid residues Ser1 to Ser108 of envelope (amino acid coordinates based on the unprocessed envelope primary translation product), was concentrated and purified by Con A and protein A affinity chromatography (43). The receptor-binding activity of the purified protein was confirmed by the proteins’ ability to bind in a dose-dependent and saturable manner to Jurkat and Sup-T1 CD4+ cells line in flow cytometry assays (43). The functional sRgp46-Fc was used to immunize CD1 mice, which responded robustly to the immunogen, and subsequently, splenocytes were recovered from three mice with high Ab titres (endpoint dilution >1:20,000). Splenocytes from individual mice were fused to SP2/0-Ag14 or, alternatively, NSO-1 myeloma cells and hybridomas selected using standard methods. Over 1700 hybridomas were recovered, of which 168 were positive for activity to the immunogen. From this group of hybridomas, based on the hybridoma fusion partner, mouse donor, Ab isotype, reactivity with the recombinant Ag, and failure to interact with human IgG, 18 independent hybridomas reactive with sRgp46-Fc were selected for detailed study (Fig. 1A). The selection criteria reduced the likelihood that multiple mAbs were derived from the same clonally expanded splenocyte. Each of the mAbs bound to sRgp46-Fc, but did not bind to the control tissue plasminogen activator (tpa)-Fc fusion protein (Fig. 1A), indicating that the binding activity was directed at the SU component of the envelope-Fc fusion protein. Isotyping assays indicated that the panel included six IgG1, six IgG2a, five IgG2b, and one IgM Abs (Table I).

Representative mAbs were purified from conditioned medium, and the relative binding activities were compared in dose-dependent sRgp46-Fc–binding assays (Fig. 1B). Although these ELISA-based assays do not allow accurate calculation of dissociation constants (Kd) for the Abs, the concentration of Ab required to give half-maximal binding can be used as a comparator.

![FIGURE 1](http://www.jimmunol.org/) Murine mAbs bind to HTLV-I SU. A, mAbs derived from immunized mice were examined for reactivity to sRgp46-Fc and the carrier control protein tpa-Fc by ELISA. The Abs (sRgp46-Fc and tpa-Fc) were coated onto the wells of a 96-well plate; following incubation with conditioned medium supernatant from each of the hybridomas, the plates were washed and bound Ab was detected. Background reactivity to the control protein tpa-Fc was subtracted from each of the data points shown. The negative control represents the signal obtained from the secondary Ab in the absence of a primary SU-specific murine mAb. B, Dose-dependent binding of purified mAbs to sRgp46-Fc. mAbs at the concentrations indicated were incubated with immobilized sRgp46-Fc. Following washing, bound Ab was detected. All data represent the mean ± SD of triplicate assays.
To begin to characterize the regions of SU recognized by SU-specific mAbs, we compared the reactivity of the mAbs to sRgp46-Fc, two envelope deletion constructs, and a control Fc protein. These constructs encode SU or its derivatives fused to the Fc region of human IgG (Fig. 2A) and are reactive with Fc-specific control antisera. In ELISA, all mAbs bound to sRgp46-Fc, but not to a control tpa-Fc product (Fig. 2B). Moreover, mAbs CSK2-71, CSK4-34, CSK11-5, CSK16-13, CSK-N30, and CSK-N37 bound efficiently to an envelope construct, CTD-Fc, which lacks amino acid residues 25–190 of SU (Fig. 2). The region deleted in these constructs encodes all of the amino acids currently known to be important for binding to the receptor Glut-1 (32). Therefore, CSK2-71, CSK4-34, CSK11-5, CSK16-13, CSK-N30, and CSK-N37 recognize epitopes that lie in the CTD of SU, in a region that is not required for interaction with Glut-1. By contrast, most of the remaining mAbs bound only to envelope proteins that included the N-terminal receptor-binding domain (RBD-Fc) of SU (residues Ser25 to Leu190), but did not bind to envelope fragments that lack these sequences (Fig. 2), indicating that the majority of the mAbs recognize the RBD of SU. Only CSK2-43 bound to both the RBD-Fc and CTD-Fc proteins, but did not bind to tpa-Fc, indicating that this Ab most likely recognizes an epitope that overlaps the junction of the RBD and CTD (Fig. 2B). In addition, each mAb was examined for detection of denatured Ag by Western blot analysis against sRgp46-Fc, rSU (without the Fc-tag), and envelope-derived fragments (Fig. 2C). Although raised against intact functional Ag, all of the mAbs displayed efficient binding to denatured envelope, including the untagged SU. Therefore, the Abs

### Table I. Isotype and affinity of HTLV-1 SU mAb

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>Isotype and Subclass</th>
<th>Half-Maximum Binding (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSK1-9</td>
<td>IgG2a</td>
<td>0.026</td>
</tr>
<tr>
<td>CSK2-43</td>
<td>IgG1</td>
<td>ND</td>
</tr>
<tr>
<td>CSK2-71</td>
<td>IgG1</td>
<td>0.144</td>
</tr>
<tr>
<td>CSK3-13</td>
<td>IgG1</td>
<td>18.40</td>
</tr>
<tr>
<td>CSK4-13</td>
<td>IgG2b</td>
<td>ND</td>
</tr>
<tr>
<td>CSK4-24</td>
<td>IgG1</td>
<td>0.016</td>
</tr>
<tr>
<td>CSK4-34</td>
<td>IgG1</td>
<td>0.222</td>
</tr>
<tr>
<td>CSK5-37</td>
<td>IgG2a</td>
<td>0.037</td>
</tr>
<tr>
<td>CSK7-12</td>
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<tr>
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<td>IgG2b</td>
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</tr>
<tr>
<td>CSK11-18</td>
<td>IgG2b</td>
<td>0.075</td>
</tr>
<tr>
<td>CSK11-81</td>
<td>IgG2a</td>
<td>0.007</td>
</tr>
<tr>
<td>CSK11-83</td>
<td>IgM</td>
<td>ND</td>
</tr>
<tr>
<td>CSK15-18</td>
<td>IgG2a</td>
<td>0.025</td>
</tr>
<tr>
<td>CSK16-13</td>
<td>IgG2b</td>
<td>0.315</td>
</tr>
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<td>0.099</td>
</tr>
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</tr>
<tr>
<td>CSK-N37</td>
<td>IgG1</td>
<td>0.275</td>
</tr>
</tbody>
</table>

FIGURE 2. Mapping of SU domains recognized by mAbs. The constructs used to map regions of SU recognized by mAbs are shown. A, Each construct is preceded by the signal sequence from human tpa to aid secretion, and the boxes represent HTLV-1 envelope sequences; sRgp46-Fc includes amino acid residues Ser25 to Ser308 of envelope; RBD-Fc includes amino acids Ser25 to Leu190, and CTD-Fc includes residues Leu190–Ser308, and each construct is fused to the Fc region of human IgG; the thin line represents deleted amino acid residues. B, Each mAb was examined for reactivity to sRgp46-Fc, or its truncated derivatives CTD-Fc and RBD-Fc, or the control protein tpa-Fc by ELISA. The Ags (sRgp46-Fc, CTD-Fc, RBD-Fc, and tpa-Fc) were coated onto the wells of a 96-well plate; following incubation with conditioned cell-free medium supernatant from each of the hybridomas, the plates were washed and bound Ab was detected. A control mAb specific to the Fc region of human IgG was included as a control. C, Each mAb was examined for recognition of denatured sRgp46-Fc, or its truncated derivatives CTD-Fc and RBD-Fc, the control protein tpa-Fc, or untagged rSU by Western blotting. Conditioned medium supernatants from untransfected S2 cells were included as negative controls for SU specificity. Typical results for a representative sample of the mAbs are shown. The reactivity of the remaining mAbs are given in Table II.
are not dependent on the conformation of the Ag for efficient binding and most likely recognize linear epitopes of SU. The Western analysis also confirmed the domain specificity of each mAb. Candidate immunogens for subunit vaccines must routinely and robustly elicit Abs that recognize the native, extensively glycosylated, and trimeric structures of viral envelope. To explore the reactivity of anti-SU mAbs with virally expressed envelope, several complementary assays were employed. First, binding of each mAb to HTLV-1–infected MT2 cells was examined by flow cytometry. The majority of the mAbs bound efficiently to the HTLV-1–infected cells (Fig. 3A, 3B), whereas none of the mAbs bound to the control Sup-T1 cells. These results indicate that the majority of the mAbs raised against sRgp46-Fc recognize viral envelope displayed on the surface of infected human T cells. By contrast, several mAbs, CSK2-43, CSK3-13, and CSK11-83, bound poorly to HTLV-1–infected cells (Fig. 3B), but bound effectively to sRgp46-Fc (Figs. 1, 2) and recognize SU by Western blotting (data not shown). The simplest and, in our view, most likely explanation for these results is that the epitopes recognized by these Abs are masked or occluded on the intact viral envelope complex and that removal of SU from TM, or denaturation of SU, increases the exposure of these epitopes. However, Env has been observed to interact with neuropilin 1 and Glut-1 in HTLV-1–infected cells (21, 22); consequently, an alternative scenario is that such interactions may prevent binding of these mAbs to envelope on infected cells. Although useful in Western analysis, due to their lack of reactivity with native viral envelope, mAbs CSK2-43, CSK3-13, and CSK11-83 were not studied further. Generally, mAbs that displayed strong relative binding to sRgp46-Fc also exhibit robust binding to infected T cells.

To visually confirm binding of mAbs to viral envelope, chronically infected MT2 cells were probed with SU-specific mAbs and examined by fluorescence microscopy. The majority of mAbs that were reactive with envelope in flow cytometry studies also demonstrated binding to viral envelope by fluorescence imaging. Typically, mAbs exhibit homogenous staining around the plasma membrane of unstimulated MT2 cells (Fig. 3C) consistent with the expected localization of viral envelope. No staining was observed with uninfected T cell lines by either fluorescence microscopy or flow cytometry (Supplemental Fig. 1), reinforcing the view that the cell staining observed on MT2 cells is due to specific recognition of the SU component of the viral envelope glycoprotein complex.

**Ab-dependent neutralization of envelope-mediated membrane fusion and viral entry**

Patient sera and mAbs derived from infected individuals interfere with viral infection of cells, and this neutralizing activity is primarily directed at viral envelope. We therefore examined the murine mAbs for the ability to block binding of SU to cells, envelope-mediated membrane fusion, and viral entry into cells. First, the ability of the mAbs to block binding of SU to uninfected T cells was examined by flow cytometry (Fig. 4). Many, but not all, of the mAbs were effective inhibitors of SU binding to uninfected Sup-T1 cells. In particular, CSK1-9, CSK7-12, and CSK11-18 were all potent inhibitors of SU binding, whereas CSK4-24, CSK4-34, CSK11-81, CSK16-47, CSKN-30, and CSKN-37 exhibit modest inhibition of SU binding, and the remaining mAbs show little, if any, inhibitory activity. Thus, one potential and predictable mechanism by which these Abs inhibit HTLV-1 viral entry is via direct steric occlusion of receptor binding. Surprisingly, a few mAbs, typified by CSK11-83, appear to enhance binding of sRgp46-Fc to cells. It is not yet known how these mAbs promote binding of soluble SU to cells, but this observation is discussed in more detail below.

Based on the above data, a group of the mAbs typical of the various Ab classes and RBD or CTD specificities was selected for high-yield purification and examined for the ability to inhibit membrane fusion in syncytium interference assays (Fig. 5A). HeLa cells transfected with the subgenomic HTLV-1 envelope expression construct pHTE-1 were cocultured with nontransfected HeLa target cells in the presence of purified mAb. In the absence of Ab, or in the presence of irrelevant anti–HIV-1 gp120 control mAb (178.1), extensive membrane fusion and syncytia production were observed (Fig. 5A, 5B). By contrast, several of the anti-SU mAbs, for example CSK7-12, CSK11-18, CSK4-24, and CSK11-9, efficiently blocked syncytium formation, as observed by low-power microscopy (Fig. 5A, 5B). Although CSK7-12 has modest relative binding activity to gp46 (Fig. 1B, Table I), mAbs CSK11-18, CSK4-24, and CSK11-9 exhibit strong relative binding to rSU, and all of these mAbs efficiently recognize viral envelope in flow cytometry assays. In contrast, some mAbs, CSK4-34, CSK15-18,
and CSK16-47, antagonized membrane fusion weakly even at high mAb concentrations. Notably, although CSK16-47 binds to the RBD of SU, is among the Abs with highest relative binding activity to monomeric SU, and very efficiently binds to virally infected cells, it is a relatively weak inhibitor of syncytium formation even at high Ab concentrations (Fig. 5A). By comparison, the most potently neutralizing Abs within this panel, CSK7-12, CSK11-18, CSK4-24, and CSK1-9, also recognize the RBD of SU. Therefore, mAbs CSK7-12, CSK11-18, CSK4-24, and CSK1-9 were compared directly for inhibition of membrane fusion (Fig. 5C). In each case, the Abs displayed potent dose-dependent inhibition of membrane fusion, and CSK7-12, an Ab with modest relative binding activity for SU, was consistently the most effective inhibitor in these assays (Fig. 5C). By comparison, the most potently neutralizing Abs within this panel, CSK7-12, CSK11-18, CSK4-24, and CSK1-9, also recognize the RBD of SU. Therefore, mAbs CSK7-12, CSK11-18, CSK4-24, and CSK1-9 were compared directly for inhibition of membrane fusion (Fig. 5C). In each case, the Abs displayed potent dose-dependent inhibition of membrane fusion, and CSK7-12, an Ab with modest relative binding activity for SU, was consistently the most effective inhibitor in these assays (Fig. 5C). By contrast, CSK1-9, a mAb with high relative binding affinity, was less effective than CSK7-12 (Fig. 5). Therefore, the ability to inhibit membrane fusion does not correlate strictly either with the efficiency of binding to envelope or recognition of the RBD.

To examine the ability of the mAbs to block envelope-mediated entry of free viral particles, envelope-deficient HIV-based luciferase-transducing viral particles were pseudotyped with HTLV-1 envelope and used in interference of infectivity assays. Each of the mAbs that efficiently blocked syncytium formation also blocked entry of HTLV-1 envelope-pseudotyped viral particles (HTLV-1pp). Robust transduction of luciferase was observed in the absence of Ab or, alternatively, in the presence of increasing doses of an irrelevant control Ab. By contrast, a strong and dose-dependent inhibition of HTLV-1pp entry and luciferase transduction was observed for mAbs CSK7-12, CSK4-24, and CSK1-9 (Fig. 6). Again, inhibition of HTLV-1pp entry did not correlate strictly with Ab affinity; although CSK1-9 is one of the strongest binding Abs, it did not inhibit as potently as CSK7-12, especially at low Ab concentrations.
Both neutralizing and nonneutralizing mAbs bind to viral particles

It was intriguing that despite recognizing SU and interacting with envelope on the surface of infected cells, some mAbs are unable to inhibit membrane fusion or neutralize the entry of HTLV-1pp. A plausible explanation for the lack of neutralizing activity of some SU-reactive mAbs is that, contrary to neutralizing mAbs, nonneutralizing mAbs do not bind effectively to trimeric envelope displayed on the surface of cells or virions. We therefore tested the ability of two neutralizing mAbs (CSK1-9 and CSK11-81), one weakly neutralizing (CSK16-47), and one nonneutralizing (CSK16-13) mAb of similar relative binding affinity to immunoprecipitate HTLV-1 particles produced by chronically infected MT2 cells. Surprisingly, both neutralizing and poorly neutralizing mAbs were effective at capturing virus particles (Fig. 7), indicating that the poor neutralizing activity is not associated with a failure to recognize viral envelope on the virion surface.

Ab-dependent recruit and activation of immune effector cells to HTLV-infected cells

Direct neutralization of virus by envelope-specific Ab is important for prevention of de novo viral infection of cells, but, in addition, Ab effector functions contribute significantly to a robust immune response. We therefore sought to test whether neutralizing and nonneutralizing mAbs produced in response to vaccination with a soluble recombinant Ag can mobilize cell-mediated immune responses to HTLV-1-infected cells. Each of the isolated mAbs was examined for the ability to enhance the Ab-dependent cytotoxic respiratory burst of PMN using an established chemiluminescence-based in vitro assay (48). Samples of HTLV-1–infected MT2 cells were opsonized with each of the independent SU-specific mAbs and dispensed into 96-well plates. Subsequently, purified PMN and luminol were added, and the plates were immediately assayed for activation of the PMN respiratory burst, as determined by hydrogen peroxide production and luminol turnover detected as light emission. Maximal relative light output developed within 5 min of PMN addition and decayed over time. PMN incubated with the uninfected T cell line Sup-T1 did not exhibit activation of the respiratory burst (Fig. 8). However, even in the absence of Ab, a very low, but reproducible level of activation of the PMN respiratory burst was noted in the presence of HTLV-1–infected T cells. By contrast, MT2 cells opsonized with anti-SU mAbs markedly enhanced the PMN respiratory burst (Fig. 8) and, significantly, both robustly and weakly neutralizing Abs induced strong responses in these assays. In particular, CSK11-18 and CSK16-47 were just as effective at stimulating a PMN respiratory burst despite the fact that CSK11-18 is one of the more robustly neutralizing Abs and CSK16-47 is only weakly neutralizing (Table II). Thus, the ability to recruit and activate immune effector cells is not tightly coupled to the neutralizing proficiency of HTLV-specific Abs.

Discussion

HTLV-1 infections induce adaptive immune responses to viral Ag, but, despite robust immune activation, infected individuals fail to clear virus and virally infected cells. The accumulating data suggest that there is an ongoing war of attrition between the immune system and the virus, wherein the immune response acts to eradicate the virus and the virus employs diverse, but inadequately understood mechanisms to avoid immune surveillance. A clear view of the immunological pathways and viral targets required for successful suppression or eradication of HTLV-1 replication is critical to the development of vaccines and novel immunological strategies to combat HTLV-1–associated disease. As a step toward this objective, we in this study demonstrate that a recombinant HTLV-1 envelope-derived immunogen displays properties of value to subunit vaccine design and induces robust humoral immune responses in mice. The immunological properties of sRgp46-Fc

FIGURE 8. Anti-SU mAbs recruit PMN to HTLV-1–infected cells and activate the neutrophil respiratory burst. HTLV-1–infected MT2 cells were opsonized with each of the indicated anti-SU mAbs and mixed with freshly isolated human neutrophils. The PMN respiratory burst was assayed by standard procedures using a luminol turnover method (48). The respiratory burst of neutrophils, shown as maximal relative light units, in the presence of opsonized MT2 cells, was compared with neutrophils incubated in the presence of MT2 cells opsonized by an irrelevant anti-murine Fc mAb, anti-Fc; or with MT2 cells in the absence of Ab, PMN + MT2; or neutrophils in the presence of uninfected Sup-T1 cells, PMN + Sup-T1; or unstimulated neutrophils, PMN.
that some mAbs (CSK1-9, CSK7-12, CSK11-18) effectively binding and entry process. In particular, our data demonstrate appears to be sensitive to neutralization throughout the receptor-

the neutralizing activity of TM-targeted mAbs. Conversely, SU

of TM is poorly accessible to Ab and is therefore protected from suggest that in the fusion-active envelope structure, the coiled coil

inhibit either envelope-mediated membrane fusion or viral entry indicate that such mAbs may interfere with coreceptor or cofactor recruitment (38, 39) or, alternatively, may prevent the receptor-induced changes in SU conformation that are required to activate the fusogenic properties of envelope. Understanding the molecular mechanism by which these mAbs neutralize viral entry will provide greater insight into envelope function and the immunology of HTLV-1 infections.

Alkylation studies coupled with elegant cryo-electron microscopy (23, 24, 41, 53) demonstrate that, upon receptor binding, the envelope protein of MLV undergoes a radical change in conformation. Receptor binding induces an isomerization and disruption of the SU-TM intersubunit thiol, which triggers the reorganization of the SU subunits to form an open ring-shaped structure. It has been suggested that the TM subunit may extend through the center of the trimeric SU complex to induce membrane fusion (41). If this is indeed the case, the critical fusion-active surfaces of TM are likely to be sterically shielded from neutralizing Ab due to the presence of the SU subunits and the proximity of the viral and cell membranes. By comparison, SU subunits are likely to have surfaces exposed to Ab in the prefusogenic state and following binding of SU to cell surface receptors. Our observations that some SU-specific Abs block the postreceptor-binding steps of entry indicate that such mAbs may interfere with coreceptor or cofactor recruitment (38, 39) or, alternatively, may prevent the receptor-induced changes in SU conformation that are required to activate the fusogenic properties of envelope. Understanding the molecular mechanism by which these mAbs neutralize viral entry will provide greater insight into envelope function and the immunology of HTLV-1 infections.

A perplexing aspect of these studies is that despite efficient recognition of the SU on infected cells and the ability to bind to envelope on viral particles, some mAbs are poor inhibitors of membrane fusion and viral entry. It is difficult to reconcile binding of a large Ab (150 kDa) to a small surface glycoprotein (46 kDa) without loss of protein function. Indeed, for HIV-1, a compelling argument has been made that if an Ab binds to the wild-type envelope complex, it will neutralize (54–57). Consistent with this notion, a heterologous Ab can inhibit and neutralize HIV-1 infectivity when the epitope recognized by that Ab is engineered into the gp120 subunit of HIV-1 envelope (56, 57). By contrast,

have enabled the selection of a diverse panel of SU-specific mAbs that will facilitate analysis of the immunological features of envelope.

Significantly, the murine mAbs exhibit properties that are typical of Abs produced during natural infections. The mAbs bind to virally expressed envelope displayed both on infected cells and, most importantly, on virion surfaces. The majority of the mAbs possess some neutralizing activity, and a few are robustly neutralizing in assays of membrane fusion and pseudovirus entry. The neutralizing activity of mAbs in syncytium interference assays is particularly pertinent to natural infections because much of the viral dissemination in infected individuals occurs by transfer of virus between cells across regions of close cell-to-cell contact (50, 51). Syncytium formation may therefore be viewed as an extreme outcome of envelope-mediated processes that naturally occur within regions of close membrane apposition. Notably, the most effective neutralizing activity is associated with Abs that recognize the RBD of SU. Nevertheless, despite similar relative binding efficiencies, there are marked differences in the neutralizing pro-
ficiencies of RBD-specific mAbs, indicating that not only affinity,

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current models of Ab activity against hepatitis C virus suggest that neutralizing activity is confined to Abs that bind to a limited set of functionally important epitopes on the viral E2 envelope protein (58, 59). Such differences in neutralizing activity may reside in the precise and mutually distinct arrangement of the envelope complexes of these highly divergent viruses. For HTLV-1, it remains to be determined whether binding of an Ab to the envelope complex is compatible with envelope function, or whether Ab must be bound to a restricted set of critical epitopes for neutralization to occur. Previously, we have provided evidence that nonfunctional forms of envelope exist on the surface of infected cells and that fusion-incompetent forms of envelope may account for the binding of nonneutralizing Ab to envelope-expressing cells (29, 30). The ability of nonneutralizing or poorly neutralizing Ab to bind to virus particles may reflect that nonfunctional forms of envelope are also found on virions. If this proves to be the case, the display of nonfunctional envelope structures may act as decoy Ags that are used by the virus to confound and evade an effective immune response. Further studies are required to test these views.

Intriguingly, a few mAbs, for example CSK11-83 (Fig. 5B), weakly, but consistently enhance binding of HTLV-1 SU to cells. At present, we do not know how this is achieved, but we suspect that such Abs lock the soluble SU in a particular conformation that is primed and highly amenable to receptor binding. In view of this possibility, it should be noted that CSK11-83 binds well to sRgp46-Fc, but poorly to native trimeric envelope displayed on the surface of infected cells. It may be that mAbs such as CSK11-83 recognize surfaces of sRgp46 that are normally buried at the interface of the SU subunits within the envelope trimer; in binding to these surfaces, the Ab may provide additional structural rigidity to the soluble SU. These ideas will be tested in future fine-mapping studies.

A significant finding of our study is that both neutralizing and weakly neutralizing Abs recruit and robustly activate the cytotoxic responses of neutrophils. A recent study also demonstrated that nonneutralizing Abs are capable of recruiting complement to envelope and envelope-expressing cells (29). Our observations indicate that, in addition to neutralizing activity, Ab effector function is likely to play a significant role in controlling HTLV-1 dissemination and viral burden during natural infections. Ab-dependent cellular cytotoxicity may be of particular benefit in suppression of HTLV-1 and HTLV-1–associated disease as the vast majority of de novo viral infections occur by direct transfer of virus from infected cell to target cell through sites of intimate cellular contact, known as the virological synapse, rather than through infection by free viral particles (50). Due to the close contact of the infected cell and uninfected cell membranes during cell-to-cell transmission, HTLV-1 may be protected from the neutralizing properties of Ab, but, because of cell surface display of envelope, infected cells should remain sensitive and vulnerable to Ab-dependent cellular cytotoxicity and Ab-mediated complement fixation. Nonetheless, despite compelling evidence that effector function has a profound impact on the success of passive immunotherapy for HIV-1, for other viral infections, and for treatment of solid tumors (60, 61), the role of Ab effector function has been largely neglected in studies of immune control and pathogenesis of HTLV-1 and other viral infections (61). Although clearly important, neutralizing proficiency is only one aspect of Ab function that should be considered in the design of effective immunotherapies to control viral load and HTLV-1–associated disease.

Vaccine strategies remain the primary means of combating viral infections of humans, and are remarkably efficient and cost effective. Indeed, vaccines have provided significant clinical success in the prophylactic treatment of virally associated neoplastic diseases (62–64). However, vaccines targeting human retroviral infections, such as HIV-1, pose unique technical challenges and have often suffered disappointing setbacks. Nevertheless, HTLV-1 is an attractive target for vaccine development. The HTLV-1 envelope glycoprotein shows relatively little sequence variation in and between infected individuals (65); envelope is a primary target for humoral and cell-based immune responses in natural infections; an attenuated strain of the related bovine leukemia virus induces sustained resistance to experimental bovine leukemia virus challenge (18); an envelope-based HTLV-1 immunogen provides protection in primate models of HTLV-1 infection (16, 17); and finally, infants born to HTLV-1–infected mothers are protected from HTLV-1 infection in the first months of life by maternally acquired Abs (15). Collectively, these observations suggest that an effective envelope-based subunit vaccine is an achievable objective for therapeutic intervention in HTLV-1 infections.

There are significant challenges to address before an effective HTLV-1 vaccine can be realized. For example, the native prefusogenic structures and fusion-active forms of envelope may both be targeted by Ab, but it is not clear which of these forms is most appropriate to vaccine design. Moreover, it is probable that a trimeric form of envelope would produce a more effective, longer-lasting response than a monomeric subunit approach, but this awaits experimental validation. Our study provides information and insight into the way that HTLV-1 envelope interacts with the humoral immune response and how these interactions may be important for viral pathogenesis and ultimately for future vaccine initiatives. A successful vaccine strategy for HTLV-1 would have considerable clinical impact and could act as a useful pathfinder for the more intractable and highly mutable viruses such as HIV-1.

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Disclosures
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References


