Elimination of Retroviral Infectivity by N-Ethylmaleimide with Preservation of Functional Envelope Glycoproteins

David R. Morcock, James A. Thomas, Tracy D. Gagliardi, Robert J. Gorelick, J. David Roser, Elena N. Chertova, Julian W. Bess, Jr., David E. Ott, Quentin J. Sattentau, Ines Frank, Melissa Pope, Jeffrey D. Lifson, Louis E. Henderson, and Bruce J. Crise

AIDS Vaccine Program, SAIC Frederick, National Cancer Institute at Frederick, Frederick, Maryland; The Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom; and Center for Biomedical Research, Population Council, New York, New York

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The zinc finger motifs in retroviral nucleocapsid (NC) proteins are essential for viral replication. Disruption of these Cys-X2-Cys-X2-His-X4-Cys zinc-binding structures eliminates infectivity. To determine if N-ethylmaleimide (NEM) can inactivate human immunodeficiency virus type 1 (HIV-1) or simian immunodeficiency virus (SIV) preparations by alkylating cysteines of NC zinc fingers, we treated infectious virus with NEM and evaluated inactivation of infectivity in cell-based assays. Inactivation was rapid and proportional to the NEM concentration. NEM treatment of HIV-1 or SIV resulted in extensive covalent modification of NC and other internal virion proteins. In contrast, viral envelope glycoproteins, in which the cysteines are disulfide bonded, remained intact and functional, as assayed by high-performance liquid chromatography, fusion-from-without analyses, and dendritic cell capture. Quantitative PCR assays for reverse transcription intermediates showed that NEM and 2,2'-dipyridyl disulfide (aldrithiol-2), a reagent which inactivates retroviruses through oxidation of cysteines in internal virion proteins such as NC, blocked HIV-1 reverse transcription prior to the formation of minus-strand strong-stop products. However, the reverse transcriptase from NEM-treated virions remained active in exogenous template assays, consistent with a role for NC in reverse transcription. Since disruption of NC zinc finger structures by NEM blocks early postentry steps in the retroviral infection cycle, virus preparations with modified NC proteins may be useful as vaccine immunogens and probes of the role of NC in viral replication.

Retroviral nucleocapsid (NC) proteins are critically involved in multiple steps of the retroviral replication cycle, both as a domain within the Gag polyprotein and in the mature protein form (reviewed in reference 19). As part of the Gag precursor, NC functions include recognition and packaging of the viral genome (50, 64) and possibly the placement of the tRNA on the primer-binding site (22). Once it is liberated from the Gag polyprotein by the viral protease, the small and highly basic NC protein functions as a chaperone during reverse transcription and integration and also protects newly synthesized viral DNA (vDNA) (10, 13, 14, 26, 49, 50, 64).

In all orthoretroviruses, the NC protein contains one or two zinc finger domains with the common sequence motif Cys-X2-Cys-X2-His-X4-Cys, which binds a zinc ion in a tetrahedral coordination complex (6, 7, 16, 58). Genetic or chemical disruption of the zinc finger domain emphasizes its critical role in virus replication. Site-directed mutagenesis studies indicate that these zinc finger structures within NC are required for essential functions in the viral replication cycle (29, 32, 33). Additionally, reagents with sufficient oxidative potential have been shown to disrupt the zinc finger structure in vitro (51, 60) and are also capable of inactivating human immunodeficiency virus type 1 (HIV-1) (52). These observations suggested that selective covalent modification to disrupt the structure of these zinc fingers can eliminate the infectivity of retroviral virions.

We previously showed that mild oxidizing reagents such as 2,2'-dipyridyl disulfide (aldrithiol-2 [AT-2]) eliminate infectivity of HIV-1 (56) and simian immunodeficiency virus (SIV) (1), both of which contain two zinc fingers in their NC proteins. This treatment oxidizes zinc-bound thiols in the NC protein and free sulfhydryl groups of internal virion proteins, inducing intermolecular disulfide bonds and extensive cross-linking of NC and other proteins containing free cysteine thiols. The disulfide-bonded cysteines of the envelope glycoproteins are unaffected, allowing preservation of functional envelope glycoproteins on the treated virions.

The zinc-coordinated thiols on purified NC protein are susceptible to alklylation by N-ethylmaleimide (NEM) (18), which reacts with protein thiol groups to form a stable S-(N-ethylmaleimido) cysteine derivative (53). NEM does not generate cross-linked oligomers of NC, in contrast to the disulfide structures induced by AT-2. Additionally, NEM has been shown to inactivate the RNase H enzymatic activity of HIV-1 reverse transcriptase (RT) while leaving the RNA-dependent DNA polymerase activity intact (35). NEM also inhibits the assembly of functional integrase-DNA complexes (20). We thus explored whether NEM could inactivate HIV-1 and SIV virions.

Our data indicate that NEM treatment modifies the core proteins of intact HIV-1 and SIV virions without disruption of glycoprotein function. Significantly, quantitative molecular assays showed that NEM treatment of virions prevented the formation of minus-strand strong-stop DNA with negligible effects on the polymerase function of RT in exogenous template assays. The vulnerability of retroviral NC proteins to
electrophilic attack has proven useful in the generation of whole killed virus preparations, and extension of this methodology to include NEM treatment may provide supplemental approaches to inactivate virus for vaccine applications as well as for probing NC function in intact, non-cross-linked virions.

**MATERIALS AND METHODS**

**HCLZ infectivity assay.** The HCLZ indicator cell line, HeLa cells containing CD4 and an HIV-1 LTR-lacZ gene responsive to Tat trans-complementation (31), was similar to previously described lines (40). HCLZ cells were grown in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) supplemented with 2 mM l-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin (Invitrogen), and 10% (vol/vol) fetal bovine serum (BioWhittaker). NEM and glutathione (reduced; GSH) stock solutions in Dulbecco’s phosphate-buffered saline (PBS; Invitrogen) were made immediately prior to treatments. On the day prior to infection, HCLZ cells were seeded into 96-well plates (2,000 cells in 100 μl of DMEM per well) and cultured overnight. On the day of infection, virus-containing supernatant was harvested from HIV-1 LAI cultured in H9 cells by centrifugation at 300 × g for 5 min and treated at 37°C with 0 to 1 mM NEM. Samples were collected from 0 to 3 h and mixed with excess GSH to quench any residual, unreacted NEM. After removing medium from the HCLZ plates, serially diluted virus samples were added to the cells in duplicate wells at 100 μl per well. After 3 days in culture, the medium was removed, the wells were rinsed with 100 μl of PBS, and the cells were assayed for β-galactosidase activity.

**Dendritic cell infectivity assay.** Infected cells expressed β-galactosidase, which was detected by reaction with a chromogenic 3,4′-diaminophenylazonitrile (Koch-Light Labs, Colnbrook, Berkshire, United Kingdom) substrate and measured at 420 nm. Two days after transfection, pseudotyped virus was harvested from the culture medium, clarified by low-speed centrifugation, passed through a 0.22-μm-pore-size Millipore G5 filter (Millipore), and then treated with 2 μg of heparin (Sigma) to trap any microparticles. The virus was then concentrated for 2 h at 37°C with DMSO alone, 4 mM NEM, or 1 mM AT-2 and then quenched with excess GSH. An aliquot was taken from each treatment for exogenous template RT assays (30). HOS cells were then infected with the treated viruses, and 24 h postinfection cells were harvested from plates and total DNA was extracted from the cells using the DNA Blood mini kit (Qiagen). The 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum (HyClone) with 2 mM L-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin per ml (Invitrogen). Jurkat-Tat-CCR5 cells were derived from Jurkat-Tat cells (Centralised Facility for AIDS Reagents, Potters Bar, Hertfordshire, United Kingdom) (54) stably transfected with CCR5 linked to a hygromycin selectable marker. Stable transfecants were selected in hygromycin (50 μg/ml), analyzed with a fluorescence-activated cell sorter, and subsequently cloned by limiting dilution.

**Lymphoid cell infectivity assay.** All lymphoid cell cultures were maintained at 37°C in 5% CO2 and grown in RPMI 1640 medium (Invitrogen) supplemented with 2 mM l-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin per ml (Invitrogen), and 10% (vol/vol) fetal bovine serum (HyClone). Jurkat-Tat-CCR5 cells were maintained in the above medium supplemented with 1 mg of G418/ml and 50 μg of hygromycin/ml. The Jurkat-Tat-CCR5 cells were derived from Jurkat-Tat cells (Centralised Facility for AIDS Reagents, Potters Bar, Hertfordshire, United Kingdom) (54) stably transfected with CCR5 linked to a hygromycin selectable marker. Stable transfecants were selected in hygromycin (50 μg/ml), analyzed with a fluorescence-activated cell sorter, and subsequently cloned by limiting dilution.

**Exogenous template RT assay.** Infected cells were then transfected with the treated viruses, and 24 h postinfection cells were harvested from plates and total DNA was extracted from the cells using the DNA Blood mini kit (Qiagen). The 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum (HyClone), and 10% (vol/vol) Triton X-100. Samples were incubated with the substrate for 3 h at 37°C, and the absorbance was measured at 570 nm with the 750-nm background subtracted (Vmax kinetic microplate reader; Molecular Devices). The HIV-1 titer was determined as the greatest dilution where the average absorbance for duplicate wells was at least three times the absorbance of control wells not inoculated with virus.

**Virion uptake studies.** To verify authentic interactions of NEM-treated HIV with immune cells, a highly purified preparation of 1,000× SIV Mne E11s/HuT 78 clone E11s, purified by C45 immunoaffinity depletion of contaminating microvesicles (62), was labeled with the fluorescent NEM analog Alexa Fluor 488 C5 maleimide (Molecular Probes) as described in detail elsewhere (E. N. Cher-tova et al., unpublished data). Dendritic cells (DCs) were prepared from freshly isolated peripheral blood mononuclear cells of normal human donors as described previously (24, 63). For quantitative analysis, immature and mature DCs were incubated with increasing amounts of labeled SIV (37°C for 1 h, followed by washing). Uptake of virions was detected by flow cytometry on a FACSCalibur flow cytometer (Becton Dickinson) (24). To evaluate colocalization of the fluorescent signal from internalized, directly labeled virions with the signal from immunostained viral proteins, preparations of DCs pulsed with labeled virus (9 ng of p27Gag/107 DCs, incubated for 1 h at 37°C followed by washing) or buffer (PBS plus 1% bovine serum albumin) were adhered to alcin blue-coated slides, fixed (30 min with 4% paraformaldehyde), and permeabilized (30 min with 0.5% saponin). Next, the murine monoclonal antibody KK64 (1:1,000; NIH AIDS Research and Reference Reagent Program), which reacts with SIV p27Carboxyl terminal (amino acids 151 to 180) (38), or the isotype control (mouse immunoglobulin G1; 2 μg/ml) was added. Bound antibodies were detected using a horseradish per-oxidase-conjugated affinity-purified donkey anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories Inc.) followed by detection with a tyramide signal amplification kit using the cyanine 3 fluorophore (NEN Life Science Products). Between each step, the slides were rinsed extensively. An antifade reagent (ProLong Gold; Molecular Probes) and the nuclear counterstain DAPI (4′,6-diamidino-2-phenylindole; Molecular Probes) were added, and coverslips were mounted onto glass slides. Dried slides were imaged on a Nikon Eclipse E800 microscope equipped with a 40× oil objective, a 2× zoom, a digital camera (SPOT RT Slider; Diagnostic Instruments), z-series sections were taken every 0.2 μm through the cell, and images from sections cutting through the middle of the cells were shown. ImagePro Plus (Media Cybernetics) was used to remove out-of-focus light and for image processing.

**Reverse transcription in intact virions**

**HIV-1 MN grown in Jurkat cells and SIV Mne Cln8 grown in CEMx174/SIV Mne CLN8 (L) were tested.** Thawed samples were treated in the presence of 10% (vol/vol) fetal bovine serum and the HIV-1 was grown in medium containing 5% (vol/vol) fetal bovine serum and 5% (vol/vol) calf serum. Quantitative real-time PCR was performed using an ABI Prism 7700 detection instrument (Applied Biosystems) using the primers, probes, and PCR conditions described previously (10). Viral DNA copy numbers were normalized for cell number (by measuring the copy number of the cellular gene porphobilinogen deaminase) and for virus particles (by RT activity). The completion of early and late steps in reverse transcription was determined by measuring copy numbers of the R-U5 and R-5′ untranslated region as described elsewhere (10).

**Exogenous template RT assay.** Frozen stocks of clarified culture supernatant from infected H9 culture, H9/HIV-1 MN, uninfected CEMx174, and CEMx174/SIV Mne CLN8 (L) were thawed. Thawed stocks were treated in triplicate with 4 μM NEM, 1 μM AT-2, or mock treated for 1 h at 37°C, washed with excess GSH, and then precipitated by 10% polyethylene glycol overnight at 4°C. Exogenous Mg2+-dependent RT activity was then assayed as described previously, using a poly(A) template (34).

**Large-volume cultures.** HIV-1 MN grown in Jurkat cells and SIV Mne Cln8 (L) were tested. Ten million CEMx174 cells were inoculated and purified as described previously (7). Twenty-liter lots of clarified culture supernatant were treated for approximately 16 h at 4°C with 10 μM NEM, 1 μM AT-2, or left untreated. Virus was then purified by continuous-flow ultracentrifugation in a sucrose gradient, diluted to reduce the sucrose concentration to 20% (wt/vol), pelletted at 100,000 ×
RESULTS

NEM inactivation of HIV-1. To assess whether NEM was effective at reducing viral infectivity, aliquots of culture fluid from chronically HIV-1-infected cells were treated with increasing amounts of NEM and subsequently plated on the indicator cell line HCLZ (CD4+ HeLa cells containing a Tat-responsive β-galactosidase reporter gene). HIV-1 titer decreased rapidly and in proportion to the NEM concentration (Fig. 1A). The rate of inactivation measured over a range of NEM concentrations from 60 to 1,000 μM fit a pseudo-first-order kinetics model. For each NEM concentration examined, the inactivation rate was calculated by fitting the data to an exponential function, summarized in Fig. 1B. Selected half-lives are shown in Table 1, as are results from similar AT-2 experiments (17). At 1 mM NEM, the titer half-life of HIV-1 was 3.3 min, approximately one-fourth the rate for AT-2.

Long-term culturing of treated HIV-1 and SIV. While the HCLZ cell assay above provided a convenient and rapid method for screening viral inactivation at different concentrations of NEM, a more sensitive test for complete inactivation of virus replication can be obtained by long-term culturing of treated, concentrated virus with lymphoid cells. Therefore, to more thoroughly assess the NEM-mediated inactivation of both HIV-1 and SIV, treated culture supernatants were concentrated 1,000-fold by sucrose density gradient centrifugation, applied to lymphoid cells, and monitored over a 3-week period. Samples were collected weekly for analysis by capsid protein ELISA. NEM treatment was effective against both HIV-1 and SIV with no viral replication detected in this assay after NEM treatment. In control assays, the 50% tissue culture infective doses of untreated HIV-1 and SIV were 6.8 × 10⁶ and 2.2 × 10⁶, respectively, thus demonstrating inactivation of greater than 6 logs of infectivity by NEM treatment. The treatment appeared as effective as AT-2 treatment of HIV-1 or SIV cultures.

TABLE 1. Inactivation half-lives of HIV-1

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>NEM Titer half-life (min)</th>
<th>AT-2* Titer half-life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>23.3</td>
<td>10.7</td>
</tr>
<tr>
<td>500</td>
<td>6.0</td>
<td>1.6</td>
</tr>
<tr>
<td>1,000</td>
<td>3.3</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* Data adapted from reference 17.

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FIG. 1. (A) HIV-1 inactivation by NEM proceeds in a time- and concentration-dependent manner. HIV-1 culture supernatants were treated with NEM at concentrations from 60 to 1,000 μM for the times indicated. For clarity of presentation, only inactivation curves at three concentrations are shown. Treated virus was applied to HCLZ indicator cells to determine the decrease in viral titer at each NEM concentration. No decrease in HIV-1 titer was observed in mock-treated samples. (B) Inactivation rates of NEM- and AT-2-treated HIV-1. Inactivation half-lives for NEM-treated virus derived from inactivation curve slopes shown in panel A were plotted relative to the NEM concentration. No decrease in HIV-1 titer was observed in mock-treated samples. (B) Inactivation rates of NEM- and AT-2-treated HIV-1. Inactivation half-lives for NEM-treated virus derived from inactivation curve slopes shown in panel A were plotted relative to the NEM concentration. 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Data adapted from reference 17.
FFWO. If NEM treatment significantly modified viral envelope proteins, then the capacity of virus to mediate FFWO would be impaired. To assess this possibility, SIV Mne E11S (chosen for its high gp120 content) was treated with 4 mM NEM for 2 h at 37°C, quenched with GSH, and mixed with CEMx174 cells; FFWO was then monitored over 4 h. Treatment with NEM did not decrease the ability of treated SIV to mediate cell-to-cell fusion, as evidenced by the many syncytia present in the assay mixture (Fig. 2). Similarly, HIV-1 treated with NEM remained equally functional in the FFWO assay, inducing as many syncytia as the untreated HIV-1 (data not shown). Consistent with previous results (1), AT-2 treatment did not alter fusogenicity of SIV (Fig. 2). These results indicate that covalent modification of internal thiols did not destroy envelope function.

Virion uptake studies. Functional envelope glycoprotein is necessary for uptake by DCs (12). Using a derivatized NEM fluorophore (Alexa Fluor 488), we labeled virions directly and then assessed their interactions with DCs. Flow cytometry showed that Alexa Fluor 488-labeled SIV virions were taken up by DCs in a concentration-dependent manner, with greater uptake seen for immature DCs compared to mature DCs (Fig. 3A). Direct fluorescent imaging and z-series analysis of deconvoluted images demonstrated that the uptake of labeled virus by DCs (Fig. 3B) was comparable to results seen for unlabeled virus (data not shown) (23, 24, 63). In both cases, SIV accumulated at the periphery of immature DCs while it was concentrated deeper within mature DCs. In addition, colocalization of the Alexa 488 signal from labeled internal virion proteins (Fig. 3B) and the signal from independently immunolabeled p27Gag (Fig. 3B) or gp120SU (data not shown) associated with the virions was observed in both immature and mature DCs (Fig. 3B). Background staining was negligible for all controls (isotype staining on virus-pulsed cells and unpulsed cells as well as p27Gag staining on buffer-treated cells [data not shown]). The ability of the virus in these assays to interact with DCs underscores that the maleimide-based labeling of virus did not disrupt the functional interactions between the viral envelope and DCs.

Reverse transcription. Having shown that treated virus remains capable of fusing with cellular membranes and entering target cells, we next examined whether subsequent steps of the viral life cycle were disrupted by NEM treatment. The first enzymatic step after cell entry is reverse transcription of the RNA genome. We tested whether NEM-treated virus would be able to generate reverse transcription intermediates after inoculation on susceptible cells. We also determined whether the RT from NEM-treated virus was enzymatically active in an exogenous template assay.

HIV-1 pseudotyped with the VSV envelope protein was generated by transfection, treated with 4 mM NEM, and applied to target cells. Twenty-four hours postinfection, the level of
FIG. 3. Uptake of Alexa 488-labeled SIV by immature (upper rows) and mature (lower rows) monocyte-derived DCs. (A) Cells were exposed to Alexa Fluor 488-labeled SIV (0, 3, or 9 ng of p28CA per 10⁴ DCs) and analyzed by flow cytometry. FL1, Alexa 488 fluorescence; FSC, forward light scatter. Fluorescence was proportional to the amount of labeled SIV. Note the increased uptake by immature DCs relative to in vitro-matured DCs. (B) Localization of virus in DCs. Nuclei were stained with DAPI (blue). Left panel: after exposure to Alexa Fluor 488-labeled SIV (9 ng p28CA per 10⁴ DCs) (green). Center panel: cells were costained for SIV p27Gag (red). Right panel: overlapping Alexa-labeled virus and p27Gag stains are evident in the overlays (yellow). Single planes of the interior of deconvoluted z-sections were imaged at an original magnification of 125×. These data are representative of results for cells from four different donors.
reverse transcription intermediates contained within infected HOS cells was measured by quantitative real-time PCR (10). Generation of minus-strand strong-stop DNA, the first product of reverse transcription, was inhibited 99.8% for NEM-treated virions, compared with untreated virus (Table 2). Consistent with previous results (56), infection of target cells with AT-2-treated virus generated far less minus-strand strong-stop DNA relative to that with untreated HIV-1. Both treatments reduced the production of second-strand transfer products to negligible amounts not distinguishable from assay background.

After treatment, the absence of reverse transcription intermediates could have resulted from direct inactivation of RT enzymatic activity or from modification of other core proteins (e.g., NC), indirectly interfering with reverse transcription. However, NEM and AT-2 had negligible effects on SIV RT polymerase activity in an exogenous template assay (Table 3). Similarly, only a modest reduction of RT activity was detected in HIV-1 samples. Although the reducing conditions of this assay could potentially reverse AT-2-induced disulfide bonds, NEM-derivatized cysteines would not be affected. These results indicate that direct inhibition of RT polymerase activity does not account for the absence of reverse transcription intermediates in NEM-treated virus, whereas the effect observed in AT-2-treated virus remains to be fully clarified.

HPLC analysis of NEM-treated virus. To identify any changes in viral proteins after NEM treatment, HIV-1 and SIV were reacted with NEM and analyzed by reverse-phase HPLC. Comparison of the nonreduced chromatograms of NEM-treated and untreated HIV-1 indicated that NC, the p1Gag spacer peptide, and CA reacted with NEM (Fig. 4). Treatment eliminated the characteristic NC peak, and a new peak, representing NC intermediates in NEM-treated virus, compared with untreated HIV-1. Both treatments reduced the production of second-strand transfer products to negligible amounts not distinguishable from assay background. After treatment, the absence of reverse transcription intermediates could have resulted from direct inactivation of RT enzymatic activity or from modification of other core proteins (e.g., NC), indirectly interfering with reverse transcription. However, NEM and AT-2 had negligible effects on SIV RT polymerase activity in an exogenous template assay (Table 3). Similarly, only a modest reduction of RT activity was detected in HIV-1 samples. Although the reducing conditions of this assay could potentially reverse AT-2-induced disulfide bonds, NEM-derivatized cysteines would not be affected. These results indicate that direct inhibition of RT polymerase activity does not account for the absence of reverse transcription intermediates in NEM-treated virus, whereas the effect observed in AT-2-treated virus remains to be fully clarified.

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Reduction of the samples revealed several NEM-derivatized species of NC (Fig. 4). Mass spectral analysis showed that unmodified as well as NC proteins adducted with one to three or with four to six NEM moieties were present (Fig. 4). After reduction, the presence of native NC in NEM-treated virus may represent oxidized protein protected from NEM.

As with HIV-1, SIV core proteins reacted extensively with NEM, as shown by nonreducing HPLC analysis (Fig. 5). In the case of SIV, NC protein reacted with NEM almost quantitatively. Mass spectral analysis of the single NEM-modified NC peak indicated that at least three species of NC—with four, five, or six adducts—were present. Additionally, the CA protein was extensively modified, apparent by its delayed elution.

Due to the limited amount of envelope glycoprotein incorporated into HIV-1 virions (65), gp120SU of HIV-1 was not readily detectable by HPLC analysis, whereas SIV envelope glycoproteins were. Under nonreducing conditions, SIV gp120SU eluted normally after NEM treatment (Fig. 5), suggesting that it was unmodified, which would be consistent with the FFWO results presented above. Under reducing conditions, the elution of gp120SU was slowed and its peak overlapped that of the CA protein. No change in retention time was observed for SIV gp41 after NEM treatment (data not shown), despite the presence of a cysteine residue in the cytoplasmic domain of the protein. However, it is unlikely that the retention time of a protein as hydrophobic as gp41 would be affected by the addition of such a comparatively small adduct.

### DISCUSSION

We have shown here that NEM treatment of either HIV-1 or SIV led to extensive modification of NC protein and elimination of detectable infectivity, with at least 6 logs of inacti-

| TABLE 2. In vivo reverse transcription activitya |
|-------------------------------|-------------------------------|-------------------------------|
| Treatment                  | Copy no. per 2,000 cells | % wt product |
|                             | (-)SSS | (+)ST | (-)SSS | (+)ST |
| HIV-1 (no treatment)       | 156,000 ± 27,000 | 51,000 ± 27,000 | 100 ± 0 | 100 ± 0 |
| +GSH                      | 124,000 | 41,000 | 71 | 78 |
| +DMSO                     | 82,000 ± 30,000 | 29,000 ± 9,000 | 52 ± 10 | 56 ± 15 |
| +NEM                      | 200 ± 220 | 30 ± 22 | 0.14 ± 0.16 | 0.059 ± 0.047 |
| +AT-2                     | 40 ± 10 | 6 ± 9 | 0.026 ± 0.002 | 0.013 ± 0.018 |
| RT-                       | 20 ± 30 | 0 | 0 | 0 |
| Neg. control              | 0 | 0 | 0 | 0 |

a (-)SSS is a target within R-US, the minus-strand strong-stop DNA. (+)ST is the R-5'-UTR target, the plus strand-transfer DNA. RT- was the HIV-1 RT polymerase-defective mutant D185K/D186L (10). Negative (neg.) control was an infection using supernatants from 293T cells transfected with sheared salmon sperm DNA (10). DNA from about 2,000 cells (as determined by phorbobilinogen deaminase content) was analyzed by real-time PCR. Standard deviations reflect duplicate experiments. wt, wild type.

| TABLE 3. In vitro RT activitya |
|-------------------------------|-------------------------------|-------------------------------|
| Sample                      | Avg. RT activity | SD | Normalized activity |
| HIV-1 (no treatment)        | 141,000 | 6,500 | 100 |
| +GSH                       | 65,000 | 52,000 | 46 |
| +DMSO                      | 82,000 | 44,000 | 58 |
| +NEM                       | 80,000 | 24,000 | 57 |
| +AT-2                      | 54,000 | 21,000 | 39 |
| Uninfected H9              | 820 | 70 | 1 |
| SIV (no treatment)          | 99,000 | 5,200 | 100 |
| +GSH                       | 105,000 | 11,000 | 107 |
| +DMSO                      | 103,000 | 8,800 | 104 |
| +NEM                       | 118,000 | 7,800 | 119 |
| +AT-2                      | 101,000 | 12,000 | 111 |
| Uninfected CEMX174         | 1,000 | 90 | 1 |

a Averages of triplicate samples are shown. Supernatants from uninfected H9 and CEMX174 cultures were included as negative controls.
Molecular analysis showed that infectivity was blocked at the initial steps of reverse transcription, complementing mutagenesis studies suggesting the need for functional NC protein during reverse transcription. The block in replication of NEM-treated virus was not due to inhibition of viral binding or entry, as treated virus was capable of mediating fusion of target cells. A further indication of preserved glycoprotein function on treated virus was obtained by showing that virus labeled with a fluorescent NEM derivative was effectively taken up by antigen-presenting DCs, a process shown to depend on functional envelope glycoproteins (12). In studies with immature and in vitro-matured DCs, uptake and subcellular localization of the labeled virus were similar to results obtained previously for unlabeled virus (24, 25, 41, 45, 63).

Labeling SIV or HIV-1 with malemide derivatives takes advantage of the relatively high number of reactive sites found in the viral core, which we have shown can be almost quantitatively modified (Fig. 5). In contrast to other techniques that require either cotransfection of reporter genes for packaging fluorescent proteins into the virus (44) or modification of the viral genome with a reporter gene that is only expressed after integration (42, 48), malemide-based labeling can be accom-
plished by treatment of virus propagated through infection, and subsequently the labeled virus can be microscopically observed in target cells prior to viral gene expression. Additionally, the virus used for labeling can be generated in lymphoid cells with different major histocompatibility complex backgrounds, and the contribution of these proteins, which are incorporated into the viral membrane (21, 55), can be assessed for their contribution in target cell binding and uptake.

In addition to showing complete inactivation of HIV-1 and SIV by NEM, the kinetics of inactivation were also determined. From the regression line graphed in Fig. 1B, we calculated that 4 mM NEM is needed to give a reaction rate equivalent to that with 1 mM AT-2 (17), a concentration well documented to provide complete inactivation of HIV-1 and SIV (1, 56). The fourfold difference in inactivation rates between NEM and AT-2 is likely due to differences in reaction mechanisms, disulfide exchange proceeding faster than alkylation. Different diffusion rates into the virion core may also contribute, with NEM likely crossing viral membranes more slowly than the more hydrophobic AT-2 (octanol-water partition coefficients are 3.8 and 85.1, respectively; Open NCI database [http://cactus.nci.nih.gov]) (36).

The presence of incompletely reacted NC after NEM treatment may have been due to oxidation, either before or concomitant with treatment, or protection of the protein in complexes with nucleic acid (18). Since NEM inactivation was slower than inactivation via AT-2-mediated cross-linking of proteins, and ejection of zinc is likely to occur after the initial modification of a cysteine residue within a zinc finger (8, 9, 39), the remaining cysteines could have reacted with NEM or another cysteine. Consistent with oxidation and formation of disulfides having taken place prior to alkylation, unmodified forms of CA protein were observed in NEM-treated preparations for both HIV-1 and SIV.

Because of their different reaction mechanisms, NEM and AT-2 could have had different effects on postentry events in the viral life cycle. Cells exposed to NEM-treated virus had very low levels of minus-strand strong-stop and gag vDNAs. Similarly, AT-2-treated virus generated very low levels of strong-stop RT intermediates, consistent with earlier results (56). The amounts of reverse transcripts detected in our assays are quite small and may be the result of endogenous intravirion reverse transcription (61) that takes place prior to treatment, particularly for minus-strand strong-stop DNA. Slightly more reverse transcription intermediates were found after NEM treatment than with AT-2 treatment. The increased levels after NEM treatment may be due to intrassay variations and may not reflect true differences in reverse transcription between the two different chemical treatments. Alternatively, the slightly greater level of RT intermediates after NEM treatment may correspond to differences to which the viral core proteins are modified relative to AT-2-treated virus. Our exogenous template RT assays of NEM-treated virus showed that RT remains active, and this is consistent with the finding that the RNA-dependent DNA polymerase activity of recombinant RT in vitro is not inhibited by NEM treatment (35). The lack of initial replication products in the presence of an active RT may reflect disruption of either NC’s chaperone function or critical nucleoprotein core structures through the modification of cysteine thiol by NEM.

Comparison of viruses inactivated by disrupting the NC zinc fingers with either mild oxidizing agents (1, 56, 60) or alkylating agents (as described here) with viruses containing point mutations in NC shows a number of similarities in their phenotypes. Mutations that alter the Cys or His residues to amino acids other than Cys or His result in zinc fingers that are no longer able to tightly bind the metal ion, and these mutant viruses are replication defective to roughly the same extent (34) as viruses treated with the sulfhydryl reagents described in this work. In contrast to the chemically modified viruses in this study, certain NC mutant viruses that maintain zinc binding by exchanging His for Cys residues in their zinc fingers are able to reverse transcribe their genomes, although at reduced efficiency, but are nevertheless markedly replication impaired. For these viruses, the vDNA synthesized appears to be protected insufficiency from cellular nucleases or ligases and thus is a defective substrate for the subsequent integration reaction, partially explaining the replication defects observed (10). The mutagenesis studies demonstrate the exquisite sensitivity of viral replication to structural alterations of the NC zinc fingers and suggest that covalent modification of the zinc fingers in retroviral NC proteins likely eliminates infectivity by interfering with the multiple steps in the viral replication cycle in which the NC protein is involved. Covalent modification of other internal viral proteins with free cysteine thiol groups may also impact infectivity, although the contribution of modifications of non-NC proteins to the overall elimination of infectivity remains to be clarified.

The use of inactivated viruses in vaccines for human use requires a high margin of safety. The stable and irreversible NEM modification of viral proteins makes this inactivation method well suited for vaccine production. An obvious point of concern for AT-2 treatment, which induces disulfide exchange and protein cross-linking, is the possibility that the treatment may be reversible. However, while vigorous chemical reduction can reduce the oxidized and cross-linked cysteines on the internal proteins of AT-2-treated virions, such treatment would also reduce the disulfide linkages of the envelope glycoproteins (Fig. 5), destroying the ability of the virions to mediate productive infection. Importantly, rigorous safety studies both in vitro and involving direct intravenous administration of large amounts of AT-2-inactivated virus to nonhuman primates indicate that there is no detectable residual infectivity following AT-2 treatment (43). Nevertheless, for any eventual clinical use of chemically inactivated virions with functional envelope glycoproteins, a combination of multiple inactivating approaches will likely be required (57). NEM and AT-2 together represent a well-defined and potent pairing for such a combination inactivation.

Cysteine oxidation has been suggested as a general antiviral strategy, targeting viral zinc fingers and other metalloproteins (15), and several reports have described successful killing of a diverse group of viruses. Beatrice and Wagner reported that maleimide-based treatments of VSV inactivated the virus only if the compound could penetrate the viral core (4). Human cytomegalovirus (2, 3) and Junin viruses (27, 28) were also vulnerable to these types of sulfhydryl reagents. An intact CCCH zinc finger motif was shown to be required for transcription in respiratory syncytial virus (59) and in Ebola virus (46). Inactivation of viruses, especially those with genomes that
cannot be directly translated, by chemically modifying free protein thiol(s), either by alkylation or inducing disulfide bond formation, is an attractive strategy.

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