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Inactivation of Murine Leukemia Virus by Compounds That React with the Zinc Finger in the Viral Nucleocapsid Protein

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All retroviral nucleocapsid (NC) proteins, except those of spumaretroviruses, contain one or two copies of the conserved sequence motif C-X_2-C-X_4-H-X_4-C. The conserved cysteine and histidine residues coordinate a zinc ion in each such motif. Rice et al. (W. G. Rice, J. G. Supko, L. Malspeis, R. W. Buckheit, Jr., D. Clanton, M. Bu, L. Graham, C. A. Schaeffer, J. A. Turpin, J. Domagala, R. Gogliotti, J. P. Bader, S. M. Halliday, L. Coren, R. C. Sowder II, L. O. Arthur, and L. E. Henderson, Science 270:1194–1197, 1995) have described a series of compounds which inactivate human immunodeficiency virus type 1 (HIV-1) particles and oxidize the cysteine thiolates in the NC zinc finger. We have characterized the effects of three such compounds on Moloney murine leukemia virus (MuLV). We find that, as with HIV-1, the compounds inactivate cell-free MuLV particles and induce disulfide cross-linking of NC in these particles. The killed MuLV particles were found to be incapable of synthesizing full-length viral DNA upon infection of a new host cell. When MuLV particles are synthesized in the presence of one of these compounds, the normal maturational cleavage of the Gag polyprotein does not occur. The compounds have no effect on the infectivity of human foamy virus, a spumaretrovirus lacking zinc fingers in its NC protein. The resistance of foamy virus supports the hypothesis that the zinc fingers are the targets for inactivation of MuLV and HIV-1 by the compounds. The absolute conservation of the zinc finger motif among oncoretroviruses and lentiviruses and the lethality of all known mutations altering the zinc-binding residues suggest that only the normal, wild-type structure can efficiently perform all of its functions. This possibility would make the zinc finger an ideal target for antiretroviral agents.

In all retroviruses (except spumaretroviruses), the nucleocapsid (NC) protein contains one or two copies of a conserved sequence motif termed the cysteine array or Cys-His box. This motif can be represented as C-X_2-C-X_4-H-X_4-C, where C is cysteine, H is histidine, and X represents other amino acids which differ in different retroviruses. The motif is also known as the NC zinc finger, since the cysteine and histidine residues coordinate a zinc ion in the virion (4, 5, 28).

The function of the NC zinc finger is not well understood as yet. All mutations in the zinc-binding residues which have been described to date have been lethal for the virus. Virions produced by these mutants are frequently defective with respect to genomic RNA content (1, 6, 7, 11, 12, 17); thus, the zinc fingers presumably participate (as part of the Gag polyprotein precursor) in RNA packaging during virion assembly. Significantly, however, the mutant particles are far more defective with respect to infectivity than with respect to genomic RNA content (11). Indeed, we have recently characterized zinc finger mutants of Moloney murine leukemia virus (Mo-MuLV) which package normal levels of genomic RNA but are nevertheless noninfectious (10). These observations imply that the zinc fingers play other roles in the viral life cycle in addition to their function in RNA packaging. It has been suggested that NC has an important role in maturation of the released virus particle (8, 9) and performs one or more functions in reverse transcription (2, 15, 18, 20, 25, 26, 31). The significance of the zinc fingers in these additional functions of NC is not known.

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The present report described the inactivation of MuLV by a class of compounds whose mechanism of antiviral action appears to be the oxidation of the zinc finger in NC. Compounds of this type have previously been shown to oxidize the zinc fingers in human immunodeficiency virus type 1 (HIV-1) NC protein in vitro (24) and to inactivate HIV-1 (23).

MATERIALS AND METHODS

Drugs and drug treatments. Disulfide-substituted benzamide-2 (DIBA-2) was previously described (24). Dicyclopentamethylene thiuram disulfide (DPMTD) and 2,2′-dithiodipyridine (Aldrithiol-2) were obtained from Aldrich Chemical Co.

Stock solutions of all drugs were made in dimethyl sulfoxide (DMSO). To treat virus with a drug, the drug solution was diluted ≥10-fold into the virus stock, and the mixture was incubated at 37°C. The mixture was then diluted further with growth medium before being placed on cell cultures for a 30- to 60-min adsorption period.

Viruses and infectivity assays. Mo-MuLV stocks consisted of culture fluids from virus-infected NIH 3T3 cells; fluids were filtered through 0.45-μm-pore-size filters before use. Infectivities were measured by the S^+L^- focus assay as described previously (3). Human foamy virus (HFV) was a kind gift of S. Yu and M. Linial and was grown on HEL299 cells (American Type Culture Collection, culture CCL-137). HFV stocks were made by scraping infected cells into their growth medium and subjecting the mixture to three cycles of freezing and thawing. HFV infectivity was assayed on the FAB indicator cell line constructed by Yu and Linial (32).

Immunoblotting. Virus pellets were analyzed by immunoblotting with the Amersham Enhanced Chemiluminescence System (Arlington Heights, Ill.) with goat antiserum against $p30^{CA}$ (29) or rabbit antiserum against $p10^{NC}$ (obtained from Patrick Wesdock and Terry Copeland, National Cancer Institute-Frederick Cancer Research and Development Center). The latter antiserum also detects a band migrating more slowly than NC, with an apparent molecular mass of 13 to 14 kDa; the identity of this band is not known, but it is not NC, since (unlike the lower band) its mobility does not change when deletions in NC (21) are tested (data not shown).

Assay for viral DNA synthesis. NIH 3T3 cells were lysed (13) 24 or 48 h after infection with drug-treated or control virus. Hirt supernatant DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and analyzed by digestion with

*Hin*dIII, followed by Southern blotting as previously described (19), with 15 μg of nucleic acid per lane. A ^{32}P-labeled fragment of the viral genome, extending from *Xho*I (nucleotide 1560) (27) to *Hin*dIII (nucleotide 4894), was used as a probe.

RESULTS

Inactivation of Mo-MuLV. The effect of the compounds on the infectivity of MuLV was tested as follows. Virus was treated with compound as described in Materials and Methods, and the mixture was then diluted and assayed on S^+L^- cells. Control experiments showed that the level of the compound placed on the assay cells did not significantly affect the number of foci observed with untreated virus (data not shown).

Figure 1 shows dose-response curves for the inactivation of MuLV by DIBA-2, DPMTD, and 2,2′-dithiodipyridine. It can be seen that, at the highest doses tested, DIBA-2 reduced the infectious titer of the MuLV by ~10,000-fold (Fig. 1A), while 2,2′-dithiodipyridine (Fig. 1B) and DPMTD (Fig. 1C) reduced the titer by ~100- to 200-fold. It is striking that the killing curves all appear to follow single-hit kinetics at low drug concentrations.

Figure 2 shows the time course of killing of MuLV by the same three agents. It is interesting to note that, in all three cases, the inactivation appeared to begin immediately upon addition of the compound to the virus and to proceed with first-order reaction kinetics.

Disulfide cross-linking of NC in Mo-MuLV. The compounds have previously been shown to induce disulfide cross-linking of the cysteines in the zinc fingers of purified HIV-1 NC protein; this reaction results in the loss of the zinc ion from the zinc finger (23, 24). In order to determine whether this reaction occurs in MuLV particles treated with these agents, we incubated virus with the three compounds and then analyzed it as follows. Treated or control virus was pelleted in the ultracentrifuge, lysed, and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in electrophoresis buffer either with or without β-mercaptoethanol (β-ME). It was then analyzed by immunoblotting, with antiserum to $p10^{NC}$.

The results of these tests are shown in Fig. 3. It can be seen that the compounds did not affect the profile obtained when virus was analyzed in the presence of the reducing agent. However, analysis without β-ME revealed that, in each case, a substantial fraction of the NC protein had been converted to a higher-molecular-weight band, evidently a dimer, by the treatment. Various amounts of larger forms are also seen in these gels. These results show that the three treatments caused intermolecular disulfide cross-linking of NC protein within Mo-MuLV particles.

Failure of killed MuLV particles to synthesize full-length viral DNA in newly infected cells. In order to gain some insight into the mechanism by which the three compounds inactivate MuLV, we tested the ability of the drug-treated MuLV to synthesize full-length viral DNA upon infection. NIH 3T3 cells were infected with drug-treated or control virus and lysed 1 to 2 days later by the Hirt fractionation procedure. The resulting Hirt supernatants were then assayed for the presence of unintegrated viral DNA by digestion with *Hin*dIII and Southern blotting with an MuLV probe. (Because the probe detects viral sequences only on the 5′ side of the *Hin*dIII site, a full-length linear molecule generates a 5.3-kb band in these Southern blots.) As shown in Fig. 4, no viral DNA was observed in cells infected with the killed virus preparations, while it was easily detectable in cells infected with control virus; indeed, lanes 5 and 8 demonstrate that 1 to 10% of the control levels of DNA were detectable in these experiments.

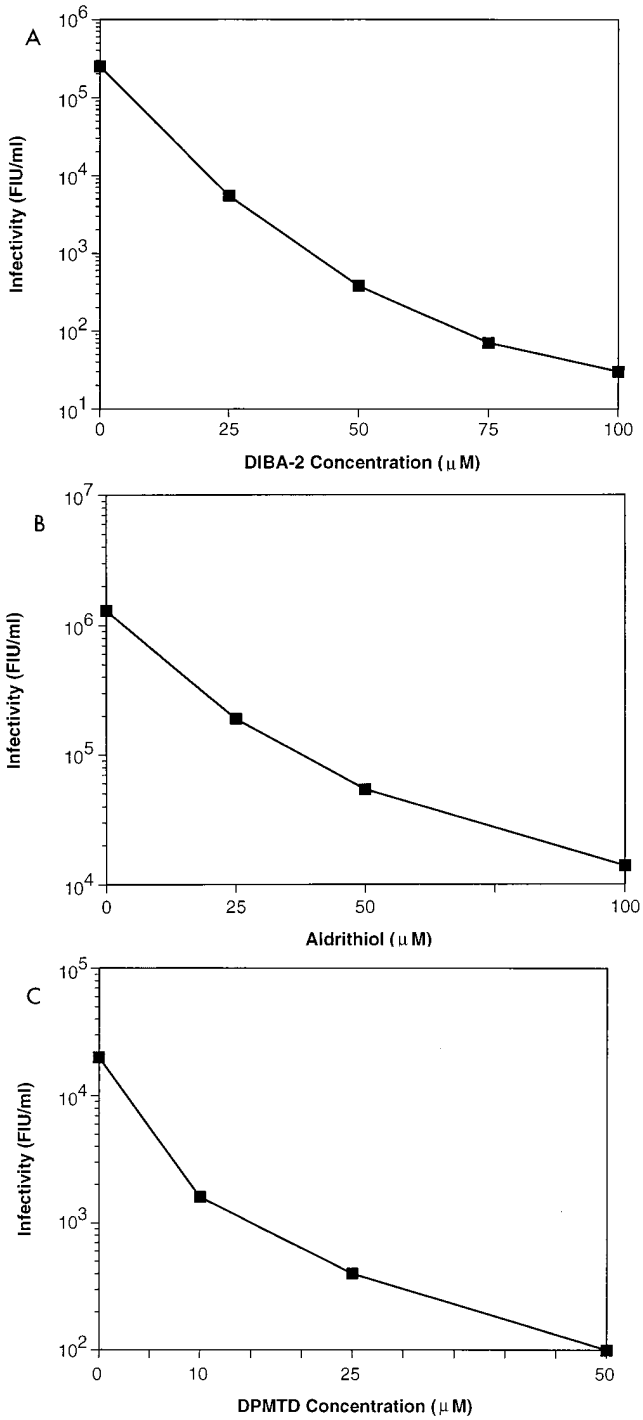

FIG. 1. Inactivation of MuLV by DIBA-2 (A), 2,2′-dithiodipyridine (B), and DPMTD (C): dose-response curves. Mo-MuLV virus stocks were treated with different doses of drug for 1 h at 37°C. The stocks were then diluted 10-fold or more and assayed for MuLV infectivity by the S^+L^- focus assay.

It also seemed possible that the residual drug added to the cells along with the drug-killed virus prevented the synthesis of DNA. To test this possibility, we applied untreated viral inocula to cells together with diluted drug and then analyzed the cells for the presence of viral DNA. The results of these control experiments demonstrated that the level of drug applied to

the cells in the diluted virus used in Fig. 4 had no effect on the ability of infectious virus to synthesize viral DNA (data not shown). Thus, treatment with each of the three agents under study here drastically reduces the ability of MuLV particles to synthesize full-length viral DNA; this reduction is presumably responsible, at least in part, for the lack of infectivity in treated virus.

Effects of treatment of chronically infected cells on virus production. The experiments described above have all dealt with the effect of the compounds on free, infectious MuLV particles. It was also of interest to determine whether the compounds have any effect on release of MuLV from MuLV-producing cells. We therefore grew MuLV-producing NIH 3T3 cells in the presence of the agents for 2 to 3 days. Twenty-four-hour harvests of culture fluid were collected during the treatment period and pooled. These fluids were then analyzed for the presence of MuLV particles by pelleting, followed by SDS-PAGE under standard (i.e., reducing) conditions and immunoblotting with anti-$p30^{CA}$ antiserum.

After approximately 48 h, the DIBA-2-treated culture contained ~40% as many cells as the control cultures; this difference reflects the modest toxicity of this compound at 100 μM. To determine whether the treatment affected the amount of virus released, we therefore compared the virus content in 2.5 ml of supernatant from the treated culture (Fig. 5A, lane 1) with that of 1.0 ml of supernatant from the control (i.e., untreated and DMSO-treated) cultures (lanes 4 and 6). (Other dilutions of the samples were also tested [lanes 2, 3, 5, and 7], as a check on the dose response of the immunoblot in this concentration range.) As shown in Fig. 5A, the three samples contained virtually identical amounts of virus particles. However, the predominant protein detected by the anti-CA antiserum in the treated sample (lanes 1 to 3) was not $p30^{CA}$ as expected but rather $Pr65^{Gag}$. We conclude that treatment of virus-producing cells with DIBA-2 has no detectable effect on the level of virus release but inhibits maturational cleavage of the $Pr65^{Gag}$ by PR following virus release.

The experiments presented above show that treatment of free, mature MuLV particles with DIBA-2 induces disulfide cross-linking of NC protein (Fig. 3) and also that the majority of $Pr65^{Gag}$ in virions assembled in the presence of DIBA-2 is not cleaved (Fig. 5A). It was also of interest to determine whether this $Pr65^{Gag}$ is cross-linked. To test this possibility, we analyzed these virions (produced by DIBA-2-treated cells) by SDS-PAGE in the absence of β-ME, followed by immunoblotting with anti-CA antiserum. As shown in Fig. 5B, the viral protein detected under these conditions migrated at an extremely high molecular weight. These results show that there is extensive disulfide cross-linking of the $Pr65^{Gag}$ in the virions released in the presence of DIBA-2.

We also performed analogous experiments with DPMTD and 2,2′-dithiodipyridine. However, virus released from cells during a 24-h treatment with these two agents had a protein profile indistinguishable from that of control virus and also exhibited significant infectivity (data not shown). The lack of a substantial effect raises the possibility that these compounds are unstable over a 24-h period in culture medium at 37°C.

Lack of effect of compounds on infectivity of HFV. The results presented above show that the compounds under study inactivate MuLV particles and induce disulfide cross-linking in NC in these particles. Others have made analogous findings for HIV-1 (12a, 24). However, this correlation between killing and cross-linking does not prove that the cross-linking is responsible for the virus inactivation which is observed. As one approach to this question, we tested the effects of these compounds on HFV, which lacks zinc fingers in its NC protein (16).

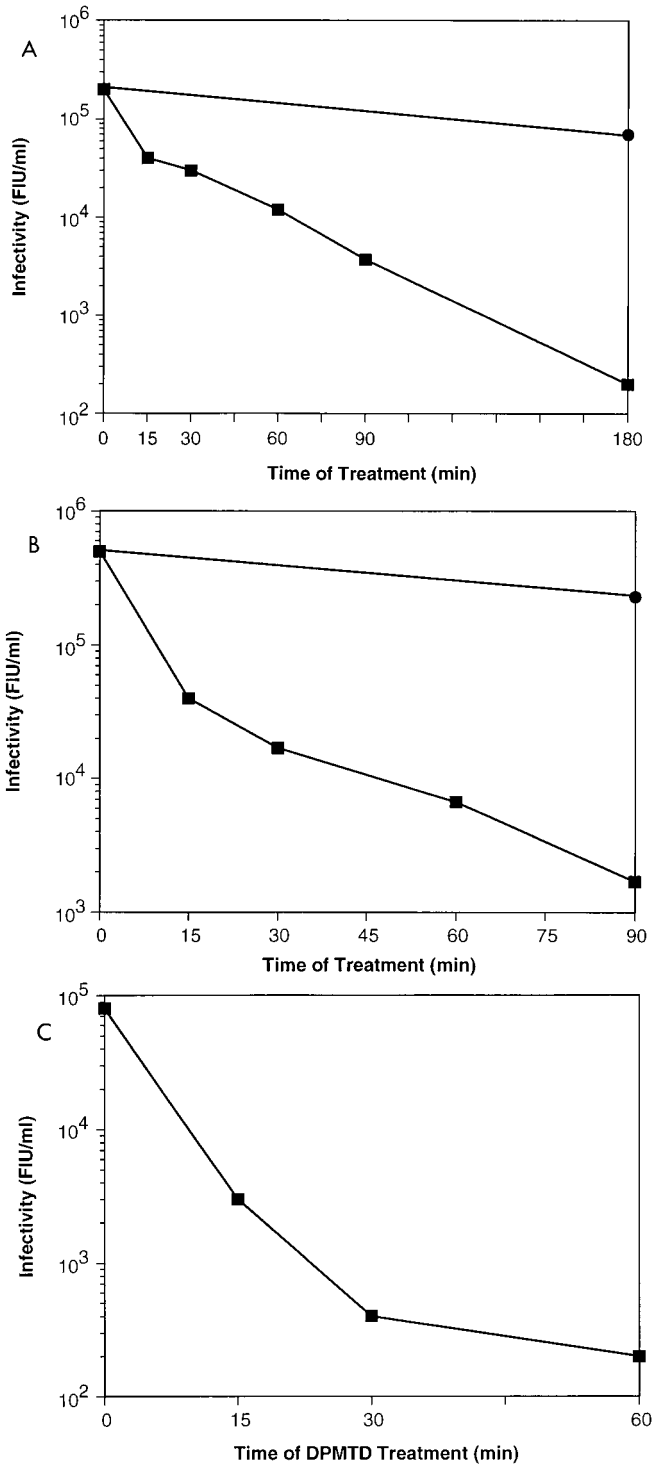

FIG. 2. Inactivation of MuLV by DIBA-2 (A), 2,2′-dithiodipyridine (B), and DPMTD (C): time course. Mo-MuLV virus stocks were treated for different times at 37°C with 20 μM DIBA-2, 100 μM 2,2′-dithiodipyridine, or 50 μM DPMTD at 37°C. The stocks were then diluted 10-fold or more and assayed for MuLV infectivity. Control incubations contained the same concentration of DMSO as that present in the drug-containing samples. ■, drug-treated samples; ●, control samples.

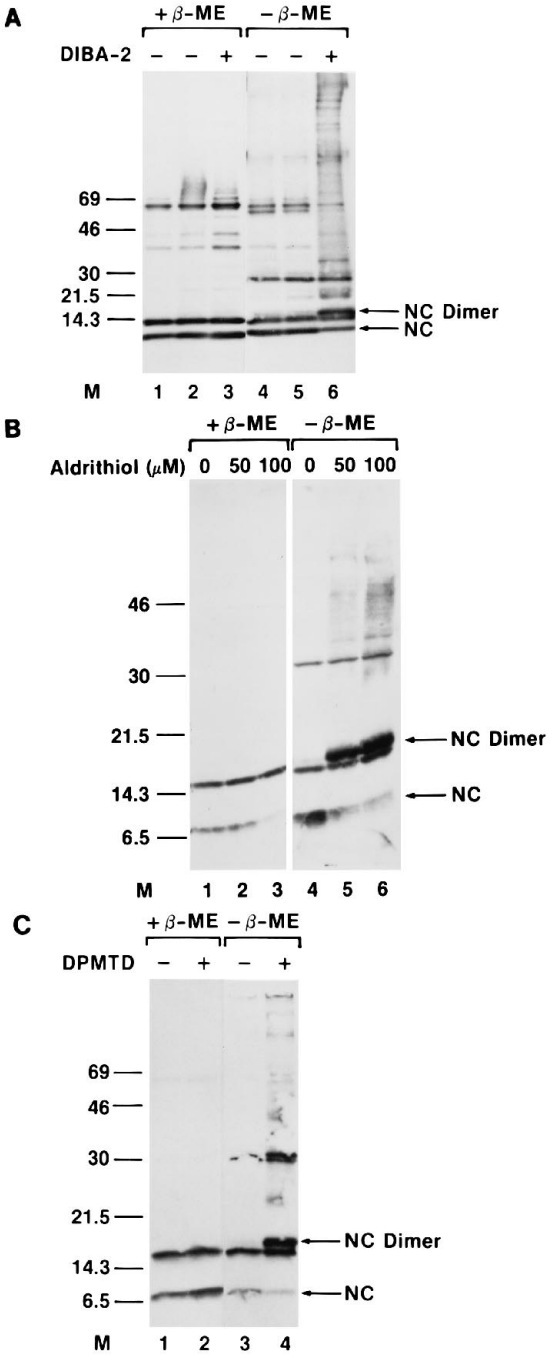

FIG. 3. Disulfide cross-linking of $p10^{NC}$ in MuLV particles by DIBA-2, 2,2′-dithiodipyridine, and DPMTD. Stocks of MuLV were exposed to drug or to control solutions of DMSO for 1 h at 37°C. They were then pelleted, lysed, and analyzed by SDS-PAGE in the presence or absence of β-ME, followed by immunoblotting with anti-$p10^{NC}$ antiserum. (A) Virus was treated with 100 μM DIBA-2 (lanes 3 and 6) or with DMSO alone (at the same concentration as in the DIBA-2 solution) (lanes 2 and 5) or not treated (lanes 1 and 4). Lanes 1 to 3 are from a gel containing β-ME, while lanes 4 to 6 are from a gel lacking β-ME. (B) Virus was treated with 50 (lanes 2 and 5) or 100 (lanes 3 and 6) μM 2,2′-dithiodipyridine or with a DMSO concentration equivalent to that present in 100 μM 2,2′-dithiodipyridine (lanes 1 and 4). β-ME was present in lanes 1 to 3. (C) Virus was treated with 50 μM DPMTD (lanes 2 and 4) or with an equivalent concentration of DMSO (lanes 1 and 3). β-ME was present in lanes 1 and 2. M, molecular mass markers in kDa.

The results of these experiments are shown in Fig. 6. In each case, HFV and MuLV were treated and assayed in parallel; as can be seen, the three compounds had no significant effect on the infectious titer of HFV, while they inactivated MuLV in the control reactions as expected.

DISCUSSION

The principal results presented here can be briefly summarized as follows. We have described a series of compounds which induce disulfide cross-linking of NC protein molecules in intact MuLV particles (Fig. 3); since the only cysteines in NC are those in the zinc finger, this chemical reaction entails the oxidation of the cysteine thiolates in the finger and the loss of the coordinated zinc ion. Treatment with these compounds destroys the infectivity of the particles (Fig. 1 and 2) and renders them unable to synthesize full-length viral DNA upon infection of new host cells (Fig. 4).

While there is no direct evidence linking the oxidation of the zinc finger with the inactivation of the virus particles, it is striking that these compounds also inactivate HIV-1 (12a, 24) and, in general, their relative potencies on MuLV parallel those on HIV-1 (data not shown). In contrast, they have no detectable effect on the infectivity of HFV, a retrovirus which lacks zinc fingers in its NC protein (Fig. 6). These correlations support the hypothesis that the zinc fingers in NC are the vulnerable targets of these compounds in MuLV and HIV-1. It should be noted, however, that HFV and other members of the spumaretrovirus family also differ from other retroviruses in a number of other ways (e.g., see reference 31a).

If it is correct that the mechanism of virus inactivation by these compounds is diffusion across the lipid bilayer into the viral core and subsequent oxidation of the cysteine thiolates in the NC zinc finger, then any mild oxidizing agent which is sufficiently hydrophobic to penetrate the virus could be considered a potential antiviral agent. Obviously, the number of such compounds is extremely large. In our experience, some of these agents exhibit severe toxicity in cell culture, but others do not. It seems possible that the toxicity of some of these agents is limited because the intracellular environment is protected

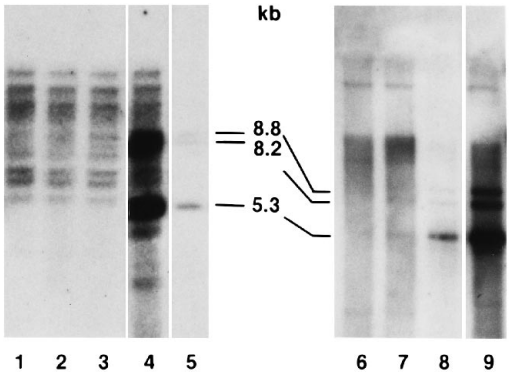

FIG. 4. Failure of inactivated MuLV particles to synthesize full-length viral DNA upon infection of new host cells. Stocks of MuLV were exposed to drug or to control solutions of DMSO for 1 h at 37°C. They were then diluted fivefold and used to infect NIH 3T3 cells. The cells were lysed and analyzed for low-molecular-weight viral DNA as described in Materials and Methods. Lanes: 1, mock infection; 2, MuLV treated with 100 μM 2,2′-dithiodipyridine; 3, MuLV treated with 50 μM DPMTD; 4, DMSO-treated MuLV; 5, 1:100 dilution of DNA used in lane 4; 6, mock infection; 7, MuLV treated with 100 μM DIBA-2; 8, 1:10 dilution of DNA used in lane 9; 9, DMSO-treated MuLV. Lanes 1 to 5 and 6 to 9 are from different experiments; lysis was at 48 h in lanes 1 to 5 and at 24 h in lanes 6 to 9.

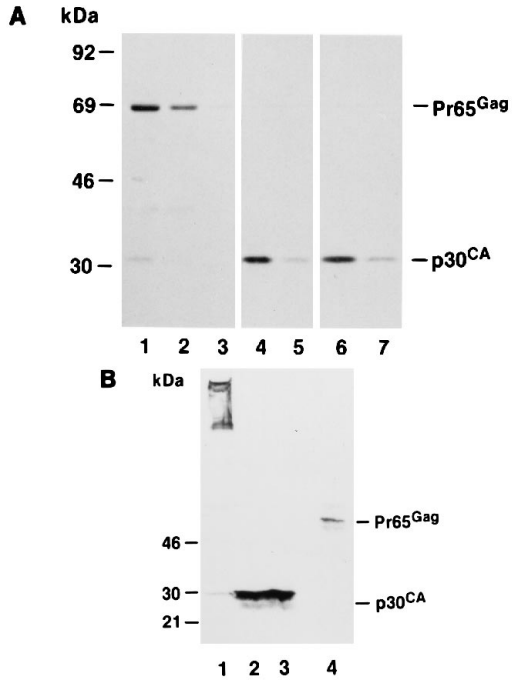

FIG. 5. Characterization of MuLV particles produced in DIBA-2. NIH 3T3 cells chronically infected with MuLV were grown in 100 μM DIBA-2 or in an equivalent concentration of DMSO, and two consecutive 24-h harvests were collected. At the end of the experiment, the DIBA-2-treated culture contained 40% as many cells as the DMSO-treated or untreated control cultures. The virus was pelleted and analyzed by immunoblotting with antiserum against $p30^{CA}$. (A) Electrophoresis in the presence of β-ME. Lanes contain virus from the indicated volume of culture fluid from cultures treated as indicated: 1, 2.5 ml, DIBA-2-treated culture; 2, 1.0 ml, DIBA-2-treated culture; 3, 0.2 ml, DIBA-2-treated culture; 4, 1.0 ml, DMSO-treated control culture; 5, 0.2 ml, DMSO-treated control culture; 6, 1.0 ml, untreated control culture; 7, 0.2 ml, untreated control culture. (B) Electrophoresis in the absence of β-ME. Lanes contain virus from DIBA-2-treated culture (lane 1), DMSO-treated control culture (lane 2), or untreated control culture (lane 3) or contain PR^- (D32L; see reference 9) MuLV, showing migration of $Pr65^{Gag}$ (lane 4).

against the destructive effects of mild oxidizing agents (14); this protection might render the compounds less damaging to the host than to extracellular virus. In vivo, the oral 50% lethal dose (LD_{50}) of DPMTD is 2.8 g/kg of body weight in mice (1a). Another drug which causes cross-linking of NC and inactivation of cell-free MuLV, i.e., tetraethylthiuram disulfide (data not shown), has an oral LD_{50} of 8.6 g/kg in rats (4a); this is so low that this compound has been approved for clinical use for other (nonviral) diseases.

Why should oxidation of the cysteine thiolates in NC to disulfides inactivate a fully matured, infectious virus particle? The fact that these particles do not synthesize full-length viral DNA (Fig. 4) is consistent with earlier findings with HIV-1 (22) and suggests that the zinc finger functions before or during reverse transcription. The nature of this function is not known, but studies with model systems in vitro suggest that NC may participate in reverse transcription, perhaps by facilitating strand transfer (2, 15, 20, 26, 31) and perhaps by rendering the DNA synthesis more processive (30a). Evidence that NC is cleaved within the zinc fingers by PR at an early stage of infection has also been presented (18, 25); experiments with purified proteins in vitro also show that oxidation of the zinc fingers prevents cleavage of HIV-1 NC by the viral PR (30). Thus, it is possible that the cleavage of NC is an essential step in infection and that cross-linking blocks infection by prevent-

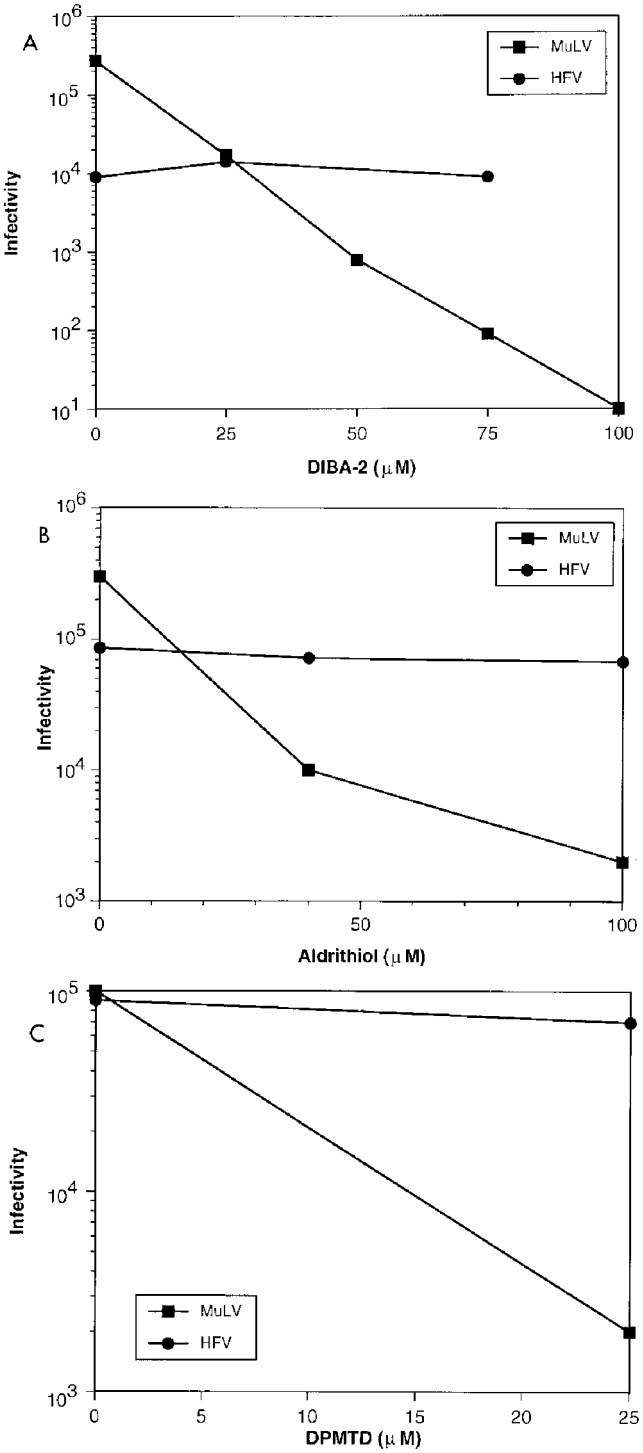

FIG. 6. Lack of effect of DIBA-2 (A), 2,2′-dithiodipyridine (B), and DPMTD (C) on infectivity of HFV. In each experiment, HFV and MuLV were incubated in parallel for 1 h at 37°C with the indicated concentrations of drug. They were then diluted, and the residual infectivities were assayed as described in Materials and Methods. Infectivities at the ordinates ("0 μM") represent virus treated with the same concentration of DMSO as was present in the highest concentration of drug used in the experiment.

ing cleavage. Experiments are now in progress to identify the point at which reverse transcription is blocked in cells infected with inactivated MuLV. These studies should shed light not only on the mechanism of action of these antiviral agents but also on the normal function(s) of NC in virus infection.

It is striking that virus inactivation by these compounds appears to follow simple one-hit kinetics (Fig. 1 and 2), with no indication of a shoulder or lag in the killing curves. This observation suggests that interaction between a single molecule of antiviral agent and its target in the virus is sufficient to destroy viral infectivity. Similarly, comparison of Fig. 1A with Fig. 3A shows that a treatment with DIBA-2 which cross-links less than 90% of the NC reduces the infectious titer of the virus preparation by about 10,000-fold. It is conceivable that oxidation of a single NC molecule is sufficient to inactivate a virus particle.

The experiments discussed above all deal with the effect of the compounds on free, infectious virus particles. However, we have also identified a second antiviral effect of one of these compounds: when virus particles are made in the presence of DIBA-2, there is no reduction in the level of particles released per cell, but the extent of maturational cleavage of the Gag precursor polyprotein is dramatically reduced (Fig. 5A). It is not known whether this effect is due to an effect on $Pr65^{Gag}$, rendering it uncleavable; on virus structure, blocking access of PR to Gag; on PR activity; or on the formation of active PR from dimers of $Pr200^{Gag\text{-}Pol}$ at the beginning of virus maturation. Turpin et al. (28a) have recently made similar observations for HIV-1. We also noted that the $Pr65^{Gag}$ in these particles is extensively cross-linked (Fig. 5B); this is also true of $Pr65^{Gag}$ in DIBA-2-treated PR^- virions (data not shown). These observations imply that DIBA-2 can penetrate immature as well as mature MuLV particles. Since the zinc fingers of NC are involved in RNA encapsidation during virus assembly (11, 17), it would be of great interest to know whether the particles released in the presence of DIBA-2 contain genomic RNA.

If it is true that these compounds inactivate HIV and MuLV by the same mechanism, then MuLV can be used as a simple, nonhazardous model system for preliminary investigations of their antiviral effects in vivo as well as in vitro. In fact, recent work shows that these compounds significantly delay the onset of Friend disease in mice (18a). The availability of this rapid, inexpensive system for in vivo screening seems particularly significant in light of the large number of oxidizing agents which might be considered as possible antiretroviral compounds.

Finally, one of the great problems with antiretroviral agents currently in use or under investigation is the ability of the virus to generate mutants which are resistant to the therapeutic agent and which are able to replicate nearly as well as the wild type. This problem could be avoided if a target could be found, for which there are no alternative structures capable of performing all of the functions of the target with good efficiency. The properties of the zinc fingers in NC raise the possibility that they might offer such a target: the sequence motif is absolutely conserved in retroviruses (except spumaretroviruses), and all mutants in the zinc-binding residues characterized to date have been profoundly defective with respect to infectivity. Thus, it is potentially of great importance that the compounds studied here appear to inactivate MuLV and HIV by reacting with the NC zinc fingers. We have not, as yet, observed the appearance of any MuLV or HIV-1 (24) mutants resistant to these compounds but are continuing to search for these mutants, both in cell culture and in animal models. The nature of these mutants, if they arise, might shed considerable light on the function(s) of the zinc fingers in the retroviral life cycle; conversely, the failure to find such mutants would suggest that oxidizing agents might be important new antiviral weapons.

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