Merocyanine 540-Sensitized Photoinactivation of Enveloped Viruses in Blood Products: Site and Mechanism of Phototoxicity

By Jill M. O'Brien, David K. Gaffney, Tian P. Wang, and Fritz Sieber

The amphipathic dye, merocyanine 540 (MC540), which preferentially photosensitizes enveloped viruses and virus-infected cells, is currently being evaluated in preclinical models as a blood sterilizing agent. In this communication, we report on an initial analysis of the site and nature of MC540-mediated photodynamic damages to human herpes simplex virus type 1 and human cytomegalovirus. The comigration of dye molecules and virions on a gel filtration column, the red-shift of the fluorescence emission spectrum of virus-containing fractions, and the distribution of MC540-treated virions in an aqueous two-phase partition system were indicative of MC540 binding to the enveloped viruses and localizing in a lipophilic environment (most likely the viral envelope). Fluorescence quenching and fluorescence resonance energy transfer experiments suggested that both dye monomers and dimers were capable of partitioning into the lipid bilayer of the viral envelope. Adsorption and penetration assays and immunohistochemical analyses of viral antigen expression showed that MC540-sensitized irradiation interfered with early phases of the infectious process, the adhesion to the host cell, the penetration of the host cell, and the translocation of the virus into the nucleus of the host cell. The inactivation of viruses was inhibited if oxygen in the medium was displaced by argon, enhanced if air was displaced by pure oxygen or if water was replaced by deuterium oxide. This suggested that the MC540-sensitized photoinactivation of enveloped viruses is an oxygen-dependent process and that singlet oxygen is one but not necessarily the only mediator of the antiviral effects of MC540.

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MATERIALS AND METHODS

Cells. African green monkey cells (Vero cells; ATCC CCL 81) were obtained from the American Type Culture Collection (Rockville, MD). Human foreskin fibroblasts were supplied by Dr. W.H. Burns (The Johns Hopkins Oncology Center, Baltimore, MD). Both cell types were grown in α-modified minimal essential medium (α-medium; Sigma Chemical Company, St Louis, MO) supplemented with 10% (for Vero cells) or 20% (for foreskin fibroblasts) fetal bovine serum (Sigma).

Viruses. Human herpes simplex virus type 1 (HSV-1; ATCC 539-VR, MacIntryre strain; American Type Culture Collection) was propagated at a low (1 to 2) multiplicity of infection on Vero cells and harvested from culture supernatants 3 days postinfection. Contaminating cells were removed by low-speed (80g, 10 minutes) centrifugation followed by filtration through a 0.45-μm polysulfone filter (Gelman, Ann Arbor, MI). For selected experiments, the virus was concentrated by centrifugation (47,800g, 2 hours) and further purified on a 10% to 40% potassium tartrate gradient as described by Richman et al.22 Virus that had been purified by the latter method is referred to as "purified" virus. Purified virus was free of cellular contaminants as judged by electron microscopic analysis.

To prepare radioactively labeled virus, 6.7 μCi of 3H-methylthymidine (specific activity, 6.7 Ci/mmol; New England Nuclear & I. Du Pont de Nemours Co, Wilmington, DE) was added per milliliter of culture medium 2 hours postinfection. For some experiments, the virus was labeled with 35S-L-methionine (100 μCi of 35S-L-methionine/mL; specific activity, 800 Ci/mmol; New England Nuclear & I. Du Pont de Nemours Co) instead of tritiated thymidine. Both methods gave equivalent results.

Virus titers were determined by infecting Vero cell monolayers in multiwell (24 wells) plates (Costar, Cambridge, MA) with small aliquots (0.25 mL) of serial fivefold dilutions of the virus suspension. After 1 hour, each well was overlayed with 1 mL of α-medium containing 5% fetal bovine serum and 0.9% methylcellulose (4000 cPs; Fluka, Buchs, Switzerland) and incubated for 4 days at 37°C in a humidified atmosphere of 5% CO2 in air. Plaques were scored using an inverted microscope. All titer estimates were based on mean plaque counts of ≥ 4 replicate wells.17

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Human cytomegalovirus (CMV; ATCC 538-VR, strain AD 169; American Type Culture Collection) was propagated at a low (1 to 2) multiplicity of infection on human foreskin fibroblasts and harvested by freeze/thawing and sonication as described previously. Contaminating cells were removed by low-speed centrifugation (800g, 10 minutes). Virus titers were determined by infecting preformed monolayers of human foreskin fibroblasts in multwell plates with small aliquots (0.25 mL) of serial fivefold dilutions of the virus suspension. After 1 hour, the cultures were overlayed with 1 mL of 0.25% methylcellulose and incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Ten days later, the cultures were fixed with cold formal saline and stained with 1% Gentian-Violet. Plaques were scored using an inverted microscope. All titer estimates were based on mean plaques counts of ≥ 4 replicate wells.

MC540-sensitized photoactivation. Cell-free virus preparations (typically 2 x 10^8 to 1 x 10^9 PFU/mL) were suspended in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; Research Organics, Cleveland, OH)-buffered (10 mmol/L, pH 7.4) α-medium supplemented with 12% of a selected lot of fetal bovine serum (Irvine Scientific, Santa Ana, CA) and placed into clear polystyrene tubes (15 mm diameter; Corning Glass Works, Corning, NY). MC540 (Kodak, Rochester, NY) was added from a 1 mg/mL stock solution in 50% ethanol to a final concentration of 15 μg/mL. For selected experiments, the virus suspension was flushed with argon or oxygen for 90 minutes before the addition of MC540 and for 15 minutes after the addition of the dye. The tubes were then mounted immediately on a plexiglass disk that rotated at approximately 60 rpm between two banks of tubular fluorescent lights (five bulbs per bank; F20T12.CW; General Electric, Cleveland, OH) and illuminated for the specified time intervals at room temperature. The fluence rate at the sample site was approximately 70 W/m2 as determined by a Kettering model 65A radiometer (Yellow Springs Instruments, Yellow Springs, OH). The reaction was terminated by transferring the virus suspension to the dark. Samples that were exposed to neither light nor dye or to light or dye only served as controls.

Immunocytochemistry. Fluorescein-labeled mouse anti-HSV monoclonal antibodies (MoAbs) were obtained from Bartels (Bellevue, WA) and used according to the manufacturer’s instructions. Murine MoAbs against late, early, and pre-early CMV antigen and fluorescein-labeled goat antiserum were obtained from Chemicon International (Temecula, CA). Cultures were air-dried 3 (pre-early antigen), 24 (early antigen), or 72 hours (late antigen) postinfection, fixed with cold acetone for 20 minutes, and then reacted with the primary antibody followed by the fluorescein-labeled secondary antibody. The specimens were washed with phosphate-buffered saline (PBS), mounted in a 9:1 mixture of glycerol and PBS, and examined with a Nikon epifluorescence microscope equipped with a 50 W DC mercury lamp and a K3 (E. Leitz) filter combination.

Virus adsorption assay. Adsorption assays for HSV-1 were performed as described by Schnipper et al.

Gel filtration chromatography. Radioactively (3H-thymidine) labeled HSV-1 was concentrated by centrifugation and further purified on a sodium tartrate gradient. Approximately 5 x 10^9 PFU (approximately 2 x 10^17 cpm) were mixed with 22.5 μg MC540 in 1.5 mL PBS and chromatographed on a 1.6 cm x 35 cm column of Sepharose CL-2B (Pharmacia, Piscatway, NJ) equilibrated with PBS (pH 7.4). Two-milliliter fractions were collected and assayed for radioactivity, virus titer, protein concentration (optical density at 280 nm), and dye concentration (optical density at 552 nm and/or fluorescence emission at 581 nm). Optical densities were measured with a Lambda 4 UV/VIS spectrophotometer (Perkin-Elmer, Norwalk, CT). Fluorescence emission measurements were performed with an LS-5 fluorescence spectrophotometer (Perkin-Elmer) using an exciting wavelength of 551 nm.

Two-phase partition. Conditions for two-phase partition experiments were similar to those described by Smith et al. In brief, 50 μL of a concentrated virus suspension (9 x 10^6 PFU/mL) was either mixed with 5.5 μL of a 0.15 mg/mL stock solution of MC540 in 50% ethanol or an equivalent volume of solvent (control), incubated for 10 minutes at room temperature in the dark, and then added to 2 mL of a charge-sensitive two-phase system consisting of dextran T500 (5%; Pharmacia), polyethylene glycol 8000 (4.5%; Sigma), phosphate buffer (pH 7.4), NaCl (0.015 mol/L), and fetal bovine serum (5%; Sigma), and thoroughly mixed. The two phases were allowed to separate for 20 minutes (at unit gravity) at room temperature. The number of infectious virus particles in the top phase was determined by plaque formation assay and expressed as a percentage (top phase partition coefficient, Knv) of the total number (top phase, bottom phase, and interphase combined) of infectious virus particles present. High Knv values indicated high negative surface charge. Neuraminidase-treated virus suspensions were prepared by incubating HSV-1 with neuraminidase (0.15 U/mL; Vibrio cholerae, 20 U/mg protein; Serva, Heidelberg, Germany) in Hanks’ balanced salt solution for 10 minutes at room temperature.

Electron microscopy. Vero cell monolayers were incubated with untreated or photoactivated (MC540, 15 μg/mL; white light, 378 kJ/m2) HSV-1 at 0°C for 45 minutes (for adsorption studies) or at 37°C for 6 hours (for penetration/uptake studies), respectively, washed five times with Tris-NaCl buffer (0.15 mol/L NaCl, 0.02 mol/L Tris, pH 7.4, 2% bovine serum albumin), and fixed with 2.5% glutaraldehyde in 0.13 mol/L sodium phosphate buffer pH 7.4. After 1 hour, the cells were scraped off the culture dish with a rubber policeman, pelleted by centrifugation, postfixed with osmium tetroxide, in block stained with uranyl acetate, dehydrated, embedded in Spur’s resin, sectioned (60 to 70 nm), poststained with Reynold’s lead citrate, and examined with a JEOL 100B (Dearborn, MA) transmission electron microscope.

Fluorescence quenching and fluorescence resonance energy transfer. Purified HSV-1 was suspended in PBS (pH 7.4) at a density of approximately 5 x 10^9 PFU/mL. Anthracene-fatty acids (ie, 2-(9-anthroyloxy)stearic acid [2-AS], 6-(9-anthroyloxy)stearic acid [6-AS], 9-(9-anthroyloxy)stearic acid [9-AS], 12-(9-anthroyloxy)stearic acid [12-AS], or 16-(9-anthroyloxy)palmitic acid [16-AP]; Molecular Probes, Eugene, OR) were dissolved in ethanol (1 mg/mL) and then added to the virus suspension to a final concentration of 20 μmol/L. After 10 minutes at 37°C, the virus suspension was diluted fivefold with PBS, and washed twice by centrifugation (47,800g, 2 hours) and resuspended in PBS. MC540 was then added from a 1 mg/mL stock solution in 50% ethanol to a final concentration of 2.7 μmol/L for fluorescence resonance energy transfer experiments. For fluorescence quenching experiments, graded dye concentrations ranging from 0.88 to 8.8 μmol/L were used. Stern-Volmer quenching constants were obtained from Stern-Volmer plots as described by Verkman. To quantify the efficiency of fluorescence resonance energy transfer, the ratio Fma/Fm was calculated for each anthracene-fatty acid, with Fma representing the height of the fluorescence emission peak of MC540 at 585 nm and Fm representing the height of the fluorescence emission peak of the anthracene fatty acid (in the absence of MC540) at 444 nm. All fluorescence emission measurements were performed with an LS-5 fluorescence spectrophotometer (Perkin-Elmer).
RESULTS

HSV-1 was used as a model enveloped virus for most experiments because high titer preparations were easily obtained, the virus could be purified and stored frozen without significant loss of infectivity, and rapid and accurate infectivity assays were available.

When mixtures of radiolabeled HSV-1 and MC540 were chromatographed on Sepharose CL-2B, infectious virions, radioactivity, and dye co-eluted, suggesting that dye molecules were binding to the virions (Fig 1). The peak fractions contained 1.37 fg of MC540 per PFU. The maximum of the fluorescence emission spectrum was redshifted from 574 to 585 nm in the virus-containing fractions, indicating that the dye had partitioned into a lipophilic environment (most likely the viral envelope).

The most compelling evidence for a direct association of MC540 with the viral envelope was derived from partition experiments in a charge-sensitive two-phase system. Control virus preparations (exposed to solvent only) showed a mean $K_{top}$ value of $75.0\pm 4.5\%$ (mean of seven determinations $\pm$ SE) whereas the corresponding $K_{top}$ value for MC540-treated virus was significantly higher ($91.2\%\pm 3.9\%$; mean of seven determinations $\pm$ SE; $P = .0056$, 2-tailed paired $t$-test). The altered partition behavior of the MC540-treated virus indicated that dye molecules associated with the virions in such a way that the negatively charged sulfonate groups of the dye molecules enhanced the surface electronegativity of the virus particles. To demonstrate the ability of our two-phase partition system to detect charge differences, aliquots of the virus preparation were treated with neuraminidase to remove negatively charged sialic acid residues from the viral envelope before the partition experiments. As expected, the mean partition coefficient of neuraminidase-treated HSV-1 was significantly lower ($54.9\%\pm 5.9\%$, mean of seven determinations $\pm$ SE; $P = .012$, 2-tailed paired $t$-test) compared with that of untreated virus. Staining neuraminidase-treated HSV-1 with MC540 restored some negative surface charge as indicated by a larger fraction of the virus particles partitioning into the top phase ($K_{top}: 64.0\%\pm 7.2\%$; mean of seven determinations $\pm$ SE; difference not significant).

To further define the localization of dye molecules within the viral envelope, fluorescence quenching and fluorescence resonance energy transfer experiments were performed with virus preparations that had been labeled with fatty acids derivatized with anthracene in the 2, 6, 9, 12, or 16 position. The efficiency of the quenching of the anthracene fluorescence by MC540 dimers and the transfer of fluorescence resonance energy from anthracene fatty acids to dye monomer is inversely proportional to the distance

![Fraction Number](image1)

**Table 1. Localization of MC540 in the Envelope of HSV-1: Fluorescence Quenching of Anthracene-Fatty Acids by MC540 and Fluorescence Resonance Energy Transfer Between Anthracene Fatty Acids and MC540**

<table>
<thead>
<tr>
<th>Probe</th>
<th>$K_d$ ($\mu$m/L)</th>
<th>$F_{644}/F_{444}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-AS</td>
<td>2.00</td>
<td>0.68</td>
</tr>
<tr>
<td>6-AS</td>
<td>0.73</td>
<td>1.22</td>
</tr>
<tr>
<td>9-AS</td>
<td>0.47</td>
<td>0.54</td>
</tr>
<tr>
<td>12-AS</td>
<td>1.59</td>
<td>0.47</td>
</tr>
<tr>
<td>16-AP</td>
<td>0.96</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Data represent means of two independent experiments. $K_d$, Stern-Volmer quenching constant.

![MC 540 (µM)](image2)

![Stern-Volmer plots of the quenching of anthracene fluorescence by MC540 dimers in HSV-1 labeled with 2-AS ( ), 6-AS ( ), 9-AS ( ), 12-AS ( ), or 16-AP ( ). The fluorescence emission of the anthracene probe in the presence (F) or absence (Fo) of the quencher was measured at 444 nm. Data points represent single determinations. Coefficients of correlation ($r^2$) for least square regression lines are $\approx .98$.](image3)
between the two molecules. As Table 1 shows, quenching was most pronounced between MC540 and 2-AS, followed by 12-AS, 16-AP, 6-AS, and 9-AS, which was indicative of dimers accumulating both near the periphery of the viral envelope and deep within the lipid bilayer. Stern-Volmer plots (Fig 2) were linear, as is typically found with a single class of fluorophores that are all equally accessible to the quencher. Fluorescence resonance energy transfer to the MC540 monomer was most efficient with 6-AS and 2-AS (Table 1), suggesting that monomers localized near the surface of the viral envelope.

MC540-sensitized photoinactivation markedly reduced the ability of radiolabeled HSV-1 to adhere to its host cell (Fig 3). Electron microscopic investigations confirmed that MC540-sensitized photoinactivation interfered with virus adhesion. In 60- to 70-nm sections, about 50% to 60% of cells had virus particles bound to their surfaces if the incubation (1 hour, 0°C) was performed with untreated virus. If a photoinactivated (MC540, 15 μg/mL; light, 90 minutes) aliquot of the same virus preparation was used, only about 25% to 30% of cells had virus particles attached to their surfaces. In the latter case, up to 50% of the cell-associated virions appeared structurally intact, although plaque assays indicated a greater than 6 log reduction of infectivity. However, the remaining virions showed evidence of massive photodynamic damage, such as ruptured envelopes and a striking lack of dense cores (Fig 4). When Vero cells were incubated with untreated or photoinactivated virus for 6 hours at 37°C to allow penetration, intracellular virus was observed in both specimens (data not shown). However, cells exposed to photoinactivated virus contained fewer virions. Nuclear virus was only observed in cells that had been exposed to untreated virus (data not shown).

The effect of dye-sensitized photoinactivation on viral antigen expression was studied in HSV-1-infected Vero cells and in CMV-infected human foreskin fibroblasts. Vero cells that were 'infected' with photoinactivated (MC540, 15 μg/mL; light, 90 minutes) HSV-1 failed to express the viral antigen detected by anti-HSV-1 antibodies (data not shown). Similarly, human foreskin fibroblasts 'infected' with photoinactivated CMV consistently showed negative reactions with antibodies against early and late antigen (Figs 5 and 6). With the exception of two very weak positive reactions obtained with high concentrations of one particular lot of antibody, reactions with antibodies against pre-early antigen were also negative (Fig 7).

The MC540-sensitized photoinactivation of HSV-1 was oxygen-dependent (Fig 8). Exhaustive (105 minutes) flushing...
Fig 5. Expression of early antigen by human foreskin fibroblast cultures 24 hours after infection with untreated (A and B) or photoinactivated (C and D; MC540, 15 µg/ml; light, 90 minutes) CMV. (A and C) Phase contrast; (B and D) fluorescence. Horizontal bar: 10 µm.

of the virus suspension with argon inhibited the reaction while displacement of air by oxygen enhanced it slightly (Fig 8). Flushing the virus suspension only briefly (30 seconds) with nitrogen (sufficient to prevent the MC540-sensitized photolysis of L1210 leukemia cells28) had no effect on the MC540-sensitized photoinactivation of HSV-1 (data not shown).

Deuterium oxide increases the half-life of singlet oxygen. Therefore, enhanced photodynamic effects in the presence of deuterium oxide are viewed as good but not definitive evidence for a type II (singlet oxygen-mediated) mechanism. As Fig 8 shows, replacing 88% of the water in the medium by deuterium oxide enhanced the dye-sensitized photoinactivation of HSV-1.

Cysteamine (10 or 30 mmol/L) and, to a lesser degree, glutathione (10 or 30 mmol/L) and a low concentration (0.1 mmol/L) of dithiothreitol (DTT) protected HSV-1 against MC540-sensitized photoinactivation (Figs 9 and 10). High concentrations (1 or 10 mmol/L) of DTT had a direct antiviral effect (Fig 10). Trolox (tetramethylchrooman-2-carboxylic acid), another antioxidant, had no effect at 0.01 mmol/L and accelerated the MC540-sensitized photoinactivation of HSV-1 when used at concentrations of 0.1 or 1 mmol/L (Fig 10). The presence of superoxide dismutase at concentrations ranging from 1.45 to 29 U/ml during the irradiation step had no effect on the MC540-sensitized photoinactivation of HSV-1 (data not shown). When the addition of the antioxidants was delayed until shortly after the completion of the irradiation step, a limited level of protection was still achieved with cysteamine (10 or 30 mmol/L) (data not shown). Glutathione (10 or 30 mmol/L), Trolox (0.01, 0.1, or 1 mmol/L), and low concentrations of DTT added after the photoirradiation step had little or no effect.

DISCUSSION

Investigations into the cytotoxic effects of MC540 have shown that the dye binds primarily to hydrophobic domains of the plasma membrane2; that the plasma membrane is a target of photodynamic damages29,32 and that the inactiva-
Fig 7. Expression of pre-early antigen by human foreskin fibroblast cultures 6 hours after infection with untreated (A and B) or photoinactivated (C and D; MC540: 15 μg/mL; light: 90 minutes) CMV. (A and C) Phase contrast; (B and D) fluorescence. Horizontal bar: 10 μm.

Fig 8. (Left) Representative example of the MC540-sensitized photoinactivation of HSV-1 in medium equilibrated with air (○), argon (□), or oxygen (▲). Data points represent mean plaque counts of ≥4 culture wells ± standard errors. (Right) Representative example of the MC540-sensitized photoinactivation of HSV-1 in water (○) or 88% deuterium oxide (■). Some virus inactivation curves did not follow first order kinetics. Deviations from first order kinetics probably reflected dye bleaching and/or the presence of virus aggregates rather than the presence of MC540-resistant mutants. Attempts to isolate mutant strains from the rare plaques formed by extensively photoinactivated virus preparations failed. Data points represent mean plaque counts of ≥4 culture wells ± standard errors. Most standard errors are smaller than the data symbols.

Fig 9. Representative example of the effects of glutathione and cysteamine on the MC540-sensitized photoinactivation of HSV-1. (○) Control; (□), glutathione, 10 mmol/L; (■), glutathione, 30 mmol/L; (▲), cysteamine, 10 mmol/L; (△), cysteamine, 30 mmol/L. Data points represent mean plaque counts of ≥4 culture wells. Standard errors are smaller than the data symbols.

The data presented in this report show several similarities but also some interesting differences between the interactions of MC540 with cells and its interactions with enveloped viruses.

The results of our two-phase partition experiments indicated that MC540 bound to the surface (envelope) of infectious virions in such a way that the sulfonate groups of adsorbed dye molecules enhanced the negative surface charge of the virus. The possibility that the altered partition behavior of HSV-1 was caused by free dye could be ruled out because the concentration of dye was much too low to have a significant impact on the anion concentration of the
charge-sensitive system. While it was possible that the virus preparation was contaminated with cellular material (eg, plasma membrane fragments) that was capable of binding MC540, such a contamination should not have interfered with our analysis because the endpoint of our assay was the distribution of infectious virions rather than the distribution of particles, dye, or particle-dye complexes.

The comigration of virions and dye molecules on the gel filtration column and the red-shifted fluorescence emission spectrum of the virus-containing fractions was consistent with the view that MC540 bound to enveloped virions. It is possible that in the latter experiments, dye binding to virions was confused with dye binding to cellular membrane fragments that happened to be copurified with the virus. However, the results of the electron microscopic analysis suggest the possibility of such an error was rather remote.

Both fluorescence quenching and fluorescence resonance energy transfer between MC540 and anthracene-fatty acids were more efficient in HSV-1 than in cells. The ready partitioning of MC540 into the lipid bilayer of the viral envelope may explain why enveloped viruses tend to be more susceptible to MC540-sensitized photoinactivation than cells. The localization of dye molecules in viral envelopes differed somewhat from that in plasma membranes. In HSV-1, fluorescence quenching by the MC540 dimer was most efficient with 2-AS and 12-AS. By contrast, cells show little evidence of dimer accumulation close to the 12-AS probe unless they are exposed to at least a moderate dose of light. With regard to the localization of the MC540 monomer, cells and HSV-1 behaved similarly. In both cells and viruses, fluorescence resonance energy transfer to the MC540 monomer was most efficient with anthracene probes located close to the surface of the lipid bilayer (eg, 2-AS and 6-AS).

The proportion of viruses showing evidence of massive structural damage was smaller than what we had expected on the basis of infectivity assays. An analogous situation has been described for the MC540-sensitized photoinactivation of leukemia cells where a ≤10% increase in trypan blue-positive cells contrasts with a ≥4 log loss of in vitro clonogenic cells. However, if trypan blue exclusion assays are delayed for several hours or even a few days, the discrepancy becomes progressively smaller, suggesting that the MC540-sensitized photolysis of a cell is a process that extends well beyond the completion of the photoirradiation step and involves the formation of potentially lethal damages that respond to treatment with antioxidants. Because all virus preparations were processed immediately after completion of the photolysis step, our electron microscopic analyses may have underestimated the extent of structural damages.

The reduced ability of photoinactivated virus to adhere to and penetrate host cells was probably a result of photodynamic damage to the viral envelope. Nonspecific adhesion of damaged virions and uptake of damaged virions via endocytotic mechanisms may explain the incomplete inhibition of adhesion and penetration in high-titer virus preparations that were rendered noninfectious by prolonged exposure to MC540 and light. Alternatively, it is conceivable that MC540-sensitized photoinactivation also affected the infectious process at a stage subsequent to penetration. Recent data show that prolonged exposure to MC540 and light leads not only to the cross-linking of envelope proteins but, eventually, also of core proteins (T.P.W., F.S., unpublished data, June 1991). It is possible that photodynamic damages to core proteins or viral DNA were in part responsible for the inactivation of the virus.

Oxygen depletion of the medium inhibited the dye-sensitized photoinactivation of HSV-1 but failed to prevent it completely. It is not clear whether the residual antiviral activity in oxygen-depleted medium was attributable to a type II (singlet oxygen-mediated) reaction that used low levels of residual oxygen or to an oxygen-independent process. Evidence of a MC540-sensitized type I (free radical-mediated) reaction has recently been observed in oxygen-free liposome preparations. However, when simi-
lar experiments are performed in the presence of oxygen, type II chemistry dominates.  

The importance of singlet oxygen in the MC540-sensitized photoinactivation of cells and viruses is sometimes questioned because of the low singlet oxygen quantum yield of MC540 in aqueous solutions. However, the antiviral and antileukemic activities of MC540 are mediated primarily by membrane-bound dye monomers, and the singlet oxygen quantum yield of membrane-bound MC540 is about one order of magnitude higher than the quantum yield in solution.  

The stimulatory effect of deuterium oxide on the MC540-sensitized photoinactivation of HSV-1 may appear modest. However, it has to be taken into consideration that only 88% of the water was replaced by deuterium oxide and that all experiments were conducted in the presence of serum proteins that are known to be efficient quenchers of singlet oxygen. Furthermore, MC540 localizes preferentially in hydrophobic domains to which deuterium oxide may have limited access.

The observed protective effect of thiol is compatible with the view that viruses are inactivated by an oxidative process. Because some thiols (eg, glutathione) are more efficient at protecting cells while others (eg, cysteamine) are more efficient at protecting viruses, thiols may provide a means to enhance the therapeutic efficacy of MC540 as a blood sterilizing agent.

All lipid-enveloped viruses that have been examined so far have been highly susceptible to MC540-sensitized photoinactivation, presumably because they all bind MC540 with high affinity. The exact nature of the viral dye-binding site remains unclear. Although experiments with liposomes have shown that fluid-like bilayers have a higher affinity for MC540 than gel-like bilayers, it seems unlikely that a high fluidity of the viral envelope is the primary reason for the high susceptibility of enveloped viruses to MC540-sensitized photoinactivation. HIV-1 is highly sensitive to MC540-sensitized photoinactivation, yet its envelope is known for its extremely low fluidity. It is conceivable that the small size of virosomes facilitates the partitioning of dye molecules into the lipid envelope because the high radius of curvature leads to a wider spacing of the polar head groups of phospholipids in the lipid bilayer.

In conclusion, the data presented in this report indicate that MC540 binds to the lipid envelope of HSV-1, the viral envelope is a major target of MC540-mediated photodynamic damages, and MC540-sensitized photoirradiation interferes with early events of the infectious process. Data obtained in the presence of oxygen are compatible with but not strictly diagnostic of a type II mechanism.

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