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Mechanism of Copper-Mediated Inactivation of Herpes Simplex Virus

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The inactivation of herpes simplex virus (HSV) by copper was enhanced by the following reducing agents at the indicated relative level: ascorbic acid ⩾ hydrogen peroxide > cysteine. Treatment of HSV-infected cells with combinations of Cu(II) and ascorbate completely inhibited virus plaque formation to below 0.006% of the infectious virus input, while it maintained 30% viability for the host mammalian cells. The logarithm of the surviving fraction of HSV mediated by 1 mg of Cu(II) per liter and 100 mg of reducing agent per liter followed a linear relationship with the reaction time, in which the kinetic rate constant for each reducing agent was $-0.87\ min^{-1}$ ($r = 0.93$) for ascorbate, $-0.10\ min^{-1}$ ($r = 0.97$) for hydrogen peroxide, and $-0.04\ min^{-1}$ ($r = 0.97$) for cysteine. The protective effects of metal chelators and catalase, the lack of effect of superoxide dismutase, and the partial protection conferred by free-radical scavengers suggest that the mechanism of copper-mediated HSV inactivation is similar to that previously reported for copper-mediated DNA damage. The sensitivity exhibited by HSV to Cu(II) and reducing agents, particularly ascorbate, might be useful in the development of therapeutic antiviral agents.

The agents that have been investigated for the treatment of the various herpesvirus infections include idoxuridine, trifluoride, vidarabine, brivudin, ganciclovir, foscarnet, 256U87, 882C87, BVaraU, HPMPC, and acyclovir (8, 9, 30). Over the past decade, acyclovir has become established as standard therapy for the management of herpes simplex virus (HSV) infection, but there are areas where improvement is desirable (7, 19). Treatment of recurrent and persistent infections in graft recipients, cancer patients, and those suffering from AIDS with acyclovir, ganciclovir, and foscarnet has resulted in both clinical benefit and the emergence of drug-resistant variants (15, 19, 23). In addition, although topical application may be beneficial, no improvement in the systemic symptoms of genital herpes simplex was reported with oral acyclovir treatment (10). Thus, there is still benefit to be gained by finding other means to treat HSV.

Cupric ions have been shown to inactivate several types of viruses, including members of the *Herpesvirus* and *Arenavirus* families, various bacteriophages treated in vitro, and free as well as intracellular human immunodeficiency virus (26–28).

Most knowledge on the mechanisms of copper-mediated biological damage comes from studies with DNA (3, 12, 24, 34), in which Cu(II) has been shown to bind specifically to DNA (25), favoring guanosine residues (24). The products of the reaction between Cu(II) and DNA include single and double DNA strand breaks as well as base modification, mainly production of 8OH-deoxyguanosine, observed in vitro and in vivo (3, 12, 34). Free radicals are believed to be involved in the chemical reaction, but copper-mediated DNA damage is only partially inhibited by free-radical scavengers (24, 29).

Hydrogen peroxide per se produces negligible damage, but this substance is known to potentiate both the DNA damage and the virucidal effects of Cu(II) ions (3, 26, 27). Other reducing substances like ascorbate and cysteine are known to potentiate oxidative damage (3, 5, 29, 33), but their role in Cu-mediated virus killing remains to be established.

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Previous reports on inactivation of herpesvirus, arenavirus, and human immunodeficiency virus (26–28), together with advances in current knowledge of the interaction of copper with biomolecules, justified this study to explore the potential of copper-based formulations for the treatment of HSV infection.

MATERIALS AND METHODS

Chemicals. Cupric chloride ($CuCl_2 \cdot 2H_2O$), L-ascorbic acid, and L-cysteine were purchased from Sigma Chemical Co. (St. Louis, Mo.). Hydrogen peroxide (30% [wt/vol]) was obtained from Mallinckrodt Inc. (Paris, Ky.). Glutaraldehyde (8%) in sealed glass ampoules was purchased from Ladd Research Industries (Burlington, Vt.). For stock solutions, the compounds were dissolved in distilled water, subsequently sterilized by passage through 0.22-μm-pore-size membrane filters (Millex GV; Millipore, Bedford, Mass.), and stored at 8°C. The copper salt was prepared to yield 7 g of Cu(II) per liter of stock solution. L-Ascorbic acid and L-cysteine were also dissolved to a concentration of 7 g/liter. Serial dilutions (1:10) in sterile distilled water were prepared fresh from the stock solutions before each experiment. Catalase (EC 1.11.1.6), superoxide dismutase (EC 1.15.1.1), DNA type VII from herring sperm, RNA from calf liver, albumin fraction V, lipoprotein from bovine plasma, glycogen, and desferoxamine mesylate were purchased from Sigma Chemical Co.

Virus. HSV type 1, MP strain, was grown and plaque assayed on Vero African green monkey kidney fibroblasts as described previously (18). HSV was used at initial concentrations of 1×10^6 to 2×10^6 PFU/ml (experiment-to-experiment variation) in Dulbecco's phosphate-buffered saline (without Ca^{2+}, Mg^{2+}) (DPBS) and was diluted from stock solutions immediately before each experiment.

Inactivation protocol. Virus was diluted 1:4 in sterile, conical 1.5-ml Eppendorf tubes in DPBS (pH 7.4) at 21°C to 20 μl, before adding 8 μl of the inactivating reagents at various concentrations. Enzymes and free-radical scavengers were added, when necessary, to the DPBS in which the virus was diluted. After 30 min of incubation at 21°C, the reaction mixture (total volume, 28 μl) was diluted to 1 ml with ice-cold Earle's minimal essential medium (EMEM)–5% fetal calf serum (FCS) and was put on ice to stop the chemical inactivation process. The surviving virus in each sample was titrated immediately (within 30 min).

For comparing the potentiating effects of the different reducing agents, 4 μl of each reducing agent at various concentrations was added to 20 μl of the virus dilution plus 4 μl of Cu(II) (final concentration, 1 mg/liter). After 30 min of incubation at 21°C, the reaction mixture (total volume, 28 μl) was diluted as described above and was immediately titrated.

For kinetic experiments, 40 μl of virus stock was diluted with 120 μl of DPBS, before adding Cu(II) and reducing agent (32 μl each) for a total reaction volume of 224 μl. At preestablished time intervals, 28 μl was withdrawn from the reaction mixture and diluted to 1 ml with ice-cold EMEM–5% FCS, and surviving HSV was immediately titrated.

Plaque-forming inhibition assay and cellular toxicity. Vero cell monolayers were grown to 95 to 100% confluence over 48 h in T-25 plastic flasks before infecting them with HSV for 1 h at 37°C. The inoculum was removed, and the

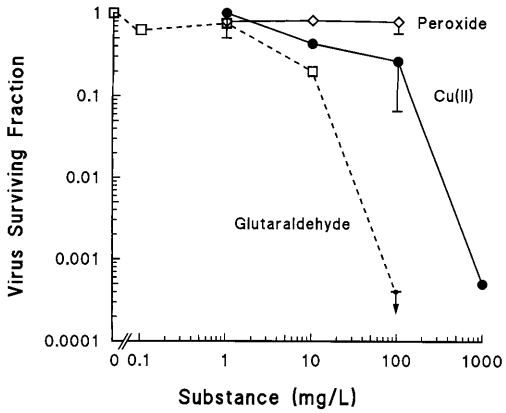

FIG. 1. Survival of HSV after treatment for 30 min at 21°C with different concentrations of Cu(II), hydrogen peroxide, or glutaraldehyde. Mean values from at least three independent experiments are presented; standard errors are shown as error bars when they extend beyond the datum symbols.

cells were then incubated in 4 ml of growth medium with 0.25% (wt/vol) gamma globulin and infection was allowed to proceed for an additional hour at 37°C. The infected cells were then exposed to several combinations of copper and reducing agent for 30 min at either 21 or 37°C. The treatment mixture was removed, and the cells were washed with 3 ml of DPBS and typically incubated for 48 h in medium at 37°C under 5% CO_2 before HSV plaques were stained with crystal violet and counted. Approximately 200 colonies were typically obtained in untreated infected cells.

The cytotoxicity of Cu(II) plus reducing agent was estimated under identical conditions in the same experiment by incubating trypsinized cells with 0.4% trypan blue (supravital stain) and calculating viability from counts (obtained with a hemocytometer) of unstained cells (cells preserving membrane integrity) divided by the total number of cells (stained, membrane-damaged cells plus unstained cells). For measuring the survival of Vero cells, approximately 5×10^5 cells in a monolayer were treated with various concentrations of cupric chloride, ascorbic acid, hydrogen peroxide, or cysteine in a final volume of 224 μl for 30 min at 21°C. After treatment, the cells were rinsed with DPBS, trypsinized, resuspended in 2 ml of fresh medium, seeded in petri dishes, and incubated for 12 to 14 days at 37°C under 5% CO_2. Colonies from surviving cells were stained with 0.1% Evans blue and counted. Approximately 100 colonies were typically obtained after seeding 1 ml of a 1:2,000 dilution from untreated cell controls.

RESULTS

Treatment with single compounds. Inactivation of HSV by Cu(II) alone or by hydrogen peroxide alone was first determined and compared with inactivation by the widely used virucidal agent glutaraldehyde (20, 36). The survival of HSV treated with different concentrations of Cu(II), peroxide, and glutaraldehyde is presented in Fig. 1.

Ninety percent of the HSV was inactivated in 30 min by 100 to 200 mg of Cu(II) per liter; a 0.1% solution (1,000 mg/liter, equal to 16 mM) of Cu(II) reduced virus survival by more than three orders of magnitude. Peroxide at concentrations of up to 100 mg/liter (or 2.9 mM) had no effect. Glutaraldehyde was roughly 10 times more efficient (on a weight-per-volume basis) at inactivating HSV than Cu(II) alone.

Cu(II) at 100 mg/liter reduced colony-forming cell survival to about 1% (Fig. 2). The toxicities of the reducing agents were also studied. None of these reducing agents altered cell viability (dye exclusion) immediately after treatment or 24 h later (data not shown). Cell survival (colony formation), displayed in Fig. 2, remained approximately constant up to the studied limit of 1,000 mg/liter for ascorbic acid and cysteine and decreased to about 10% with hydrogen peroxide at 1,000 mg/liter.

Potentiating effects of reducing agents. In an attempt to reduce the toxicity of copper alone while maintaining HSV killing, the effects of lower concentrations of Cu(II) plus reducing agents were studied. The sensitivity of HSV to inactivation by combinations of Cu(II) at levels found in normal blood, 1 mg/liter (16 μM) (11), with different nontoxic concentrations of either hydrogen peroxide, cysteine, or ascorbic acid is presented in Fig. 3.

A mixture of 1 mg of Cu(II) per liter with either 10 or 100 mg of hydrogen peroxide per liter reduced HSV survival to approximately 10 and 0.1%, respectively (Fig. 3A). These results are similar to previous data reported by this laboratory (27).

With Cu(II) at 1 mg/liter and ascorbic acid at 10 mg/liter (57 μM), HSV survival was reduced to less than 0.1% in 30 min. (Fig. 3C).

Inactivation by Cu(II) and cysteine was unusual in that Cu(II) at 1 mg/liter plus cysteine at 100 mg/liter (825 μM) inactivated approximately 90% of the HSV PFUs, while at a higher concentration, cysteine was no longer effective (Fig. 3B). With a higher concentration of Cu(II) (10 mg/liter), cysteine was synergistic at 10 and 100 mg/liter, but again, not at 1,000 mg/liter (data not shown). A "window" of effective concentration occurred only with cysteine; the other reducing agents were more effective at increasing concentrations (tested up to 1,000 mg/liter). Similar patterns of virucidal enhancement were found for inactivation of bacteriophages ϕX174, T7, and ϕ6 with these reducing agents and Cu(II) (data not shown). Over the concentration ranges investigated, none of the reducing agents alone had any effect on HSV survival.

Combinations of Cu(II) (1 mg/liter) with ascorbic acid (10 mg/liter) or hydrogen peroxide (100 mg/liter) that killed 99.9% of the HSV isolates did not substantially reduce cell viability (data not shown) or survival (Fig. 4). Combinations of Cu(II) with cysteine did not affect viability or survival within the concentration range tested.

Inhibition of HSV within infected cells. Before treatment, cells were infected with HSV (one h), and infection was allowed to proceed for an additional hour with complete medium. Any remaining extracellular HSV was neutralized with

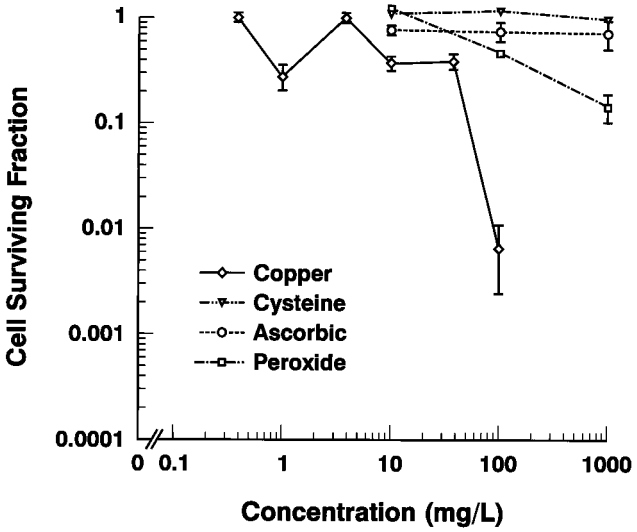

FIG. 2. Survival of Vero cells after treatment with different concentrations of Cu(II) or reducing agents. Cells were incubated with either cupric chloride, ascorbic acid, hydrogen peroxide, or cysteine at concentrations indicated on the *x* axis for 30 min at 21°C as described in Materials and Methods. After treatment, the cells were rinsed, trypsinized, resuspended in fresh medium, seeded in petri dishes, and incubated for 12 to 14 days at 37°C under 5% CO_2. The number of colonies from cells surviving treatment relative to the number of colonies in untreated controls is displayed (means ± standard error [when extending beyond symbols] from four independent experiments).

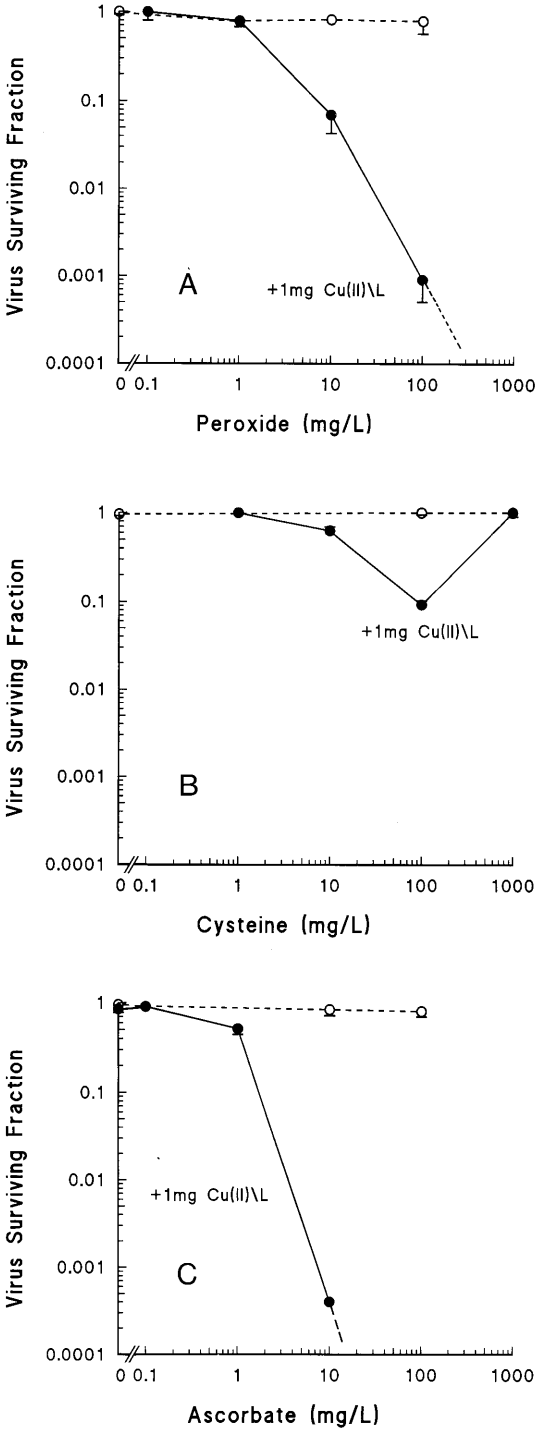

FIG. 3. Inactivation of HSV after exposure for 30 min at 21°C with 1 mg of Cu(II) per liter and different concentrations of hydrogen peroxide (A), cysteine (B), or ascorbic acid (C). Survival of HSV treated with the reducing agents in the absence of copper is indicated by open circles linked by dashed lines. Mean values from at least three independent experiments are presented; standard errors are shown as error bars when they extend beyond the datum symbols.

gamma globulin. The cells were then washed with DPBS and treated with different amounts of Cu(II) and ascorbic acid for 30 min at 37°C, after which the cells were washed with DPBS and incubated for 48 h in medium. The degrees of inhibition of

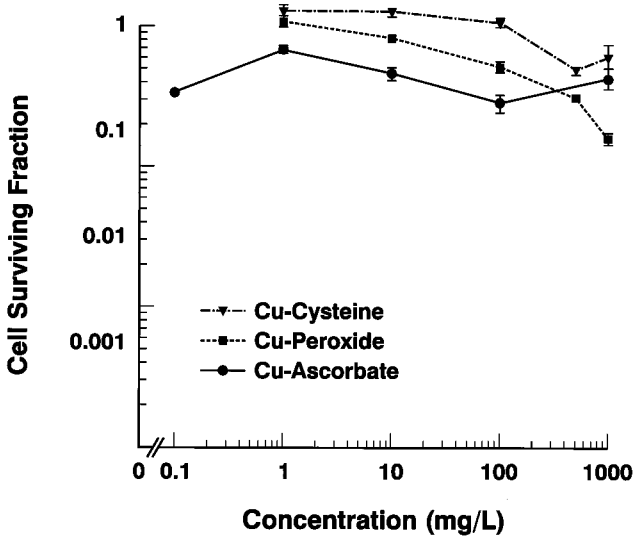

FIG. 4. Survival of cells exposed to Cu(II) at 1 mg/liter and various concentrations of cysteine, hydrogen peroxide, or ascorbic acid. The conditions used to treat cells, which were identical to those used to treat HSV in the experiment whose results are presented in Fig. 3, are described in Materials and Methods and in the legend to Fig. 2. The average ± standard error (when they extend beyond the symbols) of four experiments is displayed.

HSV plaque-forming ability by different concentrations of Cu(II) and ascorbate and concurrent cytotoxicity were compared to those for untreated controls (Table 1). No slowly growing plaques were evident since the number of plaques scored after 48 h remained constant for up to 5 days, the time at which the examination of treated-infected cells was terminated. Plaque inhibition was four to eight times lower at 21°C than at 37°C (data not shown). In all experiments (n = 11) done with Cu(II) and ascorbate at concentrations of between 0.5 and 5 mg/liter and 50 and 500 mg/liter, respectively, cell monolayers appeared to be 80% or more intact (confluent). The cells that were released from monolayers with trypsin and subcultured immediately after Cu(II) and ascorbate treatment had a relatively low (near 4%) survival, but cells subcultured 4 days later recovered to 25 to 37% survival.

In four independent experiments designed to determine the maximal HSV challenge that could be inactivated by 2.5 mg of Cu(II) per liter and 250 mg of ascorbate per liter (30 min of treatment), cells infected with 50 to 200,000 PFU (corresponding to multiplicities of infection ranging from 0.005 to 0.2)

TABLE 1. Inhibition of HSV plaque formation in infected cells[a]

Cu(II)/ascorbate (mg/liter)	% HSV plaques[b]	% Cell viability[c]
0/0	100	95
0.5/50	5.5	42
1.0/100	<0.5[d]	29
2.5/250	<0.0005[e]	24
5.0/500	<0.5[d]	14

[a] Vero cells were treated 2 h postinfection for 30 min at 37°C.
[b] The number of plaques was counted 48 h postinfection on cell monolayers that were apparently 80% or better intact.
[c] Cell viability was measured as the ratio of cells that maintained membrane integrity (by supravital staining) divided by the total number of cells.
[d] The number of plaques was 0, compared to 200 for untreated controls.
[e] No plaques developed, although cells were infected with 2×10^5 PFU of HSV before treatment.

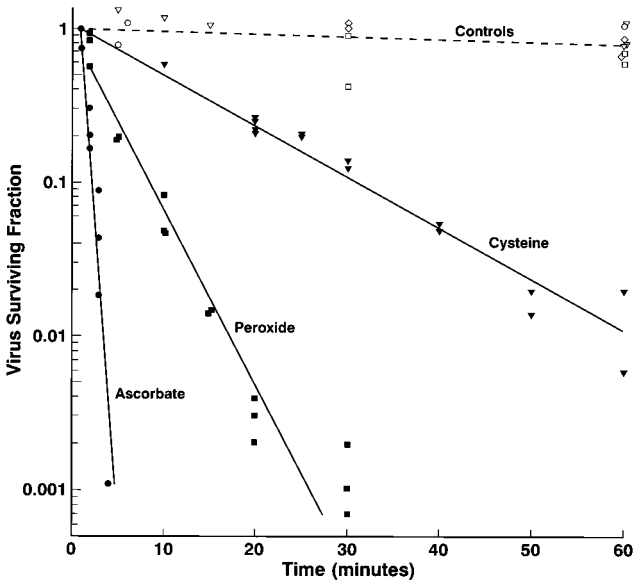

FIG. 5. Inactivation kinetics of HSV at 21°C by 1 mg of Cu(II)/liter and 100 mg of either cysteine, hydrogen peroxide, or ascorbate per liter. Data on the survival of HSV exposed to Cu(II) alone (open diamonds) or to each of the reducing agents (open triangles, squares, and circles, respectively) are included as controls.

failed to show plaques 48 h after treatment, while plaque formation in untreated controls was as expected.

To determine whether inhibition of HSV plaques by Cu(II) and ascorbate was irreversible, infected and treated cells were subcultured onto new untreated, uninfected monolayers. Nearly 100% plaque-forming ability was recovered after subculture of infected, untreated cell controls. In contrast, no HSV was recovered from infected cells subcultured after treatment with Cu(II) and ascorbate, indicating that our treatment left no HSV available to infect untreated cells.

The kinetics of HSV inactivation. The inactivation of free HSV as a function of time by Cu(II) (1 mg/liter) in the presence of ascorbate, hydrogen peroxide, or cysteine (100 mg/liter) is depicted in Fig. 5. The virucidal reaction mediated by Cu(II) followed a linear relationship of the form $\log S = kt$, where S is the surviving fraction, k is the kinetic rate constant, and t is the reaction time. The kinetic rate of HSV inactivation was estimated with commercially available software (Exel; Microsoft Corporation, Bothell, Wash.). The values of k for Cu(II) in combination with each reducing agent were -0.87 min^{-1} ($r = 0.93$) for ascorbate, -0.10 min^{-1} ($r = 0.97$) for hydrogen peroxide, and -0.04 min^{-1} ($r = 0.97$) for cysteine. The times to inactivate 90% of the HSV PFUs (D_{10} values) were 1.2, 9.8, and 24.7 min for Cu(II) in combination with ascorbate, hydrogen peroxide, and cysteine, respectively.

The mechanism of HSV inactivation. The anti-HSV reaction mediated by copper and reducing agents was investigated by adding various enzymes and chemical inhibitors (Table 2). Under these inactivation conditions (chemical concentrations and treatment times), it was possible to detect an increase or decrease in survival of Cu-cysteine-treated HSV isolates, only an increase in the survival of Cu-hydrogen peroxide-treated HSV isolates, and only a great increase in the survival of Cu-ascorbate-treated HSV isolates.

As expected, the metal chelators EDTA and desferoxamine prevented inactivation. The Cu(I) chelator bathocuproine partially precipitated in our cell incubation medium, rendering the results obtained with this substance difficult to interpret (data not shown).

Of the enzymes studied, catalase prevented most of the inactivation, while superoxide dismutase had little, if any, effect. The effect observed with native catalase was totally abolished by boiling the enzyme for 20 min (data not shown).

The free-radical scavenger dimethyl sulfoxide had a small (measurable) protective effect on Cu(II)-mediated inactivation only with peroxide. The free-radical scavengers sodium azide (0.01 M), *tert*-butyl alcohol (1 M), and mannitol (1 M) interfered with virus replication, making interpretation of the results impossible. Bovine serum albumin, DNA, and glycogen did not protect HSV from Cu(II) with any of the reducing agents. Only RNA and lipoprotein displayed even a minimal protective effect.

DISCUSSION

Anti-HSV activity of copper and reducing agents. Cupric ions alone at 100 to 200 mg/liter (1.6 to 3.2 mM) killed HSV and host cells in a similar way (over 90%; Fig. 1 and 2). The addition of any of the three reducing agents tested in this work allowed for the reduction of the concentration of Cu(II) used to a concentration similar to that in human blood, resulting in low cytotoxicity and a retention of HSV killing. Cu(II) at 1 mg/liter (16 μM) plus cysteine at 100 mg/liter (820 μM) reduced HSV by 90% without affecting cell survival or viability. Cu(II) at 1 mg/liter plus hydrogen peroxide at 100 mg/liter (2.9 mM) or ascorbate at 10 mg/liter (57 μM) killed 99.9% or more of the HSV isolates without significant cellular toxicity. Cu(II)

TABLE 2. Survival of HSV treated with Cu(II) and three reducing agents in the presence of various inhibitors

Inhibitor	% Survival[a]		
	Ascorbate	Hydrogen peroxide	Cysteine
None	≤0.02[b]	<0.03[c]	13 ± 1
Chelator			
EDTA (0.1 M)	119 ± 8	54 ± 13	101 ± 22
Desferoxamine (0.1 M)	112 ± 27	70 ± 31	88 ± 24
Enzymes			
Catalase (50 U)	1.1 ± 0.4	94 ± 0.5	90 ± 11
Superoxide dismutase (50 U)	<0.04	<0.05	22 ± 3
Scavengers			
Dimethyl sulfoxide (1 M)	<0.04	0.6 ± 0.1	13 ± 1
Bovine serum albumin (100 μg/ml)	<0.04	<0.04	12 ± 3
DNA (100 μg/ml)	<0.04	<0.05	11 ± 3
RNA (100 μg/ml)	<0.04	0.3 ± 0.1	27 ± 4
Lipoprotein (100 μg/ml)	<0.04	0.6 ± 0.5	34 ± 14
Glycogen (100 μg/ml)	<0.04	<0.05	14 ± 3
Salt, KCl (1 M)	3.0 ± 1.3	0.2 ± 0.2	116 ± 1

[a] Percent survival after treatment of HSV with Cu(II) at 1 mg/liter and reducing agent at 100 mg/liter for 30 min at 21°C.
[b] Survival after Cu(II)-ascorbate treatment for 30 min is expected to be reduced by more than 10^{20}-fold.
[c] Survival after Cu(II)-hydrogen peroxide treatment for 30 min is expected to be reduced to about 0.03%.

at 2.5 mg/liter plus ascorbate at 250 mg/liter maintained 24% cell viability, while it reduced HSV plaque formation below 0.0005% after infected cells were treated for 30 min at 37°C.

Dose-response as well as kinetic experiments demonstrated that the enhancing virucidal effect of the reducing agents that we studied followed the order ascorbate ≫ hydrogen peroxide > cysteine. The relative order of enhancement that we observed for Cu(II)-mediated HSV killing was identical to that previously reported for DNA damage mediated by ferric ions (32, 33). The window of an effective concentration for cysteine and the lack of such a window for hydrogen peroxide and ascorbic acid resembled the results found for those compounds in enhancing the DNA damage caused by Fe(III) ions (33).

Biological occurrence of the substances studied. The concentrations of the substances that killed HSV are not expected to be toxic in the human body because similar concentrations either are present endogenously or have been found to be nontoxic.

The concentration of cysteine at which we observed maximal antiviral enhancement was 100 mg/liter. The lethal toxic dose that kills half of the experimental population for cysteine is 5,580 mg/kg of body weight in rats (17). Thus, the antiviral cysteine concentration (100 mg/liter) (with copper) was well below toxic levels and is approximately 30 times lower than the amount available in normal human plasma (3.2 g/liter) (11).

Normal human peripheral blood polymorphonuclear leukocytes (PMNs) produce 8 nmol of H_2O_2 per 10^6 PMNs within 5 min of activation by a variety of antigens (21). Given the average volume of a PMN (approximately 4×10^{-12} liter), the production of peroxide must reach levels close to 2 mM. Hence, the concentrations of H_2O_2 studied, in the range of between 0.1 and 100 mg/liter (3 to 300 μM), are well within the concentrations found in some human cells. In addition, by forming spontaneous adducts with biomolecules, H_2O_2 can diffuse far from the production site to intracellular locations where the biological effect may be greater and toxicity could be lower (31).

The copper content in normal human plasma is approximately 1 mg/liter (11), similar to the concentration used in the experiments whose results are presented in Fig. 3 and 5. Although copper in plasma is bound to proteins, the equilibrium between bound and available copper could be displaced by the relative rapid reaction of Cu(II) with the reducing agents. Furthermore, killing of HSV in the presence of albumin demonstrates that Cu(II) preserves its virucidal activity even in the presence of known copper-complexing proteins.

Intravenous injection of 2.5 mg of copper per day has been used to treat many patients with rheumatoid arthritis (1). Previous experiments in our laboratory demonstrated that 4 mg of copper/kg of body weight/day can be parenterally administered to rats for several days without histopathological changes in kidney or liver (34). Various protocols in our experiments maintained the serum copper concentration at levels ranging from 30 to 120% higher than normal levels in animals, without obvious effects (34). Mice survived ingestion of up to 8 mg of copper/kg of body weight/day for a period of 45 days. An increase in copper toxicity was not shown in these mice fed a diet supplemented with 1% (wt/wt) ascorbic acid (50 mg ingested/mouse/day) (23a). This correlates with the fact that the toxicity of ascorbic acid in humans begins to be noticed at doses above 2,300 mg/kg/2 days (17).

Mechanism of copper-mediated anti-HSV activity. Metal chelators completely prevented killing of virus in the presence of any of the reducing agents studied, confirming that copper ions play a central role in HSV inactivation. The protective effect of catalase demonstrated that peroxide is an obligatory intermediary in HSV inactivation by Cu(II) and each of the reducing agents. In contrast, the lack of protection by superoxide dismutase suggests that superoxide had little relevance in this mechanism of HSV killing.

The free-radical scavenger dimethyl sulfoxide provided only a small amount of protection when peroxide was present and provided no protection when either ascorbate or cysteine was present. Bovine serum albumin, which is generally considered to have free-radical scavenging properties, also lacked a protective effect. These observations suggest that either (i) copper binds to a key HSV macromolecule, likely nucleic acid (25), and produces direct damage, (ii) any free radicals are generated near a target critical for HSV survival, giving little chance for scavenging, or (iii) free radicals do not play a major role in HSV killing.

Lipoprotein conferred a small but measurable level of protection on HSV against Cu(II)-hydrogen peroxide. This observation suggests that membrane lipoprotein could provide HSV with some protection against copper-peroxide-mediated damage. The protective effect of RNA and the lack of protection conferred by bovine serum albumin or glycogen suggest that Cu(II)-mediated HSV inactivation had a certain degree of molecular specificity.

KCl is not a free-radical scavenger, and it was originally included in our study as a negative control, without any expected effect on virucidal killing. Our results suggest that a step dependent on ionic strength is involved in copper-mediated HSV inactivation.

The order of reducing agent enhancement and the effects (or the lack of an effect) of metal chelators, catalase, superoxide dismutase, and free-radical scavengers on copper-mediated HSV inactivation were similar to those previously observed for DNA damage mediated by Cu(II) and other transition metal ions (24, 32, 33). DNA oxidation by Cu(II) was enhanced by ascorbic acid, hydrogen peroxide, and cysteine by forming complexes between Cu(I) and dehydroascorbic acid, OH^-, and cystine, respectively, producing base modification and breakage of the phosphodiester backbone in DNA (35). Hence, HSV was likely inactivated by a mechanism paralleling that observed in copper-mediated DNA damage.

We suspect that killing of HSV followed a pattern in which Cu(II) ions bound with a high affinity to nucleic acids (25) and produced oxidative base damage (35), which favors poly(G)-rich sequences, in a way similar to that demonstrated previously with plasmid DNA (24).

Potential therapeutic relevance. Approximately 50% of the number of plaques produced in HSV-infected, untreated Vero cells (50% inhibitory dose [ID_{50}]) was obtained after treating infected cells with Cu(II) at concentrations between 0.1 and 0.5 mg/liter plus ascorbic acid at between 10 and 50 mg/liter (Cu/ascorbate ratio, 1:100). This amount of copper is similar to the ID_{50} of acyclovir for HSV, also in Vero cells (0.02 to 0.2 mg/liter for HSV type 1 and 0.03 to 0.5 mg/liter for HSV type 2) (2, 4, 13, 22). The ID_{50} of cupric ascorbate is also similar to the concentrations of ganciclovir (0.05 to 0.6 mg/liter) or vidavirine (3 mg/liter) that inhibit most strains of HSV (13).

We hypothesize that a potential advantage of copper-mediated viral killing resides in a rather broad spectrum of molecular damage that the host cell can repair much faster for itself than for the virus. This hypothesis is supported by our observations showing cell recovery after treatment with Cu(II)-ascorbate. In contrast, cupric ascorbate stopped HSV replication irreversibly since no plaques appeared after subculturing infected, treated cells onto untreated cell monolayers in which the biosynthetic machinery was intact.

The emergence of acyclovir-resistant strains (15, 16) and less

resistant HSV clinical isolates due to their relative deficiency of viral thymidine kinase or DNA polymerase (6, 14) suggests that a novel approach to anti-HSV therapy would be desirable. Our findings suggest that concentrations of Cu(II) and ascorbate with adequate anti-HSV activities in vivo and relatively low toxicities could be achievable. The sensitivity exhibited by HSV to low concentrations of Cu(II) and reducing agents, particularly ascorbate, together with current knowledge of the mechanisms by which cupric ions oxidatively damage biomolecules, might be useful in the development of anti-HSV agents.

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