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Evidence for antiviral activity of glutathione: in vitro inhibition of herpes simplex virus type 1 replication

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Abstract

The role of glutathione (GSH) in the in vitro infection and replication of human herpes simplex virus type 1 (HSV-1) was investigated. Intracellular endogenous GSH levels dramatically decreased in the first 24 h after virus adsorption, starting immediately after virus challenge. The addition of exogenous GSH was not only able to restore its intracellular levels almost up to those found in uninfected cells, but also to inhibit > 99% the replication of HSV-1. This inhibition was concentration-dependent, not related to toxic effects on host cells and also maintained if the exogenous GSH was added as late as 24 h after virus challenge, i.e. when virus infection was fully established. Electron microscopic examination of HSV-1-infected cells showed that GSH dramatically reduced the number of extracellular and intracytoplasmic virus particles, whereas some complete nucleocapsids were still detected within the nuclei of GSH-treated cells. Consistent with this observation, immunoblot analysis showed that the expression of HSV-1-glycoprotein B, crucial for the release and the infectivity of virus particles, was significantly decreased. Data suggest that exogenous GSH inhibits the replication of HSV-1 by interfering with very late stages of the virus life cycle, without affecting cellular metabolism.

Keywords: Herpes simplex virus; Glutathione; Antioxidant; Antiviral agent

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1. Introduction

Glutathione is a cysteine-containing tripeptide (γ-glutamyl-cysteinyl-glycine), found in eukaryotic cells at millimolar concentrations, which has a number of important functions in cell physiology (Meister and Anderson, 1983). GSH provides cells with a large supply of reducing equivalents, thus acting as a major cellular antioxidant, via metabolic interconversion with its oxidized disulphide form (GSSG) (Reed, 1986).

There is increasing evidence suggesting that GSH and other antioxidant substances are somewhat involved in viral infections. Alteration of the endogenous levels of GSH and other antioxidants has been found in experimental infections in vitro and in vivo (Garaci et al., 1992; Hennet et al., 1992), as well as in patients infected by human immunodeficiency virus (HIV) (Buhl et al., 1989; Eck et al., 1989). Recent data from our group have demonstrated that exogenous GSH is able to induce a strong concentration-dependent inhibition of parainfluenza-1 virus (Sendai virus, SV) in vitro (Garaci et al., 1992). In this system, the antiviral activity of GSH was due to a direct inhibition of the expression of some specific virus proteins, which are crucial for virus replication and infectivity.

On the other hand, other investigators have shown that agents that increase intracellular levels of GSH can inhibit HIV replication (Kalebic et al., 1991). In the latter case, however, the antiviral effect is directed not against virus replication per se, but is essentially related to the block of oxidative stress, typically observed in patients infected by HIV (Staal et al., 1992), responsible of activation of the cellular transcription factor (NFκB), which enhances HIV-transcription and replication (Nabel and Baltimore, 1987; Griffin et al., 1989). In fact NFκB is known to be activated in response to different oxidative stresses (Staal et al., 1990; Schreck et al., 1991).

Although the mechanism(s) of antiviral activity of GSH needs to be fully elucidated, overall data suggest that GSH can be active not only against HIV or other viruses with similar replication mechanism (i.e. at least in part dependent on nuclear transcription factors), but also on other viruses involved in the pathogenesis of relevant human infections.

To our knowledge, nothing is known about the interactions between GSH (or other antioxidants) and DNA viruses such as herpes viruses. Among them, herpes simplex virus type 1 (HSV-1) has a relevant impact on morbidity in humans, because of its frequent recurrences, establishment of viral latency and development of new strains resistant to the most common anti-HSV drugs (Crumpacker et al., 1982). Therefore, we have investigated the effect of HSV-1 infection on endogenous GSH content, and the potential anti-HSV-1 activity of exogenous GSH in an in vitro experimental model.

2. Materials and methods

2.1. Cells and virus

VERO cells (African green monkey kidney (AGMK)) were grown in either T-25 flasks or in 24-well plates (Falcon Labware, Meylan Cedéx, France), in RPMI 1640

medium supplemented with 5% heat-inactivated fetal calf serum (FCS), penicillin G (100 U/ml), and streptomycin (100 μg/ml) (all reagents from Flow Laboratories, Milan, Italy) at 37°C in a 5% CO_2 atmosphere. Results obtained with either type of plasticware were superimposable. Cell counts were performed using an haemocytometer and viability was determined by vital dye (0.02%) trypan blue exclusion. Virus HSV type 1 clinical isolate TV1 was grown and titred in VERO cells. To produce virus for experiments, cells in 75-cm^2 flasks were rinsed with serum-free medium and then inoculated with 2.5 ml of RPMI containing HSV-1 at low multiplicity of infection (m.o.i.), i.e. 0.001 plaque forming units (PFU)/cell. After adsorption at 37°C for 1 h, 25–30 ml of RPMI with 2% FCS was added, and the cells were cultured as previously. Culture supernatants were harvested at 72 h after virus challenge, clarified by centrifugation at 800 *g* for 10 min and then stored in small volumes at −80°C. The final titre of virus preparation was 8×10^8 PFU/ml. VERO cells and HSV strains used in these experiments were tested for mycoplasma and found free. Results obtained with the isolate TV1 (shown in this paper) were consistent with those obtained with another HSV-1 clinical isolate, strain TV6, used in parallel experiments.

2.2. Reagents

Very pure GSH devoid of GSSG, as established by high resolution nuclear magnetic resonance (NMR) spectroscopy, obtained from Biomedica Foscama (Rome, Italy), was dissolved in distilled water and diluted at the appropriate concentration in RPMI 1640 just before use.

2.3. Assay for antiviral activity

Confluent monolayers of VERO cells were infected with HSV-1 at m.o.i. of 2 PFU/cell. After incubation for 1 h at 37°C (adsorption period), unadsorbed virus was removed, the monolayers were washed 3 times with phosphate-buffered saline (PBS), and then incubated with 1 ml (for 24-well plates) or 5 ml (for the flasks) of RPMI 1640 containing 2% FCS. If not otherwise specified, GSH was added, at the indicated doses, at established time points with respect to virus challenge, and was maintained in the culture media until the end of the experiments. Supernatants from infected cells were harvested at different time points after virus challenge and tested for their ability to form plaques in VERO cells, using a standard titration method (Mahy, 1991). Mock-infected cells treated or not with GSH were run as parallel controls. For the assessment of the potential virucidal activity of GSH, medium containing 10^8 PFU/ml of HSV-1 was incubated at 37°C with or without GSH 20 mM. After 1 h, virus aliquots diluted 1000- to 10,000-fold were used to infect VERO cells as described in the previous paragraph. It should be noted that, in this set of experiments, cell cultures were exposed to the GSH contained in the virus preparation only during the adsorption period. In addition, the final concentration of GSH was very low (i.e. between 2 and 20 μM). These concentrations of GSH have been found to be inactive against HSV-1 in our cell system, either if given only during virus adsorption or if maintained in culture throughout the whole experiment.

2.4. Intracellular GSH assay

At different time points after viral infection and treatment, cells were washed 4 times in large volumes of PBS, detached by gentle scraping and centrifuged at 700 *g* for 10 min. The pellet was resuspended in sodium dodecyl sulphate (SDS) 0.1%. The proteins were then precipitated with metaphosphoric acid 5% and centrifuged for 30 min at 12,300 *g*. The supernatants were assayed for intracellular glutathione content (GSH + GSSG) by a DTNB-glutathione reductase recycling assay as previously described (Anderson, 1985). Values are expressed as GSH equivalents/mg proteins.

2.5. DNA and protein synthesis in uninfected or HSV-infected cells

For the study of macromolecular synthesis, confluent monolayers of VERO cells (2×10^5 cells/ml) uninfected or HSV-1-infected were treated in 24-well plates with GSH and (immediately after the virus adsorption period) labelled for 24 h with 5 μCi/ml of [^{3}H]thymidine or [^{35}S]methionine (Amersham, Arlington Heights, IL, USA) for cellular DNA and protein synthesis, respectively. The radioactivity incorporated into acid-insoluble material was determined as previously described (Garaci et al., 1992). Briefly, cells were washed 3 times with PBS, and 0.4 ml 5% trichloroacetic acid (TCA) was added to each well. Acid insoluble radioactivity was measured after washing the TCA precipitates 3 times with ethanol, drying them under an infrared lamp and dissolving the samples in 0.4 ml of a solution containing 0.1 M NaOH and 0.5% SDS.

2.6. Electron microscopy

Cell monolayers were infected with HSV-1 and treated or not with GSH after the viral adsorption period, as already described. Forty-eight hours after infection, cells were extensively washed with cacodylate buffer and fixed with 1% glutaraldehyde in the same buffer. Cells were then postfixed with 2% osmium tetroxide, dehydrated and embedded in epoxidic resin. Samples were observed under a Philips CM12 electron microscope. Similar results were obtained when the electron microscopic observation was performed 24 h after virus challenge.

2.7. Immunoblot analysis

Confluent monolayers of VERO cells were infected with HSV-1 as described in a previous paragraph. After the adsorption period, RPMI containing or not GSH (20 mM), was added and the cultures were incubated for 24 h at 37°C. Equal amounts of proteins from cell lysates (obtained by treatment with 0.1% SDS) were subjected to SDS–polyacrylamide gel electrophoresis (10%) using the buffer system described by Laemmli (1970). The fractionated proteins were then electrophoretically transferred to nitrocellulose paper using the technique described by Burnette (1981). Nitrocellulose blots were then blocked in 5% non-fat dry milk in phosphate-buffered saline with 0.001% sodium azide and incubated with polyclonal anti-HSV-1 antibody (1 : 500 v : v) (Chemicon Int., Temecula, CA, USA) in TEN-Tween 20 buffer (0.05 M Tris-HCl pH 7.4, 5 mM EDTA,

0.15 M NaCl, 0.05% Tween 20). The bound antibody was detected with a horseradish peroxidase-linked sheep anti-rabbit immunoglobulin (Chemicon Int.). Molecular weight (MW) values were calculated using Bio-Rad (Bio-Rad Lab., Milan, Italy) high MW markers. Densitometric analysis was performed with a LKB ultrascan XL laser densitometer coupled to a LKB2400 gel scan XL software package. Statistical analysis was performed using the Student's *t*-test for unpaired data. Data were expressed as mean ± S.D., and *P*-values of < 0.05 were considered significant.

3. Results

3.1. Modification of GSH intracellular levels during HSV-1 infection

In order to study the effect of viral infection on the intracellular level of GSH, confluent monolayers of VERO cells were infected with HSV-1 as described in Materials and methods. At each time point either during virus adsorption or after it, the monolayers were carefully washed with PBS, the cells were gently detached, and assayed for intracellular GSH as described in Materials and methods. One set of cells was mock-infected and used as control. As shown in Fig. 1, a significant decrease in total GSH intracellular levels during the 24 h after virus infection was detected in

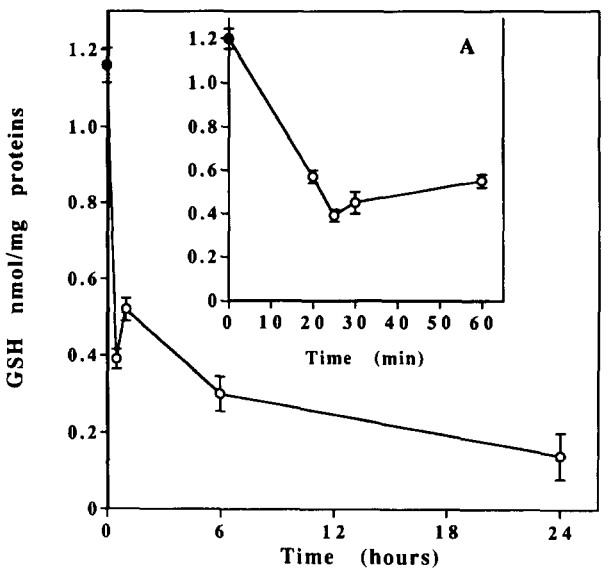


Fig 1. Intracellular GSH content in VERO cells during HSV-1 infection. HSV-1-infected cells were assayed for intracellular GSH content at different time points either during (A) or after the adsorption period. Each point represents the average ± standard error of duplicate samples. The data are from a single experiment representative of 6 consistent experiments. The difference in GSH content between infected and mock-infected cells 24 h after infection was statistically significant (*P*-value calculated on the basis of all 6 experiments: < 0.001).

HSV-1-infected cells. The fall in the GSH content started very early during the period of virus adsorption to the cells, and reached the maximum reduction (32.5% of the control level) at 25 min after virus challenge (Fig. 1A). After this period, but within the first hour, a slight increase in GSH concentration was consistently observed in all experiments: this was probably due to the stimulation of the GSH synthesis consequent to virus-induced depletion (Fig. 1). This temporary increase was followed by a further and progressive reduction of total GSH intracellular levels as compared to those measured in uninfected cells 24 h after mock infection ($P < 0.001$ between infected and uninfected cells at the same time point). No significant change in the GSH content was observed in uninfected cells after 24 h of culture.

3.2. Activity of GSH against HSV-1

To evaluate the effect of exogenous reduced GSH on viral replication, confluent monolayers of VERO cells were infected with HSV-1. One hour after infection, virus inocula were removed and GSH was added at different concentrations, in culture medium. The cells were then incubated at 37°C for 24, 48, 72 h, after which the supernatants were collected and tested for their ability to form plaques in monkey kidney cells. GSH produced a concentration-dependent inhibition of HSV replication (Fig. 2). In agreement with previous data obtained with Sendai virus (Garaci et al., 1992), microscopical examination showed a limited protection by GSH from HSV-1-in-

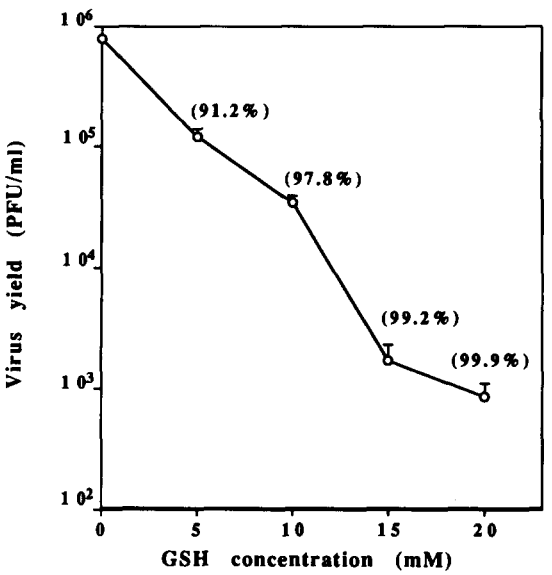


Fig. 2. Effect of exogenous GSH on HSV-1 replication. Data represent the virus yield detected 48 h after infection in the presence or absence of various concentrations of GSH. Values in parentheses indicate the percent inhibition of virus production in GSH-treated cells compared to untreated infected controls. Each point represents the average ± standard error of quadruplicate samples. Data are from a single experiment representative of 4.

duced cytopathic effect. Nevertheless, a reduction of virus yield of 91–99% (consistent in all experiments performed) occurred at concentrations of GSH between 5 and 20 mM. Comparison of the kinetics of virus growth in control-infected and GSH-treated cultures proved that the inhibition was stable for as long as 72 h postinfection (end of experiment), and it was not just a delay in virus burst. Microscopical examination, trypan blue exclusion and cell counts demonstrated that GSH (20 mM) did not produce any toxic effect, loss in viability, or modification of cell multiplication rate in uninfected cells.

3.3. *Effect of addition of exogenous GSH on intracellular glutathione levels*

In order to assess whether GSH-related virus inhibition was associated with a recovery of intracellular levels of GSH, total intracellular GSH content was assayed 24 h after infection in HSV-1-infected cells treated or not with GSH at the maximal antiviral concentration (20 mM). Mock-infected cells were used as control. Parallel plaque assays were carried out to quantitate the amount of infectious virus released in these experiments. In agreement with the results described in Fig. 1, consistent reduction of intracellular GSH 24 h after infection with HSV-1 was detected in 4 separate experiments, the average level being about 40% of that of uninfected controls (Fig. 3). The addition of GSH 20 mM increased the intracellular GSH content almost to the levels (90.8%) found in uninfected cells (Fig. 3), in parallel to a $> 99\%$ inhibition of virus release in the supernatants of infected, GSH-treated cells. By contrast, the treatment of uninfected cells with GSH 20 mM did not significantly increase the intracellular levels

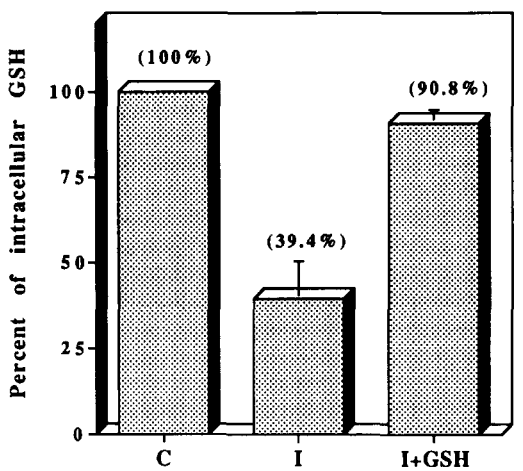


Fig. 3. Effect of addition of exogenous GSH on endogenous GSH levels. Intracellular GSH was tested 24 h after infection in VERO cells mock-infected (C), infected with HSV-1 (I), or infected and treated with 20 mM GSH (I + GSH) (added immediately after virus adsorption). Results in HSV-1-infected VERO cells represent the percent of intracellular GSH compared to that found in mock-infected cells (C). The levels of intracellular GSH in uninfected cells ranged, in different experiments, from 0.26 to 0.76 nmol/mg proteins. Results represent the percent average ± standard error, of 4 different experiments, each run in duplicate.

of GSH (data not shown). This suggests that, at least under these experimental conditions, GSH content cannot reach intracellular levels beyond those physiologically present in uninfected cells.

3.4. Characterization of antiviral activity

In order to identify which step of HSV-1 replication was affected by GSH, in a first set of experiments we added the compound at various time points, before, during, and after virus adsorption. As shown in Fig. 4A, no significant difference in the extent of inhibition of virus production was observed when treatment with GSH was started either 1, 3, 6, or even 24 h after virus challenge, and the compound was maintained in the culture until the end of the experiment. Indeed, the inhibition of virus replication, compared to untreated infected cells, was greater than 96% in all cases, including those of the addition of GSH at 6 and 24 h after virus challenge. It should be noted that, at these time points, the virus is at an advanced stage of its life cycle, and many virus particles have already been released from the cells. By contrast, no significant antiviral effect was detected either when the isolated virus was pretreated for 1 h with GSH 20 mM, or when GSH was given only during the hour of virus adsorption (and then withdrawn) (Fig. 4B). When GSH was given to the cells with a 24-h pretreatment (and washed out before infection), only a limited and not significant reduction (up to 20%) of virus production was detected (Fig. 4B). Since the antiviral effect of GSH is somewhat similar to that obtained with human α-interferon (Chatterjee et al., 1985; Chatterjee and

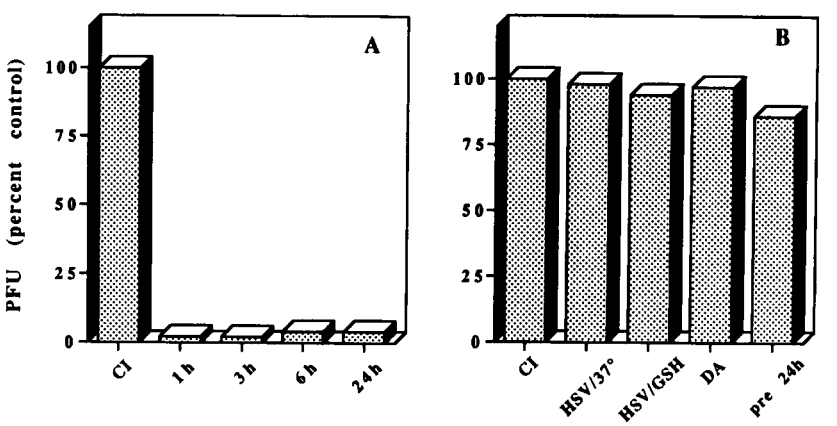


Fig. 4. Characterization of antiviral activity of GSH. A: CI, control infected cells; 1 h, 3 h, 6 h, 24 h, GSH added 1, 3, 6, and 24 h, respectively, after virus challenge. B: HSV/37°, virus maintained for 1 h at 37°C before infection; HSV/GSH, virus pretreated with GSH 20 mM for 1 h at 37°C; DA (during adsorption), GSH 20 mM given only during adsorption and then withdrawn; pre 24 h, GSH given to uninfected cells for 24 h and removed before infection. Virus production was assessed in all cases 48 h after virus challenge. Each bar represents the percentage of PFU/ml with respect to the controls (CI) at 48 h postinfection. Data are the mean of quadruplicate samples from two separate experiments. Variation within experiments did not exceed 10%. The slight decrease of virus production detected with GSH pretreatment (pre-24 h) was not significant ($P > 0.05$).

Burns, 1990), in a final set of experiments we tested whether the anti-HSV-1 activity of GSH could be reversed by the addition of a monoclonal antibody known to have a potent neutralizing activity of interferon-α (Gessani et al., 1994). No modification of the antiviral activity of GSH was detected under these experimental conditions (data not shown). Overall data suggest that GSH neither inactivates virus per se, nor inhibits virus adsorption to the cells, nor significantly affects the susceptibility of the target cells to infection by HSV. The latter point may be related to the inability of uninfected cells to increase their GSH content beyond the physiological levels (see previous paragraph). On the other hand, the data point out that the antiviral activity of GSH is substantially related to the inhibition of late stages of virus replication.

3.5. *Effect of GSH on DNA or protein synthesis in uninfected or HSV-1-infected cells*

In order to evaluate whether the antiviral activity of GSH was due to a non-specific effect upon host cells, experiments were performed to evaluate DNA and protein synthesis in uninfected or HSV-1-infected cells after treatment with 20 mM GSH. Mock-infected or HSV-1-infected cells were treated or not with GSH (20 mM) just after infection and labelled with [^{3}H]thymidine and [^{35}S]methionine. After 24 h, the incorporation into acid-insoluble material was measured as described in Materials and methods. As shown in Fig. 5, a 24-h treatment with GSH did not significantly alter the rate of DNA synthesis in uninfected cells. Cells infected with HSV-1 showed a significant increase in [^{3}H]thymidine incorporation at 24 h postinfection, conceivably due to the synthesis of viral DNA, and the addition of GSH immediately after virus adsorption did not modify this parameter (Fig. 5). These data indicate that this concentration of GSH does not affect the synthesis of DNA in both uninfected and infected cells, and further

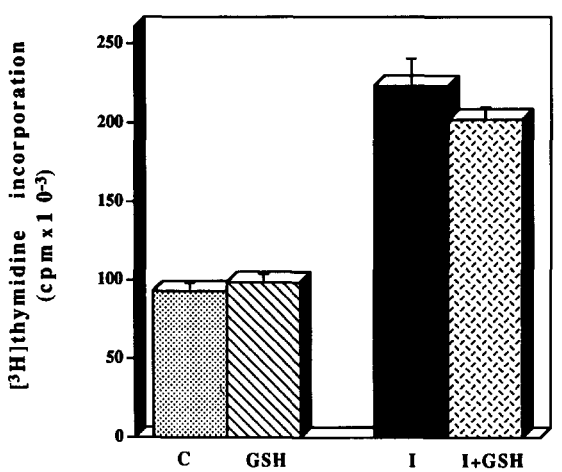
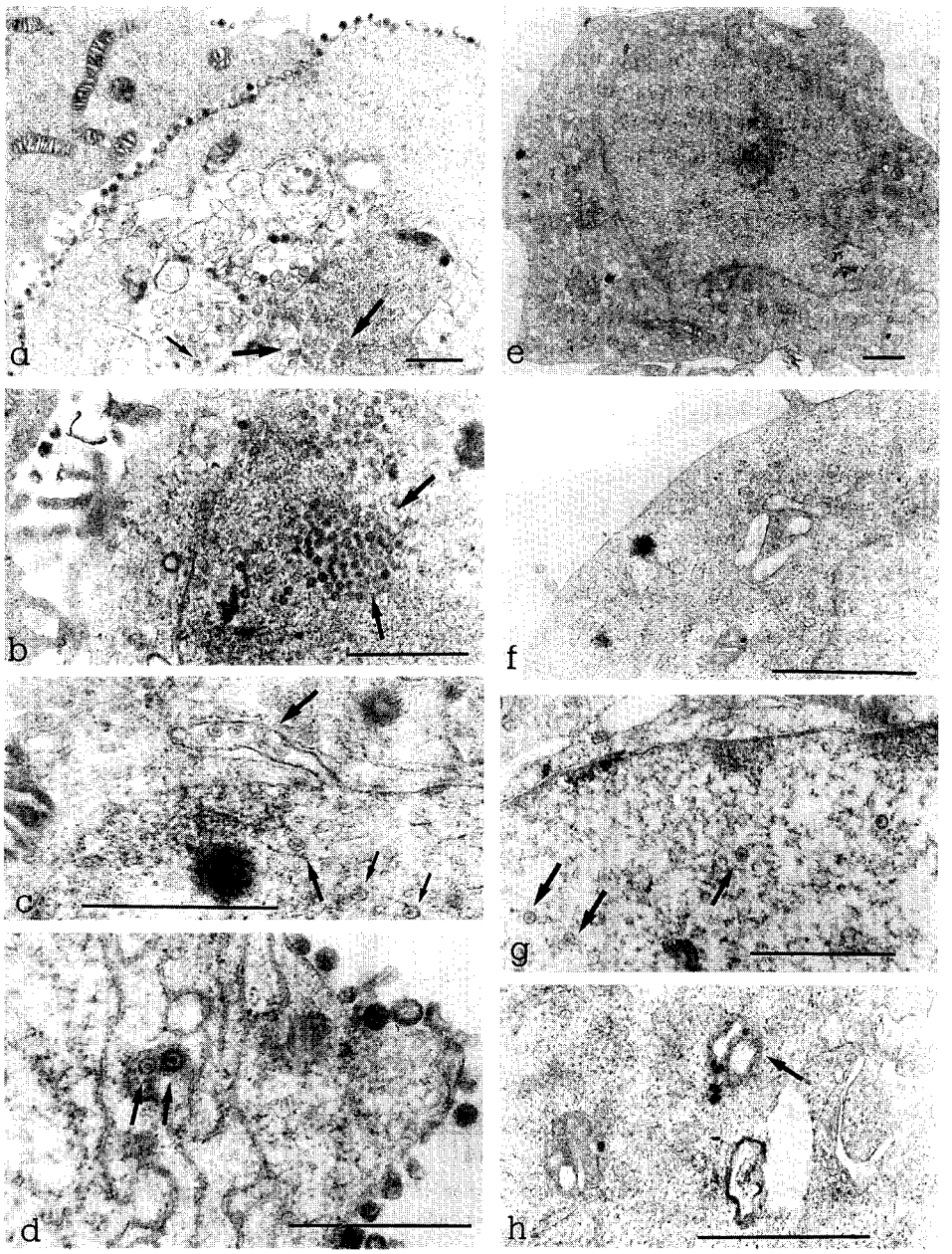


Fig. 5. Effect of GSH on DNA synthesis in uninfected or HSV-infected cells. Data (expressed in cpm $\times 10^{-3}$ $\pm$standard error) are from an experiment, run in quadruplicate, representative of two. C, uninfected cells; GSH, uninfected cells treated with GSH 20 mM; I, cells infected with HSV-1; I+GSH, cells infected and treated with GSH 20 mM.

suggests that GSH mainly inhibits steps of virus replication after DNA synthesis. When we tested the protein synthesis, we found, in agreement with previously published data (Kaplan et al., 1970), that HSV-1 infection progressively inhibited the synthesis of



cellular proteins (35 and 48.4% inhibition at 24 and 48 h postinfection, respectively, compared to uninfected cells). However, as found in the case of DNA, the protein synthesis in both uninfected and infected cells was not affected by treatment with GSH 20 mM (data not shown).

3.6. Electron microscopic examination of untreated or GSH-treated infected cells

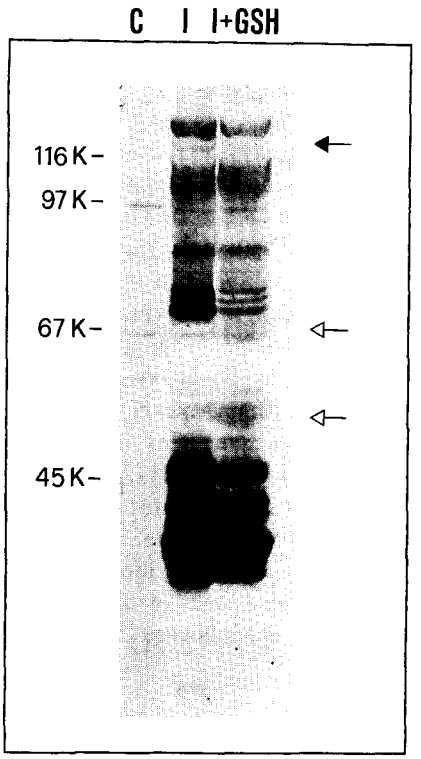
Electron microscopic studies were performed in order to assess whether the potent antiviral effect of GSH was due to a block of virus maturation and budding from infected cells and/or to a production of extracellular defective particles. VERO cells infected with HSV-1 and treated with 20 mM GSH were processed for electron microscopy 48 h postinfection as described in Materials and methods. Consistent with the other experiments, release of infectious particles, measured in the supernatants of infected cells before fixation, was inhibited $> 99\%$ by treatment with 20 mM GSH. Electron micrographs of untreated infected cells (Fig. 6) showed numerous virus particles at different stages of maturation in the nuclei, in the cytoplasmatic structures and in the extracellular space (Fig. 6a–d). By contrast, no mature virus particles were detected either in intracytoplasmic compartments or in the extracellular space of cells treated with GSH (Fig. 6e, f), while some viral nucleocapsids were found within cell nuclei (Fig. 6g). In addition, intracytoplasmatic vesicles coated with electron-dense material were observed in the cytoplasm of infected GSH-treated cells (Fig. 6h); such material was not observed in any section of either infected untreated cells, or uninfected cells treated with 20 mM GSH. These observations support the hypothesis that GSH blocks HSV-1 replication at very late stages of its morphogenesis, and in some ways inhibits the maturation and the exit of virus cores from the nucleus of treated cells.

3.7. Effect of GSH on viral proteins

In a final set of experiments, we evaluated whether the block of virus maturation could be related to a modification of the synthesis of specific virus proteins. Confluent monolayers of VERO cells were infected with HSV-1, and GSH treatment (20 mM) was started after a 1-h adsorption period. One set of cells served as uninfected control. After 24 h, the cells were processed for SDS–PAGE and immunoblot analysis was performed

Fig. 6. Electron microscopic observation of virus particles present in untreated (a–d) or GSH-treated (e–h) VERO cells infected with HSV-1. Thin sections of untreated cells show many mature particles in extracellular spaces, and normal shaped nucleocapsids within the cell nucleus (a, arrows). Higher magnifications of untreated infected cells show accumulation of intranuclear capsids (b) and viral budding through nuclear membranes (c, arrows). In the cytoplasm both enveloped and unenveloped virions are observed (d, arrows). Low magnification in samples treated with GSH shows no extracellular virus particles (e). Higher magnifications of a portion of a treated cell show a cytoplasmic compartment in which no viral particles were detected (f) and the nuclear region containing some viral nucleocapsids (g, arrows). Electron dense material is observed inside intracytoplasmic vesicles of treated cells (h, arrows): such material was never observed in infected untreated cells. At least one hundred cells were counted for each sample. Magnification: a, ×11,000; b, ×23,000; c, ×35,000; d, ×37,000; e, ×8000; f, ×27,000; g, ×27,000; h, ×37,000.

using a rabbit polyclonal anti-HSV-1 antibody, as described in Materials and methods. Parallel plaque assays were carried out to quantify the amount of infectious virus released in these experiments. A decrease of the whole expression of virus proteins



	PFU/ml	%inhibition (fold reduction)
C	0	/
I	8.93×10^5	/
I+GSH	2.30×10^3	99.7% (388)

Fig. 7. Effect of GSH on the synthesis of HSV-1-specific proteins in GSH-treated and untreated VERO cells. C, uninfected control; I, infected control; I+GSH, infected cells treated with GSH. Full arrow indicates a protein of 123 kDa, corresponding to the molecular weight of glycoprotein B. Expression of this protein was consistently inhibited by GSH treatment. Empty arrows indicate the two proteins accumulating after GSH treatment. The table under the figure shows the virus yield and the percent of virus inhibition induced by GSH treatment, detected in the supernatants of the cells used for protein analysis. This is an experiment representative of 6.

detected by polyclonal anti-HSV-1 antibody was found in cells treated with GSH, compared to untreated ones. Such a decrease was quite variable in each experiment ranging from 20 to 40% by densitometric analysis. Although the pattern of viral proteins was differently affected by GSH treatment, we found a great and consistent decrease of the amount of a 123-kDa viral protein corresponding to the molecular weight of the virus glycoprotein B, whose expression was reduced in each experiment up to 80% of the amount found in untreated infected cells. In parallel, a two-fold increase of the expression of proteins with molecular weights corresponding to the HSV-1 glycoproteins gM (53 kDA) and gD (66 kDa) (Fig. 7) was detected in GSH-treated cells. It should be noted that the supernatants of GSH-treated cells collected from the same plates immediately before cell lysis contained approximately 400-fold less infectious virus than those of infected untreated cells (Fig. 7). Thus, these results demonstrate that, at times when infectious virus release from cells is dramatically reduced by GSH treatment, only the expression of gB virus protein is substantially decreased. This is consistent with the electron microscopy observation of a number of assembled virus nucleocapsids in the nuclei of GSH-treated cells (Fig. 6), in the absence of mature enveloped virions outside the cells.

4. Discussion

In the present study, we have shown that exogenous reduced GSH acts as antiviral agent in HSV-1-infected cells by inhibiting late stages of viral morphogenesis. The latter conclusion is based on the following evidence: (1) GSH did not act by inactivating virus itself, by inhibiting viral adsorption, or by reducing the susceptibility of target cells to virus infection; (2) treatment with GSH did not significantly modify the rate of DNA and protein synthesis in HSV-1-infected cells; (3) substantial inhibition of viral production was observed when treatment was started even at late time points with respect to virus challenge (3, 6 and 24 h postinfection); (4) no extracellular particles were released from GSH-treated cells, while the assembly of nucleocapsids inside the nucleus was only slightly affected; and (5) GSH treatment only marginally inhibited the expression of the majority of virus-specific proteins, with the important exception of glycoprotein B, which is crucial for infectivity of HSV-1.

The involvement of GSH in viral infections was suggested by several lines of evidence (Buhl et al., 1989; Eck et al., 1989; Garaci et al., 1992; Hennet et al., 1992). We here show that an impairment in intracellular redox status, documented as a decrease in intracellular levels of total GSH, occurs at early time points after viral infection of VERO cells with HSV-1.

The mechanism(s) by which HSV-1 infection induces a decrease in intracellular GSH content is unclear at this moment. Nevertheless, the fact that such phenomenon occurs in different kinds of viral infections, either in vivo or in vitro systems, and very early with respect to virus challenge, suggests that the impairment of intracellular redox status is essential for the initiation and maintenance of virus replication; that is a primary event produced by viral infection other than the consequence of chronic exposure to inflammatory cytokines (as suggested in the case of HIV infection).

In fact, in the case of HIV, it has been reported that increased levels of inflammatory cytokines (interleukins 1 and 6, tumor necrosis factor α, interferon γ) are able to produce a depletion of GSH and oxidative stress (Yoshie et al., 1989; Poli et al., 1990; Roederer et al., 1992). This, in turn, may activate NFκB, a nuclear transcription factor (known to be activated in response to different oxidative stresses (Schreck et al., 1991) and thus enhance HIV replication (Nabel and Baltimore, 1987). Based on these observations, it has been proposed that the inhibition of HIV replication obtained with various antioxidants (*N*-acetylcysteine, GSH, GSH monoester, etc.) be due to the block of oxidant conditions activating NFκB factor and, thus, HIV replication (Roederer et al., 1990, 1991; Mihm et al., 1991).

An HSV-1 inducible protein able to bind to NFκB like sites in the HSV-1 genome has been recently described (Rong et al., 1992). Therefore, we cannot exclude that GSH may affect this or other mechanisms related to the transcription of HSV-1 genome. Nevertheless, our results suggest that the anti-HSV-1 activity of GSH occurs mainly at the translational or post-translational level. Indeed, electron microscopical observations show virus nucleocapsids in the nuclei of infected and GSH-treated cells, while no fully mature (enveloped) virus particles were detected within the cytoplasm and outside the cells. It should be noted that, under these conditions, the inhibition of the production of infectious particles in the supernatants of the same GSH-treated cells was greater than 99%.

With regard to the effect of GSH upon virus protein synthesis, we found consistent inhibition only of the glycoprotein B, while the expression of other virus proteins was less affected. Among herpesvirus glycoproteins, glycoprotein B (gB) is considered essential for the production of enveloped (i.e. infectious) virus particles (Spear, 1984). gB has been characterized in considerable detail and shown to exist as a dimer (Claesson-Welsh and Spear, 1987; Wealy et al., 1990), which, according to data obtained with gB homologues, is thought to be held together in a complex involving disulphide bridges (Hampl et al., 1984; Lukacs et al., 1985). These bonds are typically affected by reducing agents, and GSH is likely to interfere with the dimer formation by inducing reductive cleavage of the S–S bridges.

The reason why gB is more affected by GSH than other proteins of HSV-1 needs to be further investigated. However, these results are in agreement with previous data of our group (Garaci et al., 1992), showing that the antiviral activity of GSH on parainfluenza virus is associated with a similar inhibition of haemagglutinin-neuraminidase (HN) viral glycoprotein, while other viral proteins were only marginally affected. This protein is essential for virus infectivity (Scheid and Choppin, 1974; Portner et al., 1987), and it normally assembles into oligomers via formation of disulphide bonds (Vidal et al., 1989). Similar effects were also observed in macrophages infected with HIV-1 and in T-lymphocytes persistently infected with HIV-1 (in preparation), in which the treatment with antiviral concentrations of GSH mainly affects the expression of the CD4-binding glycoprotein gp120, which is very rich in disulphide bonds (Leonard et al., 1990).

Thus, overall data suggest that inhibition of gB expression represents an important phenomenon in the inhibition of HSV-1 replication by GSH. Similarly, the increased expression of gM and gD envelope glycoproteins might be related to an accumulation consequent to a defect in the maturation of virus particles. This is also supported by the

presence, in GSH-treated cells, of intracytoplasmic vesicles coated with electron-dense materials conceivably of protein origin. Further studies are required to better characterize this observation.

All together, these results suggest a potential use of GSH in the therapy of HSV-1-related disease. While several antioxidants are currently under investigation in patients infected by HIV, no clinical studies have been reported for administration of GSH to patients affected by diseases related to HSV-1 infection. In our experimental system, GSH showed marked anti-HSV-1 activity at concentrations substantially close to those physiologically found in the cells, with undetectable in vitro toxicity. Finally, the safety and pharmacokinetics of GSH are well established in humans (Aebi et al., 1991; Morris and Gordon, 1994). Thus, based on this favourable profile, further research concerning the potential clinical efficacy of GSH against HSV-1 infection is warranted.

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