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Inactivation of human cytomegalovirus by sodium periodate oxidation

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Summary. Human cytomegalovirus (HCMV), oxidized by sodium periodate ($NaIO_4$), is incapable of giving rise to viral progeny in cell culture. At a $NaIO_4$ concentration as low as 5 mM, there is a loss of at least 6 logs of viral infectivity which occurs very rapidly (less than 5 min). Further, the inactivation is a first-order reaction depending on the periodate concentration. Adsorption to the cell surface, penetration into cells, and penetration of the viral DNA into cell nuclei were found to occur identically in mock oxidized and oxidized HCMV. Since the carbohydrate moiety of viral glycoproteins was the target of periodate attack, these observations strongly suggest that the structural integrity of the sugar residues is not a prerequisite for adsorption and penetration. Nevertheless, no evidence for viral DNA or protein synthesis was detected in cells infected with oxidized virus, and even after 3 weeks in culture, no cytopathic effect was observed.

Introduction

Human cytomegalovirus (HCMV), a member of the herpesvirus group, is an ubiquitous pathogenic agent which can infect humans at any age.

In immunocompetent hosts, infection remains asymptomatic probably because of an efficient virus-specific host immune response. Nevertheless, following a primary infection, virus is not cleared from the body but persists either in the form of a low-grade chronic infection or in a latent state, so that reactivation or increased excretion allows further transmission to new hosts [11, 14, 30].

When the host's immune system is immature (foetus, newborn) or depressed (by immunosuppressive drugs in allograft recipients or by HIV in AIDS), HCMV is responsible for various diseases associated with significant morbidity and mortality [11, 14, 30].

Approaches to prevent and treat HCMV infections are mainly based on antiviral chemotherapy or chemoprophylaxis with nucleoside analogues such as acyclovir or ganciclovir, combined or not with immunotherapy or immunopro-

phylaxis through interferon, HCMV hyperimmune globulins or, recently, by HCMV-specific monoclonal antibodies or T-cells [3, 5, 9, 11, 24]. Although the results of these treatment strategies varied from one HCMV infection to the next, it would appear that they do reduce the incidence and the severity of HCMV diseases, although they do not obviate further infection. Furthermore, antiviral chemical agents are not free of adverse side effects and toxicity and long-term administration leads to the generation of HCMV resistant strains [3]. Finally, adoptive transfer using antibodies or T-cells remains a very difficult approach with regard to feasibility and cost, so that large-scale therapy or prophylaxis cannot be envisaged with this approach [21].

Thus, the most practical approach to prevent HCMV infection seems to be the immunization of seronegative populations with a safe, effective vaccine. Live attenuated vaccines have been prepared by serial passage in human cell culture for TOWNE [19] and AD169 [8] strains of HCMV. Vaccine virus appears to induce both humoral and cellular immune responses in healthy and in renal transplant recipients, similar to those associated with naturally acquired infection and even in the latter group, the incidence and the severity of HCMV diseases are reduced [12, 20, 25]. Although reactivation and replication of the vaccine virus has not been detected in those populations, the possibility exists that this strain may become latent and be subject to a possible shift to virulence or to transformation of the host cell. For these reasons, efforts have concentrated on the development of an HCMV subunit vaccine made up an HCMV envelope glycoprotein gB or gp55/116. Preliminary experiments showed that this protein can induce both humoral and cellular immunity when injected into healthy adults [12, 20, 25]. Nevertheless, the technology to produce and purify native viral glycoproteins in large amounts is, at present, so costly that it precludes commercial vaccine production and broad-scale immunization.

In an attempt to develop a procedure which could eventually be applied to the large-scale production of native viral proteins, we decided to investigate the periodate oxidation procedure which, thanks to the early work of Dreesman and Suriano [6] on Adenovirus types 2 and 7 (Ad2 and Ad7), had been shown to inactivate the infectious capacity, in vitro, of these two types. These authors also showed that the levels of neutralizing antibodies induced in rabbits injected with oxidized virions were similar to those elicited by injecting native virus. In our laboratory, these observations have recently been confirmed for Adenovirus type 5 (Ad5) (Ogier et al. [18a]) and extended, in that oxidized Ad5 was able to adsorb and penetrate the host cell, but viral DNA replication was partially impaired. No viral protein synthesis was detected and the production of infectious virions, as assessed in 11 different experiments was, on average, decreased by 7 log 10. This residual virus production and the ensuing risk of latency raised the question whether inactivation by periodate oxidation could be adapted so as to achieve total inhibition of viral infectivity. We therefore undertook such a study on CMV because of (i) its importance in human pathology (vide supra) (ii) its ability to become latent and (iii) the existence of a murine CMV model which allows in vivo experiments.

In this paper, we describe the reaction features and effects on infectivity in vitro of HCMV oxidized by periodate. Evidence will be presented to show that the systhesis of viral protein and viral DNA is blocked throughout infection.

Materials and methods

Cells and virus

MRC-5 human diploid cells [16], from Institut Mérieux (France), were grown to confluence at 37 °C, 5% CO_2, in 162 cm^2 Falcon tissue culture flasks, with Eagle's MEM supplemented with 7% Fetal Calf Serum (FCS), L-Glutamine (2 mM), non-essential amino-acids (Gibco), penicillin (100 U/ml), streptomycin (750 µg/ml), amphotericin B (0.25 µg/ml), HEPES (10 mM), $NaHCO_3$ (750 µg/ml). This medium will be referred to as the growth medium. Cells were used between passages 24 and 28. Confluent cells were infected with the DAVIS strain of HCMV, a kind gift of Dr R. Gibert (Laboratory of Virology and Bacteriology, Faculty of Medicine, Lyon, France), at a virus to cell multiplicity of 1 to 2. The virus inoculum was allowed to adsorb for 2 h at 37 °C, then the newly infected cells were maintained using MEM supplemented with 2% FCS (maintenance medium). When the monolayer exhibited 100% cytopathic effect (approximatively 7 days post-infection), medium from the infected cells was harvested and, after removal of cellular debris by low speed centrifugation (4 500 **g**, 20 min, 4 °C), the virus was concentrated about 100-fold by centrifugation at 30 000 rpm for 60 min at 4 °C in a 50.2 Ti Beckman rotor. Through this procedure, cellular contamination of the viral material was found to be less than 2%. The pellets were resuspended in about 3 ml of phosphate buffered saline (PBS) (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4). Aliquots were frozen at −70 °C.

Labelling of virions

20 h post-infection, [^{3}H]thymidine (1.77 TBq/mmol, Dositek, France) was added to the medium (111 kBq/ml) and the labelled virions were harvested and concentrated as described above.

Lysis of cells and radioactive counting

Cell lysis was performed in TEN buffer (0.02 M Tris-HCl pH 8, 10^{-3} M EDTA, 0.14 M NaCl) with 1% Nonidet P40 for 30 min on ice. For measuring acid-insoluble radioactivity, samples (0.1 ml) were precipitated for 30 min in the cold with 3 ml of Trichloroacetic acid (TCA) at a final concentration of 10%, collected on GF/C Whatman filters, washed 3 times with cold TCA and once with cold 95% ethanol. After drying, filters were placed in vials containing toluene scintillant and counted in a Packard liquid scintillation spectrometer.

Inactivation assays

Inactivation assays were carried out to optimise periodate concentration and time of oxidation. The viral suspension (10^6–10^7 ID/ml) was adjusted to pH 6 with 0.4 M phosphate buffer. Sodium periodate, at different concentrations in distilled water, was added and incubated for the required time at 4 °C in the dark. Sodium sulfite, at a concentration 4-fold greater than that of periodate was added to stop oxidation. The suspension was dialysed extensively against PBS to eliminate reaction products. Mock-oxidized HCMV was obtained by similarly treating samples except that sodium periodate was replaced by distilled water. Samples were titrated by the endpoint dilution method described by Reed and Muench [23].

Borohydride labelling of glycoproteins

Samples of concentrated HCMV were mock-oxidized or oxidized as described above. As a further control, a lysate of uninfected cells was treated similarly. All samples were then dialysed for 3 h against 50 mM Borate pH 9, sonicated for 1 min at 70 V and clarified by centrifugation at 10 000 **g** for 15 min at 4 °C. One MBq of tritiated borohydride (NEN, 466.2 GBq/mmol) was added to 0.3 mg protein of each sample and reduction allowed to proceed for 1 h at room temperature. At the end of this period, 100-fold excess of unlabelled borohydride was added, after which samples were dialysed overnight against 10 mM Tris-HCl pH 7.5 and subjected to immunoprecipitation.

Adsorption and penetration assays

MRC-5 cells (4×10^5) were grown to confluence in 6-well tissue culture plates (Costar). Cells were chilled for 30 min at 4 °C. Non-specific adsorption was then blocked by incubation for 30 min with cold PBS containing 5 mg/ml of sterile Bovine Serum Albumin (BSA). Cells were inoculated with [^{3}H]thymidine labelled virions either native or oxidized (5 mM $NaIO_4$, 60 min, 4 °C), to a multiplicity of 2 to 3 ID/cell. Adsorption was for 2 h at 4 °C. The inoculum was removed and monolayers were washed twice with cold 0.05 M glycine-HCl buffer, pH 3 with 0.14 M NaCl, to remove any non-specifically adsorbed virus.

For adsorption assay, cells were then harvested by scraping, lysed and acid-insoluble radioactivity was estimated on 0.1 ml of each fraction.

In assaying for penetration, cells were rapidly warmed to 37 °C by the addition of pre-warmed maintenance medium and incubated 3 h at 37 °C and 5% CO_2, to allow viral penetration. Any virus remaining on the cell surface was removed by trypsin (0.25%, 10 min, 37 °C), according to Nowlin et al. [18]. Trypsin activity was blocked by adding 3 ml of maintenance medium. Cells were suspended and washed 3 times in PBS by centrifugation (200 **g**, 10 min, 4 °C), lysed and counted.

Penetration of viral nucleic acid into cell nuclei

To avoid aggregation of nuclei with membranes or cytoplasmic material during cell fractionation, cells were embedded in agarose beads before lysis according to the method described by Jackson and Cook [15]. Cells (20×10^6) were infected as described above, with medium containing 200 µg/ml of Phosphonoacetate (PAA) to prevent viral DNA replication. At 8 h post-infection, cells were washed twice with PBS, trypsinized and washed again 3 times in PBS by low speed centrifugation and resuspended in 2 ml of PBS. Cells were warmed to 39 °C and mixed with 2.5% in PBS (w/w) of low-melting agarose (Sigma, type VII) and 5 ml of liquid paraffin (Merck, model 7174), both pre-warmed to 39 °C, in a 30 ml corex tube. After low speed vortexing for 30 sec, the tube was chilled for 5 min at 0 °C in a water/ice bath and 10 ml of PBS at 0 °C were added. After gentle mixing, the tube was centrifuged at 3 500 **g** for 5 min at 4 °C. The supernatant was removed and agarose beads were washed 3 times with cold PBS by centrifugation at 4 800 **g** for 5 min at 4 °C. Beads were finally resuspended in 1 ml of PBS and lysed in 3 ml of cold lysis buffer (130 mM KCl, 10 mM Na_2HPO_4, 1 mM $MgCl_2$, 1 mM Na_2ATP (Sigma Type II), 1 mM Dithiothreitol, pH adjusted to 7.4 by adding 100 mM KH_2PO_4, 0.5% Trition X-100). Beads were allowed to stand on ice for 30 min and then washed 3 times by centrifugation (4 800 **g**, 5 min, 4 °C) in the previous buffer without Triton X-100. Purification of nuclei was monitored by phase-contrast microscopy. Extraction of purified nuclei from beads was achieved by melting agarose at 65 °C for 5 min and pelleting nuclei by centrifugation (10 000 **g**, 30 sec, 37 °C). The pellet was then washed 3 times in PBS and radioactivity was measured in each fraction (supernatant plus washes and nuclear pellet) as previously described.

Evaluation of viral DNA and protein syntheses

MCR-5 cells (2×10^6) were grown to confluence (about 72 h) in 75 cm^2 Falcon tissue culture flasks. Infection (0.1 ID/cell), adsorption and penetration were carried out as described above. At various times after the onset of penetration, 2 µCi/ml of [^{3}H]thymidine were added for 2 h . At the end of the labelling period, cells were washed 3 times with TEN buffer and lysed. Each sample was counted to assess viral DNA synthesis and subjected to polyacrylamide gel electrophoresis (SDS-PAGE) for evaluation of viral protein synthesis by Western blotting.

SDS-PAGE

Electrophoresis of proteins was performed under reducing conditions as described by Laemmli (17). The stacking gel consisted of 5% acrylamide and the separating gel of 10% acrylamide. Samples were boiled for 5 min in a denaturing solution (50 mM Tris-HCl pH 6.8, 100 mM β-2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and subjected to electrophoresis in a buffer containing 0.2 M glycine pH 8.3, 0.25 M Tris, 0.1% SDS, at 80 V (450 mA), for 2 h in a Mighty small SE250 cuve (Hoefer Instruments). Proteins were then partially renatured, according to the technique of Dunn (7) in a renaturation buffer (50 mM Tris-HCl pH 7.4, 20% glycerol) for 30 min.

Western blot

As described by Towbin et al. [27], separated proteins were electrophoretically transferred to a nitrocellulose sheet (Schleicher and Schull) for 2 h at 80 V (450 mA) in a Mighty small TE22 cuve (Hoefer Instruments). The transfer buffer was 3 mM Na_2CO_3, 10 mM $NaHCO_3$ pH 9.9, 20% methanol. Visualization of proteins was assessed by coloration of the nitrocellulose sheet with a 0.2% (w/v) red ponceau solution in 3% (w/v) TCA. Non-specific binding sites were blocked by incubation of the nitrocellulose with a 10% skimmed-milk powder in PBS for 30 min at 37 °C. Nitrocellulose was washed 3 times with PBS. The pool of anti-HCMV sera, consisting of pooled human sera with high titers of anti-HCMV antibodies, was diluted (1:100 or 1:200) in PBS-0.1% Tween 20 and incubated with the nitrocellulose filter for 1 h 30 at 37 °C on a rocking platform. Following 5 washes in PBS-0.1% Tween 20, the filter was incubated with an alkaline phosphatase-conjugated goat anti-human IgG antibody (BioSys) at 1:1000 dilution in PBS-0.1% Tween 20 containing 0.2% BSA, for 45 min at 37 °C. After 5 washes in PBS-0.1% Tween 20, the filter was developed in the NBT (nitro blue tetrazolium)/BCIP (5-bromo-4-chloro-indolyl phosphate) substrate in AMP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) and washed in PBS-0.1% Tween 20.

Immunoprecipitation

To reduce the non specific interaction of serum antibodies with viral and cellular proteins, each sample was pretreated with 5 µl of normal human serum for 1 h at 37 °C. The mixture was then added to 50 µl of protein A-Sepharose beads (Pharmacia) previously washed twice in 50 mM Tris-HCl pH 7.5. After 1 h at room temperature, the reaction mixture was centrifuged at 3 000 rpm for 5 min and the supernatant was added to 10 µl of pooled anti-HCMV human sera, first for 1 h at 37 °C and then, for 1 h at 4 °C. At the end of this period, the mixture was added to protein A-Sepharose as described above, and the Sepharose beads with the bound antigens were washed 3 times with 50 mM Tris-HCl pH 7.5. The pellets were resuspended in 0.1 ml of the previous buffer and aliquots of 10 µl were assayed for radioactivity with 5 ml of Picofluor as scintillant.

Table 1. Effect of periodate oxidation on the infectivity of HCMV in vitro

	Non-treated HCMV	Mock-oxidized HCMV	Oxidized HCMV
Protein content (mg/ml)	2	1.3	1.3
Radioactivity associated with viral DNA (dpm/30μl)	4590	2910	3170
Viral titer (ID/ml)	6×10^6	2.3×10^6	0

Results

The effect of periodate oxidation on HCMV infectivity in vitro

Before investigating the effect of periodate oxidation on viral infectivity, it was first necessary to determine whether the oxidation procedure per se and the subsequent dialysis step had any effect on the content of viral DNA and protein. Accordingly, [^{3}H] labelled virus was either mock-oxidized or oxidized with 15 mM sodium periodate as described in the methods section. After incubation, both samples were treated with sodium sulfite to stop oxidation and dialysed. It is apparent in Table 1 that, compared to the non-treated sample, some loss of virus (about 35%) does take place on dialysis as assessed by both protein and DNA. However, the amount of virus recovered in both oxidized and mock-oxidized samples is similar. Therefore, there is no preferential loss of viral particles during oxidation. Nevertheless, when the infectious capacity was examined, 2.3×10^6 ID/ml were found for the mock-oxidized sample whereas the oxidized virus was found to be totally inactivated.

Further, cells infected with oxidized virus were kept in culture for 3 weeks without the development of any cytopathic effect.

In the former experiment, the concentration of periodate and oxidation time used were based on literature values for oxidizing glycoproteins such as IgG [22]. We therefore decided to examine the influence of these 2 parameters on the kinetics of HCMV inactivation. As shown in Fig. 1, the kinetics of inactivation by periodate was a two-stage reaction. First, the titer of the virus decreased linearly with time and then reached a plateau. The inactivation rate (estimated by slope) was closely dependent on the periodate concentration. It was a fast reaction, since it was achieved in 40 min for a periodate concentration as low as 2 mM. When the final periodate concentration was 5 mM, inhibition (6 logs) was almost instantaneous (less than 5 min). Further, the reaction was total, since on increasing the incubation time over the threshold point of the plateau (5 or 40 min for 5 mM or 2 mM periodate, respectively) there was no increase in inactivation. Finally, the amount of inactivated viral particles, as assessed by plateau height, was proportional to periodate concentration.

Since the kinetic curves seemed to show that inactivation rates and amounts of inactivated particles were closely dependent on the periodate concentration

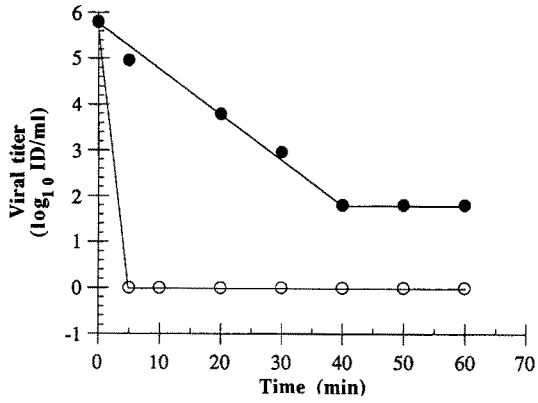

Fig. 1. Inhibition kinetics of the infectivity of HCMV in vitro by periodate oxidation. 2 mM (●) or 5 mM (○) sodium periodate

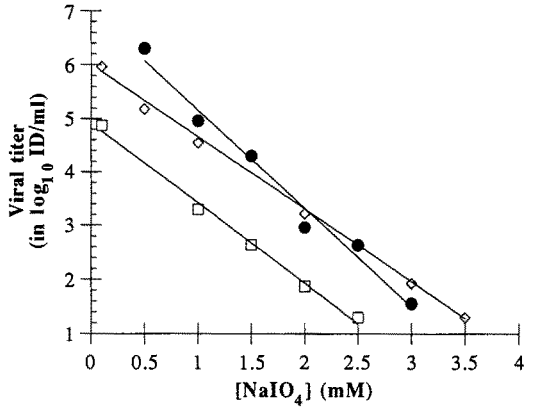

Fig. 2. Effect of the sodium periodate concentration on the infectivity of HCMV in vitro. ◇ Viral batch n°1 : $y1 = 6.0 - 1.35 \times 1$ ($r^2 = 0.992$); ● viral batch n°2 : $y2 = 6.3 - 1.50 \times 2$ ($r^2 = 0.982$); □ viral batch n°3 : $y3 = 4.9 - 1.49 \times 3$ ($r^2 = 0.994$)

used, we decided to study the effect of varying concentrations of periodate on viral infectivity. Accordingly, aliquots of viral suspension were treated with various periodate concentrations and then titrated. The results obtained with 3 different preparations of HCMV (Fig. 2) showed, in each case, a strong linear correlation ($r^2 > 0.98$) between the inhibition of viral infectivity (expressed in $\log_{10}$) and the periodate concentration. Thus, the inhibition of viral infectivity as a function of periodate concentration was a first-order reaction.

Determination of the target(s) of periodate attack

To determine whether the sugar moieties of viral glycoproteins were indeed targets for periodate attack, total aldehydes were measured using the MBTH reaction [1] or after reduction with tritiated sodium borohydride as described in

Table 2. Aldehydes generated on cellular and viral glycoproteins

Sample	Oxidized uninfected cells	Mock-oxidized HCMV	Oxidized HCMV
Protein content[a] (mg)	0.3	0.3	0.3
Aldehydes[b] (nmol/mg of protein	108	21	173
Radioactivity associated[c] with Protein A-Sepharose (dpm)	4740	4470	28116

[a] Protein content was adjusted to 0.3 mg in each sample before reduction with borohydride

[b] Aldehydes were measured using the MBTH method

[c] Radioactivity associated with protein A-Sepharose beads after reduction of aldehydes with tritiated borohydride and immunoprecipitation with an anti-HCMV serum

the methods section. Mock-oxidized HCMV and oxidized uninfected cells were used as controls.

It is evident from Table 2 that there is an 8-fold increase in the total number of aldehyde groups present in the oxidized compared to the mock-oxidized virus. However, as oxidized uninfected cells also showed a 5-fold increase, the exact contribution of viral glycoproteins to the genesis of aldehydes had to be verified. Accordingly, the reduction of oxidized, mock-oxidized and oxidized uninfected cells with tritiated borohydride was undertaken. It can be seen in Table 2 that there is a 6-fold increase in the radioactivity of the proteins in the immunoprecipitate when they are derived from oxidized virus compared to that from mock-oxidized virus, or from oxidized uninfected cells. This last observation provides good evidence for the oxidation of carbohydrate residues of viral glycoproteins by periodate.

HCMV adsorption and penetration into cells

As our first results showed that oxidized virus was incapable of giving rise to infectious progeny when added to cells, we decided to determine the step of the viral replication cycle which was blocked when oxidized virus was used. To achieve this, we first studied the ability of oxidized HCMV to adsorb specifically to the cell surface and then to penetrate the cells. Results are shown in Table 3.

About 16% of the total viral inoculum, as determined by radioactive content, was specifically adsorbed to the cell surface for mock-oxidized HCMV. These findings are similar to those obtained by Taylor and Cooper [26]. Assays carried out with oxidized virus showed that a 12.2% specific adsorption took place. The comparison of means with a t-test revealed that levels of specific adsorption between mock-oxidized and oxidized virus were not significantly different ($p = 0.01$).

78 and 80% of the specifically adsorbed particles were internalized into the host cells for mock-oxidized and oxidized virus, respectively. This difference was

Table 3. Specific binding and internalization of [^{3}H]thymidine labelled HCMV

	Mock-oxidized HCMV	Oxidized HCMV
HCMV bound[a] (% of inoculum)	16[c]	12.2
HCMV internalized[b] (% of bound virus)	78	80

[a] HCMV bound = (radioactivity specifically associated with cells at 4 °C/radioactivity in the viral inoculum) × 100

[b] HCMV internalized = (radioactivity specifically associated with cells at 37 °C after trypsinization/radioactivity specifically associated with cells at 4 °C) × 100

[c] Each value represents the mean of 4 experiments

not significant (t-test, $p = 0.01$). It would appear then that attachment and penetration into cells was quantitatively similar for both mock-oxidized and oxidized HCMV.

Viral DNA penetration into host cell nuclei

As adsorption and penetration of oxidized HCMV were not impaired, the next step to be investigated was the decapsidation process and the ensuing penetration of viral DNA into cell nuclei. We therefore tried to detect the presence of viral nucleic acid (labelled with [^{3}H]thymidine) in purified nuclei from mock-oxidized or oxidized HCMV infected cells.

As shown in Table 4, radioactivity can be detected in nuclei from mock-oxidized and oxidized infected cells, which in itself is good evidence that viral DNA had penetrated the cell nuclei. Further, when the radioactivity was expressed per μg of DNA, it was found that the percentage of nuclear-bound

Table 4. Penetration of viral DNA into host cell nuclei

Sample	Cell fraction	Radioactivity associated with viral DNA (dpm)	Total DNA amount in sample (μg)	Specific radioactivity (dpm per μg of DNA)	Nuclear/ total (%)
Mock-oxidized HCMV	cytoplasmic	12 560	—	3 019	
	nuclear	6 959	4.16	1 673	35.7
	total	19 519	—	4 692	
Oxidized HCMV	cytoplasmic	25 168	—	3 718	
	nuclear	13 452	6.77	1 987	34.8
	total	38 620	—	5 705	

material was similar in cells infected with mock-oxidized and oxidized virus: 35.7 vs 34.8%.

This result suggests therefore that the penetration of viral DNA from oxidized virions into the cell nucleus was not affected by periodate oxidation.

Viral DNA replication in host cells

Since viral DNA from both mock-oxidized and oxidized viral particles was present in cell nuclei, the question arose whether it could serve as a template for viral DNA replication and viral protein synthesis. Accordingly, viral DNA replication was studied by measuring the incorporation of [^{3}H]thymidine into viral DNA at various times following infection.

Figure 3 shows curves obtained for non-infected, mock-oxidized and oxidized HCMV infected cells. A strong increase in thymidine incorporation was observed following infection with mock-oxidized virus. This lasted until 4 days post-infection then decreased quickly. The experiment was stopped 7 days post-infection because of cell death following cytopathic effect. Such an increase was not seen with non-infected cells, which confirmed that thymidine incorporation was really due to viral DNA replication. When cells were infected with oxidized virus, the curve was similar to that obtained with non-infected cells (i.e., without any increase in thymidine incorporation) and this persisted for 10 days post-infection. It seemed therefore that oxidized viral DNA was unable to replicate in host cells.

Viral protein synthesis

To study viral protein synthesis, cells were infected with mock-oxidized or oxidized HCMV. At various times post-infection, cells were scraped and lysed

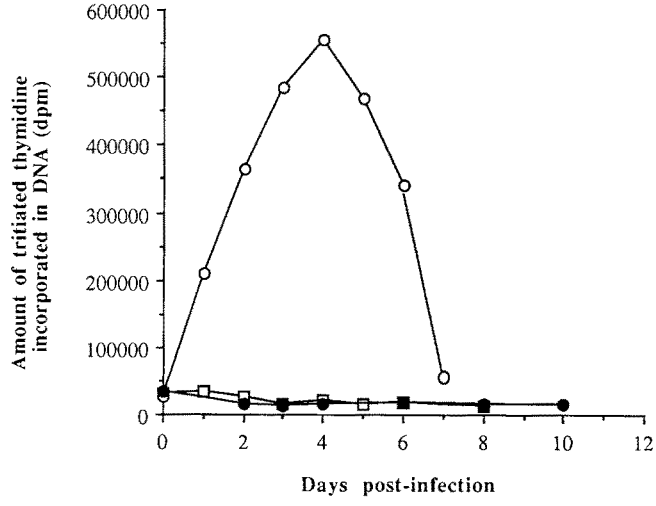

Fig. 3. Replication kinetics of HCMV in MRC-5 cells as determined by the incorporation of tritiated-thymidine into viral DNA. □ Uninfected cells; ○ cells infected with mock-oxidized HCMV; ● cells infected with oxidized HCMV

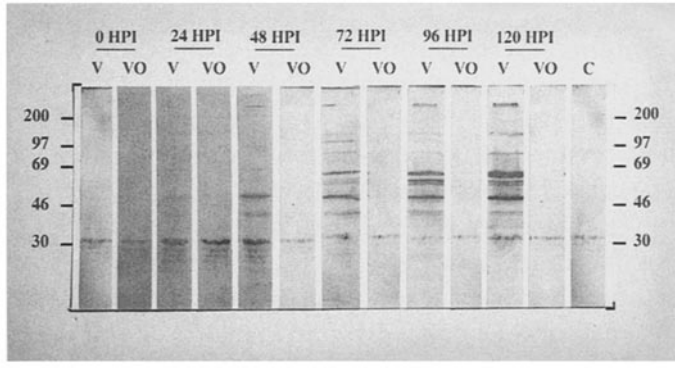

Fig. 4. Analysis by western blotting of the time course of viral protein synthesis during the infection of MRC-5 cells by HCMV. Cells were infected with mock-oxidized (*V*) or oxidized (*VO*) HCMV. At various hours post-infection (*HPI*), cells were scraped and lysed. Proteins were separated by 10% SDS-PAGE. Viral proteins were then visualized by western blotting with a pool of human anti-HCMV sera. As a control (*c*), a lysate from uninfected cells was treated similarly. Molecular weight standards (kDa) are indicated on both sides

with detergent. They were then electrophoresed in a 10% SDS-PAGE. Viral proteins were then visualized by western blotting, with a pool of human anti-CMV sera.

As shown in Fig. 4, the pool of anti-HCMV sera is specific since, apart from a band at 35 kDa found with lysates from all samples including that from uninfected control, there is no reaction at 0 h post-infection with any constituent present in the lysates from cells infected with mock-oxidized (V) or oxidized (VO) virus.

From 24 h post-infection onwards, viral proteins are present in V and exhibit both a qualitative as well as a quantitative increase with line up to 120 h post-infection. No evidence for the synthesis of viral proteins was obtained when lysates were derived from cells infected with oxidized virus.

Therefore, viral protein synthesis appears to be totally inhibited in cells infected with periodate-oxidized HCMV.

Discussion

The results presented here provide evidence for the inactivation of HCMV by periodate oxidation. Inactivation was not due to the loss of viral constituents brought about by oxidation since, as shown in Table 1, the protein concentration and the DNA content expressed as dpm [^{3}H]thymidine per mg protein were similar for both mock-oxidized and oxidized virions.

Inactivated oxidized HCMV adsorbed to and penetrated host cells to the same extent as mock-oxidized virus. This result strongly suggests that the integrity of recognition sites on the virion had been preserved by the oxidation

procedure, even though that sugar residues of viral glycoproteins were indeed oxidized (Table 2).

This preservation of recognition sites in the oxidized virions was not accompanied by the retention of functional capacity. Indeed, in infected cells, the DNA of oxidized virus was not used as a template for the syntheses of viral DNA or proteins, even when the contact time between oxidized virus and host cells was prolonged to 3 weeks post infection. It would therefore seem that the drastic reduction in infectivity is not due to any increase in the eclipse phase.

As far as inactivation kinetics is concerned, its study revealed that the rate of inactivation and the amount of inactivated viral particles were strictly proportional to the periodate concentration. Thus, in the presence of periodate at concentrations equal to or greater than 5 mM, inactivation of 6 logs of virus was almost instantaneous (less than 5 min). It is noteworthy that at lower concentrations of periodate (2 mM) no further inactivation takes place, even on increasing the contact time between periodate and virus (Fig. 1). These results are to be compared to those reported with classical chemical inactivating agents, such as formalin and binary ethylenimine (BEI). Indeed in the case of BEI, inactivation rates of the order of 1 to 4 logs virus per hour were observed for viruses such as vesicular stomatitis virus and rabies virus, respectively [2]. For formalin, the inactivation of 5 to 6 logs of poliomyelitis virus required a contact time of at least 2 to 3 days [10, 29]. Similar contact times have been published for foot-and-mouth disease virus inactivated by formalin [13, 28].

Since the amount of inactivated viral particles appeared to be dependent on the concentration of periodate, an investigation was carried out to determine the evolution of viral infectivity as a function of periodate concentration. This study showed that there is a first-order relationship between these 2 parameters, i.e., that viral titer, expressed in $\log_{10}$, bears a linear relationship to the concentration of periodate ($r^2 > 0.98$).

However, it must be borne in mind that in preparing biological material destined for prophylactic use, unequivocal evidence for total inactivation must be provided, especially in large-scale inactivation processes. In this context, some authors have recently shown the importance of using linear or first-order inactivants in controlling inactivation processes [2, 4].

It would therefore appear that in terms of its first-order inactivation characteristics, rapidity and efficacy of inactivation, ease of performance and cost benefit ratio, periodate treatment of viruses could offer distinct advantages.

However, it remains to be determined whether (i) oxidized CMV though innocuous in vitro is also devoid of any risk of integration of part(s) of the viral genome into cellular DNA (ii) the oxidized particle can induce an immune response and protect recipients against lethal CMV infection. This is the subject of ongoing investigation using the murine model of CMV.

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