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Free thiol groups are essential for infectivity of human cytomegalovirus

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The membrane-impermeable thiol blocker 5′5-dithiobis 2-nitrobenzoic acid (DTNB) blocked infectivity of human cytomegalovirus (CMV) although the virus still bound to cells. DTNB-treated CMV regained 65% of its infectivity after incubation with the disulfide bond-reducing agent dithiothreitol. These observations suggest that free thiol groups on CMV are required for infectivity and may participate in disulfide bond formation during virus entry.

Entry of enveloped viruses into host cells can be characterized by three distinct events. First, attachment of virus to a specific cell-surface receptor (Wickham *et al.*, 1993; Williams *et al.*, 1991) and then, for several viruses, binding to a secondary receptor (Spear, 1993; Tsao & Huang, 1986). This secondary binding is supposed to facilitate penetration either by fusion or endocytosis and results in irreversible conformational changes in viral proteins, including exposure of hydrophobic domains in either the receptor-binding protein or a fusogenic viral envelope glycoprotein (Haywood, 1994). The third step involves fusion between a hydrophobic domain of the viral fusion protein and the cellular membrane. It is proposed that a distance of 5–10 Å between two lipid bilayers is required for this membrane fusion (Haywood, 1994). However, viral spike proteins often protrude more than 100 Å from lipid bilayers, thereby causing significant hindrance of fusion between the virus and the lipid bilayer of the cell (Haywood, 1994). In pH-dependent fusion the conformational changes in virus spike proteins are considered to occur in endosomes, where the acidic milieu causes dramatic conformational changes in spike proteins and thereby facilitates the required interaction between bilayers. While it is well-established that enveloped viruses, which enter cells by endocytosis, fuse with endosome membranes in a pH-dependent manner (White *et al.*, 1983), the molecular mechanisms by which they fuse at the plasma membranes are poorly understood.

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Many fusogenic glycoproteins of viruses which fuse with the plasma membrane, e.g. cytomegalovirus (CMV) (Compton *et al.*, 1992), human immunodeficiency virus (HIV) (Freed & Martin, 1995; McClure *et al.*, 1988) and paramyxovirus (Lamb, 1993), are rich in disulfide bonds and it is tempting to speculate on the role that disulfide bonds play during disassembly. Ryser *et al.* (1994) have recently suggested that HIV and its target cell engage in a thiol–disulfide interchange reaction and that reduction of critical disulfide bonds in viral glycoproteins may be the initial event that triggers the conformational changes required for HIV entry. In the present study we determined whether CMV contains free thiol groups that are essential for entry and infectivity.

CMV (strain AD 169) and herpes simplex virus-1 (HSV-1; strain F9004) were cultivated on human fibroblast cells until complete CPE. Infected cells were then harvested, cellular debris removed by centrifugation, the supernatant aliquoted and titres determined as described below.

To determine whether CMV or HSV-1 possess free thiol groups critical for infectivity, $1{\cdot}2 \times 10^4$ p.f.u./10µl of virus was mock-treated or treated with various different concentrations of 5′,5-dithiobis 2-nitrobenzoic acid (DTNB; Sigma) for 1 h at 37 °C or with 500 µM DTNB for various periods of time. DTNB is a membrane-impermeable thiol-blocking reagent which covalently modifies thiol groups (Buelt & Bernlohr, 1990; Li *et al.*, 1994; Smith *et al.*, 1975) and can be used to measure free thiol groups on proteins (Ploux *et al.*, 1995; Zhi *et al.*, 1991). After DTNB treatment virus was diluted 1/100 with serum-free Eagle's MEM and 100 µl volumes (equivalent to $1{\cdot}2 \times 10^3$ p.f.u. were inoculated in duplicate in 96-well plates (Costar) containing confluent monolayers of human fibroblast cells. After 1 h incubation at 37 °C, the inoculum was removed and replaced with serum-free Eagle's MEM. At 8 h (HSV) or 24 h (CMV) post-infection, cells were fixed and the reduction in infectivity was determined by immunoperoxidase staining (Mirazimi & Svensson, 1998; Mirazimi *et al.*, 1996). Briefly, infected cells were fixed for 18 h with 2% paraformaldehyde in PBS, followed by treatment with 1% Triton X-100 for 10 min. Mouse anti-CMV early antigens [clone CCH2 + DDG9 code

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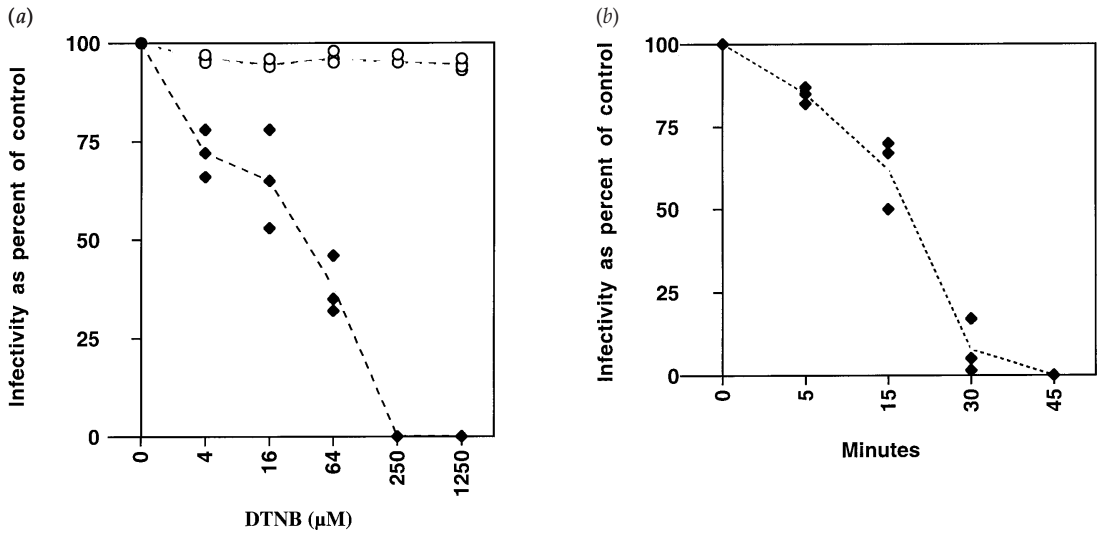

Fig. 1. (*a*) Effect of DTNB on infectivity of CMV (◆) and HSV-1 (○). CMV ($1{\cdot}2 \times 10^4$ p.f.u./10 μl) and HSV-1 ($1{\cdot}2 \times 10^4$ p.f.u./10 μl) were incubated with different concentrations of DTNB for 1 h at 37 °C and then diluted 1/100 in serum-free Eagle's MEM; $1{\cdot}2 \times 10^3$ p.f.u. of virus was then inoculated onto human lung fibroblast monolayers in 96-well plates. After 1 h incubation at 37 °C, the inoculum was removed and replaced with Eagle's MEM. After 24 h (CMV) or 8 h (HSV-1) of infection, the cells were fixed and the number of infected cells was determined. The values presented are percentages of the infectivity of mock-treated virus. All experiments were done in duplicate and the results shown are from three independent experiments. (*b*) Kinetics of the inactivation of CMV by DTNB. CMV ($1{\cdot}2 \times 10^4$ p.f.u./10 μl) was incubated with 500 μM DTNB for various periods of time at 37 °C followed by 1/100 dilution in serum-free Eagle's MEM and inoculation of $1{\cdot}2 \times 10^3$ p.f.u. virus onto fibroblast monolayers. After 1 h incubation at 37 °C, the inoculum was removed and replaced with Eagle's MEM. After 24 h infection, the cells were fixed and the number of infected cells was determined. The values presented are percentages of the infectivity of mock-treated virus. All experiments were done in duplicate and the results shown are from three independent experiments.

no. M0854 (DAKO); CCH2 reacts with CMV delayed early DNA binding protein p52 and DDG9 reacts with a 76 kDa protein of Ad 169 antigen] and rabbit anti-HSV type 1 [code no. P0175 (DAKO)] were diluted in PBS containing 0·2% BSA and 0·1% Triton X-100 and incubated for 1·5 h at 37 °C on cells followed by three washes with PBS and a second incubation for 1·5 h at 37 °C with peroxidase-labelled goat anti-mouse IgG or goat anti-rabbit IgG (Bio-Rad). After three more washes the number of infected cells was determined (Mirazimi *et al.*, 1996).

The inhibitory effect of DTNB on CMV infectivity was dose-dependent and infectivity was completely abolished at a DTNB concentration of 250 μM (Fig. 1*a*). We have also obtained the same results with purified CMV(data not shown). Analysis revealed that the inhibitory effect was rapid and almost complete inactivation was obtained after 30–45 min of treatment with 500 μM DTNB (Fig. 1*b*).

In contrast to CMV, infectivity of HSV-1 was unaffected (Fig. 1*a*), even at a DTNB concentration of 1·25 mM. This absence of effect of DTNB on HSV-1 is similar to observations previously reported for HIV and Sindbis virus (Abell & Brown, 1993; Ryser *et al.*, 1994). Both these viruses have previously been found to be insensitive to DTNB treatment when virus was treated before adsorption to cells. We therefore propose that CMV, in contrast to HSV-1, contains free accessible thiol groups, which are essential for infectivity.

To determine whether the plasma membrane possesses accessible free thiol groups which might participate in virus entry, human lung fibroblast monolayers were mock-treated or treated with 1–10 mM DTNB. After 1 h incubation at 37 °C, DTNB was washed out with Eagle's MEM and monolayers immediately infected with $1{\cdot}2 \times 10^3$ p.f.u. of CMV; the number of infected cells was determined 24 h later. The results did not reveal any difference (< 5% variation) between DTNB-treated and mock-treated cells, suggesting that if thiol groups had been accessible on the plasma membrane, they did not participate in the entry process of CMV. However, the observations do not rule out the possibility that binding of CMV to the plasma membrane causes conformational changes in cellular proteins such that thiol groups protrude and react with thiol groups on the virus.

The novel observation that CMV infectivity could be eliminated by blocking specific thiol groups on the virus surface led us to determine whether viral infectivity also could be restored if the DTNB-blocked thiol groups were reduced. To test this hypothesis, DTNB-inactivated virus with no remaining infectivity was mock-treated or treated with 1·25 or 2·5 mM of the disulfide-reducing agent dithiothreitol (DTT) for various times. Virus was subsequently diluted 1/100 in Eagle's MEM and inoculated onto cells, followed by determination of infectivity 24 h later. Most surprisingly, we found that a brief incubation with DTT restored viral infectivity

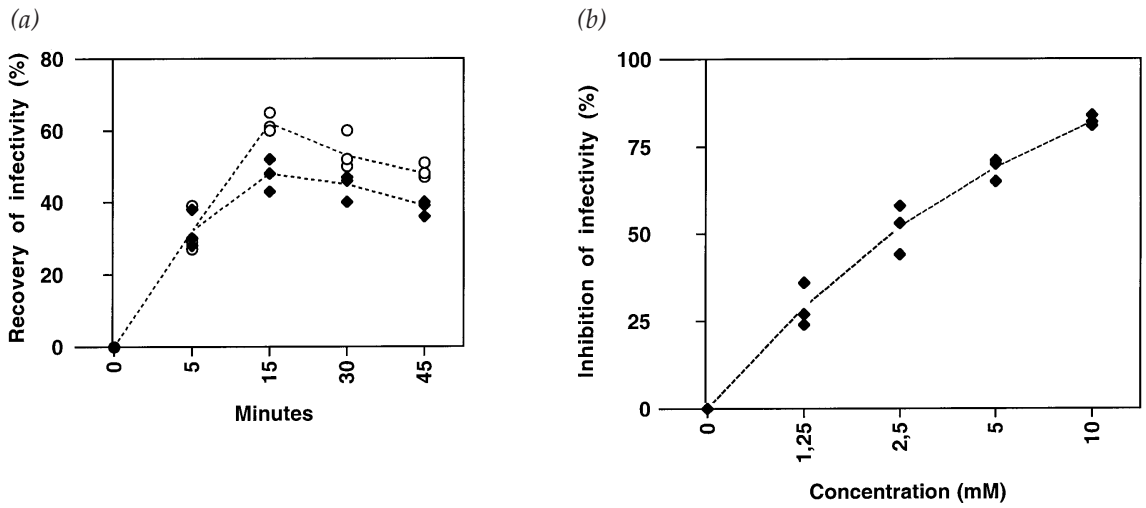

Fig. 2. (*a*) Recovery of CMV infectivity by reduction of disulfide bonds. CMV ($1{\cdot}2 \times 10^4$ p.f.u./10 μl) was first inactivated (nil infectivity) by treatment with 256 μM DTNB for 1 h at 37 °C followed by disulfide bond reduction with 1·25 (○) or 2·5 (◆) mM DTT for various times. At the end of DTT treatment, virus was diluted in serum-free Eagle's MEM and inoculated onto fibroblast monolayers. After 1 h incubation at 37 °C, the inoculum was removed and replaced with Eagle's MEM. After 24 h infection, the cells were fixed and the number of infected cells was determined. All experiments were done in duplicate and results shown are from three independent experiments. The values presented are percentages of the infectivity of mock-treated virus. (*b*) Effect of DTT on CMV infectivity. CMV ($1{\cdot}2 \times 10^4$ p.f.u./10 μl) was incubated with different concentrations of DTT for 30 min at 37 °C and then diluted 1/100 in serum-free Eagle's MEM followed by inoculation of $1{\cdot}2 \times 10^3$ p.f.u. virus onto human lung fibroblast monolayers in 96-well plates. After 1 h incubation at 37 °C, the inoculum was removed and replaced with Eagle's MEM. After 24 h infection, the cells were fixed and the number of infected cells was determined. The values presented are percentages of the infectivity of mock-treated virus. All experiments were done in duplicate and results shown are from three independent experiments.

by reducing disulfide bonds between the viral glycoprotein(s) and DTNB. As illustrated in Fig. 2(*a*), infectivity was already partly restored after 5 min incubation with DTT and increased to 65% after 15 min of treatment.

The recovery of CMV infectivity could, however, have resulted from cleavage of already-established inter- or intramolecular disulfide bonds of CMV glycoproteins, rather than reduction of the binding between DTNB and thiol groups on CMV. To investigate this possible explanation, CMV was mock-treated or treated with different concentrations of DTT for 30 min, inoculated onto cells, and the remaining infectivity determined. As shown in Fig. 2(*b*), DTT had an antiviral rather than a stimulatory effect. These results confirm a previous observation in which DTT was shown to lower HIV infectivity (Ryser *et al.*, 1994).

The conclusion that the recovery of viral infectivity was due to reduction of covalent bond interactions between thiol groups on viral protein(s) and DTNB, and not to reduction of established inter-or intra-disulfide bonds of viral glycoproteins, is therefore supported by the results presented in Fig. 2(*b*).

In order to determine whether CMV retained its capacity to bind to target cells after inactivation by DTNB, a binding experiment was performed. Fibroblast monolayers (38×10^6 cells) were infected with CMV at an m.o.i. of 0·01. When 30% CPE was observed, the medium was replaced with medium free of methionine and cysteine except for 330 μCi [^{35}S]methionine–cysteine (Trans-label; DuPont). Virus was collected 5 days later. Cell debris was removed by low-speed centrifugation and virus was pelleted by centrifugation at 25 000 r.p.m. in a Beckman SW-28 rotor for 1 h through a 5 ml layer of 20% (w/v) D-sorbitol (Soderberg *et al.*, 1993). The infectivity (p.f.u.) titre of the purified ^{35}S-labelled virus was determined by peroxidase focus reduction test (Mirazimi *et al.*, 1996). Radioactivity was quantified by liquid scintillation counting, giving a specific activity of 6·7 c.p.m./p.f.u.

Different concentrations of purified ^{35}S-labelled-CMV were mock-treated, DTNB-treated (500 μM) or heparan sulfate-treated (10 μg) and inoculated on fibroblast monolayers previously pretreated with PBS–BSA (3%). After adsorption at 4 °C for 2 h, unbound virus was washed out and cells were rinsed three times before cell lysis and scintillation counting of bound radioactivity. The heparan sulfate treatment demonstrated the specificity of attachment of ^{35}S-CMV to the cell-surface receptors, since it inhibits attachment of CMV to host cells (Compton *et al.*, 1993; Kari & Gehrz, 1992, 1993). As expected, heparan sulfate treatment (Fig. 3) inhibited attachment of CMV to the cell surface by 90% in our experiments (Fig. 3). While infectivity of CMV was completely ($< 99{\cdot}99\%$) eliminated by treatment with 0·5 mM DTNB, 70% of DTNB-treated virus still bound to the cells, suggesting that post-attachment events were inhibited by DTNB, but not the early attachment to cell receptor(s).

It has previously been proposed that the gB and gH proteins of CMV are involved in the virus entry process

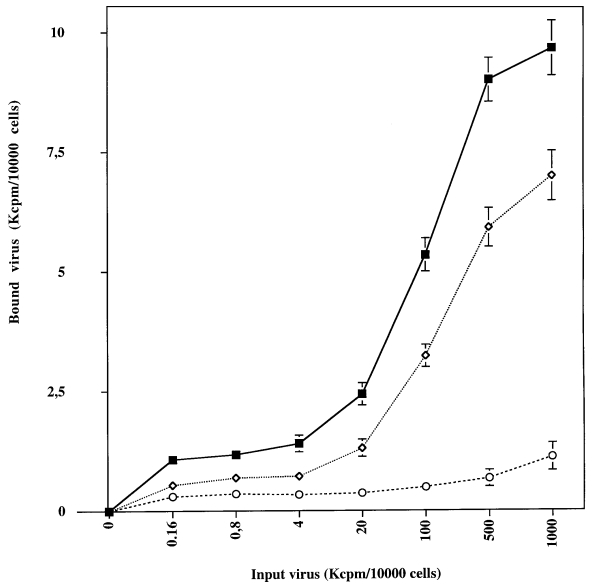

Fig. 3. DTNB-inactivated CMV binds to target cells. Different concentrations of ^{35}S-labelled CMV were mock-treated (■), treated with 500 μM DTNB (◇) or treated with 10 μg heparan sulfate (○) for 1 h at 37 °C. After adsorption to fibroblast monolayers for 2 h at 4 °C, inoculum was removed and cells washed three times before cell lysis and scintillation counting. The results are from three independent experiment and standard errors are presented.

(Compton, 1995; Soderberg *et al.*, 1993). The exact role of these proteins is as yet unknown, but it has been suggested that they recognize and interact with either Annexin II or a 92·5 kDa protein on the cell surface (Keay & Baldwin, 1992; Keay *et al.*, 1989; Wright *et al.*, 1994). The gB and gH proteins are both heterodimers and are thought to contain inter-and intra-disulfide bonds (Spaete *et al.*, 1994), of which the disulfide bonds of the gB protein are critical for folding and stability (Billstrom & Britt, 1995). In view of previous findings (Abell & Brown, 1993; Ryser *et al.*, 1994) and our present observations, it is reasonable to believe that DTNB blocks thiol–disulfide interchange within or between CMV glycoproteins (gB, gH?) or between the viral glycoproteins and cell-surface molecules, and that such interchange is critical for CMV infectivity.

As we have demonstrated in Fig. 1(*a*), the infectivity of HSV-1 was unaffected by treatment with DTNB. gB and gH of HSV-1 may have a conformation different from that of gB and gH of CMV, which could explain why HSV-1 does not expose free thiol groups critical for infectivity.

In summary, we have shown conclusively that CMV contains free thiol groups that play a critical role in viral infectivity and that after inhibition infectivity can be recovered following appropriate treatment. We believe that this new information can be useful to further explore molecular mechanisms behind entry and fusion events of CMV. Studies aimed at identifying viral and cell-surface proteins with free thiol groups are in progress.

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