Cytomegalovirus in Urine Specimens Has Host β₂ Microglobulin Bound to the Viral Envelope: A Mechanism of Evading the Host Immune Response?

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(Accepted 4 November 1986)

SUMMARY

We have previously reported that human cytomegalovirus (CMV) from urine specimens cannot be captured onto a solid phase by CMV-specific monoclonal antibodies that can capture CMV grown in vitro. We report here that CMV exists in vivo in body fluids such as urine as β₂ microglobulin (β₂m)-coated particles. We have demonstrated the presence of β₂m on CMV purified directly from urine by Western blotting and have shown that the β₂m was associated with the viral envelope. Urinary CMV could be specifically bound by an affinity column comprising a monoclonal antibody specific for β₂m bound to Sepharose. The β₂m-coated urinary CMV could not be neutralized by hyperimmune globulin, human immune sera or murine monoclonal antibodies that could neutralize CMV grown in cell culture. We conclude that the binding of β₂m by CMV masks the important antigenic sites necessary for neutralization which are recognized by man's immune response. We propose that CMV has evolved this mechanism of coating itself in a host protein as a mechanism of evading the host immune response and facilitating transmission between individuals.

INTRODUCTION

Human cytomegalovirus (CMV) in clinical specimens appears to differ from cell culture-grown virus in several respects. Firstly, the virus in urine samples appears to be much more stable than that grown in cell culture (Feldman, 1968; Stagno et al., 1980), which is reported to be thermolabile (Vonka & Benyesh-Melnick, 1966). Second, it is well recognized but to our knowledge not previously published, that CMV in urine cannot be neutralized by antisera with good neutralizing activity against CMV grown in cell culture. Third, we have recently reported that CMV in urine specimens cannot be captured onto a solid phase by CMV-specific monoclonal antibodies which can capture CMV grown in vitro (McKeating et al., 1985), suggesting some differences in surface antigens between viruses from the two sources.

We have shown that urine contains a host protein, β₂ microglobulin (β₂m) which, at physiological concentrations, inhibits the capture of cell culture-grown CMV by virus-specific monoclonal antibodies (McKeating et al., 1986). We postulated that this inhibition was due to the binding of β₂m to the virus masking the viral antigenic determinants. In an accompanying paper (Grundy et al., 1987a), we have shown that indeed cell culture-grown CMV exhibits a strong binding capacity for β₂m in vitro. We predicted that in vivo, in body fluids such as urine, CMV exists as β₂m-coated particles, and the aim of the present study was to verify that prediction using virus preparations obtained directly from infected patients.

METHODS

Preparation and purification of CMV from urine. Urine known to contain CMV by virus isolation was clarified by centrifugation at 1500 g for 15 min. The clarified urine was ultracentrifuged at 50000 g for 12 h, and the crude virus pellet was collected and resuspended in phosphate-buffered saline.
For purified urinary CMV preparations, the clarified urine was concentrated using an Amicon stirred cell concentrator with YM100 membranes with a molecular mass cut off of 100000. Concentrated urine was centrifuged through a potassium tartrate/glycerol negative viscosity/positive density gradient (Talbot & Almeida, 1977) at 400000 g for 45 min. Two bands were visible, one distinct band containing membranous material and the other a diffuse band representing the virions.

**Solubilization of viral envelope.** A preparation of CMV envelope glycoproteins was obtained by Triton extraction of purified virions as described in the preceding paper (Grundy et al., 1987a), and the Triton-soluble and Triton-insoluble fractions were stored at -80 °C prior to analysis.

**PAGE and Western blotting.** Viral proteins were separated by gradient PAGE, transferred to nitrocellulose filters and probed with monoclonal antibodies specific for β₂m (C21, kind gift from Dr A. Sanderson) as described in the preceding manuscript (Grundy et al., 1987a).

**Passage of urinary CMV through an anti-β₂m affinity column.** An 8 cm x 1 cm affinity chromatography column was prepared using the monoclonal antibody BBM.1 (ATCC Hybridoma No. HB28) specific for β₂m. The monoclonal antibody used was first precipitated from ascitic fluid by ammonium sulphate and then dialysed against 0.1 M-sodium carbonate buffer pH 8.3, 0.5 M-NaCl for 72 h with seven or eight changes of dialysate. The antibody fraction obtained was linked to cyanogen bromide-activated Sepharose 4-B (Pharmacia) according to the manufacturer's instructions and stored in PBS with 1% sodium azide at 4 °C. The column was shown to bind 17 μg purified human β₂m (kind gift from Dr A. Sanderson) per 200 μl of gel volume. The column was washed at room temperature with 10 bed vol. 50 mM-Tris–HCl, 0.5 M-NaCl, 5 mM-EDTA, 0.5% NP40 pH 8, followed by 5 bed vol. 10 mM-Tris–HCl pH 7.4.

Urine from patients excreting CMV was concentrated 500-fold by ultrafiltration as above, and 1 ml of the concentrated urine was loaded onto the column. Unbound material was washed through the column with 5 bed vol. 1 M-NaCl in PBS pH 7.4. The bound material was eluted with 50 mM-diethylamine and immediately neutralized with N-diethylmorpholine acetate. Fractions were collected and the absorbance of each fraction was measured.

**Analysis of proteins by immunoblotting.** Fractions from the β₂m affinity column were analysed for the presence of CMV proteins and β₂m by a dot immunoblot assay as follows. Five μl of each fraction was spotted onto nitrocellulose filters and air-dried. Non-specific binding sites were blocked with 3% normal rabbit serum in PBS pH 7.4 at 37 °C for 1 h. The filters were then incubated with monoclonal antibodies specific for β₂m (C21) or for CMV (CH28, CH16, CH65, CH19; Pereira et al., 1982) at room temperature for 1 h with shaking. Filters were washed five times for 10 min each with Buffer B (Grundy et al., 1987a).

The binding of the monoclonal antibodies was detected by incubation for 1 h at room temperature with a rat monoclonal antibody specific for mouse immunoglobulin kappa chain (MRC-OX20, Serotec) which had been radiolabelled with 125I using iodogen (Fraker & Speck, 1978). Filters were washed five times for 10 min each as before, air-dried, and the specific spots revealed by autoradiography.

**Virus neutralization.** Antibodies used were CH134, a neutralizing murine monoclonal antibody specific for CMV (Pereira et al., 1982), BIG6, HLA-ABC-M2 (Serotec), C23, M8, B2 (kind gifts from Dr A. Sanderson), murine monoclonal antibodies specific for β₂m, a polyclonal rabbit antiserum against β₂m (kind gift from Unilever), a human globulin against CMV (Cytotect, Biotech) and a human immune serum with titres of anti-CMV IgG of 12800 and 6400 respectively by radioimmunoassay, a non-immune serum, and SRTI4 (Serotec) a murine monoclonal antibody specific for the Fc portion of human IgG (all subclasses).

These antibodies were diluted 1/100 in either PBS containing 2% guinea-pig complement or PBS alone, added to an equal volume of virus (titrated in 10-fold dilutions to 10⁻⁵) and incubated at 37 °C for 1 h. This virus–antibody mixture was allowed to adsorb to confluent monolayers of fibroblasts in 96-well microtitre tissue culture plates, at 50 μl per well in triplicate. After 1 h adsorption at 37 °C, fresh medium was added, the cells were incubated at 37 °C for 14 days and the wells were scored for the presence of cytopathic effect.

**Presence of β₂m bound to CMV in urine specimens**

CMV was harvested from urine by ultracentrifugation and electrophoresed in an 8 to 12% gradient SDS–polyacrylamide gel. The separated proteins were transferred onto nitrocellulose filters and probed with a murine monoclonal antibody to human β₂m. Detection with 125I-labelled anti-mouse immunoglobulin revealed a β₂m band in the virus preparations from five different urine specimens (Fig. 1). In contrast such a band was not seen with cell culture-grown
Fig. 1. Autoradiograph of a Western blot of separated viral proteins showing the presence of $\beta_2$m in CMV preparations from five different urine specimens (lanes 1, 2, 3, 5 and 6). The viral proteins were separated in an 8 to 12% gradient SDS-polyacrylamide gel, transferred to nitrocellulose filters and a mouse monoclonal antibody to human $\beta_2$m (C21) was used for the detection. The arrowhead indicates the position of purified human $\beta_2$m on this filter. No $\beta_2$m band was seen in lane 4 which was loaded with cell culture-grown CMV strain AD169, or in lane 7 loaded with the ultracentrifuge pellet from the urine of a CMV-seronegative patient with the same clinical history as those excreting CMV in the urine.

Fig. 2. Autoradiograph of a Western blot of viral proteins separated in an 8 to 12% gel showing loss of $\beta_2$m from urinary CMV after Triton extraction of the viral envelope. The Triton-soluble viral protein fraction separated in lane 1 contains a $\beta_2$m band as detected with the monoclonal antibody C21, specific for $\beta_2$m. The Triton-insoluble viral proteins separated in lane 2 do not contain $\beta_2$m. The arrowhead indicates the position of purified $\beta_2$m on this filter.

CMV was harvested from urine by ultracentrifugation and the envelope components were extracted by solubilization with Triton. The presence of $\beta_2$m in the Triton-soluble or -insoluble fraction was assessed by Western blotting as above. Fig. 2 shows the presence of $\beta_2$m only in the Triton-soluble fraction of urinary CMV, thus indicating that the $\beta_2$m was associated with the viral envelope.

Demonstration that $\beta_2$m is bound to the envelope of urinary CMV

CMV was harvested from urine by ultracentrifugation and the envelope components were extracted by solubilization with Triton. The presence of $\beta_2$m in the Triton-soluble or -insoluble fraction was assessed by Western blotting as above. Fig. 2 shows the presence of $\beta_2$m only in the Triton-soluble fraction of urinary CMV, thus indicating that the $\beta_2$m was associated with the viral envelope.
Urinary CMV is bound by a monoclonal antibody specific for \( \beta_2m \)

Urine from a patient excreting CMV was concentrated by ultrafiltration and passed through an affinity column prepared with monoclonal antibody BBM.1 specific for \( \beta_2m \). Fig. 3 shows that a peak of protein was seen in the eluate from the column (fraction 5) that contained both CMV and \( \beta_2m \) as demonstrated by immunoblotting. The presence of CMV in the specific eluate from the column was further confirmed by the presence of the characteristic viral protein bands (seen in the urinary virus preparation loaded onto the column) in the eluted fractions (Fig. 4). A protein band corresponding to the molecular mass of \( \beta_2m \) (11700) was seen in the separated proteins from the eluted fractions (Fig. 4). Thus urinary CMV is bound by a monoclonal antibody specific for \( \beta_2m \). In contrast \( \beta_2m \), and not CMV proteins, was detected by immunoblotting in the eluate when urine from a CMV-seronegative individual not excreting CMV was loaded onto the column (Fig. 3).

Effect of bound \( \beta_2m \) on the ability to neutralize CMV

We investigated whether various CMV-specific antibodies could neutralize urinary CMV even though this virus was known to be coated in \( \beta_2m \). In order to maximize sensitivity and because of differences in infectivity between \( \beta_2m \)-coated and uncoated particles (see Grundy et al., 1987b), the amount of antibody was kept constant and the virus preparations were serially titrated. Thus an antibody was classed as non-neutralizing with respect to a virus preparation if it failed to neutralize even the highest dilution of virus. Table 1 shows that a pooled human hyperimmune globulin preparation, a human immune serum, and a neutralizing mouse monoclonal antibody specific for CMV (CH134), either in the presence or absence of complement, failed to neutralize urinary CMV, but showed good neutralizing activity against CMV strain AD169 grown in cell culture. The extent of the ability of these antibodies to neutralize CMV strain AD169 is shown in Table 2.
Fig. 4. Presence of CMV in the specific eluate after passage of urine containing the virus through an anti-\(\beta_m\) affinity column, as demonstrated by the characteristic viral protein profile on electrophoresis in a 9 to 16% gel. Lanes 2 and 3 were loaded with the infected urine before passage through the column and lane 1 with the flowthrough of the column. Lanes 4 to 9 were loaded with fractions 10, 9, 8, 7, 6 and 5 from the affinity column respectively (see Fig. 3). Lane 10 was loaded with human \(\beta_m\) and lanes 11 and 12 with low and high molecular mass markers respectively of the molecular masses shown \((\times10^{-3})\).

Table 1. Ability of monoclonal or polyclonal sera to neutralize CMV from cell culture or from urine

<table>
<thead>
<tr>
<th>Neutralizing antibody</th>
<th>CMV strain AD169 with C(^*)</th>
<th>without C(^*)</th>
<th>Neutralization Urinary CMV 1 with C</th>
<th>without C</th>
<th>Urinary CMV 2 with C</th>
<th>without C</th>
</tr>
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<tbody>
<tr>
<td>Hyperimmune globulin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Immune human serum</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Non-immune human serum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
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<tr>
<td>Monoclonal antibody</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH134</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>BIG6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>HLA-ABC-M2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C23</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
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<tr>
<td>M8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
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<tr>
<td>SRT14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Rabbit anti-(\beta_m)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

\(^*\) C\(^*\), Complement.
\(^\dagger\) ND, Not done.
\(^\ddagger\) CH134 is specific for CMV; BIG6, HLA-ABC-M2, C23, M8 and B2 are specific for \(\beta_m\); SRT14 is specific for the Fc portion of human IgG (all subclasses).
Table 2. Quantitative neutralization data for all antibodies used in Table 1 which showed any neutralizing activity against CMV

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Presence of C'</th>
<th>Wells (%) showing c.p.e. at various dilutions of CMV strain AD169</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10^0</td>
</tr>
<tr>
<td>CH134 +</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Nil +</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>CH134 -</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Nil -</td>
<td></td>
<td>ND*</td>
</tr>
<tr>
<td>Human immune serum +</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Nil +</td>
<td></td>
<td>ND*</td>
</tr>
<tr>
<td>Human immune serum -</td>
<td></td>
<td>ND*</td>
</tr>
<tr>
<td>Nil -</td>
<td></td>
<td>ND*</td>
</tr>
<tr>
<td>Hyperimmune globulin +</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Nil +</td>
<td></td>
<td>ND*</td>
</tr>
<tr>
<td>Hyperimmune globulin -</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Nil -</td>
<td></td>
<td>ND*</td>
</tr>
</tbody>
</table>

* ND, Not done.

We also attempted to neutralize urinary CMV using various monoclonal antibodies against \( \beta_2 \text{m} \) (HLA-ABC-M2, C23, M8, B2) or a rabbit serum against human \( \beta_2 \text{m} \); however, none of these antibodies was able to neutralize virus from this source (Table 1).

DISCUSSION

Our results clearly show that in urine specimens, CMV is closely associated with \( \beta_2 \text{m} \). Western blotting experiments directly demonstrated the presence of \( \beta_2 \text{m} \) in purified preparations of urinary CMV. Furthermore, \( \beta_2 \text{m} \) was found only in the Triton-soluble fraction after extraction of urinary CMV, indicating that \( \beta_2 \text{m} \) is associated with the envelope of urinary virus.

CMV from urine was specifically bound on an affinity column comprising a monoclonal antibody specific for \( \beta_2 \text{m} \), linked to Sepharose, thus demonstrating that \( \beta_2 \text{m} \) was present on the surface of urinary CMV. Both CMV proteins and \( \beta_2 \text{m} \) were found in the same fractions eluted from the column, reinforcing their association. Thus CMV in urine specimens has \( \beta_2 \text{m} \) bound to the surface of the viral envelope.

We have previously reported that CMV in urine specimens could not be captured onto a solid phase by CMV-specific monoclonal antibodies which could capture CMV from cell culture supernatants (McKeating et al., 1985). We have shown that \( \beta_2 \text{m} \) inhibited the capture of cell culture-grown virus and postulated that \( \beta_2 \text{m} \) bound to the virus and masked the viral antigenic determinants (McKeating et al., 1986). We have shown here that CMV in urine is coated with \( \beta_2 \text{m} \) and in an accompanying paper (Grundy et al., 1987a) we demonstrate that cell culture-grown CMV exhibits a strong binding capacity for \( \beta_2 \text{m} \) in vitro. Thus we believe our previous hypothesis to be confirmed.

If the viral glycoproteins on urinary CMV cannot be recognized by virus-specific monoclonal antibodies, it is unlikely that neutralizing antibodies produced in vivo against virus-coded proteins can bind to and neutralize urinary CMV. This explanation probably accounts for the finding reported here that a monoclonal antibody, known to neutralize cell culture-grown CMV, failed to neutralize urinary CMV. Furthermore, both pooled human high titre CMV globulin and a human immune serum also failed to neutralize CMV from urine, suggesting that the binding of \( \beta_2 \text{m} \) masks the important viral antigenic sites necessary for neutralization which are recognized naturally by the human humoral immune system. Thus the masking of viral antigens by \( \beta_2 \text{m} \) in urine explains the well accepted, but to our knowledge not previously published, finding that CMV in urine cannot be neutralized by antisera that can neutralize the same strain of virus when grown in cell culture.
Attempts to neutralize urinary CMV with several antibodies specific for $\beta_2m$ were not successful. However, we have not established whether any of these antibodies recognized CMV-bound $\beta_2m$. BBM.1 which did recognize urinary CMV was not used in the neutralization experiments.

It should be noted that our results are different from those reported in the mouse model of CMV infection where murine CMV taken from salivary glands late in infection could not be neutralized by antiviral antibodies due to the presence of non-neutralizing antibody bound to its surface (Chong et al., 1981). Polyclonal anti-mouse immunoglobulin antisera did neutralize this source of the murine virus (Inada & Mims, 1985); however, in our study with urinary human CMV a monoclonal antibody specific for the Fc portion of human IgG had no neutralizing effect.

In an accompanying paper we have shown that CMV strain AD169 binds exogenous $\beta_2m$ in vitro after the virus is released from cells (Grundy et al., 1987 a). We therefore propose that in vivo the virus binds host $\beta_2m$ after excretion into body fluids, thereby acquiring an additional protein coat. The $\beta_2m$ protein coat may also have the advantage of conferring extra stability to the enveloped virus particle. This would explain the findings of others (Krugman & Goodheart, 1964; Vonka & Benyesh-Melnick, 1966; Feldman, 1968; Stagno et al., 1980) that CMV in urine is more stable than CMV from cell culture fluid.

CMV is transmitted by direct contact with infected body fluids including saliva, urine, blood, breast milk and possibly semen. These same body fluids all contain free $\beta_2m$ (Berggard & Bearn, 1968; Evrin et al., 1971). Thus the finding that $\beta_2m$-coated urinary CMV cannot be neutralized by human immune sera has important implications in vivo, since if the virus evades neutralization by antibody in these body fluids it would facilitate transmission between individuals. Thus, we postulate that the virus has evolved this mechanism of coating itself in host $\beta_2m$ as a means of evading the host immune response. Similar mechanisms have been reported in infections with Schistosoma mansoni where the parasite adsorbs host HLA antigens onto its surface (Sher et al., 1978; Simpson et al., 1983). However, unlike the class I HLA heavy chain, $\beta_2m$ is non-polymorphic, so that the $\beta_2m$ coat acquired by the virus from one individual would not be antigenic when transmitted to another individual, whereas it is only the adult worm which adsorbs HLA in S. mansoni infection, transmission to others occurring at another stage of its life cycle. The phenomenon of 'self mimicry' by the acquisition of a 'coat' of host protein may further facilitate dissemination of CMV once infection has occurred.

In conclusion, we have shown that in body fluids such as urine, CMV is coated with the host protein $\beta_2m$, such that the viral antigenic determinants are masked and the virus evades neutralization by antibody. We propose that the virus has evolved this mechanism of coating itself as a strategy to evade host immune responses and facilitate its transmission. Further studies will elucidate whether the binding of host $\beta_2m$ by CMV can perturb in other ways the delicate balance between the virus and the host immune system.

The authors wish to thank Dr L. Pereira, Dr A. Sanderson and Unilever for donating antibodies, Philip Ward for advice in preparing the affinity column, and the staff of the Department of Nephrology and Transplantation for liaison in obtaining clinical specimens. This work was supported by project grants from the Medical Research Council and The Wellcome Trust. Part of this work has been published in the form of an International Patent Application (no. PCT/GB86/00155).

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(Received 10 June 1986)