**β₂ Microglobulin Binds to the Tegument of Cytomegalovirus: an Immunogold Study**

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(accepted 10 April 1989)

**SUMMARY**

Previous reports have provided evidence for the ability of human cytomegalovirus (HCMV) to bind the host protein β₂ microglobulin (β₂m) from body fluids or culture medium, and thus enhance infectivity of the virus, both by evasion of immune neutralization and the capacity to employ the bound β₂m for attachment to the host cell. Immunocytochemical techniques and negative stain electron microscopy were used to identify the ultrastructural components of HCMV involved in its interaction with β₂m. Probes comprising colloidal gold coupled to β₂m were seen to bind not to the envelope as previously suspected, but to material closely surrounding the nucleocapsids. It is postulated that the tegument proteins of HCMV, via their capacity to bind β₂m, play an important role in the preservation of infectivity of disrupted virions by enabling unenveloped capsids to bind to cells and gain entry by a pathway other than that normally taken by intact virions.

In recent years, interesting information has come to light regarding the affinity of human cytomegalovirus (HCMV) for β₂ microglobulin (β₂m). McKeating et al. (1985) reported that their ELISA for the detection of HCMV failed to detect the virus in fresh urine samples, but that upon storage at 4 °C for 1 to 2 weeks initially negative samples became ELISA-positive. They postulated the presence of an inhibitory substance in fresh urine which might be destroyed upon storage, and subsequently McKeating et al. (1986) identified β₂m as such an inhibitor. Furthermore, β₂m was shown to bind to HCMV strain AD169 grown in cell culture when β₂m had been added to the culture fluids (Grundy et al., 1987a), but only after the release of the virions from the cell. McKeating et al. (1987) demonstrated that the binding of β₂m to urinary HCMV was also able to prevent neutralization of the virus by various neutralizing antisera, and because the presence of β₂m could be demonstrated only in the Triton X-100-soluble fraction of HCMV harvested from urine, and not in the Triton-insoluble fraction, they concluded that β₂m was associated with the envelope, and possibly masked antigenic sites necessary for neutralization. Further studies (Grundy et al., 1987b) demonstrated that the binding of β₂m to HCMV enhanced the infectivity of the virus and the authors postulated that bound β₂m assisted in the attachment of the virus to the host cell. It was also shown that β₂m and HCMV compete for binding sites on fibroblasts, where β₂m is normally non-covalently bound to the heavy chain of class I HLA molecules. As a result of evidence which showed that β₂m-coated HCMV could bind more efficiently to Raji cells (which express class I HLA molecules) than to Daudi cells (which lack these determinants), it was postulated that HCMV could use class I HLA molecules as a virus receptor and displace β₂m from the β₂m–HLA heavy chain dimers present on the cell surface.

Although many of these earlier findings were based on the assumption that β₂m was able to bind to viral glycoproteins on the virion envelope, the exact nature of the envelope binding sites as well as the mechanism by which β₂m could exert its protective and enhancing properties remained to be conclusively explained. Previously we have successfully employed colloidal gold...
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probes for the identification of three morphologically distinct glycoprotein structures on the envelopes of herpes simplex virus (Stannard et al., 1987) and also for the detection and recognition of the major glycoprotein (gp52) of HCMV (Stannard et al., 1989). It was therefore of interest to use similar immunogold techniques in an attempt to establish the morphological nature of the \( \beta_2m \)-binding site.

Colloidal gold particles of approximately 4 nm were prepared as previously described (Stannard et al., 1987) and coupled to \( \beta_2m \) purified from human urine (Sigma) at a pH of 5.8. HCMV strain AD169 was propagated in human embryo fibroblasts (HEF) grown in MEM containing 4% foetal calf serum (FCS). When the cell cultures were seen to display HCMV-specific c.p.e. involving about 80% of cells, culture supernatant was harvested and virus particles were concentrated by centrifugation at 50 000 g for 60 min in a Spinco SW50.1 rotor. Virus pellets were resuspended in 300 \( \mu l \) phosphate buffer pH 7.2 (PB), mixed with the \( \beta_2m \)-gold probe and left to react at 20 °C for 16 h. The volume of the mixture was then increased to 5 ml with PB and it was centrifuged at 40 000 g for 30 min to sediment the virus–gold complexes. Samples were examined in a Hitachi 600 electron microscope using 2% phosphotungstic acid as negative stain.

Contrary to expectation, no \( \beta_2m \)-gold probe could be found attached to any of the viral envelopes, but in many instances where the envelope had ruptured exposing the nucleocapsid, gold probes were attached to material on the inside of the envelope, closely surrounding the nucleocapsid (Fig. 1, 2, 3). Partially released capsids (Fig. 4) appeared to be completely encased within a shell of complexed gold and protein. Some of the nucleocapsids in the preparation had been totally expelled from their envelopes (Fig. 5), and from those it was possible to ascertain that the \( \beta_2m \)-gold was not bound directly to the capsid itself, but rather to an amorphous, sometimes globular material which surrounded the capsid, presumed to be the tegument. The association between tegument and capsid was apparently not very strong, as the labelled tegument components were commonly seen to become dissociated from the capsids on the grid.

The non-attachment of the \( \beta_2m \) probes to the envelope was convincing, but to be sure that the envelope glycoproteins were still intact, the same preparation of virus was once more concentrated by centrifugation, and then reacted with 8 nm gold probes specific for the gp52 of HCMV. The anti-gp52–gold, known to attach to prominent glycoprotein spikes on the virion envelope (Fig. 6), bound in a normal way to the envelopes of the \( \beta_2m \)-treated virions (Fig. 6 and 7), indicating that the major envelope glycoprotein (gp52) was still free to combine with its specific monoclonal antibody, and that the gp52- and \( \beta_2m \)-binding sites were unrelated.

It was also necessary to ascertain whether additional binding sites for \( \beta_2m \) could have been blocked by the cross-reacting bovine \( \beta_2m \) present in the FCS in the culture medium. For this reason, HEF cultures were infected with HCMV strain AD169 which was allowed to adsorb for 1 h. Cells were then covered with MEM containing no serum. After 16 h, the culture fluid was removed, cell sheets were washed three times with serum-free medium (to ensure the removal of unattached virus particles from the initial inoculum) and overlaid with fresh MEM without serum. Culture fluids were harvested after 7 days (when cells showed 40% c.p.e.), 11 days (90% c.p.e.) and 14 days (100% c.p.e.). Virus particles from each of the three serum-free culture harvests were then allowed to react with \( \beta_2m \)-gold probes. Virions grown in the presence of 4% FCS were used in parallel control studies. Surprisingly, although the \( \beta_2m \)-gold probes attached strongly around the nucleocapsids of virions grown in the presence of FCS, there was only minimal attachment to virions from the serum-free cultures at any of the three stages of harvest. This observation remained constant in repeated experiments. Contrary to expectation, therefore, it appeared that the presence of FCS in the culture medium enhanced rather than inhibited the binding of \( \beta_2m \) to virions. This observation remains unexplained, but suggests that some component of FCS may in fact augment the \( \beta_2m \)-binding capacity of HCMV.

To establish whether the neutral pH of the reaction mixture had an inhibitory effect on the binding of \( \beta_2m \) to HCMV, experiments were repeated using a pH of 5.2 which more closely approximated the pH of urine. Results remained unchanged: \( \beta_2m \)-gold did not attach to any of the virion envelopes, but did attach around the capsids of ruptured virions from culture medium containing FCS. Disruption of virion envelopes was increased at low pH, and the \( \beta_2m \)-gold was
Fig. 1 to 5. Fig. 1, 2 and 3. HCMV virions, strain AD169, from cell cultures after reaction with β2m-gold probes. The gold is seen to attach to tegument components which closely surround the nucleocapsids. Fig. 4. Ruptured HCMV virion with a partially released capsid which is surrounded by a shell of β2m-gold. Glycoprotein projections are clearly visible on the envelope which remains unlabelled by the β2m-gold. Fig. 5. β2m-gold-labelled tegument components become dissociated from the nucleocapsid on the grid. Bar marker represents 50 nm.
Fig. 6 to 8. Fig. 6. Colloidal gold tagged with monoclonal antibody to gp52 of HCMV attaches to prominent envelope projections on an HCMV virion. Fig. 7 and 8. Anti-gp52-gold attaches in normal fashion to the envelopes of HCMV virions which have been pretreated with the smaller β2m-gold seen surrounding the capsid in Fig. 8 (arrow). Bar marker represents 50 nm.

also seen to bind to free-lying amorphous, ‘tegument-like’ material and, to a lesser extent, to apparently damaged dense bodies. Attachment of gold probes to virus from serum-free cultures remained scanty, even at low pH.

The present study using immunogold has produced evidence at an ultrastructural level to corroborate the β2m-binding capacity of HCMV virions, but has shown convincingly that the site of binding is not the virion envelope, as previously postulated, but involves proteins situated beneath the envelope in what is commonly termed the tegument. This finding should encourage a re-evaluation of previous studies with HCMV and allow a wider interpretation of the complex mechanisms involved in the infectious cycle of this major pathogen of immunocompromised individuals.

It is interesting to speculate on the implications of this unusual affinity between the tegument and β2m. Assuming the plausible postulate of Grundy et al. (1987b) that virion-bound β2m can displace the β2m associated with class I HLA molecules on the surface of host cells, the bound
fl2m could provide unenveloped capsids with a means whereby they could attach to host cells
and initiate infection. Such an attachment of nucleocapsids via bound fl2m would represent an
additional pathway of infection different from that normally taken by intact, enveloped virions,
and would play an important role when hostile environments (such as might be found in urine)
causzd the virion envelope to be partially degraded or disrupted. Enveloped virions, which we
have shown do not bind fl2m on their surface, may utilize envelope glycoproteins to attach to
host cell receptors unrelated to HLA or fl2m. This could explain the low grade infection of class I
HLA-negative Daudi cells described by Grundy et al. (1987b), as well as their observed failure to
block the binding of HCMV to fibroblasts by the addition of fl2m to the culture cells. The
hypothesis that fl2m-coated nucleocapsids may represent an additional and alternative means of
initiating infection also finds support in the results of earlier studies which have shown that the
binding of fl2m accounts for enhanced infectivity of HCMV (Grundy et al., 1987b) as well as the
evasion of neutralization by antiviral antibodies (McKeating et al., 1987). Full implications of
the interaction between fl2m and HCMV remain speculative, and the report by Beck & Barrell
(1988) that HCMV encodes a glycoprotein with homology to class I HLA antigens adds yet
another intriguing dimension to this apparently complex process.

One factor unexplained by the electron microscopy evidence regarding non-involvement of
envelope proteins in fl2m binding was the report by McKeating et al. (1985) of failure to detect
HCMV in fresh urine samples by means of their ELISA system which utilized monoclonal
antibodies said to be directed against HCMV glycoproteins. Their assumption was that fl2m in
urine bound to the viral glycoproteins thus inhibiting their attachment to the solid phase. We
obtained a urine specimen from a baby with diagnosed congenital HCMV syndrome. After
storage of the specimen at 4 °C for 16 h in the presence of ‘HCMV transport medium’ (1 M-
sucrose in 0.2 M-phosphate-buffered saline pH 7.2), HCMV virions were recovered by
centrifugation and reacted with gold probes specific either for fl2m or for HCMV gp52.
Probably as a result of competition from urinary fl2m, the fl2m-gold attached only scantily to the
tegument of ruptured HCMV virions. In addition however, no attachment of the anti-gp52-gold
could be demonstrated. After passage of the virus from the same urine specimen in HEF cells,
virions harvested from the supernatant culture fluid once more displayed a positive capacity to
bind fl2m-gold to the tegument and anti-gp52-gold to the envelope. This suggested that gp52,
normally prominent on HCMV virions from cell cultures, had either been removed (by
proteolysis) from virus particles in the urine, or that urine contains, in addition to fl2m, yet
another inhibitory substance, which binds to the envelope glycoproteins, thus masking their
detection with specific monoclonal antibody-gold probes.

The techniques of immunocytochemical electron microscopy can provide evidence of precise
locations of interactions otherwise not possible to determine, and can serve to enhance the value
of biochemical and immunological experimental investigations. The tegument proteins of
herpesviruses have not been well characterized especially with regard to their function. The
apparent ability of HCMV tegument proteins to bond with a normal host protein such as fl2m
(thus possibly ensuring the conferment of infectivity to disrupted virions) is a function hitherto
unsuspected. From the present study, it may be postulated that tegument proteins may well play
a major role in ensuring the survival of infectivity of HCMV in vitro. Further planned
ultrastructural and immunocytochemical studies may help to elucidate their function in other
members of the herpesvirus group.

I gratefully acknowledge valuable technical assistance from Mrs Margaret Lennon and Mrs Jenny
Kannemeyer. The monoclonal antibody to gp52 of HCMV was kindly supplied by Dr Janet Rider, PHLS Centre
for Applied Microbiology and Research, Salisbury, U.K. This work was financially supported by a short term
research grant from the South African Medical Research Council.

REFERENCES


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(Received 12 December 1988)