Expression in insect cells and immune reactivity of a 28K tegument protein of human cytomegalovirus

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The gene encoding the highly antigenic 28K (pp28) tegument phosphoprotein of human cytomegalovirus (HCMV) was expressed in insect cells utilizing a recombinant baculovirus. The mature intracellular form of the recombinant-derived pp28 had mobility on SDS–polyacrylamide gels similar to that of native pp28 from HCMV strain Towne-infected human foreskin fibroblasts (HFFs). In vitro labelling of recombinant Autographa californica nuclear polyhedrosis virus-infected Spodoptera frugiperda cells or of HCMV-infected HFFs with $[^{32}P]$orthophosphate followed by immunoprecipitation showed that both the insect cell-derived and HCMV strain Towne-infected fibroblast-derived pp28 were phosphorylated. The mobility of pp28 derived from these two sources as well as from extracellular HCMV virions indicated the existence of multiple charged forms of the protein, and a difference in the relative amounts of these forms expressed in HCMV-infected HFFs and recombinant baculovirus-infected insect cells. The recombinant pp28 expressed in insect cells was readily and specifically recognized by antibodies to native pp28, including HCMV-seropositive human serum, and was used in an ELISA to screen human sera for seropositivity.

Introduction

The virion of human cytomegalovirus (HCMV) is composed of at least 35 structural proteins (for review see Landini & Michelson, 1988). Approximately 25 of these viral proteins, ranging in size from 28K to 200K, appear to be important in the humoral immune response to HCMV infection (Pereira et al., 1984; Landini et al., 1986; Zaia et al., 1986; Jahn et al., 1987). Included in this group of antigenic proteins are four of the known matrix/tegument proteins of HCMV, pp28, pp65, pp71 and ppl50.

In a previous report, we described the isolation from a λgt11 expression library of a cDNA clone by utilizing a monoclonal antibody (MAb 48) specific for a 32K late structural protein of HCMV (Pande et al., 1988). An insert from this clone was used to localize the coding sequence for the 32K protein to the HindIIIIB fragment of the Towne strain and the HindIIIIR fragment of the AD169 strain. This location corresponds to that of the coding sequence of pp28 from AD169, which has been mapped and sequenced (Meyer et al., 1988; Martinez et al., 1989). The 32K protein from strain Towne has been shown by sequence analysis of the gene to be the homologue of pp28 from strain AD169 (Pande et al., 1991). The AD169 protein is present in both the cytoplasm of infected cells during the late phase of infection and in extracellular virus particles (Re et al., 1985), and has been localized to the surface of cytoplasmic capsids by immunoelectron microscopy (Landini et al., 1987). A 28K structural protein has been reported to be recognized by all highly reactive HCMV-positive human sera (Pereira et al., 1982; Landini et al., 1985; Zaia et al., 1986; Meyer et al., 1988). In addition, this protein, as well as the other matrix/tegument proteins of HCMV, pp65, pp71 and pp150, that are recognized by the immune system (Jahn et al., 1987; Landini & Michelson, 1988; Landini et al., 1985) have been identified as potentially useful diagnostic reagents (Plachter et al., 1990; Landini et al., 1990).

To investigate the structural, functional and antigenic properties of this 32K strain Towne protein further, we have cloned it into a baculovirus expression system. In this report, we describe the construction of a recombinant baculovirus containing the sequence encoding the 32K protein from strain Towne, and the initial characterization of this protein expressed in insect cells infected with the recombinant baculovirus. We show that the insect-expressed protein from strain Towne (Pande et al., 1988, 1991), heretofore referred to as pp28, is recognized by pp28-specific antibodies against the strain Towne and AD169 forms of the protein, as well as by human HCMV.
immune sera. Similarly to the strain AD169 native form, the forms of pp28 in insect cells infected with a recombinant baculovirus, fibroblasts infected with HCMV strain Towne and extracellular virions are all phosphorylated. pp28 exists in multiple charged forms, the relative amounts of which appear to differ depending on the source. In addition, we have utilized the recombinant pp28 expressed in insect cells (icr-pp28) in an ELISA for screening the presence of HCMV-specific antibodies in human sera.

**Methods**

**Cells and viruses.** The continuous cell line from Spodoptera frugiperda (Sf9; ATCC CRL 1711) was obtained from the ATCC (Rockville, Md., U.S.A.) and was maintained in TNM-FH medium (Gibco BRL) supplemented with heat-inactivated 10% foetal bovine serum (FBS) (Gibco BRL) and penicillin-streptomycin (Gibco BRL). Wild-type Autographa californica nuclear polyhedrosis virus (AcNPV) and plasmid pAc373 were the kind gifts of Dr M. D. Summers (Texas A & M University, College Station, Texas, U.S.A.). Sf9 cells and AcNPV were grown according to the methods of Summers & Smith (1987).

Experimental cultures of diploid human foreskin fibroblasts (HFFs) were plated in T-25 or T-75 flasks (Costar) containing Dulbecco's modified Eagle's medium (Irvine Scientific) supplemented with 10% FBS (Hyclone) and penicillin-streptomycin. The cells were grown to 80% confluence (4 to 7 days) before infection with HCMV Towne strain at a multiplicity of 1. Infection was allowed to continue until a 4+ c.p.e. was reached (5 to 7 days) and then infected cells were scraped into PBS and frozen at -70 °C until use. Protein concentrations were estimated using the bicinchoninic acid method (Smith et al., 1985) with BSA as a standard and Pierce reagents according to the manufacturer's instructions.

**Construction of plasmids.** The baculovirus transfer vector (pAC-pp28) was constructed by placing the entire HCMV strain pp28 DNA coding region under the transcriptional control of the polyhedrin promoter of AcNPV. Initially plasmid pBS-pp28 was constructed by inserting a 1922 bp HCMV genomic fragment, containing the entire coding region for pp28 along with 5' and 3' regulatory regions, into the phagemid Bluescript (Stratagene). Sequences encoding pp28 were then obtained from plasmid pBS-pp28 by digestion with EcoRI and AccI. The insert was treated with the Klenow fragment of DNA polymerase I, ligated with BamHI linkers, and cut with BamHI. Plasmid pAC-pp28 (see Fig. 1) was constructed by inserting the 1182 bp EcoRI-AccI insert from pBS-pp28 into the BamHI site of the baculovirus transfer vector pAc373 (Summers & Smith, 1987). The pAC-pp28 transfer vector contains all but the first 8 bp of the polyhedrin 5' untranslated region, followed by eight nucleotides from the BamHI linker, and 573 nucleotides of the pp28 coding sequence including the ATG start signal, together with 210 nucleotides of 5' and 399 nucleotides of 3' non-coding sequences of the pp28 gene (Fig. 1).

**Production of recombinant baculovirus.** Transfer of the pp28 gene into the AcNPV genome was accomplished by cotransfection of Sf9 cells with wild-type AcNPV DNA and the transfer vector pAC-pp28 DNA using calcium phosphate precipitation as described by Summers & Smith (1987). Recombinant baculovirus was purified by three rounds of plaque purification. Two rounds of plaque hybridization were followed by a single round of terminal dilution and slot-blot hybridization of DNA extracted from virus-positive, occlusion-negative cultures. The probe used for plaque hybridization and slot-blot analysis was a 32P-labelled pp28 insert from the pBS-pp28 plasmid labelled using the Random Primers DNA Labelling System (Gibco BRL), according to the manufacturer's instructions. A purified recombinant virus isolate, designated 2A4, was used for all further experiments.

**Analysis of proteins by SDS–PAGE and immunoblot.** Infected Sf9 cells and HFFs were prepared for SDS–PAGE by scraping cells into PBS, pelleting by centrifugation at 2000 r.p.m. for 10 min, disruption in SDS sample buffer (Laemmli, 1970) by 10 strokes in a syringe with a 22-gauge needle, and heating at 100 °C for 2 min. Equivalent amounts of protein were separated by SDS–PAGE on 11% gels according to the method of Laemmli (1970), and either visualized by autoradiography, as previously described (Giugni et al., 1985), or processed for immunoblot analysis.

For immunoblot analysis, the proteins were transferred electrophoretically from the gel to a nitrocellulose membrane as described previously (Zaia et al., 1986) with the exception that 10% non-fat dry milk was substituted for BSA as the blocking agent. The immunoreactive bands were detected by treating the nitrocellulose membrane with antibody diluted 1:250 to 1:500 in TMB-7.4 (20 mm-Tris–HCl pH 7.4, 150 mM-NaCl, 10 mg/ml BSA) for 1 h at room temperature. The nitrocellulose membrane was washed three times in TMB-7.4 followed by incubation with a biotinylated secondary antibody (Sigma) diluted 1:250 in TMB-7.4, washed as above and stained using the ABC kit (Vector Laboratories) according to the manufacturer's instruction with 4-chloro-1-naphthol (Sigma) as the substrate. Molecular weight standards used for calibration of the immunoblots were from a prepackaged mix of prestained proteins obtained from Bio-Rad which contained phosphorylase b (110K), BSA (84K), ovalbumin (47K), carbonic anhydrase (33K), soybean trypsin inhibitor (24K) and lysozyme (16K).

**Metabolic labelling and immunoprecipitation.** Sf9 cells or HFFs infected or not infected with baculovirus or HCMV, respectively, were labelled with [32P]orthophosphate (ICN Biomedicals) (386 µCi/ml) or Trans-35S-label (ICN Biomedicals) (254 µCi/ml) during the final 24 h
HCMV pp28 expressed in insect cells

before the cells were harvested. The cells were scraped into PBS, pelleted and solubilized in 1% SDS, 20 mM-HEPES pH 7.4, 10 mM-NaF, 1 mM-MgCl₂, 1 mM-EDTA, 1 mM-PMFSF and 4 µg/ml each of leupeptin, aprotinin and soybean trypsin inhibitor by 10 strokes in a syringe with a 22-gauge needle. Equivalent TCA-precipitable c.p.m. of the lysates were diluted to a 0.5 ml final volume with RIPA buffer (30 mM-Tris–HCl pH 7.5, 1% NP40, 150 mM-NaCl, 0.1% SDS, 0.5% deoxycholate, 2 mM-EDTA, 1 mM-PMFSF and 4 µg/ml each of leupeptin, aprotinin and soybean trypsin inhibitor), cleared by centrifugation for 5 min in a microfuge, and incubated with 3 µl MAb 48 or MAb 5 ascites fluid overnight at 4°C. Protein A–Sepharose (Sigma) (15 mg) was added and the incubation continued for 1.5 h. Samples were then centrifuged for 5 min in a microfuge. The supernatant was withdrawn and the pellet washed four times with RIPA buffer and once with Tris-saline (20 mM-Tris–HCl pH 7.5, 150 mM-NaCl). Pellets were solubilized with 100 µl SDS sample buffer, heated at 100°C for 2 min and analysed by SDS-PAGE as described above.

Two-dimensional (2D) gel electrophoresis. 32P-labelled cellular proteins from infected Sf9 cells and HFFs and 32P-labelled HCMV virions were analysed by 2D gel electrophoresis so isoelectric focusing in the first dimension followed by SDS-PAGE in the second dimension, according to the method of Garrels (1979) with modifications as indicated previously (Giugni et al., 1985). The first dimension used pH 3.5 to 10 ampholytes (Bio-Rad) and was followed by a 11% SDS–PAGE in the second dimension. The 2D gels were processed for immunoblot analysis as indicated above. After staining with 4-chloro-l-naphthol, the 32P-labelled proteins were visualized by autoradiography as indicated above.

Antisera to pp28 and HCMV. HCMV strain Towne virions were collected from extracellular fluid through a glycerol–trattate gradient as described previously (Clark et al., 1984). Purified virions plus dense bodies were used to immunize rabbits. Initial injections were administered with complete Freund's adjuvant, and booster injections were administered with incomplete Freund's adjuvant at 4 week intervals.

Murine MAbs to HCMV were of three types: (i) MAb 48 reacts strongly with two protein bands of approximately 26K to 32K from HCMV-infected cells in immunoblot assays (Pande et al., 1988); (ii) MAb P,G,1 reacts with an antigen of approximate M, 28K from HCMV-infected cells in immunoblot assays (Re et al., 1985), and was kindly provided by Dr M. P. Landini (University of Bologna, Bologna, Italy); (iii) MAb 5 is a control antibody prepared from mice immunized with HCMV virions and has weak neutralizing activity but no reactivity with pp28.

Pooled human immunoglobulin (IVIG) known to have a high anti-HCMV antibody titre was a gift from J. Hooper (Baxter Healthcare Corp., Glendale, Ca., U.S.A.). Human sera known to be either negative or positive for HCMV antibody by immunofluorescence assay utilizing the Virgo indirect fluorescence kit (Electro-nucleonics Laboratories) were obtained from volunteer blood donors or patients prior to bone marrow transplantation for purposes of comparison with conventional enzyme immunoassay (EIA) for HCMV antibody.

Detection of anti-HCMV antibody by ELISA. The HCMV antibody status of patient's sera was assessed using an ELISA. pp28 antigen was prepared from HFFs infected with strain Towne HCMV (HFF/HCMV) as indicated above, and from Sf9 cells infected with recombinant virus (icr-pp28). Control antigens were made from uninfected cells. The HFFs were scraped into a glycerine-buffered saline solution (42.5 mM-glycine pH 9.0, 150 mM-NaCl), homogenized with a Dounce homogenizer, and the supernatants were collected after centrifugation at 1000 g for 10 min. The Sf9 cells were scraped and homogenized in H buffer (20 mM-HEPES pH 7.4, 1 mM-EDTA, 2 mM-PMSF and 4 µg/ml of aprotinin, leupeptin and soybean trypsin inhibitor) followed by centrifugation at 100000 g for 30 min. The pellet was resuspended and homogenized in H buffer containing 1% NP40 and 1.6 M-NaCl, and the supernatant was collected after centrifugation as above.

ELISAs for anti-HCMV antibody were run in 96-well EIA plates (Costar) that had been coated overnight at 4°C with 400 ng/well of the above antigens diluted in a carbonate buffer (Doelken et al., 1984). Plates were then incubated with a blocking buffer (PBS with 1% BSA and 0.3% gelatin) for 1 h at 23°C, followed by the sample of human test sera for 2 h, a secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Laboratories) and finally the substrate o-phenylene diamine (Sigma). The reaction was stopped with 0.5 M-H₂SO₄, and the plates were read using an EIA reader at 490 nm (MR600, Dynatech). Plates were washed four times with PBS containing 0.05% Tween 20 between each incubation step.

The conversion of the absorbance readings to equivalents of IVIG was done using an EIA/RIA program, ImmunoFit EIA/RIA (Beckman Instruments). Utilizing this program, the conversion to equivalents of IVIG was made for each test serum by comparing the difference in absorbance values generated by the test antigens (HFF/HCMVs or icr-pp28) and the control antigens (HFFs or Sf9 cells) to those for a standard IVIG. The microgram amount of IVIG that generated an absorbance equivalent to a test serum would represent the IVIG equivalent for that test serum. This was done for each dilution of test serum, from 1:16 to 1:256, that fell in the linear portion of the absorbance spectrum, and the average of these values for the different dilutions was used as the IVIG equivalent for that serum.

Results

Identification of pp28 in 2A4-infected Sf9 cells

A recombinant baculovirus containing the open reading frame encoding pp28 of HCMV strain Towne under the control of the polyhedrin promoter of AcNPV was constructed by in vitro recombination between wild-type AcNPV DNA and the pAC-pp28 transfer plasmid (Fig. 1). Sf9 cells were cotransfected with pAC-pp28 and AcNPV genomic DNA, and recombinant virus (2A4) was recovered from the cell supernatant after three rounds of plaque purification. Sf9 cells infected with 2A4 expressed HCMV pp28 as demonstrated by the presence of immunoreactive bands after immunoblot analysis of whole cell lysates (Fig. 2). This was shown using both a MAb specific for pp28, MAb 48 (Fig. 2a) and a rabbit polyclonal antiserum raised against HCMV virions (Fig. 2b). MAb 48 reacted strongly with a 28K protein in HCMV strain Towne-infected fibroblasts (Fig. 2a, lane 1) and in Sf9 cells infected with 2A4 (Fig. 2a, lane 4), but not with uninfected or wild-type AcNPV-infected controls (Fig. 2a, lanes 2 and 3). Similarly, the polyclonal antiserum detected a wide range of proteins including a 28K protein in HCMV strain Towne-infected fibroblasts (Fig. 2b, lane 1) and a 28K protein in 2A4-infected Sf9 cells (Fig. 2b, lane 4); the 18K protein recognized by the rabbit polyclonal antiserum in lysates from 2A4-infected Sf9 cells (Fig. 2b, lane 4) was not present in all
Fig. 2. Expression of HCMV pp28 in recombinant baculovirus-infected Sf9 cells. Recombinant baculovirus was used to infect Sf9 cells for the expression of pp28. Whole cell lysates from infected or uninfected Sf9 cells and HFFs were prepared and fractionated on 11% SDS-polyacrylamide gels, and analysed by immunoblotting. The immunoreactive bands on the nitrocellulose membranes were detected by incubation with MAb 48 (a) or a rabbit polyclonal antibody to HCMV virions (b) as described in Methods. Lanes 1, HCMV strain Towne-infected HFFs; lanes 2, uninfected Sf9 cells; lanes 3, wild-type AcNPV-infected Sf9 cells; lanes 4, 2A4-infected Sf9 cells.

immunoblots and probably represents a degradation product of pp28. No immunoreactive bands were detected by immunoblot analysis of uninfected fibroblasts (data not shown) nor uninfected or wild-type AcNPV-infected Sf9 cells (Fig. 2b, lanes 2 and 3). When Sf9 cells were infected at a multiplicity of 10, the expression of immunoreactive HCMV pp28 peaked at 72 h post-infection (data not shown). The immunoreactive pp28 was completely cell-associated, and no immunoreactive material was present in the medium, as assayed by immunoblotting (data not shown).

Phosphorylation of the HCMV 28KDa tegument protein in insect cells

Icr-pp28 as well as pp28 from HCMV strain Towne-infected fibroblasts (HFF-pp28) (Fig. 2a, b, lanes 1 and 4) had an apparent Mr of 28K by SDS–PAGE. The Mr of the deduced polypeptide encoded by the pp28 gene is 20-9K (Meyer et al., 1988, Pande et al., 1991). It has been shown by others (Meyer et al., 1988; Nowak et al., 1984) that pp28 from HCMV strain AD169-infected fibroblasts is phosphorylated and not glycosylated, and Meyer et al. (1988) have proposed that the discrepancy in the Mr of the deduced polypeptide and native pp28 from infected fibroblasts is due to phosphorylation. Icr-pp28 has an Mr similar to that of the native HFF-pp28, suggesting that similar post-translational modifications occur in insect cells. To test this, we examined whether icr-pp28 is a phosphorylated protein. As a control we also tested whether pp28 is a phosphorylated protein in HCMV strain Towne-infected fibroblasts.

Sf9 cells, uninfected or infected with 2A4 or wild-type virus, were labelled with [32P]orthophosphate or Trans-35S-label and equivalent material based on c.p.m. of total cellular extracts was immunoprecipitated with MAb 48 or a control antibody, MAb 5. Sf9 cells infected with 2A4 contained a 28K protein that was labelled with both 32P and 35S (Fig. 3a, b, lanes 3). A protein of similar Mr was present in wild-type AcNPV-infected Sf9 cells (Fig. 3a, b, lanes 2), but not in uninfected Sf9 cells (lane 1). The 28K protein expressed in the 2A4-infected Sf9 cells was the HCMV 28K tegument protein, as shown by specific precipitation with MAb 48 (Fig. 3c, lane 3). The 28K protein seen in lysates from wild-type AcNPV-infected cells (Fig. 3a, b, lane 2) was not precipitated by MAb 48 (Fig. 3c, lane 2) and was most likely the polyhedrin protein which has previously been shown to be a phosphoprotein (Maruniak & Summers, 1981). The precipitation of the 32P- and 35S-labelled 28K protein from lysates of 2A4-infected cells was specific, as indicated by the lack of precipitation of a similar protein when a control MAb was used (Fig. 3c, lane 4; and data not shown). These data indicate that icr-pp28 (Fig 3c, lane 3) and the native 28K protein expressed in HCMV strain Towne-infected fibroblasts (Fig. 3c, lane 6) are both phosphorylated.

Fig. 3. Phosphorylation of pp28 in intact Sf9 cells or HFFs infected with recombinant AcNPV or HCMV, respectively. Control or virus-infected Sf9 cells or HFFs were labelled with [32P]orthophosphate (a and c) or Trans-35S-label (b). Total cellular lysates (a and b) or immunoprecipitates from cellular lysates (c) were fractionated on 11% SDS-polyacrylamide gels and processed for autoradiography as described. Lanes 1, uninfected Sf9 cells; lanes 2, wild-type AcNPV-infected Sf9 cells; lanes 3 and 4, 2A4-infected Sf9 cells; lanes 5, uninfected HFFs; lanes 6 and 7, HCMV strain Towne-infected HFFs. (c) Lanes 1, 2, 3, 5 and 6, immunoprecipitates with MAb 48; lanes 4 and 7, immunoprecipitates with MAb 5.
HCMV pp28 expressed in insect cells

Fig. 4. 2D gel electrophoresis of immunoreactive pp28. 2A4-infected Sf9 cells (a), HCMV-infected HFFs (b), and extracellular virions (c and d) were labelled with [32P]orthophosphate, fractionated by 2D gel electrophoresis, transferred to nitrocellulose membranes and processed for immunoblot or autoradiography as described. Immunoreactive spots were detected on immunoblots (a, b and c) by incubation with MAb P2G11. Following development with substrate, the immunoblot shown in panel (c) was exposed to X-ray film for 5 days (d).

To compare the extent of phosphorylation of pp28 expressed in insect cells to that expressed in HCMV strain Towne-infected HFFs, we analysed 32P-labelled cellular extracts by 2D gel electrophoresis and visualized the location of pp28 by autoradiography and immunoblotting. The 2D gels show that there is one major and one very minor form of pp28 in HCMV-infected fibroblasts, and that multiple forms occur in 2A4-infected Sf9 cells (Fig. 4 b and a, respectively). All of the immunoreactive forms corresponded to the 32P-labelled autoradiographic forms (compare Fig. 4e and d; and data not shown) and therefore are phosphorylated. There were three major phosphorylated forms of pp28 in extracellular virions and two forms, one major and one minor, in HCMV-infected HFFs (Fig. 4b and c). These data show that multiple charged species of pp28 exist and that icr-pp28 exists as multiple forms as compared to HFF-pp28. Based on these data, the pp28 associated with extracellular virions includes two species that are not present or, if present, are less prevalent than the species present in HCMV-infected HFFs.

Antigenicity of the recombinant HCMV 28K tegument protein

The antigenicity of the recombinant 28K HCMV tegument protein was indistinguishable from that of the virus-derived protein expressed in HCMV-infected human fibroblasts. The recombinant protein expressed in insect cells was recognized by immunoblotting using MAb 48, the murine MAb originally used to isolate the cDNA clone for this protein from HCMV strain Towne (Pande et al., 1988), and by MAb P2G11, used to isolate the cDNA from HCMV strain AD169 (Re et al., 1985; Meyer et al., 1988) (Fig. 5a, b, lanes 3). It is also recognized by a rabbit polyclonal antiserum directed against HCMV virions (Fig. 2b, lane 4) as is the native HCMV-infected fibroblast-derived protein (Fig. 2b, lane 1).

The reactivity of human HCMV immune serum against recombinant pp28 was analysed by immunoblotting. Serum from a seronegative individual failed to recognize icr-pp28 (Fig. 5c, lane 3), HFF-pp28 (Fig. 5c, lane 1) or any other viral antigen (Fig. 5c, lane 1). Serum from a seropositive individual, as well as IVIG, recognized icr-pp28 (Fig. 5d, e, lanes 3) and other HCMV antigens (Fig. 5d, e, lanes 1). Thus, recombinant pp28 was readily and specifically recognized by murine, rabbit and human antibodies directed to HCMV-derived pp28.

To determine the utility of this recombinant protein for detection of HCMV antibody after normal infection, we used extracts from insect cells expressing recombinant pp28 as an antigen in an ELISA and compared this to an ELISA system using conventional HCMV antigens. The initial comparison was done utilizing IVIG as the primary antibody. Icr-pp28 gave a strong specific response to increasing concentrations of IVIG (Fig. 6). The specific response generated by icr-pp28 ranged from 25 to 75% of the response seen when an equivalent
amount of total cellular protein of HFF/HCMV was used as the antigen (Fig. 6). The background immunoglobulin binding with control insect cells was similar to that of uninfected HFFs (Fig. 6). This comparison of icr-pp28 to a mixture of native antigen, HFF/HCMV, in an ELISA utilizing a mixed pool of human IgG indicates that the mixture of antigens in HFF/HCMV is more sensitive, shows a greater change in slope in the dose–response curve, for the detection of HCMV-specific antibodies at low levels of antibody (i.e. at less than 5 μg equivalents of IVIG), but at higher levels of antibody (i.e. at greater than 5 μg equivalents of IVIG) the two sources of antigen have similar levels of sensitivity (Fig. 6).

To investigate further the use of the recombinant pp28, we screened serum from seropositive and seronegative individuals in the pp28-based ELISA. For this comparison, equivalent amounts of pp28-containing and control antigen from Sf9 cells or HFFs were employed, and the reactivity of a number of sera was compared and expressed as μg equivalents of IVIG. Each human seropositive serum showed a higher titre of pp28-specific antibodies (mean of 1305 ± 1233) than antibodies to mixed antigens of HCMV (mean of 144 ± 102) (Table 1). These mean values are significantly different by a paired t-test ($P = 0.0003$). Although we have not compared the two antigens with a large number of seronegative samples, in the sera we have tested the titre of pp28-specific antibodies is very close to those of antibodies to the mixed antigens (mean of $19 ± 12$ versus $18 ± 3$, respectively) (Table 1). These results suggest that in this particular patient population the ELISA with the recombinant pp28 as an antigen may be more sensitive for testing seropositivity to HCMV.

### Table 1. Comparison of titres of antibodies in HCMV-negative and -positive human sera to HCMV mixed or insect-expressed pp28 antigens

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<tr>
<th>UPN*</th>
<th>IVIG equivalents (μg IgG)†</th>
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<td></td>
<td>HCMV</td>
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<td>Mean ± S.D.</td>
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* UPN, unique patient number.
† Values for IVIG equivalents were calculated as described in Methods.
‡ ND, Not determined.

### Discussion

The HCMV 28K structural protein (pp28) (Re et al., 1985) that was shown to be phosphorylated in HCMV strain AD169-infected fibroblasts maps to the same location as a 32K late protein of HCMV strain Towne (Pande et al., 1988; Meyer et al., 1988). The identity of these two proteins has been confirmed by comparison of nucleotide sequences (Pande et al., 1991), which reveals only five base substitutions between strains Towne and AD169, three of which result in a change in the predicted amino acid sequence. The strains share all restriction enzyme sites, with the exception of a SstII site in the
AD169 sequence that is lost in the Towne strain as a result of a C to A base change at nucleotide 144869 (Chee et al., 1990). To characterize the immunological and biological characteristics of this protein further we expressed HCMV strain Towne pp28 in a baculovirus system and compared its state of phosphorylation and antigenic properties with those of the native form from HCMV-infected fibroblasts. The expression of the strain Towne protein in Sf9 cells yields a material that is immunologically cross-reactive with the native phosphoprotein from strain AD169- and Towne-infected fibroblasts.

It has been shown by others (Meyer et al., 1988, Nowak et al., 1984) that strain AD169 pp28 is phosphorylated in infected human fibroblasts, and therefore this study sought to determine whether the recombinant pp28 was phosphorylated. We have shown that pp28 is phosphorylated in HCMV strain Towne-infected HFFs (Fig. 3c, lane 6) as is icr-pp28 (Fig. 3c, lane 3). Sequence analysis of the deduced amino acid sequence of strain Towne pp28 suggests that there are as many as 10 potential phosphorylation sites (Leader & Katan, 1988; data not shown). It remains to be determined how many and which of these sites are actually phosphorylated in pp28 derived from the various sources. The 2D gel analysis of icr-pp28 and HFF-pp28 shows the presence of at least three different charge variants of pp28 (Fig 4a, b) with icr-pp28 having three major forms as compared to HFF-pp28, which has one major and one minor form. Thus pp28 derived from insect cells exists in multiple forms that are at least partially different from the forms expressed in HFFs. Differences in the level or the sites of phosphorylation are one possible explanation for the presence of multiple forms. Under-phosphorylation of an over-expressed protein in the baculovirus system has recently been shown for the simian virus 40 T antigen (Höffs et al., 1990). In this study, the authors show that specific phosphorylation sites are under-phosphorylated. Whether different levels of phosphorylation are the explanation for multiple forms of pp28 in HFFs and insect cells, and whether similar sites are phosphorylated in icr-pp28 as in HFF-pp28 remains to be determined.

Of interest is the observation that HCMV-infected fibroblasts contain a single major and a single minor charged species of pp28, whereas extracellular virions contain three major phosphorylated forms (Fig. 5b, c, d). We do not know the reason for this difference. Among the possible explanations are (i) that pp28 is phosphorylated by a virion-associated kinase after being packaged within the virion (ii) that all charged forms exist in fibroblasts, but the relative amount of the various forms is such that only the most basic form can be detected on the immunoblot (this would imply that the more acidic forms are preferentially packaged) and (iii) that the extracts from extracellular virions and HCMV-infected HFFs are contaminated to different extents with specific phosphatases. A 24K phosphoprotein present in the virions of HCMV strain AD169 has been shown to be phosphorylated by a virion-associated kinase, and this phosphoprotein exists in multiply charged forms (Roby & Gibson, 1986). It is not known whether this protein is the same as pp28.

The 28K tegument protein of HCMV has been shown to be recognized by the majority of human sera possessing antibodies to HCMV, thus making it a candidate for use as a diagnostic tool (Landini et al., 1985, 1986, 1990). Low levels of expression of the native protein in HCMV-infected human fibroblasts precludes one from easily obtaining sufficient quantities of native protein for this purpose. Meyer et al. (1988) have expressed this protein as a fusion protein in Escherichia coli and have shown that this reacts with human sera. Use of the bacterially expressed protein as an immunological agent may be plagued with problems because of potential contamination with E. coli proteins. We have shown here that icr-pp28 appears to be as antigenic as the native protein and can thus serve as an antigen for the detection of antibodies against native pp28. Antibodies to insect proteins in human sera are less prevalent than those to E. coli proteins (T. D. Giugni & J. A. Zaia, unpublished results), so this may be a better system for high level expression of pp28 for use in immunological assays.

We employed an ELISA to compare the antigenicity of icr-pp28 with a more conventional antigen by using a number of human sera. Most of these sera were from persons with haematopoietic malignancy, many of whom undoubtedly had recent reactivation of HCMV infection. These results confirmed not only that antibodies to pp28 are present in most if not all HCMV-positive sera, but also that this antigen is particularly effective in detecting anti-HCMV antibody in this population. The relative anti-HCMV antibody titre of these sera was higher when measured using recombinant pp28 than when measured using crude HFF antigen (mean titre 1305 ± 1233 compared to 144 ± 102). Furthermore, based on the significance level for seropositivity using the method of Tijssen (1985), the pp28 antigen was associated with a cut-off level of IVIG equivalence near that of the more conventional antigen (33 μg compared to 21 μg). This suggests that an ELISA that utilizes the pp28 antigen has a greater signal : noise ratio than the HCMV-infected HFF antigen. It is possible that this is an artefact produced by the use of sera from immunodeficient patients having chronic HCMV infection. Since this selective enhancement in sensitivity was not seen when pooled human immunoglobulin was used (see Fig.
6), it will be important to determine whether the pp28 antigen is more sensitive for determining HCMV seropositivity with a larger seroepidemiological study. In addition, owing to the host immune reactivity to pp28, it will be important to explore the future role of purified baculovirus-expressed pp28 as either a diagnostic reagent or as a component of a subunit vaccine.

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References


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