Immunoaffinity Purification of a 72K Early Antigen of Human Cytomegalovirus: Analysis of Humoral and Cell-mediated Immunity to the Purified Polypeptide

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SUMMARY
An early antigen of human cytomegalovirus (HCMV) was purified from infected cells as a 72K polypeptide by immunoaffinity chromatography using a monoclonal antibody. It was located in both nucleus and cytoplasm, and was non-glycosylated and undetectable on the surface of infected cells. Known seropositive subjects had antibody against the purified protein and it elicited proliferative T cell responses in 10 of 16 subjects. Five of 14 T cell lines established in response to the purified protein were predominantly CD8+ and of these two showed major histocompatibility complex-restricted cytotoxicity against HCMV-infected cells. This provides further evidence that antigens expressed at early times may be targets for the immune response during persistent HCMV infection.

INTRODUCTION
Human cytomegalovirus (HCMV) is the largest of the human herpesviruses with a genome of $150 \times 10^6$ molecular weight. Approximately 30 structural polypeptides can be detected in HCMV virions (Kim et al., 1976; Fiala et al., 1976) and many additional virus-specific polypeptides are present in infected cells (Blanton & Tevethia, 1981). As with herpes simplex virus, HCMV polypeptides are synthesized in the infected cell in a regulated cascade of immediate early (IEA), early (EA) and late (LA) viral polypeptides and antigens (Stinski, 1978).

Serum antibodies recognizing all three synthetic classes of polypeptides can be detected after HCMV infection (Blanton & Tevethia, 1981; Pereira et al., 1982, 1983), and T cell proliferation in response to purified virions and cell-associated LA have been described (Wahren et al., 1981; Schirm et al., 1980). However, the relative importance of viral IEA, EA and LA in the immune response to infection is uncertain.

Increasing evidence suggests that the immune responses important in controlling HCMV in the persistently infected host may be directed against EA or IEA. We have shown previously that there are HCMV-specific cytotoxic T cell precursors present in the peripheral blood of normal seropositive individuals which recognize HCMV antigens expressed in cells as early as 6 h post-infection (p.i.) (Borysiewicz et al., 1983). In addition it has been shown that mice acutely or latently infected by murine CMV generate cytotoxic T cells which recognize a virus antigen expressed at immediate early times (Reddehase & Koszinowski, 1984; Reddehase et al., 1984). T cells recognizing IEA or EA might be able to limit reactivation of HCMV infection at an early stage before the onset of virus replication.

Previous studies of cell-mediated immunity to HCMV have generally been limited by the use of crude mixtures of HCMV antigens; only one report (Forman et al., 1985) has analysed cellular immune responses to an individual isolated or purified HCMV protein, i.e. the major 64K late antigen of the HCMV virion purified by HPLC. In this study we report the purification of a 72K
early nuclear protein of HCMV by immunoaffinity chromatography on monoclonal antibody-coupled Sepharose, and analyse humoral and cell-mediated immune responses to this antigen.

METHODS

Preparation of immunoadsorbent. Immunoglobulin from hybridoma H11 (Rodgers et al., 1985) was purified from ascites fluid by fractionation on Protein A-Sepharose and dialysed against 0.1 M-bicarbonate buffer pH 8.3 containing 0.5 M-NaCl. Approximately 20 mg of purified immunoglobulin was coupled to 2 g of cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions.

Preparation and radiolabelling of antigens. Flow 5000 human embryonic fibroblasts (Flow Laboratories) grown in 800 cm² roller bottles were infected with HCMV strain AD169 at a multiplicity of 5 p.f.u./cell. At the times indicated in the text monolayers were washed with phosphate-buffered saline (PBS) and the cells removed by rolling with glass beads, sonicated and incubated on ice for 30 min in PBS containing 0.5 % NP40, 0.5 % sodium deoxycholate, 0.1 % SDS and 2000 KIU/ml aprotinin. Solubilized antigen was clarified by centrifugation at 100000 g for 30 min and then preclared by incubation with normal mouse serum and Protein A before use. Virions and dense bodies were prepared by velocity centrifugation on 20 to 70 % sorbitol gradients as described by Stinski (1976).

Where appropriate, monolayers were radiolabelled with [35S]methionine by incubation in medium containing 1/20th the normal amount of methionine and 10 μCi/ml [35S]methionine (>800 Ci/mmol, Amersham). Polypeptides were separated by SDS-PAGE on 10 % polyacrylamide gels and visualized by Coomassie Brilliant Blue staining or autoradiography as appropriate.

ELISA. Microelisa plates (Dynatech) were coated with approximately 4 ng/well of antigen by overnight incubation in 0.1 M-bicarbonate buffer pH 9.5. The reactivity of human sera was then analysed by sequential incubation with dilutions of serum, peroxidase-coupled anti-human IgG (Dako, U.K.) and o-phenylenediamine as previously described (Rodgers et al., 1985).

Lymphocyte proliferation assay. Peripheral blood mononuclear cells (PBM) from eight seronegative and 16 HCMV seropositive individuals were separated from heparinized blood by centrifugation on Ficoll-Hypaque, washed, and distributed to 96-well flat-bottomed tissue culture plates at 10⁵ cells/well in RPMI 1640 containing 10 % human AB serum. Affinity-purified HCMV 72K early nuclear antigen or HCMV virions/dense bodies were added at final concentrations of 1-25, 2.5 and 5 μg/ml. Proliferation was assayed after 6 days incubation, by addition of [3H]thymidine (1 μCi/well, 25 Ci/mol) for 16 h. Proliferative responses to 10 μg/ml purified protein derivative of tuberculin (PPD; Evans) and 2 μg/ml phytohaemagglutinin (PHA; Sigma) were concurrently estimated. (The PHA response was measured after 48 h.) Results are expressed as a stimulation index (c.p.m. with antigen/c.p.m. of cells in medium alone).

Lymphocyte culture, surface phenotype and major histocompatibility complex (MHC)-restricted cytotoxicity. PBM from 18 HCMV seropositive individuals were cultured at 10⁶ cells/ml with 5 μg/ml of the early antigens in RPMI 1640 supplemented with 10 % human AB serum and 2 % interleukin 2 (IL2) (Cellular Products, New York, N.Y., U.S.A.). The cells were refed with fresh medium and IL2 at 3 day intervals and on day 7 fresh, autologous, irradiated (2.5 krad) PBM and 5 μg/ml of the early antigens were added. After 14 to 21 days the lymphocytes were harvested and viable cells isolated by Ficoll-Hypaque centrifugation.

The CD4 and CD8 surface phenotype of proliferating lymphocytes was determined with anti-T4 and anti-T8 monoclonal antibodies (Coulter, Hialeah, Fla., U.S.A.) with fluorescein isothiocyanate-conjugated goat anti-mouse Ig and the results were analysed by an EPICS C (Coulter) flow cytometer. MHC class I-restricted cytotoxic activity was determined by incubating cultured lymphocytes from 18 seropositive individuals, in various numbers, with ⁵¹Cr-labelled, 48 h HCMV-infected and uninfected HLA-matched and mismatched fibroblasts; ⁵¹Cr release was estimated after 6 h and 18 h (Borysiewicz et al., 1983).

RESULTS

Purification of the 72K early antigen of HCMV

HCMV antigen was purified by immunoaffinity chromatography using a monoclonal antibody-coupled Sepharose column prepared as described in Methods. Detergent-extracted ³⁵S-labelled antigen from 2 day HCMV-infected fibroblasts was recirculated through the column for 5 h. The column was then washed extensively with PBS containing 0-1 % NP40 and PBS alone until the background radioactivity eluted was <1000 c.p.m./ml and the absorbance at 280 nm was <0.050. Antigen was eluted by sequential washing with PBS containing 0-3 M-NaCl, 1 M-NaCl, 3 M-NaCl and 3 M-KSCN, and fractions were monitored for radioactivity and A₂₈₀.
Immune response to 72K early HCMV antigen

Fig. 1. Immunoaffinity purification of 72K HCMV early antigen. [35S]Methionine-labelled antigens from 2 day HCMV-infected fibroblasts were solubilized in PBS containing 1% NP40 and 0.5% sodium deoxycholate and applied to the monoclonal antibody-coupled Sepharose column. Antigens were eluted by sequential salt washes (a) and monitored by radioactivity (○) and absorbance (□). The composition of the KSCN peak was analysed by SDS–PAGE on a 10% polyacrylamide gel (b). Molecular weight markers (lane 1) are phosphorylase B (94000), BSA (67000), ovalbumin (43000) and carbonic anhydrase (30000). Lanes 2 and 3, Coomassie Brilliant Blue-stained total antigen and KSCN peak; lanes 4 and 5, autoradiograph of 35S-labelled total antigen and KSCN peak.

As can be seen in Fig. 1(a) a minor peak was eluted with 0.3 M NaCl and a larger peak with 3 M-KSCN. By SDS–PAGE the KSCN peak was seen to contain a single 72K polypeptide by both Coomassie Brilliant Blue staining and autoradiography (Fig. 1(b)). The 0.3 M salt peak comprised polypeptides of approximately 42000 and 150000 mol:wt. (data not shown). These polypeptides may reflect non-specific binding of cellular proteins such as actin, or possibly cooperative binding with other viral proteins as has been described for herpes simplex virus DNA-binding proteins (Vaughan et al., 1984).

For subsequent studies of immune responses, the HCMV 72K protein was purified from unlabelled cells as described above and the KSCN peak, containing a single 72K polypeptide, was dialysed against PBS containing 0.5 M NaCl and stored at -70 °C.

Characterization of the 72K antigen

We have previously reported (Rodgers et al., 1985) that monoclonal antibody H11 specifically immunoprecipitates a 72K protein from phosphonoformate-treated HCMV-infected cells (expressing IEA and EA), but not from cycloheximide/actinomycin D-blocked cells (expressing only IEA). By indirect immunoperoxidase staining this antigen is seen to be located exclusively in the nucleus at 24 h p.i., but is also seen in the cytoplasm at 72 h p.i. It is not detected on the surface of infected cells at either 24 or 72 h p.i. by immunoperoxidase staining with the monoclonal antibody.

In view of this change in the subcellular localization of the antigen we wished to determine whether the 72K protein was modified during its transport to the cytoplasm at late times in infection. Consequently fibroblasts grown in 5 cm Petri dishes were infected with HCMV at an m.o.i. of 5 and labelled for 1 h with [35S]methionine at various times after infection. The labelled antigen was then extracted in PBS containing 1% NP40, 0.5% sodium deoxycholate and 0.1% SDS and immunoprecipitated with H11 or control monoclonal antibody either directly, after labelling or after a further 24 h incubation in medium without radioactive methionine. As can be seen in Fig. 2 a single polypeptide of 72K was immunoprecipitated from cells labelled at 24 h when the antigen was seen to be nuclear by immunoperoxidase staining, and at 72 h when the antigen was seen additionally in the cytoplasm. A 24 h chase did not affect the apparent mol:wt. of the polypeptide in either case, indicating that it does not undergo major cleavage during its transport to the cytoplasm.
Fig. 2. Lack of processing of the 72K polypeptide during the period of change in its subcellular localization. Mock-infected (U) and HCMV-infected fibroblasts were pulse-labelled for 1 h with \([^{35}S]\)methionine (10 \(\mu\)Ci/ml) at the times indicated. Antigens were then immunoprecipitated with (a) monoclonal antibody H11 or (b) control monoclonal antibody either directly after the \(^{35}S\) pulse (lanes 1) or after a 24 h chase (lanes 2).

No immunoprecipitation of radiolabel was observed with monoclonal antibody H11 when HCMV-infected cells were labelled with \([^{14}C]\)mannose or \([^{14}C]\)glucosamine. In addition purified \([^{35}S]\)methionine-labelled 72K antigen did not bind to lentil lectin- or concanavalin A-coupled Sepharose, suggesting that it is not a glycoprotein. Lack of glycosylation would be compatible with our failure to detect the antigen on the cell surface.

**Immune responses to affinity-purified HCMV 72K early antigen**

Serum antibody titres to the HCMV 72K EA were studied by ELISA, and T cell proliferative responses were assayed as described in Methods. Twenty-three patients from a renal transplant unit selected as having previous serological or virological evidence of HCMV infection were compared with nine HCMV seronegative individuals by ELISA. Twenty-one of 23 individuals shown to be seropositive by a complement fixation test were positive for antibodies against both HCMV virion antigen and 72K HCMV EA at serum dilutions of 1/50 to 1/400 (Fig. 3), indicating that the affinity-purified material retained its antibody-binding site. Antibody titres to the 72K and virion antigens were usually comparable. There were only two exceptions where individuals had high antibody titres against purified virion, but not the 72K EA, or vice versa (arrowed in Fig. 3).

PBM from 10 of 16 individuals, with previous evidence of HCMV infection, proliferated in response to the 72K EA with a stimulation index of > 3 (Fig. 4). Many of those showing low responses were undergoing immunosuppressive therapy and had similar low proliferative responses to PHA and PPD. Two seropositive individuals not undergoing immunosuppressive therapy had much higher proliferative responses to the 72K antigen (stimulation index 35.6 and 109.5), whereas seronegative subjects showed no proliferative response.

The nature of the T cells responding to the 72K early antigen was further examined by growing proliferating cells from 18 HCMV seropositive individuals including those responding in the lymphoproliferation assay, in IL2-dependent culture. No T cell lines were established using PBM from seronegative donors. Fourteen of these short-term T cell lines were analysed to determine their CD4/CD8 surface phenotype. Nine of the cultures were predominantly CD4+
Immune response to 72K early HCMV antigen

Fig. 3. Antibody reactivity with affinity-purified 72K antigen. ELISA microtitration plates (Dynatech) were coated overnight with either affinity-purified 72K HCMV early nuclear antigen (a) or gradient-purified virions (b) and then incubated with serial dilutions (1/50 to 1/400) of complement fixation test (CFT) seropositive (●) or seronegative (○) human sera, developed with o-phenylenediamine and assayed at $A_{492}$. Results illustrated are at a serum dilution of 1:200. Assays were performed in triplicate. Individuals with divergent antibody responses to the 72K protein and to virions are arrowed.

Fig. 4. T cell proliferation to 72K HCMV early antigen (2.5 μg/ml) was measured by [$^3$H]thymidine incorporation in a 6 day assay as described in Methods. Proliferation in seropositive (●) and seronegative (○) individuals is expressed as a stimulation index (c.p.m. with 72K antigen/c.p.m. without antigen).

(T4+/T8+ = 0.91 to 2.72) but, in contrast to the uniformly CD4+ lines we have previously generated to ‘free’ HCMV (Borysiewicz et al., 1983), five were predominantly CD8+ (T4+/T8+ = 0.25 to 0.52).

In previous studies we have shown that HCMV-specific cytotoxic T cells were generated by co-culture with HCMV-infected autologous fibroblasts, and these lymphocytes killed HCMV-infected HLA-matched target cells which had been infected for only 6 h, by inference prior to viral DNA synthesis when only IEA and EA were expressed (Borysiewicz et al., 1983). In Fig. 5(a) we now show that a similarly generated cytotoxic T cell line killed HLA-matched target cells infected with HCMV in the presence of phosphonoformate, further suggesting that at least some of the HCMV-specific cytotoxic T cell precursors in PBM recognize HCMV non-structural antigens. To determine whether cytotoxic T cell activity could be demonstrated following stimulation with 5 μg/ml purified 72K EA in vitro, 18 T cell lines were assayed against 48 h HCMV-infected and uninfected, HLA-matched and mismatched fibroblasts in a $^{51}$Cr release assay. There was no MHC-restricted cytotoxicity of HCMV-infected cells in 16 lines but two cell lines killed HCMV-infected HLA-matched but not mismatched infected target cells, providing preliminary evidence of cytotoxic T cell activity in these two cultures (Fig. 5b).

DISCUSSION

Previous work from our laboratory has suggested that the immediate early or early phases of virus replication may be important in the recognition and lysis of HCMV-infected cells by both natural killer cells and cytotoxic T cells (Borysiewicz et al., 1983, 1985). In the case of murine CMV, Reddehase et al. (1984, 1985) have convincingly shown that immediate early gene
products can be recognized by murine cytotoxic T cells. Although the immediate early phase of HCMV replication has been extensively studied at the molecular and biochemical levels (Wilkinson et al., 1984; Stinski et al., 1983; Stenberg et al., 1985) the EA polypeptides of HCMV are relatively poorly characterized. Unlike immediate early HCMV genes, regions of early viral transcription are dispersed throughout the genome (DeMarchi, 1981; Wathen & Stinski, 1982). In addition to positive transcriptional regulation by immediate early gene products and by virion trans-activating factor(s) (Spaete & Mocarski, 1985) at least some HCMV early genes are regulated post-transcriptionally (DeMarchi, 1983a; Geballe et al., 1986).

Seven early polypeptides have been identified by pulse-labelling of infected cells (Stinski, 1978) and a further 10 by immunoprecipitation with HCMV-positive sera (Blanton & Tevethia, 1981). In both studies early polypeptides of mol. wt. comparable to the 72K protein we describe were identified. Only two HCMV early proteins have been assigned functions: a 36K DNA-binding phosphoprotein (Mocarski et al., 1985) and the virus-induced DNA polymerase (Huang, 1975). Other functions attributed to as yet unspecified early proteins are the modulation of host cell DNA synthesis and the stimulation of cellular thymidine kinase (DeMarchi, 1983b).

In this study we have purified a 72K early polypeptide of HCMV by immunoaffinity chromatography on monoclonal antibody-coupled Sepharose.

The purified 72K antigen was reactive by ELISA with HCMV seropositive human sera but not seronegative sera, and in addition was also able to induce T cell proliferative responses in vitro. The nature of the responding T cells was further studied by establishing short-term IL2-dependent T cell lines. The majority of these lines were CD4+, but in contrast to previous studies with HCMV LA (Borysiewicz et al., 1983), five of 14 lines were predominantly CD8+. Preliminary evidence suggests that two of 18 such T cell lines lysed HCMV-infected fibroblasts matched at one or more HLA class I loci, implying MHC-restricted cytotoxic T cell activity in these two lines.

Virus-specific cytotoxic T cells are usually generated by secondary stimulation in vitro with virus-infected cells and only in the case of influenza haemagglutinin (Zweerink et al., 1977), Sendai virus glycoproteins (Hale et al., 1980) and herpes simplex virus glycoproteins (in liposomes) (Lawman et al., 1981) have such cells been generated with purified viral proteins. In these instances it was possible that fusion activity may have enabled the proteins to insert into cell membranes during stimulation in vitro, but this seems an unlikely explanation in the case of the 72K EA and studies are in progress to determine the frequency of this response and the requirements for generating such cytotoxic T cells in vitro.
It is of note that the 72K EA was predominantly nuclear in distribution, not a glycoprotein and not detected by indirect immunofluorescence on the cell surface, yet is presumably recognized by cytotoxic T cells at the cell surface. In this respect it may represent another example of an intracellular viral antigen recognized by cytotoxic T cells. Influenza virus nucleoprotein (Townsend et al., 1984, 1985; McMichael et al., 1986), vesicular stomatitis virus nucleocapsid protein (Yewdell et al., 1986) and respiratory syncytial virus nucleoprotein (Bangham et al., 1986) can all be recognized by cytotoxic T cells. In the case of influenza virus nucleoprotein it has been shown that specific peptides may be detected by cytotoxic T cells on the cell surface (Townsend et al., 1986) and presumably the mature molecule undergoes some form of intracellular processing. The recognition of immediate early gene products of murine CMV by a murine T cell clone (Reddehase et al., 1985) would also be consistent with this scheme of recognition.

Thus, while the HCMV glycoproteins may be important in inducing neutralizing antibody (Rasmussen et al., 1984) non-structural viral proteins, such as the 72K EA may also have a role in inducing the development of HCMV-specific T cells. In fact our recent results suggest that the bulk of HCMV-specific cytotoxic T cell precursors in virus carriers are directed to these non-structural virus antigens expressed at immediate early or early times (L. Borysiewicz et al., unpublished results). The specific role of individual immediate early and early gene products in T cell recognition can perhaps best be addressed by expressing single HCMV genes in target cells, and we are pursuing this approach.

REFERENCES


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