The lower matrix protein pp65 is the principal viral antigen present in peripheral blood leukocytes during an active cytomegalovirus infection

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During an active infection with human cytomegalovirus (HCMV), viral antigen is consistently present in peripheral blood leukocytes. Two monoclonal antibodies (MAbs), CMV-C10 and CMV-C11, are commonly used in the HCMV antigenaemia assay to detect these cells in the peripheral blood of patients suspected of having an active HCMV infection. We demonstrate that the viral antigen detected by these MAbs is the viral structural protein pp65 and not an immediate early antigen as previously reported. Furthermore, significantly fewer leukocytes were found to be positive with MAbs specific for immediate early antigens.

Introduction

Human cytomegalovirus (HCMV) is of major clinical importance in immunosuppressed patients such as transplant recipients or patients suffering from AIDS. An active infection with HCMV is characterized by the appearance of HCMV antigen-positive leukocytes in peripheral blood (van der Bij et al., 1988a, b); HCMV nucleic acid has also been detected in various subsets of the white blood cell fraction by in situ hybridization and the polymerase chain reaction (PCR) (Schrier et al., 1985; Saltzman et al., 1988; Jiwa et al., 1989; Dankner et al., 1990). In addition, it has been shown that HCMV can be transmitted by cell fractions such as granulocytes or buffy coat cells (Winston et al., 1980; Hersman et al., 1982; Adler, 1983).

Two monoclonal antibodies (MAbs), CMV-C10 and CMV-C11 prepared in our laboratory, are commonly used to diagnose active HCMV infections by staining peripheral blood leukocytes in the HCMV antigenaemia assay (van der Bij et al., 1988a; van den Berg et al., 1989, 1991; Revello et al., 1989; Boland et al., 1990; Gerna et al., 1990; Lautenschlager et al., 1990; The et al., 1990). These antibodies were originally thought to be directed against an HCMV immediate early (IE) antigen because they display a nuclear staining pattern in HCMV-infected human embryonic fibroblasts treated successively with cycloheximide and actinomycin D. The antibodies precipitate a protein of 70K from such cells (van der Bij et al., 1988b). It was assumed that cells detected by these antibodies are actively infected with HCMV and that viral gene expression had proceeded to the IE stage of infection.

In this study we identified the viral antigen detected by MAbs CMV-C10 and CMV-C11 as the lower matrix protein pp65 by using both well characterized MAbs directed against HCMV antigens and plasmids containing sequences encoding HCMV antigens.

Methods

Cells and virus. Human embryonic lung fibroblasts (HELFs) were grown in Dulbecco's modification of Eagle's medium (DMEM; Flow Laboratories) supplemented with glutamine, gentamicin and 10% foetal calf serum (FCS; Gibco) and used between passages 10 and 25. Cell-free stocks of HCMV strain AD169 were prepared from HELFs as described previously (Middeldorp et al., 1984). Virus stocks and HELFs were routinely tested and found to be free of mycoplasma contamination.

For infection experiments, HELFs were grown and infected on 12-well Cooke slides (Nutacon). Protein synthesis was inhibited by using cycloheximide (50 μg/ml) and mRNA transcription was blocked by using actinomycin D (20 μg/ml). After the experiments, the slides were washed in PBS and sterile water. Slides were air-dried quickly and fixed in acetone at room temperature for 12 min. Finally, they were wrapped in aluminium foil and frozen at -80 °C.

Rat epithelial cell lines containing cloned IE region 1 (exons 1, 2, 3 and 4, a1 cells) or cloned IE region 2 (exons 1, 2, 3 and 5, a2 cells), encoding IE antigens of HCMV AD169, were generously given to us by
Dr R. Boom, Amsterdam, The Netherlands. These cell lines were induced to express IE antigens by treatment with sodium arsenite as described (Boom et al., 1986; Boom, 1987). They were harvested by trypsin digestion, resuspended in culture medium and seeded onto Cooke slides. Cells were allowed to grow overnight and further treated as described for HELFs. Control slides of cells not induced to express IE antigens were prepared in the same way.

**MAbs and antisera.** MAb s CMV-C10 and CMV-C11 (IgG1 subclass), originally known as 1.5.12 and 1.5.17 and prepared in our laboratory (Middeldorp et al., 1986), are routinely used to detect HCMV antigenemia in patients suspected of having an active HCMV infection (Clonab CMV, commercially available from Biotest). MAb 95/12 [clone F6B, IgG1, directed against the lower matrix protein pp65 (Amades et al., 1983; Michelson et al., 1984; Somogyi et al., 1990)] was a kind gift from Dr S. Michelson, Paris, France. MAb s 9221 [IgG2a, against IE antigens (Shuster et al., 1985; LaFemina et al., 1989)] and 9220 [IgG3, against pp65 (Shuster et al., 1985; Weiner et al., 1985; Landini et al., 1987)] were obtained from DuPont de Nemours. MAb E13 [IgG1, against IE antigens (Mazeron et al., 1983)] was purchased from Seralab. MAb 28-77 [IgG2b, against pp65 (Plachter et al., 1990)] was a generous gift from Dr W. Britt, Birmingham, Ala., U.S.A. MAb Dako-CMV [clone CCH2, IgG1, against a 43K early antigen (Niedobitek et al., 1988)] was purchased from Dakopatts. MAb HCMV16 [IgG1, directed against the gH homologue of HCMV (Cranage et al., 1985; Weiner et al., 1990)], MAb Moc31, directed against an epithelial antigen of 40K, was used as an irrelevant MAb (IgG1, MCA Development). Another MAb of the IgGl subclass, an extract of mock-infected HELFs 6 to 7 days post-infection (p.i.) as described previously (Middeldorp et al., 1984). The antigen extract was diluted in incubation buffer (10 mM-Tris-HCl pH 8.0, 0.3 M-NaCl, 3% BSA, 0.05% Tween 20) and 1% NP40 was added. Before being used, the extract was kept at 4°C for 30 min.

The antigen extract used for the immunoprecipitation and immunoblotting experiments was prepared from HCMV AD169-infected HELFs 6 to 7 days p.i. by extraction with NP40 lysis buffer (20 mM-Tris–HCl pH 9.0, 0.3 M-NaCl, 1% NP40). The extract was collected after 20 min and debris was removed by centrifugation at 50000 g for 1 h at 4°C. Control antigen extracts were prepared from uninfected HELFs in the same way.

**Antigen capture ELISA.** This was performed as described by van der Voort et al. (1989). In short, wells of flat-bottomed 96-well plates were coated with MAb s CMV-C10 and CMV-C11 as capture antibodies, diluted to a concentration of 3.0 µg/ml in carbonate buffer pH 9.6. Next, plates were incubated with the antigen extract and subsequently either with MAb 9220 in twofold dilutions and horseradish peroxidase (HRO)-labelled goat anti-mouse IgG3 (1:100-05, Southern Biotechnology Associates), or with rabbit antisera in twofold dilutions and H2O2 labelled swine anti-rabbit IgG (1:217, Dakopatts). A colour reaction was produced with 0.2 mg/ml p-phenylene-diamine dihydrochloride (Kodak) in 0.1 M-citrate buffer pH 5.05 containing 0.05% H2O2, and the absorbance was measured at 492 nm using a Titertek Multiskan.

Several negative controls were included. These were an irrelevant capture MAb of the IgG1 subclass, an extract of mock-infected HELFs, rabbit pre-immune sera and the omission of the second antibody.

**Immunoprecipitation, gel electrophoresis and immunoblotting.** The antigen extract was pre-cleared by incubation with the anti-CD8 MAb, Protein A-Sepharose beads CL-4B (PAS 10%; Pharmacia), and 0.5 mM-PMFS (Sigma) in RIPA buffer (20 mM-Tris–HCl pH 8.0, 0.3 M-NaCl, 1% Triton X-100, 4% BSA). After 4 h, the beads were removed and the pre-cleared antigen was incubated with PBS and MAb Moc31, CMV-C10, CMV-C11, 9220 or 28-77 in RIPA buffer. After 16 h, PAS 10% was added and another 4 h later the beads were collected. Beads were washed, resuspended in 20 mM-Tris–HCl pH 6.8, layered on a 20% sucrose cushion and pelleted by high-speed centrifugation.

Immunoprecipitated material was analysed by SDS–PAGE followed by immunoblotting with MAb 28-77. To this end, the beads were collected in Tris–HCl, mixed with an equal volume of 2% concentrated denaturation buffer (0.063 M-Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 0.005% bromophenol blue and 5% 2-mercaptoethanol) and boiled for 5 min. SDS–PAGE was done on an 8% polyacrylamide gel (Laemmli, 1970). Boiled extract and M, standard proteins (Bio-Rad, high range) were applied to the gel. After electrophoresis, the polypeptides were transferred to nitrocellulose membranes (BA 85, Schleicher & Schuell) using a semi-dry electroblotter (Ancon). Blocking of the residual binding capacity of the membrane was achieved by incubation in TBS (20 mM-Tris–HCl pH 8.0, 150 mM-NaCl, 0.1% Triton X-100) containing 3% BSA. The blot was incubated with MAb 28-77 and, subsequently, alkaline phosphatase-conjugated rabbit anti-mouse Ig (D314, Dakopatts). The blot was developed with nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolylphosphate (Sigma) in 0.1 M-carbonate buffer pH 9.8.

**Construction of expression plasmid pp65-RSV.** Cloning of the eukaryotic expression plasmid pp65-RSV was done in several steps. In the first step, a HindIII fragment from vector pMSG (Pharmacia) was cloned into the high copy vector pIC-20H (Marsh et al., 1984); this HindIII fragment contains the mouse mammary tumour virus (MMTV) long terminal repeat (LTR) upstream of a multiple cloning site. The MMTV LTR was replaced by the Rous sarcoma virus (RSV)
Results

Identification of the viral antigen detected by MAbs CMV-C10 and CMV-C11

To characterize MAbs CMV-C10 and CMV-C11, immunofluorescence experiments were performed in which these antibodies were compared to MAbs of known specificity, i.e. E13 and 9221, directed against IE antigens, and 95/12, 28-77 and 9220, directed against the lower matrix protein pp65.

MAbs CMV-C10 and CMV-C11 were found to give cytoplasmic and nuclear staining in HCMV late stage-infected fibroblasts similar to that of MAbs directed against pp65. In contrast, the IE antigens detected by E13 and 9221 remained localized in the nucleus during the whole virus replication cycle (data not shown).

Immunofluorescence experiments with these MAbs on α1 and α2 cell lines expressing IE antigens showed that MAbs E13 and 9221 recognized the antigens present in these cells producing nuclear staining, whereas CMV-C10 and CMV-C11, and the MAbs directed against pp65 did not show any reaction (Fig. 2).

MAbs were then compared in antigen capture ELISAs. In the first ELISA, CMV-C10 and CMV-C11 were used as capture antibodies. After incubation with an antigen extract of HCMV-infected HELFs, the captured antigen was identified as pp65 by using MAb 9220. As all controls were negative, this indicated that MAbs CMV-C10 (Fig. 3) and CMV-C11 (not shown) had specifically captured pp65.

In the second ELISA, plates were coated and incubated with antigen extract in the same way, but the captured antigen was detected by using polyclonal rabbit antiserum against fusion proteins containing defined, overlapping parts of pp65. Positive results were obtained with rabbit antiserum directed against fusion proteins 1007-35 and 1007-47 (Fig. 4). However, a rabbit antiserum directed against fusion protein 1007-74 gave negative results, suggesting that this fusion protein might contain an epitope identical to or located close to the epitope recognized by MAbs CMV-C10 and CMV-C11. Pre-immune sera (Fig. 4) and other controls (not shown) gave negative results.

As MAbs CMV-C10 and CMV-C11 are known not to react in Western blot assays (unpublished observations), immunoprecipitation experiments were performed. Immunoprecipitated proteins were analysed by SDS-PAGE and subsequent immunoblotting with MAb 28-77. The results showed that MAbs CMV-C10, CMV-C11, 9220 and 28-77 precipitated an antigen of 65K from the infected cell extract which reacted strongly with MAb 28-77 in immunodetection experiments (Fig. 5). The extra bands of 60K and 54K precipitated by MAb 28-77 might represent degradation products of pp65. No
specific precipitate was found with PBS or MAb Moc31 (an irrelevant MAb). The protein bands of <50K were shown to be non-HCMV-specific because they were also found when immunoprecipitation was performed with a mock-infected cell extract (not shown). Taken together, these results indicate that MAbs CMV-C10 and CMV-C11 are directed against the lower matrix protein pp65.

To prove this definitively, transient transfection experiments were carried out. Plasmid pp65-RSV (Fig. 1) and control plasmids encoding exons 2, 3 and 4 or exons 2, 3 and 5 from the major IE genes of HCMV under simian virus 40 (SV40) promoter control (kindly provided by Dr T. Stamminger, Erlangen, Germany) were transfected into COS cells. Control cells were transfected with Lipofectin alone. Indirect immunofluorescence assays were performed with these cells using MAbs 28-77, 9221, CMV-C10, CMV-C11, or PBS. As can be seen in Fig. 6, MAbs CMV-C10 and CMV-C11 clearly stained cells transfected with pp65-RSV, as did MAb 28-77, known to be directed against pp65. As a negative control, MAb 9221 did not stain cells transfected by pp65-RSV. In contrast, cells transfected with the IE constructs showed positive immunofluorescence.
Kinetics of the appearance of pp65 in the nuclei of infected cells

The time of appearance of pp65 in infected cells was investigated. It was found that pp65 was detectable by immunofluorescence in the nuclei of infected fibroblasts within 1 h p.i., whereas the IE antigens could first be detected 3 h p.i. Furthermore, pp65 was detectable in the nuclei of cells infected under IE conditions, e.g. subsequent treatment with cycloheximide and actinomycin D (3 h each).

To analyse whether the presence of pp65 in infected cells at 1 h p.i. was due to de novo synthesis of the protein or to uptake from the inoculum, fibroblasts were infected with 2 p.f.u. HCMV AD169/cell in the presence of cycloheximide (added 1 h prior to infection). Cells were examined 1, 2, 4, 6 and 16 h p.i. for staining with MAbs CMV-C10, CMV-C11, 95/12, 9220, 28/77, 9221 or E13. The results of these experiments showed (Fig. 7) that MAbs directed against pp65 stained the nuclei of infected cells at all times, but did not stain mock-infected cells. MAbs directed against HCMV IE antigens did not react with any of these cells, whereas they did show a good reaction with unblocked HCMV-infected cells at 4 h p.i., indicating that protein synthesis was efficiently blocked. When this experiment was repeated in the presence of actinomycin D, an mRNA transcription inhibitor, the outcome was the same.

These results demonstrate that the presence of pp65 within the nuclei of infected cells under these conditions was due to uptake from the inoculum and transport of the protein to the nucleus rather than to de novo production.

Detection of viral antigens in peripheral blood leukocytes of patients with an active HCMV infection

As pp65 belongs to the early late class of antigens, the expression of which is preceded by the production of IE antigens in a permissively infected cell (Wathen et al., 1981; Stinski et al., 1983; Depto & Stenberg, 1989), we were interested to see whether the latter antigens could also be detected in white blood cells during an active infection. Because discrepancies have been reported regarding the rate of virus isolation from, and the presence of viral DNA and mRNA in, MNCs in comparison to PMNs (Rinaldo et al., 1977; Saltzman et al., 1988; Dankner et al., 1990), we investigated the MNC and PMN fractions separately.

MNCs and PMNs were isolated from seven patients with an active HCMV infection. Cells were stained with MAbs CMV-C10, CMV-C11, 95/12 and 9220 (against pp65), and with MAbs E13 and 9221 (against IE antigens). MAbs CCH2 (reactive with an early 43K antigen) and HCMV16 (against the gH homologue of HCMV, a late 86K antigen) were included to determine whether other early or late viral antigens were present. All these MAbs have previously been shown to recognize these viral antigens in acetone-fixed, HCMV-infected fibroblasts, indicating that the recognized epitopes are not sensitive to this fixation method.

Identical results were obtained with MAbs CMV-C10, CMV-C11 and 95/12 (Table 1). Each MAb stained approximately equal numbers of cells in the MNC and PMN preparations from the same patient. In general, fewer cells stained positive in the MNC fraction than in the PMN fraction, as described earlier (van der Bij et al., 1988b). However, MAb 9220 reacted differently. In both PMN and MNC fractions, lower numbers of cells were stained with this MAb than with the others, the difference being statistically significant for PMNs only (paired Student’s t-test: P < 0.02). Since MAb 9220

Fig. 5. Immunoblot analysis of proteins precipitated with different MAbs from an HCMV-infected HELF antigen extract using MAb 28-77 as the detecting antibody. Lane 1, proteins precipitated with MAb 9220; 2, proteins precipitated with MAb CMV-C10; 3, proteins precipitated with MAb CMV-C11; 4, proteins precipitated with MAb 28-77; 5, proteins precipitated with MAb Moc31; 6, proteins precipitated with PBS. Mr's of protein standards are indicated to the left.
reacts with the same antigen as MAbs CMV-C10 and CMV-C11 in the antigen capture ELISA, it can be assumed that this MAb recognizes an epitope of pp65 different to those for CMV-C10 and CMV-C11, and this might be the reason for the difference in staining pattern observed in this experiment.

The MAbs directed against IE antigens stained significantly fewer cells in either cell fraction than did CMV-C10, CMV-C11 and 95/12 (paired Student's t-test: P < 0.03 for MNC and P < 0.01 for PMN). In addition, in two of the patients IE antigen-positive cells could not be detected at all, although pp65-positive leukocytes were present. MAbs CCH2 and HCMV16, detecting early and late proteins respectively, did not stain any of these leukocytes.

Discussion

During an active infection with human cytomegalovirus, viral nucleic acid can be found in various subsets of peripheral blood leukocytes (Schrier et al., 1985; Saltz-
Table 1. Numbers of HCMV antigen-positive MNCs and PMNs/50,000 cells in patients using anti-HCMV MAbs

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*1 to 7, Patient number.
† ND, Not done.

In most reports published, viral gene expression has been reported to be restricted to the IE genes. Expression of early or late genes has been detected by RNA in situ hybridization in only a minor fraction of cells in AIDS patients (Dankner et al., 1990).

Viral antigens have been shown to be consistently present in peripheral blood leukocytes during an active HCMV infection and a diagnostic assay, the HCMV antigenaemia assay, based on this finding has been shown to be very effective for both diagnosing and monitoring HCMV infection (van der Bij et al., 1988a, b; van den Berg et al., 1989, 1991; Revello et al., 1989; Boland et al., 1990; Gerna et al., 1990; Lautenschlager et al., 1990; The et al., 1990). However, these antigens and the viral genes that specify them have not been thoroughly characterized.

Recently, Revello et al. (1992) have shown that the viral antigen detected in the HCMV antigenaemia assay in peripheral blood leukocytes during an active HCMV infection is not the major 72K IE antigen. Furthermore, in immunoprecipitation experiments a number of MAbs prepared in their laboratory and reported to be reactive in the HCMV antigenaemia assay precipitated an antigen of 65K from HCMV-infected fibroblasts. The authors concluded that the protein detected by these MAbs was the lower matrix protein pp65. However, no direct evidence in support of this conclusion was provided.

In this study, we have provided conclusive evidence that MAbs CMV-C10 and CMV-C11, commonly used in the HCMV antigenaemia assay, are directed against the viral tegument protein designated pp65. Furthermore, we show that this viral protein is the predominant viral antigen present in peripheral blood leukocytes during an active HCMV infection in vivo, whereas IE antigens can only be detected in a significantly lower number of leukocytes.

In permissively infected fibroblasts, pp65 (Rüger et al., 1987), also termed PK68 (Somogyi et al., 1990), p64 (Pande et al., 1990) and ICP27 (Geballe et al., 1986), has
been shown to be present in large amounts (Weiner et al., 1985). It has been found as early as 2 h p.i. in the nuclei of infected cells (Britt & Vugler, 1987), and newly synthesized protein can be found as early as 3 to 4 h p.i., independent of viral DNA replication (Michelson et al., 1984; Geballe et al., 1986; Britt & Vugler, 1987; Somogyi et al., 1990). However, transcriptional activation of the pp65 promoter depends on the presence of IE gene products in infected fibroblasts (Depto & Stenberg, 1989). Therefore the gene encoding pp65 is considered to belong to the early late class of genes. Although pp65 is synthesized at early times after infection, it is one of the structural ( tegument) proteins present in virions, non-infectious enveloped particles (NIEPS) and dense bodies (Irmiere & Gibson, 1983; Roby & Gibson, 1986). In the latter it constitutes about 95% of the protein mass (Irmiere & Gibson, 1983). The number of dense bodies produced in cell cultures differs with regard to the strain of HCMV and the m.o.i. used (Jahn et al., 1987; Klages et al., 1989).

Our finding that this antigen is present in fibroblasts within 1 h after infection in vitro, even in the presence of cycloheximide or actinomycin D, indicates that the protein is not newly synthesized within these cells but that these cells acquire the antigen from the inoculum. Under these conditions, no IE antigens could be detected. The inoculum we used in our experiments consisted of the supernatant of HCMV AD169-infected fibroblasts obtained after low-speed centrifugation. Therefore it may contain cell debris and a mixture of infectious virions, NIEPS and dense bodies. Consequently, pp65 can be assumed to be present in abundance. This phenomenon of uptake and subsequent detectable nuclear localization of a viral protein, before virus replication occurs, seems to be unique for HCMV as compared to other herpesviruses. It also led to the original misclassification of CMV-C 10 and CMV-C 11 as being directed against IE antigens because these MAbs showed nuclear staining of cells infected with HCMV under IE conditions (subsequent cycloheximide and actinomycin D block). Therefore, when infected cells are used as the source of antigen to characterize the specificity of MAbs or sera, it appears to be necessary to distinguish between input virus and de novo synthesis by using a protein synthesis inhibitor. The significance of the presence of pp65 in peripheral blood leukocytes in vivo in terms of HCMV diagnosis and monitoring of infection has been established (see above). However, the pathophysiological significance of this finding is not clear. With regard to the current findings in both fibroblasts and leukocytes, the presence of pp65 in peripheral blood cells may be due to one of two possibilities.

First, these peripheral blood leukocytes or their bone marrow progenitor cells could be infected with HCMV and pp65 is produced de novo in these cells. However as the pp65 gene is an early late gene, one would expect to detect IE antigens in an equal number of peripheral blood leukocytes. We have shown here that IE antigens are detected at a much lower frequency in leukocytes compared to pp65. In addition, no other viral antigens could be detected.

An alternative explanation would be that during an active HCMV infection, a large amount of pp65 is produced at a site at which leukocytes can acquire the antigen. The cells could take up the protein by a process of virion and dense body binding, penetration and uncoating. By this process, only a subset of the leukocytes would become infected by virions and express IE antigens. Alternatively, the cells could take up the protein by phagocytosis. However, with respect to the exclusive nuclear localization of the antigen in leukocytes, this possibility appears to be less likely. Further analyses are required to investigate the origin of pp65 found in peripheral blood leukocytes during an active HCMV infection.

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