Glycoprotein gp116 of human cytomegalovirus contains epitopes for strain-common and strain-specific antibodies

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Glycoprotein gp116 of human cytomegalovirus (HCMV) is a target for neutralizing antibodies. Gp116 is a component of the gCl complex which consists of gp58 and gp116. Like its homologue, glycoprotein B of herpes simplex virus type 1, gp116 contains a highly antigenic region in the N-terminal part of the molecule, between amino acids 28 and 84. Prokaryotic expression plasmids and synthetic peptides were used to define binding sites for mouse and human monoclonal antibodies (MAbs) as well as HCMV convalescent sera. Site I, located between amino acids 68 and 77, contains an epitope recognized by the human MAb C23, which is capable of neutralizing HCMV independently of complement and the site is conserved between HCMV strains. Of HCMV-positive human sera, 53% recognized site I. Site II was mapped using mouse MAbs as well as human sera. It is located between residues 50 and 54, an area which is not conserved between strains AD169 and Towne, the two laboratory strains of known sequence. Strain-specific antibodies were detected in 25% of human sera. Site II-specific antibodies, purified from human sera by affinity chromatography, were found to be incapable of neutralizing HCMV in tissue culture.

Introduction

Investigation of the host defence against human cytomegalovirus (HCMV) has indicated that the glycoprotein complex gp58/116 represents a major target for the immune response. During natural infection the protein is capable of inducing cytotoxic T cells, as well as neutralizing antibodies (Borysiewicz et al., 1988; Masuho et al., 1987; Ehrlich et al., 1987). The humoral immune response has been characterized in some detail. In studies using immunoprecipitation or immunoblot techniques, the complex is consistently detected by human sera (Landini et al., 1985; Hayes et al., 1987; Liu et al., 1988). In addition, there is evidence that a major fraction of the neutralizing capacity of human convalescent sera is directed against this complex (Britt et al., 1990). In several laboratories neutralizing murine and human monoclonal antibodies (MAbs) reacting with the gp58/116 complex have been developed (Masuho et al., 1987; Rasmussen et al., 1985b; Britt, 1984; Banks et al., 1989). Some of these antibodies are strain-specific in that they recognize and/or neutralize a limited number of isolates (Rasmussen et al., 1984, 1985a; Masuho et al., 1987). However, the strain-specific antibodies described depend on the conformation of the glycoprotein. In contrast, antibodies directed against linear epitopes have a broad reactivity with HCMV strains (Britt, 1984; Meyer et al., 1990; Kniess et al., 1991). Gp58/116 has been proposed as a candidate for a HCMV subunit vaccine and in fact purified viral protein has been used to immunize humans (Gonczol & Plotkin, 1990). Therefore we feel that it is imperative to investigate the nature of the immune response against this polypeptide in detail.

The glycoprotein complex gp58/116, the homologue of gB in herpes simplex viruses, consists of two polypeptides, a highly glycosylated protein of 90K to 130K and a less heavily modified 58K protein. The disulphide-linked components are derived from a glycosylated precursor molecule of 150K to 160K in size by proteolytic cleavage (Britt & Vugler, 1989; Gretch et al., 1988). Cleavage occurs at position 460 of the primary translation product and is performed by a cellular endoprotease (Spaete et al., 1988, 1990). Gp58 represents the C-terminal part of the precursor protein and most likely functions as the transmembrane protein, whereas gp116 corresponds to the N-terminal half (Mach et al., 1986; Meyer et al., 1990; Kari et al., 1990). Studies utilizing immunoblot analyses of prokaryotic fusion proteins and human convalescent sera have revealed that only a limited number of linear antibody binding sites is present on the
gp58/116 gene product (Meyer et al., 1990; Kniess et al., 1991; Silvestri et al., 1991). Three major sites have been identified. Gp58 contains an antigenic domain (AD-1) on the extraviral part around amino acid 616 and a second site at the C terminus (Kniess et al., 1991; Utz et al., 1989). On gp116, an antigenic domain (AD-2) is located at the N terminus of the molecule (Meyer et al., 1990). AD-1 was originally defined using murine MAbs and in mice this domain is capable of inducing neutralizing and non-neutralizing antibodies, which can compete for binding and neutralization (Utz et al., 1989). Furthermore it has been shown that the extraviral part of gp58 contains a discontinuous neutralizing epitope (Banks et al., 1989). Antibodies against the C-terminal antigenic domain of gp58 are probably not involved in neutralization because there is evidence that this part of gp58 is localized on the inside of the virus or the infected cell (Baggoz et al., 1992).

The gp116 component of the gp58/116 complex was originally characterized using human MAb C23, which is able to neutralize HCMV independently of complement. The immunogenic region recognized by C23 has been localized to residues 28 to 84 of the primary translation product (Meyer et al., 1990). In this report we describe the detailed immunological characterization of this antigenic domain. The results indicate that there are two antibody binding sites in close proximity. Site I is conserved among strains, whereas site II is strain-specific.

Methods

Virus and cell culture. HCMV strains AD169 and Towne were propagated in human foreskin fibroblasts (HFFs) by standard procedures.

Metabolic labelling and immunoprecipitation. Subconfluent monolayers of HFF cells were infected with HCMV strain AD169 or Towne at 2 p.f.u./cell. Four days post-infection the cultures were washed twice in RPMI 1640 medium without methionine, then starved in this medium for 24 h at 37 °C. Proteins were labelled by incubation with [35S]methionine extracted and ethanol-precipitated, and incubated with 200 units exonuclease III at 37 °C in 50 mM-Tris–HCl pH 8.0, 5 mM-MgCl2, 10 mM-2-mercaptoethanol. At various times (15 s, 30 s and 45 s) aliquots were removed and the reaction was terminated by adding 175 μl 87 mM mung bean buffer (57 mM-sodium acetate pH 5.0, 34 mM-NaCl, 1.14 mM-ZnSO4), followed by heating at 68 °C for 15 min. After digestion of the single-stranded extension with 15 units of mung bean nuclease for 30 min at 30 °C, the DNA was phenol/chloroform-extracted, ethanol-precipitated and religated. The resulting deletions were characterized by determination of the nucleotide sequence (Sanger et al., 1977).

Immunoaffinity chromatography. Human antibodies specific for gp116 were isolated by immunoaffinity chromatography on fusion protein Eno-16 (residues 28 to 67 of gp116). Eno-16 was enriched in bacterial lysates as described by Urban et al. (1992) and dialysed against 0.1 M-phosphate buffer pH 7.4. Protein (10 mg) was coupled to an AminoLink column (ImmunoPure Ag/Ab Immobilization Kit, Pierce) for 6 h at room temperature following the instructions of the manufacturer. After washing with 20 ml PBS, 6 ml of a pool of human convalescent sera was applied to the column for 4 h at 4 °C. The column was washed with PBS and the bound antibodies were removed with 100 mM-glycine pH 2.8. The eluted antibodies were collected in 1 ml fractions and subsequently neutralized by addition of 50 μl 1 M-Tris–HCl pH 9.5. Fractions containing gp116-specific antibodies were pooled and passed over the column a second time. The protein content was determined by measuring the absorbance at 280 nm (A280). The antibody preparation was dialysed against PBS and the specificity of the antibodies tested by immunoblot analysis.

ELISA. Peptides were diluted to 100 to 250 μg/ml in distilled water and 50 μl was used to coat microtitre plates for 16 h at 4 °C in a humid chamber. All subsequent steps were carried out at room temperature. Reaction wells were rinsed three times (5 min each) with buffer A (PBS, 0.05% Tween 20) and blocked for 2 h with buffer containing 2% foetal calf serum (FCS). Plates were again rinsed with buffer A and incubated with MAbs or human serum (50 μl/well) for 2 h. After three additional washes with buffer A, alkaline phosphatase-conjugated anti-human or anti-mouse IgG was added at an appropriate dilution for 1 h. Plates were washed two times and 100 μl 0.7 mg/ml p-nitrophenyl phosphate was added for 30 min at 37 °C. The reaction was stopped by the addition of 50 μl 2 M-NaOH and the A405 was determined. Dilution of all antibodies was done in PBS with 2% FCS.
**Results**

**Characterization of the binding site for human MAb C23**

In a previous report we have shown that the neutralizing human MAb C23 is reactive with a bacterially derived fusion protein, HM90-7, containing amino acids 28 to 84 of the gp58/116 gene product of HCMV strain AD169 (Meyer et al., 1990). Comparison of the respective amino acid sequences of strains AD169 and Towne reveals heterogeneity in this area of the molecule (see Fig. 1). The amino acid sequence contained in the fusion protein HM90-7 is composed of the non-homologous region between amino acids 28 to 67, as well as an additional 17 amino acids which are identical in strains AD169 and Towne. Since MAb C23 is reactive with gp116 of both laboratory strains, as well as all clinical isolates tested, we predicted the binding site to be located within the stretch of conserved amino acids between residues 68 and 84. To prove this prediction a set of bacterial fusion proteins with increasing deletions at the C terminus of gp116 was produced and tested for reactivity with MAb C23 in immunoblots. Fig. 1 shows a summary of these results. Two proteins, HM90-7 (amino acids 28 to 84) and HM90-12 (amino acids 50 to 101), were included in the analyses as controls.

MAb C23 was reactive with fusion proteins Exo-5 (residues 28 to 81), Exo-4 (residues 28 to 78) and Exo-15 (residues 28 to 77) (Fig. 2a). No signal was obtained with fusion proteins ending at residue 76 and less, indicating that Lys 77 is essential for binding of MAb C23.

However, using various synthetic peptides in an ELISA, slightly different results were obtained. The specificity of the peptides is given in Fig. 1. Peptide pep90, containing amino acids 68 to 84, showed the strongest reactivity. Peptide T7-13:2, ending at Lys 77 was negative in this assay. One additional amino acid (Tyr 78) at the C terminus was required for binding of antibody, as indicated by the reactivity of peptide T7-13:3 (Fig. 2b).

Identical results were obtained when the peptides were spotted onto nitrocellulose and used as antigens for MAb C23 (data not shown). The observed differences in reactivity were most probably not due to the additional residues provided by the prokaryotic portion of the fusion protein. In no case did the bacterial part complete an epitope and constructs having identical viral but different bacterial sequences gave identical results (data not shown).

Taken together these data indicate that the binding
Characterization of a strain-specific antibody-binding site in the N-terminal part of gp116

In our attempts to characterize antibody-binding sites on gp116 we also tested a number of murine MAbs developed against strain AD169. Seven antibodies (CH45, CH86, CH386, CH396, CH404, CH408 and CH412) were chosen because they were reactive in immunoblots with the precursor molecule of gp150/160 but not with the C-terminal cleavage product, gp58. Using fusion protein HM90-7 as an antigen in an immunoblot, the binding sites of these antibodies could be localized between amino acids 28 and 84 of gp116 (data not shown). Six antibodies (CH45, CH386, CH396, CH404, CH408 and CH412) gave strong signals with proteins HM90-12 (residues 50 to 101), Exo-16 (residues 28 to 67), Exo-41 (residues 28 to 56) and Exo-27 (residues 28 to 54). No reaction was obtained with fusion protein Exo-28 (residues 28 to 50) or the β-galactosidase part derived from the vector. As a representative of this group of antibodies the reaction of CH408 is shown in Fig. 3a. Antibody CH86 gave a slightly different result. Protein Exo-27 (residues 28 to 54), which was clearly positive with the first group of antibodies, gave only a weak signal with CH86. These findings indicate that the sequence 5°RSVYS54 represents an essential part of the binding site for the first group of murine MAbs. CH86 might require residues further toward the C terminus and/or a different conformation for optimal binding. This area of the molecule was designated site II.

The binding sequence is located in a part of gp116 that is divergent between strains AD169 and Towne. The differences include one amino acid deletion and two mutations (Fig. 1). The specificity of the antibody–antigen interaction suggested that the antibodies might be strain-specific. To analyse this an additional fusion protein, HM To-16, representing amino acids 13 to 58 of strain Towne, was tested for recognition by the antibodies in immunoblots. The sequence of HM To-16 is shown in Fig. 1. Only the strain AD169-derived fusion proteins were recognized by the antibodies (data not shown). Whether this differential recognition would also be observed when the native antigen was used was tested by immunoprecipitation with infected cell lysates. The reactivity of MAb CH408 is presented (Fig. 3b). Using strain AD169 as antigen the antibody produced the expected pattern of precipitated polypeptides of 160K, 90K to 130K and 58K. In contrast, no protein was precipitated using strain Towne-infected or non-infected cell lysates. Antibody C23 recognized the glycoproteins of both strains (Fig. 3b). The 65K polypeptide which was observed in precipitations of infected cell extracts of both strains most probably represents a non-specific reaction and/or binding of antibody to an Fc receptor protein demonstrated previously (Xu-Bin et al., 1989). In either case it can be used as an internal control to demonstrate that equal amounts of protein were used for analysis. The differential electrophoretic mobility of the glycoproteins from different strains is possibly due to

site for MAb C23 is located between Glu⁶⁹ and Lys⁷⁷ or Tyr⁷⁸. We designated this antibody-binding region site I.
HCMV strain-specific epitopes

HCMV strain-specific epitopes

post-translational modification and has also been observed before (Nowak et al., 1984).

Reactivity of human sera with the antigenic domain of gp116

The results presented above identified two linear antibody-binding sites on gp116 of strain AD169 in close proximity. In addition, we have previously shown that approximately 40% of human HCMV-positive sera react with fusion protein HM90-7 (residues 28 to 84) containing both sites (Meyer et al., 1990). To analyse the antigenic capacity of either site during natural infection, human sera were tested. Eight specimens were preselected for positive reaction with proteins HM90-7 and HM90-12 (residues 50 to 101). In immunoblot analyses with the Exo proteins, two reaction patterns were observed. Five sera were reactive with fusion proteins expressing residues 28 to 77 (Exo-5, -4 and -15), and these did not recognize fusion proteins containing shorter gp116-specific sequences (Fig. 4). The complete loss of the reactivity between fusion proteins Exo-15 and Exo-17 indicates that antibodies with specificities similar or identical to that of MAb C23 were present in these sera. Three sera had specificities comparable to the murine MAbs described above. They recognized Exo-16, -41 and -27, but not Exo-28, suggesting that strain-specific antibodies are generated during natural infection (Fig. 4).

The percentage of sera containing antibodies to either site was determined employing pep90 as well as the strain-specific fusion proteins Exo-16 and HM To-16. Twenty HCMV-positive sera were tested, of which seven (33%) were found to be positive for site I-specific antibodies. Strain-specific reactions with strains AD169 and Towne were detected in three and two specimens, respectively; with the exception of a single specimen these sera also contained antibodies against site I (data not shown).

Fig. 3. Identification of the binding site for murine MAbs CH86 and CH408. (a) Western blot analyses of prokaryotic fusion proteins containing various sequences from the N-terminal part of gp116 recognized by MAbs CH86 and CH408. Cell lysates harbouring the respective expression plasmids were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Anti-mouse IgG coupled to horseradish peroxidase and diaminobenzidine were used as the detection system. Lanes 1 to 6, Exo-16, -41, -27 and -28, HM90-12 and SEM 2, respectively. (b) Immunoprecipitation with MAbs C23 and CH408. [35S]Methionine-labelled lysates of uninfected (lanes 3) or HCMV-infected (AD169, lanes 1; Towne, lanes 2) HFF cells were incubated with MAbs C23 and CH408. Following precipitation with Protein A-Sepharose, the proteins were analysed by 8% SDS-PAGE and an autoradiograph was prepared. Lane M, Mr markers.

Fig. 4. Recognition pattern of human immune sera with fusion proteins encoding the N-terminal part of gp116. Cell lysates harbouring the truncated expression plasmids were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with human convalescent sera at a dilution of 1:50 (sera 1 and 2, a and b). The immune reaction was carried out as described in the legend to Fig. 2. (a) Lanes 1 to 5, Exo-5, -4, -15, -17 and SEM 2; (b) lanes 1 to 5, Exo-16, -41, -27, -28 and SEM 2.
Neutralization capacity of site II-specific human antibodies

Several reports have described the isolation of strain-specific murine and human antibodies against HCMV (Rasmussen et al., 1984, 1985b; Masuho et al., 1987; Baboonian et al., 1989). In general these antibodies depend on conformation for recognition of antigen. Therefore it was of interest to test whether site II represents an antigenic domain capable of inducing conformation-independent neutralizing antibodies during natural infection. Antibodies specific for this site were purified from a pool of HCMV sera by affinity chromatography on fusion protein Exo-16 coupled to a solid matrix (see Methods). Purified antibody (6 ml) containing 33 mg of IgG was passed over the column several times. Bound antibodies were eluted in fractions and tested in immunoblots against three polypeptides: Exo-16, containing residues 28 to 67 of gp116, MBG58, containing residues 484 to 650 of gp58, and SEM 2, containing residues 1 to 375 of β-galactosidase. The original serum pool, as well as the flowthrough (right lane) were used as controls. A truncated β-galactosidase (SEM2, centre lane) was included as a control. For immune detection, see legend to Fig. 2.

The biological activity of these antibodies was investigated in an in vitro neutralization assay as described in Methods. MAb C23, as well as the original serum pool used for the affinity purification, blocked the infectivity of input virus strain AD169 at concentrations of 1 µg/ml (98% neutralization) and 25 µg/ml (97% neutralization), respectively, when no complement was added to the assay and the residual activity of endogenous complement in human serum was heat-inactivated. This is in agreement with previous results (Masuho et al., 1987). Purified site II antibodies (30 µg/ml) and MAb CH408 and CH86 (20 µg/ml) did not neutralize virus in the absence of complement. When 0-5% guinea-pig complement was added to the assay involving the mouse MAb, antibody CH408 produced a 15% reduction in input virus at a concentration of 20 µg/ml. MAb CH86 did not neutralize infectious virus in any of the assays (data not shown). This is also in agreement with previous results, although the neutralization capacity we found for MAb CH408 was somewhat lower (Pereira & Hoffman, 1986). Affinity-purified human antibodies did not show neutralizing activity in any of the assays.

It has been shown that gp58/116-specific neutralizing and non-neutralizing antibodies can compete for binding and biological activity (Utz et al., 1989; Lussenhop et al., 1988). Therefore it was of interest to determine whether such a phenomenon could be attributed to antibodies binding to sites I and II of gp116. Owing to the limited amounts of human antibodies obtained after affinity chromatography, these experiments were performed with the MAbs. Under our experimental conditions, MAb C23, as shown above, inhibited infection of HCMV strain AD169 at concentrations of 0.5 to 1 µg/ml. Neither MAb CH408 nor CH86 could compete for this activity at concentrations between 0.5 and 20 µg/ml (data not shown).

Discussion

Based on the available sequence data, the N-terminal part of HCMV gp116 between amino acids 30 and 90 can be divided into two domains. Residues 68 to 90 are perfectly conserved between the established laboratory strains AD196 and Towne, as well as low passage clinical isolates (Cranage et al., 1986; Spaete et al., 1988). The second domain, located between residues 30 and 68, is divergent, showing non-conservative amino acid changes as well as deletions and/or insertions (Lehner et al., 1991). In this report we have described the characterization of antibody-binding sites located in each domain.

Site I is characterized by the binding of the neutralizing human MAb C23. It is located in the conserved region of the molecule between residues Glu69 and Lys77 or Tyr78. The exact boundaries of the antibody-binding site could not be determined experimentally because the different assay systems gave conflicting results. At the N terminus, Glu69 seems to be essential, as determined by using synthetic peptides as antigens in ELISA. The C-terminal residues critical for binding are either Lys77 or Tyr78 depending on the form of antigen that is presented. Differences in immune recognition of large polypeptides and small synthetic peptides by antibodies is not uncommon and is thought to result mainly from the different structure of the antigen. In any case, the data confirm our previous prediction that the binding site of
MAb C23 is located in the conserved part of gp116. This is also indicated by the fact that MAb C23 is capable of recognizing the established laboratory strains as well as all clinical isolates tested (M. Mach, unpublished results). Human sera contain antibodies with binding specificities comparable to those of C23. However, in some specimens reactivity towards peptides T7-13:1 and T7-13:2, which did not react with MAb C23, was observed indicating the presence of multiple binding sites in this area. The difference in the amino acid requirements of the two assay systems (immunoblot and ELISA) could be reproduced with a number of sera (data not shown).

The conservation of site I between HCMV strains seems to indicate an essential function of this part of the molecule for the replication cycle of the virus. In this respect it is interesting to note that attempts to isolate antibody-resistant mutants have not been successful (Tomiyama & Masuho, 1990). The function of this part of the molecule could be associated with spread of the virus between cells because MAb C23 is capable of inhibiting cell-to-cell infection in tissue culture. In addition, similar observations have been made with antibodies against comparable regions of the homologous proteins in other herpesvirus systems (Highlander et al., 1988, 1989; Pereira et al., 1989; Kousoulas et al., 1989).

Site II was initially characterized by the use of mouse MAbs and is located in the variable part of gp116 between amino acids 50 and 54. Again, a detailed investigation of which residues are essential for antibody binding has not been performed and was not the goal of our study. The main intention was to show that epitopes are located in the variable part of the gp116 molecule. The different recognition pattern of MAb CH86 compared to the other mouse MAbs indicated that, at least in the mouse, antibodies with different binding specificities can be formed. In the immunoblot analysis with human sera, this difference was not observed. However, this does not rule out the possibility that antibodies with different specificities are developed during natural infection because in this type of assay minor changes in signal strength cannot be detected. Antibodies directed against site II are strain-specific. This was shown in immunoprecipitation analysis using as antigen the viral polypeptides derived from infected cell lysates, and immunoblots of fusion proteins expressed in prokaryotic cells.

It is believed that antigenic variation among virus strains serves to evade the immune system of the host. For site II this is not immediately clear. Neither mouse MAb CH86 nor antibodies developed against this region during natural infection seem to have a biological function in terms of neutralizing the virus in tissue culture. However, our results do not definitely rule out such a possibility. Mouse MAbs do not necessarily have the same biological function as the corresponding human antibodies and the complement-dependent neutralizing capacity of MAb CH408 is indicative of that. The human antibodies tested underwent affinity purification, which could potentially damage their function. The isolation of additional human MAbs reactive with this region will help clarify this question. In addition, antibodies which are negative in in vitro tests have been shown to be able to protect from infection in vivo (Farrell & Shellam, 1991). Alternatively, the genetic differences between strains might be unrelated to immunological pressure, but rather reflect variations independent of this selection, i.e. ability to replicate in different host cells.

In our analysis we have found 33% of randomly selected human HCMV-positive sera to be reactive with site I. This is comparable to our previous results of 40% positivity. The fraction of sera recognizing site II cannot be calculated from our study because we excluded all antibodies specific for strains other than AD169 and Towne. Therefore the observed frequency of 25% represents the lower limit of seropositivity. However, a recent analysis has shown that the variations between strains are not random and that probably only a limited number of strains circulate in the population (Chou & Dennison, 1991; Lehner et al., 1991). In most cases, an antibody response against site II seems to be accompanied by seropositivity for site I; the development of antibodies against site II alone seems to be a rare phenomenon. Serological analysis has revealed that only a limited number of individuals develop antibodies against the antigenic domain on the N-terminal part of gp116 during natural infection. The underlying mechanism, as well as the significance, of this observation for the clinical outcome of HCMV infection, needs further investigation.

Heterogeneity of HCMV isolates at the antigenic level is a well established phenomenon (Waner & Weller, 1978). However, the tools to investigate this heterogeneity at the molecular level have become available only in recent years. A number of reports have described differences between strains with respect to recognition and/or neutralization by MAbs, or differences in immunogenic parts of glycoproteins such as gp58/116 or gp86 (Baboonian et al., 1989; Rasmussen et al., 1984, 1985b; Lehner et al., 1991; Chou & Dennison, 1991; Urban et al., 1992). In addition, epitopes recognized by the cellular immune response also seem to be variable between strains, as has been shown for gp116 as well as the major immediate early protein (Alp et al., 1991; Liu et al., 1991). Therefore it seems that antigenic variability could represent an important mechanism by which HCMV survives in the human host.
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References


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