Glycoprotein H of human cytomegalovirus is a major antigen for the neutralizing humoral immune response

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A recombinant baculovirus expressing glycoprotein H (gpUL75) of human cytomegalovirus was used to examine the humoral immune response in naturally infected individuals. Recombinant baculovirus infected insect cells produced two forms of gH with molecular masses of 78–82 kDa and 94 kDa. The 94 kDa polypeptide was modified by high mannose oligosaccharide side-chains as shown by reduction in molecular mass after treatment with endoglycosidases H and F. The 78–82 kDa protein represented the non-glycosylated precursor which was resistant to the enzymes. In contrast to gH expressed in mammalian cells, the recombinant baculovirus expressed gH was transported to the cell surface. Glycoprotein H produced in insect cells was reactive with human convalescent sera and all tested neutralizing monoclonal antibodies recognizing either linear or conformational epitopes. Antibodies reacting with insect cell derived gH were detected in 96% of HCMV seropositive human sera. Using insect cells infected with the gH expressing recombinant baculovirus as immunoabsorbent, between 0% and 58% of the total virus neutralizing activity was removed from sera of individuals with a past HCMV infection. gH must therefore be considered a major antigen for the induction of neutralizing antibodies during natural infection.

Introduction

Human cytomegalovirus (HCMV) remains a significant pathogen in individuals with a compromised or immature immune system. In contrast, infection of immunocompetent individuals is of limited consequences in the vast majority of cases, indicating the importance of a functioning immune response in the control of HCMV infections (Ho, 1993). Immune responses which control HCMV are incompletely understood. By analogy to other human herpesviruses it can be assumed that both cellular and humoral immune responses play an important role (Kohl, 1992). For murine CMV it was shown that either a cytotoxic T cell response or the passive transfer of neutralizing antibodies is sufficient to protect against a lethal challenge (Rapp et al., 1993; Reddehase et al., 1987). After reactivation, neutralizing antibodies are capable of reducing virus load and dissemination (Jonjic et al., 1994). In the case of HCMV, direct evaluation of immunological effector functions is difficult since the virus is strictly species specific and no animal model system is available. Indirect support for the importance of the cellular immune response in controlling HCMV infection comes from transplant patients in whom the presence of HCMV specific cytotoxic T cells correlates with favourable outcome of the infection (Reusser et al., 1991; Riddell & Greenberg, 1994). On the other hand, the maternal humoral immune response against the virus seems to be important in preventing HCMV disease in the newborn (Fowler et al., 1992). Moreover, in a recent vaccination study it was shown that protection from reinfection is correlated with neutralizing antibodies (Adler et al., 1995).

With respect to the neutralizing humoral immune response the viral envelope proteins appear to be the principal targets of protective antibodies. Glycoprotein B (gB, gpUL55) represents a major target of this response. Antibody preabsorption experiments with recombinant derived gB have shown that in some human sera a considerable fraction of the neutralizing response is directed against gB (Britt et al., 1990; Marshall et al., 1992). The nature of additional antigens inducing significant amounts of neutralizing antibodies during natural infection is not known. In view of the clinical importance of HCMV infections the identification of these antigens seems highly desirable with regard to immunoprophylaxis. One possible candidate is glycoprotein H (gH, gpUL75). Like gB, gH is
conserved among all human pathogenic herpesviruses and is crucial for a number of steps during virus replication. During natural infection the protein induces humoral and cellular immune responses (Beninga et al., 1995; Rasmussen et al., 1991; Urban et al., 1992). An immunodominant antigenic domain (AD86) has been identified between amino acids 34 and 43 (Urban et al., 1992). AD86 is capable of inducing neutralizing antibodies and reactivity was detected in 35% of healthy HCMV seropositive blood donors. A monoclonal antibody (MAb) was developed against this site and was shown to recognize gH in denatured form (Urban et al., 1992). Interestingly, all gH specific MAbs described subsequently recognize conformational rather than linear epitopes (Baboonian et al., 1989; Cranage et al., 1988; Rasmussen et al., 1984; Simpson et al., 1993). Structures which bind these MAbs have not been characterized in detail. However, using a set of seven murine MAbs Simpson et al. (1993) have determined two non-overlapping antigenic sites that are bridged by a third site. Immunological studies on gH are complicated by the fact that in mammalian cells the protein requires additional polypeptides for correct processing and transport to the cellular membrane. During the infection process the viral glycoprotein gL (gpUL115) most probably serves this purpose since co-expression of recombinant gH and gL results in transport of gH to the cell surface (Kaye et al., 1992; Spaete et al., 1993). This feature is shared with gH homologues from other herpesviruses (Forghani et al., 1994; Hutchinson et al., 1992; Klupp et al., 1994; Liu et al., 1993; Yaswen et al., 1993). As a consequence limited information is available concerning the reactivity of human sera against the intact protein.

We have expressed gH in insect cells using a recombinant Autographa californica nucleopolyhedrosis virus (AcNPV). In contrast to mammalian systems, gH was transported to the cell surface in the absence of co-expression of gL. Our data show that antibodies against gH can be found in nearly 100% of infected individuals. More importantly, between 0–58% of the total HCMV neutralizing capacity in human sera was found to be directed against this protein.

**Methods**

- **Monoclonal antibodies (MAbs).** The MAbs that were used in this study have been described previously: AP6-SA4 (Urban et al., 1992); SDZ 89-109 (Ehrlich et al., 1988); 14-4b, 109, 5, 442 and 115 (Simpson et al., 1993); 27-287, 27-156 and 7-17 (Utz et al., 1989).

- **Construction of recombinant baculoviruses.** Plasmid pBW3 (Urban et al., 1992) was digested with NcoI and HindIII. A 2443 bp fragment was isolated, made blunt ended and inserted into the Smal site of fusion vector pAc401 (Luckow & Summers, 1988). Sf158 insect cells were cotransfected with the gH-containing plasmid (pBV-gH/AD) and BaculoGold wild-type DNA (Dianova) following the manufacturer's instructions. Expression of gH was monitored by indirect immunofluorescence of infected cells using MAbs AP6-SA4. One clone was selected following two rounds of plaque purification and designated BV-gH/AD. Baculovirus Bac 3.1 expressing glycoprotein gB of HCMV was described previously (Wells et al., 1990). Baculovirus BV-lck expressing the human T cell-specific protein tyrosine kinase Lck (p56Lck) was kindly provided by S. Lang (Institut für Virologie, Erlangen).

- **Expression of recombinant proteins, SDS–PAGE and immunoblotting.** Sf158 cell monolayers were infected at a multiplicity of approximately 10 p.f.u./cell and incubated at 28 °C for 60–70 h. Cells were pelleted by centrifugation, washed twice in PBS and resuspended in 1:5 ml PBS per 150 mm² tissue culture flask. Cells were disrupted by three rounds of rapid freezing and thawing and two additional sonication steps. The lysate was adjusted to a final concentration of 10 mM-MgCl₂, 1 mM-MnCl₂ and 10 μg/ml DNase I and incubated on ice for 2 h. Lysates were diluted 1:1 in urea–sample buffer (4% SDS, 2% β-mercaptoethanol, 8 M-urea) and incubated for at least 1 h at room temperature; samples equivalent to 1 x 10⁶ infected insect cells were run on 8% polyacrylamide gels. For immunoblot assays proteins were partially purified. Soluble insect cell and baculovirus derived proteins were removed from cell lysate by centrifugation. The protein pellet was resuspended in PBS and added slowly to 8 M-urea resulting in a 6 M-urea solution (1.5 ml per 150 mm² tissue culture flask). After stirring overnight at 4 °C and a second centrifugation step, the final protein pellet was resuspended directly in 8 M-urea, stirred overnight at 4 °C and diluted in urea–sample buffer. Immunoblot analysis following electrophoretic separation of proteins in 8% gels was done by standard procedures. The blots were blocked in PBS, 0.1% Tween 20, 5% powdered milk and incubated overnight at 4 °C with human sera diluted 1:50 in PBS, 0.1% Tween 20, 3% powdered milk. Binding of antibodies was detected after incubation with alkaline phosphatase coupled goat anti-human IgG by staining with BCIP and NBT.

- **Endoglycosidase digestion.** Digestions with peptide:N-glycosidase F (Endo F) and endoglycosidase H (Endo H) were done according to the manufacturer's (New England Biolabs) instructions. The digested protein solutions were diluted in urea–sample buffer and samples equivalent to 1 x 10⁶ BV-gH/AD infected insect cells or HCMV particles isolated from 1 x 10⁶ infected human foreskin fibroblasts (HFF) were resolved by 8% SDS–PAGE.

- **Immunofluorescence analysis.** For cell surface immunofluorescence Sf158 cells grown on Lab-Tek chamber slides (Nunc) were infected with 5 p.f.u./cell. After 40 h, cell monolayers were incubated with the respective monoclonal antibody for 1 h at 4 °C. Antibody binding was detected using FITC-conjugated goat anti-mouse IgG (Dako), diluted 1:40 in PBS, 3% BSA. After each incubation cells were washed twice in PBS, 3% BSA at 4 °C. For counterstaining of unlabelled cells, monolayers were washed in PBS, reacted with 0.001% Evan’s Blue for 5 min followed by an additional wash in PBS. For indirect immunofluorescence of permeabilized cells, infected Sf158 cells were fixed in ice-cold acetone for 15 min, dried and stored at -20 °C. Immunofluorescence was performed as described above, but with incubations at room temperature and with PBS, 0.1% Tween 20 used for dilution and washing.

- **ELISA.** Sf158 cells were infected with recombinant baculoviruses at an m.o.i. of approximately 10 p.f.u./cell and incubated for 60–70 h at 28 °C. Cells from a 150 mm² tissue culture flask were harvested by centrifugation, washed twice in PBS, resuspended in 8 ml of lysis buffer (2% Triton X-100, 0.01 M-glycine, 0.0038 M-Tris, pH 9.2) and incubated for 30 min on ice. After two additional sonication steps insoluble proteins were pelleted by centrifugation, resuspended in 2 ml of lysis buffer, sonicated and again centrifuged. The final protein pellet was resuspended in 2 ml of PBS, stirred overnight at 4 °C and stored at 4 °C. Preparations were controlled on 8% PAGE and in immunoblots. Enriched preparations from gH and gB infected cells contained between 70–80% recombinant antigens. ELISA plates were coated with protein equivalent to 0.7 x 10⁴
Fig. 1. Analysis of recombinant gH expressed in insect cells. Sf158 cells were infected with BV-gH/AD and HFF cells were infected with HCMV strain AD169. HCMV particles were purified from tissue culture supernatant of HCMV infected fibroblasts. Lysates were prepared, separated on PAGE and analysed in immunoblots. Antibody binding was detected with alkaline phosphatase conjugated anti-mouse IgG and the BCIP/NBT system. (a) Lysates from extracellular virus (HCMV), HCMV infected fibroblasts (infected HFF) and BV-gH/AD infected insect cells (BV-gH/AD) were analysed with the gH specific MAb AP86-SA4. (b) Lysates from BV-gH/AD infected cells (BV-gH/AD) and HCMV particles (HCMV) were treated with Endo F and Endo H prior to PAGE. The right-hand panel was developed with MAb 28-77, specific for phosphoprotein pp65 of HCMV, the others with the gH specific MAb AP86-SA4.
Fig. 2. Cell surface expression of gH in insect cells. Sf158 cells cultured on Lab-Tek chamber slides were infected with recombinant baculovirus expressing gH (a, b) or gB (d) or wild-type AcNPV (c) for 40 h. Unfixed living cells (b, d) and acetone fixed permeabilized cells (a, c) were analysed in an indirect immunofluorescence test. MAbs specific for gH (14-4b; a, b, c) or gB (27-156; d) and FITC conjugated anti-mouse IgG were used for detection.
smaller immunoreactive polypeptides which most likely represented truncated proteins were detected by the MAb. Lysates infected with AcNPV did not react with APB6-SA4 (see Fig. 3). The mobility of recombinant gH in SDS–PAGE differed from gH forms found in HCMV infected cells or virions. In HCMV infected fibroblasts the apparent molecular mass of gH was calculated to be 86–96 kDa and 110 kDa and in HCMV particles 94–100 kDa, respectively (Fig. 1a). The differences in the mass of gH are in accordance with published data and most likely were due to modifications of the carbohydrate side-chains during the processing of intracellular to virion associated gH (Bogner et al., 1992). For unknown reasons we observed variations in insect cell derived gH forms between different preparations. The differences were (i) the relative amount of the two prominent polypeptides of 78–82 kDa and 94 kDa, (ii) the presence of the smaller immunoreactive proteins and (iii) the separation of the 78–82 kDa polypeptide into two signals (compare Fig. 1a with 1b). The differences were independent of a number of parameters such as multiplicity and duration of infection, cell density at infection, virus stock and electrophoresis conditions.

The origins of the 78–82 kDa and 94 kDa polypeptides in BV-gH/AD infected cells were further analysed. After treatment with both endoglycosidase F (Endo F) and endoglycosidase H (Endo H), the 94 kDa protein was no longer detectable (Fig. 1b). This indicated that the 94 kDa protein represented glycosylated gH containing Endo H sensitive high mannose oligosaccharide side-chains. The mobility of the 78–82 kDa polypeptides remained unchanged suggesting that these forms were not modified by N-linked glycosylation and possibly represented precursor proteins of gH. Whether the two signals represented different protein conformations of the same polypeptide or carried modifications not sensitive to glycosidase digestion was not determined. When control reactions using HCMV virions were carried out, an increase in mobility of gH was observed after digestion with Endo F but not Endo H (Fig. 1b). This is in agreement with previous data showing that in HCMV infected cells the predominant form of gH was Endo H sensitive, whereas in extracellular virions only Endo F sensitive sugars were found (Bogner et al., 1992). The mobility of phosphoprotein pp65 (ppUL83), a polypeptide which carries no N-linked sugars, was not altered by treatment with either enzyme (Fig. 1b).

To further analyse the antigenically relevant structures of gH expressed in insect cells, indirect immunofluorescence analyses were performed with a panel of MAbS which included the murine antibodies 14-4b, 109, 5, 442, 115 and APB6-SA4, and the human MAb SDZ 89-109 (Ehrlich et al., 1988; Simpson et al., 1993; Urban et al., 1992). Since all these antibodies are capable of neutralizing HCMV it is assumed that they bind to native gH on the envelope of HCMV virions. Binding specificities of the murine MAbS to gH were assigned to at least three different antigenic sites (Simpson et al., 1993). BV-gH/AD infected insect cells were recognized by all MAbS indicating that the structure of recombinant gH was antigenically similar, if not identical, to the conformation of viral gH. It should be mentioned, however, that epitopes formed by the gH–gL complex could not be analysed in this experiment.

### Plasma membrane localization of gH in insect cells

Glycoprotein H is not transported to the surface of mammalian cells when expressed alone (Cranage et al., 1988). For efficient transport to the plasma membrane a second viral protein, gL, is required (Kaye et al., 1992; Spaete et al., 1993). Whether gH expressed in insect cells requires gL for intracellular transport was investigated by indirect immunofluorescence analysis. Cells were infected with BV-gH/AD (5 p.f.u./cell) for 40 h and either incubated directly with MAb 14-4b or permeabilized by treatment with acetone prior to incubation with antibody. Permeabilized cells exhibited diffuse cytoplasmic staining (Fig. 2a). In unfixed viable cells, readily detectable surface immunofluorescence was observed with MAb 14-4b (Fig. 2b). Staining was evenly distributed over the entire plasma membrane. In contrast, surface immunofluorescence of cells infected with baculovirus Bac 3.1 expressing HCMV gB, as detected with MAb 27-156, revealed patchy staining (Fig. 2d). No specific signals were observed in SF158 cells infected with wild-type AcNPV using either antibody (Fig. 2c).

### Recognition of insect cell derived gH by HCMV seropositive human sera

The data indicated that gH expressed in insect cells was synthesized as a protein which, with respect to antibody recognition, closely resembled viral gH. This suggested that recombinant gH could provide a suitable reagent for detection of anti-gH antibodies in human sera. A total of 30 sera from healthy HCMV seropositive individuals was studied. Four sera showed non-specific reactions in at least one of the three tests described below; the remaining 26 sera gave unequivocal results. Initially, immunoblot analyses were carried out using partially purified gH as antigen (see Methods). Control antigens included wild-type AcNPV and baculovirus derived gB. As can be seen in Fig. 3, lysates from gB infected insect cells contained mainly an immunoreactive protein of 150 kDa when analysed with a gB specific MAb. This is in agreement with previous reports demonstrating that gB expressed in insect cells consists almost entirely of the uncleaved 150 kDa precursor molecule (Wells et al., 1990). In the case of BV-gH/AD, human sera reacted with both the 78–82 kDa and 94 kDa polypeptides (Fig. 3). There was no difference in signal strength, suggesting that glycosylation did not influence antibody binding in this assay. The gH specific antibody titres varied greatly between individual sera and in relation to gB as illustrated (Fig. 3). In total, 12 of the 26 sera (46%) were positive for gH (Table 1) and all for gB (100%). The sera were
Fig. 3. Recognition of denatured recombinant gH by human sera. Human sera (19, 21, 22, 23) were analysed in an immunoblot assay with gH expressed in insect cells. Insoluble proteins from BV-gH/AD infected cell lysates were dissolved in 8 M-urea and used as antigen (gH). Baculovirus expressed gB (gB) and wild-type AcNPV (WT) served as controls. The blot shown in the left panel was developed with a combination of two MAb specific for gH (AP86-SA4) and gB (27-287). Experimental conditions were as described in the legend to Fig. 1.

Table 1. Reactivity of HCMV seropositive human sera with gH

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<th>Antigen</th>
<th>Seropositivity rate</th>
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<tr>
<td>AD86*</td>
<td>10/26 (38%)</td>
<td>+</td>
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<tr>
<td>Denatured gH†</td>
<td>12/26 (46%)</td>
<td>+</td>
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<tr>
<td>Non-denatured gH‡</td>
<td>25/26 (96%)</td>
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10/26 (38%) 2/26 (8%) 13/26 (50%) 1/26 (4%)  

* Reactivity of human sera in immunoblot assays using fusion protein Exo86-35 representing the aminoterminal linear antigenic domain (AD86) of gH as antigen.  
† Reactivity of human sera with gH expressed in insect cells in immunoblot assays.  
‡ Reactivity of human sera with gH expressed in insect cells in an ELISA under non-denaturing conditions.

also tested in immunoblot assays using a bacterial fusion protein, Exo86-35, representing the amino-terminal linear antibody binding site, AD86 (Urban et al., 1992). Ten of the gH-reactive sera (38%) also recognized AD86. Two sera (8%) were AD86 negative indicating that an additional non-conformational antibody binding site was present on gH (Table 1).

An ELISA was developed to measure conformation-dependent gH specific antibodies in human sera. The criterion for native structure of recombinant gH was reactivity with both the murine MAb 14-4b and the human MAb SDZ 89-109, both of which recognize conformational epitopes. The specificity of the ELISA was tested using HCMV negative human sera and MAbs. In all analyses an identically prepared control antigen, consisting of insect cells infected with a recombinant baculovirus expressing human protein-tyrosine kinase Lck, was included and used to determine the background activity of each human serum against insect cell or baculovirus derived antigens. We found this control antigen superior to AcNPV infected cells since in immunoblot we observed reactivity of a significant fraction of human sera with polyhedrin, which is present in large amounts in AcNPV
Neutralizing immune response against gH of HCMV

Neutralization capacity of gH specific antibodies in human sera

Neutralizing gH specific human MAbs have been derived from HCMV seropositive donors; however, the contribution of anti-gH antibodies to the HCMV neutralizing response is unknown. To analyse the relationship between neutralizing activity and gH antibody levels, gH positive human sera were absorbed with increasing amounts of BV-gH/AD. Lysates of BV-lck infected cells were included in all assays and used to correct for non-specific reduction of neutralizing activity. Results were confirmed in at least two independent assays. Specificity of the test system was controlled by the use of MAb AP86-SA4. A progressive decline in neutralizing activity was noted when lysates from $5 \times 10^4$ to $2.5 \times 10^6$ BV-gH/AD infected cells were used as absorbent, with $2.5 \times 10^6$ cells resulting in a complete depletion of neutralizing activity. Lysates from BV-lck infected cells did not reduce the neutralizing activity of antibody AP86-SA4 to a significant extent (data not shown). We then examined four human sera in an identical fashion. In this group of sera, the extent of reduction varied between individual sera. In three sera (31, 32 and 21) a progressive decline in neutralizing activity was observed after absorption with BV-gH/AD as compared to BV-lck. The low level reduction in neutralizing capacity seen with BV-lck was most probably due to non-specific absorption. Neutralizing activity for serum 25 was identical after absorption with either antigen, indicating little contribution of gH specific antibodies to the neutralization activity of this serum (Fig. 4). Additional sera were absorbed with a single dose ($5 \times 10^5$) of BV-gH/AD and BV-lck infected cells, respectively, and reduction in neutralizing activity was corrected for values obtained with BV-lck. Within our test panel of ten specimens, reductions in neutralizing activity between 0% and 58% were observed (Table 2). These results suggested that gH specific antibodies contributed significantly to the overall neutralizing capacity in some individuals infected with HCMV. Furthermore, these results also confirmed the high gH

infected cells (data not shown). Using this assay, the seropositivity rate in our serum panel increased to 96% suggesting that 50% of the sera had antibodies against gH which could not be detected with denatured antigen. ELISA determined seropositivity for gB in this panel of sera remained 100% (data not shown).
specific seropositivity rate detected using the gH ELISA since reduction in neutralizing activity was seen in nine out of ten sera.

Discussion

In this study, we have shown that following natural infection HCMV gH induced an antibody response in nearly 100% of individuals and that a considerable fraction of the virus neutralizing antibody response in some human sera consists of gH specific antibodies, thereby identifying gH as an immunodominant antigen for the humoral immune response. The results were made possible by the isolated expression of gH in insect cells resulting in an antigen which was indistinguishable from viral gH with respect to antibody recognition.

In insect cells the predominant forms of gH contained high mannose sugars, an observation that is compatible with the glycosylation pathways in insect cells (Gooch et al., 1991; Kuroda et al., 1990). Proteins containing high mannose oligosaccharides have also been reported to represent the predominant form of gH found in HCMV infected fibroblasts. The slight differences in electrophoretic mobility between recombinant gH and the gH found in infected cells was probably the result of different length of oligosaccharide side-chains added by the insect cell specific glycosylation system (Kuroda et al., 1990). Conversion of high mannose sugars to complex sugars in HCMV infected cells seems to be a slow and inefficient process since this modification can be detected only in gH derived from plasma membranes of infected cells or virions (Bogner et al., 1992). In contrast to HCMV infected cells, insect cells contained additional non-glycosylated forms of gH. The most likely explanations for the non-glycosylated forms are overexpression of gH in insect cells leading to cytoplasmic, non-glycosylated protein or inefficient usage of the gH specific signal sequence. Our findings with HCMV gH expressed in insect cells were strikingly similar to herpes simplex virus (HSV) gH (Ghiasi et al., 1991).

We were able to detect baculovirus derived gH in the plasma membrane of infected cells. This is in clear contrast to mammalian cell systems in which HCMV gH, if expressed alone, is found exclusively in the cytoplasm and/or the nuclear membrane (Cranage et al., 1988). Processing and transport of gH in mammalian cells appears to require additional polypeptides such as gL (Kaye et al., 1992; Spaete et al., 1993). There are several possible explanations for the transport in insect cells of gH to the cell surface in the absence of additional viral proteins. (i) Transport processes in insect cells are regulated differently than in mammalian cells. This explanation seems unlikely since expression of a large number of glycoproteins in insect cells has shown that cellular localization is similar to that in mammalian cells. (ii) A transporter protein is encoded by AcNPV or the insect cell. The observation that proteins other than gL could mediate transport of gH to the plasma membrane is in agreement with such a possibility (Spaete et al., 1993; Duus et al., 1995). (iii) The high expression level of gH in the insect cell results in a detectable number of gH molecules in the membrane, whereas in the mammalian systems the smaller number of molecules expressed escape detection. Again the results are in line with those reported for HSV gH. In mammalian expression systems, HSV gH also requires co-expression of gL (UL1) for surface expression, whereas expression in insect cell results in transport of the protein to the cell membrane in the absence of additional viral polypeptides (Ghiasi et al., 1991; Roberts et al., 1991; Foa-Tomasi et al., 1991; Gompels & Minson, 1989).

Analysis of recombinant gH indicated that the molecule was antigenically closely related to viral gH. This was suggested by the recognition of virus neutralizing MAbs which bind to three independent conformational domains on gH (Simpson et al., 1993). Under denaturing conditions only 38% of randomly chosen HCMV positive sera reacted with insect cell derived gH. This is in agreement with previous investigations using bacterially derived fusion proteins (Urban et al., 1992). More importantly, the seropositivity rate for gH was nearly 100% when non-denatured antigen was used in ELISAs, suggesting that during natural infection approximately 50–60% of individuals develop antibodies which are entirely dependent on higher order structure of gH. The importance of conformational epitopes for antibody recognition was also emphasized by the fact that denaturing gH with urea prior to its use in ELISA resulted in seropositivity rates comparable to the immunoblots (data not shown). This is in contrast to the second immunodominant envelope protein, gB, where seropositivity rates for native or denatured protein were identical. The high percentage of conformation-dependent antibodies against gH could also provide an explanation for the findings that gH specific MAbs, if not developed against prokaryotic fusion proteins, recognize conformational epitopes (Baboonian et al., 1991; Gompels & Minson, 1989).

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et al., 1989; Cranage et al., 1988; Rasmussen et al., 1984; Simpson et al., 1993).

Limited information is available on the immune response against gH during natural infection. L. Rasmussen and colleagues investigated gH of HCMV Towne strain, expressed in mammalian cells, for detection of antibodies in human sera. Using immunoblot analyses and immunoprecipitations with a truncated gH molecule, they reported a seropositivity rate for gH in healthy individuals between 0-10% (Rasmussen, 1993; Rasmussen et al., 1991). Several explanations could account for the significant discrepancy between our studies and those of Rasmussen. Demonstration of antibodies against gH is crucially dependent on the immunological detection system. During natural HCMV infection in some individuals, gH induces exclusively IgG3 antibodies which cannot be detected through binding of Protein A, a reagent which is routinely used in immunoprecipitation assays (Urban et al., 1994). Alternatively, depletion of parts of the primary sequence of gH in combination with expression in mammalian cells could result in a conformational change in some important epitopes on gH leading to the loss of antibody binding sites. Lack of reactivity of gH specific antibodies with recombinant protein derived from mammalian systems has been described for other herpesviruses (Foa-Tomasi et al., 1991; Forrester et al., 1991; Gompels & Minson, 1989; Roberts et al., 1991; Yaswen et al., 1993).

The neutralizing immune response against HCMV is incompletely characterized. More than 50 coding sequences for glycoproteins or exons of glycoprotein genes have been deduced from the nucleotide sequence of the HCMV genome. Only a small fraction of these have been identified as targets of neutralizing MAbs (Spaete et al., 1994). The contribution of the respective antibodies to the overall neutralizing response is not known. Experiments using insoluble gB antigen or intact cells infected with a vaccinia virus–gB recombinant resulted in removal of 0-100% of neutralizing activity from convalescent sera clearly showing that additional proteins must contribute significantly to the induction of a neutralizing antibody response (Britt & Vugler, 1992; Marshall et al., 1992). In our study, absorption of ten sera with acetone fixed BV–gH/AD infected cells resulted in a median loss of 29% of neutralizing activity. The range of reduction was between 0-58%. The lower range found with gH cannot necessarily be taken as evidence for a minor contribution of gH specific antibodies to the overall neutralizing activity. Fixation of insect cells using acetone could potentially alter antigenic structures necessary for binding of certain types of antibodies. In addition, gH specific MAbs show a wide range of neutralizing activity when different HCMV strains are compared (Baboonian et al., 1989; Rasmussen et al., 1984; Simpson et al., 1993). This could indicate that despite the high homology found between gH proteins from various isolates (Chou, 1992), strain specific recognition and neutralization takes place, mediated through minor differences between different gH molecules. In our system we could remove only antibodies cross-reacting with gH from strain AD169. It is very likely that using a homologous system higher reduction rates of neutralizing antibodies would be possible. The situation is different from gB where most neutralizing antibodies are directed against antigenic domains AD-1 and AD-2 which are conserved among strains (Chou & Dennison, 1991; Lehner et al., 1991; Roy et al., 1993).

The serological data that we have obtained must also be interpreted in the context of complex formation with gH. Many studies on different herpesviruses have demonstrated the functional interaction of gH with gL. The nature of this interaction, however, seems to be different between different herpesviruses and in at least two cases (HCMV and Epstein–Barr virus) additional proteins seem to be involved (summarized by Forghani et al., 1994). For HCMV, Gretch et al. (1988) observed a number of high molecular mass signals (the gCIII complex) when proteins from extracellular virions were immunoprecipitated using a gH-specific MAb antibody and analysed under non-reducing conditions. Upon reduction, glycoproteins of 145 kDa and 86 kDa were identified indicating disulphide linkage of the polypeptides. A gH associated glycoprotein of similar size has also been observed by Bogner et al. (1992). Both proteins seem to be involved in the humoral immune response. The gene encoding gp145 has not been identified. In the infected cell, interaction of gH with gL has been demonstrated. This interaction also seems to involve formation of disulphide bonds (Kaye et al., 1992; Spaete et al., 1993). However, it remains unclear whether these proteins are similarly associated in extracellular virions. Furthermore, the importance of the interaction between gH and gL in the formation of complex antigenic determinants which are not present on gH or gL is unclear. Antigenicity of a gH–gL complex of HSV-1 was only marginally enhanced when compared to gH alone (Browne et al., 1993). The seropositivity rate of 98% for gH that we have found is unlikely to be exceeded by more complex antigenic structures such as complexes of gH plus gp145 and/or gL. Our estimation of the contribution of gH to the overall neutralizing response must be considered a minimal estimate since it is conceivable that the complex formation and/or authentic glycosylation in mammalian cells result in a more native form of gH which could remove additional gH specific antibodies from human sera in absorption experiments.

Recent data from the murine model have provided evidence for the importance of the humoral immune response in limiting virus dissemination as well as protection from reactivation (Jonjic et al., 1994; Rapp et al., 1993). Our study has identified gH as an important component of the neutralizing antibody response to HCMV. Consequently, gH should be considered as a candidate component of HCMV subunit vaccines.

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References


Luckow, V. A. & Summers, M. D. (1988). Signals important for high-


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