Molecular analysis of the immune response to human cytomegalovirus glycoprotein B. I. Mapping of HLA-restricted helper T cell epitopes on gp93


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Human cytomegalovirus (HCMV) is one of the most common causes of congenital infection leading to birth defects, and a leading cause of serious illness in patients with impaired cell-mediated immunity. Helper T cell (Th) responses to HCMV proteins are likely to be important in limiting viral replication and preventing disease. Previous studies from this laboratory have demonstrated that the amino-terminal 513 amino acids of HCMV glycoprotein B (gB) can stimulate both B and T cell responses in humans. In the present study, the proliferative responses of HCMV-specific Th clones to recombinant proteins and synthetic peptides were examined to identify four Th epitopes on gp93, which represents the amino-terminal 460 amino acids of the gB polypeptide. Using clones of known HLA restriction specificity from several donors, it was shown that each HLA class II allele preferentially associates with a different epitope on gB. Five clones from two different donors recognized an epitope in the region of amino acids 250 to 264 restricted by DR4Dw14, two clones from different donors recognized an epitope in the region of amino acids 420 to 434 restricted by DR7Dw17, two clones from different donors recognized an epitope in the region of amino acids 178 to 194 restricted by DQw1 and a single clone recognized an epitope in the region of amino acids 190 to 204 restricted by DPw4. Although all peripheral blood mononuclear cells (PBMCs) expressing a particular HLA class II allele were able to present the appropriate HLA-restricted gB peptide to gB-specific Th clones, not all individuals expressing a given HLA allele exhibited PBMC responses to the corresponding gB peptide. The HLA-related differences in Th recognition of specific epitopes on gB described in this report may have important implications in virus-host interactions and vaccine strategies.

Introduction

Human cytomegalovirus (HCMV) is the most common cause of congenital infection leading to birth defects in the U.S.A., and is a frequent cause of serious illness in immunocompromised patients (Gehrz, 1991). Characteristically, these patients have impaired cellular immunity and prolonged HCMV viraemia or viruria (Gehrz et al., 1977, 1991; Balfour et al., 1977). Helper T cells (T_h) presumably play a major role in host defence against persistent intracellular viruses such as HCMV, both as effector cells which produce antiviral cytokines such as γ-interferon and by providing help to HCMV-specific B and cytotoxic T cells (T_c). Therefore, defects in HCMV-specific T_h responses may predispose individuals to ongoing viral replication, cell-to-cell spread and multi-system disease.

HCMV contains at least three families of glycoprotein complexes designated gcI (or gB), gcII and gcIII (or gH), each containing two or more constituent glycoproteins (Britt & Auger, 1986; Kari et al., 1986, 1990; Gretch et al., 1988a, b). The gcI complexes contain two mature glycoproteins, gp93 and gp55, representing the amino- and carboxy-terminal portions of a precursor polypeptide encoded by a single gene (UL55) exhibiting similarity to gB of herpes simplex virus (HSV) (Spaete et al., 1988; Kari et al., 1990). Several complement-independent neutralizing monoclonal antibodies (MAbs) to gp93 have been obtained, suggesting that human antibodies to this glycoprotein may be important in preventing virus infectivity and cell-to-cell spread (Kari et al., 1990; Meyer et al., 1992). We have previously demonstrated that peripheral blood mononuclear cells (PBMCs) from some seropositive individuals proliferate well when stimulated with the gB family of glycoproteins and complexes, whereas others exhibit little or no response to these proteins (Liu et al., 1988a). Furthermore, we have detected gp93-specific serum neutral-
izing antibodies and Tc activity in donors with gp93-specific Tc responses (Liu et al., 1991). A lack of gp93-specific Tc responsiveness was associated with a lack of gp93-specific antibodies and Tc activity. This suggests that Tc recognition of epitope(s) on gp93 is required to provide cognate help to gp93-specific B cells and Tc. In this report, we have identified four Tc epitopes on gp93 by analysing the proliferative responses of gB-specific Tc clones to recombinant proteins and synthetic peptides representing overlapping segments of the amino-terminal 460 amino acids of the gB polypeptide.

HCMV-specific Tc recognition involves the uptake and processing of viral proteins by antigen-presenting cells (APCs), and re-expression of antigen peptides in association with HLA class II molecules on the surface of the APCs (Kourilsky & Claverie, 1989). We previously reported that HCMV-specific Tc clones may be restricted by DR-, DQ-, and DP-encoded molecules based on selective blocking with anti-class II MAbs and panel studies using irradiated PBMCs of known HLA haplotypes as APCs (Gehrz et al., 1987). We determined that HLA-restricted Tc recognition of HCMV involves the lymphocyte-defined Dw determinants in the putative peptide binding site and not the serologically defined determinants. Moreover, more than 70% of HCMV-specific Tc clones derived from multiple donors were restricted by DR molecules, with the remaining clones equally distributed between DQ and DP. Since certain DQ-encoded alleles, and to a lesser extent DP alleles, are expressed in linkage disequilibrium with particular DR alleles (Reinsmoen & Bach, 1989), it is likely that the phenotypic Tc response to gB will be defined by the HLA-DR/Dw-DQ haplotypes expressed by a given donor. Consistent with this hypothesis, we have recently shown that the magnitude of the PBMC response to gB varies between donors expressing different HLA-DR/Dw haplotypes (Gehrz et al., 1991). In the present study, we have used gB-specific Tc clones to examine the role of specific HLA class II alleles in the immune recognition of individual Tc epitopes on gp93, and to determine the importance of HLA polymorphism in defining the polyclonal PBMC response to gB.

**Methods**

**HCMV antigen preparation.** HCMV antigen was prepared by purification of Towne strain HCMV virions and dense bodies as previously described (Gehrz et al., 1980).

**Glycoproteins expressed in vaccinia virus.** Recombinant vaccinia viruses containing the entire HCMV gB gene (vac-gB) and Tn5 transposon-induced C-terminal deletion mutants of gB (vac-gBm255, m305) were generated as previously described (Liu et al., 1991). The sites of Tn5 insertion for m255 and m305 were determined to be at 764 and 913 nucleotides after the initiation codon. Therefore, the corresponding C-terminal truncated proteins are deleted after amino acids 255 and 305 of gB. Vaccinia virus-gp93 and gBm70 recombinant viruses were generated by cloning the PCR-amplified segments of the gB gene coding for amino acids 1 to 460 and 1 to 70, respectively, into the pSF11 vaccinia virus vector (a generous gift from B. Moss, NIH, Bethesda, Md., U.S.A.). The PCR products were then introduced into vaccinia virus by homologous recombination as described previously (Mackett et al., 1987).

Recombinant glycoproteins gB, gp93, gBm305 and gBm255 were immunopurified from lysates of HuTK" cells infected with the corresponding vaccinia virus recombinants using biotinylated MAb 3C2 (which reacts with an epitope in the region of amino acids 50 to 77) according to methods previously described (Gretch et al., 1987). Recombinant proteins of the appropriate M, were identified by Western blot analysis (data not shown).

**Fusion proteins generated in prokaryotic systems.** Prokaryotic fusion protein vectors pRIT2T (Pharmacia), which produces Protein A fusion proteins, and pAX-4a" (USB Co.), which produces β-galactosidase fusion proteins, were used to clone various N-terminal and C-terminal deletions of the gB gene in the region encoding gp93. The PCR products encoding gB(71 to 162) and gB(163 to 255) were inserted into pRIT2T; PCR products encoding gB(1 to 350), gB(1 to 399) and gB(1 to 460) were inserted into pAX-4a".

Protein A fusion proteins were purified by immunopurification on a Protein G column; bacterial lysates containing β-galactosidase fusion proteins were heat-inactivated and used as antigens in proliferation assays. Recombinant fusion proteins of the correct M, were detected by SDS-PAGE (data not shown).

In vitro synthesis of gB peptides. Overlapping 15 amino acid peptides (15-mer) in regions of gB shown to contain potential Tc epitopes by analysis with recombinant proteins were synthesized on an Applied Biosystems Model 430A peptide synthesizer using the standard t-Boc solid-phase peptide synthesis chemistry developed by Merrifield (1963). Completed peptides were cleaved from the resin and protecting groups removed using trifluoroethane sulfonic acid/trifluoroacetic acid with thiouanisole:ethanediol (2:1 v/v) added as a scavenger (Bergot et al., 1986). Peptides were purified using semi-preparative reverse phase HPLC [Brownlee Prep-10, C-18 (1.0 x 25 cm)] on a Varian 5500 HPLC system. The peptides were then characterized by HPLC [Brownlee Spheri-5 ODS C-18 (4.6 x 220 mm)] and their amino acid composition was determined to confirm their purity and authenticity.

**PBMCs and HCMV-specific T cell clones.** PBMCs were obtained from heparinized peripheral blood of normal adult donors expressing known HLA haplotypes and cryopreserved in 10% DMSO in liquid nitrogen prior to use as described previously (Liu et al., 1988b). HCMV-specific T cell lines were generated from PBMCs of seropositive donors by initial stimulation with Towne HCMV virions, followed by repeated stimulation with HCMV antigen, autologous irradiated PBMCs as feeder cells, and T cell growth factor (TCGF; Biotest) every 7 to 14 days (Liu et al., 1988b). HCMV-specific Tc clones were obtained directly from positive wells of primary limiting dilution cultures of PBMCs, or by limiting dilution of HCMV-specific T cell lines at 0.3 cells/well. Clones were expanded by stimulation with HCMV antigen in the presence of autologous irradiated PBMC and Epstein-Barr virus-transformed B lymphoblastoid cell lines as feeder cells and 15% TCGF. All T cell clones used in these studies exhibited the phenotypic and functional characteristics of Tc, i.e. CD3+4+8-, proliferation specifically to HCMV and endogenous interleukin 2 production.

**APCs.** Autologous or allogeneic PBMCs expressing shared or disparate HLA class II alleles with those of the T cell donor were irradiated with 5000 rads prior to use as APCs. Alternatively, mouse L cells were stably co-transfected with HLA DR or DP α and β cDNAs to express high levels of specific class II αβ heterodimers constitutively (in the laboratory of R. W. Karr) (Klohe et al., 1988). Transfectants used in these studies included L-DR7 expressing DRaDR7/Dw17/Bl,
HLA-restricted T<sub>h</sub> epitopes on HCMV gp93

Results

Analysis of T<sub>h</sub> epitopes on gB with HCMV-specific T<sub>h</sub> clones

Glycoprotein B-specific T<sub>h</sub> clones from several different donors were tested for their reactivity with recombinant proteins expressed in vaccinia virus, pRIT2T and pAX-4a<sup>+</sup>. Results for six representative clones reacting with four different epitopes on gp93 are presented in Table 1. A non-gB-reactive, HCMV-specific T<sub>h</sub> clone, B-C4, was included as a negative control. The epitopes recognized by clones D-T2-41 and D-T3-3 from donor D were located in the region of amino acids 163 to 255, the epitope(s) recognized by clones N-A and N-T2-19 from donor N was located in the region of amino acids 256 to 305 and the epitope(s) recognized by clone 1-10 from donor I and clone K-12 from donor K was in the region of amino acids 400 to 460.

To define further the epitope(s) recognized by clones D-T2-41 and D-T3-3, overlapping 15 amino acid peptides ( pep) from the region of gB(163 to 255) were assayed for their reactivity with D-T2-41 and D-T3-3. Clone D-T2-41 responded to both pep(170 to 184) and pep(178 to 194), although the response to pep(178 to 194) was significantly better than that to pep(170 to 184) (Fig. 1a). These results suggest that the epitope lies in the overlapping region of the two peptides (178 to 184), but adjacent amino acids in pep(178 to 194) were more immunogenic than those in pep(170 to 184). Clone D-
The HLA restriction specificity of the T\textsubscript{h} clones was initially analysed in blocking studies with anti-class II MAb. Clones N-T2-19 and I-10 were restricted by DR, clone D-T2-41 was restricted by DQ and clone D-T3-3 was restricted by DP (data not shown).

The specific HLA class II alleles involved in restricted recognition of HCMV antigen by individual T\textsubscript{h} clones were then determined in panel studies using irradiated PBMCs of known DR/Dw-DQ haplotypes as APCs (Table 2). The DR-restricted clone N-T2-19 responded to HCMV presented by APCs which expressed DR4/Dw14 but not to APCs expressing disparate DR alleles, demonstrating that this clone is restricted by DR4/Dw14. The DR-restricted clone I-10 responded to HCMV presented by APCs which expressed DR7/Dw17 but not to APCs expressing DR3/Dw3 in the absence of DR7/Dw17. These data suggest that clone I-10 is restricted primarily by DR7/Dw17. The DQ-restricted clone D-T2-41 responded to APCs from donors expressing DR2/Dw2-DQw1 but not to APCs expressing DR4/Dw4-DQw3. This clone is therefore restricted primarily by DQw1/Dw2 allele. The DR-restricted clones also exhibited lower but significant responses to HCMV presented by some APCs which expressed disparate class II alleles. This probably is due to non-specific stimulation of the clones by cytokines generated by the irradiated PBMCs in response to viral antigen.

Mouse L cell transfectants that express specific HLA \textit{xp} heterodimers were then used as APCs to confirm the restriction specificity of the clones (Table 2). Synthetic peptides containing gB epitopes recognized by the T\textsubscript{h} clones were used instead of whole HCMV antigen, since L-transfectants may be unable to process the virus sufficiently to express the antigenic peptide in association with the HLA class II molecule (Celis \textit{et al.}, 1988). Transfectants expressing DR7/Dw17 (L-DR7) and DR4/Dw14 (L-Dw14) presented the appropriate gB peptides to T\textsubscript{h} clones I-10 and N-T2-19 respectively, confirming the restriction specificities observed in panel studies using the irradiated PBMCs above. Clone D-T3-3 responded to the appropriate peptide presented by a transfectant which expresses the DPw4 allele (L-DP4), and is therefore restricted by DPw4. No DQw1 transfectant was available for testing clone D-T2-41.

Several additional gB-specific T\textsubscript{h} clones restricted by DQw1/Dw2, DR4/Dw14 and DR7/Dw17 were then tested for reactivity with peptides containing the T\textsubscript{h} epitopes described above (data not shown). In summary, two DQw1/Dw2-restricted clones from different donors (B and D) reacted with gB(178 to 194), four DR4/Dw14-restricted clones from donor N and one DR4/Dw14-restricted clone from donor U reacted with gB(250 to

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**Fig. 1. Mapping of T\textsubscript{h} epitopes on gB93 using overlapping synthetic peptides.** Proliferation assays were set up using 2 x 10\textsuperscript{4} T cells, 10\textsuperscript{5} autologous irradiated PBMCs as APCs, and either HCMV at 1 lag/ml or synthetic peptides over a range of concentrations from 0.5 to 50 \mu g/ml. (a) D-T2-41; (b) D-T3-3; (c) N-A; (d) N-T2-19; (e) I-10; (f) K-12. Data presented here are the optimal responses to each peptide, which were observed at 5 \mu g/ml. The data represent the mean incorporation of [\textit{H}]thymidine in c.p.m for triplicate wells.
Table 2. Proliferative responses of HCMV-specific T\(_h\) clones to HCMV antigen presented by irradiated PBMCs and HLA transfectants as APCs

<table>
<thead>
<tr>
<th>HLA haplotype*</th>
<th>Proliferative response (c.p.m.)†</th>
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<tr>
<td></td>
<td>Antigen§</td>
</tr>
<tr>
<td>APC†</td>
<td>DR DO Dw DP</td>
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<tr>
<td>PBMC-D</td>
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<tr>
<td>PBMC-B</td>
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<td>PBMC-P</td>
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<tr>
<td>PBMC-N</td>
<td>4 3 14 ND</td>
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<tr>
<td>PBMC-I</td>
<td>3,7 2,2 3,17 ND</td>
</tr>
<tr>
<td>PBMC-K</td>
<td>5,7 3,2 5,17 ND</td>
</tr>
<tr>
<td>PBMC-Z</td>
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</tr>
<tr>
<td>L-DW14</td>
<td>4 – 14 –</td>
</tr>
<tr>
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<td>7 – 17 –</td>
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<tr>
<td>L-DP4</td>
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* HLA haplotype analysis was performed using allotype-specific antisera and mixed lymphocyte culture responses to homozygous typing cells of defined HLA specificity. ND, not determined.
† Proliferation assays were set up using 2 \(\times\) 10^4 T cells, 10^5 irradiated PBMCs or 3 \(\times\) 10^4 irradiated L-transfectant cells as APCs, and HCMV antigen or gB peptides. The cells were cultured for 3 days, and \([\text{Z}\text{H}]\text{thymidine} was included in the cultures during the last 16 h. Results are expressed as mean c.p.m. of triplicate wells.
§ Sucrose gradient-purified Towne strain HCMV was used at 1 lig/ml in panel studies with PBMCs as APCs. Synthetic peptides were used at 1 gg/well with L-transfectants as APCs.

264) and two DR7/Dw17-restricted clones from donors I and K reacted with gB(420 to 434).

Presentation of HLA class II-restricted gB epitopes to PBMCs

The results from our studies with T\(_h\) clones suggest that the number of gB epitopes associated with each HLA class II allele is limited, and that each epitope preferentially associates with a single HLA molecule. We therefore wanted to determine whether the polyclonal T cell response to gB also exhibited preferential HLA restriction. First, irradiated PBMCs from donors expressing the DQw1/Dw2 allele (B, C, D and E) responded well to the DQw1/Dw2-restricted peptide gB(178 to 194), suggesting that this epitope contributes significantly to the polyclonal T\(_h\) response to gB in individuals who express DQw1/Dw2 (Fig. 2a). PBMCs from three of five DR4/Dw14-expressing donors (P, N and U) responded to the DR4/Dw14-restricted peptide gB(250 to 264) (Fig. 2b). However, PBMCs from one DR4/Dw14-expressing donor (Q) responded well to gB but not at all to gB(250 to 264) and PBMCs from another donor (R) did not have detectable responses to either gB or gB(250 to 264), despite the fact that PBMCs from both donors were able to present this peptide to the DR4/Dw14-restricted clone. PBMCs from three of the four donors expressing DR7/Dw17 (I, K, and J) responded at low levels to the DR7/Dw17 peptide gB(420 to 434) relative to the responses to gB and a fourth donor (T) did not have a detectable response to this peptide (Fig. 2c). These data suggest that epitope(s) other than gB(420 to 434) may be immunodominant in the DR7/Dw17-restricted response to gB in certain
Fig. 2. Presentation of HLA class II-restricted gB epitopes to PBMCs. PBMCs from donors expressing DQwl/Dw2 (a), DR4Dw14 (b) and DR7Dw17 (c) were stimulated with HCMV, gB and HLA-restricted gB peptides at 10 μg/ml. Proliferation assays were set up using 10^6 PBMCs and antigens. The cells were cultured for 7 days, and [3H]thymidine was included in the cultures during the last 16 h. Results are expressed as mean c.p.m of triplicate wells. Donor HLA haplotypes are as follows: B (DR1,2/DQ1,1/Dw1,2), C (DR2,2/DQ1,1/Dw2,2), D (DR2,4/DQ1,3/Dw2,4), E (DR2,4/DQ1,3/Dw2,4), F (DR4/DQ3/Dw14), N (DR4/DQ3/Dw14), Q (DR4/DQ3/Dw14), R (DR4/DQ3/Dw14), T (DR3,7/DQ2,2/Dw3,17), U (DR4,4/DQ3,3/Dw4,14), S (DR4,4/DQ3,3/Dw4,14), I (DR3,7/DQ2,2/Dw3,17), K (DR5,7/DQ3,2/Dw5,17) and J (DR4,7/DQ2,3/Dw4,17).

Discussion

In this report, we have mapped several discrete T<sub>H</sub> epitopes on gp93, shown that these epitopes are uniquely restricted by specific DR, DQ and DP alleles and demonstrated that the HLA class II molecules expressed by the donor direct the polyclonal T<sub>H</sub> response to particular epitopes on gp93.

There is extensive evidence in guinea-pigs and mice that both T<sub>H</sub> and T<sub>C</sub> responses to given synthetic peptide or naturally occurring protein is dependent on the major histocompatibility complex (MHC)-encoded immune response genes expressed in a given strain (Adorini et al., 1979; Barcinski & Rosenthal, 1977; Heber-Katz et al., 1983; Bastin et al., 1987; Berkower et al., 1986; Townsend et al., 1986; Kojima et al., 1988; Oldstone et al., 1988; Whitton et al., 1988a, b). Recently, multiple epitopes on human immunodeficiency virus (HIV) gp120 have been defined by murine T<sub>H</sub> uniquely restricted by particular H-2 class II alleles in mouse (Kurata et al., 1989). There is more limited evidence of preferential HLA restriction of human T<sub>H</sub> responses to viral proteins. A number of epitopes on viral (glyco)proteins have been described which appear to bind to specific HLA class II molecules, including epitopes on influenza virus haemagglutinin restricted by DR1/Dw1 (Rothbard et al., 1988), rabies virus glycoprotein restricted by DR7/Dw7 (Celis et al., 1988), hepatitis B surface antigen restricted by DPw4 (Celis & Karr, 1989) and gp120 of HIV restricted by DR2/Dw2 and DR4/Dw4 (Siliciano et al., 1988). However, little is known regarding the importance of preferential HLA restriction in defining the polyclonal T<sub>H</sub> responses to these viruses.

Using gB-specific T<sub>H</sub> clones of known HLA restriction specificity obtained from several donors, we have shown that each HLA class II allele studied thus far preferentially associates with a different epitope on gB. Five clones from two different donors recognized an epitope in the region of amino acids 250 to 264 restricted by DR4/Dw14, two clones from different donors recognized an epitope in the region of amino acids 420 to 434 restricted by DR7/Dw17, two clones from different donors recognized an epitope in the region of amino acids 178 to 194 and a single clone recognized an epitope in the region of amino acids 190 to 204 restricted by DPw4. Our studies show that all PBMCs expressing a particular HLA class II allele can present the appropriate
HLA-restricted gB peptide to gB-specific Tₘ clones, irrespective of the other HLA molecules expressed on the APCs. PBMCs from several individuals expressing the same HLA class II allele for which gB epitope specificities have been defined at the clonal level responded to those peptides but not to other gB peptides restricted by unrelated HLA products. However, not all individuals expressing a given HLA allele exhibited PBMC responses to the corresponding gB peptide.

There are several possible explanations for this observation. First, it is known that the affinity of binding of a given peptide to a particular HLA allele is determined by both specific amino acid residues in the antigenic peptide, and critical residues in the binding site of the HLA molecule (Boyer et al., 1990; Krieger et al., 1991; Rothbard & Gefter, 1991; Rudensky et al., 1991; Geluk et al., 1992). Helper T cells specific for peptides that bind with high affinity to their restricting class II molecule may be preferentially expanded. Thus, the relative affinities of gB epitopes for the whole set of class II alleles expressed by a particular individual may influence the immunodominant Tₘ response to gB, and could account for the failure of some individuals to exhibit a detectable PBMC response to gB epitopes restricted by certain expressed HLA alleles. Second, it is known that the T cell receptor (TcR) repertoire is defined in a given individual. Consequently, a certain gB-class II combination may fail to stimulate significant responses owing to lack of Tₘ expressing high affinity TcR specific for that complex.

Another explanation for the lack of a PBMC response to a particular gB epitope could be that the sequence of that epitope in the infecting HCMV strain may be different from that of the HCMV Towne strain. If the donor was not primed to the Towne strain epitope sequence in vivo, it is possible that no PBMC response to this epitope would be detected in vitro. In support of this hypothesis, we have recently shown that HCMV strain sequence polymorphism of the DQw1/Dw2-restricted epitope, 178 to 194, affects the responsiveness of clone T-2-41 (data not shown).

HLA- and non-HLA-related differences in Tₘ recognition of specific epitopes on gB could be important in virus-host interactions and vaccine strategies. Expression of certain HLA haplotypes or infection with HCMV strains containing mutations in relevant HLA-restricted Tₘ epitopes may be associated with diminished gB-specific Tₘ responses, resulting in deficits in cytokine activity or Tₘ-dependent gB-specific antibody or Tₘ responses that could predispose to HCMV infection and disease. Differences in gB responsiveness could be a factor in maternal-fetal transmission of HCMV during pregnancy or dissemination of virus in patients with impaired immunity. Furthermore, preferential HLA restriction of gB (and possibly other HCMV proteins as well) could be a major obstacle in developing an effective subunit vaccine using recombinant viral proteins or peptides, since a substantial portion of the population may not develop Tₘ responses to these molecules.

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References


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