T cell proliferative responses to five human cytomegalovirus proteins in healthy seropositive individuals: implications for vaccine development

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Cell-mediated immunity plays an important role in the host response against human cytomegalovirus (HCMV) infection. For the development of HCMV subunit vaccines it is essential to identify which HCMV proteins can induce protective immune responses in humans. We have studied the T cell proliferative responses to five HCMV proteins (IE1, IE2, pp71, gpUL18 and gB). These five proteins were produced using the maltose-binding protein (MBP) fusion protein system. T cell proliferative responses in 23 seropositive and six seronegative individuals were evaluated. None of the six seronegative individuals showed significant responses to any of the proteins. Of the 23 seropositive individuals, five responded to all five proteins, 14 responded to between one and four proteins and four responded to none of the proteins. The most commonly recognized proteins were gB (17/23, 74%) and IE2 (16/23, 70%). pp71 and IE1 were recognized by 10 of 23 (43%) individuals. Nine of 22 (41%) individuals tested responded to gpUL18, providing evidence that this protein is produced during infection. Our data indicate that a subunit vaccine composed of gB alone may not be sufficient to induce protective immunity in all individuals. The combination of two or three proteins may be more efficient as a potential vaccine.

Introduction

Human cytomegalovirus (HCMV) infection involves 60–80% of individuals in developed countries and virtually 100% of those in developing countries; most of these infections usually remain entirely asymptomatic. However, HCMV infection can cause serious illness, and can even be life-threatening, in immunocompromised individuals including premature infants, transplant recipients and patients with AIDS (Griffiths & Grundy, 1987; Ho, 1990). Therefore, the development of a vaccine is an extremely important approach in prevention of HCMV disease. An experimental HCMV vaccine which consists of live attenuated virus has been tried, but this approach has not been effective (Balfour et al., 1976; Plotkin, 1991; Plotkin et al., 1976). In addition, concerns about the potential for latency, reactivation and induction of chronic disease of such whole virus vaccines have prompted many investigators to search for inert virion subunits that mimic the immunogenic capacity of live whole virus (Grundy, 1991; Plotkin, 1991; Spaete, 1991). To develop a generally applicable and effective subunit vaccine, it is essential to identify which HCMV proteins can induce a protective immune response in humans.

It has been shown that both humoral and cell-mediated immunity appear to be involved in the host defence against HCMV infection (Quinnan et al., 1984; Rasmussen, 1990). Convalescent human serum contains antibodies that will react with at least 20 different HCMV proteins, which include capsid, tegument, envelope and non-structural viral proteins. However, most of these proteins do not induce antibodies with either neutralizing activity or the capacity to bind to the virus or virus-infected cells. The most immunogenic HCMV envelope glycoprotein is glycoprotein B (gB), which can induce neutralizing antibodies in virtually all infected individuals (Britt, 1991). Although humoral immunity has been considered to be of relatively minor importance with regards to protection against HCMV infection, there is some evidence that infections during pregnancy or following renal transplantation may be less severe in the presence of pre-existing antibodies (Griffiths & Grundy, 1987).

The importance of the cell-mediated immune response to HCMV in host defence has been well documented (Greenberg et al., 1991a, b; Jonjic et al., 1990; Lindsley et al., 1991).
et al., 1986; Riddell et al., 1992; Quinnan et al., 1984).
Several specific targets for T cell recognition during HCMV infection have been recently identified; these include the envelope glycoprotein gB, the matrix protein pp65 and the immediate early IE1 protein (Liu et al., 1988; Alp et al., 1991; Gilbert et al., 1993). The initial event in an immune response involves recognition of foreign antigen by CD4+ T cells. These activated CD4+ T cells can serve a variety of functions, such as cytokine release, direct cytotoxic activity and augmentation of humoral and cytotoxic T lymphocyte (CTL) responses (Alp et al., 1991; Lindsley et al., 1986). In the presence of selective CD8+ T cell deficiency, CD4+ T cells can suppress CMV replication under specific experimental circumstances (Jonic et al., 1990). Riddell et al. (1992) demonstrated that CD4+ T cells are required for reconstitution of CD8+ CTL following bone marrow transplantation. Moreover, adoptive transfer of HCMV-specific CD8+ CTL to immunodeficient bone marrow transplant recipients can reconstitute the anti-CMV-specific CD8+ CTL response and is potentially an effective therapy (Greenberg et al., 1991a, b).

There is limited information concerning the role of CD4+ T cells in the immune response to HCMV proteins. T cell recognition of foreign antigens is determined in part by the ability of antigenic peptides to bind to MHC molecules, and allelic variation of MHC molecules greatly influences the spectrum of antigens that an individual can respond to. It is therefore possible that individuals bearing particular HLA alleles on their cells might not be able to mount an immune response to particular proteins. This may prove to be a problem when a proposed vaccine consists of one protein only.

We investigated the T cell proliferative response of 23 HCMV-seropositive individuals to a panel of five HCMV proteins which have been shown to be immunologically relevant and we attempted to see whether there is any correlation between the HLA type of HCMV-infected individuals and the reactivity of T cells to specific HCMV proteins. The proteins studied included the two immediate early proteins (IE1 and IE2), the major glycoprotein gB (gpUL55), the upper matrix protein pp71 (ppUL82) and gpUL18 (the MHC class I homologue). These proteins have either been previously shown to stimulate the immune response or have homologies with important molecules in the immune system (IE2 with HLA-DR and gpUL18 with MHC class I genes) (Fujinami et al., 1988; Beck & Barrell, 1988).

Methods

Study subjects. Twenty-three seropositive and six seronegative healthy male (19) and female (10) volunteers were analysed in this study. Serum IgG antibodies specific for CMV were quantified using a solid-phase, indirect fluorescence immunoenassay (FlAX; Whittaker Bioproducts). The subjects’ immune status to CMV was confirmed by proliferation assays to whole HCMV antigens.

Generation of HCMV-specific proteins. Five HCMV (Towne strain) fusion protein plasmids were constructed. IE1 and IE2 were cloned from plasmid pH101S1V1 (pH1D101SV1 was kindly provided by Dr Eng-Shang Huang, University of North Carolina, USA) (Davis et al., 1987). The gene used in this study, termed IE1, consists of the IE1 genomic region exon 4; the gene termed IE2 consists of exon 5/7. Using specific primers, the whole IE1 exon 4 region was amplified and EcoRV and XbaI sites were created for subcloning. IE2 was a Smal–BamHI fragment encoding 444 out of the total 492 aa, which includes the region homologous with HLA-DR (Fujinami et al., 1988). In order to subclone gB from plasmid pXaB (pXbB was a kind gift from Dr Mark Sinskey, University of Iowa, USA) (Thomsen & Sinskey, 1981), we first subcloned a 5.6 kb BamHI1 fragment from pXbB into pUC19 to get pUCgB. From pUCgB, an Hpnl–XmalI fragment was further subcloned into pMAL-c, which encodes 839 of the total 907 aa. Plasmids pp71 and gpUL18 were subcloned from pXCXH1.6 and pGXAO19, respectively (kind gifts from Dr Richard Stenberg, Eastern Virginia Medical School, USA). Plasmid pXCH1.6 was digested with HindIII at the 5' end and then filled-in to form a blunt end; the 3' end was cut by XbaI. This fragment encodes 532 of the total 559 aa for pp71. Plasmid gpUL18 is a BglII–HindIII fragment subcloned from pGXAO19, encoding 347 of the total 368 aa. All five DNA fragments have stop codons at the 3' end.

DNA fragments were inserted into the pMAL-c vector (New England Biolabs) immediately downstream of the malE gene with the 5' end inserted into the Stul site. Sequence analysis confirmed that all of the subcloned DNA fragments were inserted in the correct reading frame. The fusion proteins were produced by IPTG induction and purified by affinity chromatography with amylose resin (Maina et al., 1988). SDS–PAGE analysis of the induced proteins was performed to confirm that a protein of the expected size was produced. After dialysis against PBS and filter sterilization, the purified proteins were quantified by the Bio-Rad protein assay.

Maltose-binding protein (MBP) control antigen. The pMAL-c vector produces a fusion protein consisting of MBP plus LacZα. In order to produce MBP only, a DNA fragment which has a stop codon very close to the 5' end was inserted into the Stul site. A DNA construct was screened out to have a stop codon immediately after the malE gene and in the correct reading frame. MBP was produced as described for the production of HCMV proteins.

HCMV antigen production. HCMV antigen was prepared as previously described using a glycerine buffer extraction method following infection of human foreskin fibroblasts with the AD169 strain of HCMV (Lindsley et al., 1986). Mock antigen was prepared from the same cells without infection.

Proliferation assay. Peripheral blood mononuclear cells (PBMC) were isolated from each individual. Cells (2 × 10⁶ per well) were placed in 96-well round-bottom plates in triplicates with RPMI 1640 plus 10% human AB+ serum and appropriate antigens. A range of concentrations of HCMV antigens and controls were tested. In general, the highest antigen concentration was 10 µg/ml and serial 3-fold dilutions were performed. Cultures were incubated for 5 days at 37 °C with 5% CO₂ pulsed with 0.5 µCi [3H]thymidine per well for 18–20 h, and then harvested and counted in a liquid scintillation counter. Positive controls were whole HCMV antigen preparations and 3 day stimulations with phytohaemagglutinin (PHA). Negative controls included: MBP, mock HCMV Ag preparation, BSA and medium alone. The Δc.p.m. was calculated as: Δc.p.m. = c.p.m. with HCMV protein – c.p.m. with MBP at the relevant concentration. In order to determine whether a given individual had a positive response to a particular
protein, the Δc.p.m. and stimulation index (S.I. = c.p.m. with HCMV protein/c.p.m. with MBP) for each protein were determined. The Δc.p.m. values obtained for a particular HCMV protein in all six seronegative controls were averaged and the standard deviation (SD) was obtained. If the Δc.p.m. in an HCMV-seropositive individual was greater than 3 SD above the mean value obtained with the same protein from HCMV-seronegative individuals, and the S.I. was above 2, the response was considered positive.

**Dot blot analysis of HLA-DR and -DQ.** Genomic DNA was extracted from PBMC of each individual and HLA typing was performed using a non-radioactive DNA hybridization technique with sequence-specific oligonucleotide probing of PCR-amplified HLA-DR and -DQ genes as previously described (Morel et al., 1994).

**Western blot.** Purified proteins and MBP were separated on an SDS-PAGE gel and then transferred to PVDF membrane (Millipore). A mouse anti-MBP monoclonal antibody was applied as primary antibody. The secondary antibody was goat anti-mouse IgG antibody conjugated with horseradish peroxidase (Bio-Rad). Positive products were detected using the ECL system (Amersham).

**Results**

**Characterization of HCMV recombinant proteins**

The pMAL-c vector provides a method for expressing and purifying proteins from cloned DNA fragments in the correct reading frame. Using this system, five HCMV fusion protein DNA constructs were successfully made as determined by sequence analysis, and HCMV-specific proteins as well as the negative control protein MBP were produced and purified in large quantities. The purified HCMV proteins (IE1, IE2, pp71 and gpUL18) and the MBP are shown in Fig. 1(a). The predicted sizes for these proteins are 88 kDa for IE1, 92 kDa for IE2, 100 kDa for pp71, 82 kDa for gpUL18 and 42 kDa for MBP. The full sizes of the proteins as well as some degraded forms were detected on the SDS–PAGE gel stained with Coomassie Blue (Fig. 1a). In general, the major species observed were of the expected size. In the case of pp71 (Fig. 1a, lane 3), a minor band at 100 kDa was observed and this represents the full-length fusion protein. The major band was a 90 kDa band and this is a degradation product as all the species could be detected using an anti-MBP monoclonal antibody.

The purified gB protein could not be detected in the Coomassie Blue-stained SDS–PAGE gel (Fig. 1a, lane 5). Western blotting of the gB preparation using an anti-MBP antibody demonstrated that there were at least two bands larger than MBP (42 kDa), including a predicted

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![Fig. 1. Analysis of CMV fusion proteins by SDS-PAGE and Western blotting. (a) SDS-PAGE of HCMV proteins and MBP stained with Coomassie Blue. The predicted sizes are 92 kDa for IE2 (lane 1), 88 kDa for IE1 (lane 2), 100 kDa for pp71 (lane 3), 82 kDa for gpUL18 (lane 4), 130 kDa for gB (lane 5) and 42 kDa for MBP (lane 6). Several degraded products are also observed. The bands at 67 kDa in lanes 2 and 5 represent BSA that was added to these preparations for stability. Molecular mass markers are indicated (lane M). (b) Western blot of gB (lane 1) and MBP (lane 2) developed with an anti-MBP antibody. gB cannot be detected in the Coomassie Blue-stained gel, but Western blotting shows two bands larger than 42 kDa, including a full-size band of 130 kDa.](image-url)
Proliferation responses to HCMV proteins

The HCMV proteins were used to evaluate the T cell proliferative responses from 23 HCMV-seropositive and six seronegative healthy individuals. The six seronegative individuals did not show any significant response to any of the HCMV proteins (data not shown). The proliferative responses of PBMC, isolated from four HCMV-seropositive individuals, against a range of concentrations of HCMV proteins are shown in Fig. 2. The responses shown are representative of those observed with the other donors. All responses were dose dependent and in all cases the response to MBP alone was very low; thus positive results were readily apparent. Individual donors responded differently to the five HCMV proteins. According to the positive standard adopted in this paper, PBMC from donor #2 responded to all five proteins; donor #6 responded to IE1, IE2, pp71 and gB; donor #16 responded to gB and IE2; and donor #19 responded to gB only. In order to be able to compare responses between individuals, the optimum concentration for each protein was determined, based on the dose-response curves obtained. Based on these data, the optimum antigen concentrations were: 3 μg/ml for IE1, pp71 and gpUL18; 1 μg/ml for IE2; and 0.3 μg/ml for gB. The results are summarized in Table 1. Of the 23 seropositive individuals, five responded to all five proteins, 14 responded to between one and four proteins, and four responded to none of the proteins. The most commonly recognized proteins were gB (17/23, 74%) and IE2 (16/23, 70%). pp71 and IE1 were recognized by 10 of 12 (43%) individuals. Nine of 22 individuals tested responded to gpUL18 (41%).

Although gB was one of the most commonly recognized proteins, there were still six individuals who did not respond to gB (Table 1). These six individuals all showed a vigorous response to HCMV whole antigen.
Table 1. Summary of proliferative responses of PBMC from healthy HCMV-seropositive donors to five HCMV fusion proteins*

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<th>IE2 (3 μg/ml)</th>
<th>pp71 (3 μg/ml)</th>
<th>gpUL18 (3 μg/ml)</th>
<th>gB (0.3 μg/ml)</th>
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* Results are a summary of the proliferative responses to the optimum concentration of antigen. If Δc.p.m. was greater than 3 SD above the mean value obtained with the same protein from the HCMV-seronegative donors, and the S.I. was above 2, the response was considered positive. The positive response level was graded from + to ++ + according to the S.I. +, S.I. was below 3, but over 2; ++, S.I. 3–5; + + +, S.I. > 5; –, Negative response.

† Whole HCMV antigen preparation (c.p.m. × 103).

ND, Not determined.

Table 2. Reproducibility of proliferative assays to HCMV proteins

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* The time interval between the two experiments was 6–12 months.
† Numbers represent the Δc.p.m. (× 103) at the relevant antigen concentrations: IE2, 1 μg/ml; gB, 0.3 μg/ml. Any Δc.p.m. value above 1.25 (IE2) or 1.31 (gB) and where the S.I. was > 2, was considered positive.
‡ Whole HCMV antigen preparation (c.p.m. × 103).
§ This was classified as a negative response as the S.I. was < 2.

In an attempt to correlate these responses with the HLA type of each individual, DNA typing for HLA-DR and -DQ was done on all individuals. The results suggested that HLA-DR may play a role in unresponsiveness, as three of the four individuals who failed to respond to any of the proteins were DR2-positive and two of them were homozygous for DR2, although this did not reach statistical significance. When DRB1 alleles were identified by subtypes there was no association observed with particular DRB1 subtypes. When the responses to
individual proteins were examined, no associations were observed.

Discussion

The T cell proliferative response to five HCMV proteins (IE1, IE2, gB, pp71 and gpUL18) in 23 HCMV-seropositive and six seronegative individuals has been evaluated. The fact that four of the 23 individuals tested did not respond to any of the five proteins tested, despite vigorous responses to the whole HCMV antigen preparation, suggests that additional proteins may be involved. Likely candidates include the matrix proteins pp65 and pp150 (Gilbert et al., 1993; John et al., 1987), which have been shown to be important targets for CTL and antibodies, respectively. We have now prepared fusion proteins for pp65 and pp150 and are in the process of addressing this issue.

After HCMV infection, there are three distinct phases of viral protein synthesis, designated immediate early (including IE1 and IE2), early, and late (including gB and pp71). gB (gpUL55) is an abundant envelope glycoprotein and is also highly expressed on the surface of infected cells. After natural infection, neutralizing antibody against gB can be detected in the serum of all infected individuals (Marshall et al., 1992); for each individual, approximately one-half of the virus-specific antibodies in the serum is directed against gB (Plotkin, 1991). Based on the fact that gB plays an important role in the humoral immune response, gB is considered to be an important candidate for an HCMV subunit vaccine (Britt, 1991; Grundy, 1991; Plotkin, 1991; Spaete et al., 1987). Two groups have developed a recombinant gB subunit vaccine and tested it in animals (Plotkin, 1991; Spaete, 1991). Their experiments demonstrated that after animals were immunized with the gB protein, neutralizing antibodies could be detected and antibody titres increased after a boost dose. However, there is no evidence to demonstrate the effectiveness of such a single gB subunit vaccine in humans.

In our study, gB was one of the most commonly recognized proteins of five HCMV proteins tested for T cell proliferative responses. This confirms that gB is an important immunogenic protein in the immune response to HCMV. However, only 17 of 23 (74%) individuals studied showed T cell proliferative responses to gB. The results that we obtained with gB were reproducible, suggesting that this is not an underestimate due to test variability. The discrepancy between helper T cell responses and those of the neutralizing antibody against gB has been previously described in a study by Liu et al. (1988) in which two of nine (22%) seropositive individuals failed to respond to purified glycoprotein preparations despite having detectable anti-gB antibody.

Our results provide an independent confirmation of this finding. This discrepancy could be explained by the fact that although during a natural infection all individuals develop antibodies to gB, the T cell help required for such antibody production might not come from gB-specific T cells, but rather from T cells having specificity for other HCMV proteins. Recent data by Liu et al. (1993) demonstrated that some individuals expressing a particular HLA class II allele did not exhibit PBMC proliferative responses to the corresponding HLA class II allele restricted gB peptide, although the PBMC from these individuals were able to present the appropriate HLA-restricted gB peptide to gB-specific helper T cell clones. All these data suggest that a subunit vaccine composed of gB alone may not elicit a protective response in all individuals within the population. The 86 kDa IE2 is an immediate early protein expressed after HCMV infection and the exon 5/7 is unique to the 86 kDa IE2 protein, which includes an area of HLA-DR homology (Fujinami et al., 1988). To our knowledge, the present study is the first report demonstrating that HCMV-seropositive individuals (16/23) have a proliferative response to IE2. The average response level is as high as that to gB. Importantly, some individuals who did not respond to gB were able to respond to IE2. These data suggest that the immune response to IE2 should be further studied.

It has been previously shown that a major CTL target in HCMV-infected cells is a protein made immediately after infection (Borysiewicz et al., 1988). The immediate early protein, IE1, is the immunodominant target for the CTL immune response to murine CMV in BALB/c mice (Del Val et al., 1991). As CD4+ cells are required for the development of an effective CTL response, it is likely that CD4+ cells should respond to this protein. All the previous data about the role of IE1 were based on the use of the 72 kDa mature IE1 protein. Since IE1 and IE2 share exons 1, 2 and 3, we used exon 4 as a specific, truncated IE1 to evaluate the T cell proliferative response. From our data, only 10 of 23 (43%) individuals responded to IE1. This positive percentage is quite similar to the percentage of subjects who responded to IE1 as reported by Alp et al. (1991). Furthermore, the response level (average Δc.p.m.) was relatively low when compared to those observed with IE2 and gB. Recently, Gilbert et al. (1993) demonstrated that human IE1-specific CTL were of low frequency and of poor lytic activity compared to other HCMV proteins, such as gB or pp65. These data therefore suggest that unlike the immune response in the murine system, IE1 may not be a universal target of the immune response to HCMV.

The HCMV genome encodes the gpUL18 gene product that has MHC class I homology (Beck & Barrell, 1988). The exact role of this gene product has not been
determined, although recent data from Browne et al. (1992) indicated that the gpUL18 gene product is dispensable for the growth of HCMV in human fibroblasts in vitro. The fact that we were able to detect a T cell proliferative response to recombinant gpUL18 protein in 41% of HCMV-seropositive individuals indicates that the UL18 protein is expressed during infection and may be involved in the immune response. The upper matrix protein pp71 (ppUL82) has been shown to be the transactivating protein for IE genes and enhances transcription from viral and cellular promoters (Liu & Stinski, 1992). The role of this structural protein in the immune response is not known. Our data demonstrated that 10 of 23 seropositive individuals responded to pp71, indicating that pp71 may play a role in the immune response to HCMV in some individuals.

CD4+ T cell recognition of foreign antigen involves the binding of a peptide, derived from the antigen, into a groove on the surface of MHC class II molecules. Recent data demonstrated that each HLA-DR allele can bind a unique set of peptides which are defined by a particular structural motif (Chicz et al., 1993; Rammensee et al., 1993). We tried, therefore, to correlate the T cell responses to HCMV proteins with the HLA types of each individual. In general, there was no statistically significant association observed between the HLA-DR or -DQ type and the reactivity of T cells to the five HCMV proteins tested. This is not surprising, considering that we studied large proteins that contain, in all likelihood, several peptide epitopes. In addition, most of the individuals studied were heterozygous for HLA-DR and -DQ, which will provide them with several potential restriction elements. Further studies are underway involving the cloning of specific T cell clones specific for these proteins to identify T cell epitopes associated with particular HLA alleles.

In conclusion we have studied the proliferative T cell responses of HCMV-seropositive healthy individuals to five HCMV proteins. The results that we obtained are relevant when considering the feasibility of developing a subunit vaccine that consists of only one HCMV protein, gB (Plotkin, 1991). Our data would suggest that such a gB subunit vaccine might not be effective in all the individuals vaccinated. A combination of gB and IE2 would be able to stimulate a greater number of individuals who are responsive to one or more of the proteins tested. This suggests that a combination of two or three HCMV-specific proteins may provide a more efficient subunit vaccine. A similar situation has been identified for the hepatitis B vaccines in which DR3 has been associated with the lack of response to hepatitis B surface antigen (Alper et al., 1989). This has led to the addition of more hepatitis B proteins to the vaccine for individuals who failed to respond to the surface antigen vaccine (Kniskern & Miller, 1992). An effective vaccine should, ideally, consist of several HCMV proteins which in combination could stimulate effective CD4+ T cell and antibody responses.

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