Comparative analysis of fourteen individual human cytomegalovirus proteins for helper T cell response

J. Beninga, B. Kropff and M. Mach*

Institut für Klinische und Molekulare Virologie, Universität Erlangen-Nürnberg, Loschgestrasse. 7, 91054 Erlangen, Germany

The potential of selected proteins of human cytomegalovirus (HCMV) to induce a helper T (Th) cell immune response was investigated in healthy HCMV-seropositive donors. Recombinant derived glycoproteins B (gpUL55), H (gpUL75), integral membrane protein (pUL100), the US6–US11 glycoprotein family (pUS6–US11), the matrix proteins pp65 (ppUL83), pp28 (ppUL99) and the immediate early proteins IE1 (pUL123), IE2 (pUL122) and UL69 (pUL69) were used as stimulating antigens in a lymphocyte proliferation assay. The antigen-specific proliferative response was measured in HCMV-specific T cell lines (phenotype CD4+ CD8+) generated from five donors by stimulation of peripheral blood mononuclear cells with purified HCMV or HCMV-infected fibroblasts. A proliferative T cell response was induced by pp65, gB, gH, IE1, IE2 and UL69, with a dominant response to pp65 in all donors. Three T cell lines responded to gB and gH, respectively. For IE1, IE2 and UL69 a T cell stimulation could be demonstrated in single cell lines generated with lysate of HCMV-infected fibroblasts.

Human cytomegalovirus (HCMV) disease continues to be a formidable issue in a number of clinical situations. Firstly, the virus represents the most common cause of congenital viral infections in humans resulting in a large and varied range of disorders (Stagno et al., 1984). Secondly, the virus can cause severe problems in immunocompromised individuals with complications due to HCMV often hampering the success of transplant surgery (Rubin, 1990). Thirdly, in patients with AIDS, HCMV is the most common cause of life-threatening viral opportunistic infections (Drew, 1988).

In view of the clinical importance of HCMV infection, the development of an effective vaccine is an important long-term goal. Current efforts towards a vaccine have included immunization with tissue-culture propagated virus as well as purified glycoprotein B (Plotkin et al., 1990; Gonczol & Plotkin, 1990). Live vaccines derived from a virus that may cause latent and persistent infection and, in addition, is potentially oncogenic, will need careful consideration before widespread use. A more attractive approach is the development of subunit vaccines, based on surface glycoproteins of the virus. Induction of a potent helper T (Th) cell response to the immunogen will be crucial since the subunit preparation will be delivered as exogeneously rather than endogeneously synthesized protein. The vaccine-induced priming of the CD4+ T cell system has the potential to augment both antibody responses and CD8+ cytotoxic T cell responses to a subsequent natural infection. However, the identification of candidate antigens inducing a potent Th cell response remains a poorly understood area of research in the immunobiology of HCMV. HCMV is a complex virus containing a genome of 230 kbp with an estimated 200 open reading frames (Chee et al., 1990). More than 30 reading frames may encode glycoproteins which are candidates for the induction of neutralizing antibodies and/or a potent cellular immune response.

To date, work on cell mediated immunity to HCMV in man has largely been concerned with cytotoxic T cell responses and the proteins pp65 (ppUL83), pp150 (ppUL32), IE1 (pUL123) and glycoprotein B (gB) (gpUL55) have been identified as targets for cytotoxic T cells (Borysiewicz et al., 1988; Riddell et al., 1993; McLaughlin-Taylor et al., 1994). In the murine model adoptive transfer of murine cytomegalovirus (MCMV)-immune CD8+ T cells completely protects immunocompromised mice from lethal primary challenge with MCMV and is therapeutically effective in mice with established MCMV infection (Reddehase et al., 1985, 1987). However, CD4+ T cells compensate in situations where CD8+ T cells are depleted (Jonjic et al., 1990). To date only a few proteins have been identified as antigens for Th cells in humans, including the immediate early proteins 1 (IE1) (pUL123) and 2 (IE2) (pUL122),

* Author for correspondence. Fax +49 9131 852101.
Table 1. Proliferative response of healthy HCMV seropositive and seronegative donors to HCMV

<table>
<thead>
<tr>
<th>Donor</th>
<th>HLA type*</th>
<th>Serum titre†</th>
<th>[3H]Thymidine incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB</td>
<td>DR3 DR13 DR52 DQ2 DQ6</td>
<td>1/9300</td>
<td>287 (±90) 78865 (±9232)</td>
</tr>
<tr>
<td>EJ</td>
<td>DR1 DR7 DR52 DR53 DQ7 DQ2</td>
<td>1/12000</td>
<td>378 (±111) 61293 (±11360)</td>
</tr>
<tr>
<td>YH</td>
<td>DR11 DR7 DR52 DR53 DQ7 DQ2</td>
<td>1/20000</td>
<td>224 (±35) 46950 (±2961)</td>
</tr>
<tr>
<td>CS</td>
<td>DR15 DR17 DR52 DQ6 DQ2</td>
<td>1/13000</td>
<td>212 (±45) 31243 (±579)</td>
</tr>
<tr>
<td>AS</td>
<td>DR11 DR4 DR52 DR53 DQ7</td>
<td>1/6500</td>
<td>230 (±49) 19270 (±711)</td>
</tr>
<tr>
<td>TZ</td>
<td>ND</td>
<td>1/3900</td>
<td>433 (±123) 28661 (±5227)</td>
</tr>
<tr>
<td>RB</td>
<td>ND</td>
<td>1/7400</td>
<td>224 (±51) 20134 (±4743)</td>
</tr>
<tr>
<td>FS</td>
<td>ND</td>
<td>1/8600</td>
<td>236 (±103) 18730 (±6232)</td>
</tr>
<tr>
<td>CE</td>
<td>ND</td>
<td>1/6600</td>
<td>230 (±97) 9115 (±2828)</td>
</tr>
<tr>
<td>CH</td>
<td>ND</td>
<td>1/11000</td>
<td>203 (±33) 8879 (±3796)</td>
</tr>
<tr>
<td>JB</td>
<td>DR13 DR10 DR52 DQ1</td>
<td>0</td>
<td>232 (±50) 248 (±70)</td>
</tr>
<tr>
<td>MU</td>
<td>ND</td>
<td>0</td>
<td>230 (±60) 223 (±11)</td>
</tr>
<tr>
<td>MM</td>
<td>ND</td>
<td>0</td>
<td>236 (±69) 527 (±222)</td>
</tr>
</tbody>
</table>

* HLA class II haplotypes were determined with commercially available antibodies; ND, not determined.
† Serum titre was established with an anti-CMV/IgG ELISA (Behring).
‡ Proliferative response of 10^6 PBMC against purified HCMV (0.5 μg/ml) was measured in a 7 day lymphocyte proliferation assay by [3H]thymidine incorporation for the final 16 h. Data presented are means of triplicate cultures in c.p.m. (±SD).

We have compared 14 individual HCMV antigens, including the major antigens for the humoral immune response, for their capacity to stimulate Th cell responses. Our data indicate that the matrix protein pp65 (ppUL83) as well as glycoproteins B (gpUL55) and H (gH) (gpUL75) represent antigens for Th cells. However, the potential of the individual antigens to stimulate proliferative responses of Th cells in vitro varied between individuals.

In a first set of experiments, short term proliferative responses of freshly isolated peripheral blood mononuclear cells (PBMC) to purified HCMV virions as antigen were examined in a standard 7 day lymphocyte proliferation assay (Liu et al., 1993). Ten seropositive and three seronegative healthy adult donors were tested on two or more occasions with HCMV particles gradient purified according to Talbot & Almeida (1977). Optimal antigen concentrations were determined in a titration experiment using protein concentrations of 0.087-11.4 μg/ml. Maximal response was found with antigen concentrations of 0.5-1.5 μg/ml with slight variations between individuals (data not shown). For all subsequent analyses 0.5 μg/ml was chosen as the antigen concentration. Table 1 shows the combined results of one such experiment in which control and HCMV-stimulated cultures were assayed by [3H]thymidine labelling over the last 16 h of a 7 day culture period. Data are presented as counts per minute (c.p.m.) and the standard deviation (SD) is indicated.

A broad spectrum of proliferative responses was apparent in cultures from all seropositive donors but not in cultures of any of the seronegative donors. Repeated testing gave a similar pattern of results, with the relative levels of responsiveness among seropositive donors being generally consistent from one assay to the next. Stimulation ranged from 40-fold to 267-fold when compared to non-stimulated controls. HCMV-specific immunoglobulin titres were also examined and showed variation in the levels of antibodies between different individuals (Table 1).

In order to investigate the proliferative response with respect to specific antigens several HCMV antigens were prepared. Three types of proteins were chosen for further analysis: (i) envelope components including gH of strains AD169 (gH/AD169) (gpUL75) and Towne (gH/Towne), gB (gpUL55), the integral membrane protein IMP (pUL100) as well as proteins US6-US11 (pUS6-US11); (ii) matrix proteins including pp65 (ppUL83) and pp28 (ppUL99); (iii) regulatory proteins including IE1 (pUL123), IE2 (pUL122) and UL69 (pUL69), a protein which represents the ICP27 homologue of herpes simplex virus and is synthesized abundantly at late times in infection (Winkler et al., 1994). With the exception of gH, where proteins of strains AD169 and Towne were used, all antigens were derived from strain AD169. The proteins gB (Bac-gB), integral membrane protein (IMP) (Bac-IMP), IE1 (Bac-IE1) and IE2 (Bac-IE2), matrix protein pp65 (Bac-pp65) and gH strain Towne (Bac-gH/TO) and AD169 (Bac-gH/AD) were produced in recombinant baculovirus infected insect cells (Summers & Smith, 1987). The recombinant baculovirus for gH strain AD169 was constructed by inserting a 2443 bp
NeII–HindIII DNA fragment excised from plasmid pBW3 into the vector pAc401 (Urban et al., 1992; Luckow & Summers, 1988). Recombinant baculoviruses were produced using baculovirus Gold DNA (Dianova). The remaining recombinant baculoviruses were provided by several laboratories (see Acknowledgement). For production of baculovirus expressed antigens, 2 × 10⁷ Sf158 cells were infected with recombinant baculoviruses, or Autographa californica nuclear polyhedrosis virus (Bac-Wt) as control, at an m.o.i. of 10 for 48 h. Infected cells were tested by indirect immunofluorescence using monoclonal antibodies for antigen expression and cultures with more than 80% of cells showing positive antigen expression were harvested in phosphate-buffered saline (PBS) and lysed by three cycles of freeze–thaw followed by sonication. The cell lysate was stored in PBS at -20 °C until use. The DNA fragments encoding US6 (aa 21–150), US7 (aa 21–172), US8 (aa 13–180), US9 (aa 1–190), US10 (aa 23–156) and US11 (aa 14–180) were amplified by PCR, inserted into the plasmid pSEM...
and prokaryotically expressed as β-galactosidase fusion proteins (Knapp et al., 1990). Fusion proteins were purified as described (Ellinger et al., 1991). The proteins pp28 and UL69 were prokaryotically expressed as histidine-tagged proteins in the pQE vector system (Diagen). Synthesis of HCMV-specific proteins was analysed in immunoblots using monoclonal antibodies. As can be seen in Fig. 1(a) the various antibodies recognized specific proteins in lysates from recombinant virus infected cells but not baculovirus infected cells. In accordance with published data, gB was synthesized mainly as a 160 kDa protein which represents a glycosylated, non-processed form of gB (Wells et al., 1990). IMP, pp65 and gH were synthesized in forms closely related to the authentic antigens, indicating only minor differences in modification. For bacterial fusion proteins polyacrylamide gel electrophoresis was performed to analyse the purity of individual preparations. In general, bacterial fusion proteins were considered to be more than 90% pure (Fig. 1b).

When the individual antigens were tested in standard 7 day proliferation assays most of the antigens did not induce a reproducible response (data not shown). Therefore, short term polyclonal T cell lines were established from five seropositive donors, two of which had a serological indistinguishable HLA type (Table 1). For primary stimulation freshly isolated PBMC, 2 × 10⁵ cells per well in a 96-well round bottom plate, were incubated with 0.5 µg/ml gradient purified, heat-inactivated HCMV particles as antigen in RPMI 1640
medium with 10% autologous serum for 5 days. After 5 days medium containing human interleukin 2 (IL2) (Biotest) (10 U/ml) was added for a further 9 days with medium exchange every 3 days. T cell cultures were restimulated by incubating $2 \times 10^6$ irradiated (3000 rad) PBMC and $5 \times 10^4$ pre-stimulated T cells with HCMV antigen in normal medium for 5 days followed by incubation in IL2 containing medium for the next 9 days. Proliferation of the T cell lines was assayed by incubating $2 \times 10^6$ irradiated PBMC and $2 \times 10^4$ T cells with the appropriate antigens in optimized concentrations for 4 days. Proliferation was measured by $[^3]$H]thymidine incorporation for the last 16 h. When lysates of recombinant baculovirus-infected cells were used in concentrations from 0.16–100 µg/ml with T cells from line CS an optimal ratio of reactivity to HCMV protein containing lysates compared to wild-type lysates was found at 4 µg/ml. In a similar experiment using bacterial fusion proteins as well as the fusion partner β-galactosidase at concentrations of 0.12-4 lag/ml optimal ratios were found at 4 µg/ml was used. The T cell lines were analysed with the appropriate antigens in optimized concentrations for 4 days. Proliferation assay by $[^3]$H]thymidine incorporation for the final 16 h. Data presented are means of triplicate cultures in c.p.m. ($\pm$sn).

All lines responded to control antigens with low level of proliferation when compared to cells incubated with medium alone. Therefore, we used the values obtained with the control antigens as background proliferation response for the estimation of specific response and considered a 3-fold induction as significant.

The dominant antigen was pp65. All five T cell lines responded to this antigen with line YH reaching stimulation comparable to that with whole virus. The remaining antigens showed either donor-specific recognition or no response. gB as secondary antigen was recognized by lines YH, EJ and CS. gH/AD169 stimulated lines EJ, AS and CS. However, only a single T cell line (CS) responded to gH/Towne. The remaining antigens were negative with one exception: T cells from line AS were stimulated by UL69. The two donors with closely related HLA type both responded to gB and pp65 but only line EJ was stimulated by gH/AD169. A fluorescence flow cytometric analysis of the generated T cell lines demonstrated a CD3+ CD4+ CD8− phenotype for over 90% of the cells (data not shown).

We next determined the reactivity of T cell lines stimulated with HCMV-infected foreskin fibroblasts (HFF) as primary antigen in T cell lines from two donors (NB, YH). For preparation of HCMV infected cells as antigen, semi-confluent HFF were infected with HCMV strain AD169 at an m.o.i. of 10. After 72 h, when 80% of the cells showed a cytopathic effect, the cells were scraped off and lysed in PBS by three cycles of freeze–thaw. The lysate was stored at $-20^\circ$C until use. For stimulation of T cell lines a concentration of 1.5 µg/ml and for proliferation assays a concentration of 4 µg/ml was used. The T cell lines were analysed with the complete set of antigens and Table 2 shows representative data from selected antigens. The response that developed stimulation in both lines, indicating that non-structural HCMV proteins did not contribute significantly to the overall response. In addition, infected-cell lysates and purified virions as secondary antigens resulted in comparable stimulation of both lines, indicating that non-structural HCMV proteins did not contribute significantly to the overall response. In addition to the antigens defined by lines stimulated with HCMV particles, line YH responded to the immediate early proteins IE1 and IE2. The remaining antigens were negative for both T cell lines (data not shown).

The goal of this study was a comparison of the capacity of different HCMV antigens to stimulate Th cells. The in vitro stimulation of PBMC approximates a situation which occurs during reactivation of latent virus or reinfection, that is a primed immune system encountering free virus or virus-infected cells. In both situations, pp65 represents the most broadly reacting protein. In our study population it was the only antigen which showed significant proliferation rates with all five donors. In three cases (YH, AS, CS) pp65 was also the dominant antigen with a stimulation capacity similar to whole virus. The dominance most likely reflects a high T cell precursor frequency in the original PBMC prep-

### Table 2. Proliferative response of HCMV infected fibroblast-specific T cell lines from two seropositive donors*

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Donor</th>
<th>YH</th>
<th>NB</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCMV infected</td>
<td>34758 (±2958)*</td>
<td>63827 (±8690)</td>
<td></td>
</tr>
<tr>
<td>HFF</td>
<td>268 (±27)</td>
<td>1465 (±165)</td>
<td></td>
</tr>
<tr>
<td>HCMV</td>
<td>42538 (±1418)</td>
<td>64412 (±5998)</td>
<td></td>
</tr>
<tr>
<td>Bac-gB</td>
<td>19073 (±640)</td>
<td>5000 (±987)</td>
<td></td>
</tr>
<tr>
<td>Bac-gH/AD</td>
<td>2526 (±613)</td>
<td>4207 (±568)</td>
<td></td>
</tr>
<tr>
<td>Bac-gH/TO</td>
<td>1409 (±749)</td>
<td>2607 (±286)</td>
<td></td>
</tr>
<tr>
<td>Bac-IMP</td>
<td>1585 (±196)</td>
<td>2129 (±344)</td>
<td></td>
</tr>
<tr>
<td>Bac-pp65</td>
<td>32033 (±969)</td>
<td>51956 (±4351)</td>
<td></td>
</tr>
<tr>
<td>Bac-IE1</td>
<td>7874 (±944)</td>
<td>3876 (±1078)</td>
<td></td>
</tr>
<tr>
<td>Bac-IE2</td>
<td>6927 (±910)</td>
<td>3591 (±383)</td>
<td></td>
</tr>
<tr>
<td>Bac-Wt</td>
<td>982 (±367)</td>
<td>3214 (±1051)</td>
<td></td>
</tr>
<tr>
<td>No antigen</td>
<td>314 (±114)</td>
<td>921 (±321)</td>
<td></td>
</tr>
</tbody>
</table>

* Proliferative response of $2 \times 10^6$ T cells was measured in a 4 day proliferation assay by $[^3]$H]thymidine incorporation for the final 16 h.

Data presented are means of triplicate cultures in c.p.m. ($\pm$sd).
aration. This seems likely since pp65 is probably the most abundant HCMV protein, since dense bodies, which contain large amounts of pp65, have been identified in vivo (Grefte et al., 1993). In addition, the presence of viral DNA in plasma, as detected by PCR, indicates that free virus containing pp65 could represent an antigen during active infection (Brytting et al., 1992). Correlation between abundance of antigens and precursor frequencies of T cells has also been demonstrated in other systems such as influenza virus and varicella-zoster virus (Hurwitz et al., 1985; Arvin et al., 1986). An alternative explanation for the dominance of pp65-specific T cells could be a selective stimulation in vitro by the large amounts of pp65 present in the antigen preparations. This seems less likely since T cells from donor EJ responded better to gB than to pp65.

Previous investigations with HPLC-purified pp65 or vaccinia virus recombinants expressing pp65 have identified this polypeptide as T cell antigen for both CD4+ (Forman et al., 1985) and CD8+ T cells (McLaughlin-Taylor et al., 1994). Together with the data presented here it can be concluded that pp65 represents one of the most potent and broadly reactive antigens for the cellular immune response against HCMV and that this reactivity is largely independent of the HLA type.

In contrast, glycoprotein-specific Th cells could not be detected in all cell lines. There are several possible explanations. (i) The PBMC preparations which did not react to the glycoproteins contained very few or no precursor T cells which could be stimulated by the antigen in vitro. A loss of low frequency T cell precursors during expansion of the T cell lines cannot be excluded. However, previous investigations have also noted a low frequency of gB-specific CD8+ T cells (Borysiewicz et al., 1988).

(ii) The antigen that was used to stimulate the T cells in vitro did not sufficiently expand an existing T cell population. Such a situation could occur when some or all T cell determinants on the glycoproteins are strain specific. Several findings point to such a possibility. Although the glycoprotein sequences between different HCMV isolates show a 95-98% homology clusters with significant sequence variations can be identified (Chou, 1992a, b). Examples are AD-2 of gB, located between aa 27-84 (Meyer et al., 1990) and AD-86 between aa 34-43 of gH (Urban et al., 1992). These regions are sufficiently divergent to induce a strain specific humoral immune response (Meyer et al., 1992; Urban et al., 1992). Using gB-specific Th cell clones R. C. Gehrz and co-workers have identified epitopes on the amino-terminal part of gB which could effect T cell responsiveness through strain variation (Liu et al., 1991). Since T cell epitopes on gB have not been characterized completely it can be speculated that a number of strain specific epitopes might exist on gB. No data are available on epitopes on gH. However, the lower stimulation rates that we have obtained using gH/Towne compared to gH/AD169 as secondary antigen, after primary stimulation with HCMV/AD169, could be taken as evidence for a strain specific response against gH. Preliminary studies suggest that strain variability affects reactivity of gH-specific Th cells. More studies defining the T cell epitopes on HCMV glycoproteins and comparison of different HCMV isolates with respect to these epitopes will be necessary prior to defining the contribution of strain variability to Th cell response. In general, strain specific T cell responses against viral envelope glycoproteins are not uncommon and have been observed in a number of systems including influenza virus and human immunodeficiency virus (Mills et al., 1986; Johnson et al., 1992).

(iii) The HLA class II haplotype determines the ability to respond to the glycoproteins. Previous investigations have demonstrated the association of several HLA high responder and low responder alleles for gB. Remarkably, the two donors (YH, EJ) with strong positive response against gB express the same HLA haplotype including DR7. DR7/Dw7 has been identified as a high responder allele for gB (Curtsinger et al., 1994) and the corresponding epitope has been defined (Liu et al., 1993). The third gB responding donor (CS) expressed a DR15 haplotype which in combination with Dw2 was associated with a high responder phenotype (Curtsinger et al., 1994). Since one of the donors sharing the same HLA haplotype exhibited a strong response to gH a correlation between HLA type and T cell response was not obvious for this glycoprotein.

With respect to structural proteins stimulation of PBMC with lysates from HCMV-infected cells resulted in a reaction pattern that was not significantly different from mature virions. From this it can be concluded that viral structural proteins at different stages of post-translational processing which are present in infected cells do not contribute significantly to the proliferation of Th cells. However, immediate early proteins likely represented additional antigens. The presence of Th cells with specificity for IE1 and IE2 has also been observed by others (Rodgers et al., 1987; He et al., 1993). It is interesting to note that one of the previously identified CD4+ T cell determinants on IE1 includes one of the two amino acids which are different between strains AD169 and Towne, again raising the possibility of a strain specific contribution to the T cell response (Alp et al., 1991).

What could be the relevance of our findings for the development of a vaccine against HCMV? During natural infection a selective immune response takes place at the Th cell level. With respect to the matrix proteins, pp65 represents the dominant antigen. pp28, which is a
highly immunogenic antigen for the B cell response, did not stimulate PBMC from any of the donors. Of the envelope glycoproteins that were tested gB and gH induced a proliferative response in a fraction of the T cell lines. The US6–US11 proteins as well as IMP failed to induce a response in all donors. With respect to antibody production the failure to develop Th cells seems to have limited consequence. The fact that we could demonstrate antibodies against pp28 or glycoproteins B and H in all donors (data not shown) suggested that this antibody response bypassed antigen-specific cognate T cell help, a possibility that has been identified for other viral systems (Scherle & Gerhard, 1986; Milich et al., 1987). Although the impact of low Th cell activity for the development of cytotoxic T cells is unclear, it can be assumed that vaccination using whole virus preparations or multiple proteins will induce an immune response which, on subsequent infection with HCMV, will induce a broadly reacting immune response. However, if subunit vaccines consisting of single proteins are used a potent Th cell response will be induced only in a fraction of vaccinees.

We are grateful to the following persons and institutions for providing the following reagents: W. Britt, Birmingham, Alabama (Bac-gB), Chiron Corporation, Emeryville (Bac-gH/TO) and Th. Stamminger, Erlangen (Bac-1E1, Bac-1E2, UL69). This work was supported by grant 01 KI 9301 from the Bundesministerium für Forschung und Technologie and the Wilhelm Sander-Stiftung.

References


Short communication

protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes. *Journal of Medical Virology* 43, 103-110.


**RUBIN, R.H. (1990).** Impact of cytomegalovirus infection on organ transplant recipients. *Reviews of Infectious Disease* 12 (Suppl. 7), S754-S766.


(Received 20 June 1994; Accepted 23 September 1994)