T and B cell responses to cytomegalovirus antigens in healthy blood donors and bone marrow transplant recipients

Francesco Lolli a,b, Vivi-Anne Sundqvist a,c, Antonella Castagna a,b, Per Ljungman d, Annika Linde a, Gudrun Andersson e, Tomas Olsson b and Britta Wahren a

a Department of Virology, National Bacteriological Laboratory, Solna, Stockholm, Sweden, b Department of Neurology, Karolinska Institute, Huddinge Hospital, Huddinge, Sweden, c Department of Medical Laboratory Technology, College of Health and Caring Sciences, Stockholm, Sweden, d Department of Internal Medicine, Huddinge Hospital, Huddinge, Sweden, and e Kabi Biopharma, R&D Immunobiology, Stockholm, Sweden

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Abstract: We measured the production of interferon-gamma (IFN-γ) from single T cells and the T cell proliferative response to different cytomegalovirus (CMV) antigens in healthy blood donors and bone marrow transplant recipients. The antigens consisted of a CMV nuclear antigen (CMV na) containing the pp65-kDa matrix protein and the immediate early antigens but lacking CMV glycoproteins, and an antigen comprising native CMV glycoproteins (CMV gp). We also measured the IgG antibodies to CMV na and CMV gp. The T cells reacted to CMV na in CMV seropositive blood donors both with the production of IFN-γ and with proliferation, while bone marrow transplant recipients had a deficient T cell response. After stimulation with CMV gp, no T cell response could be observed in CMV seropositive subjects. IgG antibodies to CMV na coexisted in plasma with similar levels of antibodies to CMV gp.

Key words: Cytomegalovirus; T cell; B cell; Interferon type II; Bone marrow transplantation

Introduction

The immune response to cytomegalovirus involves both T and B cell reactivity [1,2]. The internal and external structural components of the virus and the non-structural CMV early antigens are major CMV antigens [3]. We had previously observed that T cells respond preferentially to antigens obtained from the nuclei of infected cells, while, in contrast, CMV membrane antigens contain major B cell epitopes [4]. It is now established that antigens from the CMV matrix have a central role in T cell activation [5,6], and the B cell response to CMV glycoproteins is important for antibodies with neutralizing activity [7], but the relative significance of these findings in the immune response to CMV is not yet well defined.
At present, it is not yet clear whether the viral envelope contains proteins that can stimulate both humoral and cellular immune responses. After immunization with the glycoprotein complex gA/gB, human volunteers produce neutralizing antibodies, with only a transient T cell reactivity to whole CMV [8]. A clear T cell response to recombinant gB protein expressed in vaccinia virus was, however, observed in 4 out of 5 seropositive individuals [9]. On the other hand, the T and B cell response to these antigens seems to vary greatly among individuals [10,11].

The immune response to viruses is regularly accompanied by the secretion of IFN-γ [12–15]. IFN-γ activates macrophages and natural killer cells, enhances expression of major histocompatibility antigens of class I and II, induces secretion of several other cytokines, enhances and controls IgG synthesis and has anti-viral effects [16].

The anti-CMV T cell immunity is usually studied by measurement of the proliferative response of memory T cells in vitro. However, these assays may not completely reflect the T cell immunity. Measurement of IFN-γ secretion at the single cell level in response to antigen rather than proliferation can reveal differences between proliferative responses and IFN-γ production [17] and it can measure a low grade T cell response that may be missed by other T cell assays [18].

In the present study we employed a sensitive single T cell assay to study the production of IFN-γ in response to CMV antigens. We compare the result with other measurements of the T and B cell immunity to CMV in order to clarify the immune response to the internal and envelope glycoprotein CMV antigens, both in healthy blood donors (BD) and in bone marrow transplant (BMT) recipients. In the latter group, CMV infection occurs in approximately 50% of all recipients [19] and it is associated with an inadequate immune response to CMV [20].

**Materials and Methods**

**Patients**

We examined leucocytes and sera from 17 healthy BD (age 24–52 years, median age 40 years) and 19 BMT recipients (age 4–54 years, median age 44 years). The BMT and treatment regimens were described elsewhere [21–23]. Five of the BMT recipients had aplastic anemia, 8 acute myeloblastic leukaemia, and 2 patients each had acute lymphoblastic leukaemia, lymphoma and chronic myeloid leukaemia. Twelve patients received allogeneic BMT, while seven received autologous BMT. CMV infection, as judged by a positive CMV isolation and clinical findings, developed in 5 patients, while 8 presented an acute graft-versus-host disease (GVHD), which was of grade 1 in 7 patients and of grade 2 in one patient [21]. The time since transplantation was between one month to 2 years (median 2 months). Healthy BD and BMT recipients (pre-transplant) were vaccinated for tuberculosis with BCG within a few days after birth.

**Antigens**

A CMV antigen (CMV na) was prepared from nuclei of CMV infected human lung fibroblast cells as previously described [24]. Cytomegalovirus glycoproteins (CMV gp) were obtained by lentil-lectin affinity chromatography of lysates of CMV infected human lung fibroblasts, as described by Spaete et al. [25]. In ELISA determinations, strong reactivity to CMV na antigen was seen with monoclonal antibodies to CMV phosphoproteins of 65-kDa [26] and CMV immediate early antigens (Dupont, France, code D760, cat 9221), while no reactivity was seen with monoclonal antibodies to CMV glycoprotein gB com-

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Monoclonal antibodies to:</th>
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<tbody>
<tr>
<td></td>
<td>CMV phosphoprotein 65 kDa</td>
</tr>
<tr>
<td></td>
<td>CMV immediate early antigen</td>
</tr>
<tr>
<td></td>
<td>CMV glycoproteins gB complex</td>
</tr>
<tr>
<td>CMV nuclear antigen (CMV na)</td>
<td>+</td>
</tr>
<tr>
<td>CMV glycoproteins (CMV gp)</td>
<td>−</td>
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</table>
plex [27]. The monoclonal antibody to CMV gB reacted with the CMV gp antigens but monoclonal antibodies to the CMV phosphoprotein of 65 kDa and the CMV immediate early antigens failed to react with CMV gp antigens. The reactivity of our CMV antigens (CMV na and CMV gp) in ELISA with monoclonal antibodies to CMV 65 kDa phosphoprotein, CMV immediate early antigen and CMV glycoprotein gB complex are outlined in Table 1.

As control antigens for the CMV preparations, we employed a lysate of uninfected human lung fibroblast cells and a purified derivate of *M. tuberculosis* protein (PPD, Serum Institute, Copenhagen, Denmark).

**Anti-CMV serological status**

Antibodies to CMV na and CMV gp were measured by ELISA. Microplates (NUNC, Arthus, Denmark) were coated with the two antigens, and the determination of antibodies was performed as previously described [24].

**Proliferation assay**

Peripheral blood mononuclear cells were stimulated with CMV na and CMV gp, human lung fibroblast antigens and PPD as described [4] and proliferation measured by incorporation of $^{3}$H-thymidine. The results were expressed as a stimulation index (cpm for CMV antigen-stimulated cells/cpm for cells stimulated with control uninfected human lung fibroblast antigens).

**Determination of IFN-γ production at a single cell level**

The production of IFN-γ was measured with a recently developed ELISPOT technique [18]. The technique was modified in order to optimize the assay for the antigens and cells presently employed. Peripheral blood mononuclear cells PBL, washed 3 times in tissue culture medium (TCM, consisting of Iscove’s medium containing 36 mM sodium bicarbonate, 25 mM Hepes, and supplemented with essential amino acids, penicillin and streptomycin), were depleted of the monocytes-macrophages by adherence to plastic for 2 h at 37°C in prewarmed TCM containing 10% human CMV antibody negative AB + human serum. The cells recovered were cultured in 96-well round-bottom plates at a cell density of 150 000 cells/well (total volume 200 μl) in TCM containing 10% AB + CMV antibody negative human serum and CMV na, CMV gp, control uninfected human lung fibroblast antigens or PPD. Optimal antigen concentrations were assessed in preliminary experiments to give a maximal T cell response. After 76 h of culture in a humidified atmosphere at 5% CO$_2$ in air and 37°C, the cells were washed 3 times in prewarmed TCM, resuspended in TCM with 10% human serum, and the living cells were counted with the trypan blue exclusion test. Ninety-six well plates with a nitrocellulose bottom (Millipore, USA, code Millititer HA) were coated with the anti-human IFN-γ monoclonal antibody 1-DIK [28] at a concentration of 15 μg/ml overnight at 4°C, washed 3 times with PBS and blocked with 2% BSA. After 3 subsequent washings in PBS, we applied 200 000 stimulated cells in duplicate or more for 16–20 h. Each run included leucocytes from a known T cell reactive BD. With washings between each step using PBS containing 0.05% Tween 20, we applied sequentially a polyclonal rabbit anti-human-gamma-interferon antibody (Interferon Science, New Jersey, USA, code 3700; dilution 1 : 500) as second antibody, biotinylated goat anti-rabbit IgG (Dakopatts, Copenhagen; dilution 1 : 1000), and an avidin-biotin-peroxidase complex (Dakopatts; dilution 1 : 200). A substrate solution containing amino-ethyl-carbazole was applied for 3 min and, after washing and drying of the plate, the number of spots (each spot corresponding to a single IFN-γ secreting cell) were calculated in a dissection stereomicroscope under low magnification (×25).

The depletion of adherent cells was found to be important for obtaining a good reproducibility of the results. The time of culture (4 days) was chosen on the basis of the kinetics of IFN-γ production shown in preliminary experiments. A linear correlation was found between the number of cells applied per well for ELISPOT detection and the IFN-γ spots detected in the range of $1 \times 10^3$ to $2 \times 10^5$ cells. No spots appeared when any of the reagents were omitted. Addition of the monoclonal anti-human-IFN-γ at a concentration
of 100 μg/ml to the culture medium in the last day of culture blocked the appearance of IFN-γ spots (> 95–99%).

Statistics
The Mann-Whitney U-test was employed to evaluate differences between groups and the Wilcoxon paired test to compare differences between antigens. Correlations between continuous variables were studied with the Spearman rank correlation test (r_s = Spearman correlation coefficient).

Results

Anti-CMV serological status
Ten BD had IgG antibodies to CMV na, with titers between 2900 and 46000 (median 8600). The remaining 7 BD had no measurable CMV antibodies (titers < 100). Nine of the 19 BMT recipients displayed anti-CMV na IgG antibodies (titers between 780 to 52 000, median 6200). In all the patients with IgG antibodies to CMV na, we detected comparable levels of antibodies to CMV gp. CMV seropositive BD had titers to CMV gp between 1170 to 49 700 (median 8450) and CMV seropositive BMT recipients had titers between 4500 and 83 000 (median 6800). There was a significant correlation between the antibody titer to CMV na and to CMV gp in serum (r_s = 0.85, n = 36, P < 0.0001).

Proliferation assay
All CMV seropositive BD had a stimulation index (SI) > 2 (range 2.1–106, median 40) when stimulating with CMV na, while all CMV seronegative BD had SI below 2. Five out of 9 CMV seropositive BMT recipients had an SI over 2 (3 to 329, median 55). In CMV seropositive BD or BMT recipients, no proliferation was observed when stimulating with CMV gp in a concentration range from 10 to 1 μg/ml. In response to PPD, blood donor leucocytes from both CMV seropositive and CMV seronegative individuals proliferated with SI between 3 and 128 (median 62). No response to PPD was detected in BMT recipients (SI between 0.2 and 1).

Table 2
Gamma-interferon secreting cells x 10^5 blood mononuclear cells

<table>
<thead>
<tr>
<th>Antigen stimulation:</th>
<th>CMV na</th>
<th>CMV gp</th>
<th>PPD</th>
<th>Medium only</th>
<th>Human fibroblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV seropositive BD</td>
<td>9/9 (62)</td>
<td>2/5 (1.5)</td>
<td>8/9 (34)</td>
<td>2/9 (1)</td>
<td>1/9 (3)</td>
</tr>
<tr>
<td>CMV seronegative BD</td>
<td>6–107</td>
<td>0–2</td>
<td>0–79</td>
<td>0–1</td>
<td>0–3</td>
</tr>
<tr>
<td>CMV seropositive</td>
<td>4/6 (7)</td>
<td>2/5 (1.5)</td>
<td>5/6 (53)</td>
<td>3/6 (1)</td>
<td>4/6 (2)</td>
</tr>
<tr>
<td>BMT recipients</td>
<td>0–12</td>
<td>0–2</td>
<td>0–177</td>
<td>0–7</td>
<td>0–6</td>
</tr>
<tr>
<td>CMV seronegative BMT</td>
<td>5/7 (15)</td>
<td>2/4 (1.5)</td>
<td>3/7 (2)</td>
<td>0/6</td>
<td>0/7</td>
</tr>
<tr>
<td>recipients (n = 7)</td>
<td>0–30</td>
<td>0–2</td>
<td>0–15</td>
<td>0–0</td>
<td>0–0</td>
</tr>
</tbody>
</table>

The results are reported as number of subjects with IFN-γ secreting cells > 1/ number of subjects analyzed (median result in positive subjects), and range. BD = healthy blood donors, BMT = bone marrow transplant recipients.

Statistical differences:
CMV seropositive BD: medium or human lung fibroblast vs. CMV na (P < 0.003), medium or human lung fibroblast vs. PPD (P < 0.002).
CMV seronegative BD: medium or human lung fibroblast vs. PPD (P < 0.05).
CMV seropositive BMT recipients: medium or human lung fibroblast vs. CMV na (P < 0.009).
CMV na: CMV seropositive BD vs. CMV seronegative BD (P < 0.005); CMV seropositive BD vs. CMV seropositive BMT recipients (P < 0.02).
PPD: CMV seropositive BD vs. CMV seropositive BMT recipients (P < 0.03); CMV seronegative BD vs. CMV seronegative BMT recipients (P < 0.03).
IFN-γ secreting cells × 10^5 blood mononuclear cells

The results obtained for IFN-γ secreting cells are summarized in Table 2. The optimal antigen concentration for this assay was in the range from 10 to 1 μg/ml (Fig. 1). After 72 h of culture, no cytotoxic effects were observed with any of the antigen preparations employed, as evaluated by trypan blue exclusion tests. The results from individual patients for PPD and CMV na stimulated IFN-γ production are plotted in Fig. 2. The CMV seropositive BD showed a median of 62 IFN-γ secreting cells when stimulated with CMV na, while CMV seronegative BD presented no or few (median 7) IFN-γ secreting cells, with no differ-

![Graph showing IFN-γ secreting cells vs antigen concentration](https://example.com/graph.png)

**Fig. 1.** Effect of different CMV antigen concentrations on IFN-γ production in 3 CMV seropositive (closed symbols: ■, ●, ▲) and 2 CMV seronegative subjects (open symbols: ○, ◆). The cells were stimulated with different concentrations of CMV nuclear antigens. The number of IFN-γ secreting cells (Y-axis) detected is plotted for each antigen concentration employed (X-axis). The optimal IFN-γ production corresponded, in seropositive subjects, to concentrations of 10–1 μg/ml of antigens. We could not induce IFN-γ production in CMV seronegative blood donors using antigen concentrations between 0.05 and 10 μg/ml.

![Graph showing IFN-γ secreting cells in healthy blood donors and BMT recipients](https://example.com/graph2.png)

**Fig. 2.** Cytomegalovirus and PPD antigen-stimulated IFN-γ secreting cells per 10^5 blood mononuclear cells in healthy blood donors and bone marrow transplant (BMT) recipients. The results are grouped in CMV seropositive and CMV seronegative subjects. The values for CMV nuclear antigen-stimulated IFN-γ secreting cells are reported after subtraction of the IFN-γ secreting cells observed after stimulation with uninfected human lung fibroblast antigens.}

dence in comparison to the number of spots obtained with the control antigen from uninfected human lung fibroblast cells. IFN-γ secreting cells were observed after stimulation with PPD, with no difference between CMV seropositive or seronegative blood donors. No IFN-γ production was observed in unstimulated cells.

In CMV seropositive BMT recipients, only a few IFN-γ secreting cells were evident after stimulation with CMV na or PPD. CMV na stimulated IFN-γ secreting cells were found in 5 out of seven seropositive patients examined, whenever the T cells also reacted in the proliferation assay. Two of these patients had been transplanted more than one year before and had evidence of previous clinical CMV infection: they presented the highest number of CMV stimulated IFN-γ secreting cells among BMT recipients (15 and 30 spots) and had PPD stimulated IFN-γ secreting cells. The 3 other patients with IFN-γ production after CMV stimulation were at 1–3 months after transplantation. Two of them had an acute CMV...
infection and a positive CMV isolation at the time of the sampling. No IFN-γ secreting cells were detected in any group after stimulation with CMV gp.

**Correlation between T cell immune responses to CMV**

The T cell proliferative response and the number of IFN-γ secreting cells obtained with CMV na were significantly associated ($r_s = 0.71$, $n = 28$, $P < 0.0002$).

**Discussion**

Studies of the anti-CMV cell-mediated immunity in the immunosuppressed host have shown that CD8 + CMV-specific class I-MHC restricted T cell responses are critical for the resolution of CMV infection [29]. The CMV antigens recognized by cytotoxic lymphocytes include the major immediate early antigen and the glycoprotein gB complex [10]. Different mechanisms of endogenous viral antigen presentation to class I MHC-restricted T lymphocytes may exist [30]. However, the antigen specificity of the majority of CMV-specific cytotoxic lymphocytes and the role of T and B cell responses to different CMV antigens in humans must be better defined.

When evaluating the T cell response to CMV at the single cell level, our results confirm that IFN-γ production, as well as proliferation, is a distinctive function of memory T cells in response to CMV in seropositive individuals. In our assay, T cells were activated to produce and secrete IFN-γ only by the CMV antigen containing CMV matrix proteins and the immediate early antigen and not by CMV glycoproteins. The present data confirm previous results [4] and are in contrast with the findings of Gonczol et al. and Liu et al. [8,9]. Differences in the purity of the antigens employed and the use of native compared to recombinant CMV glycoproteins, as well as the high variability observed in anti-viral T cell responses to glycoproteins [31] may account for part of these discrepancies.

We efficiently detected CMV-induced IFN-γ production after the depletion of adherent mononuclear cells, as already reported by Andesson et al. [32]. This observation is not surprising. Activated macrophages can suppress IFN-γ secretion [33] as well as interleukin 2 production [34]. The depletion of adherent cells has already been shown to potentiate the anti-CMV T cell response in vitro [35].

The present method could be easily applied to the study of T cells from transplanted patients. In BMT recipients, IFN-γ is produced after mitogenic stimulation [36]. However, mitogenic stimulation may not be very sensitive in analyzing the defect of T cell function, as this function disappears very late and it can be preserved in patients with a deficient T cell response to antigens. For instance, Ljungman et al. and Levin et al. have shown that lymphocytes from BMT recipients can be easily stimulated by mitogen at a time when they are not able to respond to CMV [20,36,37]. With our method and with PPD, an antigen to which all of the patients had been previously immunized, the numbers of antigen-stimulated IFN-γ secreting cells were profoundly deficient, and could be barely detected in 2 patients. CMV-induced IFN-γ production was reduced even in the presence of a relatively recovered anti-CMV T cell activation, as demonstrated by a good proliferative capacity. Our finding is in accordance with that recently described by Bowden et al. [38], who has observed a deficient IFN-γ secretion in allogeneic marrow transplant patients for many months after transplantation. Our evaluation of the function of single T cells shows, at a greater level, the depression of the T cell response to CMV antigens. Van Tiel et al. had shown that IFN-γ production in response to CMV is impaired in vitro up to 6 months post transplantation to a greater extent than T cell proliferation [39]. We have confirmed this finding revealing that it depends upon a relative reduction in the number of cells which can respond with IFN-γ secretion after antigen activation.

When evaluating the anti-CMV B cell response with the measurement of free antibodies in plasma, we have shown that the antibody response to cytomegalovirus is equally directed to internal and external viral components both in healthy seropositive subjects as well as in seropos-
itive BMT recipients with a significant association between the responses to these antigens. The current data is in accordance with that reported by Ljungman et al. [4] who observed anti-CMV IgG secreting cells among peripheral lymphocytes in healthy subjects and in BMT recipients, especially after CMV reactivation, with a preponderant reaction to a crude CMV membrane preparation. On the other hand, the 2 results are not completely comparable, since we have employed a further purified fraction from CMV membranes containing CMV gp without detectable CMV nucleocapsid, matrix or immediate early antigens.

In conclusion, we have shown that the B and T cell response to CMV during natural immunization differs with regard to antigenic specificity. While the B cell response is directed against internal and external viral antigens, the T cell response is exclusively directed to internal CMV components both when considering the proliferative capacity of T cells and the secretion of IFN-γ from single T cell. This pattern is observed in healthy subjects as well as in BMT recipients. IFN-γ production by single T cells is therefore a useful measure of the T cell response to CMV. This response, as well as the response to PPD, is severely impaired in BMT recipients. Whether the different T and B cell response to CMV antigens is related to the control of the CMV infection is at present not completely understood, but the severe reduction in the number of IFN-γ secreting cells observed in BMT recipients must be considered an important T cell defect of this condition.

Acknowledgements

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References


