The Humoral Immune Response against Human Cytomegalovirus Is Characterized by a Delayed Synthesis of Glycoprotein-Specific Antibodies

Konrad Schoppel, Barbara Kropff, Christian Schmidt, Rolf Vornhagen, and Michael Mach

Infections with human cytomegalovirus (HCMV) continue to be an important clinical problem. The virus represents the most common cause of congenital viral infections in humans and is the leading infectious cause of central nervous system damage in newborn infants [1]. In addition, the virus can cause life-threatening infections in immunosuppressed persons, particularly in transplant recipients and in patients with AIDS [2–4].

Although cellular immunity may be responsible for the recovery from HCMV infection, humoral immunity likely also has an important role in protection against primary infection and in limiting the severity of the disease. For example, it has been documented that preconception seroimmunity to HCMV provides substantial protection against symptomatic infection of the newborn [5–7]. In transplant recipients, passive transfer of antibodies also seems to have a beneficial effect on the clinical outcome of the infection (reviewed in [8]). Moreover, in the closely related murine cytomegalovirus system, it has repeatedly been demonstrated that antibodies can limit viral spread and confer protection from a lethal challenge [9–11].

However, the immunologic events that follow primary and secondary infection or endogenous reactivation are incompletely characterized. A major problem in defining the role of antibodies during the course of HCMV infection is the lack of defined assay systems. HCMV is a highly complex virus potentially encoding close to 200 proteins [12]. The humoral immune response against individual antigens has only been partially investigated but appears to involve a considerable number of structural and nonstructural proteins [13–16]. In recent years, a number of studies have used HCMV-specific recombinant antigens to overcome these limitations [17–20]. The major conclusion from these studies was that phosphoproteins, in particular the basic phosphoprotein of 150 kDa (pp150, ppUL32), represent the most immunogenic antigens for the humoral immune response against HCMV [18, 21–23]. Antibodies against these polypeptides are non-neutralizing and therefore can be expected to contribute little to limitation of viral dissemination [24]. Comparably detailed studies involving glyco- and phosphoprotein antigens are lacking. Since glycoproteins can induce neutralizing antibodies, the kinetics of antibody formation against these antigens could be important in the clinical course of the infection.

For this study, we developed an ELISA using recombinant HCMV-specific peptides to individually determine antibody titers in human sera. Antigens included domains from structural phosphoproteins pp65, pp150, pp28, and pp71, envelope glycoproteins gB and gH, and nonstructural proteins IE1 and p52. The ontogeny of antibodies of the IgG type was analyzed in transplant recipients and in patients with AIDS [2–4].

Materials and Methods

ELISA antigens. Selected HCMV antigens were expressed either as glutathione-S-transferase (GST) fusion proteins (pp65/3, pp28, pp71/2, p52/3, and IE1) or with β-galactosidase (Sem2) as fusion partner (pp150, gH/AD169, gH/Towne, gB/AD-1, gB/
Table 1. Specification of antigens used in the ELISA and serologic results of analyses with HCMV-positive healthy persons.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Amino acids</th>
<th>Peptide</th>
<th>Reactive epitope</th>
<th>Strain specificity</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>pp65 (ppUL83)</td>
<td>372–546</td>
<td>65/3</td>
<td>UK</td>
<td>No</td>
<td>33</td>
</tr>
<tr>
<td>pp28 (ppUL99)</td>
<td>3–190</td>
<td>28</td>
<td>UK</td>
<td>No</td>
<td>23</td>
</tr>
<tr>
<td>pp71 (ppUL82)</td>
<td>188–389</td>
<td>71/2</td>
<td>UK</td>
<td>No</td>
<td>1</td>
</tr>
<tr>
<td>S2 (pUL64)</td>
<td>297–433</td>
<td>52/3</td>
<td>UK</td>
<td>No</td>
<td>47</td>
</tr>
<tr>
<td>IE1 (pUL123)</td>
<td>1–234</td>
<td>IE/1</td>
<td>UK</td>
<td>No</td>
<td>33</td>
</tr>
<tr>
<td>pp150 (pUL32)</td>
<td>555–705</td>
<td>XP1</td>
<td>UK</td>
<td>No</td>
<td>98</td>
</tr>
<tr>
<td>gH/AD169 (pUL75)</td>
<td>15–142</td>
<td>Ap86*</td>
<td>AD86</td>
<td>AD169</td>
<td>40.5</td>
</tr>
<tr>
<td>gH/Towne (pUL75)</td>
<td>14–43</td>
<td>To86*</td>
<td>AD86</td>
<td>Towne</td>
<td>24</td>
</tr>
<tr>
<td>gB/AD169 (pUL55)</td>
<td>28–67</td>
<td>Exo16*</td>
<td>AD2</td>
<td>AD169</td>
<td>34</td>
</tr>
<tr>
<td>gB/Towne (pUL55)</td>
<td>12–57</td>
<td>HMTo16*</td>
<td>AD2</td>
<td>Towne</td>
<td>2.5</td>
</tr>
<tr>
<td>gB/AD2 (pUL55)</td>
<td>67–84</td>
<td>pep90</td>
<td>AD2</td>
<td>No</td>
<td>47</td>
</tr>
<tr>
<td>gB/AD1 (pUL55)</td>
<td>484–650</td>
<td>Mbg58</td>
<td>AD1</td>
<td>No</td>
<td>97</td>
</tr>
<tr>
<td>gH/AD86 (pUL75)</td>
<td></td>
<td></td>
<td>AD86</td>
<td>AD169 + Towne</td>
<td>16.5*</td>
</tr>
<tr>
<td>gB/AD2 (pUL55)</td>
<td></td>
<td></td>
<td>AD2</td>
<td>AD169 + Towne</td>
<td>19*</td>
</tr>
<tr>
<td>gH (pUL75)</td>
<td></td>
<td></td>
<td>AD86</td>
<td></td>
<td>82*</td>
</tr>
<tr>
<td>gB (pUL55)</td>
<td></td>
<td></td>
<td>AD1 + AD2</td>
<td></td>
<td>97*</td>
</tr>
</tbody>
</table>

NOTE. UK = reactive epitope is unknown; no = no discrimination between AD169-like and Towne-like strains possible; AD86 = antigenic domain 86; AD2 = antigenic domain 2; AD1 = antigenic domain 1.

* Peptide containing strain-specific epitope that allows discrimination between AD169-like and Towne-like viruses.

† % of reactive samples containing antibodies against AD169-type and Towne-type viruses.

‡ % of reactive samples containing antibodies against AD86 localized on gH (AD169 + Towne).

AD169, and gB/Towne). The construction of GST-plasmids and purification of the respective proteins has been described [18]. The final purity of the GST fusion proteins was >95%. The construction of β-galactosidase–fusing plasmids has been described elsewhere: gB/AD-1 [25], gB/AD169 and gB/Towne [26], gH/AD169, and gH/Towne [27]. The purification of Sem2 fusion proteins was described previously [20]. Purified polypeptides were analyzed on polyacrylamide gels and estimated to be >80% pure (data not shown). The peptide gB/AD-2 was chemically synthesized [25]. The origins of the individual antigens within the respective HCMV proteins are summarized in table 1.

**ELISA with selected HCMV antigens.** Polystyrene 96-well microtiter plates were coated with 50 μL/well purified fusion protein at 1–1.5 μg/mL. The peptide gB/AD-2 was used at a concentration of 0.5 μg/mL. Antigens were tested and found optimal at these concentrations. GST fusion proteins and gB/AD-2 were solubilized in 0.01 M carbonate buffer, pH 9.5, while Sem2 fusion proteins were diluted in 6 M urea, pH 9.5. Microtiter plates were incubated for 16 h at 4°C in a humid chamber. All subsequent incubation steps were carried out at 37°C. Reaction wells were rinsed three times with buffer A (PBS, 0.05% Tween 20) and blocked for 2 h with PBS containing 2% fetal calf serum. Plates were again rinsed with buffer A and incubated with human sera (dilution 1:50) or monoclonal antibodies for 2 h (50 μL/well). After 4 additional washes with buffer A, 50 μL of peroxidase-conjugated anti-human or anti-mouse IgG was added in appropriate dilutions for 45 min. Plates were washed four times, and 100 μL of substrate (o-phenylenediamine; 2 mg/mL) was added for 20 min. The reaction was stopped by addition of 100 μL of 2 N H2SO4, and the optical density was determined at 492 nm. Dilution of all antibodies was done in the dilution buffer provided with the human immunodeficiency virus (HIV)–1/2 ELISA kit (Biotest).

To determine the cutoff for this ELISA, 13 HCMV-negative human sera and 2 independent pools (20 HCMV-negative sera each) were analyzed with all antigens, and reactivity was measured as A490. A reactivity index (RI) was calculated according to the formula A490-antigen/A490-respective fusion partner. The RI cutoff for each individual antigen was defined as median + 2 SD. To define a sample positive with an individual antigen, two different criteria had to be met: reactivity with GST and Sem2 between A490 = 0.1 and A490 = 0.4, and RI higher than the antigen-specific RI cutoff.

To determine the variability of the assay, individual samples were analyzed at least three times in different experiments. The SD calculated for each antigen was 10% at the maximum.

**Serum samples.** In this study, recombinant HCMV antigens were evaluated with human sera from different sources, including healthy blood donors (n = 220), pregnant women with primary HCMV infection (n = 7), liver transplant patients with different donor/recipient (D/R) seropositivity combinations (D+/R+, n = 12; D+/R− , n = 9; D−/R+ , n = 8), and patients after kidney transplantation (n = 9). All serum samples from transplant recipients were analyzed for viral replication by polymerase chain reaction (PCR) as described previously [28].

**Limiting dilution analysis in the EL-4 culture system.** B cells were obtained from peripheral blood mononuclear cells by positive selection with the CD19-Dynabead system (Dynal, Hamburg, Germany). The purity of the prepared B cells was controlled during flow cytometry by use of phycoerythrin-coupled anti-CD19, fluorescein isothiocyanate (FITC)–coupled anti-CD3, and FITC-coupled anti-
CD14 (Becton Dickinson, Mountain View, CA) on a FACStaRe (Becton Dickinson) and was in the range of 95%. For limiting dilution analysis of anti-HCMV B cells, 200, 500, and 800 B cells were pipetted into a series of 96-well flat-bottomed plates (200 µL cultures) containing 5 x 10^5 irradiated (5000 cGy) murine thymoma cells/well (mutant EL-4 cell clone 6.1.5.5 [29]) plus 5% supernatant from human T cells (T-SN), which had been produced by stimulation with phytohemagglutinin plus phorbol myristate acetate as described [30]. After 10 days of culture at 37°C, supernatants were analyzed by ELISA for antibody production. Polystyrene microculture plates (96 wells each; Nunc, Roskilde, Denmark) were coated with gB/AD-2 (amino acids [aa] 67–84; 0.5 µg/mL), pp150/7 (aa 862–1048; 0.02 µg/mL), and gB/AD-1Δ (aa 483–651; 0.5 µg/mL) to detect HCMV-specific antibody production. The prokaryotically expressed constructs pp150/7 and gB/AD-1Δ contain 13 aa at their N-terminal parts that are not HCMV-derived. gB/AD-2 was used as a synthesized peptide. Overall IgG production was detected with plates coated with rabbit anti-human γ-chain antibody (Dako, Carpenteria, CA) using a peroxidase-coupled sheep anti-human Fab antibody (Dako). EL-4 + B cell cultures were scored positive if the A490 exceeded the mean level +3 SD as determined with 18 control cultures (EL-4 + B cells without 5% T-SN; EL-4 + T-SN without B cells) for each experiment. The antigen-specific B cell frequencies (pASC) were calculated by Poisson statistics from the fraction of negative wells [31].

Statistical analysis. Statistical analyses of precursor B cell frequencies were done as described [31]. Significance of the correlation between antibody titers against gB/AD-1 and PCR positivity was calculated using the χ² test.

Results

Antibody Profiles in Healthy HCMV-Seropositive Persons

For the purpose of this study, the HCMV-derived antigens were divided into two broad categories: 1) structural phosphoproteins and nonstructural proteins and 2) glycoprotein antigens.

Structural phosphoproteins, including pp150 (ppUL32), pp65 (ppUL83), and pp28 (ppUL99), and nonstructural proteins IE1 (pUL123) and p52 (pUL44). Phosphoproteins are located at inner structures of virions or infected cells (or both) and are not present on the surface of infected cells (reviewed in [24]). Available evidence suggests that antibodies against the antigens used in this study lack neutralizing capacity. Therefore, we will refer to these antigens collectively as non-neutralizing antigens and to the corresponding antibodies as non-neutralizing antibodies.

Glycoprotein antigens, including the immunodominant envelope glycoproteins B (gB, gpUL55) and H (gH, gpUL75). From gB, antigenic domains AD-1 (gB/AD-1) and AD-2 (gB/AD-2) were used [25, 26, 32–34]. AD-2 encompasses two antigenic sites located between aa 68–77 (site I) and aa 50–54 (site II). Site I is conserved between HCMV isolates, and the corresponding antigen was designated gB/AD-2. Site II shows variations, and the prototype sequences are encoded by the HCMV strains AD169 and Towne [26]. The corresponding antigens were designated gB/AD169 and gB/Towne, respectively. The gH antigen source consisted of the domain AD-B6. This domain also shows heterogeneity between strains [27]. The prototype sequences were included in the antigens gH/AD169 and gH/Towne, respectively. Antibodies to the strain-specific epitopes do not cross-react [26, 27]. For detailed description of the individual antigens see table 1. Since these domains have been shown to induce neutralizing antibodies during natural infection [20, 25, 27, 34], we will refer to these antigens as neutralizing antigens and to the respective antibodies as neutralizing antibodies.

We are aware that this classification represents an oversimplification, but it greatly facilitates description, interpretation, and discussion of the data in the context of this study.

In a first set of experiments, 79 sera from healthy HCMV-seropositive persons were analyzed with the entire set of antigens. The optical density obtained for the individual samples in the ELISA was converted to RI. The RI was found to be constant for a given serum, thus eliminating variations in absolute concentration of IgG resulting from long-term storage or handling of serum samples and also day-to-day variations between assays. Nevertheless, sequential sera from individual patients in general were analyzed in one assay.

Different antibody titers against the individual antigens were observed, which allowed us to identify a characteristic profile for each person. Profiles of 3 donors are shown in figure 1. In general, sera were positive for more than one antigen. In the entire sample group, antibodies that were most consistently detected were directed against pp150 and gB/AD-1, with positivity rates of 98% and 97%, respectively (table 1). The remaining strain-common antigens were recognized with considerably lower frequency (table 1). With respect to the strain-specific antigens on gB and gH, the majority of sera reacted with either the AD169- or the Towne-derived antigen. We observed seropositivity for gH/AD169 in 40.5% of samples and for gH/Towne of 24%. However, we also found that 16.5% of sera were positive for both antigens. In total, 81% of sera reacted with gH. The strain-specific epitope on gB/AD169 was recognized by 34% of the sera, whereas the gB/Towne antigen reacted only in 2.5%. The rate of sera that were positive for both gB-derived antigens was 19%, which was similar to results for gH. Strain-common and strain-specific seropositivity rates for glycoproteins B and H were confirmed with additional sera from 141 healthy persons (data not shown).

Next we wanted to determine whether antibody titers against the individual antigens showed fluctuations over time in healthy HCMV-seropositive persons. To this end, consecutive serum samples from 7 donors, collected over a period of up to 70 months, were tested. In 4 cases, the antibody titers were quantitatively stable with differences between the individual samples that could be explained by the variation of the assay, which was in the range of 10%. The profile of 1 of these donors is shown in figure 1 (donor H2). The remaining 3 donors showed fluctuation in antibody titers that exceeded variations between
assays. This was most pronounced in donor H3; differences of 50% between the highest and lowest titers against the major antigens pp150 and gB/AD-1 were seen. The changes appeared in a timely, coordinated manner in that HCMV-specific antibody titers increased between 0 and 11 months and thereafter declined. However, as can be seen from figure 1, antibody profiles were qualitatively remarkably stable. We did not observe cases in which antibody titers against single antigens

Figure 1. Antibody profiles of 3 healthy persons (H1, H2, H3) against selected HCMV antigens. Bars indicate antibody reactivity, expressed as reactivity index (RI), at first time point (0 months). Subsequent time points (months) are shown as symbols connected by lines.

Figure 2. HCMV-specific antibody response after primary infection in pregnant women. Antigen-specific antibody reactivity (reactivity index [RI]) against non-neutralizing antigens is shown as solid lines; glycoprotein-specific antibodies are shown by dashed lines.
Table 2. Kinetics of appearance of neutralizing and non-neutralizing antibodies in patients after liver or kidney transplantation and in pregnant women.

<table>
<thead>
<tr>
<th>Patient group, antigen</th>
<th>At primary infection*</th>
<th>Day 50</th>
<th>Day 100</th>
<th>&gt;100 days</th>
<th>At reactivation†</th>
<th>Day 50</th>
<th>Day 100</th>
<th>&gt;100 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver transplant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-neutralizing</td>
<td>7/9</td>
<td>7/9</td>
<td>6/7</td>
<td>6/6</td>
<td>5/6</td>
<td>5/6</td>
<td>5/6</td>
<td>6/6</td>
</tr>
<tr>
<td>Neutralizing</td>
<td>0/9</td>
<td>0/9</td>
<td>2/7</td>
<td>4/6</td>
<td>5/6</td>
<td>5/6</td>
<td>5/6</td>
<td>6/6</td>
</tr>
<tr>
<td><strong>Kidney transplant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-neutralizing</td>
<td>4/5</td>
<td>2/2</td>
<td>ND</td>
<td></td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>1/1</td>
</tr>
<tr>
<td>Neutralizing</td>
<td>0/5</td>
<td>0/2</td>
<td>ND</td>
<td></td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>1/1</td>
</tr>
<tr>
<td><strong>Pregnant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-neutralizing</td>
<td>7/7</td>
<td>7/7</td>
<td>6/6</td>
<td>5/5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutralizing</td>
<td>0/7</td>
<td>0/7</td>
<td>2/6</td>
<td>2/5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-neutralizing</td>
<td>18/21 (86)</td>
<td>16/18 (89)</td>
<td>12/13 (92)</td>
<td>11/11 (100)</td>
<td>9/10 (90)</td>
<td>9/10 (90)</td>
<td>9/10 (90)</td>
<td>7/7 (100)</td>
</tr>
<tr>
<td>Neutralizing</td>
<td>0/21</td>
<td>0/18</td>
<td>4/13 (31)</td>
<td>6/11 (55)</td>
<td>9/10 (90)</td>
<td>9/10 (90)</td>
<td>9/10 (90)</td>
<td>7/7 (100)</td>
</tr>
</tbody>
</table>

NOTE. ND, not determined. *Days” refers to days after primary infection or reactivation, respectively. “Reactive” means \( \geq 3 \) times the reactivity index.

* As determined by polymerase chain reaction (PCR) for transplant patients or after first seropositive sample for pregnant women.
† As determined by PCR.

changed in relation to the rest. Taken together, these data indicate that in healthy persons, an individual antibody profile against the various HCMV antigens is developed that is qualitatively stable over long periods of time.

Serologic Response during Primary HCMV Infection

**Pregnant women.** Antibody titers were analyzed in 7 women experiencing a primary HCMV infection during pregnancy as defined by seroconversion established by a commercially available ELISA. Figure 2 shows representative data for 2 women. In subject PW1, seroconversion was characterized by a 10-fold increase in RI against pp150 within 50 days, followed by a drop to constant levels by day 85. Antibodies against any of the other non-neutralizing antigens did not develop. Glycoprotein-specific antibodies were not elevated by day 50 and showed increases of 2-fold by day 100 and 2.8-fold by day 140. Similarly, antibodies against non-neutralizing antigens IE1, pp65, and p52 developed at day 50 in subject PW2, whereas glycoprotein-specific antibodies could not be detected at this time. Primary infection in the entire group of women was uniformly characterized by the predominant development of high titers of antibodies against non-neutralizing antigens within the first 50 days. In contrast, glycoprotein-specific antibodies were not detected during this time and developed in only 2 of 6 cases at day 100 and 2 of 5 at times later than day 100 (table 2). In contrast to results in sera from healthy persons, we repeatedly observed serum samples that had detectable antibody levels against only one antigen (e.g., day 50, subject PW1).

**Transplant patients.** Primary infection in immunosuppressed patients was assayed in recipients of liver and kidney transplants. Collectively, the liver transplant group of patients consisted of 9 HCMV-seronegative transplant recipients. Three patients received a transplant from an HCMV-seronegative donor (the D−/R− group) and developed an active HCMV infection between days 25 and 39 (median, 27) after transplantation as demonstrated by a positive PCR result. Six patients in this group received a transplant from an HCMV-seropositive donor (the D+/R− group). In this group, all patients tested positive by PCR between days 19 and 49 (median, 38) after transplantation.

Figure 3 shows antibody responses that are typical of those seen in D−/R− patients who responded serologically to viral infection. In patient LTX1, PCR became positive at day 39 after transplantation. Antibody titers against phosphoproteins pp65 and pp150 started to rise at the same time, with pp65 showing elevated levels already at day 39. Titers against other antigens, including glycoproteins, remained at a low level until day 100 and were only slightly elevated at day 138. The pattern of antibody response was similar in patients from the D+/R− group. For example, patient LTX4 became PCR-positive at day 31 and started to develop antibodies against pp150 at day 37 (figure 3). Within the observation period (day 114 after transplantation), this patient did not make antibodies against any of the other antigens, including glycoproteins. Development of glycoprotein-specific antibodies was found in patient LTX26. At day 180 after transplantation, high titers against gH/AD169 and gB/AD-1 were noted. Initially, this patient did not respond to viral replication, which was detected at days 49 and 62 after transplantation, with a relevant increase in RI. We interpret the low titers of pp150-specific antibodies in the early phase after transplantation as a weak response against low-level viral replication, which did not produce a
positive PCR signal. We have repeatedly detected serologic response in the absence of PCR positivity (see patient LTX35, figure 4).

When we analyzed sera from 5 patients experiencing a primary infection after kidney transplantation, we found a situation that was identical to patients undergoing liver transplantation. All patients were seronegative and received an organ from a seropositive donor (D+/R-). An example is shown in figure 3. This patient (KTX1) responded to viral replication with the production of antibodies against IE1, pp52, and pp28, whereas glycoprotein titers were not elevated.

Taken together, the data derived from the immunosuppressed patients were very similar to the situation found in pregnant women. Following primary infection, exclusively antibodies reactive with non-neutralizing antigens were synthesized within the first 50 days (table 2). Glycoprotein-specific antibodies started to appear around day 100 in a fraction of patients (table 2).

Serologic Response during Reactivation/Reinfection

In the liver transplant group, we analyzed 6 patients who were seropositive for HCMV before transplantation and who developed an active infection after transplantation as determined by PCR. Figure 4 shows a typical antibody profile of such a recipient. Patient LTX20 was tested PCR-positive at days 15, 22, and 29 after transplantation. Elevated antibody titers were observed at day 29. The antigens to which the patient responded included non-neutralizing antigens (pp150, pp65, p52, IE) as well as glycoproteins (gB/AD-1, gB/AD-2). There was no delay in response against the

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Figure 3. Serologic analysis of patients after liver (A–C) or kidney transplantation (D) during primary HCMV infection. Antigen-specific antibody reactivity (reactivity index [RI]) against non-neutralizing antigens is shown as solid lines; glycoprotein-specific antibodies are shown by dashed lines. Arrows indicate times at which polymerase chain reaction was positive for HCMV. "Days" refers to before or after transplantation.
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Figure 4. HCMV-specific serologic analysis of patients after liver (A, B) or kidney transplantation (C) during virus reactivation or reinfection. Antigen-specific antibody reactivity (reactivity index [RI] against non-neutralizing antigens is shown as solid lines; glycoprotein-specific antibodies are shown by dashed lines. Arrows indicate times at which polymerase chain reaction was positive for HCMV. “Days” refers to before or after transplantation.

In summary, the kinetics of antibody production in the reactivation/reinfection group indicated that in contrast to primary infection, production of antibodies against neutralizing and non-neutralizing antigens was synchronized (table 2). The results were confirmed in ELISAs using only pp150 and gB/AD-1 antigens in 14 additional liver transplant patients (D+/R+ = 5; D+/R- = 3; D+/R+ = 6) (data not shown).

Precursor Frequencies of B Cells Specific for Phospho- and Glycoproteins

One possible explanation for the delayed appearance of antibodies directed against glycoproteins during primary infection could be a lower precursor frequency of glycoprotein-specific B
cells. We therefore determined the pASC of B cells in 2 healthy persons against pp150, gB/AD-1, and gB/AD-2, using the limiting dilution analysis as described by Wen et al. [30]. Antibody production against HCMV antigens was determined in an ELISA using HCMV-specific antigens, and frequencies were calculated [31]. In the HCMV-seropositive person, pASC of 1/618, 1/678, and 1/690 were obtained for antigens gB/AD-1, gB/AD-2, and pp150, respectively. The HCMV-seronegative donor showed a slightly lower B cell frequency, which was ~1/757 and 1/1153 for gB/AD-2 and pp150, respectively. Figure 5 shows data from a representative experiment and table 3 summarizes three independent analyses. Precursor frequencies for gB/AD-1 were confirmed in 2 additional HCMV-seronegative donors (data not shown). In conclusion, these data suggest that the precursor frequencies against the individual structural antigens were not significantly different.

Inverse Correlation of High Antibody Titers against Glycoproteins and PCR Positivity

Antibodies against glycoproteins potentially neutralize virus and therefore could inhibit dissemination of free virus [9, 11].

Table 3. Precursor frequencies of HCMV-specific B cells isolated from HCMV-seropositive and HCMV-seronegative donor.

<table>
<thead>
<tr>
<th>HCMV-specific antigens</th>
<th>HCMV-seropositive</th>
<th>HCMV-seronegative</th>
</tr>
</thead>
<tbody>
<tr>
<td>gB/AD-1</td>
<td>618 ± 164</td>
<td>1001 ± 271</td>
</tr>
<tr>
<td>gB/AD-2</td>
<td>678 ± 143</td>
<td>1153 ± 364</td>
</tr>
<tr>
<td>pp150</td>
<td>690 ± 131</td>
<td>757 ± 202</td>
</tr>
</tbody>
</table>

NOTE. Statistical analyses were done according to [31]. Data are mean frequency as determined from 3 independent experiments (±SD).

We and others have shown that anti-gB antibody titers correlate with neutralizing capacity, whereas phosphoprotein antibodies do not [20, 24, 35]. This was also confirmed in this study. When we tested selected samples from all groups, high HCMV neutralizing titers were observed only in those samples that also had high titers of glycoprotein-specific antibodies (data not shown).

In addition, during our analyses we repeatedly have seen patients with consistently high levels of glycoprotein-specific antibodies and no sign of viral replication. However, a negative PCR signal could also be the result of an absence of viral replication in these patients. In general, it is not possible to experimentally discriminate between these possibilities. However, for cases in which PCR positivity was observed, a correlation could be analyzed. To do this, it was necessary to determine the absolute amount of gB-specific antibodies in the serum samples rather than to calculate an RI. To establish a

Table 4. Inverse correlation of antibody titers against gB/AD-1, determined in patients after liver transplantation, with polymerase chain reaction (PCR) positivity against HCMV (P < .001, χ² test).

<table>
<thead>
<tr>
<th>Liver transplant patients' donor/recipient status</th>
<th>No. PCR-positive</th>
<th>gB/AD-1 titer (A490)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>D+/R+</td>
<td>27</td>
<td>2</td>
</tr>
<tr>
<td>D+/R−</td>
<td>41</td>
<td>0</td>
</tr>
<tr>
<td>D−/R+</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>82</td>
<td>2</td>
</tr>
</tbody>
</table>

* Antibody titer against gB/AD-1 measured by ELISA (absorbance at 490 nm).

Figure 5. Limiting dilution analysis of frequencies of HCMV-specific B cells from blood of seronegative donor. Data from 1 experiment are shown. % of cultures negative for antigens gB/AD-1, gB/AD-2, and pp150 by ELISA are plotted against no. of B cells added per culture. Intersection of dashed lines with x axis represents frequencies calculated according to [31]: gB/AD-1 = 1/974, gB/AD-2 = 1/1075, pp150 = 1/733.
reference point for antibody titers against gB that effectively could neutralize free virus, a pool of healthy HCMV-seroposi-
tive donors was tested against gB/AD-1. In our assay system, this pool created a net absorbance value of 1.7 A492 when corrected for the value obtained with an HCMV-seronegative pool (data not shown). This value was then correlated with PCR positivity in the samples. A total of 82 specimens were available for which the PCR assay gave a positive result (table 4). In only 2 cases, gB/AD-1 titers were >1.7 A492. These 2 samples were derived from patients in the early antibody response phase during a reactivation and who had PCR-negative results for the following samples when AD-1 titers were well above 1.7. The lack of PCR positivity in the presence of high titers of anti-gB antibodies was statistically highly significant \( (P < .001, \chi^2 \text{ test}) \).

**Discussion**

In this study, we have analyzed domain-specific antibodies against a variety of HCMV-derived structural and nonstructural antigens. As expected, we found individual titers against the antigens, resulting in an antibody pattern that is characteristic for each healthy donor. Our results confirm and extend those of previous investigations. For example, it has been reported before that antibodies against pp150 and gB can be detected in nearly 100% of infected persons [18, 20, 22, 36]. In contrast to previous investigations, we could show that overall seropositivity against gH of HCMV is also high (>80%) when epitopes from strains AD169 and Towne are used as antigens. In addition, with the use of antigens that bind strain-specific antibodies, we could demonstrate exposure to multiple HCMV strains in ~20% of the healthy population. The individual antibody profiles are qualitatively constant over years. However, quantitative changes do occur in some infected persons. The most plausible explanation for these fluctuations in titer is increased antibody production in response to viral replication (reactivation or reinfection). Since we determined relative antibody titers in healthy persons as well as different groups of transplant patients, it is extremely unlikely that our study population biased our results.

A second possibility is that there may be a delay in development of antibodies against rather linear epitopes on glycoproteins. This also seems highly unlikely, since we could not detect a difference in the kinetics of antibody formation when native or denatured baculovirus-derived gB was used as antigen in ELISAs, an antigen that in its native form is capable of binding conformation-dependent antibodies (Urban M, Mach M, unpublished data). In addition, gB/AD-1 is an antigen that requires conformation for antibody binding [25, 32, 34].

A third possibility is that glycoprotein-specific antibodies were trapped in immune complexes with antigen and therefore escaped detection. Such a mechanism has been postulated for other viral infections, such as hepatitis B virus (HBV), in which antibodies against the surface antigen can be detected only with considerable delay compared to capsid-specific antibodies [47]. However, primary HBV infection is characterized by the production of excessive amounts of surface antigen, which makes such a complex formation possible. For HCMV, such a mechanism seems highly unlikely, since the amount of antigen during primary infection is most probably not sufficient to bind all glycoprotein-specific antibodies.

A fourth possibility is that frequencies of precursor B cells with antibody specificities for HCMV glycoproteins are signifi-
cantly lower than those against other antigens, resulting in a delay in the appearance of detectable amounts of glycoprotein-specific immunoglobulins. This assumption is based on studies that have shown a \( \sim 10 \)-fold increase in pASC between naive and primed immune systems. Examples are patients infected with HIV or \textit{Plasmodium falciparum} and persons after immunization with tetanus toxin [30, 48]. Delayed production of neutralizing antibodies against LCMV has also been discussed as a result of low precursor B cell frequencies [40]. Our data did not reveal a significant difference in pASC-producing antibodies against HCMV glycoproteins or phosphoproteins, indicating that the number of precursor B cells is not the cause for the delayed synthesis of glycoprotein-specific antibodies during natural infection. We feel that testing 3 HCMV-seronegative donors for pASC was sufficient, since the delayed synthesis of glycoprotein-specific antibodies was seen in 100% of our study populations. The fact that we observed only a marginal difference in pASC between an HCMV-primed and a naive immune system is most probably the result of the high numbers of HCMV-specific precursor B cells. Comparable high frequencies have been reported for precursor B cells producing rheumatoid factors in patients suffering from rheumatoid arthritis or for the transferrin receptor in patients with Crohn’s disease [49, 50]. pASC for these antigens were comparably frequent in normal controls, indicating that in situations in which the number of precursor B cells is high, circulating memory B cells do not significantly increase the absolute number of pASC specific for a given antigen.

In general, we exclude the possibility that passively transferred antibodies did influence the data obtained with transplant patients, since we did not observe a change in antibody status in 3 HCMV-seronegative patients who were treated with immunoglobulin preparations but remained PCR-negative (data not shown). In addition, the different antibody profiles seen in individual patients argued against a significant contribution from antibodies provided by treatment with intravenous immunoglobulins.

Because of the above reasons, the most plausible explanation for the delayed occurrence of glycoprotein-specific IgG antibodies during primary HCMV infection is delayed synthesis. We hypothesize that it is caused by a mechanism that is active during clonal development of B cells (activation, somatic maturation, immunoglobulin class switch). The fact that it is not seen during reactivation or reinfection events indicates that this mechanism is not active on memory B cells.

What could be the consequences of the delayed synthesis of glycoprotein-specific antibodies for the course of the HCMV infection? The role of antibodies during HCMV infection has been a matter of debate for decades. However, only in recent years have their specific functions been investigated in more detail. The importance of antiviral antibodies in different clinical situations is a complex problem, and these situations should be considered separately.

Passively transferred antibodies can protect from a subsequent infection. This has been demonstrated in the closely related murine cytomegalovirus (MCMV) [9–11]. In addition, vaccination with glycoproteins has demonstrated protection from infection in other herpesvirus systems, such as HSV (reviewed in [51]). For HCMV, evidence for the role of antibodies in prevention of infection is indirect (reviewed in [8]). However, in a recent vaccination study involving the attenuated Towne strain, protection from reinfection was correlated with presence of neutralizing antibodies and not cellular immunity [5].

Since primary infection in immunocompetent persons is usually asymptomatic, data on the role of antibodies mainly come from MCMV. Mice that are incapable of immunoglobulin synthesis clear virus and establish viral latency with kinetics that are indistinguishable from normal mice [9]. This has led to the conclusion that antibodies play a minor role in primary infection.

Antibodies cannot prevent reactivation. This is evident from epidemiologic data on HCMV reactivation in HCMV-seropositive immunocompetent persons. However, antibodies effectively limit viral spread during reactivation. Mice that are B cell deficient have a 100- to 1000-fold-higher virus load after reactivation of latent virus than their B cell–containing littermates. The extent of reduction is dependent on antibody titer [9].

Regarding prevention from disease, virulent congenital HCMV infection with clinically apparent multiorgan involvement, rarely, if ever, follows fetal infection in women with preexisting seroimmunity [52, 53]. It is believed that transfer of maternal antibody to the fetus plays the key role in this protection. In addition, in allograft recipients, the importance of antiviral antibodies in the prevention of severe HCMV disease has been demonstrated (reviewed in [8]).

With the exception of primary infections, it seems therefore established that antibodies can have a protective effect on HCMV infection and progression to disease. The quantitative relationship between antibodies and virus will likely influence the course of the infection. A number of studies have indicated a correlation between virus load and risk for disease [54, 55]. On the other hand, it has also been shown that virus neutralization in vivo is dependent on the titer and biologic activity of antibodies [10, 56]. Therefore, the delayed synthesis of neutralizing antibodies during primary infection could lead to viral dissemination, with higher virus load in visceral organs and, consequently, increased risk of disease. It is well-established that primary HCMV infection in transplant patients is associated with a higher risk of developing severe HCMV disease than reactivation [2]. During reactivation of HCMV, the neutralizing antibody response is prompt and the titers are often higher, resulting in an effective control of viral dissemination and reduction in the risk for disease. Our data on the correlation between glycoprotein-specific antibody titers in transplant patients and absence of viral DNA in peripheral blood support this assumption.

Primary maternal infection represents a consistent risk factor associated with symptomatic congenital HCMV infection.
Transmission of HCMV to the fetus follows ~40% of primary maternal infections. In contrast, preconception seroimmunity to HCMV provides substantial protection against intrauterine transmission and damaging fetal infection [5–7]. The mechanism(s) responsible for the increased severity of fetal HCMV infection during primary infection of the mother remains unknown. However, the lack of neutralizing antibodies, as we have seen it in the early phases of the primary infection, might represent one important risk factor for efficient viral dissemination and, consequently, for transmission to the fetus. Previous studies have also noted low titers of anti-gB antibodies in the early phase after primary infection during pregnancy, although titers against other viral antigens were not determined [57, 58].

In summary, we have shown that the humoral immune response against primary HCMV infection is characterized by a delayed synthesis of glycoprotein-specific antibodies, whereas during reactivation or reinfection, these antibodies are promptly synthesized. Since levels of glycoprotein-specific antibodies correlate with virus neutralizing capacity, these observations provide an explanation for the efficient viral dissemination during the early phases of the infection. Further studies are needed to explore the mechanism for the delayed synthesis in order to devise strategies to overcome these limitations. Whether the separate determination of antibodies against glyco- and phosphoproteins could represent a useful diagnostic tool to discriminate between primary HCMV infection and reactivation or reinfection is currently under investigation.

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