CYTOMEGALOVIRUS (CMV) - SPECIFIC ANTIBODIES INHIBIT THE IN VITRO GENERATION OF CMV PP65 ANTIGENEMIA IN POLYMORPHONUCLEAR CELLS


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submitted
Abstract
Cytomegalovirus (CMV) pp65 antigenemia can be mimicked by coculture of polymorphonuclear granulocytes (PMN) with CMV infected endothelial cells. We questioned whether CMV virus specific antibodies including neutralizing antibodies could inhibit the development of CMV antigenemia. Sera from CMV seropositive kidney transplant recipients inhibited the generation of pp65 positive PMN to 58.6 ± 26.9% of the control. Sera from CMV seronegative patients had no effect. The inhibitory effect could be attributed to the immunoglobulin fraction of the sera, as purified serum IgG of CMV seropositive sera and CMV hyperimmune globulins reduced the production of pp65 positive PMN to 42.5 ± 4.8% and 54.2 ± 7.0% of the control, respectively. All CMV seropositive sera tested had virus neutralizing capacity. Thus, to our knowledge we are the first to report that CMV specific antibodies reduce the generation of pp65 positive PMN in vitro and thus may inhibit CMV antigenemia-mediated viral dissemination.

Introduction
Since the introduction of the cytomegalovirus (CMV) pp65 antigenemia assay, CMV infections after transplantation can already be diagnosed from early stages of infection [1]. The assay is based on the detection of CMV protein pp65 in nuclei of polymorphonuclear granulocytes (PMN) [2]. The CMV antigenemia shows a good correlation with viral load and development of HCMV disease [1].

Recently, in vitro models for CMV antigenemia became available in which CMV pp65 positive PMN were obtained after coculture with fibroblasts or endothelial cells infected with clinical strains of CMV [3,4]. In these models only clinical strains of CMV could evoke positive PMN. These pp65 positive PMN could retransmit the virus to fresh layers of fibroblasts [3]. Antibodies against ICAM-1 or LFA-1 inhibited the uptake of pp65 by PMN. Furthermore the incubation of PMN with viral supernatant did not result in pp65 positive PMN [4]. These data indicated that a close cell-cell contact between infected cells and PMN was required for the transfer of viral particles and viral proteins.

We hypothesize that apart from the ICAM-1-LFA-1 interaction, viral molecules expressed at the surface of infected cells play a role in the cell-contact mediated transfer of viral particles to the PMN. If this would be the case, CMV specific antibodies exert an inhibiting effect on the transfer of CMV to PMN, which can be measured as a decreased pp65 antigenemia. We used human umbilical vein endothelial cells infected with the endothelial adapted CMV strain TB42E as a source of infectious virus and cocultured the infected endothelial cells with PMN from healthy volunteers. We examined the effect of CMV specific antibodies, present in sera of patients after a CMV infection, on the uptake of pp65 by PMN in an in vitro model for CMV antigenemia.
Material and Methods

Human umbilical vein endothelial cells (HUVEC) were grown at 1% gelatin coated tissue culture flasks in endothelial growth medium (RPMI 1640, 20% FCS, endothelial cell growth factor 50 µg/ml, heparin 5 U/ml, glutamine 2 mM, penicillin 100 U/ml and streptomycin 0.1 mg/ml). HUVEC were used between passage 1 and 4 [5]. Human embryonic lung fibroblasts were used between passage 8 and 17. HUVEC were infected with the endotheliotropic CMV clinical isolate TB42E [6] by seeding trypsinized CMV-infected HUVEC and uninfected HUVEC at a ratio 1:10 in 24 wells plates at subconfluent density. After 5 days more than 85% of the HUVEC were infected as determined by immunofluorescent staining with monoclonal antibody E13 (Seralab, Sussex, UK) directed against CMV immediate early antigens.

Heparinized blood was obtained from healthy volunteers and PMN were isolated by Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway, d = 1.077 g/cm²) density-gradient centrifugation. Contaminating erythrocytes were removed by hypotonic lysis (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA.2H₂O). Experiments were performed in EC assay medium (EC growth medium without ECGF or heparin). In the experiments with patient sera, HUVEC were preincubated with EC assay medium containing 10% FCS and 10% patient serum for 30 minutes. In case of purified IgG, endothelial cells were preincubated with IgG diluted in EC assay medium for 30 minutes. After preincubation, PMN in EC assay medium were added at a PMN to EC ratio of 10:1 and cocultured for 2 hours at 37°C. PMN were removed by gentle pipetting and aspiration. The aspirated fraction was collected, counted and suspended at a concentration of 2 x 10⁶/ml. Cytospots were prepared in duplicate with 100 µl of the cell suspension. Cytospots were stained according the procedure of the HCMV antigenemia test [1]. All cytospots of one series of samples were stained in parallel to circumvent variations in yield of positive PMN by differences in staining intensity.

Sera were obtained from kidney transplant recipients 6 to 12 month after transplantation. Ten sera of 8 patients recovering from a HCMV infection and 5 sera of 3 CMV seronegative transplant recipients with a kidney allograft from a seronegative donor were used. The titers of IgG and IgM were determined using a CMV specific ELISA method as described previously [7]. IgG from patient sera was isolated using a Protein G column (MAbTrap G II, Pharmacia Biotech, Woerden, The Netherlands) according the instructions of the manufacturer. After isolation, the protein content was determined using a Biorad protein assay (Bio-rad Laboratories, Munich, Germany). The IgG fractions were dialyzed against 0.01M phosphate buffer and used for experiments.

The titers of neutralizing antibodies in the patient sera were determined in a virus neutralization assay, as described elsewhere (unpublished data). Statistical analyses were performed using Student’s t-test for differences between two groups and one-way analysis of variance for differences between multiple groups.
Results

After two hours of coculture 12.0 ± 1.3% (mean of control reactions ± SEM, n=33) of the PMN were positive for pp65.

Addition of sera from CMV seropositive patients to the coculture system inhibited the generated number of pp65 positive PMN to 58.6 ± 26.9 % of the control without human serum (Fig. 1A). In contrast, sera from seronegative patients caused no detectable reduction in the generated number of pp65 positive PMN. Sera from patients with CMV specific IgG as well as patient sera containing only CMV specific IgM inhibited the generation of pp65 positive PMN too. In a virus neutralization assay we demonstrated the neutralizing capacities of all sera that contain CMV specific antibodies. However, the reduction in generated number of pp65 positive PMN did not correlate with the titer of CMV specific antibodies or the IC50 of virus neutralization in vitro (Table 1).

Table 1: Data on CMV IgG and CMV IgM levels in serum of patients, the respective IC50 and inhibitory activity in generation of pp65 positive PMN

<table>
<thead>
<tr>
<th>Patient</th>
<th>CMV IgG</th>
<th>CMV IgM</th>
<th>IC501</th>
<th>% of control2</th>
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<tr>
<td>1</td>
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<td>130</td>
<td>7313</td>
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<td>2</td>
<td>200</td>
<td>45</td>
<td>6483</td>
<td>66.9</td>
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<td>390</td>
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<td>19500</td>
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<td>4</td>
<td>410</td>
<td>n.d.4</td>
<td>21570</td>
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<tr>
<td>5</td>
<td>560</td>
<td>9</td>
<td>15220</td>
<td>38.3</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>62</td>
<td>1183</td>
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<td>7</td>
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<td>325</td>
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</tr>
<tr>
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<td>5</td>
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<td>8035</td>
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</tr>
<tr>
<td>2-23</td>
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<td>56 (+Rf)5</td>
<td>4210</td>
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<tr>
<td>9</td>
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<td>CMV-Ig</td>
<td>160</td>
<td>&lt;1</td>
<td>1307</td>
<td>45.8</td>
</tr>
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</table>

1IC50: virus neutralization capacity
2% of control are the generated numbers of pp65 positive PMN in the presence of serum compared to the control without serum.
31-2, 2-2, 9-2,10-2: Samples of patient 1,2,9 and 10 obtained at a different time point from the samples without additional number.
4n.d. not determined
5Rf is reuma factor [7].
To assess whether this effect was specific for CMV Ig, we added CMV hyperimmune globulins to the coculture system. Addition of 5 U of CMV-Ig (Biotest Pharma, Dreiech, Germany) inhibited the generation of pp65 positive PMN to 54.2 ± 7.0% of the control without CMV Ig. Next, we used purified serum IgG from 2 seropositive and 2 seronegative transplant recipients. Addition of IgG equivalent to 15% serum, resulted in a mean reduction to 42.5% ± 4.8 % of the control of the generated number of pp65 positive PMN for both seropositive patient sera, whereas the seronegative sera resulted in a slight increase of 7.1% (Fig.1B)(not significant). Since, the amount of IgG per ml in the seronegative transplant recipients was two and four times lower than in serum of both seropositive transplant recipients, additional experiments were performed with 0.2 mg IgG equivalent to 8.1%, 19.4%, 4.8% and 4.2% serum of patient 1 to 4, respectively. This latter procedure also resulted in a significant inhibition of pp65 positive PMN, whereas CMV seronegative sera did not (data not shown).

**Figure 1**
The generation of pp65 positive PMN in the presence of serum (Fig. 1A) or IgG (Fig. 1B) expressed as percentage of the control, i.e. without serum or IgG addition. The number of pp65 positive PMN produced in the presence of serum containing CMV specific antibodies (closed dots) was lower (* P<0.05) than sera without CMV specific antibodies (open dots). IgG isolated from CMV seropositive sera (hatched bars) and CMV hyperimmune globulins (crosshatched bars) caused a significant inhibition of pp65 positive PMN compared to the control (** P<0.01). IgG isolated from CMV seronegative sera did not affect pp65 uptake of the PMN from EC (open bars). Data in Fig. 1B represent mean ± SEM of three experiments. For explanation of sera annotation see Table 1.

**Discussion**
In this study we present a mechanism for CMV specific human serum antibodies by which they are able to reduce the generated number of pp65 positive PMN obtained in an *in vitro* model of CMV antigenemia. IgG or IgM serum antibodies obtained from CMV patients reduced the number of *in vitro* generated pp65 positive PMN as compared to the number of pp65 positive PMN generated in the absence of antibodies. This effect could not be
attributed to a serum factor because the CMV seronegative sera did not inhibit generation of pp65 positive PMN. Moreover, purification of the IgG fractions resulted in an even higher inhibition of pp65 positive PMN. Although the purified IgG contained an equivalent of 15% serum instead of 10%, preliminary experiments had indicated that addition of 10% or 20% serum caused no major differences in the uptake of pp65 by PMN. Additional proof of inhibition of the transfer of pp65 to the nucleus of the PMN was obtained by addition of CMV hyperimmunoglobulins, that reduced the number of pp65 positive PMN to approximately 50% of the control.

In our hands, coculture of PMN with CMV infected HUVEC for 2 hours resulted in detection of pp65 in the nucleus of PMN to 12,0 ± 1,3% of the PMNs added. This number is considerably higher than reported by Grundy et al [3] and Revello et al [4]. Possibly, CMV strain differences, coculture variations, donor variations in PMN, and/or yet unidentified factors may contribute to these variations in pp65 uptake efficiency.

Several lines of evidence indicate an important role for humoral immunity in protection against CMV infection, such as in newborns where maternally derived high IgG levels protect against symptomatic CMV infection [8,9]. In transplantation patients, treatment with CMV immunoglobulins reduces the risk to develop symptomatic CMV disease [10]. Why hyperimmunoglobulin could exert such an effect for an intracellular harboring virus has, so far, not been fully elucidated, although it has been suggested, theoretically, that it might play a role in the dissemination of virus. This is, to our knowledge, the first report providing experimental evidence how hyperimmune globulin might have such a beneficial effect in the outcome of CMV infection after organ transplantation as has been suggested in the literature [11]. Experiments in B cell deficient mice, infected with mouse CMV revealed that the course of a primary infection was not influenced by the absence of CMV specific antibodies, but only dissemination of reactivating virus was enhanced [12,13]. All these studies have in common that they demonstrate that CMV specific antibodies do not affect primary infection, but prevent viral expansion to some extent. A fraction of the antibodies produced during the humoral immune response has neutralizing capacity for these antibodies are directed against the viral glycoproteins that are involved in viral entry. However, in vivo, during infection the virus is mainly cell-associated. Also during reactivation of virus from the different sites of latency [14,15] the virus may remain cell-associated. Thus, although CMV specific antibodies reduce the severity of the infection after reactivation of latent virus, the mechanism of action is still unclear.

In the present study we provide evidence for the inhibitory role of CMV specific antibodies in cell-contact mediated transfer of viral proteins and particles to PMN.

The inhibitory effect of CMV specific antibodies is particular interesting, because, once PMN have taken up the virus they are able to retransmit the virus to uninfected monolayers of fibroblasts and endothelial cells (data not shown, [4]). Thus, the CMV antigenemia may provide a dissemination route for the virus, in which CMV specific antibodies can play a limiting role. The early presence of neutralizing antibodies after viral reactivation may limit dissemination of the virus.
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References


