Vascular pathophysiology of cytomegalovirus infection after kidney transplantation
Kas-Deelen, Adriana Mariska

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2000

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
UPTAKE OF PP65 IN IN VITRO GENERATED PP65 POSITIVE POLYMORPHONUCLEAR CELLS MEDIATED BY PHAGOCYTOSIS AND CELL FUSION?

A.M. Kas-Deelen¹,*, T.H. The¹, N. Blom², B.W.A. van der Strate⁴, E.F. de Maar³, J. Smir², W.J. van Son³, M.C. Harmsen¹.

¹Department of Clinical Immunology, ²Central Laboratory of Clinical Haematology, ³Department of Nephrology, University Hospital Groningen, ⁴Groningen University Institute for Drug Exploration (GUIDE), Department of Pharmacokinetics and Drug Delivery, Groningen, The Netherlands.

Submitted
Abstract
Cytomegalovirus (CMV) antigenemia reflects the severity of infection and viral load in CMV patients. It consists of the detection of CMV pp65 in the nucleus of polymorphonuclear granulocytes (PMN), but it is unclear where and how the PMN pick up virus particles or proteins. In an in vitro model for CMV antigenemia we investigated the mechanism of pp65 uptake by PMN that results in its expression in the nucleus. A series of inhibitors of different mechanisms were used to study the uptake of pp65 by PMN during coculture with CMV infected endothelial cells and we performed morphological analysis by light and transmission electron microscopy.
After two hours of coculture 12.0 % of the PMN showed to be positive for pp65. Nodocazole and cytochalasin B inhibited uptake of pp65 by PMN with 59.4 ± 14.1% and 73.3 ± 12.7%, respectively. A small number of the pp65 positive PMN obtained after coculture were fused with neighboring positive PMN and had formed large cells with multi-lobed nuclei. The presence of anti-CMV hyperimmune globulin or lactoferrin during coculture reduced the number of pp65 positive PMN with 45.8 ± 7.0% or 40.6 ± 3.2%. PMN were observed that enclosed viral particles as well as free viral particles containing PMN in the cytoplasm. We discuss that fusion of viral particles with PMN and phagocytosis are both involved in the uptake of pp65, but doubt whether both routes are responsible for the presence of pp65 in the nucleus of PMN.

Introduction
One of the most common strategies to diagnose and monitor human cytomegalovirus (HCMV) infections is determination of the number of polymorphonuclear granulocytes (PMN) positive for the CMV phosphoprotein 65 (CMV pp65 antigenemia assay) [1]. The levels of the CMV antigenemia correlate well with viral load and development of HCMV disease [2]. However, the presence of pp65 in the nucleus of polymorphonuclear granulocytes (PMN) [3] does not reflect viral replication. This was demonstrated by the failure to detect viral mRNA of immediate or late viral genes in PMN of patients, and therefore it was suggested that pp65 might to enter PMN through uptake of viral material [4].
With the development of an in vitro model of CMV antigenemia, high levels of CMV pp65 positive PMN were obtained after coculture with fibroblasts or endothelial cells infected with freshly isolated clinical strains [5] or endothelial adapted strains [6]. It appeared difficult to obtain pp65 positive PMN after coculture with fibroblasts that were infected with laboratory strains [6,7]. Furthermore, cell-to-cell contact was a prerequisite. Coculture of PMN with viral supernatant did not result in pp65 positive PMN [6] and experiments in the presence of antibodies against ICAM-1 or LFA-1 inhibited the uptake of pp65 by PMN [6]. In electron microscopic studies dense bodies and viral particles seemed to be phagocytozed by PMN [6].
Recently, we demonstrated that CMV specific antibodies in patient sera inhibited the transfer of pp65 from infected endothelial cells to PMN (submitted). It is still inconclusive how neutralizing antibodies may inhibit the uptake of pp65 by PMN if this is mediated by phagocytosis. Furthermore, we questioned how phagocytosis of viral material into vesicles
that are directed to the lysosomal pathway should eventually end up by expression of pp65 in
the nucleus of PMN.
In the present investigation we describe a series of parameters involved in the transfer of
viral proteins from infected endothelial cells to PMN, as detected by pp65 expression in the
nucleus and we discuss possible fusion as an additional mechanism to phagocytosis for the
uptake of pp65.

Material and Methods

Cell culture
Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord
veins [8,9]. HUVEC were grown in endothelial cell growth medium (RPMI1640
supplemented with 20% foetal calf serum, 50 µg/ml endothelial cell growth factor, 5 U/ml
heparin, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin). Culture
flasks were precoated with 1% gelatin and endothelial cells were used at passage 1 to 3.

Virus
HUVEC were infected with the endotheliotropic CMV clinical isolate TB42 [10] by seeding
trypsinized CMV infected HUVEC and uninfected HUVEC at a ratio 1:10. After 5 days
more than 85 % of the HUVEC were infected. The percent infection was determined by
immunofluorescent staining with monoclonal antibody E13 (Seralab, Sussex, UK) directed
against CMV immediate early antigens.

Isolation of PMN
Heparinized blood was obtained from healthy volunteers and PMN were isolated by
Lymfoprep™ (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), followed by two wash steps with Hank’s
balanced salt solution (HBSS) without calcium and magnesium and 1% bovine serum
albumin. After the last wash, PMN were diluted in EC growth medium without ECGF and
heparin (EC medium), counted and assessed for coculture.

Coculture experiments
CMV infected HUVEC monolayers were washed with EC medium and a suspension of
PMN was added at a ratio of PMN:EC is 10:1. Cells were cocultured at 37°C for two hours.
After that, 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. Inhibition experiments were performed with
10 µM Nodocazole (Sigma Chemical Co, St Louis MO, USA), 1 µM Cytochalasin B (Serva,
Heidelberg, Germany), 5 Units of anti-CMV hyperimmune globulin (Cytotect, Biotest
Pharma, Dreieich, Germany) or 0.5 mg/ml bovine lactoferrin (Numico Research B.V.,
Wageningen, The Netherlands). All agents were diluted in EC medium and preincubated at
CMV infected endothelial layers for 30 minutes, whereafter the PMN were added to the reaction mixture.

Staining of PMN
Cytospots were stained according the procedure of the HCMV antigenemia test [11], i.e. cytospots were fixed with paraformaldehyde, followed by a permeabilization step with NP40. Indirect peroxidase staining was performed with C10/C11, a mixture of two mouse monoclonal antibodies directed to HCMV pp65. Two cytospots were stained for each sample and all samples of one experiment were stained in parallel to circumvent variations in yield of positive PMN by differences in staining intensity.

Figure 1
Time and culture characteristics of coculture of PMNs with CMV infected endothelial cells. PMN were harvested 5, 15, 60 or 120 minutes after coculture and expressed as % pp65 positive PMN (Fig. 1A). The percentage positive PMN after a 2 hours coculture period at different PMN to endothelial cell ratios (1:1, 5:1, 10:1 and 50:1, respectively), is shown in Fig. 1B. Data represent mean ± SEM of three (Fig. 1A) or two (Fig. 1B) experiments.

Electron microscopy
CMV infected HUVEC were grown in small petri-dishes and cocultured with PMN for 2 hours at 37°C. The monolayer of CMV infected HUVEC incubated with PMN was fixed overnight at 4°C in 2% glutaraldehyde in 0.1M phosphatebuffer (pH =7.4).
After embedding in Epon, ultrathin sections were cut and stained with uranyl acetate and lead citrate. Sections were examined in a 201 Philips transmission electron microscope (Philips, Eindhoven, The Netherlands).
Figure 2
Appearance of *in vitro* generated PMN. PMN were stained for pp65 by indirect immunochemical staining. Cells with dark staining in the nucleus represent pp65 positive PMN. Small number of PMN harvested after coculture had formed syncytia of PMN (arrows) with separated nuclei (A) or a clump of multiple lobes in the nucleus (B).

**Results**

*Yield and kinetics of pp65 expression in the nucleus*

Already 5 min after the addition of PMN suspension, pp65 positive PMN were detected among the harvested PMN from the supernatant, albeit in low frequencies. After 1 to 2 h, the yields of positive PMN increased only little (Fig. 1A). Therefore in our experiments, we cocultured PMN together with CMV infected endothelial cells for 2 h. After two hours of coculture 12.0 % ± 1.3% (mean ± SEM, n=33, i.e. total of all control reactions) of the PMN were positive for pp65. The optimal ratio of PMN to endothelial cell was 10:1 (16.4% pp65+PMN) while PMN to endothelial cell ratios at 1:1, 5:1 and 50:1 resulted in lower percentages of pp65 positive PMN (Fig. 1B). A small number of the pp65 positive PMN obtained by coculture showed multi-lobed nuclei, i.e. more than the normal number of nuclear lobes (Fig. 2).

The cell surface of such a cell had considerably increased. Two types of nuclei were observed: nuclei with individual clusters of lobes (Fig. 2A) and nuclei with a massive clump of nuclear lobes (Fig. 2B). The enlarged cytoplasmic area with multi-lobed nuclei indicated that the PMN could form syncytia in the process of pp65 uptake. Pp65 positive PMN were never observed after coculture with supernatant of infected endothelial cells, although this supernatant did contain infectious virus and dense bodies full of pp65 (data not shown).

*Active cytoplasmatic transport*

The next issue we addressed was the requirement of active transport in PMN enabling transfer of pp65 from infected endothelial cells to the nucleus from PMN. We added nodocazole or cytochalasin B to the coculture of CMV infected endothelial cells and PMN. Nodocazole inhibits microtubuli-associated transport by depolymerizing microtubuli.
Cytochalasin B inhibits F-actin polymerization and thus inhibits endocytosis and cytoplasmatic transport. Both nodocazole (10 µM) and cytochalasin B (1 µM) inhibited uptake of pp65 by PMN with 59.4±14.1% (n=4) and 73.3±12.7% (n=2), respectively (Fig. 3A).

Figure 3
Effect of a series of inhibitors. Number of pp65 positive PMN produced in the presence of 10 µM nodocazole, 1 µM cytochalasin B (Fig. 3A), 0.5 mg/ml lactoferrin or 5U CMV hyperimmune globulin (Fig. 3B). The number of pp65 positive PMN is expressed as percentage of the control without inhibitors. Data represent mean ± SEM of four (nodocazole), three (lactoferrin and CMV-Ig) or two (cytochalasin B) experiments.

Inhibition of cell-cell contact
To study whether CMV specific antigens are required for the uptake of pp65 by the PMN we added anti-CMV hyperimmune globulin to the coculture system. 5 U/ml anti-CMV hyperimmune globulin, added 30 min. before and during coculture of PMN and CMV infected endothelial cells inhibited the uptake with 45.8 ± 7.0% compared to the control reaction without anti-CMV hyperimmune globulin. The antiviral activity of lactoferrin is probably effectuated at the level of entry [12] and might therefore be involved in uptake of pp65. Addition of 0.5 mg/ml bovine lactoferrin caused an inhibition of 40.6 ± 3.2% (Fig. 3B).

Electron microscopy
Monolayers of CMV infected endothelial cells with at least 25 PMN near to endothelial cells were examined. Several PMN in the process of engulfment and uptake of viral material were detected (Fig. 4A). The closed membrane around the partially degraded dense body indicated that uptake occurred in vesicles (Fig. 4B). We also observed complete viral particles free in the cytoplasm of PMN (Fig. 4C). The viral particles and dense bodies were present inside infected endothelial cells (not shown) as well as outside the infected cells (Fig. 4A).
Figure 4
Electron microscopy graph of PMN and CMV infected endothelial cells. Dense bodies in the process of uptake by the PMN are indicated by arrows. Fig. 4B is a close-up (30,000x) of a dense body in the PMN as shown in Fig. 4A (10,000x). A virion free in the cytoplasm is shown in Fig. 4C (arrow)(30,000x).

Discussion
In the present study we have examined a series of parameters involved in the transfer of viral proteins from CMV infected endothelial cells to PMN, as reflected by the presence of pp65 in the nucleus.
The PMN contacted the CMV infected endothelial cells by gravity and probably also chemotaxis. Already after a very short time interval (5 min), non-adherent PMN could be harvested that showed nuclear presence of pp65. Anti-CMV hyperimmune globulin as well as lactoferrin inhibited the generation of pp65 positive PMN. Transfer of pp65 to the nucleus of the PMN required active cytoplasmatic transport, as demonstrated by inhibition with nodocazole or cytochalasin B. Finally, electron microscopical analysis of monolayers of CMV infected endothelial cells incubated with PMN showed PMN that were including viral particles. In the PMN, dense bodies were enclosed in cytoplasmic vesicles. In contrast, naked viral particles were present in the cytoplasm of these cells as well.
CMV can enter its target cells by at least three different entry routes, which may depend on the cell type to be infected. In fibroblasts, the cell type commonly used for \textit{in vitro} CMV infection, the virus enters via adsorption to heparan sulphate proteoglycans, followed by binding to a specific receptor and fusion with the cell membrane [13]. In a retinal pigment epithelial cell line, two entry routes may function in one cell. At the apical site, infection could be inhibited by neutralizing antibodies, indicating that fusion plays a role [14]. Via the basolateral surface of retina epithelial cells, CMV spreads from cell to cell using collateral connections via the cytoskeleton [15]. A group working with endothelial cells and primary retinal pigment epithelial cells reported that both cell types were infected at the apical surface by endocytosis as entry mechanism. This was demonstrated by electron microscopy and inhibition of CMV infection after treatment with cytochalasin B [16]. Hence, entry of CMV is not restricted to one particular mechanism for all cell types and even not to one mechanism for a typical cell type. Therefore PMN might take up virus via phagocytosis, but also may use fusion to obtain pp65.
Our data obtained after electron microscopy as well as the EM-data from Revello et. al. [6] clearly demonstrate phagocytosis, therefore it is very likely that phagocytosis plays a role in
the uptake of viral material by the PMN. However, whether pp65 derived by phagocytosis is transported to the nucleus is unknown.

Should phagocytosis be the major mechanism for the transfer of viral material, from endothelial cells to the nucleus of PMN, it might be expected that 1) viral material is degraded in the lysosomal route and thus may not reach the nucleus. 2) Moreover, should pp65 reach the nucleus after being phagocytosed, the time course to detect pp65 in nuclei of PMN would probably be longer than 5 min or even an hour. 3) It is unlikely that anti-CMV hyperimmune globulin would inhibit uptake if only phagocytosis is involved. If this would be the case, Fc-receptor mediated mechanisms could be expected enhancing phagocytosis. However, by using anti-CMV hyperimmune globulin, lower levels of in vitro obtained pp65 antigenemia levels were found. We presume that if Fc-receptor mediated phagocytosis occurs in the experiments with pre-added anti-CMV hyperimmune globulin, it could compete with the other route of pp65 uptake. Furthermore, we think that the latter route of pp65 uptake is responsible for pp65 expression in the nucleus. 4) Finally, lactoferrin, acting via charge interactions that interfere with fusion and entry into CMV target cells, would have no effect.

All the above mentioned arguments brought us to postulate that besides phagocytosis another mechanism is involved.

The neutralizing capacity of CMV specific antibodies is based on masking of viral proteins involved in viral entry. After infection, some of these viral molecules are highly expressed at the surface of the infected cell. For instance, gB is involved in virion entry into cells, cell-to-cell spread of infection and syncytium formation [17]. Virus-neutralizing antibodies block these effects [18]. Our finding that CMV-specific antibodies suppress the transfer of viral material to PMN indicates that fusion of PMN with infected viral particles or virally infected cells may play a role.

Until now, the precise mechanism of the antiviral activity of lactoferrin has not been unravelled. Both mixing of cell-free virus with lactoferrin and incubation of target cells with lactoferrin prior to infection results in the inhibition of CMV infection in vitro [12]. By binding to the negatively charged cell surface, lactoferrin probably prevents the adherence of virus to its target cells thus inhibiting steps leading to fusion. Moreover, charge-modified lactoferrin with additional positive charges inhibits CMV stronger than does ‘neutral’ lactoferrin in vitro [19]. The inhibition of pp65 transfer from endothelial cells to PMN by lactoferrin presumes cell-cell contact resulting in a fusion step.

The possibility to inhibit the expression of pp65 in the nucleus by nodocazole indicates that microtubuli-associated transport is used for the transfer of pp65 to the nucleus. Other herpesviruses, of which HSV-1 is well studied, utilize microtubuli-associated transport as well [20,21]. HSV-1 capsids that enter the cytosol via glycoprotein-mediated fusion with the cell membrane are transported by dynein to the nuclear pore. Specific nuclear localization signals, expressed by HSV-1, bind to receptors that mediate transport and release of the viral genome through these nuclear pores [22]. Pp65 harbours at least two nuclear localization signals enabling efficient transport to the nucleus [23,24]. It could be that the transport route towards the nucleus utilized by CMV has similarities to HSV-1.

The clinical implication of the rapid uptake of pp65 by PMN might be that the CMV
antigenemia may serve as a monitor of the cellular viral load, in particular of endothelial cells. Inhibition of CMV antigenemia levels by CMV specific antibodies indicates that the humoral immune response may have a modulatory effect in viral dissemination and thus the course of infection.

In conclusion, the uptake of pp65 by PMN from CMV infected endothelial cells can occur very rapidly resulting in expression of pp65 in the nucleus. The uptake of pp65 is probably mediated by two different mechanisms, phagocytosis and cell fusion, whereof the latter might be responsible for the presence of pp65 in the nucleus.

Acknowledgements
We thank Roelie van Wijk and Henk Moorlag for endothelial cell culture assistance; Ilby Bouwman and Boelo Meedendorp for technical assistance and C. Sinzger for HCMV clinical isolate TB42. We thank mr. Ed de Moura-Correia (Biotest) for providing anti-CMV hyperimmune globulin and Numico Research B.V., Wageningen for supplying bovine lactoferrin. Grant support: Dutch Kidney foundation (C94.1386), European Commission Grant (ERB BMH4CT-0471 (DG12-SSMA)).

References