Experimental cytomegalovirus infections in a rat model : pathogenesis and treatment

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Experimental Cytomegalovirus Infections in a Rat Model

- Pathogenesis and Treatment -

Jeroen Steven Kloover

Levenswijze
infecties

Levenswijze

Levenswijze

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Experimental Cytomegalovirus Infections in a Rat Model

- Pathogenesis and Treatment -

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht, op gezag van de Rector Magnificus,
Prof.Dr. A.C. Nieuwenhuijzen Kruseman, volgens het besluit van het
College van Decanen, in het openbaar te verdedigen op
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Aan
Susan
Papa en Mama
Annemieke

Ter herinnering aan mijn grootouders

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List of abbreviations

AIDS	acquired immune deficiency syndrome
BMT	bone marrow transplantation
CADI	chronic allograft damage index
CMV	cytomegalovirus
CNS	central nervous system
CTL	cytotoxic T lymphocytes
DFO	desferioxamine
DHPG	9-(1,3-dihydroxy-2-propoxymethyl)guanine
DTPA	calcium trisodium diethylenetriaminepentaacetic acid
E	early
EBV	Epstein Barr virus
ER	endoplasmic reticulum
gp	glycoprotein
GvHD	graft versus host disease
HAART	highly active anti-retroviral therapy
HHV	human herpes virus
HIS	hyper immune serum
HIV	human immune deficiency virus
HLA	human leukocyte antigen
HPMPC	(s)-1-(3-hydroxy-2-phosphononylmethoxypropyl)cytosine
HSV	herpes simplex virus
IE	immediate early
ICAM-1	intracellular adhesion molecule-1
IL-1	interleukin-1
IL-2-R	interleukin-2 receptor
IFN-gamma	Interferon gamma
IRV	immune recovery vitritis
iv	intravenous
L	late
LFA-1	leukocyte function antigen-1
MCMV	murine cytomegalovirus
MHC	major histocompatibility complex
mRNA	messenger RNA
NK	natural killer
PBSCT	peripheral blood stem cell transplantation
RCMV	rat cytomegalovirus

TAA	transplant associated arteriosclerosis
TNF-alpha	tumor necrosis factor-alpha
US	unique short
UL	unique long
VCAM-1	vascular adhesion molecule-1
VLA-4	very late antigen-4
VZV	varicella zoster virus
WT	wild type

General introduction and aims of the study

Cytomegalovirus (CMV) is an important human pathogen due to the high seroprevalence of the virus in the normal population and the severity of CMV disease in the immunocompromised patient, like in transplant recipients and AIDS patients. The best hypothetical therapy for CMV infections in the immunocompromised patient is restoration of immune effector functions. For example, a drastic decrease in the incidence of CMV disease in AIDS patients could be appreciated since the introduction of HAART. Reconstruction of immune function can be realized in a subgroup of AIDS patients, but it is unfeasible in transplant recipients, since inadequate immunosuppression is a risk factor for allograft rejection. Current treatment modalities for CMV disease include potent inhibitors of CMV replication, such as ganciclovir, cidofovir and foscarnet. However, the treatment of severe CMV disease remains difficult due to the limited specificity and serious side effects of the compounds and deterioration of the patient is often seen, despite the administration of high dose antiviral agents. Therefore, the search for new treatment modalities capable of reducing viral replication and/or recurrence and subsequently CMV disease is of great importance. Animal models have been shown to be extremely useful for studying viral pathogenesis and the development of new antiviral treatments. The rat model, using rat CMV (RCMV), has been used extensively in various fields of research and is now widely accepted as a good model for CMV infections in humans. The aim of this thesis was to study some aspects of CMV pathogenesis and treatment of CMV infection in various experimental settings, using the RCMV/rat model.

The outline of the study is as follows. **Chapter 2** and **3** focus on RCMV replication in the salivary gland, the primary organ for RCMV replication during chronic infection and the biological significance of RCMV replication in salivary gland cells on the systemic infection was studied. **Chapter 4** describes infection of neonatal animals with RCMV of which the MHC class I homolog (r144) has been disrupted (RCMV Δ r144). In contrast to adult rats, in which immunosuppression is mandatory for disseminated infection, the use of neonatal rats enabled us to study the interaction of the r144 gene product with an unaffected, but immature natural immune system. **Chapter 5** is devoted to RCMV-mediated enhancement of chronic renal allograft rejection. In this chapter, the additive effect of RCMV infection on immune activation was subject of study. Treatment of CMV disease is described in **chapter 6** and **7**. In **chapter 6**, direct inhibition of viral replication in brains of immunocompromised rats was studied, whereas in **chapter 7**, two drugs with putative antiviral activity and supposed ability to modulate RCMV-induced immunopathogenesis were studied. In **chapter 8**, the results and conclusions of this thesis are summarized and discussed.

~ Chapter 1 ~

Review of the literature

Viral Structure and characteristics

Cytomegalovirus (CMV) is a member of the herpesviridae. Membership of the herpesviridae is based on the composition of the virion: a core containing linear double-stranded DNA, an icosadeltahedral capsid (diameter 100-110 nm) containing 162 capsomeres, presence of an amorphous material that surrounds the capsid, called the tegument and an envelope containing viral glycoprotein spikes ⁽⁴³⁴⁾. Cytomegaloviruses are classified in a subfamily of the beta-herpesviridae, due to the restricted host range, long reproduction cycle, the characteristic cellular morphological alteration during viral replication (cytomegaly, owls eye appearance) and the ability to persist in the host in a latent form.

The first step in CMV infection, is attachment of the virion to the membrane of the cell. CMV enters fibroblasts by a process involving pH-dependent fusion ⁽¹¹²⁾. The initial event in this process is attachment to extracellular heparan sulfate ⁽¹¹³⁾. This binding is followed by attachment of CMV envelope glycoproteins (gp), such as gpB and gpH with cell membrane components. Other host membrane molecules, such as CD13 ⁽⁴⁷⁶⁾, a 92.5 kDa phosphoprotein ⁽²⁶⁰⁾ and annexin II ⁽⁵⁵⁴⁾ have been suggested to be involved in CMV attachment to the target cell.

The mechanism of cell penetration by CMV appears to differ between cell types. In contrast to fibroblasts, cell entrance into endothelial and astrocytoma cells appears to be mediated by endocytosis, rather than fusion with the cytoplasmic membrane ⁽⁵⁶⁾. After penetration of the cell membrane, the virus is uncoated and the viral genome is transported to the nucleus. Expression of the CMV genome is controlled by a cascade synthesis of proteins ⁽¹⁹⁷⁾. The first proteins to be synthesized are immediate early (IE) proteins. These proteins have a regulatory function and permit transcription of early (E) messenger RNA (mRNA). During the E phase of replication, DNA replication occurs. The transcription of late (L) mRNA takes place after completion of DNA replication and L mRNA encodes structural proteins, such as viral capsides (UL86), tegument protein (UL83) and envelope glycoproteins.

During virus assembly, the CMV DNA is packaged in the nucleus into preformed capsids and a viral envelope and the viral matrix (tegument) is acquired at the nuclear cell membrane. However, it is thought that the final viral envelopement occurs at the cellular membrane ^(185, 359). Transport to the cellular membrane may be mediated by cytoplasmic vesicles derived from the Golgi apparatus ⁽³⁵⁹⁾. The characterized owls eye appearance is due to an excessively expanded Golgi apparatus.

Epidemiology of human cytomegalovirus infections

Presence of CMV in the normal population is common and seroprevalence in healthy individuals can be as high as 80-90% (183, 221, 368, 377). However, these data probably even underestimate the real prevalence of CMV since 5-20% of serologically negative individuals will harbour latent viral DNA (48, 291, 470, 501). Seroprevalence is directly correlated with socio-economic status (280, 328) and age (86, 192, 368). Most individuals become infected during childhood (255, 460) or adolescence (18, 436).

Congenital CMV infection is unquestionably the most common intra-uterine infection in humans and has been reported to vary between 0.2%-6.8% of all new-borns (8, 128, 129, 204, 447). After birth and during the first months of life, children are most frequently infected by vertical transmission of the virus (contaminated genital secretions and/or breast milk) (221, 368). Thereafter a significant number of children is infected at day care centers in which close contact and thus (horizontal) viral transmission, is inevitable (5, 6, 369, 384). In addition, infected children can be a source for viral transmission to their parents (386) or day care employees (4, 6, 363, 385). However, in a recent report by Murph et al., day care workers were not found to be at risk for acquiring CMV (364). This is possibly due to an increased awareness about (personal) hygiene in these institutions.

Another period during which CMV infection is common, is during adolescence when infection has been associated with heterosexual (168, 364, 479, 518) and homosexual (109, 136) activity.

Cytomegalovirus infections in the immunocompetent host

CMV infection of the immunocompetent host is usually asymptomatic or may result in a mononucleosis like syndrome in approximately 10% of infected individuals. The dominant symptoms are fever, malaise, myalgia, atypical lymphocytosis and abnormal liver functions (251, 265). However, also splenomegaly and lymphadenopathy have been reported. The syndrome is further characterised by a persistent viruria in almost all patients (251, 265).

Although few data are available concerning the incidence of CMV mononucleosis, it is thought that the condition is relatively common and runs a mild course. However, few cases progress into life threatening CMV disease (141). In these patients, the most commonly infected organs are liver, CNS and lungs. Patients with severe CMV disease respond well to ganciclovir or foscarnet treatment (141, 337).

The host immune response to CMV infections can be divided in non-specific and specific immune surveillance. The humoral immune response does not appear to play a significant role in controlling acute CMV infections, since viral infection or recurrence is rapidly cleared by the cellular immune system. However, anti-CMV immunoglobulins are able to limit recurrent CMV disease effectively (11, 12, 395). Cytotoxic CD8+ T lymphocytes are important for controlling CMV infections (359, 426). The number of CD8+ cells in blood

correlates with the severity of CMV disease ⁽³⁰⁸⁾. The importance of these cells in controlling CMV disease is further shown by results of adoptive transfer studies in post-transplant disease ^(431, 432). The biological significance of CD4⁺ cells is demonstrated by the rapid increase in incidence of CMV disease in AIDS patients with a progressive decline in CD4 counts ^(117, 240). Besides the specific cellular immune response, natural killer (NK) cells, which are part of the non-specific cellular immune system, were also shown to be important for CMV immune surveillance ⁽⁵²⁾.

The physiological significance of the humoral ^(154, 459) and cellular, i.e. CD4⁺ T cells ^(248, 249), CD8⁺ T cells ^(278, 420, 421) and NK cells ^(80, 544), components of the immune system on controlling CMV infection was corroborated extensively in the murine/MCMV model.

Cytomegalovirus and latency

The ability to enter a latent state, i.e. absence of infectious virus as measured by plaque assay but persistence of viral DNA, is a characteristic shared by all herpesviruses ⁽⁴³⁵⁾. CMV has been known to be capable of infecting a wide range of organs in immunosuppressed patients, resulting in multiple potential sites for CMV latency ⁽²¹²⁾. In humans, emphasis has been laid on peripheral mononuclear cells ^(64, 454, 466, 514) and their progenitors ^(203, 271, 272, 346, 357, 461) as sites for CMV latency. The number of viral genomes per latently infected hematopoietic cell of healthy blood donors is very low with only 2-13 viral genome copies per cell ⁽⁴⁶⁶⁾. However, CMV replication in mononuclear cells may be abortive or restricted to early events of gene expression ^(231, 429, 515). Interestingly, differentiation of mononuclear cells makes them fully permissive for CMV ^(203, 231, 477).

Recent data suggest that CMV reactivation and replication in myeloid cells largely depends on immune activation. Cytokines, i.e. tumor necrosis factor-alpha (TNF-alpha) and interferon-gamma (IFN-gamma) and the presence of CD8⁺ T cells enhances CMV replication in monocyte-derived macrophages ⁽⁴⁷⁷⁾. In addition, TNF-alpha is able to enhance the immediate early (IE) promotor of CMV in myeloid cells ^(412, 504). Furthermore, reactivation of latent CMV from latently infected peripheral blood mononuclear cells was established by allogeneic stimulation of these cells *in vitro* ⁽⁴⁷⁸⁾.

Another mechanism for viral reactivation has been described, which acts independently of inflammatory mediators ⁽⁴¹¹⁾. Prösch et al. described that catecholamines, measured in patients with an acute myocardial infarction, stimulates the major IE enhancer-promotor of CMV, similar to TNF-alpha, although via different cellular pathways ⁽⁴¹³⁾.

In the murine model using murine CMV (MCMV), latent MCMV has been detected in blood leukocytes, endothelial cells, bone marrow, salivary gland, heart, liver, brain, kidney, adrenal glands and spleen ^(111, 269, 270, 358). However, the lungs were shown to be the major organ site for viral latency ^(31, 283). Reactivation of MCMV could be established by tissue explant cultures ^(347, 406) or, *in vivo*, after immunosuppression of latently infected mice ⁽³¹⁾.

284, 419, 503). It was shown in this model that 1) reactivation does not accumulate over time after initiation, 2) reactivation is distributed in the murine lung in a patchwork pattern and 3) that reactivation of latent virus is a multistep process⁽²⁸⁵⁾. Latency-associated transcripts were found in the murine model^(200, 284) and in humans^(273, 289, 322). These findings suggest that induction of CMV reactivation is a tightly regulated, non-stochastic process.

Cytomegalovirus and immune evasion

During a period of thousands of years, the intimate relation of herpesviruses with their host, has resulted in multiple mechanisms to elude to host immune system⁽³⁴⁰⁾. An effective mechanism to escape from immune surveillance is to limit the number of antigens expressed on the cellular surface. The immune system cannot kill an infected cell when it is not able to identify this cell as being infected. Therefore, the ultimate form of immune evasion is latency. The key characteristic of latency is the persistence of viral DNA without production of infectious virus with minimal cellular expression of viral antigens^(48, 291, 470, 501). However, it should be noted that latency-associated transcripts of CMV may elicit a humoral immune response^(273, 289, 322).

Another mechanism for avoiding detection by the immune system, is by preventing major histocompatibility complex (MHC) class I-mediated presentation of antigens to T lymphocytes. The gene product of UL 83, pp65, is able to inhibit immediate-early peptide presentation⁽¹⁸⁶⁾. In addition, CMV infection is associated with a down-regulation of two cellular aminopeptidases⁽⁴⁰¹⁾, i.e. CD10 and CD13, which are responsible for trimming peptides during their insertion into MHC class I and II molecules^(290, 362).

However, the most effective way of inhibiting MHC class I recognition, is interference with the MHC class I assembly itself. It has been known that human CMV (CMV) down-regulates cellular human leukocyte antigen (HLA) class I molecules in order to protect infected cells from MHC class I restricted cytotoxic T Lymphocytes mediated lysis⁽⁴⁰⁾. Gene products of US2⁽²⁴⁶⁾, US3^(9, 247), US6^(10, 213) and US11⁽⁵⁴⁵⁾ have been shown to interfere with the cellular expression of MHC class I. Genes with a similar function were found on the genome of MCMV (m152⁽⁵⁶⁵⁾, m06⁽⁴²⁴⁾) and m04⁽²⁶⁴⁾.

According to the missing self hypothesis, absence of HLA / MHC class I molecules on the cellular membrane renders a cell susceptible for NK cell lysis⁽³¹⁶⁾. For optimal evasion of immune surveillance the virus must encode factors which are able to elude the innate immune response. It was hypothesized that a virally encoded MHC class I homolog could function as a decoy for NK cell surveillance.

CMV encodes an HLA class I homolog, named UL18⁽³⁹⁾. Similar genes were found in the genome of murine (m144,⁽⁴¹⁸⁾) and rat (r144,⁽⁵³¹⁾) CMV. CMV encoded HLA class I homolog associates with beta-2-microglobulin⁽⁷²⁾, is able to bind peptides⁽¹⁵¹⁾ and was found in peripheral blood mononuclear cells of organ transplant recipients⁽²⁰⁷⁾. In contrast

to CMV, MCMV derived "class I" molecules are not able to bind endogenous peptides, which may be mediated by a deletion within the m144 alpha-2 domain encoding the predicted peptide-binding groove (92).

UL18 expression on target cells has been shown to interact with the CD94/NKG2 receptor (418) and the leukocyte immunoglobulin-like receptor-1 (LIR-1) receptor (114). CD94 is a member of the C-type lectin superfamily and associates with NKG2 family of C-type lectins (87, 295). CD94/NKG2 heterodimers are expressed on NK cells and have been shown to recognize MHC class I and to inhibit NK cell function. Another viral protein, gpUL40, which contains an HLA-E ligand, also interacts with the CD94/NKG2 complex and is able to inhibit NK cell surveillance (517, 523). LIR-1 is a member of the Ig superfamily and is predominantly expressed on B cells and monocytes (114).

There is strong evidence from the murine model that expression of the MCMV-encoded MHC class I molecule on the cellular surface protects the infected cell from NK cell lysis *in vivo* (155) as well as *in vitro* (282). It should be noted that *in vitro* only a partial inhibition of NK cell cytotoxicity was observed (282).

Furthermore, it was shown that m144 protected lymphoma cells transfected with m144 from NK cell mediated rejection *in vivo* (116). Interestingly, expression of m144 reduced the recruitment of leukocytes and NK cells and the level NK cell activation (116). This suggests that the MHC class I homolog may influence the local interaction between cells of the innate immune system, such as antigen presenting cells (APC) and NK cells. Especially, since it is known that monocyte-derived cytokines are important for NK cell activation and infiltration (443, 473). Leong et al. proposed a similar role for UL18 (306), based on the interaction of this molecule with LIR-1, which is predominantly expressed on monocytes and B lymphocytes (114).

Recent studies have shown that also CD4+ T cells are important in controlling CMV infections (122, 249, 321). There is evidence that besides interference with HLA/MHC class I complexes, CMV has also been shown to inhibit HLA class II presentation by inhibition of IFN-gamma (354).

CMV encodes 4 genes which show homology to chemokine receptors, namely UL33, US27, US28, UL78 (93, 94). Scavenging of chemokines by US27 and US28 may influence infiltration of leukocytes (26). The ligands and functions of UL33 and UL78 are still unknown. However, deletion mutant viruses (MCMV-d-m33, RCMV-d-r33, RCMV-d-r78) have been generated for the rat (41, 44) and murine system (124). Disruption of the r33 and m33 gene was associated with an absence of viral replication in salivary glands, whereas deletion of the r78 resulted in a lower replication efficiency than wild type (WT) virus and infected cells developed a syncytium-like appearance *in vitro*. Furthermore a significantly higher survival was seen in RCMV-d-r78 infected rats than in WT RCMV infected animals (41).

Although our insights in immune evasive strategies by CMV are accumulating, further research is required, since full knowledge about these strategies will result in novel diagnostic and therapeutic modalities.

Clinical aspects of cytomegalovirus infection

Congenital cytomegalovirus infection

CMV is the most common congenital infection in humans and the prevalence of congenital CMV ranges from 0.2% to 6.8% of all new-borns (8, 32, 89, 128, 393, 447). Of all infected newborns, 5% are born with CMV disease, which is characterized by splenomegaly, hepatomegaly, prolonged neonatal jaundice, thrombocytopenic purpura and/or pneumonitis (128, 393). Other consequences of intra-uterine CMV infection are fetal growth retardation, preterm birth and intra-uterine death. Prognosis in this group is poor and during follow-up, children mainly suffer from neurological deficits, such as microcephaly, cerebral palsy, epilepsy, mental retardation, chorioretinitis, optic atrophy, delayed psychomotor development and especially sensorineural hearing loss (65, 106, 128, 387, 393).

The majority of the asymptomatic CMV infected newborns will develop normally. However, 5% will suffer from long term sequelae of which sensorineural hearing loss is the most common (166, 167, 393). Sensorineural hearing loss is fluctuating, albeit progressive and is in 50% bilateral (167). Of concern is the fact that population wide screening of acoustic function in asymptomatic CMV infected neonates fails in over 50% (166). A careful follow-up of these patients is thus warranted.

The factors that influence the occurrence of congenital CMV infection and those that influence the occurrence of primary CMV infection in pregnant women include race, sexual activity, young age, unmarried status and lower socio-economic status (168, 364, 479, 495, 518). CMV infection during pregnancy is either from reactivation of latent virus, infection with a different strain or a primary CMV infection. One in every 100 women will encounter a primary CMV challenge during pregnancy and approximately 40% will transmit the infection to their foetus (393). Newborns with CMV disease as a result from maternal reactivation or re-infection with a different strain have a better prognosis than new-borns from mothers with a primary infection (128, 169, 496).

A reliable diagnostic tool for predicting which newborn will develop CMV disease is still missing. Serological status of the mother is of little value for predicting vertical transmission of virus, since not all seropositive women will transmit the virus to their child in utero. However, negative serostatus will define a subgroup of women who are at low risk for viral transmission to their fetus.

Culture of the virus (296) is considered diagnostic for CMV infection of the fetus, since

amniotic fluid is mostly produced by the fetus and culture of which thus closely represents fetal CMV status. Also detection of viral DNA in amniotic fluid by PCR ⁽²⁴⁵⁾ has been used for diagnosing CMV infected fetuses. However, cases are described in which children with CMV DNA containing amniotic fluid remained unaffected ^(286, 297, 312, 441). Those cases are associated with a low number of viral DNA copies. Although the results from amniotic fluid sampling should be handled with care, this technique will be one of the most important diagnostic tools for congenital CMV disease in utero ^(286, 297, 312, 441) since the amount of viral DNA present may be an indicator for the severity of CMV disease ⁽²⁹⁷⁾. However, the true value of the PCR technology for the diagnosis of congenital CMV disease will have to be established.

Cytomegalovirus infection in the HIV infected patient

Since its discovery in the early 1980's ^(33, 175, 243), human immune deficiency virus (HIV) infection is a major cause of morbidity and mortality on a world-wide basis. HIV selectively infects peripheral CD4+ T lymphocytes and interferes with normal T cell function, resulting in a depletion of CD4+ lymphocytes ⁽¹⁵⁶⁾. Below the threshold of 100-400 CD4+ T cells/ μ l blood, patients are prone to be infected with opportunistic pathogens, such as CMV and are likely to develop opportunistic malignancies ⁽¹¹⁵⁾.

CMV end-organ disease occurs approximately in 21-44% of patients suffering from AIDS and is primarily associated with low CD4+ counts (<50 cells/ μ l) ⁽²³⁷⁾. Retinitis accounts for over 85% of active CMV disease, followed by gastro-intestinal (16%) and central nervous system disease (1%) ^(174, 240, 400).

CMV retinitis may be the initial manifestation of AIDS. Onset of CMV retinitis may be unilateral or bilateral and usually starts with a focal discrete lesion, which eventually will progress in a diffuse retinal involvement ⁽²¹⁴⁾. The initial lesions are characterized by retinal edema, yellow-white granular dots and vascular cuffing (due to perivascular mononuclear cell infiltrates) and attenuation. The clinical presentation depends on the ocular structures involved ⁽²¹⁴⁾. Lesion of the peripheral retina is usually found at indirect fundoscopy in the asymptomatic patient. Vitreous involvement results in a blurred vision and floaters ⁽²³⁹⁾. Infection of the macula leads to a drastic decrease in visual acuity, metamorphopsia and micropsia. The end result of CMV retinitis is a thin atrophic and/or necrotic retina, prone to retinal detachment ^(70, 170).

Gastro-intestinal involvement encompasses infection of the oral cavity, esophagus / stomach ^(244, 400) and the intestines ^(132, 258, 400). Neurological manifestations include ventriculo-encephalitis ^(223, 442), polyradiculopathy ^(16, 108), spinal cord and peripheral nerve involvement ^(16, 191) and may complicate AIDS dementia complex ⁽¹⁶¹⁾. In addition, also CMV induced adrenalitis ⁽⁴¹⁴⁾ and pneumonitis ⁽⁵⁴³⁾ have been described.

CMV can be considered exclusively as an opportunistic pathogen but its relation with

HIV, however, is more complex. It is known that CMV can augment HIV infection *in vitro* (34, 35, 123, 361) as well as *in vivo* (36, 159). Additionally, co-infection of HIV with CMV will result in an increased lysis of cells (88). This indicates that CMV might have an additional effect to the HIV-mediated rapid decrease in CD4+ count.

Since the introduction of therapeutic regimens that include protease inhibitors, termed: highly active anti-retroviral therapy (HAART), a striking reduction of CMV related morbidity in AIDS patients has been seen, which is associated with an increase in CD4 cell count (225, 239, 301, 383, 457). Conversely, failure of HAART (i.e. decrease in CD4+ count) is associated with CMV relapses (482). Cessation of CMV prophylaxis is possible in AIDS patients with HAART related immuno-reconstruction (352, 522, 538). Viral load and CD4+ count should be monitored thoroughly since relapses have been described within 4 months after initiation of HAART with adequate suppression of HIV (241).

On the other hand, morbidity that does occur, is no less serious since the introduction of HAART. Immune-mediated tissue damage at the site of infection (225) may occur due to the HAART induced improved immune function (22, 239, 383). For example, CMV retinitis before initiation of HAART was characterized by minimal intra-ocular inflammation whereas in the post-HAART era, inflammation is more prominent and may result in tissue destruction. This immune recovery vitritis (IRV) is characterized by posterior segment inflammation, including vitritis, papillitis and macular changes and an absence of active CMV retinitis (256, 257).

Cytomegalovirus and vascular pathology

CMV has been associated with the process of atherosclerosis, restenosis, transplant associated atherosclerosis (TAA) and inflammatory aortic disease. These conditions encompass different clinical and histopathological entities. For example, atherosclerosis develops over years, whereas restenosis after angioplasty usually progresses in a period of weeks or months. TAA differs from classical atherosclerosis by a generalized, concentric involvement of the vascular structures in the allografted organ. Although there are a lot of differences between these conditions, some pathophysiological mechanisms are similar.

Atherosclerosis is a chronic inflammatory disease of the large and medium sized arteries (437, 438). It is now generally accepted that atherosclerosis develops as a response to injury (439). The first and perhaps the most important step is dysfunction of the endothelial cells. The first histological characteristic of atherosclerosis is the presence of the fatty streak, an inflammatory lesion consisting of monocytes derived macrophages and T lymphocytes (437, 438). In persons with hypercholesterolaemia, the influx of these cells is preceded by extracellular deposition of lipids (437, 438). These fatty streaks are already detectable in coronary arteries and aortas of children before the age of 10 (341, 502). Fatty

streaks will progress into atherosclerotic plaques (149, 150, 335, 336) and progression is characterized by a continued inflammation, smooth muscle cell proliferation and formation of fibrous tissue. Eventually, a fibrous cap is formed covering a core of lipid and necrotic material. Rupture of the plaque or thrombolisation may lead to acute vascular accidents (437, 438). The etiology of atherosclerosis is multi-factorial and include high levels of serum cholesterol, smoking, diabetes mellitus, hypertension, genetic predisposition and infectious micro-organisms, especially herpesviruses and *Chlamydia pneumoniae* (219, 311). Multiple micro-organisms may be present in the atherosclerotic lesion (96) and it was recently shown that the risk of coronary artery disease is related to the total pathogen burden through inflammatory responses (564).

CMV has been linked to atherosclerotic disease based on serological data (3, 484, 485, 532, 563), although not all authors found this association (7, 139, 433). It has been shown that CMV is able to elicit a subclinical inflammatory response, as measured by C-reactive protein, in a subgroup of patients and that these particular patients are at a higher risk for developing atherosclerosis than infected patients without the subclinical inflammatory response (563).

CMV-DNA has been detected in the atherosclerotic vascular wall (96, 211, 344, 345). Indeed, vascular structures have been suggested to be a potential site for latency (211, 344) and/or persistence (163). Data from *in vitro* studies showed that a wide variety of cell types are permissive for CMV, such as monocytes/macrophages, fibroblasts, smooth muscle cells and endothelial cells. However, in contrast to viral replication in fibroblasts, permissiveness of endothelial cells for CMV is restricted (536) and depends on the activation state of the cell (465, 537) and on the virus strain used (182, 323, 464). From studies in animal models it is known that CMV increases the adhesion of leukocytes to and augments lipid accumulation in endothelial cells (487). Also, virus-induced morphological alteration of endothelial cells and subendothelial space of the large vessels could be detected by electron microscopy (45, 120, 486). These data indicate that CMV mediated endothelial cell dysfunction contributes significantly to the inflammation-mediated progression of atherosclerosis. Another important event in the development of atherosclerosis is the proliferation and migration of smooth muscle cells (SMC). This process may be mediated by the production of growth factors and cytokines from macrophages and/or endothelial cells in response to vessel injury. Of special interest is the fact that the US28 gene product, a chemokine receptor encoded by CMV, is able to induce SMC migration (509).

CMV also enhances the development of restenosis after atherectomy or balloon angioplasty (490, 559). It was shown that CMV was able to inhibit the transactivational activity of the p53 suppressor gene by the interaction of p53 with IE2-84, an immediate early gene product of CMV. Inhibition of p53 abolishes p53-mediated cell cycle arrest of SMC and the interaction between p53 and IE2-84 inhibits CMV induced apoptosis (562). Both factors contribute to excessive SMC proliferation, which in turn leads to lumen narrowing. Besides

SMC proliferation, CMV-induced immune activation after balloon injury also contributes to the process of restenosis in a similar manner as was described for the process of atherosclerosis. Animal experiments, using rodents subjected to balloon denudation and infected with the appropriate virus, support these findings in humans (397, 398, 560, 561).

TAA, i.e. chronic rejection, is associated with an acute onset, generalized and concentric involvement of the entire vessel, persistent perivascular inflammation and an intact internal elastic lamina (50, 51, 530). Cytomegalovirus has been suggested to be associated with TAA, leading to an increased rate of graft rejection, more severe graft atherosclerosis and lower survival rates (138, 148, 193, 277, 319, 339). However, some groups failed to find a positive correlation between CMV infection and TAA (445). A positive correlation between TAA and CMV infection was found in cardiac allografted rats (302) and mice (85). Also, in rats receiving an aorta allograft, it was demonstrated that CMV infection enhanced the generation of a prominent inflammatory response with presence of inflammatory cells and SMC proliferation in the allograft, resulting in neointima formation (304). The RCMV induced enhancement of TAA could be diminished by administration of triple therapy consisting of cyclosporin, methylprednisolone and azathioprine (305) or antiviral therapy (303). This indicates that viral replication, as well as the immune response following transplantation are important for the development of TAA.

Atherosclerosis is a multifactorial disease, although inflammation plays a key role in this process. Several reports have suggested a relationship between CMV infection and enhancement of vascular pathology. However, further research on how CMV enhances these vascular changes is warranted.

Cytomegalovirus and hemapoietic stem cell transplantation

Bone marrow transplantation (BMT) is a widely accepted component of the chemotherapeutic regimen for hematologic malignancies, bone marrow failure and congenital disorders of the lymphohemopoietic system (19). Hematopoietic stem cells can be derived from bone marrow, peripheral blood, cord blood and fetal liver (at 10-14 weeks of gestation) of a donor. In the absence of a syngeneic donor, an HLA matched sibling donor is the best choice. However, marrow derived from non-related HLA-matched donor is also widely used. The incidence of graft rejection and graft-versus-host disease (GvHD) is directly proportional to the level of mismatch (121, 325, 492).

CMV disease is a major cause of morbidity and mortality in both allogeneic BMT (68, 348) and peripheral blood stem cell transplantation (PBSCT) (224). During the 1980s, reactivation of CMV infection in CMV seropositive BMT recipients has been estimated to occur up to 65% of those patients (355, 548). The incidence of CMV disease was reported 20-35 % in seropositive bone marrow allografted patients (348, 350). Pneumonia is the most frequent manifestation with a high mortality rate up to 80% (145, 348, 425). The clinical presentation

includes fever, nonproductive cough, tachypnea and hypoxemia (46, 366). Besides the lungs, also the gastro-intestinal tract and retina can be involved, although less frequently (507). Previous to the introduction of CMV prophylaxis, CMV disease occurred 1-4 months after transplantation (350). However, with current potent anti-CMV prophylaxis, the peak incidence of CMV disease occurs not until 100 days after transplantation (63, 279).

CMV seropositive bone marrow recipients are at the highest risk for CMV disease, regardless of the serological status of the donor (187, 229, 349, 355). Seronegative patients receiving marrow from seropositive donors are at a lower risk for CMV associated disease than their CMV seropositive counterparts with either bone marrow from seropositive- or seronegative donors. The importance of allogeneic stimulation of latent virus in the recipient for developing CMV disease was shown by Soderberg-Naucleer (478). The fact that the serostatus of the donor is of little importance is surprising, since it is thought that bone-marrow progenitors may serve as a reservoir for CMV (203, 271, 272, 346, 357, 461). Seronegative patients receiving haemopoietic stem cells from seronegative donors are at the lowest risk for CMV infection (317). Other risk factors for developing CMV disease include high viral load (61, 350), GvHD (229, 550), transplants from volunteer unrelated donor (327, 512) or mismatched related donors (317) and a delayed reconstitution of the CMV specific cytotoxic T cell response (308, 417).

The CTL response is important in controlling CMV infections. For example, patients receiving T-cell depleted grafts have an increased risk for developing CMV infections (218, 327). Also, reconstitution of CMV specific CD8+ lymphocytes has been shown to be protective in BMT recipients (431, 432, 467). Also viral factors may influence onset and/or severity of CMV disease. There is increasing evidence that strain differences based on the CMV glycoprotein B may contribute to the virulence of CMV in BMT recipients (171, 520).

Cytomegalovirus infection and solid organ transplantations

Solid organ transplantations, such as liver, heart and lung transplantations, are currently available for treatment of various organ end-stage diseases in humans. Outcome is related to the surgical procedure, immunosuppression, allograft rejection and infections (253). The post transplant period can be divided into three phases, depending on the type of microbiological challenge (389). During the first month, infectious complications are related to surgical complications. The microbiological challenge in solid organ transplant recipients during this period is similar to the microbiological threat after general surgery. The second period, i.e. the period between the second and sixth month post transplantation, opportunistic infections, such as CMV, occur. From 6 months after transplantation onward, patients encounter the 'normal' microbiological challenge as seen in the general community.

CMV infections are common in solid organ transplant recipients (137). CMV infection may

be the result from viral transmission via an infected organ or blood products (primary infection or super-infection) or may be due to reactivation of latent virus already present in the recipient (secondary infection). Symptomatic CMV disease occurs in 8% of kidney-, 29% of liver-, 25% of heart-, 50% of pancreas- (or kidney/pancreas), 22% of small bowel- and 39% of heart/lung transplant recipients ⁽³⁸⁹⁾.

Risk factors for CMV disease include transplanting an organ from a CMV-seropositive individual to a CMV -seronegative recipient, the level of immunosuppression (especially with antilymphocyte preparations, such as OKT3 monoclonal antibodies) and clinical condition of the patient ⁽⁵²⁹⁾. CMV disease can involve lung, liver, gastro-intestinal, renal and/or retinal disease.

CMV infection has been associated with acute renal allograft rejection ^(407, 423, 535) and with chronic rejection of heart ^(193, 226, 277, 319), lung ⁽²⁸¹⁾ and liver transplants ^(20, 292, 379).

Although CMV infection is thought to be a risk factor for chronic renal rejection as shown in animal studies ^(209, 228), little is known about the role of CMV in chronic rejection of renal allografts in humans. The histological diagnosis of chronic kidney allograft rejection in humans is well defined and consists of focal interstitial lymphocytic inflammation and fibrosis, glomerular mesangial matrix increase and sclerosis, vascular intimal proliferation and tubular atrophy ^(391, 392). End-stage chronic rejection is predominantly characterized by interstitial fibrosis.

A positive correlation between CMV and the development of chronic renal rejection has been reported in animals ^(293, 294, 526). In the model used by Lautenschlager et al., rats received an allogeneic kidney transplantation under triple drug immunosuppression, which consisted of azathioprine, methylprednisolone and cyclosporin ⁽⁴⁸³⁾. Rats were subsequently challenged with RCMV or were mock infected. Viral antigens could be demonstrated in endothelial cells of capillaries and arterioles of RCMV infected animals. In contrast, mock-infected animals demonstrated characteristic histological changes of chronic rejection 60 days after transplantation, whereas those changes could already be demonstrated in RCMV-infected animals at 20 days after transplantation. The accelerated development of chronic rejection was associated with an increased and prolonged inflammatory response ⁽²⁹⁴⁾.

In liver transplantation, CMV infection has been associated with late acute rejection ⁽⁸²⁾ and chronic rejection (vanishing bile duct syndrome, VBDS) ^(147, 292, 380). The role of CMV infection in acute liver allograft rejection was studied in a rat model ⁽³²⁹⁾, which was developed by the group of Lautenschlager ⁽³³⁰⁾. CMV was shown to significantly increase portal inflammation and caused more severe bile duct damage when compared to uninfected control rats during acute liver allograft rejection ⁽³²⁹⁾.

Additional rat studies have shown a positive correlation of CMV and solid organ rejection in lung ⁽⁵⁰⁶⁾, heart ⁽³⁰²⁾, aorta ^(309, 310) and tracheal ^(274, 276) transplantation.

Several mechanisms for the CMV mediated enhancement of solid organ rejection have been postulated. Graft failure due to viral or immune-mediated destruction of infected cells, or activation of the immune system by CMV, which mounts an additional inflammatory response to the alloresponse, has been suggested in this matter. Evidence supporting the latter hypothesis is increasing ⁽²³⁰⁾.

The process of inflammation is crucial for organ transplant rejection. Extravasation of leukocytes into the allograft is mediated by an intimate interaction of leukocytes with endothelial cells. Firm adhesion of leukocytes to endothelial cells is mediated by binding of intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), both members of the Ig superfamily, to the integrin molecules, lymphocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4), expressed on leukocytes ^(493, 494). CMV infection has been shown to enhance adhesion molecule expression on endothelial cells *in vitro* ^(199, 455) as well as *in vivo* ^(24, 84, 275, 329, 505, 506, 513, 557). Induction of expression of adhesion molecules is mediated by cytokines, such as IFN-gamma, TNF-alpha and interleukin-1 (IL-1) ⁽⁴⁹⁴⁾. It was shown *in vitro* by Waldman et al., that CMV infected endothelial cells were able to activate host T cells. Production of IFN-gamma and TNF-alpha by these T cells was responsible for the observed enhancement of adhesion molecule expression on endothelial cells ⁽⁵⁴¹⁾.

The role of MHC class I and II antigens in the inflammatory process leading to organ transplant rejection has also been subject of several studies. CMV has been shown to enhance MHC class II *in vivo* ⁽⁵³⁵⁾ and MHC class I *in vitro*, using cultured tubular epithelial cells ⁽⁵²⁸⁾ and endothelial cells ⁽⁵²⁷⁾. Upregulation of MHC molecules by CMV could not be demonstrated in human umbilical vein endothelial cells ⁽⁴⁵⁶⁾ and in human kidney cells of tubular, glomerular or endothelial origin ⁽⁵²⁴⁾. However, it should be noted that the virus interferes with MHC class I ⁽⁵⁴⁶⁾ and class II ⁽³⁵⁴⁾ molecule expression and that viral homologs of MHC class I ⁽³⁹⁾ and class II ⁽¹⁷³⁾ molecules have been identified in the genome of CMV.

Treatment of cytomegalovirus infection

Compounds used for treatment of cytomegalovirus infections

Ganciclovir, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG), is an acyclic analogue of the natural nucleoside 2-deoxyguanoside. After intracellular phosphorylation of ganciclovir to ganciclovir-triphosphate, the compound competes with deoxyguanoside triphosphate and this results in an inhibition of viral DNA synthesis. The antiviral activity of ganciclovir against CMV, HSV-I and HSV-II has been extensively evaluated. Ganciclovir also inhibits replication of VZV, EBV, human adenovirus and HHV-6. Ganciclovir is used for treatment of CMV-associated syndromes, such as retinitis, prevention and treatment of CMV disease in transplant recipients, CMV pneumonia, CMV-induced esophago-

gastrointestinal infections and CMV hepatitis. The most frequent side effect of ganciclovir treatment is bone marrow depression (especially neutropenia). Ganciclovir treatment has also been associated with impairment of CNS function, abnormal serum liver protein values and allergic reactions, such as fever and rash ^(157, 378).

Foscarnet, trisodium phosphonoformic acid (PFA), is a pyrophosphate analogue. Foscarnet inhibits non-competitively and binds reversibly to the DNA polymerases of herpesviruses, including CMV ⁽¹⁰⁰⁾. The compound reduces CMV replication significantly *in vitro* as well as *in vivo*. Foscarnet is used for the treatment of CMV retinitis (systemical as well as intra-ocular administration of the drug) and gastro-intestinal CMV infection. The major side-effects of foscarnet therapy, are the relatively short half-life of the drug which necessitates intravenous administration of the drug, renal function impairment, disturbances in serum calcium and phosphate levels and anemia. Renal impairment is reversible when foscarnet administration is ceased ⁽²³⁶⁾.

Cidofovir is a acyclic analogue of the corresponding monophosphate of deoxycytidine. The compound possesses a phosphonomethylether moiety that is resistant to metabolism. After diphosphorylation, the compound competitively competes with incorporation of deoxycytosine-5'-triphosphate. This process decreases drastically the efficiency of viral DNA transcription ⁽²⁹⁹⁾. Cidofovir shows a broad spectrum antiviral activity against DNA viruses, including papillomaviruses, poxviruses and herpesviruses ⁽¹¹⁸⁾. Cidofovir has been shown to be a potent inhibitor of CMV replication *in vitro* ^(372, 373) as well as *in vivo* ^(287, 374, 498, 500). Cidofovir is mainly used for the treatment of CMV retinitis.

Despite the potent antiviral properties of the drug, clinicians should be aware of the following disadvantages. Firstly, in contrast to intravenous infusion of the compound, oral administration is characterized by a low bioavailability ⁽⁵³⁹⁾. Secondly, the major adverse effect of cidofovir is nephrotoxicity and as a consequence usage is limited in patients with renal function impairment ⁽²⁶¹⁾. After intravenous infusion, cidofovir is excreted unchanged over 90% in the urine ⁽¹¹⁸⁾. Clearance of cidofovir exceeds the glomerular filtration rate, indicating that tubular excretion is involved. It has been shown that accumulation of the drug within the proximal tubulus cells results in a dose limiting toxicity. However, administration of probenecid, a drug which inhibits active renal excretion, decreases cidofovir induced nephrotoxicity ⁽¹¹⁹⁾. This also suggests a direct relationship between tubular accumulation of cidofovir and nephrotoxicity. Minor adverse effects are neutropenia and uveitis.

Acyclovir, 9-(2-hydroxyethoxymethyl)guanine, is a nucleotide analogue, with minimal *in vitro* activity against CMV ⁽¹¹⁰⁾. Therefore clinical usage is restricted. It has been reported that high dose of acyclovir could have a prophylactic effect in transplant recipients at risk for CMV infection ^(131, 143, 178). However, it should be noted that not all authors were able to confirm these findings ^(27, 189, 266, 463, 553).

Antisense oligonucleotides are synthetic, short, single-stranded sequences of DNA or RNA that are designed to target and bind to messenger RNA. This attachment disrupts gene expression and inhibits protein synthesis. Fomivirsen is a novel antisense phosphorothioate oligonucleotide, which binds to major IE1 and IE2 mRNA of CMV. However, the observed antiviral effect of the compound *in vitro* ^(23, 130) cannot be explained by an antisense mechanism alone, since sequence-independent inhibition of virus absorption to host cells was also observed ⁽¹⁷⁾. In addition, intra-vitreal injection of fomivirsen decreased CMV activity in previously untreated patients suffering from CMV retinitis as well as in patients with CMV retinitis which was refractory to treatment with established anti-CMV compounds ^(127, 396). Although the results of fomivirsen administration for CMV retinitis are promising, clinical experience and application are limited. Unfortunately, there are several side-effects of intra-vitreal administration of the drug such as increased ocular pressure, bull's-eye pigmentary changes in the macula and mild to moderate intra-ocular inflammation of the anterior and posterior compartments of the eye ^(15, 127, 508).

Treatment of CMV infections in AIDS patients

Ganciclovir ⁽¹⁵⁸⁾, foscarnet ^(153, 462, 542) and cidofovir ⁽²⁸⁸⁾⁽²⁾ have successfully been used in the treatment of CMV disease in AIDS patients. Before HAART therapy became available, the incidence of CMV retinitis was reported up to 85% in AIDS patients. The potent inhibition of HIV replication by HAART, resulted in a significant increase in CD4 count. Restoration of immune competence of the immune system enabled effective control of CMV infections.

Initial treatment of CMV retinitis in AIDS patients consists of intravenous (i.v.) ganciclovir or i.v. foscarnet plus HAART in HAART-naïve patients ⁽³⁷⁵⁾. If CMV retinitis occurs in a patient who is already receiving HAART, high dose of i.v. ganciclovir, sometimes in combination with intra-ocular treatment may be used ^(333, 375). With progression of retinitis, intra-ocular treatment should be considered ⁽³⁷⁵⁾. In case of optimal HAART-related immune-reconstruction, oral ganciclovir ^(1, 134, 488) or even cessation of anti-CMV treatment may be optional.

Intra-ocular injections of foscarnet ⁽³⁷⁵⁾, ganciclovir ⁽¹⁰⁷⁾, cidofovir ^(177, 263) and fomivirsen ^(127, 396, 403) have been effective in patients with a decrease in visual acuity due to CMV despite HAART or i.v. anti-CMV compounds. The intra-ocular ganciclovir implant ⁽³³²⁾ has

been shown to be superior to all other intra-ocular therapies ⁽³⁷⁵⁾. However, local therapy does not prevent systemic disease and/or progression into bilateral acuity loss ^(107, 334, 365). These patients may benefit from oral ganciclovir ⁽³³³⁾.

Treatment of cytomegalovirus infections in transplant recipients

Hematopoietic stem cell transplantation has been associated with a high incidence of and severe outcome of disseminated CMV disease, especially in CMV seropositive patients. Prevention of CMV infection in seronegative- and CMV recurrence in seropositive recipients can be achieved by using CMV seronegative or leukocyte-depleted blood products ^(66, 425). In addition, seropositive recipients or seronegative patients receiving bone marrow from a seropositive donor should receive prophylactic or pre-emptive antiviral therapy ^(375, 425, 507). Prophylactic treatment consists of treating all patients at risk, whereas pre-emptive treatment is based on laboratory evidence of viral replication, such as viral culture, pp65 antigenemia and detection of viral DNA. Pre-emptive treatment reduces unnecessary drug exposure and allows immunological recovery ^(62, 142, 317).

CMV pneumonia is lethal in 80% of the patients and high dose of i.v. immunoglobulins in addition to ganciclovir have been shown to improve survival ⁽⁵⁰⁷⁾, with a response rate of 60% and a long term survival of 30% ^(375, 425). Usage of immunoglobulin infusions as monotherapy is controversial ^(425, 507, 549).

CMV induced gastro-intestinal disease and CMV retinitis can be treated with i.v. ganciclovir or i.v. foscarnet. Intra-ocular treatment for CMV retinitis may be considered in affected eyes refractory to treatment.

A major drawback of ganciclovir treatment is the increased incidence of neutropenia in BMT patients (41-58%) when compared to AIDS patients (34%) and solid organ transplant patients (10%) ⁽⁴⁴⁴⁾. An alternative for ganciclovir is administration of a high dose of acyclovir ^(351, 408, 409) or foscarnet ^(232, 318, 427). Ganciclovir is the treatment modality of choice for treatment of CMV infection of solid organ transplant recipients ⁽³⁷⁵⁾. Initial treatment is usually given until active CMV replication has ceased. However, after cessation relapses are common and the incidence of recurrence has been estimated to be 25% ⁽⁴⁴⁹⁾. Current diagnostic tools for detecting CMV infection allow safe pre-emptive treatment in solid organ transplant recipients with a lower risk for CMV infection (seronegative donor / seropositive recipient or seronegative donor / seronegative recipient) ^(81, 388, 394, 463). Prophylactic therapy is particularly attractive for patients at a high risk for CMV infection, i.e. seronegative recipient receiving an allograft from a seropositive donor ^(176, 320).

Drug resistance

The active metabolites of ganciclovir, cidofovir and foscarnet inhibit the DNA polymerase (UL18) of CMV. Phosphorylation of ganciclovir into its active metabolite form, depends on the UL97 gene product ^(315, 511). In contrast to ganciclovir, cidofovir does not require viral gene products for its activation into cidofovir-diphosphate ⁽¹⁰¹⁾. Foscarnet is an direct non-competitive inhibitor of the UL54 gene product ⁽¹⁰⁰⁾.

Resistance to ganciclovir is associated with mutations in UL97 alone ^(30, 98, 152, 551, 552, 558) or in combination with mutations in UL54 ^(468, 510). Mutations in UL54 alone are rare. Resistance to foscarnet occurring during first line treatment is seldom ⁽³⁰⁷⁾ and occurs predominantly after extensive ganciclovir treatment ^(97, 448, 468).

Recently, also CMV strains were isolated which showed resistance to cidofovir ^(95, 99, 140, 206). Also, treatment of *in vitro* CMV infected cells yielded viral strains which were less sensitive to the drug ^(102, 475).

Isolation of multiple resistant viral strains has important clinical implications and may result in uncontrollable deterioration of the patient. Follow-up of antigenemia levels may be a guideline for suspecting infection with resistant CMV strains. A decrease in antigenemia levels during anti-viral therapy indicates a good response of the virus to the drug. In case of increasing antigenemia values, a drug resistant CMV strain should be suspected ⁽²⁸⁾.

Rat cytomegalovirus as a model for cytomegalovirus infections

RCMV (Maastricht strain) has been isolated from salivary glands extracted from wild brown rats (*Rattus norvegicus*) ⁽⁷⁸⁾. Infection of immunocompetent rats with RCMV resulted in a disseminated infection of liver, spleen, kidney, bone marrow and thymus at very low concentrations (<12 plaque forming units (PFU)/ml), during the first week after infection ^(75, 77). From 1 week p.i. onward, the salivary gland yields high amounts of infectious virus ⁽⁷⁵⁾. The amount of infectious virus in this organ depends on the rat strain used ⁽⁷⁵⁾. Excretion of RCMV occurred predominantly via the saliva, although also low level of virus were found in the urine during the first weeks p.i. ⁽⁷⁷⁾.

Infection of rats with irradiation-induced immunosuppression increases viral load in 'internal' organs, such as spleen, kidney, liver and lungs ⁽⁴⁹⁷⁾. In addition, irradiation-induced immunosuppression (5Gy) is required for lethal CMV infections ⁽⁴⁹⁷⁾.

Recently, the complete DNA sequence of the rat cytomegalovirus genome was described ⁽⁵³¹⁾. The length of the genome was found to measure 229,896 bp. The RCMV genome encodes at least 166 protein-encoding open reading frames (ORFs), of which 113 and 76 have significant homology to ORFs of MCMV (Smith) and CMV (AD169),

respectively. The RCMV genome encodes homologs of cellular proteins. In this regard, it is interesting that the RCMV genome encodes for homologs of G protein coupled receptors (proteins R33 and R78), as well as for homologs of chemokines and MHC class I molecules (proteins r131 and r144) ^(41, 43, 44, 531).

The rat/RCMV model has been generally accepted as an appropriate model for studying CMV infections in humans based on its viral characteristics and biological behavior and has been used for studying the role of the virus in vascular pathology and in allograft rejection ^(302, 309, 330, 483). In addition, this model has also been used for elucidating pathogenesis of RCMV infection, especially in the immunocompromised host, and evaluation of novel antiviral compounds ⁽⁴⁹⁷⁾.

~ Chapter 2 ~

**Rat cytomegalovirus (RCMV)
replication in the salivary glands is
exclusively confined to striated duct
cells.**

Summary

The salivary gland is the preferent organ for cytomegalovirus replication and viral persistence. To identify the nature of infected cells and to study viral replication in more detail, several experiments were conducted. For this purpose, using the rat CMV (RCMV) model, acutely infected young adult rats (6 weeks of age) and new-born rats (3 days of age) were infected and submandibular, parotid and sublingual glands were harvested at different time points after infection. For identification of the nature of infected cells, immunohistochemistry, *in situ* hybridisation and electron microscopic techniques were used. In young adult animals, the submandibular gland was the preferent organ for RCMV replication. The parotid and sublingual gland, contained less virus. In contrast, in new-born rats the main site of RCMV replication was the sublingual gland while the submandibular and parotid gland contained low amounts of virus. In all glands of RCMV-infected animals, the infection was exclusively confined to striated duct cells. Infection resulted in a cellular inflammatory response which was mostly located in the interlobular duct region, whereas only few inflammatory cells were found in the neighbourhood of infected striated duct cells. This phenomenon may contribute to the long persistence of the virus in this organ.

Introduction

Cytomegalovirus (CMV) is one of the most common viruses affecting humans. Infection of the immunocompetent host is usually associated with mild disease or asymptomatic infection. After primary infection the virus persists in its host in a latent state from which it can be reactivated as was shown for human CMV (CMV) ^(54, 55, 73), murine CMV (MCMV) ⁽⁴¹⁹⁾ and rat CMV (RCMV) ⁽⁷⁴⁾.

The rat model provides an appropriate model for CMV, since RCMV has a virion structure similar to CMV and the pathology it induces both *in vitro* as *in vivo* is similar to the CMV induced pathology ^(76, 410, 497). Infection of immunosuppressed rats with RCMV results in a generalised infection with presence of infectious virus in most organs, such as liver, spleen, heart, kidney and lungs ⁽⁴⁹⁷⁾. In these organs infectious virus is already detectable at day 3 post infection (p.i.). Within these organs the virus is found in many cell types, among them brown fat cells, endothelial cells and monocytes/macrophages. After this period, the so called acute phase, which lasts for 1-2 weeks, no infectious virus is detectable in these organs. An exception is the salivary glands, where the virus persists for a long period i.e. several months up to one year p.i. ^(75, 77). The persistence of the virus in this organ is characteristic for RCMV and is believed to be one of the principal routes by which the virus is spread within the population of susceptible hosts. In summary, in the salivary glands, RCMV infection is characterised by two main properties: 1) infectious virus can be detected at a later time point than in all other organs, starting at day 7 p.i. and 2) infectious virus is produced in very high concentrations during a long period of time.

The tropism of this virus for the salivary glands and the long term shedding from this organ is one of the main characteristics of all CMVs. During evolution, the virus has been adapted to this target organ as shown by studies using deletion mutants of the virus ^(44, 90, 326, 446). In rats and mice infection with deletion mutants can result in a generalised infection with presence of virus in internal organs but without infectious virus and virus-infected cells in the salivary glands. This indicates that the virus harbours within its genome genes, that code for proteins/antigens which are necessary for infection of and replication in salivary glands.

Over the years many research groups have studied the course of CMV infection in both humans and animal models. Although the salivary gland is one of the main organs involved in replication of CMV and spread of the virus within the population, little is known of the cells that are infected and are producing infectious virus.

To study RCMV-infection of the salivary glands more thoroughly and to identify the nature of infected cells, we injected RCMV into the peritoneal cavity of young adult rats and followed the infection in the salivary glands. Since it is known that during the early phase of life organs are in development, we tested whether the age of the host is of importance for the infection of salivary gland cells. To study this, we included acutely

infected new-born animals in this study and followed the infection in their salivary glands.

Materials and methods

Animals, virus and immunosuppression

Inbred male specific pathogen free (SPF; according to ⁽⁴²²⁾) Lewis rats were used in all experiments. The animals were purchased from the Department of Experimental Animal Service at the University of Maastricht, the Netherlands. The animals were infected at the age of 3 days (new-born rats) or 6 weeks (young adult rats). The RCMV inoculum was derived from submandibular / sublingual salivary gland homogenate obtained as described previously ⁽⁷⁵⁾ and was intraperitoneally (i.p.) administered at a dose of 1×10^4 and 1×10^5 plaque forming units (PFU), respectively ⁽⁴⁹⁷⁾. Negative salivary gland homogenate was used for mock infection. CMV infection is known to be enhanced by immunosuppression. Therefore, the 6 weeks old rats were immunosuppressed using 5 Gy total body irradiation (TBI) ⁽⁴⁹⁷⁾. New-born rats have an immature immune system and were considered as "naturally" immunosuppressed.

Prior to the experiments, permission was obtained from the Ethical Commission for Laboratory Animals of the University of Maastricht, Maastricht, The Netherlands and all experiments were performed according to the Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) and the Dutch Law on Laboratory Animals.

Experimental design

The 6 weeks old animals were divided in three groups. The first group (n=5) received immunosuppression and was subsequently infected with RCMV. A second group consisted of immunosuppressed mock-infected animals (n=3), while a third group consisted of immunocompetent (non-irradiated) RCMV-infected rats (n=3). The rats were sacrificed at 7, 14 and 28 days post infection (p.i.). New-born rats (n=3) were infected at an age of 3 days and were subsequently sacrificed at 21 days p.i. At time of sacrifice, the submandibular, parotid and sublingual gland of young adult and new-born rats were sampled and processed for histological analysis and assessment of infectious virus. None of the infected rats showed symptoms of CMV disease as was defined previously ⁽⁴⁹⁷⁾.

Immunohistochemistry

Tissue samples were fixed in a phosphate buffered 3.7% formaldehyde solution in PBS for 24 hours and subsequently embedded in paraffin. RCMV-induced antigens were detected in 4µm tissue sections using a mouse monoclonal antibody (MoAb) 8 directed against an early nuclear RCMV antigen ⁽⁷⁹⁾. For evaluation of the inflammatory response MoAb ED-1, W3/13 and 3.2.3. were used, with specificity for monocytes-macrophages ⁽¹³³⁾, T lymphocytes ⁽⁹¹⁾ and NK cells ⁽⁷¹⁾, respectively. Positive cells were visualised using

diaminobenzidine (DAB) substrate and slides were counterstained with haematoxylin-eosin. For inflammatory assessment, positive cells in 5 representative high power fields (HPF, 400 x magnification) were counted. The presence of inflammatory cells was assessed semi-quantitatively. Absence of cells was scored as -; sporadic cells as $\pm$; small numbers of cells as +; considerable number of cells as ++; high numbers of cells +++.

Images of tissue sections were analysed using a computerized morphometric analysis system (Qwin Software, Leica, Cambridge). Total cellular and nuclear area was assessed at 28 days p.i. in samples harvested from young adult animals as well as from new-born rats and were expressed as mean $\pm$ SEM.

In Situ Hybridisation (ISH)

Detection of RCMV DNA was performed using the ISH technique as described previously ⁽⁴⁹⁷⁾. In short, paraffin embedded sections (4 μ m) were dewaxed and proteolytic digestion was carried out (5 min, 37°C) with pepsin (2mg/ml). Denaturation (80°C for 5 min) was followed by hybridisation with the DNA probe overnight at 42°C. The DNA probe consisted of the XbaI fragments C,D and E labelled with biotin 11-dUTP ⁽³⁴³⁾. Positive cells were visualised with DAB substrate. The ISH stained sections were used for viral load assessment since ISH is more sensitive than immunohistochemistry, as described before ⁽¹⁹⁶⁾.

Ultrastructural analysis by electron microscopy

Rats were anesthetized by injection of 0.1 ml Pentobarbital (6%) / 100 g body weight intraperitoneally. Subsequently, rats were perfused with 0.1 M Na-cacodylate buffer + 2% PVP (MW 40.000) pH 7.4 for 3 minutes (flowrate 15 ml/min), followed by perfusion fixation using fixative containing 4% Paraformaldehyde, 2% Glutaraldehyde and 0.2% Picric-acid in 0.1 M Na-cacodylate buffer pH 7.4. After perfusion, salivary glands (submandibular, sublingual and parotid) were removed and fixed overnight using the same fixative. Thereafter, tissues were washed 0.1 M Na-cacodylate buffer pH 7.4 containing 6.8% sucrose (3 x 5 min.) followed by another 2 hr fixation in 1% OsO₄ + 1.5% K₄Fe(CN)₆ in 0.1 M Na-cacodylate buffer at 4°C. Again, tissues were washed in 0.1 M Na-cacodylate buffer pH 7.4 containing 6.8% sucrose (3 x 5 min.) at room temperature and subsequently dehydrated in 30%, 50% and 70% EtOH (10 min. each) followed by 2 hr 100% EtOH. Tissues were embedded in Epon 812 and semi-thin (1 μ m) tissue sections were stained using Toluidine Blue. Areas containing RCMV infected cells were selected by light-microscopy. Selected areas were processed for ultra-thin sectioning (70 nm) and ultra-thin sections were contrasted according to Reynolds, using 2% uranylacetate (15 min.) and leadcitrate (2 min.). Finally, TEM analysis was performed at 60 kV using a transmission electron microscope (Philips, EM201, The Netherlands).

Assessment of infectious virus

The amount of infectious virus was determined in salivary glands by plaque assay using rat embryonic fibroblasts (REFs) as described before ⁽⁷⁸⁾. In short, the organs were homogenised and suspended in minimal essential medium containing 2% fetal calf serum. Tenfold dilutions of 10% homogenates (w/v) were inoculated on confluent REF monolayers. After an incubation period of 7 days, under 0.25% agarose, the number of plaques was determined microscopically after fixation and methylene blue staining.

Statistics

For statistical analysis the two-tailed non-parametric Mann Withney U test was used. Differences associated with $p < 0.05$ were considered statistically significant. Data were presented as mean $\pm$ SEM.

Results

Localisation of RCMV infection in the salivary glands of young adult rats

Infection of salivary glands was studied in young adult rats using several virological and histological techniques at 7, 14 and 28 days p.i. Presence of infectious virus in submandibular, parotid and sublingual glands was tested by plaque assay at 14 and 28 days p.i. The submandibular and parotid gland yielded high concentrations of infectious virus: 8.09 ± 0.25 and 4.98 ± 0.22 $^{10}\log$ PFU per gram tissue at 14 days p.i., respectively (table 1). Production of infectious virus increased in these organs during the course of infection, being, at 28 days p.i., 10.90 ± 0.17 $^{10}\log$ PFU for the submandibular and 8.54 ± 0.40 $^{10}\log$ PFU for the parotid gland. In contrast, the amount of infectious virus found in the sublingual gland decreased from 8.51 ± 0.28 $^{10}\log$ PFU at 14 days to 6.91 ± 0.67 $^{10}\log$ PFU at 28 days p.i. The number of infected cells within these glands was determined using the ISH technique and is expressed as number of positive cells per 1000 striated duct cells, as is shown in table I. The number of infected cells during the course of infection correlated accurately with the data from the plaque assays. Data from both techniques indicate that at 28 days p.i., the submandibular gland is the preferent organ for RCMV replication, while the parotid and the sublingual gland contained significantly lower concentrations of virus.

Immunohistochemistry and ISH revealed that infection was confined to striated duct cells in submandibular, parotid and sublingual gland of immunosuppressed young adult animals. Granular convoluted duct cells, acinar cells, intercalated duct cells and interlobular duct cells remained negative during the course of infection.

Table 1.Presence of RCMV in the salivary glands of 6 week old rats ^a

<i>Salivary glands</i>	<i>days p.i.</i>	<i>RCMV positive cells</i> ^b	<i>viral load</i> ^c
submandibular gland	7	0 ± 0	ND ^d
	14	114 ± 18	8.09 ± 0.25
	28	116 ± 19	10.90 ± 0.17
parotid gland	7	0 ± 0	ND
	14	10 ± 4 ^e	4.98 ± 0.22 ^e
	28	55 ± 24 ^e	8.54 ± 0.40 ^e
sublingual gland	7	0 ± 0	ND
	14	95 ± 35 ^e	8.51 ± 0.28
	28	53 ± 21 ^e	6.91 ± 0.67 ^e

a The rats received 5 Gy TBI before i.p. inoculation with 10⁵ PFU RCMV at the age of 6 weeks

b RCMV-infected cells were detected by in situ hybridisation (ISH) and the number of positive cells is expressed per 1000 striated duct cells (mean ± SEM)

c Viral load was measured by plaque assay and expressed as log PFU/g tissue (mean ± SEM)

d Not Determined

e Significant (p<0.05) when compared to the submandibular gland

Infection of immunocompetent animals resulted in the same viral tropism for striated duct cells, although the concentration of infectious virus and virus infected cells was lower or even undetectable (data not shown).

Uninfected striated duct cells showed a rectangular shape ($17.22 \pm 0.13 \times 9.21 \pm 0.45 \mu\text{m}$) with their small basis attached to the basal membrane. The cells contained a centrally located round nucleus ($7.40 \pm 0.13 \times 6.55 \pm 0.14 \mu\text{m}$). At the basis of the cell multiple invaginations could be appreciated. The most prominent finding in RCMV-infected striated duct cells was the enormous increase in total cell size (p<0.05 when compared to non-infected) (figure 1A). Cellular and nuclear size assessment was conducted at 28 days p.i. The cytoplasm protruded into the lumen of the duct and in some cases multiple infected cells resulted in a partial or even complete obliteration of the duct. The cytoplasm was filled with granular material, which gave the cells a foam-like appearance. This increase in cytoplasmic volume resulted in a more basally located nucleus and disappearance of the invaginations. In contrast to non-infected striated duct cells, the

nucleus was of oval shape ($16.04 \pm 0.64 \times 8.96 \pm 0.55 \mu\text{m}$) and significantly enlarged (figure 1A). In the duct, infected cells are most frequently lined by non-infected cells (figure 2A) and infection was focally distributed throughout the gland.

In contrast to the submandibular gland, the parotid gland was characterised by a more lobular structure of the gland and the absence of granular convoluted duct cells. The sublingual gland also lacks this duct segment. In both glands infection was also confined to striated duct cells. There were no differences in morphology of infected cells between the submandibular, parotid and sublingual gland.

In addition to light microscopy, electron microscopy was used as a golden standard for identification of infected cells in the submandibular, parotid and sublingual gland. The observations found by light microscopy were confirmed by EM. Infected cells were characterised by an enlarged ovally shaped nucleus and by an increase in total cell size as described above. The surrounding cells are characterised by basal invaginations, the presence of many mitochondria and a centrally located nucleus indicating that the infected cells are part of the striated duct (figure 2A). In the nucleus of infected cells many immature virus particles could be visualised. Figure 2B suggests the budding of a mature virus particle from the nuclear membrane (arrowhead 8). Additionally, several relatively small vesicles containing mature and immature viral particles and dense bodies assemble in the cytoplasm. As depicted in figure 2D, the mature virus particles consist of a nucleocapsid with an electron dense DNA structure. The nucleocapsid is surrounded by tegument and viral envelope. The large vesicle fuses with the cellular membrane and releases its contents into the lumen (figure 2C). In conclusion, RCMV replication in salivary glands of young adult Lewis rats is exclusively confined to striated duct cells as shown by immunohistochemistry, ISH and EM.

Localisation of RCMV in the salivary glands of the newborn rat

Preliminary data in our laboratory suggested that infected cells were of granular convoluted duct origin due to the characteristic appearance of infected cells. Data from young adult animals shown above suggested otherwise. To confirm our data from the young adult animals, a second model was employed which lacks the putative target cells. This model consisted of infection of newborn rats at the age of 3 days. The animals were sacrificed 21 days p.i., i.e. just before onset of granular duct development. As shown in table 2, infectious virus could be detected in the salivary glands of these animals, especially in the submandibular and sublingual gland.

Figure 1. Total cell- and nuclear area of RCMV-infected (measured by ISH) and non-infected striated duct cells was assessed in the submandibular, parotid and sublingual gland from young adult (A) and new-born rats (B). Salivary glands harvested at 28 days p.i were used for assessment. (black bar) = total cell area of RCMV-infected striated duct cells; (dark grey bar) = nuclear area of RCMV infected striated duct cells; (light grey bar) = total cell area of non-infected striated duct cells; (white bar) = nuclear area of non-infected striated duct cells. Data are expressed as mean $\pm$ SEM (mm²). All differences between infected and non-infected cells are significant ($p < 0.05$). By ISH, no viral DNA could be detected in parotid glands (*) extracted from new-born rats.

Table 2.Presence of RCMV in the salivary glands of new-born rats ^a

<i>Salivary glands</i>	<i>RCMV positive cells</i> ^b	<i>Viral load</i> ^c
Submandibular gland	23 ± 8	5.6 ± 0.5
Parotid gland	0 ± 0 ^d	3.1 ± 0.4 ^d
Sublingual gland	66 ± 17 ^d	7.0 ± 0.3 ^d

- a rats were infected with 10⁴ PFU RCMV at day 3 after birth and were sacrificed at day 21 p.i.
b measured by in situ hybridisation (ISH) and expressed per 1000 striated duct cells (mean ± SEM).
c Viral load was measured by plaque assay and expressed as ¹⁰log PFU/g tissue (mean ± SEM)
d Significant when compared to the submandibular gland (p<0.05)

In contrast to young adult animals, the amount of infectious virus and the number of RCMV-infected cells in new-born animals was significantly higher in the sublingual gland than in the submandibular glands while low concentrations of infectious virus, measured by plaque assay but no viral DNA, using ISH, was found in the parotid gland of these animals. In concert with their young adult counterparts, viral replication was restricted to striated duct cells and similar cytopathology of the infected cells was found (figure 1B). In conclusion, these experiments in new-born rats show that despite differences in salivary gland tropism, RCMV replication in these animals is also confined to striated duct cells and indeed independent of granular convoluted duct cells.

Inflammatory cell influx in the RCMV-infected salivary glands

RCMV infection of the salivary glands of young adult rats generated a cellular inflammatory response as shown by the presence of monocytes/macrophages, lymphocytes and NK cells at day 14 and 28 post infection (table 3). At these time points the number of inflammatory cells in the submandibular and in the parotid glands was significantly higher than in the glands of mock-infected animals. In the sublingual glands of the infected rats, no increased influx of inflammatory cells was observed compared to controls. The influx of monocytes/macrophages preceded the influx of T lymphocytes in the submandibular and the parotid glands. A similar observation was reported previously by Persoons et al. ⁽³⁹⁹⁾ using the subcutaneously-infected hind paw model.

The influx of NK cells was rather low and was maximal at 2 weeks p.i. (table 3). This is of importance since it has been suggested that especially NK cells are responsible for the clearance of CMV infection ^(52, 544).

Figure 2. Striated duct cells are characterised by basal striations, a centrally located nucleus and many mitochondria. The infected cell (IC) is located within the striated duct (A). Assembly of small vesicles containing mature and immature viral particles at the Golgi apparatus is shown in figure B. The mature virus particles consist of a nucleocapsid with an electron dense DNA structure (D). The nucleocapsid is surrounded by tegument and viral envelope. Release of virus particles in the lumen of the duct is shown in figure C. Arrowheads indicate the following structures: 1 = basal striations; 2 = mitochondrion; 3 = uninfected nucleus; 4 = virus containing vesicle; 5 = RCMV-infected nucleus; 6 = lumen; 7 = nuclear membrane; 8 = budding of a virus particle from the nuclear membrane; 9 = Golgi apparatus; 10 = cellular membrane; 11 = nucleocapsid; 12 = tegument and viral membrane; 13 = dense body.

Table 3.

Presence of monocytes/macrophages, T-lymphocytes and NK cells in the salivary glands of RCMV-infected rats ^{a, b}

	day	Submandibular		Parotid		Sublingual	
		RCMV	Mock	RCMV	Mock	RCMV	Mock
monocytes/	7	+ ^f	+	+	+	+	+
macrophages ^c	14	+++	+	+++	+	+	+
	28	+++	+	++/+++	+	+	+
T lymphocytes ^d	7	+	+	+	+	+	+
	14	+	+	+	+	+	+
	28	+++	+	++/+++	+	+	+
NK cells ^e	7	+	+	+	+	+	+
	14	+ / ++	+	+ / ++	+	+	+
	28	+	+	+	+	+	+

- a Rats received a total body irradiation of 5 Gy before inoculation with 10⁵ PFU RCMV.
b The animals were sacrificed at different time points and the salivary glands were used for this study.
c Cells reactive with MoAb ED-1, specific for monocytes/macrophages
d Cells reactive with MoAb W3/13, specific for T lymphocytes
e Cells reactive with MoAb 3.2.3, specific NK cells
f The presence of cells was assessed semi-quantitatively. Absence of cells was scored as -; sporadic cells as +; small numbers of cells as ++; considerable number of cells as +++; high numbers of cells +++

Another interesting finding in this study was that inflammatory cells detected in the infected glands were not localised around the infected striated duct cells, as expected (figure 3A), but were confined to the interlobular duct region in the submandibular and parotid gland (figure 3B). This was true for both immunocompetent as immunosuppressed animals (data not shown). Only few inflammatory cells were present in the sublingual gland (table 3). This is an intriguing observation since it could be one of the mechanisms involved in the persistence of infected cells in this organ.

Discussion

In this study a detailed description of CMV replication in the submandibular, parotid and sublingual salivary gland of the rat is reported. The main objective of the present study was to assess the identity of the type of RCMV-infected cells in the organs. Initially, preliminary data suggested that infected cells were of granular convoluted duct origin due

to the characteristic appearance of the infected cells. By using different techniques (immunohistochemistry, *in situ* hybridisation and electron microscopy) it became clear that not the granular convoluted duct cells but the striated duct cells were infected. This finding was further supported by the results obtained in intraperitoneally infected newborn rats, which were sacrificed before onset of granular duct development (at 4 weeks of age). Our study clearly shows that RCMV replication was exclusively confined to striated duct cells in all three salivary glands and that acinar cells, intercalated duct cells, granular convoluted duct cells and the interlobular duct cells remained free of virus. This is of interest, since striated duct cells differentiate into granular convoluted duct cells starting at 4 weeks of age ⁽²⁴²⁾ and as a result the ability for RCMV to replicate in these cells is lost in the process. Restriction of CMV replication to (a segment of) ductal cells in the rat model is in concert with the data obtained in the guinea pig infection model using guinea pig CMV (GPCMV), in which infection was limited to ductal segments ^(49, 164, 165, 196). In humans, CMV infected ductal cells, but also infected acinar cells, were found ^(194, 402, 471, 540), while in mice only infected acinar cells are described ^(215-217, 227, 248, 356, 382). No clear explanation for these differences in infection of different cell types in salivary glands between different models has been given. Especially, the difference between RCMV and MCMV in their behaviour for salivary gland cell tropism is fascinating since it is known that these viruses possess a lot of similarities and that the genomes of both viruses show the highest homology of all known CMVs ^(42-44, 47).

Another interesting observation of this study is that, although RCMV replicates only in the striated duct cells in the salivary glands, the virus content is significantly different among the different glands, suggesting that other yet unexplained factors are also involved in the infection and production of virus in these glands.

An important characteristic of CMV infection is its persistence in salivary glands. In the rat model persistent infection accompanied by continuous shedding of virus from this organ has been shown earlier ⁽⁷⁷⁾. The cells responsible for this persistence and shedding are striated duct cells, since only these cells contain high amounts of virus particles, as shown by immunohistochemistry, ISH and electron microscopy.

From our *in vivo* data and from results obtained with other cells, such as endothelial and smooth muscle cells, we know that at least two factors contribute to persistent infection; first the type of cell that is infected and secondly the immune response of the host to this infection. Depending on the type of cell that is infected, an infection results in cell death (fibroblasts ⁽⁷⁸⁾), an abortive infection (endothelial cells, ⁽⁵⁷⁾) or a persistent infection (smooth muscle cells and monocytes- macrophages ^(162, 521)). In the present study we detected not only that striated duct cells are the preferent cells for CMV infection in the salivary glands but also that in these cells the production of virus particles follows a different course than in fibroblasts.

Figure 3. A) Detection of viral DNA by ISH in the submandibular gland of young adult rats at 28 days p.i. The nuclear staining of RCMV-infected cells (arrowhead 1) can be distinguished from the cytoplasmic staining of virus-containing vesicles (arrowhead 2). Note the relative absence of inflammatory cells. B) Parotid gland of a young adult rat at 28 days p.i. Note that the influx of inflammatory cells is predominantly confined to the interlobular duct region. Arrowheads indicate the following structures: 3 = acinus; 4 = uninfected striated duct segment; 5 = intercalated duct segment; 6 = artery; 7 = vein; 8 = acinus; 9 = interlobular duct.

While in fibroblasts, as in neo-intimal smooth muscle cells, high concentrations of virus particles were found in the nucleus ^(397, 519), we detected large amounts of virions in vacuoles in the cytoplasm of striated duct cells. Such observations have thus far not been performed in other cell types of rat origin. It suggests that replication, production and excretion of the virus in striated duct cells is different from replication in fibroblasts and is cell type dependent. However, it should be noted that virus containing vesicles is a common observation in the human system ⁽³⁵⁹⁾.

In vivo the situation is even more complex since not only the cell type is important but also other factors such as the immune response of the host. In our study it was found that in the infected salivary glands, inflammatory cells were not localised directly around the infected cells but were localised at the interlobular duct regions. This is an interesting

finding since it might explain the persistence of CMV in salivary glands. A similar observation has also been described for the mouse model ⁽²¹⁶⁾ indicating that, in contrast to other CMV infected organs, the inflammatory cells in the salivary glands do not reach the infected cells. Thus they are not able to eliminate virus infected cells and by this way limit the infection. The reason for this remains unknown. However, it is known that eventually the virus is removed from the gland ⁽⁷⁵⁾. In addition, electron microscopy showed that viral excretion from the infected cell is by apical cell budding of the larger virus containing vesicles. It should be noted, however, that transmission of virus to other cells via direct cell-cell contact could not be excluded by electron microscopy. A possible mechanism for viral clearance from these organs might be that infected cells can only infect downstream-located striated duct cells. During the course of infection, infected cells located in the proximal striated duct segment would die and would be replaced by non-infected counterparts. As a result, the infection would be removed from the gland in the direction of saliva flow, without intervention of the immune system. Adding to this is the fact that the number of striated duct cells decreases with aging of the rat due to the differentiation of striated duct cells into granular convoluted duct cells ⁽²⁴²⁾.

Also other mechanisms could be involved in the persistence of the infection in the salivary glands, for example the observation that, in contrast to other organs, the NK cell influx in this organ is only slightly enhanced, while in other infected organs a high influx of NK cells is found ⁽⁵²⁵⁾. Since it is known that these cells are important cells for the clearance of CMV infected cells ⁽⁴⁵⁸⁾, diminished influx of NK cells could play a role in persistence of the virus in the salivary glands.

In conclusion, we showed that RCMV exclusively infects striated duct cells in the salivary glands. In these cells, high levels of infectious virus are produced during a long period p.i. Persistence in the salivary glands is partly due to the type of cell that is infected, but probably also to a failure of the immune reaction in removing the virus-infected cells in these organs. The difference between MCMV and RCMV in preference of infection site is an interesting observation that cannot yet be explained. The use of *in vitro* cell culture in the near future will give us more answers about infection and persistence of CMV in salivary glands.

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~ Chapter 3 ~

**Persistent rat cytomegalovirus (RCMV)
infection of the salivary glands
contributes to the anti-RCMV antibody
response.**

Summary

The salivary glands are the major sites of persistent replication of rat cytomegalovirus (RCMV). At several months post infection (pi), infectious RCMV is usually still produced in the salivary glands but not in any other organ or tissue of the rat. To investigate whether the persistence of RCMV in the salivary glands is crucial to the pathogenesis of viral infection, we monitored the progression of RCMV-induced disease in rats from which the salivary glands were surgically removed (desalivated) as well as in sham-operated rats, both after a lethal and sublethal challenge with RCMV. Interestingly, desalivation did not have a significant effect on either RCMV-induced morbidity or mortality. As expected, at one year pi, relatively high levels of infectious virus were detected in the salivary glands of sham-operated rats, whereas neither infectious virus nor RCMV DNA could be detected in liver, spleen and lungs of these animals. Infectious virus and viral DNA were also undetectable in organs from desalivated animals at one year pi. Interestingly, a difference was found between desalivated and sham-operated rats in the titers of anti-RCMV IgG antibodies, which were significantly higher in sham-operated rats than in desalivated animals at 183, 295 and 365 days pi. This finding indicates that the persistence of RCMV in the salivary glands may contribute significantly to the anti-RCMV humoral immunity of infected rats.

Introduction

Cytomegalovirus (CMV) infection is common in the human population and seroprevalence has been reported to be as high as 80-90%. In immunocompetent individuals, infection is usually asymptomatic. However, when the host's immune system is compromised (i.e., in AIDS patients or transplant recipients), CMV infection may cause life-threatening disease and even lead to death ^(210, 400).

In our laboratory, a rat cytomegalovirus (RCMV)/rat model has been developed in order to study the pathogenesis of CMV infections ^(75, 77, 78). RCMV infections in this model can be characterized by two distinct phases. In the first phase, during the first week post infection (pi), a generalised infection of internal organs, such as liver, kidney, pancreas, lung and spleen occurs ⁽⁴⁹⁷⁾. Although viral DNA can be detected in the salivary glands by PCR, neither infectious virus nor viral antigens are found in these glands during the first phase of infection ^(42, 43). The second phase of infection initiates at approximately 8 days pi. During this phase, the salivary glands start to produce high levels of infectious virus for a prolonged period of time ^(75, 77). The infection of the salivary glands can therefore be regarded as a persistent infection. By contrast, infectious virus can no longer be detected in 'internal' organs after the first week pi, whereas viral DNA can occasionally be detected ⁽⁴³⁾. Thus, RCMV may reside in these organs in a latent form.

In the rat salivary glands, RCMV replication was found to be confined to striated duct cells of the submandibular, parotid and sublingual glands ⁽²⁶⁷⁾. Within these cells, virus particles are deposited in large cytoplasmic vesicles, which release their contents in saliva by apical cell budding ⁽²⁶⁷⁾. The virus-containing saliva is generally regarded as a crucial vehicle for horizontal transmission of RCMV. However, the production of high virus titers in the salivary glands and saliva for longer periods of time could also have other consequences. It can be envisaged that the continuous production and accumulation of virus particles in the salivary glands may have an influence on the overall physiology of the infected host. Shedding of virus from the salivary glands and virus-containing saliva to other sites of the body may result in re-infection of internal tissues and organs, providing a continuous challenge for the host's immune system.

In order to study the role of the salivary glands in the pathogenesis of RCMV infection, we employed an experimental infection model in which viral replication and dissemination were monitored in rats in which the salivary glands had been surgically removed prior to infection. It has previously been demonstrated that desalivation of rats only results in relatively mild alterations in the normal physiology of the animals ^(69, 367, 376, 469). Here, we describe two separate experiments. In the first experiment, the effect of desalivation on RCMV-induced disease and mortality was studied after administration of a lethal dose of RCMV. In the second experiment, desalivated rats, sham-operated rats as well as mock-infected rats were challenged with a sublethal dose of RCMV.

Figure 1. Schematic representation of the experimental design of the study. (A) Mortality experiment. The effect of desalivation on mortality after a lethal RCMV challenge was studied. (B) Long-term follow-up experiment. Rats were sublethally challenged with RCMV. The general condition of the animals was recorded. Blood samples were taken from the tail vein at 14, 29, 84, 117, 183, 245, 295 and 365 days p.i. in order to determine anti-RCMV IgG levels. At 1 year pi, various organs were harvested for detection of infectious virus and viral DNA.

Subsequently, the generation of anti-RCMV antibodies was measured during the course of infection until one year pi. In addition, the presence of infectious virus as well as viral DNA was assessed in various organs. Surprisingly, we found significantly higher titers of anti-RCMV antibodies in sham-operated rats than in desalivated animals, indicating that persistently infected salivary glands may contribute to the anti-RCMV humoral immunity of infected rats.

Materials and Methods

Animals and virus

Specific pathogen-free, inbred male Lewis rats were obtained from the Experimental Animal Facilities of the University of Maastricht, Maastricht, The Netherlands. The RCMV (Maastricht strain) inoculum was derived from submandibular and sublingual salivary gland homogenates, which were generated as described previously ⁽⁴⁹⁷⁾. Salivary gland homogenates from uninfected rats were used for mock infection.

Extirpation of salivary glands

Salivary glands were removed from either 3- or 4-week-old rats. Rats were anaesthetized with a mixture of ketamine (100 mg/kg, ip) and xylazine (10 mg/kg, subcutaneously (sc)). Both drugs were purchased from Eurovet (Bladel, The Netherlands). After a transverse incision, the submandibular, sublingual and parotid glands of the rats were removed and the skin was subsequently closed. An equal number of rats received a sham operation. Buprenorphine (0.5 mg/kg, sc; Schering-Plough, Maarssen, The Netherlands) was used to alleviate post operative pain ⁽³¹³⁾. All animals were fed acidified tap water and moistened, grinded rat chow (Hope Farms, Woerden, The Netherlands) ad libitum. The rat chow was suspended (600 g/l) in 2% granulated agar (Becton, Dickinson and Company, Cockeysville, MD, USA) and enriched with sugar (12 g/l). All rats quickly recovered and started to gain weight within 2 days after surgery. After 3 months, all rats returned to a diet of normal (ungrinded) rat chow ad libitum.

Experimental design

To study the influence of the salivary glands on the course of RCMV infection, two experimental models were employed (Fig. 1). In the first model (Fig. 1A), the effect of salivary gland extirpation on mortality after a lethal RCMV challenge was studied. Three weeks-old rats were subjected to a radical salivary gland extirpation.

Figure 2. The onset of RCMV-induced disease and mortality in groups (n=7) of RCMV-infected, sham-operated (A) and desalivated rats (B). The dashed lines represent the period in which symptoms of RCMV infection could be appreciated. † indicates time of death. The experiment was carried out as outlined in figure 1A.

Subsequently, at one day before infection (day -1), rats were immunocompromised by total body X-ray irradiation (TBI, 5 Gy). At day 0, RCMV was administered intraperitoneally (ip) at a dose of 1×10^5 plaque forming units (PFU)⁽⁴⁹⁷⁾. RCMV-infected, desalivated rats (n = 7) were compared to RCMV-infected, sham-operated animals (n = 7). Desalivated, mock-infected rats (n = 7) were used as control animals. Rats were checked for CMV disease⁽⁴⁹⁷⁾ and weighed on a daily basis. In addition, survival was recorded.

The second experimental model (Fig. 1B) consisted of a long-term follow-up of RCMV infection in salivary gland-extirpated rats and sham-operated rats as well as in mock-infected control animals. In this model, salivary glands were removed from 4 weeks-old rats ($n = 8$). Another subgroup of rats ($n = 8$) received sham surgery. Control rats were mock-infected and were not subjected to surgery ($n = 3$). After a recuperation period of 4 days, rats were irradiated and infected, similarly as described above. The rats were weighed daily, and the general condition of the animals (weight, dental health, behavior and condition of oral mucosa) was recorded. In order to assess anti-RCMV IgG levels, blood samples were taken from the tail vein at 14, 29, 84, 117, 183, 245, 295 and 365 days pi. At 1 year pi, organs (submandibular glands, liver, spleen and lung) were harvested for detection of infectious virus (by plaque assay) and viral DNA (by PCR).

Detection of anti-RCMV IgG antibodies

Blood samples were centrifuged for 10 min at 4500 x g and stored at -20°C until use. An enzyme-linked immunosorbent assay (ELISA) was developed to monitor total anti-CMV IgG in rat serum. To prepare the antigen, rat embryo fibroblasts (REF) were cultured in minimal essential medium (MEM) containing 10% newborn calf serum (NCS). The cells were infected with RCMV at a multiplicity of infection (MOI) of 0.05 in MEM containing 2% NCS. After an incubation period of 4 hours at 37°C , the medium was replaced with fresh MEM containing 2% NCS and the cells were cultured until all cells showed cytopathic effect (CPE). The medium and the cells were collected and centrifuged for 10 minutes at 900 x g to remove cell debris. PEG 6000 was added to the supernatant at a concentration of 15 % (w/v) and the solution was stirred at 4°C for 4 hours. After centrifugation at 1400 x g for 10 minutes, the pellet was resuspended in dialysis buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.1) and dialyzed overnight at 4°C . The solution was placed on a solution of 30% sucrose in dialysis buffer and centrifuged for 75 minutes at 1400 g at 4°C . The pellet was diluted in dialysis buffer and stored at -20°C until use. As control antigen, mock-infected cells were processed. The ELISA was performed in 96-well vinyl assay plates. The wells were washed with coating buffer (2.5 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 7.5 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.15 M NaCl, pH 7.2) and incubated overnight at 4°C with 0.1 ml of antigen diluted 1:10 in coating buffer. After three washes with washing buffer (2.5 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 7.5 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.5 mM NaCl, 1 mM Tween 20, pH 7.2), 0.1 ml of 1:10 diluted rat serum was added to each well. After incubation for 1 hour at room temperature (RT), the wells were washed and biotinylated, sheep anti-rat IgG (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) was added, followed by another incubation step of 1 h at RT. Subsequently, the wells were washed and incubated with peroxidase-conjugated streptavidine (Amersham Pharmacia Biotech).

Figure 3. Relative weight increase during long-term follow-up of groups of RCMV-infected sham-operated (closed circles) rats, RCMV-infected desalivated rats (open circles) and untreated and non-infected (closed squares) animals. Experiments were conducted as outlined in figure 1B. Data are expressed as mean $\pm$ SEM.

Immediately before use, the substrate solution was prepared (12 mg O-phenyldiamine and 15 μ l 30% H_2O_2 added to 36 ml substrate buffer (35 mM $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$, 67 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, pH 5.0)), and 0.1 ml was added to each well. The plate was incubated in the dark for 10 minutes and the reaction was stopped by adding 0.1 ml of 4 M H_2SO_4 . The extinction at 492 nm was measured using an ELISA reader (340 ATCC). All samples were processed simultaneously and each ELISA was performed in duplicate. The extinction values of the control-coated wells were subtracted from the extinction values of RCMV antigen-coated wells.

Plaque titration assay

The amount of infectious virus was determined in salivary glands by plaque assay as described previously ⁽⁷⁸⁾. In short, the organs were homogenised and suspended in MEM containing 2% NCS. Tenfold dilutions of 10% homogenates (w/v) were inoculated on confluent REF monolayers. After an incubation period of 7 days under 0.25% agarose, the number of plaques was determined microscopically, after fixation and staining with methylene blue.

Figure 4. RCMV antibody response of sham-operated (black circle), desalivated (open circle), and control (black square) rats. Sham-operated and desalivated rats were irradiated (5 Gy TBI) and infected with 1×10^5 PFU of RCMV at day -1 and day 0, respectively. Rats were sacrificed at 365 days p.i. Differences marked with * were considered to be statistically different when compared to sham-operated rats. Data are expressed as mean $\pm$ SEM.

RCMV PCR

Organ samples were minced mechanically and DNA was isolated by using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). During the isolation procedure, organ homogenates were incubated with proteinase K at 60°C for 60 to 90 minutes. Viral DNA was detected by using a sensitive, single-tube, nested PCR which amplifies a part of the major immediate-early region of the RCMV genome. The PCR was carried out as described previously ⁽⁴³⁾. In short, the PCR mixtures, with a total volume of 50 μ l, contained 1 μ g of target DNA, 0.05 μ M of primers RIE3F (5'-CCA GAG TGA CGT TGC AGA TGT TGG AAA TCA-3') and RIE3R2 (5'-GGT CAC GAC CCT GCT GCC GTC TAG GT-3'), 1 μ M of each primer RIE4F (5'-ATG AAA TGG TGA TGA GAT-3') and RIE4R (5'-CTT CTA GTG ATT TGG CAT-3'), 100 μ M concentrations of each dNTP, 15 mM MgCl₂, HotStar Taq DNA polymerase buffer and 1.25 U of HotStar Taq DNA polymerase (Qiagen, Leusden, The Netherlands). Amplification was performed with a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer, Nieuwerkerk aan de IJssel, The Netherlands). PCR products were visualized by ethidium bromide-staining after gel electrophoresis.

Samples were tested both undiluted and 1:10 diluted. In addition, samples were tested with or without spike (10 copies of viral DNA).

Statistical analysis

For statistical analysis, the two-tailed non-parametric Mann Withney U test was used. Differences associated with $p < 0.05$ were considered statistically significant. Data are presented as mean $\pm$ SEM.

Results

Desalivation does not have an influence on RCMV-induced disease and mortality

To study the role of the salivary glands in the pathogenesis of RCMV infection, we employed an experimental model in which the course of the infection was compared between groups of desalivated rats and normal, sham-operated rats. In an initial experiment (Fig. 1A), we compared the mortality rates of these groups of animals after infection with a lethal dose of RCMV. As shown in Fig. 2, there is no significant difference in mortality rate between desalivated rats and sham-operated animals. In both groups, the rats died between day 8 and 15 pi. In most animals, symptoms of RCMV-induced disease, such as mucosal haemorrhages, development of ascites and weight loss, were found approximately 3 days before death (Fig. 2). These symptoms, which have been described previously ⁽⁴⁹⁷⁾, appeared in a similar fashion in either group of animals. As a control, mortality was also investigated in a group of desalivated, mock-infected animals. As expected, each of the animals of this group survived the surgical procedure and did not have a mortality significantly different from that of untreated, mock-infected rats (data not shown). In addition, desalivation did not lead to detectable side effects in any of these animals. Taken together, our data indicate that desalivation does not have a significant effect on either onset or course of a lethal RCMV infection.

Desalivated rats have a significantly lower titer of anti-RCMV antibodies than normal rats

It was previously shown that desalivation does not result in significant alterations in the physiology of rats ^(69, 367, 376, 469). Nevertheless, in a second, long-term experimental model, in which rats were subjected to sublethal doses of RCMV (Fig. 1B), we monitored the health status of the animals on a daily basis, by using the following parameters: weight increase, dental health, the condition of the oral mucosa and behavior. At the time of surgery, the weights of the RCMV-infected, desalivated rats, RCMV-infected, sham-operated animals and mock-infected control rats did not differ significantly (117 ± 11 g, 133 ± 13 g and 109 ± 8 g, respectively). During long-term follow-up, the weight of the animals increased steadily. However, significant differences in relative weight increase between the

three experimental groups were not found (Fig. 3). In addition, differences in either dental health, oral mucosa or behavior could not be detected between these groups (data not shown). Thus, in accordance with previous reports (69, 367, 376, 469), our results indicate that surgical removal of the salivary glands does not have a significant influence on either growth or health status of the animals up to one year after surgery.

To investigate the dissemination of RCMV in desalivated rats after long-term infection, these animals, as well as RCMV-infected, sham-operated rats, were sacrificed at one year pi. Subsequently, the presence of both infectious virus and viral DNA was investigated in liver, spleen, lungs and submandibular glands (of sham-operated rats). As expected, infectious virus was detected in the salivary glands of sham-operated animals ($3.6 \times 10^4 \pm 2 \times 10^3$ PFU/g tissue), but not in any other tissue of either desalivated or sham-operated rats. This finding is in agreement with previous reports in which RCMV replication at several months after infection was detected exclusively in the salivary glands (43, 75, 77). In order to detect viral DNA in the rat organ samples, we employed a previously described single-tube, nested PCR assay, which can detect 1 to 10 copies of RCMV DNA per 2.4×10^5 cells (43). Previously, we could occasionally detect RCMV DNA in organs samples from RCMV-infected rats at 330 days pi (43). We therefore anticipated that viral DNA might also be found in some of the organ samples from rats sacrificed at 365 days pi. However, RCMV DNA was exclusively detected in the salivary glands of sham-operated animals (data not shown). The apparent discrepancy between this result and that of the previous study (43) may be due to the use of a 10 times lower RCMV inoculum in the present study. Nevertheless, our results underline the importance of the salivary glands as the predominant site of long-term, persistent RCMV replication.

As described previously, an anti-RCMV IgG antibody response is elicited during long-term, persistent RCMV infection in rats (75). To investigate whether persistent RCMV infection of the salivary glands has an effect on this humoral immune response, we regularly collected serum samples over a one-year period from both desalivated and sham-operated, RCMV-infected animals. Subsequently, the presence of IgG antibodies directed against RCMV virion proteins in the rat sera was monitored by using an in-house developed ELISA (see Materials and Methods). Fig. 4 shows that the anti-RCMV IgG titers in sera from sham-operated animals are similar to those reported for normal, RCMV-infected rats in previous studies (75). By contrast, significantly lower antibody titers were found in desalivated rats than in sham-operated animals at days 183, 295 and 365 pi. This finding indicates that the RCMV-infected salivary glands stimulate the production of anti-RCMV antibodies. An explanation for this phenomenon may be that the continuous production of viral proteins and virions in the salivary glands provides a constant trigger, either directly or indirectly, to the immune system. Since this constant trigger is absent

from desalivated rats, these animals may eventually develop a less efficient humoral immune response than normal RCMV-infected rats.

Discussion

The salivary glands are the preferred sites for persistence of CMVs ⁽²²²⁾. The persistent viral shedding via saliva over a long period of time is characteristic for all CMVs and is believed to play a major role in horizontal transmission of these viruses. In order to study the role of the salivary glands during the course of RCMV infection of rats, we employed an experimental animal model in which the pathogenesis of infection was compared between normal rats and rats from which the salivary glands were surgically removed (desalivation). Previous reports demonstrated that desalivation does not result in significant changes in the physiology of rats. Nevertheless, desalivation was found to result in delayed wound healing after surgical infliction of large (3 to 5 mm) circular wounds in the palate ⁽⁵⁸⁻⁶⁰⁾. In addition, desalivation was reported to alter eating patterns and food preference of the animals ^(69, 367, 376, 469). It is important to note, however, that in previous studies with desalivated rats, different methods of desalivation were employed. In some cases, the desalivation procedure only consisted of ligation of the parotid duct, whereas the parotid gland was left *in situ* ⁽⁵⁸⁻⁶⁰⁾. However, in the present study, we relied on radical desalivation in which the submandibular, sublingual and parotid glands were surgically removed. This approach was chosen since RCMV is able to replicate in each of these glands ⁽²⁶⁷⁾. Following desalivation, the course of RCMV infection was monitored both after a lethal and sublethal challenge with RCMV. After a lethal challenge with RCMV, we were unable to observe significant differences between desalivated and normal, sham-operated rats, both in disease progression and mortality induced by the virus. However, after a sublethal challenge with RCMV, significantly higher titers of anti-RCMV IgG were found in sera from sham-operated rats than in those from desalivated rats. These differences in titers appeared at relatively late times after infection (183, 295 and 365 days pi). On the basis of these observations, we suggested that the RCMV-infected salivary glands augment the production of anti-RCMV IgG antibodies. However, the mechanism by which this augmentation is effectuated is currently unknown. It is possible that the anti-RCMV IgG titers are higher in sham-operated rats than in desalivated rats because the immune system of the sham-operated rats is constantly 'boosted' as a consequence of the relatively high level of replication of RCMV in the salivary glands. It was previously demonstrated that RCMV particles are collected in large cytoplasmic vesicles of striated duct cells and that the virions are released into the lumen of the duct via apical cell budding of the vesicles ⁽²⁶⁷⁾. Re-infection may subsequently occur, possibly via small lacerations, of the oral/pharyngeal and/or esophageal mucosa. Shedding of virus or viral

proteins into the circulation, either directly from the salivary glands or from virus-containing saliva, may account for boosting of the humoral immune response.

In general, viral infections are controlled by both the cellular and humoral immune response ^(426, 432). The importance of the humoral immune response in the control of RCMV-induced disease has previously been demonstrated. Treatment of RCMV-infected, immunosuppressed rats with hyperimmune serum provided full protection against virus-induced mortality ⁽⁴⁹⁷⁾. Similarly, anti-HCMV antibodies were found to prevent severe HCMV disease in newborns ⁽¹⁶⁹⁾, AIDS patients ⁽¹³⁾, solid organ transplant recipients ⁽²⁹⁸⁾ and allogeneic bone marrow transplant recipients ⁽⁴⁵³⁾. The protective effect of antibodies was also demonstrated in the murine CMV/mouse model system ^(154, 250, 459). In conjunction with these previous reports, the present study indicates that persistent infection of the salivary glands by RCMV may have at least two major consequences. First, the production of infectious virus by the salivary glands, and subsequent shedding of infectious virus in the saliva, provides an important means for the virus to spread horizontally through the population. Second, the high level production of viral proteins and particles may boost the humoral immune response of the infected host and thereby protect the host against repeated challenges by either endogenous or exogenous RCMV.

In conclusion, the productively RCMV-infected salivary glands contribute significantly to the formation of anti-RCMV IgG antibodies. Viral shedding from this organ or from virus-containing saliva may be responsible for boosting the anti-RCMV antibody response, which may subsequently protect the host from either endogenous or exogenous reinfections. The elucidation of the mechanisms that underly the augmentation of the anti-RCMV humoral immune response will be the challenge of future studies.

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~ Chapter 4 ~

A rat cytomegalovirus strain with a disruption of the r144 MHC class I-like gene is attenuated in the acute phase of infection in neonatal rats.

Summary.

We previously generated an RCMV strain in which the r144 gene, encoding a major histocompatibility complex class I homolog, had been deleted (RCMV Δ r144). To investigate the role of r144 during acute infection of neonatal rats, we infected three days-old neonatal rats with either RCMV Δ r144 or wild type (wt) RCMV and the presence of infectious virus as well as viral DNA in various organs was determined at either 3, 5 or 21 days p.i. In addition, we assessed both type and number of inflammatory cells in these organs. Interestingly, a significantly lower concentration of infectious virus as well as viral DNA was found in spleens of RCMV Δ r144-infected rats than in those of wt RCMV-infected animals at 3 days p.i. At the same time point, a significantly lower amount of infiltrating NK cells and monocytes/macrophages was seen in the spleens of RCMV Δ r144-infected rats than in spleens of rats infected with wt RCMV. At 21 days p.i., RCMV Δ r144-infected rats were found to have lower virus titers in the salivary glands than wt RCMV-infected animals. No significant differences between RCMV Δ r144- and wt RCMV-infected rats were detected at other time points nor at other sites. We conclude that after infection of neonatal rats, the replication of RCMV Δ r144 is severely restricted compared to wt RCMV.

Introduction

Cytomegalovirus (CMV) is an important human pathogen causing severe disease in the immunosuppressed host. AIDS patients, transplant recipients and especially newborns are prone for severe HCMV disease. Viral shedding is common during early childhood and may persist for two years or more ⁽³⁸⁴⁾. Thereafter the virus persists in the host in a latent state ^(64, 203, 212, 271, 272, 466). The ability to enter latency is a characteristic shared by all CMVs. Immune-evasive strategies of HCMV are likely to play an important role in the process of latency and currently several virus-encoded immune-modulators were identified (reviewed by S. Michelson ⁽³⁵³⁾).

One of the strategies of human CMV (HCMV) to evade immune surveillance is the downregulation of cellular MHC class I molecules. This strategy may protect infected cells from MHC class I-restricted cytotoxic T lymphocytes (CTL)-mediated lysis ⁽⁴⁰⁾. HCMV genes that are involved in MHC class I downregulation are US2 ⁽²⁴⁶⁾, US3 ^(9, 247), US6 ^(10, 213) and US11 ⁽⁵⁴⁵⁾. MHC class I down-regulation was also observed in the murine system ⁽⁸³⁾, in which gene products of m152 ⁽⁵⁶⁵⁾, m06 ⁽⁴²⁴⁾ and m04 ⁽²⁶⁴⁾ were shown to affect MHC class I complexes.

Although the downregulation of MHC class I expression may protect the cell from CTL restricted immune surveillance, absence of MHC class I molecules on the cellular membrane renders a cell susceptible for NK cell lysis, termed the 'missing self' hypothesis ⁽³¹⁶⁾. HCMV encodes an MHC class I homolog, named UL18 ⁽³⁹⁾. Similar genes, designated m144 and r144, were found in the genome of murine CMV (MCMV) ⁽⁴¹⁸⁾ and rat CMV (RCMV) ⁽⁵³¹⁾, respectively. It was shown in the murine model, using an MCMV strain carrying a disruption in the m144 gene, that infection with this strain was associated with restricted replication in vivo ⁽¹⁵⁵⁾. Further analysis revealed that NK cells were responsible for this attenuated phenotype ⁽¹⁵⁵⁾. Inhibition of NK cell immune surveillance was also observed in vitro in both the human ⁽⁴²⁸⁾ and murine system ^(116, 282). However, it should be noted that expression of m144 only partially inhibited NK cell-mediated lysis ^(116, 282). Although UL18 gene product protected UL18-transfected cells in vitro from NK cell attack ⁽⁴²⁸⁾, Leong et al. did not find a positive correlation between UL18 and inhibition of NK cell immune surveillance using fibroblast infected with either WT HCMV or UL18 knockout virus ⁽³⁰⁶⁾.

Interestingly, m144 protected m144-transfected lymphoma cells from NK cell-mediated rejection in vivo ⁽¹¹⁶⁾ and expression of m144 was associated with a reduction of leukocyte and NK cell recruitment and NK cell activation ⁽¹¹⁶⁾. These data are in concert with the evidence from the human and rat model, using virus mutants lacking the UL18 and r144 gene, respectively. The gene product of UL18 was found to associate with LIR-1, a member of the Ig receptor superfamily, which is predominantly expressed on monocytes and B lymphocytes and only on a subset of NK cells ⁽¹¹⁴⁾. Additionally, data from the rat

model demonstrated that subcutaneous infection with an RCMV strain carrying a disruption of r144 gene (RCMVΔr144) resulted in a significantly lower number of monocytes/macrophages compared to wild-type (wt) RCMV-infected animals. However, differences in the number of recruited NK cells between wt RCMV- and RCMVΔr144-infected tissue were not detected ⁽⁴³⁾. These data suggest that the MHC class I homolog of RCMV is more likely to interfere with the innate immune response as a whole, rather than solely affect NK cell immune surveillance.

RCMVΔr144 showed a similar phenotype in infected immunosuppressed rats as wt RCMV ⁽⁴³⁾. Since it is thought that the MHC class I homolog may interfere with cells of the innate immune system, it is possible that potential differences between wt RCMV and RCMV-d-144 were concealed by the high level of irradiation-induced immunosuppression. We therefore employed a rat model with a natural, but still immature unaffected immune system. In this model, neonatal rats were infected with either wt RCMV or RCMVΔr144. Rats were sacrificed at either 3, 5 or 21 days p.i. and the amount of infectious virus and the presence of viral DNA was determined in various organs. In addition, the type and number of infiltrating leukocytes was determined by using immunohistochemistry.

Table 1.
The presence of infectious virus in wt-RCMV- or RCMVΔr144-infected neonatal rats

	Spleen		Liver	
	wt RCMV	RCMVΔr144	wt RCMV	RCMVΔr144
3 ^d	170 ± 86 ^a	3 ± 3 *	0 ± 0	3 ± 3
5	30 ± 6	10 ± 7	0 ± 0	7 ± 7
21	0 ± 0	0 ± 0	0 ± 0	0 ± 0

	Lung		Salivary gland	
	wt RCMV	RCMVΔr144	wt RCMV	RCMVΔr144
3 ^d	0 ± 0	3 ± 3	0 ± 0	0 ± 0
5	0 ± 0	3 ± 3	0 ± 0	0 ± 0
21	0 ± 0	0 ± 0	45000 ± 3200	12500 ± 740 *

a Data is expressed as PFU/g tissue and is presented as mean ± SEM.
b Days p.i.
* Significant when compared to wt RCMV (p<0.05).

Materials and methods

Virus

RCMV (Maastricht strain) was derived from salivary gland homogenates from immunosuppressed RCMV-infected rats as described previously ⁽⁷⁸⁾. Prior to injection (see below), the salivary gland homogenate underwent several in vitro passages. RCMV Δ r144 was previously generated in our laboratory ⁽⁴³⁾ and was propagated in similar fashion as wt RCMV. Mock infection consisted of supernatant from uninfected rat embryonal fibroblasts (REF).

Animals

Inbred specific-pathogen-free (SPF) male and female Lewis rats were used at the age of 3 days. The animals were purchased from the Department of Experimental Animal Service at the University of Maastricht, Maastricht, The Netherlands. Animals were nourished by their mother until they reached the age of 21 days. Thereafter, the rats were fed normal rat chow and tap water ad libitum.

Experimental design

Rats were injected intraperitoneally (i.p.) with 200 μ l of REF supernatant, WT-RCMV or RCMV Δ r144. Viruses were administered at a dose of 3×10^4 PFU. Animals (n=5 per group) were subsequently sacrificed at either 3, 5 or 21 days post infection. At the time of sacrifice, spleen, liver, lung, salivary gland, kidney, heart, pancreas, thymus and bone marrow were harvested for the detection of infectious virus and viral DNA by plaque assay and PCR, respectively. The presence of inflammatory cells (NK cells, monocytes/macrophages and T cells) was assessed by immunohistochemistry.

Plaque titration assay

The titer of infectious virus in organs was determined by plaque assay using REF, as was described before ⁽⁷⁸⁾. In short, the solid organs were homogenised and suspended in minimal essential medium (MEM) containing 2% fetal calf serum (FCS). Tenfold dilutions of 10% homogenates (w/v) were inoculated on confluent REF monolayers. Bone marrow was extracted from both femurs and was dissolved in PBS and stored at -70°C. Before incubation on confluent REF, MEM containing 2% FCS was added to the bone marrow homogenate. A minimum of 1×10^6 cells was added to each well. All plaque assays were performed in triplicate. After an incubation period of 7 days, under 0.25% agarose, the number of plaques was determined microscopically after fixation and methylene blue staining.

PCR

Organ samples were minced mechanically and DNA was isolated by using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). During the isolation procedure, organ homogenates were incubated with proteinase K (60 mg / 600 µl) at 60°C for 60-90 minutes. Viral DNA was detected by using a sensitive, single-tube, nested PCR which amplifies a part of the major immediate-early region of the RCMV genome as described previously ⁽⁴³⁾. The PCR mixtures, with a total volume of 50 µl, contained 1 µg of target DNA, 0.05 µM of primers RIE3F and RIE3R2, 1 µM of each primer RIE4F and RIE4R, 100 µM of each dNTP, 1.25 U of HotStar Taq DNA polymerase, HotStar Taq DNA polymerase buffer (Qiagen, Leusden, The Netherlands) and 15 mM MgCl₂. Amplification was performed with a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer, Nieuwerkerk aan de IJssel, The Netherlands). PCR products were visualized by ethidium bromide-staining after gelelectrophoresis. To determine the relative concentration of RCMV DNA, the samples tested in serial 10-fold dilutions of target DNA. In addition, samples were tested with or without spike (10 copies of viral DNA).

Immunohistochemistry

Tissue samples were snap frozen and mounted in Tissue-Tec (Sakuro Finetek Europe BV, Zoeterwoude, The Netherlands). Immunohistochemistry was performed using 4-µm tissue sections. Monoclonals (MoAbs) ED-1, W3-13 and 3.2.3. were used for the detection of monocytes/macrophages ⁽¹³³⁾, T-cells ⁽⁷¹⁾ and NK cells ⁽⁹¹⁾, respectively. Positive cells were visualized using HRP-labeled rabbit anti-mouse immunoglobulins (P260, Dako A/S, Glostrup, Denmark) and diaminobenzidine (DAB) substrate. The sections were counter-stained with hematoxylin and eosin. For the assessment of inflammation, the amount of inflammatory cells was counted in 5 independent high power fields (HPF, 400x) were counted and expressed as mean + SEM.

Statistics

For statistical analysis, the non-parametric Mann Withney U test was used. Differences associated with $p < 0.05$ were considered statistically significant. Data were presented as mean ± SEM.

Figure 1. The relative concentration of viral DNA in organs from RCMV $\Delta r144$ - and wt RCMV-infected rats. The relative DNA concentration was determined in spleen (A), liver (B) and lung (C) at 3 days p.i, using 10 fold serial dilutions of target DNA. Each bar indicates the highest dilution of target DNA that was still found to be PCR-positive. Black bars represent wt-RCMV-infected rats whereas white bars represent RCMV $\Delta r144$ -infected animals. *= significantly different when compared to wt RCMV ($p < 0.05$).

Results

RCMV Δ r144 shows restricted replication in salivary glands and spleen of neonatal rats

It was shown previously, that there are no significant differences in tissue distribution and virus titers between wt RCMV- and RCMV Δ r144-infected immunosuppressed adult rats ⁽⁴³⁾. Since it is thought that the r144 gene might play a role in virus-induced evasion of immune surveillance, it is possible that the high level of irradiation-induced immunosuppression was responsible for those results. In order to study the role of r144 in the pathogenesis of RCMV infection in rats with a normal, unaffected immune system, neonatal rats were challenged with either wt RCMV or RCMV Δ r144 and the presence of infectious virus as well as viral DNA was assessed in various organs at different time points p.i.

As expected, infectious virus could be demonstrated in spleen samples of wt RCMV-infected neonatal animals at 3 and 5 days p.i. (table 1). The amount of infectious virus declined significantly in this organ from 170 ± 86 PFU/g at day 3 to 30 ± 6 PFU/g at day 5 and 0 ± 0 PFU/g at day 21 p.i. Interestingly, at day 3 p.i., the amount of infectious virus in the spleen was significantly lower in RCMV Δ r144-infected animals (3 ± 3 PFU/g) than in wt RCMV-infected rats (170 ± 86 PFU/g). Significant differences in virus titer between both experimental groups were only found in spleens harvested at this time point. Very low virus titers were found in liver and lung of challenged animals at 3, 5 and 21 days p.i. In these organs, there were no significant differences between wt RCMV- and RCMV Δ r144-infected rats in the amount of infectious virus. In accordance with previous studies ^(75, 77, 267), infectious virus could not be detected in the salivary gland at 3 and 5 days p.i.

At day 21 p.i., infectious virus was detected exclusively in the salivary glands of infected rats. Interestingly, the amount of infectious virus was significantly lower in RCMV Δ r144-infected rats ($12,500 \pm 740$ PFU/g) than in wt RCMV-infected animals ($45,000 \pm 3,200$ PFU / g). Infectious virus (wt RCMV and RCMV Δ r144) was not detected in kidney, heart, pancreas, thymus and bone marrow samples at any of the time points measured (data not shown).

To further investigate the viral load in organs of wt RCMV- and RCMV Δ r144-infected rats, the relative concentration of viral DNA was assessed by a nested PCR assay ⁽⁴⁴⁾, which was carried out on serial 10-fold dilutions of the target DNA. As expected, both wt RCMV and RCMV Δ r144 DNA could be detected in internal organs, such as spleen, liver and lung samples of all infected rats at 3 days p.i. However, at this time point, the concentration of viral DNA in the spleen was significantly lower in RCMV Δ r144-infected rats than in wt RCMV-infected animals (fig. 1a). In contrast, differences in viral DNA load between both experimental groups were not observed in liver and lung (fig. 1b and 1c).

Table 2.
Presence of inflammatory cells ^a in organs of wt RCMV- or RCMVΔr144-infected neonatal rats.

Organ	Virus used	Monocytes/macrophages			T lymphocytes		
		3	5	21	3	5	21
Spleen	wt RCMV ^b	90±8	111±10	95±13	94±13	111±7	97±13
	RCMVΔr144 ^b	60±8*	110±9	90±7	93±6	118±17	88±11
	Mock ^c	55±2*	53±1*	56±4*	58±1*	60±1*	57±0*
Liver	wt RCMV	58±3	61±2	34±4	12±4	10±2	7±1
	RCMVΔr144	58±5	60±8	37±7	11±2	11±3	7±2
	Mock	26±6*	21±0*	8±3*	4±2*	2±0*	2±1*
Lung	wt RCMV	28±2	34±1	35±2	25±2	26±3	50±2
	RCMVΔr144	28±5	30±2	36±2	25±1	24±3	55±1
	Mock	11±1*	9±1*	10±3*	11±0*	17±4*	20±2*
Salivary gland	wt RCMV	13±3	15±1	17±2	1±0	2±1	3±1
	RCMVΔr144	13±2	14±2	15±3	1±0	1±1	2±2
	Mock	10±6	4±1*	2±2*	1±0	0±0	0±0*

Organ	Virus used	NK cells		
		3	5	21
Spleen	wt RCMV ^b	17±1	24±3	29±4
	RCMVΔr144 ^b	12±0*	25±2	24±8
	Mock ^c	7±0*	5±0*	11±1*
Liver	wt RCMV	11±4	15±4	1±0
	RCMVΔr144	9±3	12±3	2±0
	Mock	2±1*	1±0*	0±0
Lung	wt RCMV	9±1	14±1	7±1
	RCMVΔr144	8±2	13±1	7±1
	Mock	1±1*	1±1*	2±1*
Salivary gland	wt RCMV	1±0	1±1	4±0
	RCMVΔr144	1±0	1±0	4±1
	Mock	0±0	0±0	0±0*

a The number of inflammatory cells was counted in 5 independent HPF fields. Data are expressed as mean ± SEM.

b Rats were ip infected at a dose of 3 x 10⁴ PFU

c Mock infection consisted of REF supernatant

* significantly different when compared to wt RCMV (p<0.05)

Taken together, these data show that in comparison with wt RCMV-infected rats, RCMV Δ r144-infected animals have a significantly lower viral load in spleen and salivary gland. These organs have previously been shown to be the most important target organs for RCMV replication ⁽⁴⁹⁷⁾.

The number of leukocytes in the spleen corresponds with the viral load in this organ

The RCMV r144-encoded MHC class I homolog may interfere with cells of the innate immune system in order to evade immune surveillance. To explore this possibility, we studied the number monocytes/macrophages, T lymphocytes and NK cells in spleen, liver, lung and salivary gland of wt RCMV- and RCMV Δ r144-infected rats (table 2). Similar to the situation in immunocompromised adult rats ^(399, 497), wt RCMV infection resulted in a significantly higher number of inflammatory cells in spleen, liver, lung and salivary gland than did mock infection (table 2). Interestingly, at 3 days p.i., significantly lower numbers of monocytes/macrophages and NK-cells were found in spleens of RCMV Δ r144-infected rats than in spleens of wt RCMV-infected animals. Thus, the number of monocytes/macrophages and NK-cells corresponds with the viral load in these organs. At 5 days p.i., we did not find significant differences between spleens of wt RCMV- and RCMV Δ r144-infected rats in the number of recruited monocytes/macrophages, T cells and NK-cells. In addition, the number of inflammatory cells present in liver, lung and salivary gland does not differ between both experimental groups at all three time points measured (table 2).

In conclusion, these data indicate that the number of monocytes/macrophages and NK cells present in the spleen of infected neonatal rats is directly proportional to the amount of infectious virus in this organ.

Discussion

Experimental infection of immunocompetent rats with RCMV is predominantly characterised by replication of virus in salivary gland cells ^(75, 77). Dissemination of high levels of virus is more widespread in animals receiving total body-irradiation (TBI) prior to injection of virus and involves spleen, liver, lung and salivary gland ⁽⁴⁹⁷⁾. In addition, RCMV disease can only be observed in lethally infected rats with irradiation-induced immunosuppression ⁽²⁶⁸⁾. The administration of a single dose of 5 Gy TBI as immune suppressive regimen affects the number of granulocytes, NK cells, monocytes, B cells and T (CD4+ and CD8+) cells in peripheral blood of irradiated animals significantly and leads to a transient state of severe immunosuppression ⁽⁵²⁵⁾. Peripheral white blood cells are suppressed during the acute phase of infection and the number of cells return to control levels at approximately 3-4 weeks after irradiation ⁽⁵²⁵⁾.

We previously demonstrated that the r144 gene is not essential for virus replication shortly after infection, nor for long-term infection in immunocompromised rats ⁽⁴³⁾. Since it is thought that the r144-encoded MHC class I homolog may interfere with the function of the innate immune system, the immunosuppressive regimen that we previously used may mask potential differences in replication between wt RCMV and RCMV Δ r144. We therefore employed an alternative model in which neonatal rats were used with a natural, unaffected immune system and infected them with either wt RCMV or RCMV Δ r144. In this model, we found that: (i) the deletion mutant virus, RCMV Δ r144, replicated to significantly lower virus titers than wt RCMV in spleens and salivary glands at 3 days and 21 days p.i., respectively and (ii) the number of monocytes/macrophages and NK cells in the spleen was significantly lower in RCMV Δ r144-infected rats than the wt RCMV-infected animals at 3 days p.i.

RCMV Δ r144 has previously been shown to have similar replication characteristics as wt virus in vitro ⁽⁴³⁾. In addition, significant differences in virus titers between spleens of RCMV Δ r144- and wt RCMV infected immunosuppressed adult rats were not detected ⁽⁴³⁾. These data suggest that RCMV Δ r144 replicates as efficiently as wt RCMV in the absence of a fully functional immune system. In neonatal rats with a normal, unaffected immune system, the viral load in both salivary gland and spleen were significantly lower in RCMV Δ r144- than in wt RCMV-infected rats. This implies that differences in phenotype between both viruses in this model may be governed by the status of the immune system.

As described above, spleens of neonatal rats infected with RCMV Δ r144 harbored significantly lower numbers of monocytes/macrophages and NK cells than those infected with wt RCMV. In contrast, T lymphocytes were present in similar amounts in this organ in both experimental groups. This indicates that the r144 gene product may be involved in the interaction with cells of the innate immune system, rather than with cells of the cell-mediated immune response. Furthermore, we previously demonstrated that subcutaneous infection of the rat hind paw with either wt RCMV or RCMV Δ r144 resulted in a significantly lower number of monocytes/macrophages in subcutaneously infected rat hind paws of RCMV Δ r144-infected rats than in those of wt RCMV-infected animals ⁽⁴³⁾. In this model, the viral load in the rat hind paw was similar between both experimental groups ⁽⁴³⁾. However, differences in the number of NK cells in the rat hind paw between wt RCMV- and RCMV Δ r144-infected animals could not be detected ⁽⁴³⁾. Our data from the neonatal model and rat hind paw model are in agreement with previous findings from the MCMV/murine model ⁽¹¹⁶⁾. It was shown that the MCMV homolog of r144, m144, protects lymphoma cells from NK cell-mediated rejection in vivo by regulation of the total leukocyte- and NK cell recruitment and NK cell activation ⁽¹¹⁶⁾. Since cytokines are important for the recruitment of NK cells and other leukocytes ^(381, 443, 473), it was hypothesized by Cretney et al., that interaction of the murine MHC class I homolog with mononuclear cells may interfere with

the cytokine production of these cells, which eventually diminishes the innate immune response as a whole ⁽¹¹⁶⁾. Similarly, the MHC class I homolog encoded by HCMV, UL18, may be more important in affecting monocyte and dendritic cell function during CMV infection, rather than mediate NK cell immune surveillance ⁽³⁰⁶⁾.

As expected, the salivary gland harbored high amounts of infectious virus at 21 days p.i. Interestingly, the amount of infectious virus in this organ was significantly lower in RCMV Δ r144-infected rats than in wt RCMV-infected animals. In contrast to what was seen in the spleen, virus titers did not correlate with the numbers of infiltrating leukocytes in the salivary glands: similar numbers of these leukocytes were detected in the glands of wt RCMV- and RCMV Δ r144-infected animals. This apparent discrepancy can be explained by the observation that the inflammatory response in the salivary gland is restricted to the interlobular duct region, whereas the virus replicates in the striated duct cells of the intralobular duct ⁽²⁶⁷⁾.

The exact nature of the attenuation of RCMV Δ r144 as opposed to the wt virus in neonatal rats is still unknown. Also, it is not clear whether the attenuated growth of the recombinant virus in salivary glands and spleen is either specific for these organs or the result of a more 'general' inability of the virus to replicate efficiently. These and other questions on the role of r144 in the replication of RCMV will have to be addressed in future studies.

~ Chapter 5 ~

Rat cytomegalovirus infection in kidney allograft recipients is associated with increased expression of ICAM-1, VCAM-1 and their ligands LFA-1 and VLA-4 in the graft.

Summary

Cytomegalovirus (CMV) infection is suggested to be a risk factor for chronic rejection. We have recently shown that rat CMV (RCMV) increases the inflammatory response and accelerates chronic rejection in a model of rat kidney allograft ⁽²⁹³⁾. In this study, the early inflammatory response and time-related expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and their ligands, leukocyte function antigen-1 (LFA-1) and very late antigen-4 (VLA-4), were assessed in kidney allografts of RCMV infected rats and compared to non-infected rats developing chronic rejection. Kidney transplantations were performed in a rat strain combination of DA (RT1^a) to BN (RT1ⁿ) receiving triple drug immunosuppression. One group of rats was infected with RCMV and the other group was left uninfected. The grafts were harvested at different time points after transplantation. The adhesion molecules, their ligands and activation markers, i.e. MHC class II antigen expression and interleukin-2-receptors (IL-2-R), were demonstrated by monoclonal antibodies and immunoperoxidase staining of frozen sections of the grafts. Graft histology was evaluated according to the Banff criteria. RCMV caused a significant, prolonged increase of VCAM-1 and ICAM-1 expression in the vascular endothelium compared to the non-infected grafts. Also, the number of cells expressing activation markers, LFA-1 and VLA-4 was significantly enhanced in these animals. Significantly enhanced histological changes indicative for chronic rejection were seen in the RCMV infected group. In conclusion, increased expression of ICAM-1 and VCAM-1 and increased numbers of inflammatory cells expressing their ligands in the CMV infected grafts, were associated with accelerated chronic allograft nephropathy.

Introduction

The etiology of chronic rejection is multifactorial and several risk factors have been described (14, 38, 209, 390), such as acute rejection episodes, ischemia/reperfusion injury and viral infections, especially cytomegalovirus (CMV) infections. CMV infection has previously been suggested to trigger acute rejection of kidney allografts in clinical transplantation (407, 423, 535). Although CMV has been shown to be associated with chronic rejection of heart (193, 277, 339), lung (281) and liver transplants (20, 292, 379), little is known about CMV and chronic rejection in renal transplantation.

Histological findings of chronic rejection are well defined and are characterised by focal interstitial lymphocytic inflammation and fibrosis, glomerular mesangial matrix increase and sclerosis, vascular intimal proliferation and tubular atrophy (391, 392, 516). These variables can be summarised in the chronic allograft damage index (CADI) which can be used as a predictive parameter for chronic rejection (233, 234).

The inflammatory response and T-cell activation seem to be necessary for the development of chronic rejection (209, 390). Vascular adhesion molecules are important in the early phase of the alloresponse, cell-cell interactions, T-cell activation and extravasation of inflammatory cells into the organ (67, 172, 220, 534). Firm adhesion of leukocytes to the endothelial cells is established by the binding of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) to the integrin molecules expressed on leukocytes, such as lymphocyte function associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4) (324, 405, 494). The induction of adhesion molecules is mediated by cytokines, such as IFN-gamma, TNF-alpha and IL-1, produced during the inflammatory process of the alloresponse.

We have recently developed an experimental model, in which rat renal allografts after an early inflammatory episode, develop chronic rejection under triple drug therapy of steroids, azathioprine and cyclosporin, within 40-60 days after transplantation (483). We have also studied the early phase of the process in detail and recorded that the peak of inflammation, seen 5-10 days after transplantation, is associated with lymphoid activation and induction of vascular adhesion molecules, such as ICAM-1 and VCAM-1. However, the early expression of adhesion molecules vanishes together with immune activation associated with acute rejection, while the changes associated with chronic rejection increase (259).

In this experimental model, we have also used the appropriate rat virus (RCMV) in order to study the processes involved in chronic rejection complicated by CMV infection (294, 483). RCMV increases and prolongs the early inflammatory response up to 20 days and accelerates the development of chronic rejection. In this model, RCMV infects the graft and RCMV specific antigens were found in the endothelial cells of the small vessels as well as in the smooth muscle cells of the large arteries (294). The CMV infected rats

developed an end-stage chronic rejection with graft vasculopathy and remarkable fibrosis within 20 days after transplantation ⁽²⁹⁴⁾. In this study, we have investigated in detail the impact of RCMV on the early phase of the immunological process, at the level of lymphoid activation and induction of vascular adhesion molecules.

Materials and methods

Animals

Male inbred Dark Agouti (DA; RT1^a) and Brown Norway (BN; RT1ⁿ) rats weighing 200-300 g were used in the experiments. The rats were housed according to the international principles of laboratory animal care and were fed normal rat chow and tap water ad libitum. The experiments were approved by the Committee for Experimental Research of Helsinki University Central Hospital and the regional authorities.

Transplantation and immunosuppression

Transplantations were performed from DA to BN as previously described ^(300, 483). In short, the rats were anaesthetised with midazolan (Dormicum^R) and fentanyl-fluanisone (Hypnorm^R). After dissection of the kidney to be transplanted, the organ was flushed with ice cold Euro-Collins solution, containing 500 IU of heparin/ml. The renal artery, renal vein and proximal ureter of the kidney from the donor were anastomosed to the aorta, inferior vena cava and distal ureter of the recipient, respectively. Total ischemia time was 30±10 min. The recipient's native right kidney was removed after implantation of the graft. The left native kidney of the recipient was left intact and used as an internal control. Immunosuppression consisted of methylprednisolone (2 mg/kg), azathioprine (2 mg/kg) and cyclosporin (5 mg/kg), which was administered subcutaneously on a daily basis during the experiment. Autograft transplantations with the same cold and warm ischemia times were performed, also triple drug immunosuppression was given and the autografts were used as control. Rats receiving an autograft were left uninfected.

Viral infection

One group of the allografted animals was infected with RCMV (Maastricht strain). The RCMV inoculum was derived from homogenised salivary glands and was injected 1 day post transplantation, intraperitoneally at a dose of 1x10⁵ plaque-forming units. The characteristics of RCMV infection and the method of viral inoculation, have been described in detail previously ^(75, 77). The infection was confirmed by viral cultures from the grafts on day 7 after inoculation as described ⁽²⁹⁴⁾. The infectivity of rat kidneys and the appearance of viral antigens in the grafts have been described in detail in our previous article ⁽²⁹⁴⁾.

Histology

The grafts were harvested at 3, 5, 7, 10, 20 and 30 days after transplantation and fixed in 10% buffered formalin. Each experimental group contained 4 animals at each time-point. Specimens were paraffin embedded, cut (4 μ m) and stained with haematoxylin-eosin and Masson's trichrome. The Banff criteria ⁽⁴⁸⁰⁾, without grading, were used to assess the graft histology. The numerical chronic allograft damage index (CADI) was used to quantify the chronic alterations characteristic of chronic rejection, as described previously ^(233, 234): focal interstitial lymphocytic inflammation, fibrosis, glomerular sclerosis, mesangial matrix increase, vascular intimal proliferation and tubular necrosis. The graft histology of this model with and without CMV is described in detail in our previous articles ^(294, 483).

Immunohistochemistry

The expression of vascular adhesion molecules and the number of inflammatory cells expressing their ligands and activation markers were immunohistochemically evaluated from frozen sections (3-5 μ m) of the kidneys. After fixation in cold acetone, an indirect immunoperoxidase technique and monoclonal antibodies against ICAM-1 (R&D Systems Europe LTD, Abingdon, UK), VCAM-1 (a gift from Dr. R.Lobb, Biogen, Cambridge, MA), LFA-1 (R&D Systems), VLA-4 (Pharmingen, San Diego, CA), IL-2R (Sera-lab, Sussex, UK), and rat MHC class II (Sera-Lab, Sussex, UK) were used. The frozen sections were first incubated with the monoclonal antibodies. A peroxidase conjugated rabbit-antimouse (Dako, Glostrup, Denmark) and a peroxidase-conjugated goat anti-rabbit (Zymed Laboratories, San Francisco, CA) were used as second and third antibodies. The reaction was revealed by 3-amino-9-ethyl carbazole solution containing hydrogen peroxide. Mayer's hemalum was used for counterstaining. The expression of vascular adhesion molecules in kidney samples was assessed and the intensity of expression was graded from 1 to 3. The numbers of infiltrating inflammatory cells positive for the ligand molecules, IL-2-R and class II were counted separately in different high power visual fields (magnification x 400). Three randomly chosen high power fields were analysed in each kidney. All analyses were carried out blindly by two or three observers.

Statistics

Intensity of adhesion molecule expression (ICAM-1 and VCAM-1 were assessed on different tissue sections) was expressed as median values. The number of cells (each marker was assessed on different tissue sections) which showed positive for LFA-1, VLA-4, IL-2R and MHC class II were expressed as mean $\pm$ SEM. For statistical analysis, the student *t*-test was used and *p*-values < 0.05 were considered to be significant.

Figure 1. Time related follow-up of the intensity of ICAM-1 and VCAM-1 expression, graded from 1 to 3 (median), on endothelia of renal allografts during the development of chronic rejection in RCMV infected and non-infected animals. A) ICAM-1 expression on endothelium of larger vessels. B) ICAM-1 expression on capillary endothelium. C) VCAM-1 expression on endothelium of larger vessels. D) VCAM-1 expression on capillary endothelium. Straight lines = RCMV infected rats; dashed lines = non-infected rats; # = significantly different when compared to controls, $p < 0.05$.

Results

Expression of vascular adhesion molecules and their ligands in the graft in relation to the development of chronic rejection

In the phase shortly after transplantation, at day 7, grafts showed signs of vascular damage and strong interstitial inflammation, which consisted predominantly of lymphocytes. As was already described in our previous study ⁽²⁹⁴⁾, in non-infected grafts, the early inflammation was seen 5-10 days after transplantation and CMV prolonged the inflammation so that it was seen from day 5 onwards, until the grafts ended-up with chronic rejection at day 20 after transplantation. The grafts from non-infected rats showed less intense inflammation and only mild vascular changes at the early post transplantation course. The CMV infected grafts developed chronic rejection within 20 days after

transplantation, according to the histological criteria of chronic rejection. In grafts harvested from RCMV infected animals, characteristic findings of chronic rejection were recorded, as described previously with significantly higher CADI values also than in the non-infected grafts (9.0 ± 0.5 versus 5.8 ± 1.7 , $p < 0.05$). The histological findings of this experiment were described in detail in our previous article⁽²⁹⁴⁾ and are not repeated here.

In the RCMV infected group, expression of ICAM-1 and VCAM-1 of endothelium in the larger vessels, increased to a maximum at 7 days and virtually remained at this level during the follow-up until 30 days post transplantation (Figure 1). In the non-infected grafts, induction of the adhesion molecules was also seen shortly after transplantation, but the expression decreased during the course of the development of chronic changes. The prolonged expression of both ICAM-1 and VCAM-1 was significant ($p < 0.05$) in the vascular endothelium of larger vessels compared with the non-infected grafts (Figure 1). In the capillary endothelium, a significant ($p < 0.05$), prolonged VCAM-1 expression was also observed and ICAM-1 showed the same tendency, although the difference was not statistically significant. Induction of tubular expression of ICAM-1 and VCAM-1 was also recorded with the expression of ICAM-1 (median 2.0) being more intense than that of VCAM-1 (median 0.5), but no statistically significant difference was found between the RCMV infected and the non-infected animals (data not shown).

Expression of LFA-1 and VLA-4, the ligands for ICAM-1 and VCAM-1 respectively, on infiltrating lymphocytes was assessed. LFA-1 and VLA-4 positive cells were abundantly present in non-infected animals at 3-4 days after transplantation and the number of these cells decreased steadily during the development of histological changes indicative for chronic rejection. However, RCMV infection resulted in a significant increase in the number of LFA-1 positive and VLA-4 positive cells present in the allograft when compared to non-infected animals. (Figure 2).

Expression of lymphoid activation markers IL-2R and MHC class II

In the inflammatory infiltrate, the number of IL-2R positive cells in allografts harvested from RCMV infected animals was significantly increased during the early phase of the process, compared to the non-infected rats. Approximately the same pattern of cellular expression was also recorded for MHC class II. (Figure 3).

Figure 2. The time related follow-up of the number of allograft infiltrating inflammatory cells counted per high power visual field (mean $\pm$ SEM) expressing LFA-1 (A) and VLA-4 (B) during the development of chronic rejection with and without concomitant RCMV infection. Straight lines = RCMV infected rats; dashed lines = non-infected rats; # = significantly different when compared to controls, $p < 0.05$.

Controls: Autografts and own kidneys

In autografted kidneys, only a slight temporal expression of ICAM-1 and VCAM-1 was recorded, located in the vascular and capillary endothelium at 5 days after transplantation (not shown). Thereafter the autografts showed a similar staining pattern as control kidneys i.e. the own left native kidney. Autografted kidneys were characterised by an absence of inflammatory cells and the number of cells expressing LFA-1, VLA-4 and IL-2R was similar to that of the normal own kidneys (not shown). During follow up, kidneys did not show any signs of chronic rejection (CADI < 2.0). Minor surgical inflicted injuries were seen in the tubuli and vascular structures immediately after transplantation, at days 3-5, but the only long-term histological finding of the autografts was slight diffuse fibrosis of the kidney in

two animals at day 20 and in one animal at day 30 after transplantation. No significant vascular, tubular or glomerular changes were recorded at days 20 and 30.

The own kidneys of the CMV infected rats showed the same expression pattern as the own control kidneys of the non-infected animals at each time point up to 30 days post transplantation. No induction of adhesion molecules was recorded and no inflammatory cell infiltration with the expression of their ligands were seen in the own kidneys of CMV-infected or non-infected animals. No histological changes were recorded in the control own kidneys of both animal groups (CADI=0).

Discussion

CMV infection increased the inflammatory response in our rat renal allograft model and accelerated the development of chronic rejection, as was reported in our previous series (294). As was also described previously, in non-infected grafts there was an early inflammatory response between 5-10 days after transplantation, but in the CMV infected grafts an increased inflammation was seen (294). CMV also prolonged the inflammation so that it was still prominent at day 20 after transplantation, when the histological criteria of chronic rejection were fulfilled (294). Enhanced chronic rejection with vascular changes and prominent fibrosis and also with high CADI values, on day 20 in the CMV infected animals, corresponded with the findings of non-infected grafts in our chronic rejection model of days 40-60 post transplantation (259, 294).

In the present study, we have further demonstrated that CMV infection also resulted in a significant and prolonged increase of VCAM-1 and ICAM-1 expression on the vascular endothelium in the renal allografts. The difference between the groups was clear from day 5 onward, but the statistical significance was reached later together with the histological criteria of chronic rejection. In addition, the number of infiltrating cells expressing the ligand molecules LFA-1 and VLA-4, as well as activation markers, was significantly increased in these animals corresponding to the prolonged inflammatory response. Autografted kidneys harvested from non-infected rats were used as controls. Autografts did not show a significant induction of adhesion molecules and no inflammatory response was seen either. In histology, only slight fibrosis was recorded at the end of the follow-up. The native own kidneys of CMV-infected and non-infected animals demonstrated the same expression pattern and no inflammatory infiltrate or histological changes were recorded.

The induction of these adhesion molecules is mediated by cytokines, such as IL-1, TNF-alpha and IFN-gamma, which are produced by activated lymphocytes during the alloresponse. The role of adhesion molecules and cytokines in the induction of the process of chronic rejection has been shown in rat renal allograft models (205, 259).

Figure 3. Time related follow-up on the number of allograft infiltrating inflammatory cells counted per high power visual field (mean $\pm$ SEM) expressing activation markers MHC class II (A) and IL-2R (B) molecules during the development of chronic rejection in RCMV infected and non-infected rats. Straight lines = RCMV infected rats; dashed lines = non-infected rats; # = significantly different when compared to controls, $p < 0.05$.

On the other hand, it has been shown *in vitro* that T lymphocytes which are activated by CMV infected endothelial cells, may trigger cytokine mediated expression of adhesion molecules on non-infected endothelial cells ⁽⁵⁴¹⁾. In addition, it has been demonstrated that one week after inoculation, RCMV is predominantly found in capillary endothelium of allografted kidneys, also in the smooth muscle cells of the arterial wall, but not in the endothelium of larger arteries ⁽²⁹⁴⁾. So this might also explain why the difference in ICAM-1 and VCAM-1 expression between RCMV infected rats and non-infected animals was more noticeable on larger vessels than on capillary endothelium in our assessment. CMV has also been found to upregulate IL-1-beta gene expression, which may lead to production of IL-1 by mononuclear cells ⁽²³⁵⁾. CMV induces the production of TNF-alpha in monocytes and macrophages as well ⁽⁴⁷²⁾. The same kind of two-way activation also regulates the

CMV-TNF-alpha co-reactivity. However, the CMV induced T-cell activation, production of cytokines and the increase of adhesion molecule expression may thus be involved in the alloresponse and trigger the process of chronic rejection. In the own native kidneys, no changes were demonstrated, which shows the importance of the immunological triggering in the graft. Thus, the alloresponse seems to be necessary for the CMV associated changes, as was already recorded in our previous study on rat aorta allografts ⁽³⁰⁴⁾.

The involvement of inflammation, adhesion molecules and cytokines has been studied *in vitro* and *in vivo* in animal models. In experimental models of lung ⁽⁵⁰⁶⁾ and liver ⁽³²⁹⁾ allografts, CMV infection has been reported to induce lymphocyte dominated inflammation, T-cell activation and adhesion molecules in the graft. In another experimental model of renal allografts, in which the animals underwent acute rejection episodes and received cyclosporin only as immunosuppressive treatment, increased ICAM-1 expression was recorded in the graft at the end-stage of the process in the CMV infected animals ⁽⁵⁵⁷⁾. Our sequential follow-up of the development of chronic rejection demonstrated that the involvement of CMV infection occurs already at the induction phase of the immunological cascade in the graft, at the level of T-cell activation and lymphocyte extravasation. CMV associated peripheral T cell activation in long term renal transplant patients has been described ⁽²⁶²⁾. However, these immunological events should also be studied in the clinical series of human kidney allografts complicated with CMV infection. Although the role of adhesion molecules in chronic rejection has been studied in human renal allografts ^(184, 481, 533), no clinical report exists describing the impact of CMV on these findings.

In conclusion, prolonged, increased expression of adhesion molecules ICAM-1 and VCAM-1 on the vascular endothelium and increased numbers of cells positive for their ligand molecules LFA-1 and VLA-4 in kidney allografts of CMV infected rats, was linked with the acceleration of chronic nephropathy in this model. The CMV associated, prolonged inflammation correlated with the increased number of infiltrating cells expressing activation markers. These findings indicate that the prolonged activation of the immunological cascade in the CMV infected grafts was associated with accelerated chronic nephropathy.

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~ Chapter 6 ~

**Effective treatment of experimental
CMV-induced encephalo-meningitis in
immunocompromised rats with
HPMPC.**

Summary

Cytomegalovirus (CMV) -induced encephalomeningitis is a dramatic complication in patients with the acquired immunodeficiency syndrome (AIDS) and treatment of this infection remains a major clinical problem. In order to study the pathogenesis and treatment of CMV-induced encephalomeningitis, we experimentally induced intracranial rat-CMV (RCMV) infection in rats that were immunosuppressed by total body X-irradiation. CMV infection was monitored by viral plaque assay for estimation of the viral load. CMV-induced pathology, the presence of CMV-infected cells, as well as the presence of T-lymphocytes and monocytes/macrophages were studied by histopathologic and immunohistochemical staining techniques. The meninges showed CMV infection in mononuclear infiltrating cells and in endothelium of small blood vessels eight days after intracerebral inoculation. This was accompanied by multiple haemorrhages and inflammatory cell infiltration. The infection and inflammatory response persisted for at least 21 days p.i. Animals were treated with (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC), 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG), hyperimmune serum (HIS) and both DHPG and HIS combined. Treatment with one dosage of HPMPC at 20 mg/kg effectively reduced virus titers. However, all other treatment modalities were not effective. In conclusion, the pathology of RCMV-induced encephalomeningitis in immunocompromised rats closely resembles that of AIDS patients. The infection is effectively treated with HPMPC.

Introduction

Cytomegalovirus (CMV) infections are a major problem in immunocompromised patients especially in transplant recipients ⁽³¹⁴⁾ and in patients with the acquired immunodeficiency syndrome (AIDS). The prevalence of CMV infections in individuals infected with the human immunodeficiency virus (HIV) is high. ^(109, 136, 416). In AIDS patients CMV infections are the most frequent opportunistic infections during the late phase of disease ^(342, 442, 547). Those infections are associated with symptoms of fever, pneumonitis, hepatitis and retinitis ⁽³⁴⁸⁾. A significant percentage of these patients ^(254, 474) experience neurological complications, such as encephalitis, meningo-encephalitis and radiculomyelitis ^(21, 338, 452, 474). Although it has been shown that neural tissue obtained from these patients contains CMV antigens or nucleic acids, the role of the virus in the pathogenesis of meningo-encephalitis is not fully understood ^(160, 370).

For treatment of CMV infections in these patients commonly used antiviral drugs are 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) and phosphonoformate (PFA) ^(144, 198). The use of both compounds is limited by serious side effects such as neutropenia and kidney failure. The effect of alternative treatment modalities like hyperimmune serum (HIS) in AIDS patients is not known.

More recently, it has been described that (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine (HPMPC)^(125, 126) is a very potent inhibitor of herpes viruses ⁽⁵⁵⁶⁾. The advantage of HPMPC over DHPG is the very high selectivity and antiviral activity against human CMV. Other advantages are that HPMPC is highly specific and active against rat and mouse CMV ^(373, 498, 499). The intracellular half-life of the active metabolite is long and in animal studies the effect of HPMPC is long lasting^(373, 498). In immunocompromised rats HPMPC completely inhibited viral replication in all internal organs and effectively reduced the mortality from RCMV infection.

In this report we describe CMV infection in rats at day 8 and day 21 p.i. after intracerebral infection of rat CMV (RCMV). We compared the therapeutic effect of HPMPC on this infection to the effects of DHPG, HIS and both HIS and DHPG combined.

Material and methods

Animals

Inbred specified pathogen free (SPF) male Brown Norway (BN) rats of 8 weeks old were bred at the department of experimental animal services at the University of Maastricht, The Netherlands. Animals were used for the experiment at a total body weight between 140 and 180 g.

The rats were immunosuppressed one day before infection by 5 Gray total body irradiation (TBI) as described previously ⁽⁴⁹⁷⁾.

Table 1.
Experimental groups and treatment regimens used.

Treatment regimen of rats sacrificed at day 8 p.i.	n ^c	Treatment regimens of rats sacrificed at day 21 p.i.	n ^c
HPMPC (10 mg/kg) ^a	5	-	-
HPMPC (20mg/kg) ^a	4	-	-
HPMPC (40 mg/kg) ^a	5	HPMPC (40 mg/kg) ^a	5
DHPG (40 mg/kg) ^b	5	DHPG (40 mg/kg) ^b	3
HIS (NT 640) ^a	5	HIS (NT 640) ^a	5
DHPG (40 mg/kg) ^b	5	DHPG (40 mg/kg) ^b	5
+ HIS (NT 640) ^a		+ HIS (NT 640) ^a	
Infected, but not treated	5	Infected, but not treated	4
Not infected	5	-	-

a treatment administered at day 0

b treatment administered daily from day 0 to day 5

c n=number of rats per group

RCMV infection

The rat cytomegalovirus (RCMV) Maastricht strain, was obtained from a pool of salivary glands of infected laboratory rats (75, 78). The virus pool used underwent 7 passages in laboratory rats. The salivary glands were homogenised and the supernatant was collected after centrifugation and stored at -70°C. Each rat received 10⁵ plaque forming units (PFU) of RCMV in a volume of 20 µl by intracerebral injection. Controls received 20 µl of a noninfected salivary gland supernatant.

Antiviral treatment

The antiviral agents ganciclovir, 9-(1,3-dihydroxy-2-propoxymethyl)guanine was purchased from Syntex (Palo Alto, CA, USA). (S)-1-(3-hydroxy-2-phosphonyl-methoxypropyl)cytosine (HPMPC) (Gilead Sciences, Foster City, CA, USA) was a gift from Dr E. De Clercq, Leuven, Belgium. Hyperimmune serum (HIS) was prepared in rats as described by Stals et al. 1990. In this study HIS was used at a neutralization titer of 640 and was given intravenously (i.v.). The antiviral compounds (HPMPC and DHPG) were administered by intraperitoneal (i.p.) injection. All treatments were started one day after RCMV infection. The treatment regimens used for each group are outlined in Table 1.

Survival, specimen collection and detection

All rats were examined daily for the presence of disease, as described by Stals et al.⁽⁴⁹⁷⁾ and survival was recorded. To study the effect of treatment on the course CMV infection, brain tissue was obtained at two time points after infection (day 8 and 21 p.i.). To study the effect of treatment on the spread of virus within the body, the spleen was also sampled at these two time points. At that time the presence of infectious virus and viral antigens was measured. In addition, brain tissue was analysed for histopathological changes. The number of infectious virus particles determined in organs was counted by plaque assay using rat embryonic fibroblasts, as described previously⁽⁷⁸⁾. The presence of viral antigens was evaluated by immunohistochemistry. For this purpose organs were fixed in paraformaldehyde-lysine-periodate and embedded in parafin. For detection of RCMV antigens mouse monoclonals (McAb) were used, directed against cytoplasmic (McAb 35) and nuclear (McAb 8) antigens⁽⁷⁹⁾. The infection load was graded by two independent observers based on the number of RCMV immunoreactive cells in the sections. This number was expressed as point score units (PSU), as described previously⁽³¹⁰⁾.

Table 2.
Effect of antiviral treatment on the RCMV infection in brain and spleen at day 8 post infection.

Treatment of rats	n ^a	Virus titer	
		Brain ^b	Spleen ^b
HPMPC (10 mg /kg)	5	1.19 (2.67) ^c	1.31 (1.80) ^c
HPMPC(20 mg/kg)	5	0.72 (1.44) ^c	0.00 (0.00) ^c
HPMPC (40 mg/kg)	5	1.36 (1.87) ^c	0.00 (0.00) ^c
DHPG (40 mg/kg)	5	6.58 (0.22)	4.64 (0.50) ^c
HIS (NT 640)	5	6.79 (0.18)	4.73 (0.71) ^c
DHPG (40 mg/kg) +HIS (NT640)	5	6.22 (0.18)	2.10 (1.95) ^c
none (control)	5	6.54 (0.47)	6.05 (0.41)

- a n = number of animals per group
- b Virus titer in brain and spleen at day 8 p.i. was measured and is expressed as ¹⁰log PFU/g organ tissue (mean (standard deviation))
- c Significant difference (p < 0.05) compared to the nontreated (control) group

The quantity of CMV reactive cells scored from 0 to 5 (0: no staining; 1: sporadic cells; 2: few cells; 3: moderately dense; 4: dense; 5: very dense). Parafin embedded sections of brain tissue (4 μ m) were analysed for the presence of inflammatory cells by the immunoperoxidase technique. Monoclonal antibody W3/13 (sera-lab, Crawly Brown, U.K.) were used for the detection of T-lymphocytes and monoclonal antibody ED-1 (kindly supplied by Dr. C. Dijkstra, University of Amsterdam, The Netherlands) for detection of monocytes and macrophages were used. In addition, routine haematoxylin and eosine staining was carried out.

Statistical analysis

The score of positive cells in different groups is expressed as median (range) and is compared with the aid of the two tailed non-parametric Mann-Whitney U test. For comparison of the number of infectious virus particles measured by plaque assay the non parametric Mann-Whitney U test was also used. P values < 0.05 were regarded as statistically significant.

Table 3.
Effect of antiviral treatment on the RCMV infection in brain and spleen at day 21 post infection.

Treatment of rats	(n) ^a	Virus titer	
		Brain ^b	Spleen ^b
HPMPC (40 mg/kg)	5	1.26 (1.76) ^c	0.00 (0.00) ^c
DHPG (40mg/kg)	6	6.31 (0.43)	2.79 (2.41)
HIS (NT 640)	5	6.37 (0.52)	3.80 (0.74)
DHPG+HIS	5	5.84 (0.61)	3.72 (0.30)
none (control)	5	5.61 (1.45)	4.79 (1.25)

a n = number rats per group
b Virus titer in brain and spleen harvested at day 21 p.i. is expressed as ¹⁰log PFU/g organ tissue (mean (standard deviation))
c Significant difference (p<0.05) compared to the nontreated (control) group

Table 4.
Effect of antivirals on the presence of RCMV-infected cells within the meninges and in blood vessels within the meninges.

Treatment	Virus infected cells ^a in			
	Meninges		Blood vessels within the meninges	
	8 p.i.	21 p.i.	8 p.i.	21 p.i.
HPMPC ^b	0.2 (0.2) ^d	0.0 (0.0) ^d	0.0 (0.0) ^d	0.0 (0.0) ^a
DHPG ^b	3.0 (0.3)	1.6 (0.2)	1.4 (0.2)	0.6 (0.1)
HIS ^c	1.2 (0.3) ^d	1.4 (0.2)	0.8 (0.1) ^d	0.8 (0.0)
HIS ^c + DHPG ^b	2.8 (0.2) ^d	2.4 (0.3)	1.6 (0.3)	2.8 (0.4)
None (control)	3.7 (0.3)	1.7 (0.4)	2.2 (0.3)	1.2 (0.1)

a Virus infected cells expressed as median (range)

b Drug administered at a dose of 40 mg/kg

c 1 ml of HIS (NT 640)

d Significant difference $p < 0.05$ compared to infected not treated (control) rats

Results

Effect of treatment on virus titers at day 8 p.i.

At 8 days after intracerebral inoculation of 10^5 PFU RCMV, the brains harbored high numbers of infectious virus. In these animals generalised infection was observed by the presence of high numbers of infectious virus in the spleen (Table 2). Treatment with HPMPC at a dosage of 20 and 40 mg/kg significantly reduced the virus titers in both brain tissue and spleen. HPMPC treatment at 10 mg/kg reduced the virus titer in both organs, but not completely. The other treatment regimes (DHPG, HIS and both, DHPG and HIS combined) all failed to reduce the number of infectious CMV in the brains significantly. In contrast, these compound had a moderate effect on the number of infectious virus particles in the spleen.

Effect of treatment on the persistence of the virus in brain and spleen at day 21 p.i.

Intracerebral injection of RCMV resulted in persistence of infectious virus for at least a period of 3 weeks (Table 3). At that time CMV was also present in the spleen, indicating general infection during this period of disease. No significant difference was found in the infectious virus load between day 8 and day 21, which indicates continuous viral replication

in these immunosuppressed animals for a period of at least 3 weeks.

As described for day 8 p.i., HPMPC treatment at 40 mg/kg significantly decreased the number of RCMV in the brain. In addition, at that dosage HPMPC completely inhibited RCMV infection in the spleen, indicating that the drug prevented further spread of the infection through the body. The other treatment modalities (DHPG, HIS and both combined) did not significantly reduce virus titers in the brain and the spleen.

Pathology of the brain and meninges and the effect of antiviral therapy

CMV in meninges and brain tissue was localised immunohistochemically using RCMV-specific monoclonal antibodies. As presented in Table 4, the meninges of untreated rats harbored high numbers of virus-infected cells day 8 p.i. (cell score = 3.7 (0.3) median (range) at day 8 p.i.). At 21 days p.i. the number of virus-infected cells declined to a score of 2 (1) median (range). In the brain tissue only few virus-infected endothelial cells and monocytes/macrophages were observed (median PSU (range) 1 (0) at day 8 and 1 (1) at day 21).

In the meninges, and to a significant lesser extent in the brain, scattered hemorrhages with foci of necrosis were present. CMV antigen was often detectable within these necrotic regions, as shown immunohistochemically. In the blood vessels numerous giant endothelial cells were noticed (data not shown). The enlarged cells contained basophilic intranuclear and intracytoplasmic inclusions which are typical for an active CMV infection. Immunohistochemical staining proved the presence of RCMV antigens in these cells.

While in non-infected rats virtually no inflammatory cells were noticed, in the CMV-infected animals numerous inflammatory cells infiltrated the meninges and brain. These infiltrates predominantly consisted of monocytes/macrophages and to a lesser extent T-lymphocytes. Nearly half of the macrophages present in the meninges and brain was reactive with monoclonals 8 and 35, indicating that these cells contained viral antigens. Infiltrates were extensively present at day 8 p.i. Thereafter, the inflammatory response declined. In addition, endothelial cells, mainly those present in the small vessels, reacted with McAb 8 and 35.

The effect of treatment with HPMPC, DHPG, HIS and both, DHPG and HIS combined, on the quantity of virus-infected cells in brain and meninges was evaluated. As presented in Table 4, HPMPC at a dosage of 40 mg/kg significantly reduced the number of virus-infected cells in the meninges. In the blood vessels of these organs the effect was even more pronounced. In contrast, treatment with DHPG, HIS and both, DHPG and HIS combined, had hardly any effect on the number of CMV-infected cells. In the brains of untreated animals the number of virus-infected cells was low. Therefore, evaluation of the effect of antivirals was not performed. None of the four antiviral regimes had any effect on the inflammatory response in the meninges and in the brain of RCMV-infected rats. This

was true for both measure points: day 8 and 21 p.i.

Survival

All animals submitted to this study survived at least during a period of 21 days. Only the HIS treated group and the control group became ill, but they all survived.

Discussion

This study describes the pathologic features of CMV infection in the brains and meninges of immunosuppressed rats after intracerebral inoculation of RCMV. In addition, it describes the effect of administration of antiviral treatment modalities, HPMP, DHPG, HIS and both, DHPG and HIS combined, on the course of infection. The rat model described mimics the pathology of CMV-induced meningo-encephalitis in AIDS patients. Histochemical evaluation of brain and meninges from RCMV-infected rats revealed acute infection accompanied with signs of inflammation. Particularly, in the meninges high amounts of virus-infected cells were present. The virus-infected cells were localized mainly in and around the blood vessels and consisted predominantly of monocytes/ macrophages and endothelial cells. The presence of CMV-infected cells present in blood vessels of infected organs is also described in AIDS-patients ⁽²⁰⁸⁾. CMV infection in and around blood vessels is accompanied by a local inflammatory response. Earlier, it has been described that after inoculation of RCMV in the foot-pad of rats polymorphonuclear granulocytes infiltrated the perivascular region during the first days post infection, followed by infiltration of CMV-infected monocytes/macrophages. The inflammatory response reached the highest level at about one week after infection ⁽³⁹⁹⁾. Another interesting observation is the detection of viral antigens in the endothelium of meninges and brain during the acute phase of infection. The presence of CMV in the endothelium of the brain is also described in postmortem specimens of AIDS patients ⁽³⁶⁰⁾. It suggests that endothelium could act as a portal for entry of the virus into the central nervous system. An important role of endothelial cells in the pathogenesis of CMV infection is suggested by ⁽¹⁹⁵⁾, who indicates that infected endothelial cells are detectable in systemically infected immunocompromised patients. Predominantly, endothelial cells of small vessels become infected by CMV, while the endothelium of large vessels is exceptionally infected ⁽⁴⁸⁶⁾. Recent data of Vossen et al. ⁽⁵³⁶⁾ indicate that endothelial cells consist of a very heterogenous population that differently interact with CMV. Infection of these cells seems to be relevant for the pathogenesis. The finding of CMV specific antigens and nuclear inclusions of cells in the brain and meninges indicates local replication and is related with brain damage.

Treatment of RCMV-infected rats with DHPG, HIS or both DHPG and HIS combined had hardly any effect on the virus titers in spleen and brain of the animals. This finding is in contrast with the synergistic effect of DHPG and HIS during generalised infection ^(440, 499).

In patients with severe CMV disease combined treatment with DHPG and immunoserum appears to be beneficial ^(180, 415). One explanation for therapeutic failure in this model could be poor penetration of the antivirals into the brain area, which permits initial replication cycles of the virus. It should also be mentioned that antibody does not cross the blood brain barrier unless there is inflammation. Since there is nearly no inflammatory response in the brains this explains the fact that HIS alone or in combination with DHPG does not lead to a reduction in virus titer.

HPMPC treatment significantly reduced virus titers in brain and spleen. This is in agreement with earlier reports about the highly effective and specific effect of the drug ^(372, 499). We found, using immunohistochemical methods, that CMV antigens were localised in endothelial cells and monocytes/macrophages of the brains and the meninges from rats. In contrast with the lack of antiviral effect of HIS and both HIS and DHPG combined, the number of virus-infected cells was reduced in the meninges at day 8 p.i. by these treatment regimens. Whether this effect was a result of a decreased number of infected cells, or a decreased sensitivity of the immunohistochemical detection technique by interference of the HIS, remains unknown.

High dosages of HPMPC reduced effectively and long lasting the virus titers as well as the number of virus-infected cells in both brain and spleen, while a lower dosage (10 mg/kg) of the drug showed a less pronounced effect. It should also be mentioned that intracerebral infection may injure the blood brain barrier and that this may possibly alter the penetration of the compound(s) in the brain. Yang et al. ⁽⁵⁵⁶⁾ demonstrated however that HPMPC is still able to reduce titers of HVS-2 in brains of mice that were injected intraperitoneally and in which treatment was only started at the time the virus had spread to the brains. Neyts et al. ⁽³⁷¹⁾ demonstrated that HPMPC efficiently prevents MCMV-associated morbidity and mortality upon intracerebral infection. However, following long-term but infrequent treatment (once a week) with HPMPC in SCID mice that had been infected intraperitoneally with MCMV, virus replication was found in the brain. In contrast, little virus replication was found in other organs at that time. Thus brain infections may be more refractory to systemic treatment with HPMPC than visceral infections. This may suggest that perhaps higher and more frequent dosages of HPMPC are needed to treat brain infections with HPMPC than those doses used in the present study.

In conclusion, we showed that intracerebral RCMV infection in immunocompromised rats closely resembles severe human CMV infection in AIDS patients. It displays pathology with pronounced local infection of mononuclear infiltrates and endothelial cells of the small vessels. The treatment model seems suitable to study the effect of candidate antiviral drugs on CMV infection of meninges and brain in the severe immunocompromised host. HPMPC significantly reduces the viral load in brain, meninges and spleen. Therefore, we recommend to study the effect of HPMPC on CMV-induced encephalitis in AIDS patients.

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Summary

Cytomegalovirus (CMV) infection is a major problem in the immunosuppressed patient. It is thought that besides direct CMV induced cell lysis, immunological damage is part of CMV pathogenesis. New antiviral drugs, which combine immunomodulating and antiviral qualities, could be beneficial. Recently, it has been described that desferioxamine (DFO) and calcium trisodium diethylenetriaminepentaacetic acid (DTPA) exhibit both properties. In this report the antiviral effects of both compounds against rat CMV (RCMV) infection are described *in vitro* and *in vivo* using a generalised and local infection model. *In vitro*, both compounds inhibited viral replication significantly, with DTPA being more potent than DFO. However, in the generalised infection model no effect was seen on mortality, morbidity or presence of virus in internal organs. In rats infected subcutaneously in the hind paw, no effect was seen locally on paw thickness, presence of viral antigens and inflammatory response. In addition, these rats suffered from a generalised infection of low magnitude at 15 days post infection. In this case both DFO and DTPA were able to lower the level of viral replication. In conclusion, our data indicate that despite previous *in vitro* reports, *in vivo* usage of DFO or DTPA as monotherapy for an acute CMV infection is questionable. The use of both compounds in combination with either ganciclovir or foscarnet should be tested in further studies.

Introduction

Cytomegalovirus (CMV) infection is common in the general population. Clinical symptoms are observed in immunosuppressed patients. Since the onset of the Human Immunodeficiency Virus (HIV) epidemic, the number of patients suffering from a disseminated cytomegalovirus (CMV) disease has increased markedly. Currently, this progression has been halted by the use of Highly Active Antiretroviral Therapy (HAART) but nevertheless, it is still a major problem in medical health care. The brain, retina and the gastro-intestinal tract are frequently involved during CMV infection in these patients ^(21, 132, 400). Infection may impair the patient's homeostasis drastically and may eventually evolve into a life threatening event. Also, in transplant (TX) patients and especially in heart TX recipients CMV infection is a major cause of morbidity and mortality ⁽¹⁹³⁾. Similar outcomes are found in liver and kidney TX recipients ^(294, 379, 407, 423) and bone marrow TX (BMTX) patients ⁽¹⁴⁴⁾.

Attempting to control the CMV induced pathology several treatment regimens have been developed. Nowadays, mostly 9-(1,3-dihydroxy-2-propoxymethyl)guanine (ganciclovir) or trisodium phosphonoformic acid (foscarnet) are the treatment modalities of choice for combating CMV disease. In BMTX patients, ganciclovir is able to prevent disseminated CMV disease when the patient is treated prophylactically ⁽¹⁸⁸⁾. Both compounds have been shown to drastically improve CMV retinitis in immunocompromised patients although relapses are common when treatment is stopped ^(25, 158, 462, 542). In 1986, De Clercq et al. ⁽¹²⁵⁾ described a nucleotide analogue (s)-1-(3-hydroxy-2-phosphononylmethoxypropyl)cytosine (HPMPC, cidofovir) which is active against various DNA viruses, including CMV. Cidofovir has been tested in animal models ^(371, 498, 500) and is now available for treating patients ⁽²⁹⁹⁾.

Despite the strong antiviral capacities of these compounds, usage is limited due to unwanted side effects. One of the major side effects of ganciclovir is neutropenia especially when ganciclovir is given to patients receiving other drugs such as trimethoprim-sulfamethoxazole ⁽¹⁹⁰⁾ or azidothymidine (AZT) ⁽⁴³⁰⁾. Anemia, proteinuria and an elevated serum creatinin level indicating nephrotoxicity are adverse effects of foscarnet and cidofovir treatment. Besides this, foscarnet should be administered intravenously hereby limiting its clinical use ⁽⁵⁴²⁾.

Another complicating factor is that some strains of CMV are less sensitive or even resistant to ganciclovir ^(135, 146, 181) and to a lesser extent to foscarnet ^(238, 307). Alarming are the reports of Sarasini et al. ⁽⁴⁴⁸⁾ and Baldanti et al. ⁽²⁹⁾ who described multiple CMV strains resistant to both ganciclovir and foscarnet. In addition, also a cidofovir resistant strain has been described *in vitro* ⁽²⁹⁹⁾ and *in vivo* ⁽²⁰⁶⁾.

Recently, progress has been made in the search for new antiviral drugs. Cinatl et al. described two compounds desferioxamine (DFO) and calcium trisodium

diethylenetriaminepentaacetic acid (DTPA), which were able to inhibit human CMV replication *in vitro* ^(103, 105). Both compounds are metal chelators and clinically DFO is already used in case of an iron overload ⁽⁴⁰⁴⁾. Besides data of their antiviral capacities *in vitro* also an *in vivo* effect is described in an AIDS patient suffering from a progressive CMV retinitis. This patient did not respond to combination therapy consisting of ganciclovir and foscarnet but when DFO was added to the treatment regimen, the progression was halted ⁽²⁰¹⁾.

Although the cited references suggest an antiviral effect of DFO and DTPA on human CMV replication *in vitro*, hardly any data are available concerning the antiviral properties of both compounds on *in vivo* replicating virus. For this purpose we used a rat model and the appropriate virus (rat CMV, RCMV) and studied the effects of DFO and DTPA on viral dissemination after systemic or local inoculation of virus.

Materials and methods

Animals and immunosuppression

Specific pathogen-free (SPF) Lewis rats were used in the *in vivo* experiments. The animals were bred at the Department of Experimental Animal service at the University of Maastricht, the Netherlands. Rats were kept under normal housing conditions and were fed normal rat chow ad libitum. Depending on the experiment, animals were used at an age of 4 (60-90g), 6 (120-140) or 8 (180-200g) weeks. In order to enhance the effect of the infection rats received a total body irradiation (TBI) of 5 Gy 8 hours before infection as discussed by Stals et al. ⁽⁴⁹⁷⁾.

Virus, infection and treatment

Rats infected intraperitoneally (i.p.) were given 3×10^5 plaque forming units (PFU) of RCMV. The RCMV inoculum was derived from a pool of salivary glands from acutely infected rats as described previously ⁽⁷⁵⁾. Local infection was established by injecting rats subcutaneously (s.c.) in the right hind paw with 1×10^5 PFU (dissolved in 200 μ L) of RCMV, which had undergone several *in vitro* passages. The left hind paw was injected with supernatant of uninfected fibroblasts (200 μ L) and served as an internal control.

Calcium trisodium diethylenetriaminepentaacetic acid (DTPA) purchased from Heyl, Berlin, Germany and desferioxamine (DFO) purchased from Ciba-Geigy, Wehr, Germany were tested for their antiviral effect. For *in vivo* experiments both compounds were dissolved in distilled water and were given i.p. in a dose of 100 mg/kg dissolved in 0.2 ml twice a day. Control (non-infected) rats received sterile Phosphate Buffered Saline (PBS) twice a day.

Experimental design

For the *in vivo* experiments two models depending on the mode of inoculation are used. Intraperitoneal inoculation will lead to a fast dissemination of virus in the body as described earlier ⁽⁴⁹⁷⁾. A subcutaneous infection in the hind paw, however, will concentrate the viral load to the hind paw with slow release of virus to the systemic circulation ^(77, 399). In the i.p. infected rats a lethal and a sub-lethal infection was established by infecting 4 and 8 weeks old rats with 3×10^5 PFU of RCMV, respectively.

All rats were weighed and checked for CMV induced illness daily and survival was recorded. The sub-lethally infected rats were sacrificed at 4 and 21 days post infection (p.i.). Effect of treatment was determined in the sub-lethal experiment by measuring viral load by plaque assay and viral DNA by polymerase chain reaction (PCR) in important target organs such as salivary gland, spleen and liver. In the second model a local infection was established in the rat hind paw using rats of 6 weeks old ⁽³⁹⁹⁾. The paw thickness of the right and left hind paw was measured at a fixed point on both hind paws on a daily basis. Rats were sacrificed at 4, 8 and 15 days p.i. Effect of DFO or DTPA on the presence of viral antigens in the rat hind paw and on the amount of infectious virus in internal organs was measured. In addition the effect of treatment on local inflammatory response (paw thickness and presence of inflammatory cells) was followed.

Plaque assay and PCR

The amount of infectious virus was determined by plaque assay using rat embryonic fibroblasts (REFs) as described before ⁽⁷⁸⁾ and is expressed as the number of PFU/g tissue homogenate.

Presence of viral DNA in the extracted organs was determined by a semiquantitative nested PCR as described previously ⁽⁵²⁵⁾.

Immunohistochemistry

Harvested tissues were fixed in a 3.7% formaldehyde solution in PBS for 24 hours and embedded in paraffin. RCMV antigens were detected on 4µm tissue sections with a mixture of 2 mouse monoclonal antibodies (MoAb) 8 and 35 ⁽⁷⁹⁾. MoAb W3-13 (Sera-lab, Crowley Down, UK) reacting with rat T-lymphocytes and MoAb ED-1 (gift from Dr. C. Dijkstra, Amsterdam, The Netherlands) specific for rat monocytes and macrophages were used to characterise infiltrated cells. Expression of interleukin-2 receptor (IL-2R) reflecting T-cell mediated immune activation was determined using the appropriate antibody (OX39, Sera-lab, Crowley Down, UK). Positive cells were detected by using the immunoperoxidase method as described earlier ⁽⁴⁹⁷⁾. Slides were evaluated semiquantitatively (- = no positive cells, +/- = sporadic cells, + = few cells, ++ = average number of cells, +++ = many cells, ++++ = very many cells) by two independent observers.

Figure 1. Antiviral properties of DTPA (indicated in black) and DFO (indicated in white) in vitro. REFs were infected with RCMV at a MOI of 0.01 and fixed at 5 days p.i. (figure A) or infected with a MOI of 0.1 and fixed at 3 days p.i. (figure B). Viral nuclear antigens were stained with MoAb8. The bar charts show the ratios between infected versus total number of counted cells (infection index). The line chart (figure C) shows the number of uninfected cells treated with DFO or DTPA. Control cells were fixed at 3 days p.i.

Table 1

Presence of infectious virus ^a in organs of intraperitoneal infected rats ^b
with and without drug treatment.

Treatment	Salivary gland ^c		Spleen ^d		Liver ^d	
DFO ^e	7.63 ± 1.6 ^g	5/5 ^h	1.04 ± 1.43	7/7	0.00 ± 0.00	0/7
DTPA ^e	7.98 ± 1.5	5/5	2.50 ± 1.42	7/7	0.00 ± 0.00	0/7
None ^f	8.65 ± 0.6	5/5	0.00 ± 0.00	0/7	0.00 ± 0.00	0/7

^a Measured by plaque assay

^b Rats received 3 x 10⁵ PFU RCMV by intraperitoneal injection

^c Harvested at day 21 post infection (n=5/group)

^d Harvested at day 4 post infection (n=7/group)

^e Compound given intraperitoneally at a dose of 100 mg/kg twice a day

^f Control rats received 0.2 ml PBS twice a day intraperitoneally

^g Expressed as ¹⁰log PFU per gram tissue

^h Number of rats harbouring infectious virus per total number of rats.

Antiviral properties of DTPA and DFO

For detection of an antiviral effect *in vitro* an immunofluorescence assay was used. For this purpose REFs were cultured in 96 well culture dishes and were used at confluency. RCMV was added at a multiplicity of infection (MOI) of 0.01 or 0.1. For optimal infection the cells were centrifuged at 700g for 45 minutes at 20°C. Directly after infection, cells were treated with DTPA or DFO at the concentrations of 0, 4, 8, 16, 32, 64, 128 and 256 µM. Cells were fixed at 3, 5 or 7 days post infection and the presence of RCMV antigens was detected by indirect immunofluorescence using RCMV specific monoclonal antibodies as described above. Cellular DNA was counterstained with 0.5 µg/ml 4'-6-diamino-2-phenyl indole (DAPI). The number of RCMV positive cells per well was scored by counting 5 visual fields (200 x magn.) and the ratio of the number of positive cells over the total number of cells (infection index) was determined. Each experiment was performed in quintuplo.

Statistical analysis

In all experiments the two sided non parametrical Mann Whitney U test was used and p<0.05 was regarded as significantly different.

Table 2.
Presence of infectious virus ^a in organs of locally RCMV infected rats ^b with and without drug treatment.

	Liver			Spleen			Salivary gland		
	4	8	15	4	8	15	4	8	15
DFO ^c	0/5 ^a	0/5	1/5 ^f	0/5	0/5	0/5 ^f	0/5	0/5	5/5
DTPA ^c	0/5	0/5	0/3 ^f	0/5	0/5	0/3 ^f	0/5	0/5	3/3
Control ^d	0/5	0/5	5/5	0/5	1/5	5/5	0/5	0/5	5/5

a Measured by plaque assay

b Rats were infected with 1×10^5 PFU in the subcutis of the hind paw

c Compound given intraperitoneally at a dose of 100 mg/kg twice a day

d Control rats received 0.2 ml PBS twice a day intraperitoneally

e Expressed as number of rats harbouring infectious virus per total number of rats

f Significant ($p < 0.05$) when compared to control rats.

Results

In vitro

Viral inhibition and cytotoxicity. Confluent REFs were infected with a MOI of 0.01 and were fixed at day 5 p.i. respectively. Addition of DTPA and DFO at a concentration of 4 μ M to cells infected at a MOI of 0.01 resulted in a significant reduction of the infection index when compared to control (i.e. non treated) cells (figure 1A). When DTPA was used at a concentration of 8 μ M a complete reduction in the number of RCMV infected cells was found. In contrast, infection index seen with DFO treatment did not change at this concentration. Even when high concentrations (16 and 32 μ M) of DFO were used, no effect was seen.

The antiviral effect of DFO and DTPA was dependent on the concentration of input virus as is shown in figure 1B. REFs were infected at a MOI of 0.1 and fixed at day 3 p.i. At this MOI, DFO was not able to decrease infection index significantly at a concentration of 4 μ M. However, a significant reduction in infection index was observed in cells treated with 8 μ M of DTPA (0.9 ± 0.1) when compared to control cells (0.7 ± 0.1). Cellular cytotoxicity was determined by counting the number of (non-infected) cells treated with DFO or DTPA at various concentrations. The concentration at which the compounds gave a 50% reduction in cell number (CC50%) was calculated. For DTPA the CC50% were 150, 52 and 20 μ M after 3, 5 or 7 days of incubation respectively, while for DFO the CC50% were 288, 100 and 84 μ M.

As shown in figure 1C, the number of cells did not change when concentrations of DFO up to 16 μ M were used after 3 days of culture in the presence of DFO. For DTPA no effect on the number of cells was observed when concentrations up to 8 μ M were used (after 3 days of culture). At these concentrations both compounds exhibited their antiviral effect as described above.

In vivo

Lethal intraperitoneal infection

To investigate whether DTPA and DFO could avert a lethal CMV challenge, immunosuppressed rats of 4 weeks old were inoculated i.p. with a lethal dose of 3×10^5 PFU of RCMV as described before ⁽⁴⁹⁷⁾. At 6 days p.i., rats started to show symptoms of CMV disease, such as subcutaneous and mucosal haemorrhages, development of ascites, hypokinesia and weight loss. The increase in weight per day during the experiment was 1.6 g, 2.0g and 2.6g for DFO treated, DTPA treated and control rats, respectively. There was no significant difference between the DFO and DTPA treated and the control rats in severity of clinical symptoms and in time of onset of these symptoms. Infection led to death starting at 9 days after infection in 4 out of 6 control rats, in 6 out of 7 DFO treated and in 5 out of 7 DTPA treated rats. No significant difference could be detected in the time of death. Rats surviving the CMV inoculation were followed for a period of 2 weeks and all these animals recovered completely.

Sublethal intraperitoneal infection

Presence of virus in liver, spleen and salivary gland. In order to investigate the antiviral properties of DTPA and DFO in rats suffering from a generalised infection, rats of 8 weeks old were i.p. infected and the effect of DFO or DTPA treatment on viral replication in internal organs was investigated. As is shown in table 1, salivary gland homogenates yielded high amounts of infectious virus in non treated animals (8.65 ± 0.6 log PFU/ml) at 21 days p.i. Although treatment with DFO or DTPA showed a slight decrease in viral load (7.63 ± 1.6 and 7.98 ± 1.5 respectively) in this organ, this decrease was not significant. In spleens harvested at day 4 p.i. a low amount of infectious virus could be detected in DFO or DTPA treated rats while in the non treated rats no infectious virus was detectable in this organ. No infectious virus could be detected in spleen samples harvested at day 21 and in all liver samples.

Presence of viral DNA in organ samples was determined semiquantitatively. Spleen and liver samples yielded viral DNA in 10^{-5} and 10^{-1} dilutions at day 4 p.i., respectively. In salivary gland samples harvested at day 21 p.i. viral DNA was found in 10^{-8} dilutions. No differences were seen in amount of viral DNA between non treated, DFO treated or DTPA treated rats (data not shown).

Detection of viral antigens by immunohistochemistry showed that RCMV positive cells were present in the salivary glands in all experimental groups at 21 days p.i. Treatment with DFO and DTPA did not result in a reduction of the number of RCMV positive cells. In conclusion, using the intraperitoneal infection route, no antiviral effect was observed in DFO and DTPA treated animals when compared to controls.

Subcutaneous infection in the hind paw

Macroscopy. As depicted in figure 2, in this infection model the course of infection can be divided in 4 phases. Phase 1 (day 1-5) is the steady state with no increase in paw thickness. Phase 2 (day 6-9) is characterised by a sharp increase in thickness and massive inflammation. Although the infected paw was painful, functionality was preserved until 12 days p.i. The next phase (day 9-12) is the plateau phase in which loss of functionality and edema of the leg are the most eminent phenomena. In this period the inflamed hind paw starts to loose its hair indicating tissue destruction. The last phase (day 13-15) is the healing period in which paw thickness and inflammation gradually decrease. In this period also functionality returns. The observed effects are all RCMV induced, since mock infection in the left paw did not show any sign of inflammation or increase in paw thickness. Treatment with DTPA or DFO did not lead to a significant reduction in paw thickness increase (figure 2).

Viral dissemination from the hind paw infection locus. Subcutaneous injection of RCMV led to a generalised infection as shown by the presence of infectious virus in spleen, liver and salivary gland at 15 days p.i. (table 2). At 4 and 8 days p.i. no infectious virus was detected in all organ samples except in the spleen of one animal. At day 15 p.i. all liver and spleen samples of the infected (non treated) rats contained RCMV. Treatment with DFO or DTPA reduced the number rats harbouring infectious virus in both the spleen and liver at 15 days p.i.

Plaque assays of the salivary glands extracted at 4 and 8 days p.i. remained negative. However, at day 15 p.i. the salivary glands yielded high amounts of infectious virus and in contrast to the spleen and liver no effect of antiviral therapy on viral replication was seen in this organ.

In spleens of control and DFO treated rats viral DNA was found in 10^{-2} dilutions whereas spleen samples of DTPA treated rats remained negative at 15 days p.i. In liver and salivary glands no differences in RCMV DNA content were found between DFO treated, DTPA treated and control rats. Viral DNA was present in 10^{-2} dilutions for liver samples and in 10^{-9} dilutions for salivary gland samples respectively.

Table 3.
Table showing the inflammatory response at 4, 8 and 15 days after
RCMV^a injection in the rat hind paw.

	8&35 ^b			W3-13 ^c		
	4	5	15	4	8	15
DFO ^f	+ ^b	+++	++	+	+/++	+++
DTPA ^f	+	+++	++	+	+/++	+++
none ^g	+	+++	++	+	+/++	+++

	ED-1 ^d			IL-2R ^e		
	4	8	15	4	8	15
DFO ^f	+	++	++++	-/+	+	+/++
DTPA ^f	+	++	++++	-/+	+	+/++
none ^g	+	++	++++	-/+	+	+/++

- a Rats were subcutaneously infected with 1×10^5 PFU
b Cells reactive with MoAb 8 & 35, specific for RCMV early antigens
c Cells reactive with MoAb W3-13, specific for T-lymphocytes
d Cells reactive with MoAb ED-1, specific for monocytes/macrophages
e Cells expressing the Interleukin-2 receptor
f Compound given intraperitoneally at a dose of 100 mg/kg twice a day
g Control rats received 0.2 ml PBS twice a day intraperitoneally
h The presence of cells was semiquantitatively analysed and expressed as median (- through ++++).

Although infectious virus and viral DNA could be detected in liver and spleen, no RCMV induced antigens were found in these organs by immunohistochemistry. Only the salivary glands at 15 days p.i. yielded positive staining. DFO or DTPA treatment did not result in a decline in the number of cells expressing viral antigens.

Viral antigen detection in the hind paw. After staining with MoAb 8 and 35, the amount of viral antigens was determined. In the hind paw of RCMV infected rats, cells with a cytoplasmic staining were observed in the hypodermis and the reticular layer of the dermis. In control, i.e. non treated, animals the amount of viral antigens could be correlated to the macroscopic findings in the hind paw as described above. In the phase before onset of paw thickness increase, the amount of RCMV positive cells was low (-/+). Maximal increase in paw thickness correlated with a large number of infected cells (+++) at day 8 p.i. In the last phase (i.e. 15 days p.i.), when paw thickness decreased, also the presence of virus decreased, leading to a low level (+) of RCMV positive cells. Treatment with DFO or DTPA could not reduce the number of viral antigen positive cells when compared to control rats.

Figure 2. Paw thickness of rats after sub-cutaneous injection of 1×10^5 PFU RCMV in the dorsum of the right hind paw. (day 0 to 4: $n=15$ /group, day 5 to 8: $n=10$ /group, day 9 to 15: $n=5$ /group). Black triangle indicates 100 mg/kg DTPA i.p. twice a day; White circle indicates 100 mg/kg DFO i.p. twice a day; black circle indicates 0.2 ml PBS i.p. twice a day.

Inflammatory response. Influx of T-lymphocytes and monocytes/macrophages at the inoculation site gradually increased and the amount of both cell types present reached a maximum at 15 days p.i. Also the expression of IL-2R increased over time until day 15 p.i. No differences were found in the amount of T-lymphocytes, monocytes/macrophages and expression of IL-2R between DFO treated, DTPA treated and control rats (table 3).

Discussion

Recently it has been reported that DFO ⁽¹⁰³⁾ and DTPA ⁽¹⁰⁴⁾ are able to reduce CMV replication in human cells. In a case report, Gumbel et al. ⁽²⁰¹⁾ have shown that the antiviral effect was also observed *in vivo*. In order to further study this effect *in vivo* a well defined animal model with the appropriate virus (RCMV) was used. Our *in vitro* data are in agreement with the previous reports on human cells and indicate that both DFO and DTPA are able to lower viral replication. However, only DTPA could reduce the amount of infected cells completely. Bearing in mind previous results ^(103, 105) one could state that the magnitude of the decrease of infection index by DFO and DTPA was dependent on the viral load.

In vivo, two infection models, i.e. the intraperitoneal and the subcutaneous infection model were used. With the intraperitoneal infection model characterised by a fast spread of the virus into internal organs, the effect of both compounds on dissemination of virus was studied. By using the local, i.e. subcutaneous infection model, it was possible to study not only the spread of the infection in the host but also to follow the local inflammatory response. The latter is important in the case of DFO and DTPA since it is known that both compounds have anti-inflammatory effects ^(450, 451).

In the generalised (intraperitoneal) infection model no effect of DFO and DTPA was observed, neither on morbidity and mortality nor on the presence of virus in internal organs. The salivary gland is the preference organ for this virus and contains high amounts of infectious virus. In this organ, no effect of either both compounds was observed in all animals tested. In contrast, in the local infection model an antiviral effect, as shown by a decrease in the number of rats harbouring infectious virus in liver and spleen at day 15, was found. Also no viral DNA in spleens of DTPA treated rats could be detected. The difference in effect in both models could be due to differences in viral load: in spleen samples of control (i.e. non treated) rats, viral DNA present in the intraperitoneally infected rats exceeded the amount of viral DNA detected in the subcutaneously infected animals. Besides the viral load, the difference in effect might also be explained by biological variations.

The mechanism by which both compounds act has not been fully elucidated but it is thought that the inhibition of an iron dependent ribonucleotide reductase plays an important role in inhibiting RCMV replication as was shown for other herpes viruses ⁽⁴⁸⁹⁾. In order to evaluate the effect of DTPA and DFO on the inflammatory response, paw thickness was measured daily and the number of inflammatory cells (T-lymphocytes and monocytes and macrophages) and the IL-2R expression reflecting T-cell mediated immune activation in the hind paw was determined. In control (i.e. non treated) animals the observed inflammatory response was consistent with the previous report of Persoons et al. ⁽³⁹⁹⁾. Treatment with DFO or DTPA did not diminish the amount of T-lymphocytes, monocytes/macrophages nor the expression of IL-2R. Also macroscopically, both compounds were not able to reduce the increase in paw thickness. The latter is in contrast to the report of Blake et al. ⁽⁵³⁾ in which a bolus of 30 mg DFO i.p. (instead of 100 mg/kg i.p. twice a day used in our experiments) was able to reduce foot pad swelling after monosodium urate monohydrate cristal or carrageenan injection in the rat foot pad. In addition to the inhibitory actions of iron depletion on viral replication as discussed above, iron itself has been associated with increase in free radicals resulting in an increase in oxidative stress ⁽²⁵²⁾. In auto-immune uveitis this increase in oxidative stress will lead to retinal tissue destruction. The magnitude of retinal cell loss can be diminished by DFO treatment ⁽⁵⁵⁵⁾. It is not known why both DFO and DTPA failed to have an effect on the

inflammatory response in the hind paw. However, one should bear in mind that immunologically induced damage and direct viral induced cell lysis are not totally separable. For it is known that CMV infection not only increases intracellular reactive oxygen intermediates (ROIs), but also benefits from a ROIs mediated enhancement of viral gene expression and thus viral replication ⁽⁴⁹¹⁾. Both the viral replication and the inflammatory process could result in a vicious circle. This could account for the differences in the observed immunomodulatory capacities of DFO and DTPA between our data and the papers of Wu et al. ⁽⁵⁵⁵⁾ and Kadiiska et al. ⁽²⁵²⁾.

Recently Gumbel et al. reported that addition of DFO to conventional treatment was able to reduce CMV induced retinitis refractory to ganciclovir and foscarnet in an AIDS patient ⁽²⁰¹⁾. It is unclear whether the improvement of visual acuity was the result of the immunomodulatory or the CMV inhibiting capacities of the drug or a combination of both.

In conclusion, our data indicate that despite previous *in vitro* reports, *in vivo* usage of DFO or DTPA as monotherapy for an acute CMV infection is questionable. The use of both compounds in combination with either ganciclovir or foscarnet should be tested in further studies.

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~ Chapter 8~

Summary and general discussion

Introduction

The pathophysiological and clinical characteristics of CMV infection are reviewed in **chapter 1**. Seroprevalence of CMV infections in the normal population is high and CMV infection usually follows a subclinical course. In contrast, patients with a defective immune system are at risk for developing severe CMV disease. For example, AIDS patients with a progressive decline in CD4+ T cell counts, suffer from severe CMV infections at CD4+ T cell counts below 100 cells/ μ l blood. Additionally, when immune effector functions are restored by HAART, the incidence of opportunistic infection, such as CMV, drastically diminishes.

CMV is able to infect a wide range of tissues and one of the main characteristics of CMV infection is the ability to persist at these sites, i.e. during chronic infection or latency. Peripheral blood mononuclear cells and their progenitors have been suggested as the main site of viral latency in humans, although CMV DNA can be found in multiple cell types in several organs of healthy trauma victims. Allogeneic stimulation of latently infected peripheral blood mononuclear cells has been shown to result in reactivation of virus. In addition, CMV seropositive patients receiving hemapoietic stem cell transplantation are at the highest risk for developing CMV disease, regardless of the CMV serostatus of the donor. These findings stress the importance of immune activation on RCMV replication and reactivation. Data from the murine model suggest that reactivation of virus from the lungs occurs stochastically via a patchwork pattern and does not depend on a decrease in immune effector functions. The significance of these findings for the human situation remains to be determined.

The interaction between CMV and the host's immune system is complex. It is generally accepted that atherosclerosis is an chronic inflammatory disease and develops as a response to injury. There is increasing evidence that systemic CMV infection or CMV replication in the atherosclerotic vascular wall itself accelerates atherosclerotic changes. CMV-mediated endothelial dysfunction and virus-mediated proliferation and migration result in increased inflammation, lipid accumulation and lumen narrowing. Additionally, CMV is thought to be an important risk factor for chronic rejection. The development of chronic rejection in the CMV-infected host is mediated by an increased and prolonged immune response, which is triggered by the viral challenge. On the other hand, however, TNF- α has been shown to directly enhance CMV replication via stimulation of the CMV IE enhancer/promotor mediated by transcription factor NF- κ B. These data suggest that CMV replication enhances inflammation and vice versa.

Current treatment of CMV infections with either ganciclovir, foscarnet or cidofovir is effective, although drug resistant strains have been reported. Furthermore, evidence is increasing that also CMV-triggered inflammation may partly account for the observed tissue damage. This points out the need for new antiviral therapies in which antiviral

activity and the ability to modulate CMV-triggered immune activation are combined. Insight in viral pathogenesis is essential for developing new modalities of intervention. RCMV has been isolated approximately 20 years ago ⁽⁷⁸⁾ and experimental RCMV infections have been accepted as models for CMV infections in humans.

RCMV replication in salivary gland cells

The salivary gland is the preferred organ for CMV infections. RCMV replication in salivary gland cells is detectable approximately 8 days after infection, whereas during the first week after infection, infectious virus is predominantly produced in internal organs, such as liver and spleen ^(75, 77, 497). Infection of salivary glands is characterized by a long term production of high amounts of infectious virus. The salivary gland consists of three major glands, designated the submandibular, parotid and sublingual gland. Although some data were available about RCMV replication in the submandibular gland, little was known about virus replication in the parotid and the sublingual salivary gland. Replication of RCMV in the major salivary glands is described in more detail in **chapter 2**. Assessment of viral load in the submandibular, parotid and sublingual gland of young adult animals, revealed that the submandibular gland was the preferred organ for RCMV replication. In contrast, in newborn rats, the main site of viral replication was the sublingual gland. It is possible that the maturation process of the sublingual gland enables optimal RCMV replication shortly after birth, whereas this permissiveness for the virus is lost when the maturation process is completed. Interestingly, it was shown by immunohistochemistry, *in situ* hybridization and electron microscopy, that, in all infected animals, viral replication was restricted to striated duct cells. This is in sharp contrast to MCMV where replication is exclusively found in acinar cells. Differences in cell tropism between RCMV and MCMV are especially fascinating since it is known that both viruses possess a lot of similarities and the genome of RCMV and MCMV show the highest homology of all known CMVs. Infection of rats resulted in a cellular inflammatory response, which was predominantly located in the interlobular duct region. Only a few inflammatory cells were found in the neighborhood of infected striated duct cells. A similar observation was made in the murine system ⁽²¹⁶⁾. The inability of inflammatory cells to reach infected striated duct cells may be one of the explanations for the persistence of CMV in the salivary glands.

It was shown that infectious virus was produced in large cytoplasmic vesicles and is excreted into the lumen of the salivary gland by apical cell budding of the vesicle. The chronic production of high amounts of infectious virus could have other consequences, rather than solely providing infectious virus for horizontal transmission. Shedding of virus from the salivary gland may result in re-infection of the host. In order to study the biological significance of RCMV replication in the salivary gland cells, we employed a model in which the salivary glands were removed in a subset of rats prior to infection (**chapter 3**).

Surgically desalivated rats were compared to sham-operated animals after a lethal and sublethal infection with RCMV, respectively. Survival was recorded and presence of infectious virus was determined by plaque assay and the presence of viral DNA was assessed by PCR. We demonstrated that desalivation did not have an effect on the clinical course of RCMV disease and on RCMV-induced mortality, but a significantly higher titer of anti-RCMV antibodies was found in sham-operated rats than in desalivated animals during long-term follow-up. These data indicate that shedding of virus or viral antigens to the systemic circulation occurs from the salivary gland via virus-containing saliva and that this re-introduction of virus or its components is responsible for boosting the humoral response, resulting in high antibody titers. Interestingly, we were unable to detect viral DNA and infectious virus in spleen, liver and lung of infected animals at 1 year p.i., but as expected, the submandibular glands still yielded high amounts of infectious virus. These data suggest that boosting of the humoral response is caused by shedding of viral antigens from the salivary glands and that infectious virus shed from the salivary gland is neutralized by already existing circulating anti-RCMV antibodies, a phenomenon described previously ⁽²⁵⁰⁾.

The r144 gene product interferes with the innate immune system

CMV infection is the most common congenital infection in humans. Approximately 5% of infected newborns are born with CMV disease ^(128, 393). Prognosis in this group is poor and the clinical presentation during follow-up is mainly characterized by neurological deficits ^(106, 387). A significant proportion of the asymptomatic infected newborns (5%) will suffer from long term sequelae of which sensorineural hearing loss is most common ^(166, 167). Sensorineural deterioration due to asymptomatic infection usually progresses undetected and it has been reported that screening of these patients fails in over 50% of the cases ⁽¹⁶⁶⁾. In contrast to the human situation, RCMV is not able to cross the placental barrier (unpublished results). It is therefore likely that vertical transmission of virus in rats is of little importance and that newborn rats become infected after birth. Thus, an experimental rat model for congenital infection in humans can only be achieved by infecting newborn rats shortly after birth. Experimental RCMV infection in newborn rats is described in **chapter 4**. RCMV infection of newborn rats resulted in similar distribution of virus during the course of infection as did infection of immunocompromised adult rats. RCMV was present in liver, spleen and lung at 3 and 5 days p.i., whereas RCMV could only be detected in the salivary glands at 21 days p.i. Furthermore, RCMV infection resulted in an inflammatory response in infected organs, which consisted of monocytes/macrophages, T cells and NK cells. The immune system of neonatal rats was able to control CMV infection to some extent, since animals did not show symptoms of CMV disease and did survive for at least 21 days after inoculation of 10^4 PFU of virus. In

contrast, it is known that survival rates are greatly impaired when rats of 4 weeks old are subjected to 5 Gy of TBI prior to injection of 10^5 PFU of RCMV. The usage of TBI as immune suppressive regimen affects the number of granulocytes, NK cells, monocytes, B cells and T (CD4+ and CD8+) cells in peripheral blood of irradiated animals significantly and leads to a transient state of severe immunosuppression ⁽⁵²⁵⁾. Peripheral white blood cells are suppressed during the acute phase of infection and the number of cells return to control levels at approximately 3-4 weeks after irradiation ⁽⁵²⁵⁾. The natural, unaffected immune system of neonatal rats enables disseminated RCMV infection, but prevents uncontrolled viral replication and CMV-induced multi-organ failure. This optimal equilibrium between on the one hand the level of RCMV replication and on the other hand the control of RCMV infection by immune effector functions in neonatal rats enables us to study the interaction of the virus with the immune system of the host.

CMV has developed several mechanisms to evade immune surveillance ⁽³⁵³⁾. One of those mechanisms is to downregulate cellular MHC class I expression. However, according to the 'missing self hypothesis', downregulation of MHC class I renders a cell susceptible to NK cell killing ⁽³¹⁶⁾. Viral encoded MHC class I homologs have been identified on the genome of human CMV (UL18, ⁽³⁹⁾), MCMV (m144, ⁽¹⁵⁵⁾) and RCMV (r144, ⁽⁴³⁾).

An RCMV strain in which the r144 gene is disrupted was generated in our laboratory ⁽⁴³⁾ and infection of immunocompromised adult rats with this deletion mutant showed that there were no significant differences in tissue distribution and virus titers between wt RCMV- and RCMV Δ r144-infected animals ⁽⁴³⁾. Since it is thought that the r144 gene might play a role in virus-induced evasion of immune surveillance, it is possible that the high level of irradiation-induced immunosuppression was responsible for those results. In order to study the role of r144 in the pathogenesis of RCMV infection in rats with a normal unaffected immune system, neonatal rats were challenged with either wt RCMV or RCMV Δ r144 and the presence of infectious virus as well as viral DNA was assessed in various organs at different time points p.i. Replication of RCMV Δ r144 in the spleen and the salivary gland was severely restricted compared to wt RCMV at 3 and 21 days p.i., respectively. RCMV Δ r144, has previously been shown to have similar replication characteristics as wt virus *in vitro* as well as *in vivo* ⁽⁴³⁾, suggesting that RCMV Δ r144 replicates as efficiently as wt RCMV in the absence of a fully functional immune system. In addition, also a significant lower number of monocytes/macrophages and NK cells were found in spleens of RCMV Δ r144-infected rats than in those of wt RCMV-infected animals at 3 days p.i. These results are in concert with previous results, using a local infection model, in which either RCMV Δ r144 or wt RCMV were subcutaneously injected in the hind paw of immunocompromised adult rats. In this model, both viruses were shown to replicate with similar efficiency locally in the hind paw. However, a significantly lower number of

monocytes/ macrophages was found at the site virus injection. In this case, however, a similar number of NK cells was found in the rat hind paw of RCMV Δ r144- than in those of wt RCMV-infected animals. These data from the neonatal- and rat hind paw model are in concert with previous reports obtained from the human ^(116, 306) and murine system ⁽²⁸²⁾. The UL18 gene product was shown to interact with a membrane receptor, designated LIR-1, which is predominantly expressed on monocytes and B lymphocytes, but only on a minor subset of NK cells ⁽¹¹⁴⁾. Cretney et al. showed that the m144 gene product provided some protection against NK cell-mediated killing of lymphoma cells transfected with m144 *in vitro*, but that *in vivo*, the major effect of m144 is to regulate NK cell accumulation and activation ⁽¹¹⁶⁾. These data show that the CMV-encoded MHC class I homolog is more likely to compromise the innate immune system as a whole, rather than to solely inhibit NK cell-mediated killing.

RCMV infection of renal allografts results in an increased and prolonged expression of adhesion molecules.

Current immunosuppressive regimens and anti-rejection therapies prevent renal allograft failure due to acute rejection in the majority of cases. However, several years after transplantation, a significant number of renal allografts are lost as a result of chronic rejection. The level of histoincompatibility, inadequate immunosuppression, acute rejection and viral infections, especially CMV infections, have been suggested as risk factors for chronic rejection ^(14, 38, 209, 390). Although it is generally accepted that CMV infection plays a role in the acceleration of chronic rejection ^(20, 193, 277, 281, 292, 339, 380) the mechanism involved is unclear. To study the role of CMV infection chronic renal allograft rejection, the rat model developed by Soots et al. ⁽⁴⁸³⁾ was used.

Chapter 5 focuses on the accelerated development of chronic rejection in RCMV infected rats receiving a kidney allograft under triple drug immunosuppression. It was shown previously that renal allografts harvested from rats receiving triple drug immunosuppression showed histological signs of chronic rejection at 40-60 days after transplantation ⁽⁴⁸³⁾. The histological diagnosis of chronic rejection includes perivascular and interstitial inflammation, fibrosis, glomerulosclerosis, vascular intimal thickening and tubular atrophy ^(391, 480). The peak of inflammation in these allografts was seen 5-10 days p.i. and was associated with lymphoid activation and induction of adhesion molecules ⁽²⁵⁹⁾. Thereafter, renal inflammation gradually decreases while the histological characteristics of chronic rejection became more and more prominent. RCMV antigens could be detected in endothelial cells of renal allografts at 7 days p.i. ⁽²⁹⁴⁾. Strikingly, RCMV infection increased and prolonged inflammation and accelerated the development of chronic rejection significantly. The histological criteria for chronic rejection were already fulfilled before 20 days after transplantation ⁽²⁹⁴⁾. RCMV infection of renal allografts was associated with an

increased and prolonged expression of adhesion molecules ICAM-1 and VCAM-1 on endothelial cells and their ligand molecules, LFA-1 and VLA-4, expressed on leukocytes. Expression of adhesion molecules is mediated via cytokines, such as IL-1, TNF-alpha and IFN-gamma, which are produced by activated lymphocytes. On the other hand, CMV has been shown to upregulate IL-1 β gene expression, which may lead to production of IL-1 by mononuclear cells ⁽²³⁵⁾. In addition, IE genes of CMV have been found to upregulate IL-2 and IL-2R genes ⁽¹⁷⁹⁾. Furthermore, CMV induces TNF-alpha in monocytes/macrophages ⁽⁴⁷²⁾, as well as *in vivo* ⁽²⁰²⁾. RCMV mediated production of cytokines may result in a generalized increased and prolonged expression of adhesion molecules, even on uninfected endothelial cells of the graft ⁽⁵⁴¹⁾. These data suggest that RCMV influences the induction phase of the alloresponse and determines the magnitude of this process from that point onward.

Treatment of CMV infections in immunocompromised rats.

Despite the drastic decrease in CMV related morbidity since the introduction of HAART, CMV-induced encephalomeningitis and its treatment are still a major clinical problem in patients with end-stage HIV infection. The effect of several antiviral compounds on RCMV-induced encephalomeningitis in immunocompromised rats is described in **chapter 6**. Rats were intracerebrally infected and were systemically treated with either HPMP, DHPG, HIS, or DHPG in combination with HIS. After intra-cerebral infection, the viral antigens could be detected in mononuclear cells in the meninges and endothelial cells of small vessels. In addition, an inflammatory response consisting of mononuclear cells and T cells and scattered foci of necrosis harboring RCMV antigens could be demonstrated in the meninges and to a lesser extent in the brain. RCMV infection of endothelial cells is associated with a local inflammatory response, in a similar manner as described previously in the rat hind paw model ⁽³⁹⁹⁾. The observed histological alterations in our rat model closely resemble the pathology of CMV-induced meningoencephalitis in AIDS patients ^(208, 360). Viral replication in endothelial cells may be a portal for entry of CMV into the central nervous system. Treatment with a single dose of 20 mg/kg HPMP significantly reduced the amount of infectious virus in brain and spleen compared to all other treatment modalities, which were administered daily at the appropriate doses for 5 days. One explanation for this therapeutic failure in the model used could be poor penetration of the antiviral compounds into the brain area, which permits initial replication cycles of the virus.

Tissue damage due to CMV infection is at one hand caused by direct virus-induced cell damage and on the other hand mediated by the mounted immune response. For example, the HAART-associated reconstruction of immune effector functions in AIDS patients renders patients with a history of CMV retinitis susceptible to IRV, a condition

characterised by retinal inflammation without any signs of CMV relapse ^(256, 257). In addition, CMV pneumonitis in AIDS patients is rare and follows a mild course, whereas CMV pneumonitis in BMT is a frequent and lethal condition ⁽³⁷⁾. This suggest that the inflammatory immune response after CMV infection plays an important role in causing tissue dysfunction. Drugs with anti-CMV and anti-inflammatory properties may be beneficial. **Chapter 7** describes the evaluation of 2 compounds, DFO and DTPA, which are thought to inhibit viral replication and decrease oxidative stress. The mechanism by which both compounds act is not fully understood, but it is thought that the inhibition of an iron-dependent ribonucleotide reductase may be responsible for the inhibition of RCMV replication. The antiviral effect of both compounds was assessed *in vitro* as well as *in vivo*, using a generalized and a local infection model. In concert with previous results ^(103, 105), DFO and DTPA exhibited an antiviral effect *in vitro*, although DTPA was more potent than DFO. In immunocompromised rats, DFO and DTPA were unable to prevent RCMV mortality and RCMV disease. Neither DFO nor DTPA reduced virus replication in organs significantly after a sublethal RCMV challenge. A local infection model, in which RCMV was injected in the rat hind paw of immunocompromised animals, enabled us to study the effect of DFO and DTPA on the spread of virus from the infection site as well as the effect of both drugs on the local inflammatory response. Treatment with DFO or DTPA was unable to reduce the swelling of the hind paw, did not affect the viral load as measured by the number of RCMV antigens expressed in rat hind paw tissue and failed to diminish the number of infiltrating leukocytes. However an antiviral effect was observed in the local infection model, as shown by a significant decrease in the number of rats harboring infectious virus in spleen and liver after treatment with DFO or DTPA. The *in vivo* transplantation study using rat liver allografts by Martelius and co-workers ⁽³³¹⁾ supported this finding. In their study an inhibitory effect of metal chelators on CMV replication, inflammation and on bile-duct damage was detected. In addition, DFO has been shown to reduce auto-immune-mediated retinal inflammation ⁽²⁵²⁾ and iatrogenic inflammation of the rat hind paw ⁽⁵³⁾ significantly. Both compounds were shown to inhibit 1) mitogen- and allogen- induced proliferation of peripheral blood lymphocytes, 2) NK cell function and 3) adhesion molecule expression ⁽⁴⁵⁰⁾. More importantly, in a case report, administration of DFO to an AIDS patient suffering from CMV retinitis, refractory to ganciclovir and foscarnet, inhibited progression of ocular changes. These data indicate that metal chelators as DFO and DTPA have to some extent antiviral capacities *in vivo*, but that these compounds as reported by Martelius et al. are especially usefull in settings with evident immune activation, such as organ transplantation recipients.

Concluding remarks

In this thesis several aspects of CMV infection and CMV disease have been studied in a rat model. The model used in this thesis gave us the opportunity to study the replication of the virus and the pathology it induced in more details. Although, RCMV infections closely resemble CMV infections in man, one should bear in mind that translation of data from CMV-infected laboratory animals to the human situation should be made with care, even when the genome of both strains shows a high level of homology. Nevertheless, the ability to control most variables in an experimental setting using animals and the appropriate viruses enable us to unravel mechanisms of CMV disease. Data from these models may provide new potential targets for antiviral treatment.

Samenvatting

De seroprevalentie van CMV is hoog in de gezonde populatie. Een eerste infectie met CMV is normaliter asymptomatisch. In een minderheid van de gevallen kan een griepachtig beeld ontstaan. Levensbedreigende ziekte door CMV infectie bij gezonde personen komt voor, maar is uiterst zeldzaam. Patiënten met een immuundeficiëntie, zoals AIDS- of transplantatiepatiënten, lopen risico op ernstige CMV ziekte door een primaire infectie of reactivatie van latent aanwezig CMV. Ganciclovir, cidofovir en foscarnet zijn middelen die gebruikt worden voor de behandeling van ziekte door CMV infectie.

Ongeveer 20 jaar geleden werd uit ratten CMV geïsoleerd (RCMV). Het RCMV model wordt gebruikt voor onderzoek naar pathogenese en het testen van nieuwe potentieel antivirale middelen. In dit model wordt het virus meestal intraperitoneaal toegediend, maar ook subcutane en intraveneuze toediening kunnen gebruikt worden. Na intraperitoneale toediening wordt het virus in de eerste week na infectie voornamelijk gevonden in abdominale en thoracale organen zoals lever, milt, nieren en longen. Na de eerste week kan geen infectieus virus in deze organen meer worden aangetoond. Wel blijft het DNA van CMV in deze organen aantoonbaar (latentie). Vanaf ongeveer 8 dagen na infectie wordt er infectieus virus aangetroffen in de speekselklieren. De productie van infectieus virus houdt lange tijd (enkele maanden) aan. **Hoofdstuk 2** beschrijft de replicatie van RCMV in de speekselklieren van de rat. Met in situ hybridisatie, electronen microscopie en immunohistochemie is gebleken dat, ondanks verschillen in speekselkliertropisme tussen pasgeboren- en jongvolwassen ratten, uitsluitend striatale cellen geïnfecteerd zijn. Het virus wordt met het speeksel uitgescheiden. Dit is een van de mechanismen waarmee het virus zich kan verspreiden tussen soortgenoten. Het is onduidelijk of het uitscheiden van speeksel met CMV een re-infectie veroorzaakt. In **hoofdstuk 3** worden ratten met en zonder speekselklieren geïnfecteerd met RCMV om het infectieverloop in beide groepen te vergelijken. Het acute infectieverloop na een letale dosis RCMV verschilt niet in ratten met en zonder speekselklieren. In een tweede experiment, waarbij ratten gedurende een jaar gevolgd werden, werd een verschil in antilichaamtiter gevonden. Ratten zonder speekselklieren hadden lagere antilichaamtiters na infectie dan ratten met speekselklieren. Dit suggereert dat uitscheiding van het virus of virale antigenen plaatsvindt via speekselklieren en dat dit mede verantwoordelijk is voor activatie van de humorale immuunrespons.

In tegenstelling tot jongvolwassen ratten heeft men bij pasgeboren ratten geen algehele lichaamsbestraling nodig om een ernstig verlopende RCMV infectie te bewerkstelligen. Hoewel het immuunsysteem van pasgeboren ratten nog onrijp is, is het echter in staat om de infectie uiteindelijk het hoofd te bieden en overlijden te voorkomen. Het gebruik van pasgeboren ratten is dus een uitermate geschikt model voor het bestuderen van de interactie van het virus met het immuunsysteem. Eerder onderzoek

wijst erop dat een aantal genen in het CMV genoom coderen voor eiwitten die interfereren met het immuunsysteem. Door het immuunsysteem te misleiden en een geïnfecteerde cel als "gezond" te presenteren kan de virusgeïnfecteerde cel ontsnappen aan het immuunsysteem en er derhalve voor zorgen dat het virus overleeft. De door CMV gecodeerde MHC klasse I homoloog is geassocieerd met het proces van immuun evasie. Het cellulaire MHC klasse I eiwit wordt tot expressie gebracht op de celmembraan van elke kernhoudende lichaamscel en speelt een cruciale rol in de afweer tegen virusinfecties. In **hoofdstuk 4** wordt de bijdrage van de virale gecodeerde MHC klasse I homoloog aan de pathogenese nader bestudeerd door virussen met (wt RCMV) en zonder de MHC klasse I homoloog (RCMV Δ r144) met elkaar te vergelijken. Hiervoor werden pasgeboren ratten intraperitoneaal geïnfecteerd met een van beide virussen. Eerder onderzoek liet zien dat beide virussen in vitro even efficiënt repliceerden. Daarnaast konden er geen verschillen in virusconcentratie worden aangetoond tussen wt RCMV- en RCMV Δ r144-geïnfecteerde immuundeficiënte ratten. In deze experimenten werd de virale replicatie niet beïnvloed door een aanwezig immuunsysteem. Echter in het RCMV-infectiemodel met pasgeboren ratten wordt er een significant lagere hoeveelheid infectieus virus gevonden in de milt en speekselklieren van RCMV Δ r144- dan in milt en speekselklieren van wt RCMV-geïnfecteerde ratten. Deze resultaten in de verschillende modellen suggereren dat het verschil in virusreplicatie tussen beide virussen veroorzaakt wordt door de activiteit van het immuunsysteem. De functie van de virale MHC klasse I homoloog is echter nog onduidelijk. Er zijn aanwijzingen dat de viraal gecodeerde MHC klasse I homoloog, naast zijn functie als vervanger van het cellulaire MHC klasse I, ook een interactie aangaat met cellen van het niet-specifieke immuunsysteem.

CMV speelt een belangrijke rol bij orgaantransplantaties. De mate van histo-incompatibiliteit, inadequate immunosuppressie, episodes van acute afstoting en virale infecties (waaronder CMV) worden geassocieerd met de chronische afstotingsreactie. Hoewel het verband tussen CMV infectie en chronische afstoting algemeen geaccepteerd is, is het achterliggende mechanisme onduidelijk. Uit eerder onderzoek in het RCMV model is gebleken dat RCMV infectie de chronische afstotingsreactie van een allogeen niertransplantaat versnelt. De versnelde afstotingsreactie door RCMV gaat gepaard met verhoogde expressie van de adhesiemoleculen ICAM-1 en VCAM-1 op endotheelcellen van de getransplanteerde nier en hun ligand moleculen LFA-1 en VLA-4 op de aanwezige leukocyten (**Hoofdstuk 5**). Het is bekend dat CMV de expressie van sommige cytokines, zoals IL-1 β , IL-2, IL-2R en TNF- α , kan verhogen. De langdurig verhoogde expressie van adhesiemoleculen op geïnfecteerde en niet-geïnfecteerde endotheelcellen kan verklaard worden door de verhoogde cytokine productie. Adhesiemolecuul expressie is essentieel voor het uittreden van inflammatoire cellen uit de bloedbaan en staat als dusdanig aan de basis van de afstotingsreactie van de getransplanteerde nier.

De behandeling van ziekte door CMV infectie is complex. Aangezien CMV infectie voornamelijk een probleem is in immuundeficiënte patiënten zou theoretisch de beste behandeling het opheffen van de immuundeficiëntie zijn. Een voorbeeld hiervan betreft het behandelen van AIDS patiënten met HAART. Door de replicatie van HIV te reduceren zal het immuunsysteem (T-helper cellen) van de patiënt tijdelijk kunnen herstellen. De incidentie en de ernst van CMV infecties in deze populatie daalt dan drastisch. In een aantal patiënten wordt echter een immunologisch gemedieerde orgaanschade gemeld. Dit treedt voornamelijk op ter hoogte van de retina. Het immuunsysteem gaat dan massaal het geïnfecteerde weefsel te lijf en schiet hierbij zijn doel voorbij waardoor zogenaamde immuunpathologische schade optreedt. In **hoofdstuk 6** wordt de werking van enkele antivirale middelen bestudeerd. Deze middelen remmen direct de virale replicatie. Om dit te onderzoeken werden immuundeficiënte ratten intracerebraal geïnfecteerd met RCMV en vervolgens behandeld met HPMPC (cidofovir), DHPG (ganciclovir), Hyper Immune Serum (HIS) en een combinatie van HIS en DHPG. In het beschreven ratmodel kwam het histopathologisch beeld van de hersenen overeen met de pathologie zoals die gezien wordt in CMV-geïnduceerde meningo-encephalitis in AIDS patiënten. Behandeling met HPMPC was, met betrekking tot inhibitie van virale replicatie, superieur aan de andere geteste antivirale middelen. Daarbij werd HPMPC slechts eenmalig gegeven, terwijl DHPG gedurende 5 dagen dagelijks toegediend werd. In **hoofdstuk 7** worden de resultaten beschreven van experimenten waarbij middelen getest worden waarvan naast een directe antivirale werking, ook een immuunmodulerende werking worden toegeschreven. DFO en DTPA zijn metaalchelatoren en hun antivirale werking wordt toegeschreven aan inhibitie van een ijzerafhankelijk ribonucleotide reductase. Gebruik van deze middelen in RCMV-geïnfecteerde ratten toont aan dat het antivirale effect van beide middelen in ratten gering is: DFO en DTPA konden overlijden ten gevolge van een letale dosis CMV niet voorkomen. Ook waren beide middelen niet in staat om de concentratie van infectieus virus in organen van niet-letaal geïnfecteerde ratten te verminderen. Wel werd er een beperkt antiviraal effect gezien van beide middelen in een lokaal infectiemodel. In dit model wordt virus subcutaan in de achterpoot van een immuundeficiënte rat gespoten. Infectieus virus wordt nu geleidelijk aan de lichaamscirculatie afgegeven. Behandeling met DFO en DTPA resulteerde in een significante vermindering van het aantal dieren met geïnfecteerde interne organen zoals lever en milt in vergelijking met controle dieren die geen behandeling kregen. DFO en DTPA hebben dus een beperkte waarde als antiviraal middel. Deze resultaten staan echter in schril contrast met de resultaten die door de groep van Lautenschlager verkregen werden in een allogeen levertransplantatiemodel. DFO bleek in dit model de afstotingsreactie en de virale replicatie te verminderen. Dit suggereert dat metaalchelatoren geïndiceerd kunnen zijn in patiënten met een duidelijke immuunactivatie. Verder onderzoek naar de werking van metaalchelatoren in

transplantatiemodellen is geïndiceerd.

In dit proefschrift worden enkele aspecten van CMV infectie en CMV ziekte bestudeerd in een ratmodel. De ratmodellen die in dit proefschrift gebruikt werden, gaven ons de mogelijkheid om de virale replicatie en de pathologie die het induceert te bestuderen. Hoewel ziekte door RCMV en CMV niet veel van elkaar verschillen, is echter voorzichtigheid geboden bij het extrapoleren van gegevens uit het RCMV model naar de humane situatie. Het voordeel van diersmodellen is de mogelijkheid om veel variabelen te controleren en systematisch de mechanismen van CMV ziekte te ontrafelen.

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Kloover, J.S., A. E. van den Bogaard, J.G. van Dam, M.J. Blok, G. Grauls, C. Vink, C.A. Bruggeman. Persistent rat cytomegalovirus (RCMV) infection of the salivary glands contributes to the anti-RCMV antibody response in infected rats. *Submitted*.

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Curriculum vitae

Jeroen Steven Kloover werd geboren op 19 december 1975 te Rotterdam. Na het behalen van zijn VWO eindexamen in 1994 aan de CSG Blaise Pascal te Spijkenisse, startte hij in dat zelfde jaar met de studie geneeskunde aan de Faculteit der Geneeskunde van de Universiteit van Maastricht. Na het propedeutisch examen in 1995 volgde het doctoraal examen in 1998. Tijdens zijn studie raakte hij als student betrokken bij het wetenschappelijk onderzoek van de afdeling Medische Microbiologie van het Academisch ziekenhuis Maastricht (AzM). Aanvankelijk was de begeleiding in handen van Dr. F. Stals en later van prof. dr. C.A. Bruggeman. Dit onderzoek resulteerde in promotieonderzoek met prof. dr. C.A. Bruggeman als promotor. Van 1998 tot en met 2001 volgde een periode van co-schappen in het Academisch ziekenhuis Maastricht. In 2001 werd het arts-examen behaald. Vanaf mei 2001 werkt hij als arts-assistent niet in opleiding op de afdeling longziekten van het Erasmus Medisch Centrum Rotterdam onder prof. dr. H. Hoogsteden. Per september 2002 start hij aan de Universiteit van Utrecht met de opleiding tot huisarts.

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