Chapter 4

Factors determining the Cytomegalovirus-enhanced Development of Transplant Arteriosclerosis: Effect of Timing of Infection and Recipient Genotype

Jan-Luuk Hillebrands¹, Joanne G. van Dam², Flip A. Klatter¹, Gert Grauls², Cathrien A. Bruggeman² and Jan Rozing¹

¹Department of Cell Biology, section Histology & Immunology, Faculty of Medical Sciences, University of Groningen, Groningen, The Netherlands
²Department of Medical Microbiology, Cardiovascular Research Institute Maastricht (CARIM), University Hospital Maastricht, Maastricht, The Netherlands

Submitted
Chapter 4

Abstract

Background. CMV infection appears to be an important risk factor in the development of transplant-related vasculopathy (i.e., transplant arteriosclerosis, TA). In this paper, we studied the severity of TA development, with or without additional RCMV infection, in different donor/recipient combinations. Moreover, the relation between timing of infection and enhanced development of TA was studied.

Methods. Allogeneic aorta transplantation was performed in the BN to Lew, Lew to BN, PVG to AO, and DA to WF rat strain combinations, and the severity of TA was analyzed. To study the effect of timing of RCMV infection on TA, Lew recipients were infected at several time points before and after BN aortic allografting. Moreover, transmission of infectious virus from BN donors to Lew recipients by the graft was studied.

Results. Lew recipients only developed weak TA 4 weeks after transplantation. AO and WF recipients developed also weak TA which was similar to Lew recipients. In contrast, BN recipients showed a four-fold increase in the amount of TA compared to Lew recipients. RCMV infection resulted in enhanced TA only when Lew recipients were infected 1 day after transplantation (i.e., coinciding with the beginning of the rejection response). This effect was not present in the other weak TA strain combinations (PVG to AO and DA to WF). Although transmission of virus from the graft to the recipient occurred after transplantation of a graft from an infected donor 5 and 10 days p.i., CMV-enhanced development of TA was not observed.

Conclusions. RCMV enhances the development of TA in Lew recipients only when acute rejection and acute infection are present simultaneously. However, this effect cannot be generalized, and obviously there are more factors than timing of infection and weakness of TA that determine the CMV-enhanced development of TA.
Introduction

Solid organ transplantation has become an established therapy for patients with end-stage organ failure. However, despite the introduction of new immunosuppressive agents in the last two decades, development of chronic transplant dysfunction (CTD) is still a complicating factor for long-term graft survival. To date, CTD has become the most important problem in clinical organ transplantation after the first perioperative year which cannot be prevented by current immunosuppressive protocols. Depending on the type of organ grafted, 4% to >50% of solid organ transplants show functional deterioration and histological changes characteristic of CTD three years after transplantation. Irrespective of the organ grafted, graft vessels eventually develop so-called transplant arteriosclerosis (TA), which is, however, most prominent in cardiac allografts. This vascular remodeling process consists of progressive concentric intimal thickening (intimal hyperplasia or neointima formation) coinciding with ongoing perivascular inflammation (perivasculitis). TA has generally been accepted as the main cause for progressive deterioration in graft function. Pathogenesis of TA seems to be multifactorial but precise mechanisms involved in the development of this remodeling process still remain obscure. Risk factors appear to include cold ischemia time and reperfusion injury, MHC disparity between donor and recipient and number of rejection episodes. In addition, cytomegalovirus (CMV) infection has been associated with the development of TA. Studying human cardiac allografts, Grattan et al. showed a positive correlation between the presence of cytomegalovirus and graft atherosclerosis. In addition, Almond et al. showed CMV to be a risk factor for the development of CTD in human kidney allografts. However, also data have been described suggesting that CMV does not contribute to TA after solid organ transplantation. A possible explanation for these conflicting results is that in some studies patients received prophylactic treatment with antiviral drugs preventing viral replication immediately after transplantation, whereas in other studies antiviral therapy was started when active CMV infection was already manifest. Also in several experimental transplant models in rats (heart, kidney, liver and trachea) the role of CMV infection on the development of CTD related pathology has been studied. Using Rat Cytomegalovirus (RCMV), it was shown that viral infection accelerates the development of CTD related pathology. In the aortic transplant model in rats, which is a well established and reproducible model to study development of TA, we and others have shown that RCMV infection (injected 1 day after transplantation) results in enhancement of both the perivascular inflammatory response and neointima formation. The increase in perivascular inflammation observed after RCMV infection peaks around 1 month after transplantation, whereas the effects on neointima formation can be detected 2-3 months after transplantation. Therefore, it has been hypothesized that the increased perivascular inflammatory response after viral infection is the cause of enhanced neointima formation. This is strengthened by the observation that an increase in the viral load results in increased perivasculitis. Inhibition of viral replication and
inflammation using antiviral\textsuperscript{22,25} and immunosuppressive drugs\textsuperscript{26}, respectively, prevent RCMV enhanced development of TA. These observations further indicate that CMV may play a role in the process of TA development.

Clinical data suggest that CMV infection enhances development of CTD related pathology only when active CMV infection and acute rejection are present simultaneously\textsuperscript{27,28}. We previously showed that RCMV infection of aortic allograft recipients resulted in enhanced TA only in a donor/recipient combination in which only weak TA develops in the absence of RCMV\textsuperscript{24}. In combinations that develop strong TA, additional RCMV infection seems not be able to further accelerate or enhance the development of TA.

In this paper we investigated the role of the donor/recipient combination and the timing of CMV infection on the CMV-enhanced development of TA after allogeneic aorta transplantation in rats. We first analyzed the development and severity of TA in various MHC-incompatible donor/recipient combinations without additional RCMV-infection. Based on these results we selected a donor/recipient combination with weak TA development to study the effect of timing of CMV infection on the development of TA. Finally, to test whether our results can be generalized, we tested whether CMV infection at the time of acute rejection resulted in enhanced TA in other ‘weak combinations’. However, to our surprise, both other combinations tested failed to show the CMV effect on the development of TA, even after sublethal irradiation of the recipients prior to transplantation and infection. Obviously, there are more factors involved in determining whether CMV enhances development of TA, than the timing of infection and the ‘weakness’ of TA.

Materials and Methods

Animals

Specified pathogen free male PVG (RT-1\textsuperscript{a}), AO (RT-1\textsuperscript{a}), DA (RT-1\textsuperscript{a}), WF (RT-1\textsuperscript{a}), Lew (RT-1\textsuperscript{a}), and BN (RT-1\textsuperscript{n}) rats were obtained from Harlan (Zeist, the Netherlands). Lew and BN rats used for RCMV infection experiments were bred at the central animal facilities of the Maastricht University. The rats were 8-10 wk of age, maintained under clean conventional conditions, and were fed standard rat chow and acidified water ad libitum. All animals received humane care in compliance with the \textit{Principles of Laboratory Animal Care} (NIH Publication No.86-23, revised 1985) and the Dutch Law on Experimental Animal Care.

Aorta transplantation

Aortic allografts (10-12 mm) were transplanted as described previously\textsuperscript{18}. Briefly, the abdominal aorta between the left renal artery and the bifurcation was removed from the donor rat and perfused with saline to remove blood cells. Subsequently, the aortic graft was orthotopically transplanted into the recipient rat via end-to-end anastomosis using 9-0 nylon suture. Total ischemic time was consistently less than 30 minutes during which the grafts were kept in ice-cold saline. Transplantations were performed in the following MHC incompatible strain combinations: PVG to AO, DA to WF, BN to Lew, and Lew to BN. Isograft controls consisted of aortic grafts transplanted from BN to BN rats.
Experimental groups
To study whether the development of TA differs among different donor/recipient combinations (in the absence of RCMV), aortic transplants were performed in the strain combinations as listed in Table 1A. BN allografts transplanted in Lew recipient rats were explanted 4, 8, 12 and 24 weeks after transplantation (groups 1-4) and processed for histological analysis. In addition, aortic allografts were transplanted in the Lew to BN (group 5), PVG to AO (group 6) and DA to WF (group 7) strain combinations, and explanted 4 weeks after transplantation. BN isografts (group 8) served as controls and were sacrificed 12 weeks after transplantation.

To study the effect of timing of RCMV infection on the development of TA, recipient rats received 5 Gy total body irradiation (TBI) 6 hours before administration of virus to promote viral replication. Recipient rats were divided into 6 different treatment groups (groups 9-14) as listed in Table 1B. Allograft recipients were mock-infected (group 9) or RCMV-infected shortly after transplantation (day 1 or day 1+5; groups 10 & 11) or late after transplantation (day 21 or day 21+25; groups 12 & 13). In addition, one group of recipient rats was infected 21 days prior to transplantation (group 14) to study the effect of chronic RCMV infection of the host on the development of TA.

To study whether RCMV is transferred after aortic transplantation and grafts were removed and processed for histological analysis and virus detection.

Finally, we studied whether RCMV infection influences TA development in other combinations than the BN to Lew strain combination (Table 1C). Therefore, aortic transplantation and subsequent RCMV infection (with or without TBI) was performed in the PVG to AO (groups 18-21) as well as the DA to WF (groups 22 & 23) strain combination. Grafts were removed at 12 weeks after transplantation and processed for histological analysis.

Infection with RCMV
RCMV infection was performed by intraperitoneal (i.p.) injection of $3 \times 10^5$ plaque forming units (pfu) RCMV (Maastricht strain). The virus-pool used consisted of a salivary gland homogenate which was prepared as described elsewhere. Most recipients that were RCMV-infected were sublethally irradiated (5 Gy) 1 day after aortic transplantation. This method of immune suppression is standard for the animals used in our infection experiments and is routinely used to enhance the replication and dissemination of RCMV in adult rats. To analyze whether irradiation is a prerequisite for RCMV-enhanced TA development, recipients from groups 18, 20, 22, and 23 were not irradiated before infection (Table 1C).

Transfer of infectious virus by aortic allografts
To study whether aortic allografts carries the risk to transfer RCMV to recipient rats resulting in systemic RCMV infection of the host, a sensitive infection transfer assay was used as described elsewhere. Donor BN rats were sublethally irradiated.
Table 1. Groups used for aorta transplantation to study the effect of recipient genotype, RCMV infection of the recipient, and transfer of RCMV from the donor on the development of transplant arteriosclerosis.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Graft donor</th>
<th>Graft recipient</th>
<th>5 Gy TBI recipient</th>
<th>RCMV donor b</th>
<th>RCMV recipient b</th>
<th>Day of infection c</th>
<th>Sacrifice d</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>BN</td>
<td>Lew</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>BN</td>
<td>Lew</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>BN</td>
<td>Lew</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>BN</td>
<td>Lew</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Lew</td>
<td>BN</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>PVG</td>
<td>AO</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>DA</td>
<td>WF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>BN</td>
<td>BN</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>B</td>
<td>9</td>
<td>BN</td>
<td>Lew</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>1 (mock)</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>BN</td>
<td>Lew</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>BN</td>
<td>Lew</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>1 &amp; 5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>BN</td>
<td>Lew</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>BN</td>
<td>Lew</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>21 &amp; 25</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>BN</td>
<td>Lew</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-21</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>BN</td>
<td>Lew</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>BN</td>
<td>Lew</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-10</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>BN</td>
<td>Lew</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-21</td>
<td>7</td>
</tr>
<tr>
<td>C</td>
<td>18</td>
<td>PVG</td>
<td>AO</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>PVG</td>
<td>AO</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>PVG</td>
<td>AO</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>PVG</td>
<td>AO</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>DA</td>
<td>WF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>DA</td>
<td>WF</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>1</td>
<td>12</td>
</tr>
</tbody>
</table>

a number of animals analyzed

b animals were infected with 3x10^5 plaque forming units (pfu) RCMV salivary gland homogenate

c animals were non-infected (-) or infected before or after transplantation (day 0)
d weeks after transplantation

(5 Gy) followed by i.p. injection of 3x10^5 pfu RCMV. After 5, 10, and 21 days post infection, aorta allografts were transplanted into sublethally irradiated Lew recipient rats. At the time of transplantation, parts of the donor aorta and salivary glands were removed and processed for RCMV detection (plaque assay, immunohistochemistry and PCR analysis). Recipient rats were sacrificed 7 weeks after transplantation. Salivary glands and aortic tissue (both the graft and autologous
part) were removed and processed for RCMV detection.

**Detection of infectious virus by plaque-assay**

Presence of infectious virus was quantitated using a plaque-assay as described previously. Salivary glands and aortic tissue (graft as well as autologous part) were suspended in EMEM (supplemented with 2% fetal calf serum (FCS), 2 mM L-glutamine, 1% non-essential amino acids (all obtained from Gibco BRL, Life Technologies B.V., Breda, The Netherlands) and 0.1 mg/ml gentamycin (Centrafarm B.V., Etten-Leur, The Netherlands) in a 10% weight/volume ratio. Tissues were homogenized and sonicated, and subsequently 10- and 100 fold dilutions of the supernatants were inoculated on a confluent rat embryonic fibroblast monolayer in 24-well tissue culture plates (Greiner B.V., Alphen a/d Rijn, The Netherlands). After 7 days of incubation in culture medium containing 0.25% agarose (Seakem ME, FMC Bioproducts, Rockland, Maine, USA), cells were fixed in 10% PBS buffered formalin and stained with methylene-blue. The number of plaques was quantitated microscopically, and the virus titer was calculated and expressed as plaque forming units (pfu).

**Detection of RCMV antigens**

For detection of RCMV antigens, 4 µm formalin-fixed paraffin embedded tissue sections were stained with RCMV monoclonal antibody (mAb 8). Briefly, after deparaffination tissue sections were incubated with mAb8 for 60 minutes. Subsequently, sections were incubated with a second step alkaline-phosphatase-conjugated rabbit anti-mouse antibody (DAKO A/S, Glostrup, Denmark) for 30 minutes. The chromogen Fast Red was applied for 30 minutes, and subsequently sections were counterstained with Mayer’s hematoxylin, dehydrated and coverslipped.

**Detection of RCMV DNA**

To determine the presence of viral DNA, salivary glands and aortic tissue were cut in small pieces and stored at -80°C until analysis. After DNA extraction, a semi-quantitative nested PCR was performed on the RCMV DNA polymerase gene (GenBank accession no. U50550). Total cellular DNA was extracted from the salivary glands and aorta with a DNA extraction kit (Gull Laboratories, Salt Lake City, Utah, USA). The DNA samples were serially diluted from $10^0$ to $10^{-8}$ µg. Each of the diluted DNA samples was incubated for 10 min. at 95°C, immediately cooled on ice, and subjected to a two round (nested) PCR. The sequences of the primers as well as the PCR conditions are described elsewhere. The first and second round PCR resulted in DNA fragments of 536 bp and 431 bp, respectively. The PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

**Quantitation of transplant arteriosclerosis**

Grafts removed at autopsy were fixed in 10% PBS buffered formalin and embedded in paraffin. Tissue sections (4 µm) were taken from the center of each graft and were stained with Lawson solution (Klinipath, Duiven, The Netherlands) to visualize elastin fibers. TA was quantitated using a computerized morphometric analysis system (QWin Software,
Chapter 4

Leica Microsystems B.V., Rijswijk, The Netherlands). Presence of TA was expressed as the surface neointima/surface media ratio.

Statistical analysis
To analyze differences in the ratio surface neointima/surface media for statistical significance, the Kruskal-Wallis One-Way ANOVA was performed. If this test revealed a significant difference, a Mann-Whitney U test was performed to analyze differences between subgroups. Differences were considered to be statistically significant when P-values were <0.05.

Results

TA development in different strain combinations
Development of TA after aortic transplantation was analyzed in different donor/recipient combinations (Table 1A). The development of TA was quantitated 4 weeks after transplantation and the results are shown in Figure 1A. Of the four strain combinations analyzed, three developed weak TA, whereas one developed strong TA. BN allografts transplanted into Lew recipients (group 1) resulted in development of only weak TA. In contrast, Lew recipients (group 1) resulted in development of only weak TA. In contrast, Lew allografts transplanted into BN recipient rats (group 5) showed a four-fold increase in the amount of TA compared to Lew recipients (P<0.05). Although slightly higher compared to Lew recipients, also AO recipients of PVG allografts (group 6) and WF recipients of DA allografts (group 7) showed weak TA 4 weeks after transplantation (P<0.05 vs. Lew to BN). Since Lew recipients developed only weak TA 4 weeks after transplantation, we analyzed whether there is a further increase in TA at later time points. Aortic allografts were transplanted in the BN to Lew combination, and grafts were removed 4, 8, 12 and 24 weeks after transplantation (n=4/time point, groups 1-4) and the amount of neointima formation was quantitated. BN isografts served as syngeneic controls (group 8) and were removed 12 weeks after transplantation (n=5). Figure 2 shows the development of TA (expressed as the ratio NI/media) at different time points after transplantation. One month after transplantation, BN allografts showed mild TA. An increase in the amount of TA is observed at 8 and 12 weeks after transplantation, reaching maximal TA 24 weeks after engraftment. In contrast to Lew recipients, BN recipients developed the similar amount of TA within 4 weeks after transplantation and no further increase in time was observed (data not shown). BN isografts hardly show any signs of TA 12 weeks after transplantation.

Effect of timing of RCMV infection on the development of TA
We recently showed that RCMV infection enhances development of TA only in strain combinations that develop weak TA after allogeneic aorta transplantation24. Figure 1A shows that Lew recipients develop weak TA after transplantation of BN allografts developing with time (Figure 2), whereas BN recipients are ‘high-responders’ and develop maximal TA al-
ready 1 month after transplantation. Therefore, the BN to Lew combination was used to study the effect of timing of RCMV infection on the development of TA. Recipient rats were mock-infected (group 9) or infected with RCMV 1 day (group 10) or 21 days (group 12) after transplantation. In addition, one group of rats was infected 21 days prior to transplantation (group 14). Grafts were removed 7 weeks after transplantation and the amount of neointima formation was quantitated (Figure 3A). Only infection shortly after transplantation (day 1, group 10) showed a significant increase (P<0.05) in neointima formation com-

---

**Figure 1.** Development of TA (1 month after transplantation) after allogeneic aorta transplantation in different rat strain combinations. (A) Neointima formation is expressed as the ratio surface neointima/surface media. (* P<0.05 vs. BN to Lew, PVG to AO, and DA to WF combinations, Mann-Whitney U test). Representative photomicrographs after Lawson-staining (elastin) of aortic allografts transplanted in the (B) BN to Lew and (C) Lew to BN strain combinations. Grafts were explanted 4 weeks after transplantation. Abbreviations: a, adventitia; iel, internal elastic lamina; l, lumen; m, media; ni, neointima. Magnification x50
pared to mock-infection. Infection 21 days before (group 14) and 21 days after (group 12) transplantation did not result in increased neointima formation. To analyze whether the absence of increased neointima formation in animals infected 21 days after transplantation was due to a low viral load, Lew recipients were infected twice shortly (days 1 & 5, group 11) or late (days 21 & 25, group 13) after transplantation. Again, only infection shortly after transplantation resulted in increased neointima formation 7 weeks after transplantation (ratio neointima/media: 1.68±0.14 (group 11) vs. 1.17±0.26 (group 13) and 1.11±0.25 (group 9), P<0.05). These results indicate that the viral load is not responsible for the differences observed, and that RCMV infection enhances TA only when infection is given around the time of transplantation.

**Effect of RCMV infection of the donor on the development of TA**

Since primary CMV infection after transplantation of an organ from a seropositive donor into a seronegative recipient has been associated with an increased risk for the development of TA, we analyzed

![Figure 2](image.png)

**Figure 2.** Kinetics of the development of TA after allogeneic aorta transplantation in the BN to Lew strain combination. Neointima formation is expressed as the ratio surface neointima/surface media.

### Table 2

<table>
<thead>
<tr>
<th>Transplantation (days p.i.)</th>
<th>Donor (BN)</th>
<th>Recipient (Lew) 7 weeks post transplantation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IHC</td>
<td>Plaque-assay</td>
</tr>
<tr>
<td></td>
<td>aorta</td>
<td>SG</td>
</tr>
<tr>
<td>5</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>10</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>21</td>
<td>0/2</td>
<td>0/2</td>
</tr>
</tbody>
</table>

(Abbreviations: IHC, RCMV specific immunohistochemistry; p.i., post infection; SG, salivary gland)
Cytomegalovirus-enhanced development of transplant arteriosclerosis

whether reactivation of RCMV transferred by the graft enhances neointima formation after transplantation. We first analyzed the presence of virus in aortic tissue at several time points (5, 10, and 21 days) after infection. Moreover, transfer of infectious virus after transplantation, resulting in systemic infection of the recipient, and its possible effect on the development of TA was analyzed. BN donors were infected with RCMV and 5, 10, and 21 days p.i. aortic allografting was performed into irradiated Lew recipient rats (groups 15, 16, and 17, respectively). At the time of transplantation, also parts of (non-transplanted) donor aorta and salivary glands were removed and processed for virus detection. Aortic grafts (BN) as well as autologous recipient aorta (Lew) and salivary glands were removed 7 weeks after transplantation and processed for virus detection and neointima formation. Results of RCMV-specific immunohistochemistry (mAb8) and plaque-assay are summarized in Table 2. Using specific immunohistochemistry RCMV infected cells could not be detected in donor aortic tissue and salivary glands 5, 10 and 21 days post infection. However, results from the plaque-assay indicated presence of infectious virus in aortic tissue 5 and 10 days after infection (1/4 animals at both time points). In the salivary glands of the donor-rats infectious virus was present in all animals analyzed 10 and 21 days post infection. These data indicate that aortic tissue of some of the animals did contain infectious virus relatively shortly after infection (5 and 10 days p.i.). Three weeks after infection, RCMV could not be detected in aortic tissue by immunohistochemistry and plaque-assay, whereas the salivary glands were RCMV positive. Although RCMV could not be detected by plaque-assay in aortic tissue transplanted 5 or 10 days post infection in most animals, transplantation of these grafts resulted in transmission of virus to the recipient. Seven weeks after transplanta-
Figure 4. Transfer of RCMV after transplantation of an aortic allograft 5 days after infection of the donor. (A) HE staining of recipient salivary gland 7 weeks after transplantation, and (B) mAb8 (RCMV) staining of recipient salivary gland 7 weeks after transplantation. Arrowheads indicate RCMV infected ductal epithelial cells. Magnification x400. (C) Nested RCMV PCR performed on aortic and salivary gland tissue of the donor at the time of transplantation, and performed on the aortic graft and salivary gland tissue of the recipient 7 weeks after transplantation. Grafts were transplanted 5, 10, and 21 days after infection of the donor with RCMV.
Cytomegalovirus-enhanced development of transplant arteriosclerosis

6/6 and 3/4 recipient rats (transplanted 5 and 10 days p.i., respectively) showed signs of systemic infection as indicated by the presence of infectious virus in their salivary glands. Figures 4A and B show representative photomicrographs of RCMV infected cells in salivary glands of recipient rats transplanted with aortic allografts 5 days after infection of the donor. Twenty-one days p.i. donor aortic tissue did not contain detectable levels of RCMV, whereas donor salivary glands contained infectious virus as determined by the plaque-assay. After transplantation, no virus could be detected in the recipient salivary glands and aortic tissue (graft and autologous part) suggesting that no virus transmission had occurred. However, since RCMV specific immunohistochemistry as well as plaque-assay are relatively insensitive techniques to detect virus infected cells, a PCR-based approach was followed to detect viral DNA in aortic tissue and salivary glands after transplantation.

RCMV specific nested PCR was performed on donor (aorta and SG) and recipient (autologous aorta, aorta graft, and SG) tissues. The results obtained by the nested PCR confirmed the results obtained by IHC and plaque-assay and are shown in Figure 4C. Five and ten days p.i., viral DNA could be detected in donor aortic tissue, whereas the donor salivary glands did not contain detectable levels of viral DNA. Twenty-one days p.i., however, no viral DNA could be detected in donor aortic tissue, whereas at this time point viral DNA was present in the donor salivary glands. After transplantation, viral DNA could be detected in the recipient salivary glands only when transplantation was performed at 5 and 10 days p.i.. When transplanted 21 days p.i., no viral DNA could be detected in the recipient salivary glands 7 weeks after transplantation. This indicates that indeed no viral transmission had occurred, confirming the results obtained by plaque-assay. BN donors were infected 21 days prior to transplantation (group 17) and after 7 weeks, grafts were removed and the amount of neointima formation was quantitated. As expected, infection of the donor 21 days prior to transplantation did not enhance neointima formation compared to mock-infection (ratio neointima/media: 1.10±0.18 (group 17) vs. 1.11±0.25 (group 9)), since we showed that 21 days p.i. no detectable amounts of virus were present in aortic tissue, and no viral transmission was observed after transplantation. Although we clearly showed effective viral transmission after transplantation of aortic allografts 5 or 10 days p.i., no enhancing effect on the development of TA was observed after transplantation (data not shown). One explanation is that the amount of virus which is being transferred from the aortic allograft to the recipient at the beginning of the rejection response is not sufficient to induce this enhancing effect.

Effect of RCMV infection on the development of TA in other strain combinations

RCMV infection enhances neointima formation after aortic allografting in the BN to Lew rat strain combination only when RCMV is administered shortly after transplantation (day 1 and/or 5 post transplantation), indicating that active RCMV infection and acute rejection should be present simultaneously. To study whether this effect can be generalized and is also
present in other strain combinations that show weak TA in the absence of RCMV, we performed similar experiments in the MHC incompatible PVG to AO (groups 19-21) and DA to WF (groups 22 & 23) strain combinations. In the PVG to AO combination, we also tested whether irradiation of the recipient 6 hr before infection would facilitate the possible RCMV mediated increase in TA development. Therefore, recipient rats were left untreated (group 18) or received 5 Gy TBI (group 19). Two other groups included RCMV infected recipient rats without (group 20) or with (group 21) 5 Gy TBI. Grafts were removed at 12 weeks after transplantation and the surface neointima was quantitated. Results are shown in Figure 3B. In contrast to RCMV infection after transplantation in the BN to Lew strain combination, RCMV does not enhance neointima formation in the PVG to AO combination.

In the DA to WF combination, we also observed development of weak TA 4 weeks after transplantation. To investigate the effect of RCMV infection on the development of TA in this particular strain combination, DA aortic allografts were transplanted into WF recipient rats which were left untreated (group 22) or RCMV infected (group 23) 1 day after transplantation. Grafts were removed 12 weeks after transplantation and neointima formation was quantitated. Results are shown in Figure 3B. Also in this strain combination, RCMV infection did not result in enhanced neointima formation.

**Discussion**

Chronic transplant dysfunction (CTD), histologically characterized by obliteration of the graft vasculature (i.e., transplant arteriosclerosis, TA), is the primary cause of allograft loss after the first perioperative year and is today’s most important problem in clinical organ transplantation. The etiology of TA is multifactorial and alloantigen-dependent as well as independent risk factors have been identified. Cytomegalovirus (CMV) infection is one of the alloantigen-independent factors associated with increased risk for the development of TA. The role of CMV infection in the process of TA development has been studied after human solid organ transplantation, but results are conflicting. Using the aorta transplant model in rats, we and others showed that Rat Cytomegalovirus (RCMV) infection results in enhanced perivascular inflammation as well as in enhanced neointima formation, indicating that also in this animal model CMV enhances the development of TA. Several studies suggest that CMV enhances development of TA only when active CMV infection and acute rejection are present simultaneously. In the experimental transplant models, recipient rats generally are infected 1 day after transplantation, indeed suggesting that active infection and the beginning rejection response should be present simultaneously for CMV-enhanced development of TA.

In this study we analyzed whether active infection at the time of acute rejection is indeed a prerequisite for the enhancing effect on TA after allogeneic aorta transplantation in rats, and therefore the timing of RCMV infection on the develop-
ment of TA was studied. However, since our previous results suggest that CMV only enhance TA in rat strain combinations with weak TA development after allogeneic aorta transplantation, we first analyzed the development of TA in several donor/recipient combinations in the absence of RCMV infection. Lew recipients of BN grafts developed weak TA 4 weeks after transplantation, and reached maximal neointima formation 24 weeks after transplantation. In contrast, in the reciprocal Lew to BN combination maximal neointima formation was already observed 4 weeks after transplantation, indicating that Lew rats are ‘low-responders’ whereas BN rats are ‘high-responders’. Similar observations were reported by Geerling et al. Also AO and WF recipient rats showed development of weak TA compared to BN recipients. Recently we reported that the rate of development of TA is for a major part determined by the recipient genotype. Such differences might also explain the differences in TA development found in this study. Similar genetic differences might actually also contribute to the variety in the incidence and severity of CTD and TA observed among human transplant recipients.

Of the four strains tested, Lew recipient rats showed the weakest development of TA 4 weeks after transplantation, followed by AO and WF recipients. Therefore, we used the BN to Lew strain combination to study the effect of timing of RCMV infection on the development of TA. Lew recipient rats were infected with RCMV 21 days before or 1 day or 21 days after transplantation. Only infection shortly after transplantation (1 day) resulted in enhanced neointima formation. These results suggest that indeed active infection and acute rejection should be present simultaneously to get enhanced neointima formation, resembling clinical data on the effect of CMV on CTD. Moreover, these results suggest synergistic effects of CMV infection on the developing alloresponse shortly after transplantation as the underlying mechanism for CMV enhanced development of TA.

In clinical organ transplantation, primary CMV infection after transplantation of a seropositive donor in a seronegative recipient increases the risk for CTD and TA. To mimic this situation, transplantation of a graft from an infected donor into an uninfected recipient was performed. We analyzed whether also in this situation CMV enhanced neointima formation could be observed. Infection of the donor 3 weeks before transplantation did not result in increased neointima formation. Since analysis of donor aortic tissue by RCMV specific immunohistochemistry, plaque-assay and RCMV-specific PCR revealed that 3 weeks after infection no virus is present anymore in aortic tissue, it is not surprising that transplantation of an aortic allograft from a RCMV infected donor 3 weeks after transplantation did not show enhanced neointima formation. The course of RCMV infection can be divided in 3 distinct phases: acute phase (1-2 weeks p.i.), chronic phase (3-12 weeks p.i.) and latent phase (>12 weeks p.i.). In the acute phase several internal organs can be infected (e.g., kidney, liver, heart), whereas in the chronic phase infectious virus can only be detected in the salivary glands. Indeed, the donor salivary glands contained infectious virus 3 weeks after infection. In contrast to aortic (and cardiac) allografts, how-
ever, kidney and lung allografts can effectively transfer virus to the recipient rats, also in the chronic phase of infection. Even in the latent phase, kidney allografts have been shown to effectively transfer RCMV to the host.

We also transplanted allografts 5 and 10 days after infection (i.e., in the acute phase of infection). Plaque-assay as well as nested PCR analysis showed presence of virus in aortic tissue, and allografting resulted in systemic infection of virtually all recipient rats. Although RCMV can be effectively transferred by aortic allografts if transplanted within 10 days after infection of the donor, the development of TA was not enhanced 7 weeks after transplantation. A possible explanation is that the viral load is not sufficient during the acute rejection episode to enhance the development of TA. Alternatively, the acute infection phase is somewhat delayed after transplantation of an infected aortic allograft compared to intraperitoneal administration of high dosages of RCMV. In this situation, acute rejection and acute infection are not present simultaneously and consequently, RCMV does not enhance the development of TA.

Finally, we tested whether our results in the BN to Lew strain combination could be generalized. Therefore, aortic transplantation in two other strain combinations, both showing weak TA in the absence of RCMV, was performed. Recipient rats were mock- or RCMV infected 1 day after transplantation. However, to our surprise, in both the PVG to AO and DA to WF rat strain combinations, RCMV infection did not result in enhanced neointima formation.

The effect of RCMV on the development of TA after allogeneic aorta transplantation was for the first time described in the DA to WF rat strain combination by Lemström et al. We attempted to reproduce these data, however RCMV infection did not result in enhanced neointima formation 12 weeks after transplantation. Although we used the same strain combination and the same RCMV strain (Maastricht strain), we were not able to reproduce the data as described previously. Possibly, minor genetic differences between the rats used in our study and the rats used in studies described by Lemström et al. may account for this. In addition, differences in the microbiological status of the rats used in the different studies may also have influenced the experimental outcome.

In conclusion, this study shows that the development of TA after allogeneic aorta transplantation differs between different rats strains. The kinetics and severity of TA are primarily determined by the recipient genotype. RCMV infection enhances the development of TA only when active RCMV infection and acute rejection are present simultaneously, favoring synergy between the alloreponse and CMV infection as the underlying mechanism. This RCMV effect, however, cannot be generalized, indicating that obviously there are more factors than time of infection and recipient-genotype, determining RCMV enhanced development of TA.

Acknowledgements
The authors thank Peter Kelderman and Ming Yin for performing aorta transplantations, and Prof. Paul Nieuwenhuis for carefully reading the manuscript and helpful discussion.
Cytomegalovirus-enhanced development of transplant arteriosclerosis

References


