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Effects of cytomegalovirus infection and prolonged cold ischemia on chronic rejection of rat renal allografts

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Abstract Previous studies have demonstrated that both cytomegalovirus (CMV) infection and prolonged cold ischemia of the allograft (CI) are associated with chronic rejection of renal transplants. The purpose of this study is to investigate the effect of CMV infection, of CI and of the combination of both, on the progression of chronic rejection, and to obtain a more detailed insight in their effects on the expression of adhesion molecules. Therefore, a rat transplantation model was used. Lewis recipients of renal allografts (with and without CI) from MHC-incompatible Brown Norway rats were inoculated with rat CMV or left uninfected. CMV infection alone resulted in an increased influx of CD4+ cells and macrophages early after infection, and in an increase in glomerular sclerosis and intima proliferation. CI caused an increase in infiltrating NK cells and an effect on intimal proliferation, glomerular sclerosis, and tubular atrophy. When CMV infection and CI were combined, an additive effect could be measured. This was however not the case for the function of the kidney. The creatinin showed a synergistic effect of the two influencing factors. Due to the CMV infection, an increase in CD49 d cells was detected. CI resulted in an increase in CD18 cells and an increase in the expression of CD62P on vessels, and CD54 and CD44 on tubules. When CMV infection and CI were combined, all the effects caused by CMV and CI alone were present in an additional way.
The results of the present study suggest that special attention should be paid to the recipient of an ischemically injured graft when either the donor or the recipient is CMV-infected. The patterns seen in histology, the infiltration of leukocytes and the expression of adhesion molecules, suggest that CI and CMV infection both have an effect on rejection, but act by different mechanisms.

Key words Kidney transplantation · Rat · Chronic rejection · Cytomegalovirus · Adhesion molecules

Introduction

With the current immunosuppressive regimen, the incidence of graft loss due to acute rejection has been dramatically reduced. However, the expected half-life of the allograft has remained the same. This late allograft loss is mainly due to transplant arteriosclerosis, or so called "chronic rejection" [7]. In renal allografts, chronic rejection has been defined as progressive functional deterioration occurring months or years after engraftment, and has proved resistant to therapeutic attempts. Although the pathogenesis of chronic rejection still re-

mains largely unknown, several risk factors have been implicated, e. g. repeated episodes of acute rejection, viral infection, ischemia/reperfusion injury, and loss of functional mass [1, 33].

In immunocompromised transplant recipients, cytomegalovirus (CMV) infection is the most common and important viral infection [10, 12]. There is evidence of CMV infection in up to two thirds of all recipients, although clinical disease does not occur in all the infected patients [23]. Unfortunately, CMV infection has been found to be associated with the development of chronic rejection both clinically and experimentally [10, 17, 19]. Donor kidneys usually undergo a period of warm and cold ischemia followed by reperfusion. Ischemia/reperfusion injury has also been found to play a role in chronic rejection [1, 10, 18].

In rejection, leukocytes play an important role. For the leukocytes to enter the allograft, expression of adhesion molecules on both leukocytes and endothelial cells is necessary. Expression on other cells of the allograft can facilitate the interaction between leukocytes and their target cells, and thereby contribute to rejection of the allograft [5, 6, 30]. A very promising method of preventing allograft rejection is treatment with monoclonal antibodies to various cell adhesion molecules [13, 42, 43]. These monoclonal antibodies inhibit the interaction between adhesion molecules, and can thereby prevent the leukocytes to enter the allograft. To be able to optimize this therapy, the influence of multiple factors on the expression of adhesion molecules has to be investigated.

It has previously been described that kidney transplantation causes upregulation of the expression of adhesion molecules such as CD54 (ICAM-1), CD62 E (E-selectin, ELAM-1), CD106 (VCAM) and LFA-1 [2, 16, 21]. In-vitro studies have shown that CMV is able to induce upregulation of CD54, LFA-3 and CD44 (Pgp-1, HCAM) [8, 15, 27, 29, 37, 40]. In vivo, the CD54 expression in transplanted kidneys of rat CMV (RCMV)-infected animals is increased, compared to the allografts of non-infected animals [17]. Cold ischemia (CI) followed by reperfusion causes an upregulation in CD62P and E-selectin early after transplantation (< 24 h) [32, 45]. At 16 and 52 weeks after transplantation, CD54 is upregulated in kidneys subjected to 30 min of cold, and 30 min of warm ischemic time [34]. However, it is not known whether prolonged cold ischemia together with CMV infection increases the expression of adhesion molecules and thereby increases the severity of chronic rejection.

The purpose of this study is twofold: 1. To determine whether CMV infection and CI have an additional effect on the development of chronic allograft rejection. 2. To obtain a more detailed insight into the effect of CMV infection and CI on the expression of adhesion molecules in the transplanted organ.

Materials and methods

Animals and surgery

All experiments were performed in accordance with the law governing the protection of animals. The 'Principles of laboratory animal care' (NIH publication No.85–23, revised 1985) were followed. Specified pathogen free (SPF, according to the recommendations of the Federation of European Laboratory Animal Science Association) [22] male inbred rats, weighing 240–260 g, obtained from the Central Animal Facility of the Maastricht University, were used in all experiments. Lewis (LEW; $RT1^l$) rats were used as graft donors and Brown Norway (BN/M; $RT1^n$) rats as recipients. The animals were housed under standardized conditions, fed with commercially available pellet diet, and had free access to acidified demineralized water (pH ± 3).

Orthotopic kidney transplantations were performed from Lewis to the MHC-incompatible Brown Norway rat strain using a technique described previously [44]. Briefly, the animals were anesthetized, the donor kidneys were harvested, cooled with UW (University of Wisconsin) solution (Dupont Pharma, USA), and transplanted into the recipient. The right native kidney was removed at the time of engraftment, and the left 10 days later. The allograft recipients were treated with cyclosporine A (CsA, 15 mg/kg per day, Novartis, Switzerland) subcutaneously for the first 10 days after engraftment, to suppress an initial acute rejection episode.

Experiment protocol

The recipient rats were randomly allocated into 4 groups ($n = 6$ per group). In the control group (CI-CMV-), the donor kidneys were transplanted immediately into recipients. In this group, the total period of cold ischemia varied from 35–45 min. The renal allograft recipient in the second group (CI-CMV+) received an intraperitoneal inoculation of 10^5 plaque forming units (PFU) of Maastricht stain RCMV [3] on the day of transplantation. In the third group (CI+CMV-), renal allografts were stored for 24 h in University of Wisconsin solution (UW solution) at 4 °C before transplantation. In the fourth group (CI+CMV+), the recipients received renal allografts with 24 h of CI, and were subsequently injected with RCMV. Rats were sacrificed on day 10 and 60 after transplantation. Tissue samples of the renal allografts and other organs (livers, spleens and salivary glands) were removed and processed either for frozen and paraffin sections, or for plaque assay.

Virus detection

For the detection of systemic virus infection, the salivary glands, spleen, and liver of the recipients were examined for the presence of infectious virus particles. The organs were homogenized in a tissue grinder and suspended in MEM with 2% foetal calf serum (FCS). Infectious virus particles were quantified by plaque assay [3]. For this, 10- and 100-fold dilutions of 10% homogenates (wt/vol) were inoculated on a confluent rat embryonal fibroblast monolayer. After 7 days of incubation under 0.25% agarose, the number of plaques was monitored microscopically, following fixation and methylene-blue staining. For detection of RCMV antigens, 4 μm-thick formalin-fixed paraffin-embedded tissue sections were stained with RCMV monoclonal antibody 8 [4].

Determination of serum creatinine

Blood samples were taken from the 60-day surviving rats at days 7 and 14 post transplantation and at sacrifice. Serum creatinine was measured to determine the function of the transplanted kidney. Normal levels of serum creatinine were obtained by measuring the blood samples taken a few days before transplantation.

Histological examination

Formalin-fixed and paraffin-embedded kidney specimens were stained with hematoxylin and eosin for routine histology, and with elastic van Giesen for evaluation of vascular changes. The general histological changes in renal allografts were assessed semi-quantitatively in both a longitudinal and a transversal section of each kidney. Different parameters were scored blindly from 0 to 3 (0 = normal, 1 = mild, 2 = moderate, 3 = severe changes), without knowledge of group assignment. The intensity of chronic changes in the transplants was then expressed as a single numerical figure by the chronic allograft damage index (CADI), which is the sum of 6 parameters: interstitial inflammation and fibrosis, glomerular mesangial matrix increase and sclerosis, vascular intimal proliferation, and tubular atrophy [14].

Immunohistochemistry

Four-micron-thick sections of frozen grafts were used for immunohistochemistry and stained with the two-layer indirect immunoperoxidase technique, using monoclonal antibodies (mAbs). MAbs against CD4 + lymphocytes (W3/25) were obtained from Sera-Lab (Crawley Down, UK); mAbs against major histocompatibility complex (MHC) class II (OX-6), CD54 (ICAM-1, clone 1A29) and the polyclonal antibody against CD62P (P-selectin, clone AK-6) were provided by Serotec (Wiesbaden, Germany); mAbs against monocyte/macrophages and dendritic cells (ED-1) were kindly supplied by Dr. Dijkstra (Department of Immunology, Free University, Amsterdam, The Netherlands); NK cell markers (3.2.3) were supplied by the Department of Pathology of the University of Leiden, The Netherlands. The mAbs directed against CD11a (LFA-α_L chain, clone WT.1), CD44 (Pgp-1, H-CAM, clone OX-49), CD49d (Integrin α_4 chain, clone MRα4–1) and CD18 (β_2 integrin, clone WT.3) were purchased from PharMingen (San Diego, USA). As second step labeling antibodies, rabbit-anti-mouse-HRP and goat-anti-rabbit-HRP (DAKO, Denmark) were used.

Quantification

Quantification of positive immunohistochemical staining for infiltrating leukocytes and MHC class II expression was done semi-quantitatively by scoring blindly the intensity of staining from 0–3 (0 = no visible staining, 1 = few cells with faint staining, 2 = moderate intensity with multifocal staining, 3 = intense diffuse staining of cells analyzed). The evaluation of intensity of positively-stained infiltrating leukocytes was focused on perivascular and interstitial areas. The analysis of expression of MHC class II antigens was focused on endothelial cells of arterioles. Immunohistochemical stainings of the adhesion molecules were evaluated blindly. The presence of positively-stained interstitial infiltrating leukocytes was determined by counting the number of positive cells per field of view (magnification 400 ×). Three fields were scored per section for the presence of CD18, CD49 and CD11a positive leukocytes. Expression of CD62P and CD54 on endothelial cells was determined by counting the number of positive vessels per field of view. In the cortex, three (CD62P, magnification 100 ×) or five (CD54, magnification 200 ×) fields were scored in each section. The expression of adhesion molecules on epithelial cells (CD54 and CD44) was scored by determining the percentage of positive tubules (magnification 200 ×), in three fields per section.

Statistics

All data were presented as mean ± SEM. The significance of the differences within one staining group was determined using the Kruskal-Wallis 1-way Anova. If this test revealed a significance, the differences between the groups were determined using the Mann-Whitney U-Wilcoxon Rank Sum W test. P-values < 0.05 were taken to be statistically significant.

Results

Systemic CMV infection in allograft recipients

The allograft recipients inoculated with RCMV did not show any clinical symptoms of CMV disease. Infectious RCMV was, using plaque assays, detected in salivary glands of all the CMV-inoculated recipients (CI-CMV+ and CI+CMV+) at days 10 and 60, indicating a systemic CMV infection. No RCMV could be detected in any organ of the non-infected animals (CI-CMV- and CI+CMV-). The allografted kidneys were all but one (CI+CMV+ group, day 10 post transplantation) negative in the RCMV immunohistochemical staining.

Function of renal allografts

Figure 1 shows the effects of CMV infection, prolonged cold ischemia and their combination on the levels of serum creatinine. The creatinine levels in the non-ischemic groups (CI-CMV- and CI-CMV+) remained constantly low throughout the entire experimental period (60 days), compared to the normal (pre-operative) values. A moderate elevation of serum creatinine was observed in the recipients of renal allografts that were subjected to prolonged cold ischemia (CI+CMV-). The CMV infected animals with ischemically injured renal grafts (CI+CMV+) demonstrated over 3-fold higher values than the groups 1 and 2 ($P < 0.05$).

Morphological changes in allografts

Effects of CMV infection and prolonged CI on the pathology scores of renal allografts 60 days after transplantation were shown in Table 1. Compared with the control group, CMV infection alone (CI-CMV+) caused marked vascular lesions, i.e. increased glomerular sclerosis and intimal proliferation in afferent and efferent glomerular arterioles. Prolonged cold ischemia alone

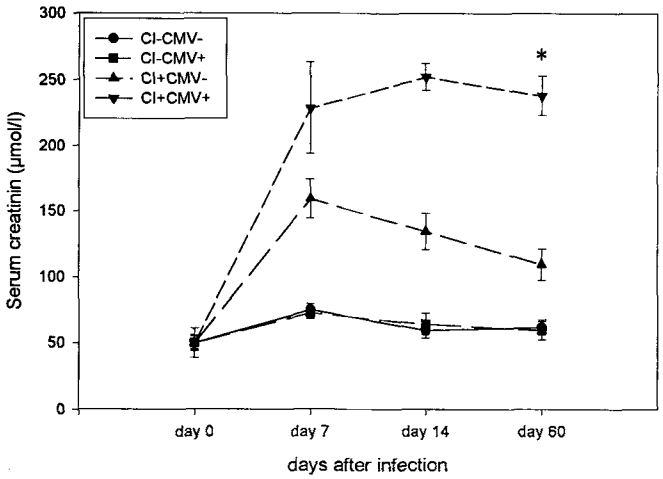

Fig. 1 Effect of CMV infection and prolonged cold ischemia on the level of serum creatinin in renal allograft recipients. Data are presented as mean ± SEM. * $P < 0.05$ compared to all other groups

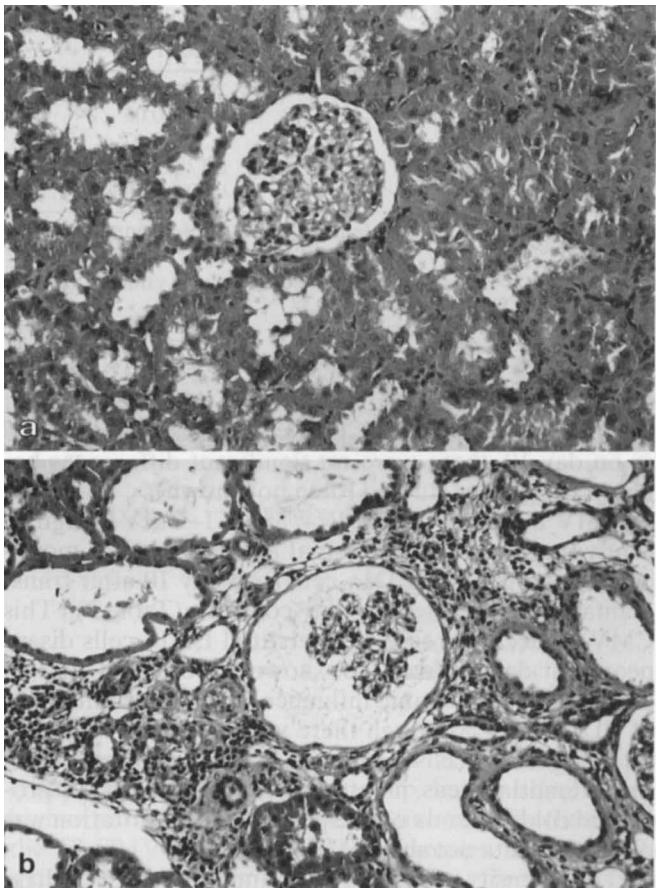

Fig. 2 Hematoxilin-eosin staining of a kidney from the CI-CMV+ group (**a**) and the CI+CMV+ group (**b**). In the CI+CMV+ group interstitial inflammation and fibrosis, glomerular sclerosis, intimal proliferation and tubular atrophy are found (original magnification 200 ×)

(CI+CMV-) resulted in a significant augmentation of interstitial fibrosis, glomerular sclerosis and tubular atrophy, leading to a dramatic increase of the CADI. The CI+CMV+ group exhibited significantly more interstitial fibrosis, glomerular sclerosis and tubular atrophy than the CI-CMV- and CI-CMV+ group, and demonstrated significant enhancement of interstitial inflammation comparing to the CI+CMV- group. As a result, the CADI of the CI+CMV+ group was increased approximately two-fold compared to the CI-CMV- group ($P < 0.05$, Fig. 2). The CADI scores of the CI+CMV+ group were also significantly increased compared to the scores of the CI-CMV+ and CI+CMV- group ($P < 0.05$, Fig. 2).

Infiltration patterns of inflammatory cells

CD4+ cells, monocytes/macrophages, and NK cells were observed in the renal allografts. The most prevalent infiltrating cell was the CD4+ cell, which was found predominantly in perivascular areas. CMV infection increased the perivascular accumulation of CD4+ cells on day 10 after transplantation, compared to the controls (Table 2), while prolonged cold ischemia alone did not influence the extravasation of CD4+ cells. Sixty

Table 1 The effects of CMV infection and prolonged cold ischemia on histological changes of renal allografts (*CADI* represents chronic allograft damage index)

	CI-CMV-	CI-CMV+	CI+CMV-	CI+CMV+
Interstitial inflammation	1.7 ± 0.2	1.9 ± 0.1	1.7 ± 0.1	2.2 ± 0.1***
Interstitial fibrosis	0.4 ± 0.2	0.4 ± 0.1	1.8 ± 0.1*, **	2.0 ± 0.2*, **, ***
Mesangial matrix increase	1.5 ± 0.1	1.4 ± 0.2	1.3 ± 0.1	1.5 ± 0.1
Glomerular sclerosis	0.1 ± 0.1	0.5 ± 0.1*	1.0 ± 0.1*, **	1.1 ± 0.1*, **
Intimal proliferation	1.3 ± 0.1	1.9 ± 0.1*	1.3 ± 0.2	1.9 ± 0.2*, ***
Tubular atrophy	0.6 ± 0.2	0.5 ± 0.1	1.9 ± 0.1*, **	2.0 ± 0.3*, **
CADI	5.6 ± 0.5	6.8 ± 0.5	8.9 ± 0.3*, **	10.7 ± 0.1*, **, ***

Data are presented as mean ± SEM
* $P \leq 0.05$ compared to the CI-CMV- group
** $P \leq 0.05$ compared to the CI-CMV+ group
*** $P \leq 0.05$ compared to the CI+CMV- group

Table 2 The effects of CMV infection and prolonged cold ischemia on infiltrating cells and MHC class II expression in renal allografts

	CI-CMV-	CI-CMV+	CI+CMV-	CI+CMV+
Perivascular CD4 + cells[a]	1.50 ± 0.02	1.92 ± 0.10*	1.70 ± 0.09	2.10 ± 0.10*
Interstitial macrophages[a]	0.50 ± 0.05	1.69 ± 0.11*	1.00 ± 0.20	1.41 ± 0.08*
Perivascular NK cells[a]	0.33 ± 0.17	0.58 ± 0.15	0.80 ± 0.12*	1.25 ± 0.11*, **
Interstitial NK cells[a]	0.17 ± 0.17	0.33 ± 0.10	0.70 ± 0.10*	1.00 ± 0.18*, **
MHC class II[b]	0.90 ± 0.30	1.58 ± 0.30*	0.20 ± 0.12	1.50 ± 0.39*

[a] The number of infiltrating leukocytes was determined at day 10 post-Tx
[b] MHC class II expression was scored on arterial endothelial cells at day 60 post-Tx

* $P \leq 0.05$ compared to the CI-CMV- group
** $P \leq 0.05$ compared to the CI-CMV+ group

days after transplantation, although the magnitude of perivascular invasion of CD4+ cells remained the same as on day 10, there were no significant differences between the groups studied (data not shown).

CMV infection (CI-CMV+ and CI+CMV+) significantly increased the interstitial accumulation of monocytes/macrophages (ED1+ cells), on day 10 after transplantation, compared to the controls (Table 2). This CMV-induced increase in interstitial ED1+ cells disappeared at day 60 (data not shown). Prolonged cold ischemia did not exhibit influence on the accumulation of ED1+ cells. Although there were relatively more infiltrating ED1+ cells in the perivascular regions than in the interstitial areas, no effect of CMV infection or prolonged cold ischemia on the perivascular infiltration was observed (data not shown).

The intensity of perivascular and interstitial infiltration of NK cells at 10 days was increased significantly by prolonged cold ischemia, and further exacerbated by the combination of CMV infection with prolonged cold ischemia (CI+CMV+), compared to the controls (Table 2). The differences between the groups could no longer be detected by day 60 (data not shown).

Expression of MHC class-II antigens

The scores of MHC class II antigens in arteriolar endothelium at day 60 after transplantation is shown in Table 2. CMV infection (CI-CMV+ and CI+CMV+) significantly increased the expression of MHC class II antigens. Interestingly, a down-regulated expression of the antigens was observed in the allografts with prolonged cold ischemia alone (CI+CMV-). There was no endothelial expression of MHC class II antigen in any experimental group at day 10 after transplantation.

CD11a, CD18 and CD49d expression on the interstitial infiltrating leukocytes

The influence of CMV infection, CI or both, on the number of leukocytes expressing CD11a, CD18 and CD49d was determined using immunohistochemistry. The number of CD11a (LFA α-chain)-positive cells present in the cortex (Fig. 3A)was not influenced by CMV infection or CI. The number of CD18-positive cells in the cortex at day 10 post transplantation was higher in the CI+CMV- and the CI+CMV+ groups compared to that of the CI-CMV- group. The number of positive cells in the CI+CMV+ group was also significantly higher compared to that of the CI+CMV- group. At day 60, these differences could no longer be seen (Fig. 3B).

At day 10 post transplantation, the number of CD49d (VLA-4α-chain) positive cells in the cortex was significantly higher in the CMV-infected groups (CI-CMV+, CI+CMV+) compared to that of the CI-CMV- group (Fig. 3C). At day 60, no differences in the numbers of CD49d positive cells were found between the groups.

In summary, CMV infection results in a significant increase of the CD49d-positive leukocytes, while CI results in an increase of CD18-positive leukocytes.

CD54 and CD62P expression on endothelial cells in the cortex

At day 10 and 60 post transplantation, no significant differences in the number of CD54-positive vessels could be detected between the different groups (Fig. 4A). However, the number of positive vessels at day 60 (2.7 ± 1.0) compared to that at day 10 (0.3 ± 0.3) was increased.

At day 10, no significant differences in the number of CD62P positive vessels could be detected between the different groups (Fig. 4B). However, at day 60 after transplantation, a moderate increase ($P < 0.05$) in the CI+CMV- compared to the CI-CMV- group, and a large increase ($P < 0.05$) in the CI+CMV+ compared to the CI-CMV- group and the CI-CMV+ group was detected.

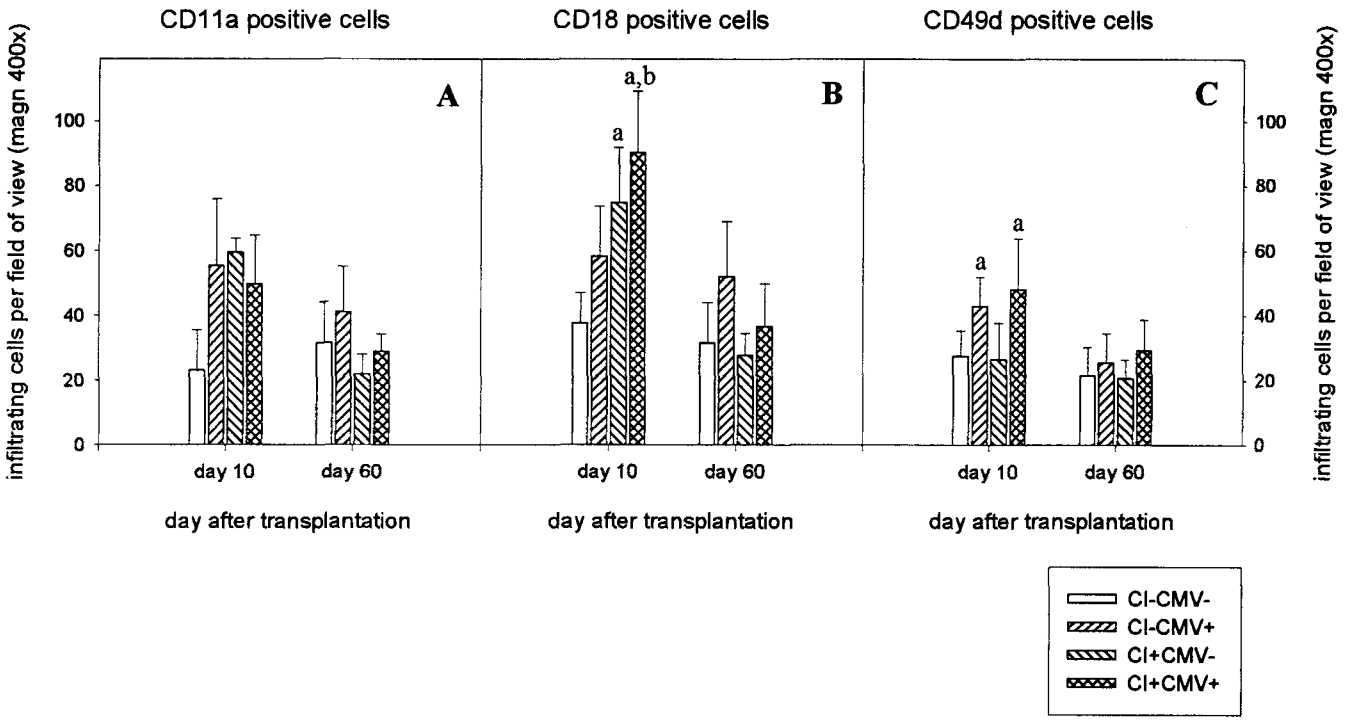

Fig. 3 A–C The number of infiltrating CD11 a, CD18 and CD49 d positive cells in the cortex of that allografted kidney at day 10 and 60 post transplantation was determined per field of view (magnification 400 ×). Per section three fields were scored. Data are presented as mean ± SEM. $a = P < 0.05$ compared to the CI-CMV- group; $b = P < 0.05$ compared to the CI-CMV+ group. **A** The number of CD11 a positive leukocytes is not influenced by CMV infection or CI. **B** At day 10 post transplantation CMV infection and CI have a synergistic effect on the presence of CD18 positive leukocytes. **C** CMV infection causes an increase in the number of CD49 d positive leukocytes in the allografted kidney

CD54 and CD44 expression on epithelial cells in the cortex

At day 10 after transplantation, there was no significant difference between the four groups in the percentage of CD54-positive tubules in the cortex (Fig. 5A). At day 60, the percentage of positive tubules was higher in the CI+ groups (CI+CMV- and CI+CMV+) compared to that of the CI-CMV- group ($P < 0.05$).

The percentage of CD44-positive tubules presented a similar pattern early and late after transplantation (Fig. 5B). At both time points, the CI-CMV+ group showed a small increase, the CI+CMV- group showed a moderate increase, and the CI+CMV+ group a large increase, in the percentage of positive tubules, compared to the CI-CMV- group. At day 10, the percentage of CD44-positive tubules in the CMV+CI+ group was significantly higher compared to that of all other groups. At day 60, the percentage in the CI+CMV- group was significantly higher compared to that of the CI-CMV- group, and the CI+CMV+ group had a significantly higher percentage of CD44 positive tubules than both the CI-CMV- and the CI-CMV+ groups. This suggests that CMV infection has a moderate effect and CI a stronger effect on CD44 expression. Thus, CI increases the expression of CD44 (day 10 and 60) and CD54 (day 60).

Discussion

The present study demonstrates that CMV infection and prolonged cold ischemia (CI) have additive effects on deterioration of the function of renal allografts. However, these histological effects were not the same for CMV infection and CI. CMV infection tended to induce vascular changes (intimal proliferation and glomerular sclerosis), while CI increased interstitial fibrosis, tubular atrophy, and glomerular sclerosis. Both CMV infection and CI also resulted in a different pattern in the expression of adhesion molecules. CMV infection led to an increase in the presence of CD49d-positive leukocytes, while CI increased the expression of CD18 on leukocytes, of CD54 and CD62P on tubules, and of CD44 on epithelium. The combination of CMV infection and CI resulted in an additional effect on the expression of the adhesion molecules.

The increased intimal proliferation or allograft arteriosclerosis by CMV infection has been found not only in kidney transplants, but also in cardiac, pulmonary,

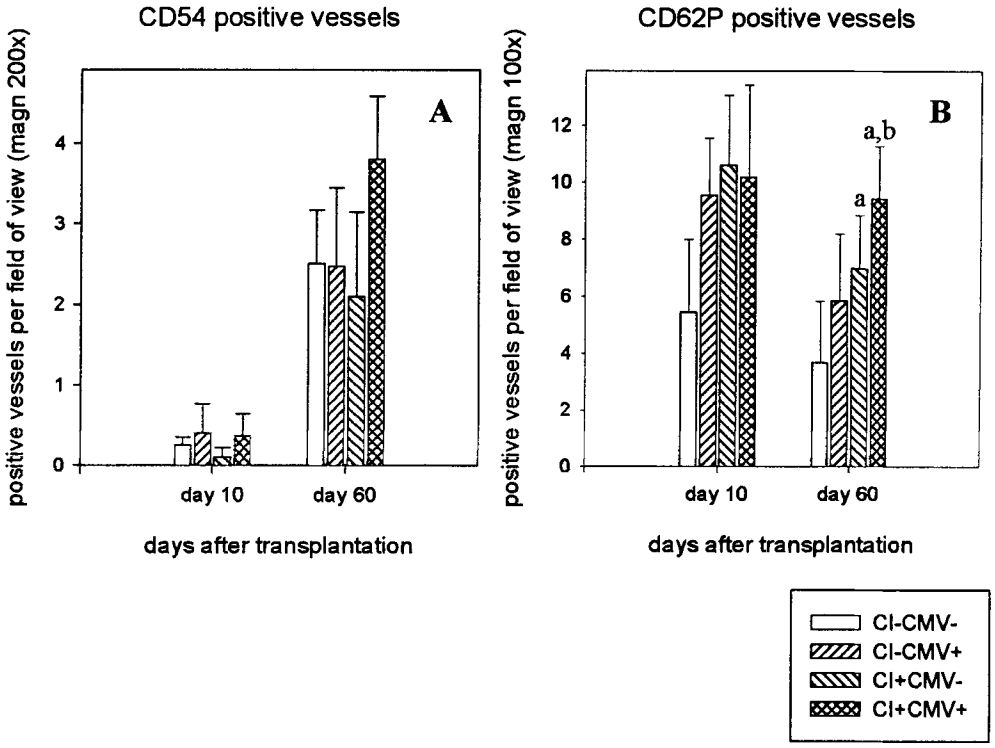

Fig. 4 A, B At day 10 and 60 post transplantation, the number of CD54 and CD62P positive vessels in the cortex of the allografted kidney was determined per field of view. For CD54, five fields were scored (magnification 200 ×), while for CD62P three fields per section were scored (magnification 100 ×). Data are presented as mean ± SEM. *a* = $P < 0.05$ compared to the CI-CMV- group; *b* = $P < 0.05$ compared to the CI-CMV+ group. **A** CMV infection and CI have no effect on the expression of CD54 on the endothelium of allografted kidneys. **B** At day 60 post transplantation, CMV infection and CI have a synergistic effect on the expression of CD62P on endothelium

and aortic allografts [10, 20, 31], suggesting that the effect of CMV on the allograft vasculature is a general phenomenon. It has been well established that T-cell mediated endothelialitis is the hallmark of vascular rejection [25] and that repeated episodes of acute rejection accelerate the development of chronic rejection [33]. It is, therefore, postulated that the accelerated transplant arteriosclerosis in CMV-infected recipients may be a process mediated by leukocytes that have been activated specifically by the early CMV infection. This hypothesis is supported, at least partly, by the fact that the early infiltration of CD4+ cells and macrophages was enhanced in the renal grafts of CMV-infected hosts. It is also possible that allograft endothelium harboring CMV has the potential to activate host cells, and that the consequent release of cytokines has the potential to raise surrounding endothelium to a fully activated, highly immunogenic state [24], which may promote the development of chronic rejection in allografts without active virus particles being present in the grafts as seen in this study [39]. However, previous studies have shown that, although 14 days after RCMV-infection of immunosuppressed rats, infectious virus particles and viral antigens could only be detected in the salivary gland, all tested organs (including the kidney) contained viral DNA [36]. Therefore, RCMV might be able to induce expression of adhesion molecules in the host cells without expressing detectable levels of viral antigen in these cells. Thus, the increased expression of adhesion molecules in CMV-infected animals can be either a direct or an indirect effect of CMV.

In vitro, human CMV (HCMV) is known to induce an increase in the expression of CD44 and CD54 on fibroblasts [8, 15], of CD54 and CD62 E on endothelial cells [27, 40] and of CD54 on proximal tubular epithelial cells [37]. In vivo, RCMV infection causes an increased CD54 expression on endothelial cells and an increased infiltration of CD11a and CD49d positive leukocytes in allografted lungs [31]. In a kidney transplantation model, RCMV infection significantly increased the CD54 expression on endothelial and tubular epithelial cells [17]. We could not detect a statistically significant effect of CMV infection on CD54 and CD11a, but the CD49d expression was increased in the CMV-infected groups at day 10.

Prolonged cold ischemia led to interstitial fibrosis, glomerular sclerosis, and tubular atrophy, the histological alterations typically seen in biopsies of renal allografts undergoing chronic rejection [1, 33]. It is known that the prolonged ischemia may cause the necrosis of tubular cells due to hypoxia and reperfusion injury early after transplantation [28]. This may cause loss of functional mass, a factor held responsible for the development of chronic rejection [33]. Endothelium represents the interface between the host immune system and allograft tissue [26], and endothelial injury plays an important role in the development of arteriosclerosis [24]. Endothelial injury caused by prolonged ischemia and

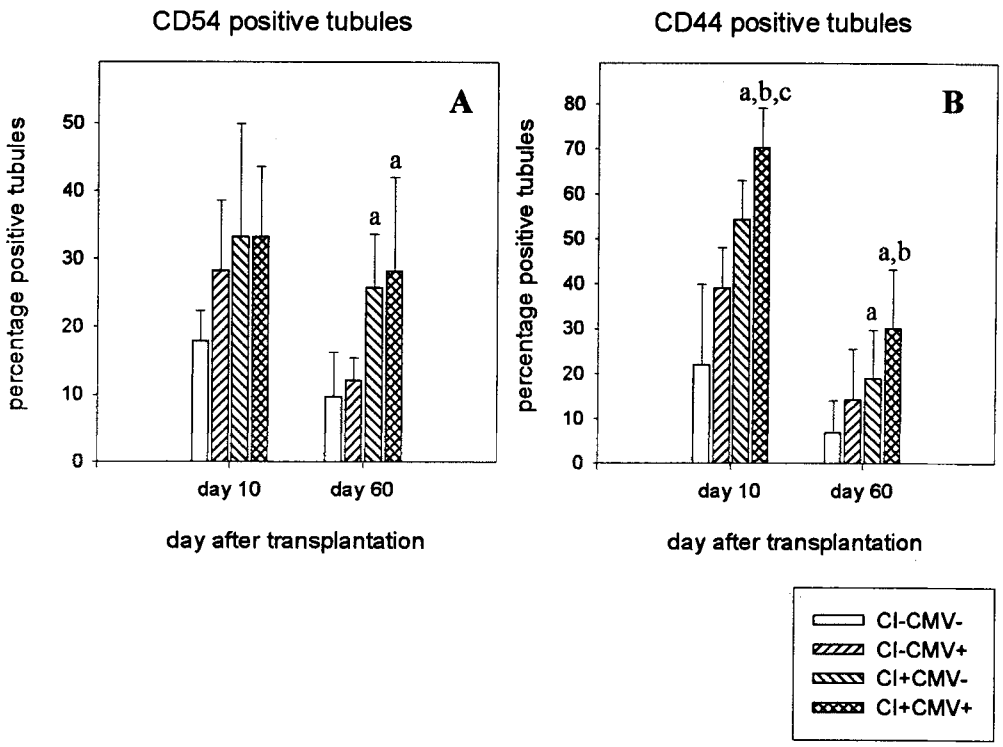

Fig. 5 A, B At day 10 and 60 post transplantation, the percentage of CD54 positive tubules in the cortex of the allografted kidney was determined using a magnification of 200 ×. Per section three fields were scored. Data are presented as mean ± SEM. $a = P < 0.05$ compared to the CI-CMV- group; $b = P < 0.05$ compared to the CI-CMV+ group; $c = P < 0.05$ compared to the CI+CMV- group. **A** CI increases the percentage of CD54 positive tubules in the allografted kidney. **B** CMV infection and CI have a synergistic effect on the expression of CD44 on epithelial cells

subsequent reperfusion may result in upregulation of adhesion molecules.

The recruitment of leukocytes from the circulation is regulated by adhesion molecules (reviewed in [5, 6, 30]). Since individual receptors often participate in multiple leukocyte-endothelial cell interactions, it is believed that leukocyte recruitment involves multiple steps. Previously, Butcher [5] described a three-step model for this process, in which the first step involves primary adhesion ('rolling'), the second step is activation of the leukocyte and the third step is an activation-dependent adhesion, resulting in stable binding of the leukocyte to the endothelium. The increased expression of adhesion molecules on the tubules might be considered as a 'fourth step', resulting in an increase of the efficiency of the immune reaction towards the target cells.

If we incorporate our data into the multiple step model, we can see that CI leads to an increase in the number of blood vessels expressing CD62P. The second step, activation of the leukocyte, can occur by a large number of factors like cytokines and chemoattractants that are not studied here, but are known to be upregulated by both CMV infection and CI [9, 38, 41]. In the third step, activation-dependent adhesion, the expression of adhesion molecules like LFA-1 and VLA-4 on leukocytes and CD54 on endothelium is involved. The molecule CD49d we detected is a part of the adhesion receptor VLA-4. The number of CD49d positive cells was increased in CMV-infected animals. An increased number of CD18 positive cells was detected in allografted kidneys of rats subjected to CI. Finally in the 'fourth step', interaction of the leukocyte with the target cells, the expression of CD54 and CD44 on the tubules might be important. CD44 and CD54 expression was increased in the CI+ groups compared to the CI- groups. Thus, CI causes an increase in adhesion molecule expression in all steps necessary, whereas CMV infection causes at least upregulations in the second and third step.

The upregulated expression of MHC class II antigens is one of the factors known to be important in the development of allograft rejection [11, 35]. Moreover, ischemia/reperfusion injury was recently shown to involve components of an inflammatory reaction, which may increase graft immunogenicity via events such as upregulation of MHC as well as of the cytokine-adhesion molecule cascade, followed by subsequent extravasation [18]. In the present study, CMV infection alone, or in combination with prolonged cold ischemia, caused a significant upregulation of MHC class II antigen expression on endothelial cells at day 60 post infection. This phenomenon was previously found in vitro [35]. In vivo, however, this effect is seen while no viral antigens can be detected. This suggests that instead of a direct viral effect this is an indirect effect that starts late after infection. The enhanced expression of MHC class II may enable a direct presentation of foreign antigens to alloreactive T-cells, and the local macrophages that were in-

creased, as noted in the study, may process donor alloantigens for indirect T- and B-cell activation, leading to further recruitment of CD4+ and CD8+ cells and macrophages into the graft, which in turn may induce local cytokine and growth factor production and ultimately graft deterioration.

In summary, the results of the present study suggest that special attention should be paid to the CMV-infected recipient receiving an ischemically injured kidney graft, since an accelerated development of chronic rejection may occur in this situation. CMV infection accelerates renal graft deterioration probably through pathways different from ischemia-reperfusion injury. The knowledge about the expression of adhesion molecules due to CMV infection and CI may be useful when judging the cause of rejection in biopsies and to develop new therapeutic approaches for control and prevention of graft rejection.

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