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Triple Drug Immunosuppression Significantly Reduces Immune Activation and Allograft Arteriosclerosis in Cytomegalovirus-Infected Rat Aortic Allografts and Induces Early Latency of Viral Infection

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The effect of triple drug immunosuppression (cyclosporine A 10 mg/kg/day + methylprednisolone 0.5 mg/kg/day + azathioprine 2 mg/kg/day) on rat cytomegalovirus (RCMV)-enhanced allograft arteriosclerosis was investigated applying WF (AG-B2, RT1^v) recipients of DA (AG-B4, RT1^a) aortic allografts. The recipients were inoculated intraperitoneally with 10^5 plaque-forming units of RCMV 1 day after transplantation or left noninfected. The grafts were removed on 7 and 14 days, and at 1, 3, and 6 months after transplantation. The presence of viral infection was demonstrated by plaque assays, cell proliferation by [^{3}H]thymidine autoradiography, and vascular wall alterations by quantitative histology and immunohistochemistry. Triple drug immunosuppression reduced the presence of infectious virus in plaque assays and induced early latency of viral infection. It significantly reduced the peak adventitial inflammatory response ($P < 0.05$) and reduced and delayed intimal nuclear intensity and intimal thickening ($P < 0.05$) in RCMV-infected allografts. The proliferative response of smooth muscle cells was reduced by triple drug immunosuppression to 50% of that observed in nonimmunosuppressed RCMV-infected allografts, but still the proliferative peak response was seen at 1 month. Only low level immune activation, ie, the expression of interleukin-2 receptor ($P < 0.05$) and MHC class II, was observed under triple drug immunosuppression in the adventitia of RCMV-infected allografts, whereas there was no substantial change in the phenotypic distribution of inflammatory cells. In conclusion, although RCMV infection significantly enhances allograft arteriosclerosis also in immunosuppressed allografts, triple drug immunosuppression has no additional detrimental effect but rather a protective one on vascular wall histology. These results further suggest that RCMV-enhanced allograft arteriosclerosis may be an immunopathological condition linked to the host immune response toward the graft and/or the virus rather than a direct virus-induced phenomenon. (Am J Pathol 1994, 144:1334–1347)

Cytomegalovirus (CMV) infection is an important cause of morbidity and mortality among heart allograft recipients.[1] In addition, strong clinical evidence exists indicating the role of CMV infection in the pathogenesis of acute allograft rejection[2] and in the development of accelerated allograft arteriosclerosis in heart transplant patients.[2–5] We have previously demonstrated in our aortic allograft model[6] that early rat CMV (RCMV) infection accelerated and enhanced the adventitial inflammatory episode and doubled the arteriosclerotic changes in the allograft intima. If the time point of RCMV infection was postponed by 2 months, the effect was entirely lost. RCMV infection

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showed no effect in syngeneic grafts. This suggested that acute alloimmune response must coincide with acute RCMV infection to induce accelerated allograft arteriosclerosis.[7]

It is common knowledge that immunosuppression exposes to the development of CMV infection. In this study we describe that cyclosporine A (CsA)-based triple drug immunosuppression has no detrimental effect but rather a protective one to chronic rejection in the RCMV-infected aortic allografts. Triple drug immunosuppression reduces the presence of infectious RCMV in plaque assays and induces early latency of viral infections. It significantly reduces the generation of aortic allograft arteriosclerosis in RCMV-infected allografts. However, the enhancing effect of RCMV infection on allograft arteriosclerosis still exists under triple drug immunosuppression and is associated with prolonged and up-regulated immune response in the aortic allografts.

Materials and Methods

Rats

Inbred DA (AG-B4, $RT1^a$) and WF (AG-B2, $RT1^v$) rat strains were used as donors and recipients, respectively. The rats were purchased from Laboratory Animal Center, University of Helsinki, Helsinki, Finland. They were 2 to 3 months of age and weighed 200 to 300 g. The rats were fed with regular rat food (altromin, standard diet, Chr. Petersen A/S, Ringsted, Denmark) and tap water ad libitum. All animals were maintained on a 12-hour light/dark cycle.

All animals received humane care in compliance with the *Principles of Laboratory Animal Care* and the *Guide for the Care and Use of Laboratory Animals* prepared and formulated by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication no. 86-23, revised 1985).

Aortic Allografts

A 3-cm long segment of descending thoracic aorta was removed, thoroughly perfused with phosphate-buffered saline and used as transplant.[6] End-to-end anastomosis was performed using 9-0 continuous nylon suture. The graft was transplanted into heterotopic position below renal arteries and above bifurcation, forming a loop in the recipient abdominal cavity. Total ischemic time varied from 45 to 60 minutes, during which time the graft was kept in an ice bath at 4 C for 15 minutes. The experimental animals were anesthetized with chloral hydrate 240 mg/kg intraperitoneally and given 0.25 mg/kg buprenorphine subcutaneously (Temgesic; Reckitt & Colman, Hull, UK) for postoperative pain relief.

The grafts were removed on 7 and 14 days and 1, 3, and 6 months after transplantation. Usually five successful transplantations were included in each time point. The grafts were processed for morphometry, autoradiography, and immunohistochemistry.

Immunosuppressive Regimens

One group of allograft recipients infected with RCMV received triple drug immunosuppression by mouth for the entire observation time. Perioperatively, the rats received CsA (Sandimmun; Sandoz Pharma AG, Basel, Switzerland) 15 mg/kg subcutaneously in the neck. For the injection, 50 mg/ml of CsA infusion substance was dissolved in Intralipid 200 mg/ml (KabiVitrum, Stockholm, Sweden) to a final concentration of 3 mg/ml. Thereafter, CsA (Sandimmun mixture 100 mg/ml; Sandoz) at a dose of 10 mg/kg was given by mouth with regular rat food. Methylprednisolone 0.5 mg/kg (Solu-Medrol 40 mg/ml; Upjohn s.a., Puurs, Belgium) and 2 mg/kg azathioprine (Imuran; Wellcome, London, UK) was administered with drinking water.

Radioimmunoassay (Sandimmun-Kit; Sandoz) was applied to determine whole blood CsA levels. The blood was drawn from the tail tip of the rat once a week for the first month and then monthly.

RCMV

Nonimmunosuppressed and immunosuppressed allograft recipient rats were inoculated intraperitoneally with 10^5 plaque-forming units (PFU) of RCMV[8] 1 day after transplantation. The noninfected control rats were kept in separate colonies with otherwise similar diet and conditions.

RCMV Maastricht strain[9,10] was passaged by infecting rat embryo fibroblasts from 17-day-old DA rat embryos. The cells were cultured in flasks containing modified Eagle's medium (Flow Laboratories, Irvine, UK) supplemented with 200 mmol/L L-glutamine (Northumbria Biologicals LTD., Cramlington, UK), 10,000 IU/ml penicillin/1,000 μg/ml streptomycin solution (GIBCO Ltd., Paisley, Scotland, UK), and 2% fetal calf serum (Sera-Lab, Sussex, UK) according to standard viral culture techniques.[11] The stocks of virus were stored at −70 C until use.

Plaque Assays

Quantitations of RCMV stock were done by plaque assays.[12] Confluent rat embryo fibroblast cell monolayers were infected with appropriate virus dilution and incubated for 60 minutes at 37 C in a 5% CO_2 incubator. After incubation, the infected cells were overlaid with 1 ml D-BME (Eagle) (GIBCO) with a final concentration of 0.25% agarose (Sigma, St. Louis, MO). After an incubation for 7 days, the cells were fixed with 10% formalin overnight. After fixation, the solid base layer was removed and the cell monolayers were stained with 1% aqueous crystal violet, and the plaques were counted by light microscopy.

To gain insight into the course of the RCMV infection, the presence of infectious virus was determined by plaque assays in some important organs (liver, spleen, and salivary glands). The biopsies of salivary glands, liver, and spleen were removed aseptically in modified Eagle's medium with 2% fetal calf serum at graft removal and stored at −70 C for plaque assays. In short, organs were homogenized in a tissue grinder and 10-fold dilutions of 10% homogenates (w/v) were inoculated on confluent rat embryo fibroblast monolayers. After the 7-day incubation, the number of plaques was monitored microscopically after methylene blue staining.

Histology

A piece of graft was fixed for 24 hours in 10% phosphate-buffered formalin then dehydrated and embedded in paraffin. Four-micron thick cross-sections were stained with Mayer's hematoxylin and eosin for the evaluation of histological changes in the vascular wall of the graft. There was no difference observed, whether or not *in situ* fixation was performed. Nontransplanted DA thoracic aortas were used as controls.

Morphometry

The morphological changes were quantitated according to standard morphometric principles and are expressed as point score units (PSU), ie, the mean number of points falling over a given anatomical area using straight cross-sectional lines and a 0.02-mm grid.[13] The number of adventitial, media, and intimal cell nuclei and intimal thickness in the allograft vascular wall were evaluated. The mean ± SEM was used as final scores.

In Vivo *Labeling and Autoradiography*

All rats received 300 μCi of [methyl-^{3}H]thymidine (Amersham International plc, Amersham, UK) intravenously 3 hours before graft removal. The histology was processed from paraffin cross-sections and emulsion film autoradiography (Ilford L.4; Ilford, Mobberley, Cheshire, UK) was performed. The number of labeled nuclei was counted in the adventitia, media, and intima of the allograft vascular wall.

Immunohistochemistry

Four-micron thick frozen cross-sections of aortic allografts were stained with three-layer indirect immunoperoxidase technique[14] using the following monoclonal antibodies:

- OX 1 (Sera Lab, Sussex, UK), a mouse IgG1 monoclonal antibody to rat leukocyte common antigen (LCA);
- W3/25 (Sera Lab), a mouse IgG1 monoclonal antibody to rat T helper cells (CD 4 equivalent);
- OX 8 (Sera Lab), a mouse IgG1 monoclonal antibody to rat T cells (nonhelper subset; Lyt 2/Lyt3, CD8 equivalent);
- OX 33 (Sera Lab), a mouse IgG1 monoclonal antibody to a subfraction of rat B cell LCA;
- OX 42 (Sera Lab), a mouse IgG2a monoclonal antibody to rat macrophages (160, 103, 96 kd polypeptide);
- 3.2.3., a monoclonal antibody to rat natural killer cells (a generous gift from William H. Chambers, Pittsburgh Cancer Institute, Pittsburgh, PA);
- Interleukin (IL)-2R (CD25), a monoclonal antibody to rat IL-2 receptor (a generous gift from Dr. J. Kupiec-Weglinski, Harvard Medical School, Boston, MA);
- OX 6 (Sera Lab), a mouse IgG1 monoclonal antibody to rat MHC class II common determinant;
- The α smooth muscle cell (SMC) actin (Bio-Makor, Rehovot, Israel).

After cutting, the frozen sections were air-dried for 2 hours, acetone-fixed for 20 minutes, and stored at −20 C until used. Before incubation with monoclonal antibody, the sections were refixed with chloroform for 30 minutes. Thereafter, they were incubated with appropriately diluted (usually 1:100, class II 1:200) monoclonal antibodies, washed with Tris buffer (pH 7.4), and incubated consecutively with peroxidase-conjugated rabbit anti-mouse Ig and goat anti-rabbit Ig, followed by treatment with the substrate solution containing the chromogen 3-amino-9-ethylcarbazole. Hydrogen peroxidase was added to the 3-amino-9-

ethylcarbazole solution immediately before use. The specimens were counterstained with hematoxylin and mounted in aquamount.

Immunohistochemical Quantitation

Positive staining for IL-2R was quantitated as a number of positive cells in the cross-section of the allograft adventitia. The adhesion molecules and inflammatory cell subsets in the allograft adventitia were scored from 0 to 5 (0, no positive staining; 1, very weak; 2, weak; 3, moderate; 4, intense; 5, very intense positive staining). Data are expressed as mean ± SEM.

Statistics

Data are expressed as mean ± SEM and for nontransplanted DA aortas as mean ± SD. In the statistical analysis, RCMV-infected immunosuppressed allografts were compared with RCMV-infected nonimmunosuppressed allografts, and RCMV-infected immunosuppressed allografts were compared with noninfected immunosuppressed allografts. For comparison, the values in noninfected nonimmunosuppressed allografts are given in the figures and tables.[7,16,17] The Mann-Whitney *U* test (Z for corrected ties) was used to evaluate the significances. Values of $P < 0.05$ were regarded as statistically significant.[15]

Results

RCMV Infection in Triple Drug Immunosuppressed Allograft Recipients

The immunosuppressed and nonimmunosuppressed allograft recipients were inoculated intraperitoneally with 10^5 PFU of RCMV 1 day after transplantation.

As shown in Table 1 in nonimmunosuppressed RCMV-infected allograft recipients, the liver and spleen usually contained infectious virus in plaque assays on day 7 (acute infection) and were negative thereafter. In contrast, the salivary glands were usually RCMV negative on days 7 and 14, whereas they appeared almost 100% positive at 1 and 3 months after RCMV inoculation (chronic infection). At 6 months, no infectious virions could be recovered from the rat organ biopsies (latent infection).

Under triple drug immunosuppression less infectious virus was demonstrated in plaque assays from the organ biopsies (Table 1). None of the immunosuppressed rats had infectious virus in the liver or spleen on day 7. Of the nonimmunosuppressed rats, 80% (4/5) had a high titer of infectious virus in the salivary glands at 1 month after infection in the quantitative plaque assay. However, only 40% (2/5) of the immunosuppressed rats had infectious virus in the salivary glands at 3 months after infection. At 6 months, no infectious virus was present in any organ.

Effect of Triple Drug Immunosuppression on RCMV-Enhanced Allograft Arteriosclerosis

Aortic allografts were done from the DA to the WF rat strain, and allograft recipients were infected with 10^5 PFU of RCMV 1 day after transplantation. One group of infected rats received triple drug immunosuppression and the other was left nonimmunosuppressed. Whole blood CsA levels are depicted in Figure 1.

Adventitial Nuclei

There were only a few fibroblasts in the adventitia of nontransplanted DA aortas (0.5 ± 0.5, mean ± SD). In noninfected nonimmunosuppressed allografts, LCA-positive white inflammatory cells infiltrated the adventitia with a peak of 11.5 ± 1.2 PSU at 1 month after transplantation and thereafter declined gradually.[6] In RCMV-infected nonimmunosuppressed aortic allografts, a rapid infiltration of LCA-positive cells

Table 1. *The Presence of RCMV in Plaque Assays in Nonimmunosuppressed and Triple Drug Immunosuppressed Allograft Recipients*

Group	Number of Rats Positive in Plaque Assays/Tested Rats				
	7 Days	14 Days	1 Month	3 Months	6 Months
RCMV					
Liver/spleen	3/5	1/5	0/4	0/5	0/5
Salivary glands	0/5	2/5	4/4	4/5	0/5
RCMV + triple					
Liver/spleen	0/5	0/5	1/5	0/5	0/5
Salivary glands	0/5	2/5	4/5	2/5	0/5

Note that no infectious virus could be demonstrated from the liver/spleen on 7 and 14 days after infection, and that only 40% of salivary glands were positive at 3 months under triple drug immunosuppression. These findings indicate that triple drug immunosuppression induces early latency of RCMV infection.

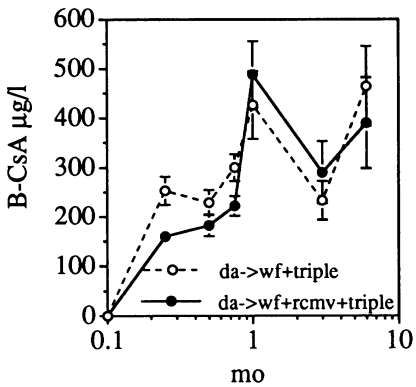

Figure 1. *CsA concentration in whole blood measured by radioimmunoassay.*

was observed already on 7 days after transplantation with 11.6 ± 1.5 PSU, reaching a peak of 15.7 ± 1.5 PSU at 1 month after transplantation.[7] In RCMV-infected immunosuppressed allografts, the inflammatory response was reduced by 50%, ie, to 6.9 ± 0.8 on 7 days ($P < 0.025$) and to 8.0 ± 1.2 PSU at 1 month ($P < 0.05$) after transplantation (Figure 2a).

Media Nuclei

The number of media cell nuclei in nontransplanted DA aortas was 3.1 ± 0.1 PSU (±SD). A rapid reduction in the number of media cell nuclei down to 0.8 ± 0.2 PSU was seen in noninfected nonimmunosuppressed allografts at 3 months after transplantation.[6] In RCMV-infected nonimmunosuppressed allografts, the loss of media cell nuclei occurred with the same pace as in noninfected nonimmunosuppressed allografts.[7] In RCMV-infected allografts, triple drug treatment entirely abolished the development of media necrosis during the whole observation time and the number of media nuclei remained within the normal variation (Figure 2b).

Intimal Nuclei

The vessel wall of nontransplanted DA aortas is normally lined by a single cell monolayer (0.9 ± 0.2 PSU, ± SD). The number of intimal cell nuclei in noninfected nonimmunosuppressed allografts reached 2.3 ± 0.4 PSU at 1 month, was almost the same at 3 months, and peaked with 3.7 ± 0.8 PSU at 6 months after transplantation.[6] In RCMV-infected nonimmunosuppressed allografts, the number of intimal cell nuclei reached 3.6 ± 0.7 PSU already at 1 month after transplantation, remained unchanged till 3 months, and slightly declined at 6 months after transplantation.[7] In RCMV-infected immunosuppressed al-

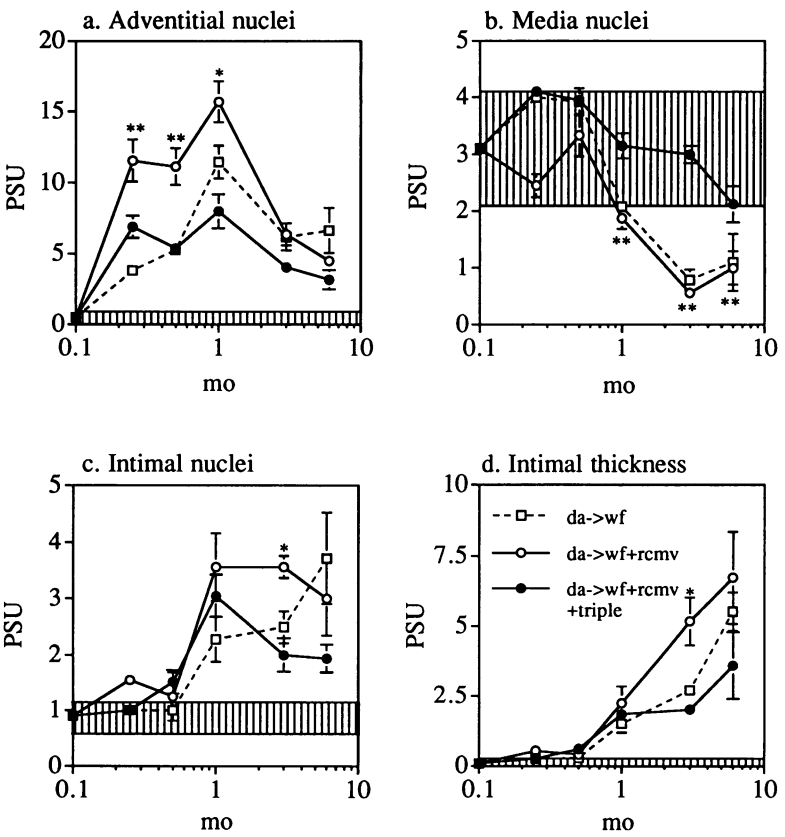

Figure 2. *Effect of triple drug therapy on RCMV-enhanced allograft arteriosclerosis. Aortic transplantations were done from the DA to the WF rat strain and the rats were infected intraperitoneally with 10^5 PFU of RCMV 1 day after transplantation. The rats received by mouth 10 mg/kg CsA, 2 mg/kg AZA, and 0.5 mg/kg MP for the entire observation period. The responses are quantitated as PSU in the adventitia, media, and intima ± SEM. The RCMV-infected immunosuppressed rats were killed 7 (n = 5), and 14 (n = 5) days, 1 (n = 5), 3 (n = 5), and 6 (n = 5) months after transplantation. The corresponding numbers in the RCMV-infected nonimmunosuppressed allografts were 5, 5, 4, 4, and 3. For comparison, the values in noninfected nonimmunosuppressed DA→WF allografts (n = 5, 3, 21, 11, and 5) are shown and derived from Reference 7. Framed area = thoracic aorta of nontransplanted DA rats ± SD. Mann-Whitney U test was used to evaluate the significances; *P < 0.05, **P < 0.025, and ***P < 0.01 when RCMV-infected immunosuppressed rats are compared with RCMV-infected nonimmunosuppressed rats. Note that the time is given in logarithmic scale to emphasize the early posttransplant period.*

lografts (Figure 2c) there was also an increase in the number of intimal nuclei up to 3.1 ± 0.4 PSU at 1 month after transplantation. Thereafter, a gradual decrease in intimal nuclear density down to 2.0 ± 0.3 PSU at 3 months ($P < 0.025$) and 1.9 ± 0.3 PSU at 6 months (P = NS) occurred.

Intimal Thickness

In nontransplanted DA aortas, there is no intimal thickening (0.1 ± 0.1 PSU, ± SD). In noninfected nonimmunosuppressed allografts, intimal thickness increased gradually reaching 2.7 ± 0.3 PSU at 3 months and 5.5 ± 0.7 PSU at 6 months.[6] In RCMV-infected nonimmunosuppressed allografts, there was a gradual increase in intimal thickness, reaching 5.2 ± 0.9 and 6.7 ± 1.6 PSU at 3 and 6 months after transplantation.[7] Under triple drug immunosuppression (Figure 2d), the intimal thickness was significantly reduced in RCMV-infected allografts to 2.0 ± 0.2 PSU at 3 months ($P < 0.05$) and to 3.6 ± 1.2 PSU at 6 months ($P = 0.05$) after transplantation.

Thus, taken together, triple drug immunosuppression in RCMV-infected allografts compared with nonimmunosuppressed infected allografts reduced adventitial inflammation by 50%, prevented the development of media necrosis, and reduced the number of intimal nuclei and intimal thickness by approximately 30 to 50%.

Effect of RCMV Infection on Allograft Arteriosclerosis under Triple Drug Immunosuppression

Immunosuppressed recipients of aortic allografts were infected intraperitoneally with 10^5 PFU of RCMV 1 day after transplantation, and the infected allografts were compared with similarly immunosuppressed noninfected DA→WF allografts.

Adventitial Nuclei

In noninfected immunosuppressed allografts, the number of inflammatory white blood cells infiltrating the adventitia was very low during the entire observation time, reaching 4.9 ± 0.5 PSU on 7 days and 5.4 ± 0.6 at 1 month. In RCMV-infected immunosuppressed allografts compared with noninfected immunosuppressed allografts (Figure 3a) an upregulated inflammatory response in the adventitia

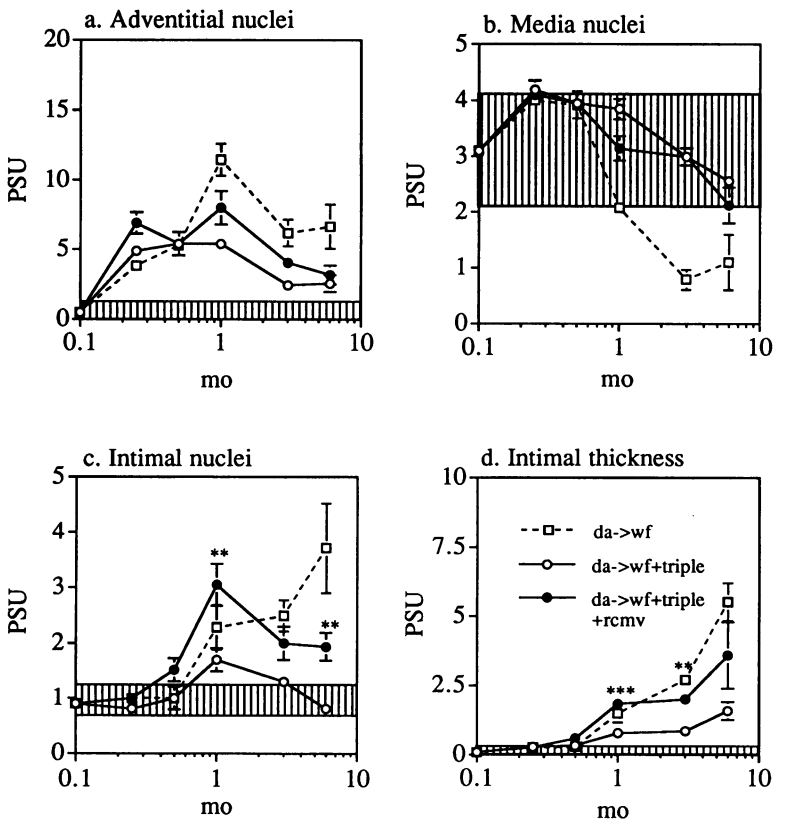

Figure 3. *Effect of RCMV infection on allograft arteriosclerosis under triple drug therapy. Aortic transplantations were done from the DA to the WF rat strain. The rats were infected intraperitoneally with 10^5 PFU of RCMV 1 day after transplantation or left noninfected and used as controls. The responses are quantitated as PSU in the adventitia, media, and intima ± SEM. The rats received by mouth 10 mg/kg CsA, 2 mg/kg AZA, and 0.5 mg/kg MP for the entire observation period. The RCMV-infected immunosuppressed rats were killed 7 (n = 5) and 14 (n = 5) days and 1 (n = 5), 3 (n = 5), and 6 (n = 5) months after transplantation. The corresponding numbers for the noninfected immunosuppressed rats were 4, 5, 5, 5, and 4. For comparison, the values in noninfected nonimmunosuppressed DA→WF allografts (n = 5, 3, 21, 11, and 5) are shown and derived from Reference 7. Framed area = thoracic aorta of nontransplanted DA rats ± SD. Mann-Whitney U test was used to evaluate the significances; *P < 0.05, **P < 0.025, and ***P < 0.01 when RCMV-infected immunosuppressed rats are compared with noninfected immunosuppressed rats. Note that the time is given in logarithmic scale to emphasize the early posttransplant period.*

was observed, peaking with 6.9 ± 0.8 PSU on 7 days and 8.0 ± 1.2 PSU at 1 month after transplantation (P = NS).

Media Nuclei

No significant loss of media cells was seen either in noninfected immunosuppressed or RCMV-infected immunosuppressed allografts during the entire observation time. (Figure 3b).

Intimal Nuclei

In noninfected immunosuppressed allografts, the number of intimal nuclei peaked with 1.7 ± 0.2 PSU at 1 month after transplantation, returning thereafter to the normal control level. After RCMV infection (Figure 3c), the number of intimal nuclei gradually increased in immunosuppressed allografts, peaking with 3.1 ± 0.4 PSU at 1 month ($P < 0.025$) after transplantation. It both groups this was due to the proliferation of α-actin-positive SMC and infiltration of LCA-positive white inflammatory cells in the intima as demonstrated by immunohistochemistry. The number of intimal cell nuclei in RCMV-infected immunosuppressed allografts declined to 1.9 ± 0.3 PSU at 6 months after transplantation but there was still a significant difference ($P < 0.025$) compared with noninfected immunosuppressed allografts.

Intimal Thickness

There was a slow progressive intimal thickening in noninfected immunosuppressed allografts, reaching 0.8 ± 0.1 PSU at 1 month, 0.9 ± 0.1 PSU at 3 months, and 1.5 ± 0.8 PSU at 6 months after transplantation. In RCMV-infected immunosuppressed allografts (Figure 3d), a significant increase in intimal thickness was seen compared with noninfected immunosuppressed allografts, to 1.9 ± 0.2 PSU ($P < 0.01$) at 1 month, 2.0 ± 0.2 PSU ($P < 0.025$) at 3 months, and 3.6 ± 1.2 PSU ($P = 0.09$) at 6 months after transplantation.

Thus, taken together, RCMV infection in immunosuppressed allografts compared with noninfected immunosuppressed allografts induced early inflammatory episode in the adventitia and doubled intimal thickening.

Effect of Triple Drug Immunosuppression on Cell Proliferation in the Vascular Wall of RCMV-Infected Allografts

All rats received 300 μCi of [^{3}H]thymidine (TdR) intravenously 3 hours before graft removal. The number of labeled nuclei was counted from autoradiograms performed from paraffin sections and is expressed as [^{3}H]TdR$^+$ nuclei/cross-section.

Adventitial Cell Proliferation

In noninfected nonimmunosuppressed allografts, a gradual increase in the number of [^{3}H]TdR$^+$ nuclei in the adventitia was observed, up to 225 ± 25 positive cells at 1 month after transplantation, lasting for 3 months and declining thereafter.[6] In RCMV-infected nonimmunosuppressed allografts, a rapid and prominent proliferative peak was already observed 7 days after transplantation, with 227 ± 21 [^{3}H]TdR$^+$ nuclei in the adventitia. Thereafter, a gradual decline occurred.[7] In RCMV-infected immunosuppressed allografts, inflammatory cell proliferation was significantly depressed during the entire observation time (Figure 4a). Thus, in immunosuppressed recipients compared with nonimmunosuppressed allografts RCMV infection was associated with a dramatic decrease in the number of proliferating inflammatory cells in the allograft adventitia.

In the adventitia of noninfected immunosuppressed allografts, only a low level proliferating response was observed, peaking with 32 ± 11 [^{3}H]TdR$^+$ nuclei at 1 month after transplantation compared with nonimmunosuppressed allografts. As depicted in Figure 5a, no significant difference in the proliferating response of white inflammatory cells was observed when noninfected and RCMV-infected allografts were compared under triple drug immunosuppression.

Media Cell Proliferation

In noninfected nonimmunosuppressed allografts, there was a gradual increase in the number of proliferating nuclei in the media, peaking to 23 ± 14 [^{3}H]TdR$^+$ nuclei at 1 month after transplantation.[6] In RCMV-infected nonimmunosuppressed allografts, the media cells showed a rapid proliferating response 16 ± 4 and 21 ± 2 [^{3}H]TdR$^+$ nuclei already on 7 and 14 days after transplantation, respectively.[7] Triple drug immunosuppression significantly reduced the number of proliferating SMC in the media of RCMV-infected allografts on 7 ($P < 0.01$) and 14 days ($P <$

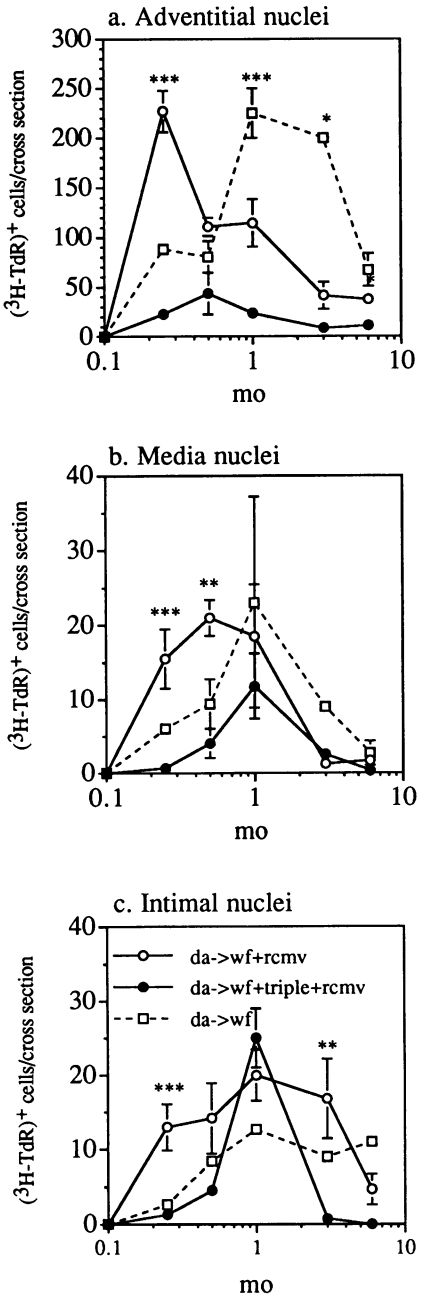

Figure 4. *Effect of triple drug treatment on cell proliferation in RCMV-infected allografts. All rats received [^{3}H]TdR intravenously 6 hours before graft removal. The autoradiograms were quantitated as [^{3}H]TdR$^+$ nuclei/cross-section ± SEM in different layers of aorta. For explanations see Figure 2.*

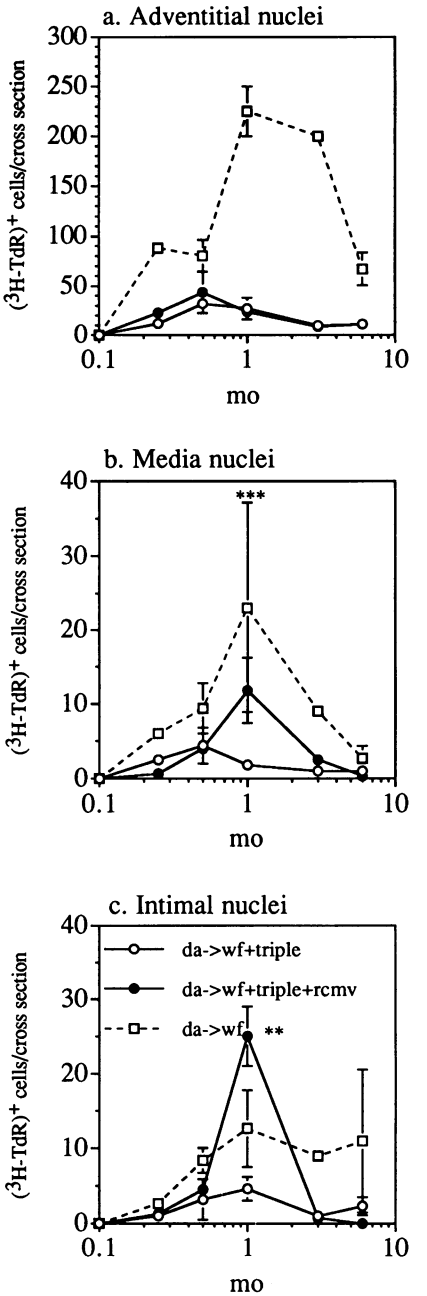

Figure 5. *Effect of RCMV infection on cell proliferation during triple drug treatment in the three layers of allograft vascular wall. For explanations see Figure 3.*

0.025) after transplantation but peak response at 1 month was still obvious (Figure 4b).

In immunosuppressed allografts, RCMV infection induced a significant ($P < 0.01$) proliferating peak in media cells with 11.8 ± 4.3 [^{3}H]TdR$^+$ nuclei at 1 month after transplantation compared with noninfected immunosuppressed controls with 2 ± 1 [^{3}H]TdR$^+$ nuclei at 1 month (Figure 5b).

Intimal Cell Proliferation

The intimal cell proliferation in noninfected nonimmunosuppressed allografts began on the second week and lasted for at least 6 months.[6] In RCMV-infected nonimmunosuppressed allografts, there was an intensive proliferating response in the intima during the entire experimental period.[7] Under triple drug immunosuppression, the proliferating response in RCMV-

infected allografts was significantly reduced during the entire observation time, except at 1 month when it reached the level observed in RCMV-infected nonimmunosuppressed allografts (Figure 4c).

RCMV induced a significant ($P < 0.025$) proliferating response of intimal cells in immunosuppressed allografts at 1 month after transplantation compared with noninfected immunosuppressed allografts (Figure 5c).

Thus, taken together, triple drug immunosuppression in RCMV-infected allografts compared with infected nonimmunosuppressed allografts reduced the proliferation of adventitial inflammatory cells and the rate of media proliferation of SMC to 30%. The rate of intimal proliferation was least affected. When RCMV-infected immunosuppressed allografts were compared with noninfected immunosuppressed allografts, a weak media and intimal proliferative responses were observed in RCMV-infected animals compared with the noninfected immunosuppressed controls.

Effect of Triple Drug Immunosuppression on the Immune Response in RCMV-Infected Allograft Adventitia

Frozen sections of the allografts were stained with immunoperoxidase technique using monoclonal antibodies to IL-2R and MHC class II to evaluate the effect of triple drug immunosuppression on the immune activation in RCMV-infected allograft adventitia, and to evaluate the effect of RCMV infection on immune activation in immunosuppressed allografts.

IL-2R

In the adventitia of nontransplanted DA aortas, there was no expression of IL-2R. In noninfected nonimmunosuppressed allografts a remarkable up-regulation of IL-2R with 255 ± 52 positive cells/cross-section was recorded at 1 month after transplantation, thereafter, the response declined to 46 ± 19 positive cells at 3 months after transplantation.[16] In RCMV-infected nonimmunosuppressed allografts compared with noninfected nonimmunosuppressed allografts, the expression of IL-2R was already up-regulated on 7 days after transplantation and remained up-regulated during the entire observation time.[17] In RCMV-infected allografts, triple drug immunosuppression significantly reduced the expression of IL-2R to 7 ± 1 ($P < 0.05$) on 7 days and to 44 ± 15 ($P < 0.05$) at 1 month after transplantation (Table 2).

In noninfected immunosuppressed allografts, only few IL-2R-positive cells were seen on day 7, reaching a peak at 36 ± 16 at 1 month.[16] In RCMV-infected immunosuppressed allografts, a significant ($P < 0.05$) increase to 7 ± 1 in the IL-2R level was observed on day 7 compared with noninfected immunosuppressed allografts. Thereafter, the IL-2R expression remained slightly elevated compared with noninfected immunosuppressed allografts (Table 3).

MHC Class II

There were only very few MHC class II-positive cells in the adventitia of nontransplanted DA aortas. In noninfected nonimmunosuppressed allograft adventitia, the level for MHC class II molecule expression was

Table 2. *Effect of Triple Drug Immunosuppression on the Expression of Activation Markers and on the Presence of Inflammatory Cell Subsets in RCMV-Infected Allograft Adventitia as Demonstrated by Immunoperoxidase Staining*

Time after Transplantation	IL-2R* (CD25)	Class II† (OX6)	Th† (W3/25)	Tc† (OX8)	NK† (3.2.3.)	MØ† (OX42)
DA→WF (n = 3)						
7 days	10±6	1.3±0.3	1.7±0.8	1.0±0.0	1.0±0.0	2.2±0.9
1 month	255±52	4.7±0.3	3.8±0.8	2.1±0.4	2.0±0.4	4.0±0.5
3 months	46±19	2.8±0.3	0.8±0.3	0.8±0.2	0.5±0.0	1.5±0.5
DA→WF+RCMV (n = 3)						
7 days	29±9	3.2±0.2	3.3±0.4	2.7±0.8	3.0±1.0	3.8±0.5
1 month	299±46	4.5±0.3	4.3±0.3	2.3±0.3	2.0±0.4	4.5±0.3
3 months	77±14	3.2±0.4	1.8±0.3	0.8±0.2	1.8±0.3	2.0±0.5
DA→WF+RCMV+triple (n = 3)						
7 days	7±1‡	1.5±0.3‡	2.2±0.7	1.6±0.4	0.8±0.3	2.8±0.5
1 month	44±15‡	4.0±0.0	3.0±0.6	0.8±0.1‡	1.7±0.2	3.0±0.0‡
3 months	56±5	3.0±0.0	1.8±0.3	0.8±0.2	0.8±0.3	2.5±0.3

Th, T helper; Tc, T cytotoxic; NK, natural killer.
* Positive cells/cross-section ± SEM.
† Scored from 0 to 5 ± SEM.
Significances by Mann-Whitney *U* test. ‡ $P < 0.05$ when compared with DA→WF+RCMV. For comparison, the values in DA→WF allografts are shown.[16]

Table 3. *Effect of RCMV Infection under Triple Drug Immunosuppression on the Expression of Activation Markers and Inflammatory Cell Subsets in Allograft Adventitia as Demonstrated by Immunoperoxidase Staining*

Time after Transplantation	IL-2R* (CD25)	Class II† (OX6)	Th† (W3/25)	Tc† (OX8)	NK† (3.2.3.)	MØ† (OX42)
DA→WF (n = 3)						
7 days	10±6	1.3±0.3	1.7±0.8	1.0±0.0	1.0±0.0	2.2±0.9
1 month	255±52	4.7±0.3	3.8±0.8	2.1±0.4	2.0±0.4	4.0±0.5
3 months	46±19	2.8±0.3	0.8±0.3	0.8±0.2	0.5±0.0	1.5±0.5
DA→WF+triple (n = 3)						
7 days	2±1	1.0±0.0	1.7±0.4	1.2±0.2	0.5±0.0	1.8±0.5
1 month	36±16	3.0±0.0	2.0±0.0	1.0±0.0	1.5±0.3	2.8±0.4
3 months	30±17	1.8±0.6	0.5±0.0	0.2±0.2	0.2±0.2	1.7±0.3
DA→WF+triple+RCMV (n = 3)						
7 days	7±1‡	1.5±0.3	2.2±0.7	1.6±0.4	0.8±0.3	2.8±0.5
1 month	44±15	4.0±0.0‡	3.0±0.6	0.8±0.1	1.7±0.2	3.0±0.0
3 months	56±5	3.0±0.0	1.8±0.3	0.8±0.2	0.8±0.3	2.5±0.3

Th, T helper; Tc, T cytotoxic; NK, natural killer.
* Positive cells/cross-section ± SEM.
† Scored from 0 to 5 ± SEM.
Significances by Mann-Whitney *U* test. ‡ $P < 0.05$ when compared with DA→WF+triple. For comparison, the values in DA→WF allografts are shown.[16]

1.3 ± 0.3 on day 7 and 4.7 ± 0.3 at 1 month after transplantation.[16] In RCMV-infected nonimmunosuppressed allografts, a significant up-regulation to 3.2 ± 0.2 on day 7 in the class II molecule expression was observed. Thereafter, the level of class II in RCMV-infected nonimmunosuppressed allografts was quite similar to that seen in noninfected nonimmunosuppressed allografts.[17] The class II molecule level was significantly ($P < 0.05$) down-regulated by triple drug immunosuppression in RCMV-infected allografts on day 7 but not thereafter (Table 2). When compared with noninfected immunosuppressed allografts,[16] RCMV infection significantly ($P < 0.05$) enhanced the class II expression to 4.0 ± 0.0 at 1 month after transplantation (Table 3).

Effect of Triple Drug Immunosuppression on the Presence of Leukocyte Subsets in RCMV-Infected Allograft Adventitia

Frozen sections were stained with immunoperoxidase technique using monoclonal antibodies to rat T helper, T cytototoxic, natural killer (NK), and monocyte/macrophages (Mϕ) to evaluate the impact of triple drug immunosuppression on the presence of leukocyte subsets in RCMV-infected allografts and to evaluate the effect of RCMV infection on the presence of leukocyte subsets in immunosuppressed allografts.

T Cells

T helper cells were not present in nontransplanted DA aortas. In noninfected nonimmunosuppressed allografts, a significant up-regulation in the presence of T helper cells was observed at 1 month.[16] The expression of T helper cells was significantly stronger on day 7 after transplantation among RCMV-infected nonimmunosuppressed allografts compared with noninfected nonimmunosuppressed allografts. The presence of T helper cells in RCMV-infected allografts reached a peak at 4.3 ± 0.3 at 1 month and remained up-regulated still at 3 months compared with noninfected nonimmunosuppressed allografts.[17] Triple drug immunosuppression down-regulated the presence of T helper cells in RCMV-infected allografts but the difference was not significant (Table 2). When compared with noninfected immunosuppressed allografts,[16] RCMV infection had a slight enhancing but not significant influence on the presence of T helper cells (Table 3).

T cytotoxic cells were not present in nontransplanted DA aortas. The expression of T cytotoxic cells in noninfected nonimmunosuppressed allografts was mild to moderate.[16] In RCMV-infected nonimmunosuppressed allografts, the expression of T cytotoxic cells was significantly up-regulated on day 7 compared with noninfected nonimmunosuppressed allografts.[17] Triple drug immunosuppression significantly reduced the presence of T cytotoxic cells in RCMV-infected allografts (Table 2). When compared with noninfected immunosuppressed allografts,[16] RCMV infection had no effect on the expression of T cytotoxic cells (Table 3).

NK Cells

There were no NK cells observed in nontransplanted DA aortas. Only a mild expression of NK cells occurred in noninfected nonimmunosuppressed allografts.[16] In RCMV-infected nonimmunosuppressed

allografts, NK cells were significantly up-regulated on day 7 after transplantation and the expression remained elevated up to 3 months compared with noninfected immunosuppressed allografts.[17] Immunosuppression reduced the expression of NK cells in RCMV-infected allografts but not significantly (Table 2). When compared with noninfected immunosuppressed allografts,[16] RCMV infection had no extra effect on the presence of NK cells (Table 3).

Mϕ

No Mϕ were seen in nontransplanted DA aortas. In noninfected nonimmunosuppressed allografts, there was a moderate presence of Mϕ on 7 days after transplantation, peaking with a score of 4.0 ± 0.5 at 1 month after transplantation.[16] In RCMV-infected nonimmunosuppressed allografts, the Mϕ response occurred earlier and the intensity was already on 7 days as high as that seen in noninfected allografts at 1 month.[17] Triple drug treatment significantly reduced the number of Mϕ at 1 month in RCMV-infected allografts (Table 2). When compared with noninfected immunosuppressed allografts,[16] RCMV infection slightly up-regulated the presence of Mϕ during the entire period (Table 3).

Thus, taken together, triple drug immunosuppression in RCMV-infected rats compared with infected nonimmunosuppressed rats brought down IL-2R and class II expression of inflammatory leukocytes and reduced the number of T cytotoxic cells and Mϕ in the inflammatory infiltrate. When RCMV-infected immunosuppressed allografts were compared with noninfected immunosuppressed allografts, virus infection resulted only in a marginal increase in the expression of IL-2R and class II as well as T helper cells and Mϕ.

Discussion

Accelerated allograft arteriosclerosis, ie, chronic rejection, is the most common cause for late death and retransplantation among heart transplant recipients.[18] The major factors predisposing for accelerated allograft arteriosclerosis include humoral and cellular immune response, hyperlipidemia, and viral infections.[2–5,19–24] Clinical studies have demonstrated the role of CMV infection in the pathogenesis of cardiac allograft arteriosclerosis.[2–5]

We have earlier demonstrated that RCMV infection in nonimmunosuppressed recipients induces an early accelerated adventitial inflammatory cell proliferation and doubles the rate of SMC proliferation and intimal thickening.[7] The enhancing effects of RCMV infection were also evident in the triple drug immunosuppressed rats but with much reduced intensity; compared with noninfected allografts, the early RCMV-linked inflammatory episode in the adventitia was still visible, as was the increase in intimal thickness. Also significant, though weak, RCMV-linked media and intimal proliferative responses were observed in infected immunosuppressed allografts compared with noninfected immunosuppressed allografts.

The main message of this communication is that RCMV infection in immunosuppressed animals did not enhance chronic vascular wall changes. Compared with infected nonimmunosuppressed allografts, adventitial inflammation was reduced by 50%, there was no media necrosis, and the number of intimal nuclei and intimal thickness in infected immunosuppressed allografts was reduced by approximately 30 to 50%. The proliferation of adventitial inflammatory cells was also reduced and the rate of media proliferation of SMC was reduced by 70%. The intimal proliferation of SMC was least affected. Although immunosuppression is considered a risk factor for the development of viral infection, our findings suggest that triple drug immunosuppression seems to protect against virus-enhanced allograft arteriosclerosis rather than to enhance it.

The impact of immunosuppression was also evident in the cellular and molecular level when we quantitated the expression of several acute inflammatory markers and the frequency of different inflammatory cell types in the allograft adventitia. Triple drug immunosuppression brought down in RCMV-infected allografts the IL-2R and class II expression. Concomitantly, the levels of T helper cells, T cytotoxic cells, NK cells, and Mϕ were reduced.

The second message of this communication is that during triple drug immunosuppression the latent phase of RCMV infection is reached earlier than in nonimmunosuppressed rats. In nonimmunosuppressed rats, the liver and spleen were positive for RCMV only during the first weeks (acute infection), whereas the salivary glands appeared positive 1 month after infection (chronic infection). No infectious virus could be demonstrated 6 months after infection (latent infection). Under triple drug immunosuppression less infectious virus was demonstrated in plaque assays from organ biopsies. No infectious virus could be demonstrated from the liver or spleen during the first weeks; of the salivary glands, 40% were positive already on day 14, 80% at 1 month, and only 40% at 3 months after infection.

The reduction of infectious virus by immunosuppression, especially during the acute phase of RCMV

infection, might be due to reduced amounts of inflammatory cells and therefore shedding of the virus into inner organs is decreased. Human CMV is able to infect peripheral monocytes and lymphocytes, and in the latent state of CMV infection these cells express immediate early genes of human CMV.[25,26] Findings concerning CsA and replication of CMV vary.[27] In *in vitro* studies, high dose CsA inhibited murine CMV replication.[28] Human CMV replication was inhibited *in vitro* when the cells were simultaneously treated with the drug and infected with CMV.[29] On the other hand, CsA enhanced murine CMV infection in mice with the prerequisite that the animals were pretreated with a gigantic dose of CsA by mouth for 14 days before infection.[30] If both CsA and the virus were administered intraperitoneally at the same time, CsA reduced viral replication.[28] In guinea pig, orally administered CsA prolonged and exacerbated CMV infection when compared with CsA plus corticosteroid-treated control animals but the inflammatory response to virus was most reduced in CsA-treated guinea pigs.[31]

If the recipient is overimmunosuppressed, the constellation may be different. ATG and high dose prednisolone cause more clinical complications of viral infections than does basic triple drug immunosuppression. CMV infection in total body-irradiated rats, like in human bone marrow transplant recipients, is often detrimental without any anti-viral treatment. After total body irradiation of the rat, RCMV infection is prolonged, higher virus titers are reached in various organs, and all animals die within 14 days if infected with only 10^4 PFU.[32]

In this study, triple drug immunosuppression seemed to protect against RCMV infection in the rat. The following may explain this observation. First, one or more of the components of the triple drug treatment might inhibit viral replication, eg, by inhibition of *de novo* viral DNA synthesis, or virus assembly, thus protecting the infected cell from cell death by a lytic course of infection. Second, by reducing the T cell response by CsA there is less T cell proliferation that further reduces viral replication. The dose and combination of immunosuppressants used, associated with a relative mild acute rejection and with very little graft-*versus*-host reaction, might be responsible for early latency of CMV. Thus, the harmful effects of acute CMV infection on the intimal formation are reduced.

There are two possible mechanisms by which CMV infection may increase allograft arteriosclerosis: either directly via a pro-proliferative effect on SMC and/or indirectly via a pro-proliferative effect on inflammatory cells. Favoring direct effect, CMV is capable of infecting endothelial cells[33,34] and SMC[35] of the vascular wall and may transform the infected cells by incorporating into cell genome and by inducing local changes in the metabolism of these cells.[36] The direct hypothesis is, however, unlikely because we have been unable to demonstrate viral antigens or fragments of viral genes in the SMC of vascular wall of the allograft by immunohistochemistry or by *in situ* hybridization (unpublished observations).

Several experimental data are compatible with the proinflammation role of CMV infection. It has recently been reported that CMV immediate early genes prevent the inhibitory effect of CsA on IL-2 gene transcription, thus possibly enhancing the immune responses against the grafted organ.[37] The same group showed earlier that the immediate early genes of human CMV up-regulate the expression of IL-2 and IL-2R genes.[38] Evidence exists that human CMV infection up-regulates IL-1β gene expression leading to increased production of IL-1 by the Mϕ cell lineage and to enhancement of inflammatory responses, eg, against foreign antigens.[39] Studies in AIDS patients with CMV-induced severe colitis have shown that CMV induces the production of tumor necrosis factor-α (TNF-α) by monocytes and mucosal macrophages.[40] Furthermore, the immediate early gene of human CMV is able to code for a protein that has sequence homology and immunological cross-reactivity with HLA-DR β-chain,[41] and human CMV encodes a glycoprotein that is homologous to MHC class I antigens.[42] Direct induction of class II molecule by CMV in rat heart endothelium *in vitro* has been shown.[43] In heart transplant recipients, the association between the induction of vascular cell adhesion molecule-1 and CMV antigenemia has been described.[44] These findings, as our present results, suggest that CMV infection up-regulates immunological responses, which may have a role in the generation of allograft arteriosclerosis. On the other hand, our results also demonstrate that triple drug immunosuppression efficiently interferes with this process in RCMV-infected animals.

Thus, a likely mechanism by which CMV infection enhances allograft arteriosclerosis is the enhancement of several molecular inflammatory cascades, ie, the indirect effect. The enhancement of inflammatory response by CMV might be either CMV-specific and/or an enhanced lymphoid cell activation against the allograft. T helper cells and Mo are predominating cell subclasses in the inflammation and may be of pivotal importance in the development of arteriosclerotic process. These cell types are capable of producing a large variety of cytokines such as IL-1, IL-2, IFN-γ, and TNF, as well as peptide growth factors such as PDGF and TGF-β, which all may promote

SMC proliferation and lead to an increase in intimal thickening. The indirect hypothesis is still supported by the fact that CMV is capable of infecting inflammatory cells and that viral antigens are occasionally found in the inflammatory leukocytes by immunohistochemistry (unpublished observations). Taken together, our findings postulate that CMV-enhanced allograft arteriosclerosis in the rat might be an immunopathological condition, as suggested by Grundy et al[45] in the pathogenesis of CMV interstitial pneumonitis rather than a direct virus-induced phenomenon.

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