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Correlation between the intensity of cytomegalovirus infection and the amount of perivasculitis in aortic allografts

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Abstract We previously demonstrated that cytomegalovirus (CMV) infection enhanced perivascular inflammation in rat aortic allografts. In this study, we investigated the relationship between the CMV infection load and the magnitude of perivasculitis (chronic rejection) in aortic transplants. Rats received orthotopic abdominal aortic grafts, different degrees of total body irradiation (TBI) for immunosuppression and CMV inoculation. The spleens of the rats receiving 5 Gy of TBI contained more infectious virus and viral antigens than those of rats receiving 3 Gy of TBI or no TBI. Although the number of inflammatory cells infiltrating the perivascular area was decreased after TBI, CMV infection resulted in increased perivasculitis in rats that received 5 Gy of TBI as compared to non-infected animals. This virus-induced effect was characterized predominantly by an increased T-cell infiltration, including CD4 and CD8 T-cells. It is concluded that an enhanced systemic CMV infection during severe immunosuppressive therapy can accelerate the development of chronic rejection, which seems to be mediated mainly by T-cells.

Key words Chronic rejection · Transplant-associated · arteriosclerosis (TAA) · Cytomegalovirus (CMV) infection · Perivasculitis · Immunosuppression

Introduction

Transplant-associated arteriosclerosis (TAA) or chronic rejection is the major cause of diminished long-term survival of transplanted organs [13]. In cardiac allograft recipients the incidence of TAA is nearly 50 % at 5 years posttransplantation [7]. The most common pathological features of TAA are persistent perivascular inflammation (perivasculitis) and intimal thickening in arteries (arteriosclerotic changes) of grafts [9].

The factors responsible for initiation and progression of TAA are not completely understood. Among others, TAA may be induced or accelerated by virus infections, such as cytomegalovirus (CMV) infection [11]. There is strong clinical evidence to show that CMV infections play a role in the pathogenesis of cardiac allograft arteriosclerosis [12]. CMV nucleic acids have been identified in the coronary arteries of heart allografts with severe accelerated arteriosclerosis [10, 18].

Previous work in our laboratory showed that immunosuppression enhanced the replication of CMV in the host leading to massive viral infection in almost all the organs [14] and that treatment with antiCMV therapy reduced the development of intimal thickening in the vascular wall of allografts [5]. However, it has not been elucidated whether the systemic CMV infection load, dependent on the degree of immunosuppression, influences the extent of transplant perivasculitis. For this purpose we used the rat aorta transplantation (Tx) as a specific model to investigate the effects of enhanced viral infection on the perivascular inflammatory response.

Table 1 Summary of experimental groups

Group	Transplantation	Number of rats	TBI[a]	RCMV[b] infection	Day of sacrifice
1	Syngeneic	5	No	+	7
2	Syngeneic	5	No	–	7
3	Allogeneic	5	No	+	7
4	Allogeneic	5	No	–	7
5	Allogeneic	4	3Gy	+	7
6	Allogeneic	4	3Gy	–	7
7	Allogeneic	3	5Gy	+	7
8	Allogeneic	4	5Gy	–	7
9	Syngeneic	5	No	+	28
10	Syngeneic	5	No	–	28
11	Allogeneic	5	No	+	28
12	Allogeneic	5	No	–	28
13	Allogeneic	3	3Gy	+	28
14	Allogeneic	3	3Gy	–	28
15	Allogeneic	6	5Gy	+	28
16	Allogeneic	5	5Gy	–	28
17	Allogeneic	4	5Gy	iRCMV	28

[a] Total body irradiation (TBI) was performed on day 1 after transplantation
[b] RCMV inoculation was performed intraperitoneally at 6 h after TBI

Materials and methods

Animals and aorta transplantation

Male inbred Brown Norway (BN/M; RT_{1n}) and Lewis (LEW/N; RT_{1l}) rats, weighing 250–300 g, were used as donors and recipients. In the donor operation, a segment of the abdominal aorta (1.8–2 cm) between the left renal artery and the bifurcation was removed with all side branches ligated after intravenous administration of heparin (50 IU). The grafts were transplanted orthotopically into recipients by the end-to-end anastomosis technique. Total ischemic times varied from 25 to 35 min, during which the grafts were kept cold with 4 °C PBS.

Experimental design

The aortic grafts from BN/M rats were transplanted into LEW/N recipients (allogeneic) or into BN/M recipients (syngeneic). After Tx, the animals were randomly divided into several groups (Table 1). A total body irradiation (TBI) of 3 or 5 Gy was used as immunosuppression therapy. The rats of the RCMV-infected group were inoculated intraperitoneally with 10^5 plaque forming units of rat CMV (RCMV, Maastricht strain [1]). The animals used as controls received either inactivated RCMV (iRCMV), which was derived from the same virus pool and inactivated by UV irradiation, or no virus at all. The recipients were sacrificed either on day 7 or day 28 after Tx. Tissue samples of the grafts and other organs were removed and divided into several pieces, which were processed either for frozen and paraffin sections, or for plaque assay.

Virus detection

Immunocytochemical techniques [14] were used for the detection of rat CMV antigens in grafts using RCMV monoclonal antibodies [4]. For the detection of general viral infection, the salivary glands, spleen and liver were placed aseptically into culture medium at sacrifice. These organs were homogenized in a tissue grinder and suspended in MEM with 2 % FCS. Quantification of infectious virus was done by means of plaque assay [1, 2]. For this, ten-fold dilutions of 10 % homogenates (wt/vol) were inoculated on a confluent rat embryonal fibroblast monolayer. After an incubation period of 7 days, the number of plaques was monitored microscopically after fixation and methylene blue staining.

Histological and immunohistochemical staining of grafts

A segment of the graft fixed in 3.7 % buffered formalin was embedded in paraffin, and examined histologically after sectioning and staining with haematoxylin and eosin. The aortas from non-transplanted rats were used as normal controls.

Four-micron-thick cross-sections of grafts were stained with three-layer (paraffin sections) or two-layer (frozen sections) indirect immunoperoxidase technique using monoclonal antibodies: W3/13 (Sera-lab, Crawley Down, UK), a mouse monoclonal antibody to rat pan T-cells; ED-1 (kindly supplied by Dr. Dijkstra, Department of Immunology, Free University, Amsterdam, The Netherlands), a mouse monoclonal antibody to rat monocytes/macrophages (mo/mΦ); Ox-8 (Sera-lab, Crawley Down, UK), a mouse IgG_1 monoclonal antibody to rat CD8 T-cells; and W3/25 (Sera-lab, Crawley Down, UK), a mouse IgG_1 monoclonal antibody to rat CD4 T-cells.

Quantification of the extent of perivasculitis

The total number of nuclei (TNN) and positively stained cells for W3/13 (T-cells), ED-1 (mo/mΦ) and Ox-8 (CD8 T-cells) in aortic adventitia was quantified in the cross-sectioned grafts and expressed as point score units (PSU), i.e. the mean number of points falling over given anatomical areas using straight, cross-sectional lines, and a 100 mm^2 square eye piece micrometre under a magnification of 400 ×. Under this magnification, a total area of 400 mm^2 was counted. The positively stained cells for W3/25 were scored from 0 to 5 (0, no staining; 1, very weak; 2, weak; 3, moderate; 4, intense; 5, very intense specific staining). Data are expressed as mean ± SEM. The number of positive cells or TNN in different groups were statistically compared with the aid of the non-parametric Mann-Whitney U-test. P values < 0.05 were regarded as statistically significant.

Results

RCMV infection

Two out of four RCMV-inoculated rats that received 3 Gy of TBI (3 Gy-TBI) and five out of six rats that received 5 Gy of TBI (5 Gy-TBI) harboured infectious RCMV in their spleens at 7 days after Tx. Viral culture from the salivary glands and liver was negative. On day 28, only the salivary glands harboured infectious RCMV, while no infectious virus was found in the liver and spleen of both syngeneic and allogeneic recipients that received 3 or 5 Gy-TBI. In iRCMV-inoculated control rats, no virus was cultured from any organs.

Sporadic RCMV-antigen-harbouring cells in the adventitia were detected in the allografts at 7 and 28 days

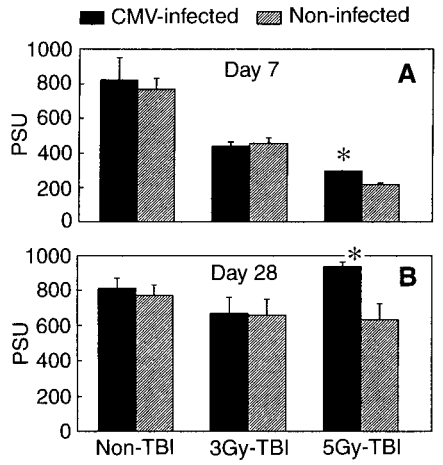

Fig. 1 A, B Total number of nuclei in the adventitia of aortic allografts at **A** 7 and **B** 28 days after transplantation. Data are expressed as mean ± SEM/PSU. (* $P < 0.05$ when compared with the non-infected group)

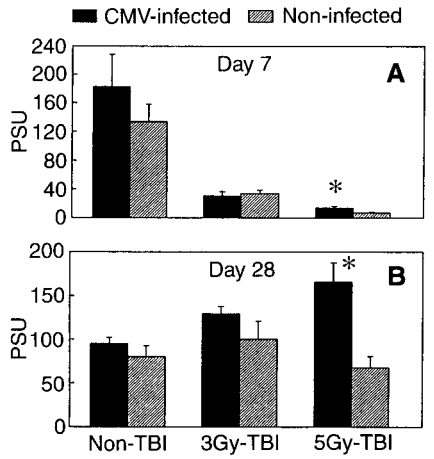

Fig. 2 A, B The number of total T-cells in the adventitia of aortic allografts at **A** 7 and **B** 28 days after transplantation. Data are expressed as mean ± SEM/PSU. (* $P < 0.05$ when compared with the non-infected group)

after Tx. There was a tendency that slightly more RCMV-reactive cells were present in recipients with TBI than without TBI. No viral antigens were detectable in isografts and allografts of iRCMV-inoculated rats irrespective of TBI.

Magnitude of perivasculitis

Without the application of TBI, the TNN in the allografts of the RCMV-infected group did not differ from that in the non-infected group at 7 days after Tx (Fig. 1 A). The TNN was decreased in rats that received 3 Gy-TBI as compared to that of non-TBI rats ($P < 0.05$). Nevertheless, after 3 Gy-TBI no significant difference in TNN was observed between the RCMV-infected and non-infected rats. In animals that received 5 Gy-TBI, the TNN was further decreased as compared to animals that received 3 Gy-TBI ($P < 0.05$). However, after 5 Gy-TBI, a significant difference in the TNN was observed between the RCMV-infected and non-infected groups ($P < 0.05$). At 28 days after Tx in the animals that received either non-TBI or 3 Gy-TBI, no significant difference in TNN could be found between the RCMV-infected and non-infected rats (Fig. 1 B). However, in the rats that received 5 Gy-TBI the TNN in the RCMV-infected group was significantly higher than in the non-infected group ($P < 0.05$). Moreover, after RCMV infection, the TNN was higher in the animals that received 5 Gy-TBI than in those that received 3 Gy-TBI ($P < 0.05$). Meanwhile, the TNN in the rats that received iRCMV did not differ from that in the non-infected rats, but was significantly less than in the RCMV-infected rats that received 5 Gy-TBI ($P < 0.05$).

The number of infiltrating T-cells in the allografts is shown in Fig. 2. At 7 days after Tx (Fig. 2 A), the number of infiltrating T-cells decreased significantly with an increase in the degree of TBI from non-TBI to 3 Gy-TBI and 5 Gy-TBI, respectively ($P < 0.05$). Nevertheless, no significant difference in the number of T-cells could be found between the RCMV-infected group and the non-infected group that received either no TBI or 3 Gy-TBI. In contrast, after 5 Gy-TBI, there was a significant increase in the number of T-cells in the RCMV-infected group as compared to the non-infected group ($P < 0.05$). At 28 days after Tx (Fig. 2 B), there was no significant difference in T-cell infiltration between the RCMV-infected and non-infected rats that received either no TBI or 3 Gy-TBI. However, when the animals received 5 Gy-TBI, the number of T-cells in the RCMV-infected group was more than twice that in the non-infected group ($P < 0.05$). Moreover, the number of T-cells in animals that received RCMV infection and 5 Gy-TBI was higher than that in rats that received RCMV infection without TBI and in animals that received iRCMV inoculation and 5 Gy-TBI ($P < 0.05$). The latter group was comparable to the non-infected animals with 5 Gy-TBI (PSU of 65 ± 21 and 67 ± 13, respectively).

In the allografts of animals that received RCMV infection and 5 Gy-TBI, the number of CD4 T-cells was higher than in the non-infected animals that received 5 Gy-TBI at 28 days after Tx only ($P < 0.05$, Fig. 3 A). The number of CD8 T-cells, however, was significantly higher in the RCMV-infected group as compared to the non-infected group both at 7 and 28 days after Tx ($P < 0.05$, Fig. 3 B).

Although with TBI the number of mo/mΦ in the allograft was decreased on day 7, no significant difference could be found between the RCMV-infected and the non-infected rats (Fig. 4 A). Likewise, at 28 days after

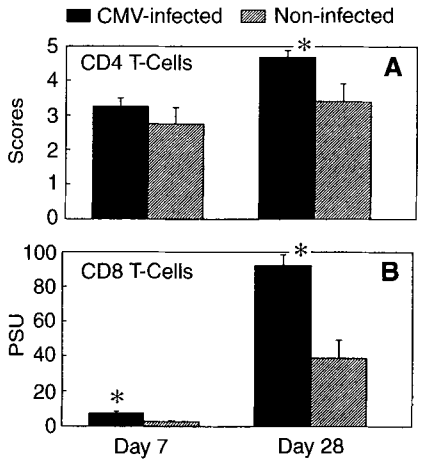

Fig. 3 A, B The number of CD4 and CD8 T-cells in the adventitia of aortic allografts in animals that received 5 Gy-TBI at 7 and 28 days after transplantation. Data are expressed as mean ± SEM/PSU or Scores. (* $P < 0.05$ when compared with the non-infected group)

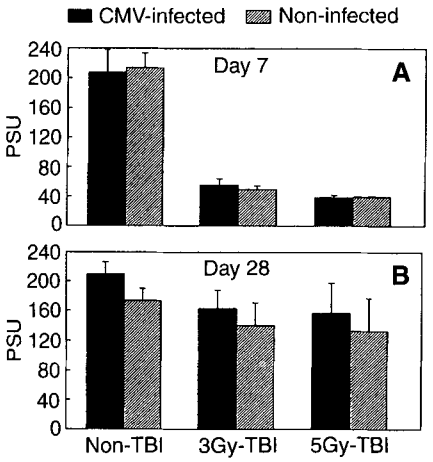

Fig. 4 A, B The number of monocytes/macrophages in the adventitia of aortic allografts at **A** day 7 and **B** day 28 after transplantation. Data are expressed as mean ± SEM/PSU. No statistical difference is found between the RCMV-infected and non-infected groups

Tx, no effect of TBI and RCMV infection on the number of mo/mΦ (Fig. 4 B) was observed.

In the adventitia of non-transplanted normal aortas, almost no infiltrating cells (T-cells or mo/mΦ) were found, only some fibroblasts were present. In syngeneic grafts (with or without RCMV infection), the TNN at 7 and 28 days after Tx was comparable with the normal aorta (data not shown). Sporadic, specifically stained T-cells and mo/mΦ in the adventitia were observed.

Discussion

The present study demonstrates the effect of acute generalized RCMV infection on allograft perivasculitis in immunosuppressed recipients. Although previous work in rats and data obtained from human material suggested that CMV infection leads to an enhanced perivascular inflammation in the transplanted organs, this report describes, for the first time, the relationship between the active replication of the virus in the host and the enhanced perivasculitis in the allografts. The fact that the perivasculitis was not affected by the inoculation of iRCMV, as seen in our study, indicates that replication of the virus in the host is necessary for the increased inflammatory response in the allografts. In line with previous work in our laboratory [14], this study also demonstrates that increased TBI enhances RCMV replication in the host, resulting in massive RCMV infection in almost all the organs of the animals after 5 Gy-TBI.

It is noticeable that CMV infection enhances the perivascular inflammatory response in allografts but not in isografts. This result suggests that the virus-induced perivasculitis is not the result of direct interactions of the virus with vessel wall cells, but the result of interactions between the virus and the immune cells, such as the allospecific T-lymphocytes. Although the cellular and molecular factors leading to TAA are largely unknown and the mechanism of the effect of CMV infection on TAA remains unclear, there is some evidence that immune inflammatory cells and the factors they produce are important for the process [3]. In the vessel wall of immunocompetent (non-TBI) and immunocompromised (TBI) rats, almost no viral antigen and infectious virus were detectable, while the internal organs, such as the spleen, contained infectious virus and viral antigens during the acute infection period (the first week postinfection).

The spleen is known as an important lymphoid organ in the immune system and in the course of viral infection [2, 14]. In the immunosuppressed host, replicating virus is detectable in large amounts in this organ [14]. Studies in mice have shown that CMV infection can lead to polyclonal T-cell activation and upregulation of allospecific cytotoxic T-cells [15, 19]. In addition, it has been shown that CMV infection enhances the graft versus host reaction in mice [6]. The activated T-cells and the mediators they produce, such as IL-2, TNF [8], may contribute to the process of allograft rejection. Thus, it might be speculated that the CMV-associated effect is an immune-mediated process rather than direct viral damage [3]. In support of the above hypothesis, we did find that mainly T-cells, including both CD4 and CD8 T-cells, were involved in the perivasculitis.

Besides the effect of CMV infection on the T-cell response, the interaction of CMV with endothelial cells (EC) could also be of importance for the development

of chronic rejection. In an in vitro study, Waldman [17] has demonstrated that CMV-infected EC provide a powerful stimulus for the activation of T-cells derived from CMV-seropositive donors, and that T-cells thus activated release IFN-γ sufficient to induce the expression of HLA-DR on the surrounding uninfected EC [16]. In addition, cytokines elaborated by CMV-activated T-cells are capable of enhancing endothelial HLA class I and ICAM-1 expression [17]. Therefore once CMV infects EC, several molecular inflammatory cascades may be triggered, and continue in the absence of the virus.

In conclusion, our results indicate that enhanced general RCMV infection has a detrimental effect on development of TAA of aortic allografts in highly immunocompromised recipients. This CMV-associated process, which appears to be T-cell mediated, is accelerated by an increased immunosuppressive regimen.

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