Transpl Int (2003) 16: 37–44
DOI 10.1007/s00147-002-0504-z

ORIGINAL ARTICLE

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Busulfan depletes neutrophils and delays accelerated acute rejection of discordant xenografts in the guinea pig-to-rat model

Received: 15 October 2001
Revised: 29 May 2002
Accepted: 8 July 2002
Published online: 10 December 2002

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Abstract Complement factor C6 plays a critical role in mediating hyperacute rejection of discordant xenografts. In order to explore the mechanism of discordant xenograft rejection, we investigated kinetics and phenotypes of the cellular infiltrate in xenografts in untreated and leukocyte-depleted recipients, in relation to graft survival. Guinea pig cardiac xenografts were heterotopically transplanted to totally C6-deficient PVG (C-) rats. Grafts were removed after 0, 6, 12, and 24 h ($n=6$). Histological evaluation was performed with hematoxylin and eosin (H & E) and immunoperoxidase staining. The agents fucoidin and busulfan were applied to delay xenograft rejection further. Within 6 h, minimal perivascular edema with isolated infiltrating CD11b/c- and ED1-positive cells were found. An intense infiltration of CD11b/c- and ED1-positive cells with interstitial hemorrhage was present after 24 h, though with little CD161 and CD3 cell infiltration. Inhibition of cell adhesion by fucoidin did not prolong xenograft survival (34 ± 15 h, $n=4$, $P<0.47$), but the depletion of granulocytes by injection of busulfan did prolong survival of the discordant xenografts, to 62 ± 22 h ($n=7$, $P \leq 0.0039$). These results demonstrate a significant effect of specific depletion of granulocytes and macrophages by busulfan therapy on guinea pig cardiac xenograft survival in PVG (C-) rats, suggesting the participation of these infiltrating cells in the xenoreactive rejection process.

Keywords Xenograft · C6 deficiency · Neutrophil depletion · Accelerated acute rejection

Introduction

Successful discordant xenotransplantation (DXTx) between phylogenetically distant species is prevented by hyperacute rejection (HAR) [3, 21, 30, 31]. HAR is a highly destructive rejection mechanism, with endothelial cell injury, hemorrhage, edema, and infarction occurring within minutes to hours after graft reperfusion. This process is mainly initiated by the presence of pre-formed natural antibodies (mostly of the IgM type) in the host, that, after graft reperfusion, bind to xenogeneic antigens expressed on the endothelial cells and activate the classic complement pathway. The alternative complement pathway is activated by different a endothelial surface [3, 21, 30, 31].

Subsequent activation of the cascade culminates in the formation of the membrane attack complex (MAC), a structure consisting of the complement (C) compounds (C5b–C9). Cell lysis or cell activation of endothelial cells may result, depending on the quantity of MAC deposition on the cell membrane [21].

An important role of C in HAR has been shown by prolonged survival in recipients depleted of C by treatment with cobra venom factor (CVF) [20, 24, 39] and inhibition of the classic and alternative complement pathway with human recombinant soluble C-receptor type 1 (sCR1) [32, 33, 34, 35, 43]. The application of CVF delays discordant xenograft rejection by uncontrolled consumption and activation of complement factors. For this reason, treatment of recipients with CVF is not suitable in the study of discordant xenograft rejection mechanisms.

Inhibition of terminal MAC formation effectively prevents HAR [3, 7, 21, 22]. We have previously reported that cardiac xenograft survival was significantly prolonged to 1–2 days in a unique strain of PVG (C–) rats fully lacking the C6 component of complement due to an isolated genetic deficiency in the C6 gene [42], compared with the C6-sufficient congenic controls that rejected their grafts hyperacutely within minutes [5, 21, 22]. The role of C6 in discordant xenograft survival has also been demonstrated in Lewis ($RT1^l$) rats. Depletion of C6 in Lewis rats with a polyclonal rat-anti-rat C6 antibody of IgG subclass 1 prolonged discordant xenograft survival similarly to that in C6-deficient PVG (C–) rats [8].

Prolongation of discordant xenograft survival was histologically related to a shift from HAR – characterized by edema, interstitial hemorrhage, disrupted vessels, and intravascular platelet aggregation [3] – to accelerated acute rejection [3, 21].

In the C6-deficient xenograft model, endothelial cell activation takes place through the binding of natural antibodies and complement factors up to C5b [3]. One of the early markers of endothelial cell activation is CD62P (P-selectin) expression [18]. Several authors have shown that inhibition of P-selectin by administration of anti-P-selectin antibody [12] or fucoidin (an algal polymer of fucose-4-sulfate that binds to both L- and P-selectin) inhibits selectin-dependent leukocyte–endothelial cell interaction [23, 26, 27]. The in vivo capacity of fucoidin to delay discordant xenograft rejection has not yet been investigated.

Activation of the early complement components results in the production of the split products C4a, C3a, and C5a. C3a acts primarily on mast cells and eosinophils. C5a is quantitatively much more potent as a chemotactic agent than C3a and affects a wider range of cells, including neutrophils, monocytes, basophils, and eosinophils [15]. It has previously been shown that the drug busulfan, which has been used for experimental and clinical bone marrow (BM) transplantation, is an extremely effective anti-leukemic and myeloablative agent, involving little immunosuppressive activity because of its minimal impact on lymphoid cells [16, 36, 37]. Intra-peritoneal injection of busulfan in sub-lethal concentrations selectively depletes the myelopoietic stem cells of BM and therefore reduces the circulation of peripherally circulating granulocytes and monocytes without affecting the lymphoid cell population.

The present study was undertaken to investigate the kinetics and phenotypes of the cellular infiltrate after 0, 6, 12, and 24 h in guinea pig cardiac xenografts. Using a panel of monoclonal antibodies in immunoperoxidase staining, we further dissected the rejection process of DXTx in the absence of the MAC. We used the effect of pretreatment with busulfan and fucoidin to investigate the participation of the infiltrating cells in accelerated acute rejection as well as to assess possible new therapeutic strategies.

Materials and methods

Animals

Male 9 to 12-week-old homozygous PVG (C–)($RT1^c$) rats weighing 200–250 g were obtained from Prof. Mohamed R. Daha, Department of Nephrology, University Hospital Leiden, The Netherlands. Sera of the PVG (C–) rats were tested by hemolytic complement assays and gel electrophoresis to confirm C6 deficiency as described previously [3, 6, 9]. Inbred male virus-free Hartley guinea pigs (GPs), 8–12 weeks of age, from Charles River Laboratories (Kisslegg, Germany) served as cardiac xenograft donors. The animals were housed in our own isolated colony in micro-isolator cages and received standard laboratory chow and water ad libitum, in accordance with standard guidelines. Principles of laboratory animal care were followed as well as the current version of the German law on the protection of animals.

Heterotopic cardiac xenotransplantation

GP cardiac xenografts were transplanted heterotopically into rat recipients under ether inhalation anesthesia by the microsurgical technique of Ono and Lindsey [28] with anastomosis of the donor aorta to the recipient infra-renal aorta and the donor pulmonary artery to the recipient infra-renal vena cava. Cardiac xenografts were evaluated visually over the first 45 min following reperfusion, and, subsequent to abdominal closure, every 60 min by abdominal palpation, until rejection. Rejection was defined as total cessation of contractions, confirmed by direct visualization as well as histological examination of the cardiac xenograft.

Histological assessment

Sample segments of cardiac xenografts were collected after rejection or according to the protocol at 0, 6, 12, or 24 h ($n=6$ per group) after reperfusion for evaluation of the kinetics of cellular infiltration. For light microscopy, portions of xenograft tissue were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H & E). The xenografts were evaluated for intravascular platelet aggregation, congestion, vessel disruption, interstitial hemorrhage, myocardial necrosis, and leukocyte infiltration. Tissue samples of the same grafts were covered with tissue-tec, snap-frozen in liquid nitrogen, and stored at −80 °C for immunohistochemical analysis.

Antibodies

The following monoclonal antibodies (mAbs) were used in this study: mouse anti-rat CD11b/c mAb for granulocytes (22081D, PharMingen, San Diego, Calif.), mouse anti-rat ED1 for macrophages (MCA341R, Serotec, Oxford, UK), mouse anti-rat CD161 for natural killer (NK) cells (MCA1427, Serotec) and mouse anti-rat CD3 for T cells (22011D, PharMingen). Rabbit anti-mouse IgG peroxidase conjugate (A-9044, Sigma, St. Louis, Mo.) was used as secondary antibody. Polyclonal goat anti-human C6 for hemolytic complement assays was obtained from Accurate (Westbury, N.Y.) and anti-human IgG from PharMingen.

Immunohistochemistry

Cryostat sections were prepared at 5 μm, fixed in cold acetone for 20 min, dried, and stored at −80 °C. Endogenous peroxidase activity was blocked by pre-incubation of the sections with rat serum and H_2O_2. Thereafter, the sections were incubated for 45 min with 100 ml of the diluted mAbs (CD11b for granulocytes, ED1 for macrophages, CD161 for NK cells, CD3 for T cells were obtained from Serotec). Subsequent to three washes in PBS, 100 ml of secondary goat anti-mouse IgG antibody (dilution 1:150) labeled with horseradish peroxidase (PharMingen) was added for 45 min. Non-specific background staining was reduced by pre-incubation (20 min) of the peroxidase-conjugated antiserum with 5% FCS PBS. All incubation was conducted in a moist, light-protected chamber at room temperature. The sections were rinsed again in PBS and stained for 3–5 min in 50 mM acetate buffer containing 0.005% H_2O_2 and 5 mg/ml 3-amino-9-ethylcarbazole (Sigma), which was dissolved in N,N′-dimethylformamide. Following extensive washing in PBS, the slides were counterstained with Mayer's hematoxylin for 5–10 min and mounted with glycerol gelatin. Evaluation was performed by three investigators with blind labeled slides. All infiltrating leukocytes were counted in ten different high-power fields from three sections of every tissue sample ($n=6$). The number of infiltrating cells was expressed as mean value with standard deviation.

Pretreatment with busulfan

Busulfan (Sigma, Germany) inhibits the myelopoietic stem cells within BM in rats in vivo [10, 17, 37, 41]. Prior to use in xenotransplantation, the effect of busulfan on stem cells in BM of PVG (C−) rats was investigated by evaluation of BM staining after intraperitoneal (i.p.) injection. Busulfan was dissolved in PBS and injected i.p. in PVG (C−) rats ($n=6$) in concentrations of 30 mg/kg b.w. and 60 mg/kg b.w. As previously described, BM was removed from femurs and tibias [6, 17, 41] after 3, 7, and 14 days and stained with Pappenheim's stain by a standard technique. Following injection of busulfan (30 mg/kg b.w.), evaluation of the BM by light microscopy showed a reduction in myelopoietic stem cells with a right shift of the granulopoietic cells on day 3 post-injection. Seven days after injection, a further decrease in myelopoietic stem cells was found that did not affect the erythropoietic cell lineage. On day 14, regeneration of the BM had been re–initiated. The use of busulfan at a higher concentration of 60 mg/kg b.w. did not prove to be of any advantage. This dose was found to be lethal and did not allow regeneration of the BM on day 14. Based upon these findings and those from reports in the literature [6, 17, 36, 41], busulfan was used at a concentration of 30 mg/kg b.w. and injected i.p. on day 3 prior to xenografting (Table 1).

Pretreatment with fucoidin

Fucoidin (Sigma, Germany), an algal polymer of fucose-4-sulfate, is known to inhibit the selectin-dependent leukocyte–endothelial cell interaction in vitro [19, 23, 26, 27]. In view of this, the question arose as to whether fucoidin might have a beneficial effect on discordant xenograft survival. Fucoidin was dissolved in PBS and injected i.p. at concentrations of 15 and 7.5 mg/kg b.w. according to the literature [19, 23, 26, 27] at the time of xenografting (Table 1).

Statistical analysis

Xenograft survival data are expressed as mean survival time ± standard deviation. A two-tailed Student's t-test was used to compare xenograft survival among groups. Significance was accorded to values of $P \leq 0.05$.

Results

Xenograft survival

GP-to-PVG (C−) rat cardiac xenograft survival is summarized in Table 2 and illustrated in Fig. 1. Untreated PVG (C−) ($RT1^c$) rats rejected their discordant GP cardiac xenografts in an accelerated acute fashion in 29.1 ± 3.5 h ($n=6$), which is similar to previously published results [3, 21]. Pretreatment of PVG (C−) recipients by i.p. injection of busulfan (30 mg/kg b.w.) at day 3 before DXTx significantly prolonged survival to $62 \bullet 21.5$ h ($n=7$, $P<0.0039$).

The effect of i.p. fucoidin application at the time of xenografting (intended to inhibit leukocyte adhesion to endothelial cells) was disappointing in terms of its delaying cardiac xenograft rejection. The injection of

Table 1 Study design

Group	n	Pretreatment	Guinea pig xenograft	DXTx
1	6	None	+	Rejection
2	6	None	+	t=0
3	6	None	+	t=6 h
4	6	None	+	t=24 h
5	6	Saline	+	t=24 h
6	7	30 mg/kg b.w. busulfan	+	Rejection
7	5	30 mg/kg b.w. busulfan	+	t=24 h
8	4	7.5 mg/kg b.w. fucoidin	+	Death
9	4	15 mg/kg b.w. fucoidin	+	Death

Table 2 Graft survival

Treatment	Individual graft survival (h)	*n*	Graft survival (mean ± SD)	*P*
Saline	26, 26, 28, 28, 33, 34	6	29.1 ± 3.5 h	
Busulfan	44.5, 45, 48, 57, 65, 66, 106	7	61.6 ± 21.5 h	$P < 0.0039$
Fucoidin	18, 24, 43, 51	4	34 ± 15 h	$P < 0.47$

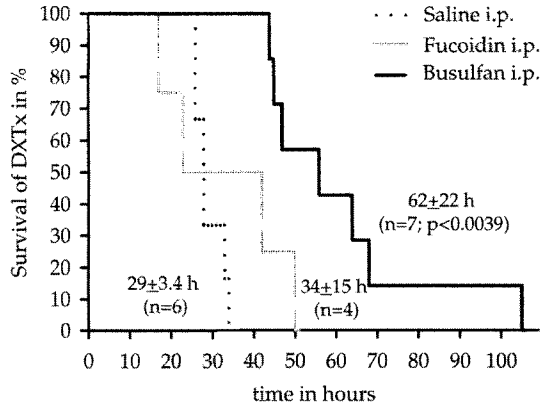

Fig. 1 Discordant xenograft survival following i.p. pretreatment of PVG (C–) recipients with fucoidin (7.5 mg/kg b.w.) and busulfan (30 mg/kg b.w.). Busulfan injected on day 3 prior to grafting significantly delayed DXTx rejection in comparison with i.p. treatment with fucoidin

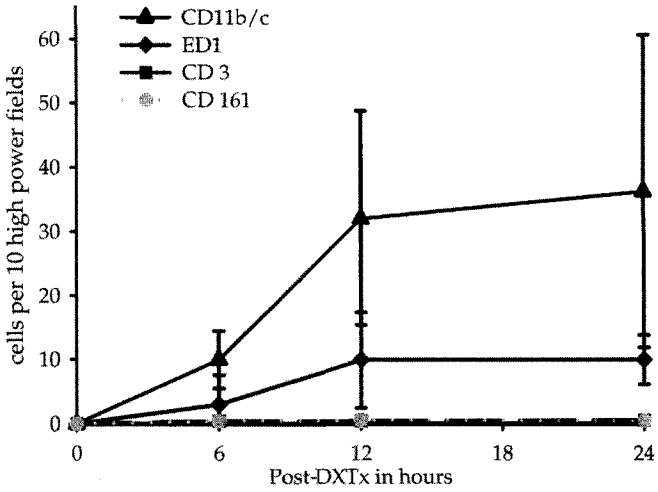

Fig. 2 Kinetics of cellular infiltration ($n = 6$) in DXTx after 6, 12, and 24 h. Values are expressed as mean ± SD of positive cells of ten high-power fields. Granulocytes and macrophages are predominant. NK and T cells are not present during this period of the rejection process of DXTx in the absence of the MAC

fucoidin in concentrations of 15 and 7.5 mg/kg b.w. resulted in a high death rate of xenografted recipients with beating GP hearts (> 50%) after 12–24 h post-treatment. The remaining survivors rejected their xenografts at a similar tempo, when compared with the untreated PVG (C–) rats (34 ± 15 h, $n = 4$, $P < 0.47$). Post-mortem examination showed massive intra-abdominal bleeding of the recipients. The anastomosis did not show any evidence of dehiscence, aneurysmal dilatation, or thinning of the vessel walls. The high mortality was due to diffuse intra-abdominal bleeding, possibly induced by fucoidin.

Kinetics of cellular destruction

To explore further the mechanism of rejection within discordant xenografts lacking the MAC, we determined the kinetics of vascular and cellular destruction 0, 6, 12, and 24 h after grafting without pretreatment. Six hours after DXTx, little perivascular edema, with only isolated perivascular hemorrhage, was found in the saline-treated group ($n = 6$). Arteries as well as veins did not show any intravascular thrombosis. Initial cellular infiltration consisting mainly of polymorphonuclear cells was evident by H & E staining. Immunoperoxidase staining demonstrated that the cellular infiltrate was composed mainly of granulocytes (CD11b/c) and macrophages (ED1), especially within the perivascular area. Some isolated NK cells (CD161) and T cells (CD3) were additionally found. Twelve hours after DXTx, adhesion of polymorphonuclear cells (PMNs) was observed under light microscopy, particularly to the endothelial cells of veins as well as within partially infiltrated perivascular tissue. Hemorrhage was more prominent in the perivascular area and also detected in the interstitial tissue. Immunoperoxidase staining defined most of the cellular infiltrate as granulocytic. Macrophages presented the second largest population of infiltrating cells. The infiltration of NK and T cells remained limited.

These histomorphological changes were strongly present 24 h after DXTx, with granulocytic infiltration into the perivascular area as well as into the myocardium, combined with a pronounced interstitial hemorrhage and destruction of the myocardium with disruption of the myocardial fibers. Immunoperoxidase staining revealed massive interstitial infiltration of granulocytes (CD11b/c) and macrophages (ED1) within different areas of the myocardium. The isolated occurrence of NK and T cells remained constant during the rejection process. The overall cellular infiltration of CD11b/c-, ED1-, CD161-, and CD3-positive cells ($n = 6$ grafts per group) is expressed in Fig. 2 as the mean ± SD number of cells per ten high-power fields. These data suggest that NK and T cells are not involved in the early

phase of the rejection process of DXTx in absence of the MAC. The massive increase of CD11b/c- and ED1-positive cells, with concurrent destruction of the xenograft, implies the functional participation of these cells in the discordant rejection process in absence of the MAC.

Histological effect of busulfan, saline, and fucoidin

Histological changes within discordant xenografts removed 24 h after grafting of a GP cardiac xenograft into saline-pretreated PVG (C−) recipients (5/6 grafts sampled) were compared with those removed at the same time from busulfan-pretreated (30 mg/kg b.w.) PVG (C−) recipients (5/7 grafts sampled). Macroscopically, discordant xenografts removed from busulfan-pretreated PVG (C−) rats were substantially less damaged than those removed at the same time from untreated PVG (C−) recipients. Histologically, perivascular edema and perivascular hemorrhage were significantly less extensive, as was interstitial damage of the myocardium, which contained only minimal interstitial hemorrhage and cellular infiltration, compared with the untreated hosts. Associated with the significant delay in accelerated acute rejection of discordant xenografts after pretreatment with 30 mg/kg b.w. busulfan was less interstitial cell infiltration. In particular, granulocyte and macrophage numbers were decreased in the rejection process of DXTx. The phenotypes and numbers of infiltrating cells after pretreatment with busulfan are expressed in Fig. 3 as mean ± SD of cells per ten high-power fields. The number of CD11b/c-positive cells in the myocardium was reduced from 36.3 ± 24.3 ($n=6$) to 19.7 ± 13.3 ($n=5$; $P<0.348$) and ED1-postive cells from 10.4 ± 4.4 to 4.6 ± 4.4 ($n=5$, $P<0.06$). The numbers of infiltrating NK and T cells remained unchanged.

The harvested discordant xenografts from fucoidin-pretreated recipients were not histologically evaluated because of the high lethality associated with this treatment.

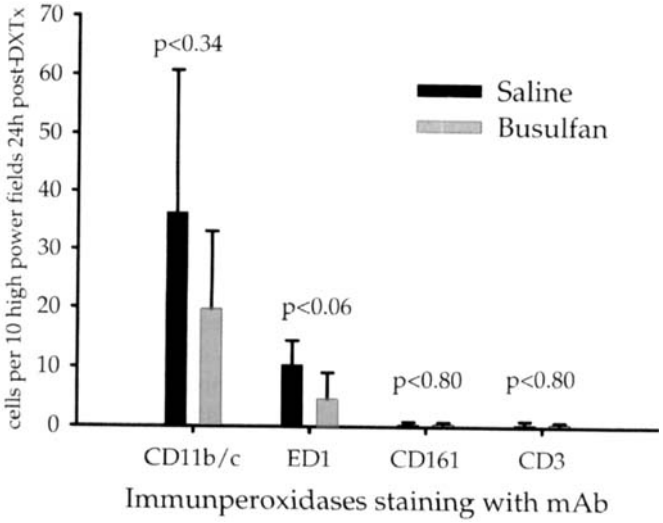

Fig. 3 Phenotypes of cellular infiltration ($n=5$) in DXTx 24 h after grafting in untreated and busulfan-pretreated recipients. Values are expressed as mean ± SD of positive cells of ten high-power fields. Granulocytes are predominant over macrophages, but reduced by 50% when compared with the untreated PVG (C−) hosts ($n=6$)

Discussion

HAR can be successfully prevented by inhibition of the activation of the complement system at different levels of the cascade. Various approaches include the use of endogenous complement regulation in transgenic organs [11, 29, 38, 40] as well as exogenous complement inhibition by agents such as CVF [24], sCR1 [32, 34, 35], or anti-C6-directed polyclonal antibodies [8].

Each of these approaches prevents the hyperacute onset of events that leads to immediate and irreversible graft destruction within minutes. Only little is known about the mechanism and immunosuppressive requirements after overcoming HAR when cellular response becomes effective during delayed xenograft rejection [1, 2, 4, 25].

Complement C6-deficient PVG (C−) recipients, unable to construct the terminal MAC (C5b–C9) of the complement cascade due to an isolated genetic deficiency in C6 [42], reject discordant GP cardiac xenografts in an accelerated acute fashion within 1–2 days [3, 13, 21, 22, 44, 45]. The C6 deficiency blocks a late step in complement activation of the classic and alternative complement pathway. The complement cascade is activated up to C5b, without formation of the MAC (C5b–C9). Vascular staining of C3 within the GP xenograft [3, 45] as well as consumption of C3 and C5 during the rejection process [3] support the presumption that activation of the chemotactic complement split products C3a, C4a, and C5a is responsible for the cellular infiltration of PMNs and subsequent damage to the xenograft.

To assess further the mechanism of discordant xenograft rejection, we investigated the kinetics of the inflammatory infiltrate into GP cardiac xenografts transplanted into PVG (C−) recipients after 0, 6, 12, and 24 h in the absence of the MAC, by H & E and immunoperoxidase staining. Within 6 h after grafting, an initial cellular infiltrate predominantly made up of granulocytes (CD11b/c) and macrophages (ED1) was found. This infiltrate increased significantly in the next 6 h of the rejection process, with a greater number of granulocytes than macrophages. Interstitial hemorrhage and damage to the myocardium intensified during the 24 h after grafting, without further increase in the numbers of infiltrating granulocytes and macrophages. Only iso-

lated T cells (CD3) and NK cells (CD161) infiltrated during the rejection process, but did not increase in number over time. T and NK cells do not appear to be involved in the first response of cellular infiltrate during discordant xenograft rejection.

The drug busulfan, which has been used for experimental and clinical BM transplantation because of its anti-leukemic and anti-myelopoietic effects [10, 16, 17, 36, 37, 41], was effective in the depletion of myelopoietic stem cells within the BM of PVG (C−) rats at a concentration of 30 mg/kg b.w. when given 3 days before xenografting, without any evidence of morbidity or mortality. A higher concentration of busulfan (60 mg/kg b.w.) provided no additional beneficial effect. In comparison with the untreated control group at 24 h after grafting ($n=6$), the i.p. application of busulfan (30 mg/kg b.w.) in PVG (C−) recipients 3 days prior to xenografting significantly prolonged the survival of discordant GP cardiac xenografts, by up to 100 h (61.6 ± 21.5 h, $n=7$) with less interstitial hemorrhage and myocardial damage as well as infiltration of granulocytes and macrophages. The decreasing number of granulocytes and macrophages in the xenograft correlated with the increased survival time of the graft. These results imply that not only does the composition of the cellular infiltrate consist primarily of granulocytes and macrophages, but that these cells also contribute to the first phase of cellular response in the rejection of discordant xenografts in the absence of the MAC. These results are consistent with those of Wu et al., who primarily found macrophages (ED1 and ED2) and only few NK and T cells in the grafts, without staining for CD11b/c cells [44, 45]. Wu et al. demonstrated that the agent Lip-Cl_2MDP, a liposome-encapsulated dichloromethylene diphosphonate, was not only effective in depleting macrophages, but also significantly prolonged discordant xenograft survival on its own or in combination with other agents [44].

Endothelial adhesion and migration of PMNs from blood vessels into tissues is an early event in the immunological response to inflammation or tissue injury. Strategies combining the inhibition of complement activation and standard immunosuppressive therapy prevent HAR in the pig-to-primate model, but not rejection due to cellular infiltration of neutrophils [14]. Several studies appear to indicate that a further delay in this type of discordant xenograft rejection may be possible by blocking the adhesion of PMNs to endothelial cells [13, 21, 46]. Although the exact mechanism is not fully understood, anti-inflammatory agents such as leumedins and nactins appear to be effective in the further delay of discordant xenograft rejection within the C6-deficient model by blocking the neutrophil function dependent on the CD11b/CD18 pathway [13, 21].

It has been shown in several in vitro models that the agent fucoidin, an algal polymer of fucose-4-sulfate, inhibits the cellular adhesion of leukocytes [19, 26, 27]. Therefore, it may be assumed that fucoidin might have a beneficial effect on discordant xenograft survival. We applied this drug at two doses (7.5 and 15 mg/kg b.w.), but even at the reduced dose fucoidin had a high lethality in PVG (C−) rats, possibly due to intra-abdominal bleeding. The surviving recipients did not profit from a longer-surviving graft; instead, graft rejection was not even significantly different from that of the untreated PVG (C−) recipients.

In conclusion, our results indicate that the GP cardiac xenografts were heavily infiltrated, predominantly by granulocytes and macrophages, after overcoming HAR. Depletion of myelopoietic stem cells by pretreatment with busulfan reduced the number of infiltrating granulocytes and macrophages, as demonstrated by H & E and immunoperoxidase staining. The reduced number of infiltrating cells correlated with a further delay of discordant xenograft survival and implies the participation of these cells in discordant xenograft rejection in the absence of the MAC.

Acknowledgements We wish to thank Dr. William M. Baldwin III for his advice and for carefully reading the manuscript.

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