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Immunosuppressive protocol with delayed use of low-dose tacrolimus after aortic transplantation suppresses donor-specific anti-MHC class I and class II antibody production in rats

Authors' Contribution:
 Study Design A
 Data Collection B
 Statistical Analysis C
 Data Interpretation D
 Manuscript Preparation E
 Literature Search F
 Funds Collection G

ABCDEF 1 **Ivan Matia***
 ADEFG 1 **Peter Fellmer***
 ABCDEF 2 **Katrin Splith***
 BDEF 3 **Martin Varga**
 ADEG 3 **Milos Adamec**
 BCD 2 **Ines Kämmerer**
 BD 1,2 **Linda Feldbrügge**
 BD 1 **Felix Krenzien**
 BD 1 **Hans-Michael Hau**
 BD 1 **Georgi Atanasov**
 ADEG 1,2 **Moritz Schmelzle**
 ADEG 1,2 **Sven Jonas**

1 Department of Visceral, Transplantation, Thoracic and Vascular Surgery, University Hospital Leipzig, Leipzig, Germany
 2 Translational Centre for Regenerative Medicine, University of Leipzig, Leipzig, Germany
 3 Department of Transplant Surgery, Institute for Clinical and Experimental Medicine, Prague, Czech Republic

* I. Matia, P. Fellmer and K. Splith contributed equally to this work.

Corresponding Author: Moritz Schmelzle, e-mail: moritz.schmelzle@medizin.uni-leipzig.de

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Background: Arterial allografts are used as vascular conduits in the treatment of prosthetic graft infection. Immunosuppression decreases their rupture risk rate. However, immunosuppression can be unprofitable in florid infection. Previously, we confirmed inhibition of cell-mediated destruction of rat aortic grafts by delayed use of tacrolimus. In this work, we studied the influence of this protocol on the antibody-mediated rejection.


Material/Methods: Flow cytometry was used for the retrospective analysis of day 0, 14, and 30 sera obtained from Lewis rat recipients of isogeneic fresh infrarenal aortic grafts (group A) or Brown-Norway rat aortic grafts (group B,C,D) for the presence of donor-specific anti-MHC class I and II antibodies. Tacrolimus in daily dose of 0.2 mg/kg was administered from day 1 to day 30 (group C) or from day 7 to day 30 (group D).

Results: Inhibition of fluorescence-labeled anti-BN MHC class I and MHC class II antibodies binding to BN-splenocytes was observed only by day 14 and day 30 sera of allogeneic non-immunosuppressed Lewis rats (group B). The day 30 sera significantly decreased anti-MHC I ($42\pm 3\%$) and anti-MHC II antibody binding ($56\pm 3\%$) compared to day 0 ($76\pm 9\%$, $p=0.005$ and $79\pm 5\%$, $p=0.003$, respectively). Deposition of immunoglobulins G into the tunica media was observed only in non-immunosuppressed aortic allografts on day 30.

Conclusions: Fresh aortic allografts induce donor-specific anti-MHC class I and anti-MHC class II antibody production. Delayed administration of tacrolimus completely suppressed antibody production and antibody-mediated destruction of aortic allografts.

Keywords: Antibody-Mediated Rejection • Arterial Allografts • Tacrolimus • Anti-MHC Class I Antibody • Anti-MHC Class II Antibody • Arterial Rejection

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Background

Infection of vascular prostheses remains a serious problem in vascular surgery. Replacement of an infected prosthesis with an arterial allograft represents the therapy of choice in this life-threatening condition [1,2]. However, strong homograft antigenicity triggers immune response similar to rejection processes evident in solid organ transplant recipients [3]. A higher incidence of graft-related death, graft ruptures or thrombosis and graft aneurysm formation was observed in non-immunosuppressed patients after arterial implantation [1].

In contrast, good long-term patency rates and no aneurysmal formation of arterial allografts were observed in patients receiving triple immunosuppression after simultaneous organ and arterial transplantation [4–6]. However, the use of immunosuppression in patients after arterial allograft implantation is not generally accepted by vascular surgeons [7]. This is probably caused by a reluctance to use immunosuppressive treatment in patients with ongoing infection [8].

In our previous experimental work, we investigated cell-mediated destruction of rat aortic grafts by the recipient's immune system in the field of no immunosuppression [8]. The main features of aortic graft rejection were adventitial inflammatory infiltration, the disappearance of medial smooth muscle cells, and intimal proliferation. However, we showed no graft destruction by the use of low-dose tacrolimus immunosuppression. Moreover, we evaluated the possibility of delaying this low-dose tacrolimus immunosuppression for 1 week without any negative influence on morphological signs of acute arterial wall rejection. These data led to the introduction of delayed immunosuppressive protocol with tacrolimus in allografted patients with prosthesis infection, with good results [9].

Destruction of the tunica media seems to be crucial in the process of aneurysmal dilatation of rejected allo-arteries and can lead to graft rupture with life-threatening bleeding [10].

Humoral antibodies against major histocompatibility complex (MHC) class I play a very important role in the rejection of solid organs [11]. The verification of humoral theory of transplantation led to new therapeutical approaches, mainly in patients after kidney transplantation [12].

The objectives of the present study were to determine 1) donor-specific antibody production in recipients of aortic allografts with the correlation to histological findings of rejected arteries, and 2) suppression of antibody-mediated rejection after aortic grafts implantation by delayed administration of low-dose tacrolimus (FK506) therapy.

For this purpose, we assessed the presence of donor-specific anti-MHC class I and anti-MHC class II antibodies in the sera of recipient animals obtained previously in our Brown-Norway (BN) to Lewis (LEW) aortic transplantation model. In addition, we compared the level of antibody production with histological findings obtained previously.

Material and Methods

Animals

Adult male inbred Brown-Norway (BN; RT1ⁿ) and Lewis (LEW; RT1^y) rats were obtained from Charles River (Sulzfeld, Germany). Principles of laboratory animal care were followed and all rats were maintained according to the National Institute of Health Guidelines. Male LEW rats (n=23, 240–380 g) were used as recipients of allogeneic or syngeneic abdominal aortic grafts. Male BN rats (n=12, 220–280 g) were used as donors of allogeneic abdominal aortic grafts. Male LEW rats (n=4, 300–420 g) were used as donors of syngeneic abdominal aortic grafts. Each transplanted animal was held in a separate cage during the 30-day follow-up period.

Only animals that passed the whole follow-up period were included into the study.

Operative procedure

Orthotopic fresh aortic graft transplantation was described in detail in our previous publication [8]. Briefly, the donor animals were anaesthetized by an intramuscular injection of ketamine (Narkamon[®], Spofa a.s., Prague, Czech Republic) at 100 mg/kg and xylazine (Rometar[®], Spofa a.s., Prague, Czech Republic) at 10 mg/kg. A 2–2.5 cm long segment of the infrarenal aorta was excised and divided into 2 pieces, which were stored separately at 4°C in Custodiol[®] solution (Custodiol[®], Dr. Franz Köhler Chemie GmbH, Bensheim, Germany) for 25 h. The recipient animals were anaesthetized with less invasive anesthesia (intramuscular injection of sufentanil (Sufenta[®], Janssen Pharmaceutica Inc., Beerse, Belgium) at 20 µg/kg and azaperone (Stresnil[®], Janssen Pharmaceutica Inc., Beerse, Belgium) at 1 mg/kg) to ensure more natural awakening. The aortic grafts were implanted orthotopically into the recipient's infrarenal aorta after a midline laparotomy using a 10/0 mono-filament suture (Ethicon Inc., Sommerville, New Jersey, USA). No anticoagulants or anti-platelet drugs were used in the experiment.

Immunosuppressive therapy

Two different protocols (day 1–30 or day 7–30) of tacrolimus immunosuppression were used in animals after allogeneic aortic transplantation [8]. Lewis recipients were divided into 4 groups.

Group A (n=4) was the syngeneic control (LEW to LEW) and group B (n=8) was the allogeneic control (BN to LEW) with no immunosuppression. Animals in group C (n=5) (BN to LEW) were immunosuppressed from day 1 to day 30 after transplantation and animals in group D (n=6) (BN to LEW) were immunosuppressed from day 7 to day 30 after transplantation (delayed immunosuppression). Tacrolimus (Prograf®; Astellas Pharma Inc., Munich, Germany) was administered IM in a daily dose of 0.2 mg/kg.

On day 30, tacrolimus blood levels were evaluated in all immunosuppressed animals using an enzyme-enhanced immunoassay technique (Emit® 2000 Tacrolimus assay, Dade Behring Inc., Deerfield, Illinois, USA).

Blood samples

Blood samples were collected in all groups on day 0, 14, and 30 by orbital sinus puncture as described by van Herck.[13]

Splenocytes

As a source of splenocytes, we used spleens from other male BN rats (n=20, weight 200–250 g) obtained from Charles River (Sulzfeld, Germany). The spleens were removed after midline laparotomy in anesthetized animals (intramuscular anesthesia with sufentanil and Stresnil as described above). The animals were then killed by an intracaval administration of a lethal dose of thiopental (Thiopental®, Spofa, Czech Republic). Removed spleens were immediately processed according to our protocol for splenocyte preparation. Briefly, the excised spleen was minced into small pieces, pressed through a strainer using the plunger end of a syringe, and washed with phosphate-buffered saline solution (PBS). The cell suspension was added on Biocoll separating solution (BioScience, Nottingham, UK) and centrifuged at 2000 rpm for 20 min. The supernatant was resuspended in 10% fetal calf serum (FCS) with RPMI 1640 (Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) and centrifuged at 1200 rpm for 10 min. This step was done twice. A cell count and viability check using trypan blue was performed afterwards. Cells were then stored in tubes with freezing medium (Iscove's Modified Dulbecco's Medium +20% fetal calf serum) in liquid nitrogen until processing.

Flow cytometry analysis

In vitro binding of obtained sera to quiescent Brown-Norway splenocytes was determined by flow cytometry, as described previously [14]. Briefly, cells were thawed, washed in phosphate-buffered saline (PBS), and resuspended in PBS with 1% fetal bovine serum (FBS). We incubated 100 000 cells for 30 min at 4°C with 10 µL of rat serum. Cells were washed twice in PBS (1% FBS) and then incubated with original antibodies as follows: MHC expression on quiescent BN splenocytes was evaluated using a Biotin-MHC class I (anti-RT1.Ac, OX-27, Acris

Antibodies GmbH, Herford, Germany) or a Biotin-MHC class II (anti-RT1.D, OX-17, BD Biosciences, Heidelberg, Germany) primary antibody and a PE-Cy7-Streptavidin secondary antibody (BD Biosciences, Heidelberg, Germany). For double-staining experiments, splenocytes were stained with a PE-CD3 (anti-CD3, G 4.18, BD Biosciences, Heidelberg, Germany) and a FITC-CD45RA antibody (anti-CD45, OX 33, BD Biosciences, Heidelberg, Germany) to distinguish between T- and B-cells. We acquired 10 000 cells on a FACSCanto II flow cytometer (BD Biosciences, Heidelberg, Germany) and analyzed them using FACSDiva™ software (BD Biosciences, Heidelberg, Germany). Dead cells were excluded from analysis using propidium iodide staining. Graphic presentation in histograms allowed the determination of mean fluorescence intensity on a log scale. MHC class I or class II antibody binding of the cells without previous serum incubation was set to 100%.

Immunohistochemical analysis

The aortic grafts were embedded in Sakura Finetek Tissue Tek® Cryomold holders (Sakura Finetek, Tokyo, Japan) and Sakura Finetek Tissue Tek® O.C.T. compound (Sakura Finetek, Tokyo, Japan). The samples were frozen in 2-methylbutane (Fluka Chemika, Buchs, Switzerland), cooled by liquid nitrogen and then stored at -80°C.

Detection of CD8+ cells was performed on 8-µm thick sections with a 2-step indirect method. Briefly, the sections were fixed in cold acetone for 10 min. After rinsing in 0.2% Triton X100 and phosphate-buffered saline, the specimens were incubated with anti-CD8 primary antibody (OX-8, Cymbus Biotechnology LTD) for 60 min. Endogenous peroxidase was blocked by incubating in 0.3% hydrogen peroxide (H₂O₂) and 70% methanol for 30 min. Next, the sections were incubated with a secondary antibody (Histofine® Simple Stain Rat MAX PO, Nichirei, Japan) for 30 min, and then incubated with Dako Liquid DAB+ Substrate-Chromogen System (Dako Denmark A/S, Glostrup, Denmark) for 5 min. The specimens were counterstained and dipped in Entellan® (Merck KGaA, Darmstadt, Germany).

Detection of immunoglobulins was performed on 8-µm-thick sections using a direct method. Briefly, after being rinsed in phosphate-buffered saline, the tissues were air-dried and incubated with an antibody directly conjugated with Fluorescein isothiocyanate (anti rat IgG, Chemicon International Inc., Temecula, California, USA) for 30 min. The specimens were then dipped in glycerine medium and immediately analyzed by fluorescence microscopy.

Statistical analysis

The values are expressed as mean ±SEM. Comparison between 2 groups was made with Student's t-test. Values of p<0.05 were considered statistically significant.

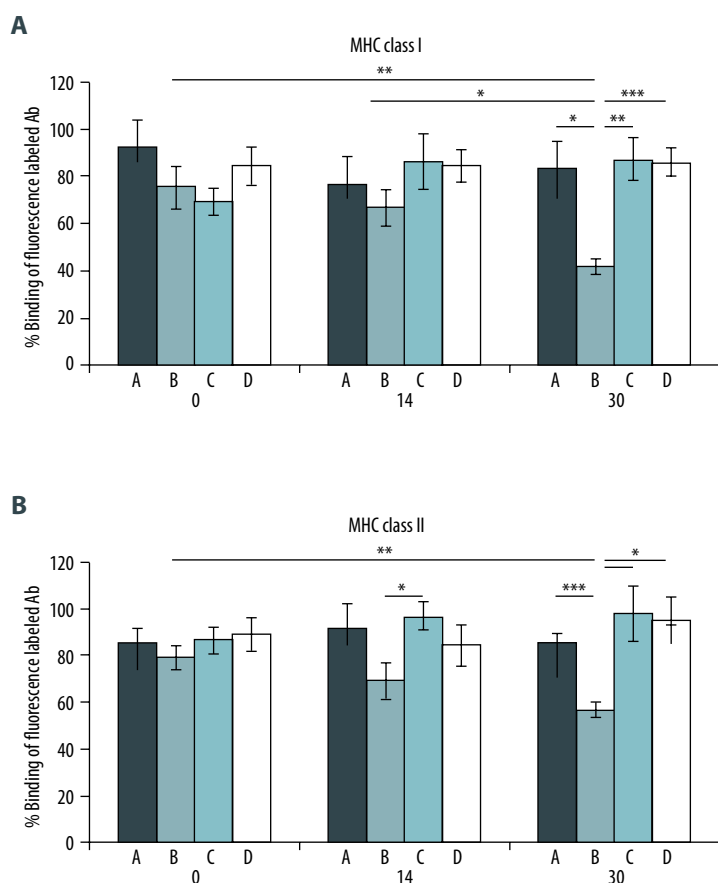


Figure 1. MHC class I and II allorecognition on splenocytes. The percentage of binding of the anti-MHC class I (anti-RT1.Ac, OX-27) (A) and anti-MHC class II (anti-RT1.D, OX-17) antibody (B) to quiescent BN splenocytes in the presence of sera obtained from rats of all 4 different experimental groups (A–D) on day 0, 14, and 30 after transplantation. Error bars represent SEM, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Group A – LEW to LEW ($n = 4$ for each time point), no immunosuppression, group B – BN to LEW ($n = 8$ for each time point), no immunosuppression, group C – BN to LEW ($n = 5$ for each time point), tacrolimus day 0–30, group D – BN to LEW ($n = 8$ for each time point), tacrolimus day 7–30.

Results

The mean tacrolimus blood level on postoperative day 30 was 5.0 ± 0.7 ng/ml, with no statistical difference between the immunosuppressed groups.

We determined the presence and dynamics of alloantibodies recognizing MHC complexes on quiescent BN splenocytes, splenic B-cells and T-cells in the sera of LEW recipients of BN aortic grafts using different fluorescence-labelled antibodies. The serum antibodies from allografted LEW rats, when presented, were bound comparatively to MHC class I and MHC class II molecules on quiescent BN splenocytes and splenic B-cells and T-cells.

MHC class I positive splenocytes

Blood samples were collected preoperatively (day 0) and on day 14 and 30 after transplantation. Syngeneic group A sera, as well as both allogeneic immunosuppressed groups C and D sera, showed no significant inhibition of fluorescence-labelled MHC class I antibody binding to BN-splenocytes during the entire follow-up period (Figure 1A).

By contrast, sera from allogeneic non-immunosuppressed group B animals obtained on day 14 and 30 after transplantation showed inhibition of fluorescence-labelled MHC class I antibody binding to spleen cells. Sera obtained on day 30 significantly decreased the binding ($42 \pm 3\%$), compared with day 0 sera ($76 \pm 9\%$, $p = 0.005$) and day 14 sera ($67 \pm 8\%$, $p = 0.01$), respectively (Figure 1A).

In addition, sera from the allogeneic non-immunosuppressed group B obtained on day 30 showed significant inhibition of fluorescence-labelled MHC class I antibody binding to spleen cells ($42 \pm 3\%$) compared with syngeneic group A sera ($83 \pm 11\%$, $p = 0.03$), as well as both allogeneic immunosuppressed groups C ($87 \pm 9\%$, $p = 0.005$) and D sera ($85 \pm 6\%$, $p = 0.0003$) obtained on day 30 (Figure 1A).

MHC class II positive splenocytes

Syngeneic group A sera, as well as both allogeneic immunosuppressed groups C and D sera, showed no inhibition of the fluorescence-labelled MHC class II antibody binding to BN-splenocyte during the entire follow-up period (Figure 1B).

Table 1. Percentage of fluorescence-labelled MHC class I and MHC class II antibody binding to Brown-Norway splenic B-cells and T-cells in the presence of sera of four different animal groups obtained preoperatively (day 0) and on postoperative day 14 and 30. Allogeneic non-immunosuppressed group B sera obtained on day 14 and 30 showed statistically higher inhibition of fluorescence-labelled MHC class I and MHC class II antibody binding to B-cells and T-cells compared to all other animal groups. Group A – LEW to LEW (n=4 for each time point), no immunosuppression, group B – BN to LEW (n=8 for each time point), no immunosuppression, group C – BN to LEW (n=5 for each time point), tacrolimus day 0–30, group D – BN to LEW (n=8 for each time point), tacrolimus day 7–30.

Group	Day 0				Day 14				Day 30			
	B-cells MHC		T-cells MHC		B-cells MHC		T-cells MHC		B-cells MHC		T-cells MHC	
	Class I	Class II	Class I	Class II	Class I	Class II	Class I	Class II	Class I	Class II	Class I	Class II
A	102±13%	96±10%	81±12%	69±6%	88±13%	96±9%	65±11%	72±10%	92±14%	83±10%	69±16%	55±7%
B	89±8%	92±6%	65±9%	58±3%	77±8%*	73±9%	56±7%	56±7%	46±3%**	54±4%***	38±4%#	58±7%##
C	85±6%	98±6%	58±6%	61±4%	101±11%	103±5%	76±14%	71±5%	101±7%	102±11%	78±11%	78±14%
D	95±7%	95±8%	75±9%	70±6%	97±8%	94±9%	75±8%	65±13%	99±7%	100±10%	76±7%	81±14%

* p=0.0007 compared with day 0 sera from non-immunosuppressed group B; ** p=0.005 compared with day 0 sera from non-immunosuppressed group B; p=0.04 compared with day 30 sera from syngeneic group A; p=0.0007 compared with day 30 sera from immunosuppressed group C; p=0.0002 compared with day 30 sera from group D with delayed immunosuppression; *** p=0.0003 compared with day 0 sera from non-immunosuppressed group B; p=0.02 compared with day 30 sera from immunosuppressed group C; p=0.006 compared with day 30 sera from group D with delayed immunosuppression; # p=0.02 compared with day 0 sera from non-immunosuppressed group B; p=0.02 compared with day 30 sera from immunosuppressed group C; p=0.002 compared with day 30 sera from group D with delayed immunosuppression; ## quiescent splenic T-cells do not express MHC class II antigens, we did not observe any significant binding inhibition of the fluorescence-labelled MHC class II antibody in the presence of sera obtained during the entire follow-up period in all four experimental groups.

By contrast, allogeneic non-immunosuppressed group B sera obtained on day 14 and 30 showed inhibition of fluorescence-labelled MHC class II antibody binding to spleen cells. In the presence of day 30 sera, the binding significantly decreased (56±3%) compared with day 0 (79±5%, p=0.003). In addition, allogeneic non-immunosuppressed group B sera obtained on day 30 showed significant inhibition of fluorescence-labelled MHC class II antibody binding (56±3%) compared with syngeneic group A sera (85±4%, p=0.0006), as well as both allogeneic immunosuppressed groups C (98±12%, p=0.02) and D sera (95±10%, p=0.01) obtained on day 30 (Figure 1B).

MHC class I and MHC class II positive splenic B-cell and T-cell

Quiescent splenic T-cells do not express MHC class II antigens. The separate flow cytometry for splenic T and B cells was done to control the adequacy and feasibility of inhibition flow cytometry analysis used in our experiment.

Quiescent BN splenic B-cells were identified as CD45RA-positive cells and quiescent BN splenic T-cells were identified as CD3-positive cells.

Syngeneic group A sera, as well as both from day 1 immunosuppressed allogeneic group C sera and from day 7

immunosuppressed allogeneic group D sera, showed no inhibition of the fluorescence-labelled MHC class I and MHC class II antibody binding to BN B-cells and T-cells during the entire follow-up period (Table 1).

By contrast, allogeneic non-immunosuppressed group B sera obtained on day 14 and 30 showed statistically higher inhibition of fluorescence-labelled MHC class I and MHC class II antibody binding to B-cells and T-cells when compared to all other animal groups (Table 1).

Immunoglobulins in the aortic wall

Immunofluorescent staining revealed IgG deposition in the media only in non-immunosuppressed allogeneic aortic allografts obtained on day 30 after transplantation. The immunosuppressive regimen with delayed administration of tacrolimus was able to suppress IgG deposition completely in all animals from group D (Figures 2A and 3A).

CD8+ cells in the aortic wall

The CD8+ cells were detected mainly in the thick tunica intima and in the adventitial layer of non-immunosuppressed allografts. In contrast, only a few CD8+ cells were detected in the tunica media of these allografts. Syngeneic group A aortic

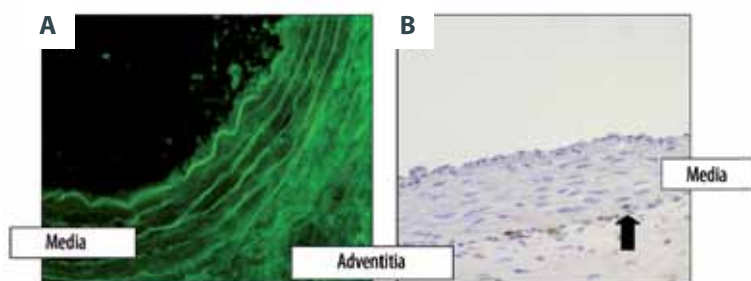


Figure 2. Representative examples of histological features from day 7 immunosuppressed aortic allografts obtained on day 30 after transplantation. (A) Aortic allograft stained for immunoglobulins with a fluorescein isothiocyanate-conjugated antibody. No IgG depositions in tunica media was detected on day 30. (B) Brown-Norway aortic allograft stained for the CD8+ cells. A small number of the invading CD8+ cells was detected, mainly in the tunica adventitia of explanted allografts (CD8+ cells are stained brown, black arrow). Original magnification $\times 400$.

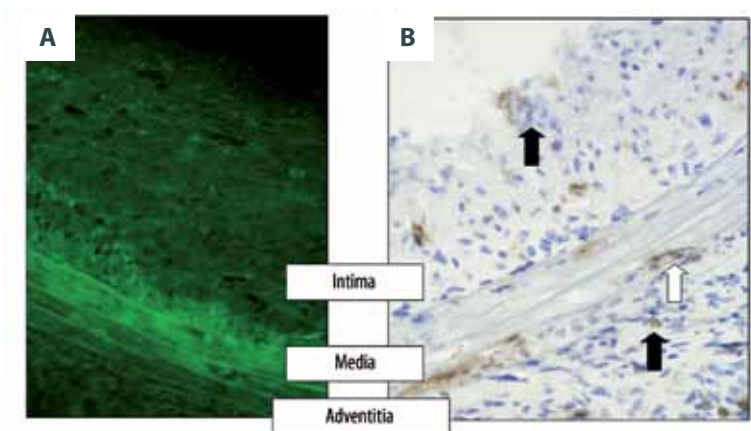


Figure 3. Representative examples of histological features of non-immunosuppressed aortic allografts obtained on day 30 after transplantation. (A) Deposition of IgG immunoglobulins into the tunica media was detected. (B) Infiltration of CD8+ cells was detected in the thick tunica intima and tunica adventitia of explanted allografts, but few CD8+ cells were detected in the tunica media. (CD8+ cells are stained brown, black arrow in tunica intima and adventitia, white arrow in tunica media). Original magnification $\times 400$.

grafts, as well as both from day 1 immunosuppressed allogeneic group C aortic grafts and from day 7 immunosuppressed allogeneic group D aortic grafts, showed minimal adventitial infiltration of CD8+ cells (Figures 2B and 3B).

Discussion

The results of the present experimental study concerning antibody-mediated rejection of aortic allografts during the 30-day follow-up period revealed induction of donor-specific anti-MHC class I and anti-MHC class II antibody production in allotransplanted, but not in immunosuppressed, animals. The general immune response by recipients resulted in intimal proliferation, medial smooth muscle cells disappearance with immunoglobulin deposition, and a massive adventitial infiltration by immunocompetent cells of donor origin [8]. However, antibody-mediated rejection and the destruction of aortic grafts were completely suppressed by the use of low-dose tacrolimus therapy started as early as day 7 after aortic allograft implantation.

Reports in the literature support the hypothesis that arterial allografts used in humans are immunogenic and that this

immune response is triggered by cellular immunity followed by production of antibodies [3]. We found histological phenomena of aortic rejection only in the allogeneic non-immunosuppressed animals. Signs of cell-mediated aortic grafts rejection were confirmed by massive infiltration of tunica adventitia by donor MHC class II positive cells, and CD4+ and CD8+ cells [8]. The rejection of tunica media was characterized by the presence of IgG deposition and smooth muscle cell disappearance. We correlated the deposition of IgG in the tunica media of non-immunosuppressed recipients of aortic allografts with the serum concentration of donor-specific anti-MHC antibodies.

The importance of anti-MHC class I antibodies production in the rejection process of aortic grafts was documented experimentally. Thauat et al. reported in a BN to LEW aortic transplant model that anti-MHC I alloantibodies play a key role in the arterial remodeling during the graft rejection [15,16].

By using a model of human arteries grafted into immunodeficient SCID/beige mice, Galvani et al. demonstrated that antibodies towards MHC class I antigens are able to provoke neointimal thickening in the grafted artery, even in the absence of

immune cells [17]. However, they were not able to show accumulation of antibodies in the graft with the destruction of tunica media in this model.

In our experimental work, we observed donor-specific anti-MHC class I production in allografted animals without immunosuppression. This antibody production was connected with IgG deposition into the tunica media and their subsequent destruction. In contrast, even delayed administration of low-dose tacrolimus therapy was able to inhibit this antibody production and the IgG deposition into the media.

Moreover, we showed that allotransplanted non-immunosuppressed animals also developed donor-specific anti-MHC class II antibody production. The importance and origin of anti-MHC class II production in the process of arterial allograft rejection is not clear. Plissonnier et al. failed to find any cell membrane expression of MHC class II molecules on quiescent vascular wall cells of BN origin [18]. In rejected aortic grafts in a BN to Lewis model, Thauinat et al. observed, that neither SMCs from the donor nor from the recipient expressed the MHC II molecules [15]. However, the expression of MHC class II antigen in the graft coronary arteries as a sign of immune activation of the allograft was observed by others [19].

Endothelial cells and SMCs normally do not present detectable levels of MHC class II antigen, but will express this protein after immunological activation, particularly by interferon- γ [19,20]. Moreover, recent clinical studies have reported that the presence of anti-MHC class II antibodies in the serum of renal recipients was the most predictive for microcirculation injuries, suggesting that they might have a higher capability than anti-MHC class I to trigger graft failure [21,22].

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Many alloantigens eliciting antibody responses in clinical practice are proteins (e.g., MHC antigens) [23]. Antibody responses to protein antigens require antigen-specific T-cell help. In this way T-cells targeting tacrolimus not only prevent T-cell, but also antibody (B-cell)-mediated immune responses [24]. This mechanism explains why tacrolimus was sufficient to suppress cell- as well as antibody-mediated rejection of transplanted aortic grafts in our experiment.

Conclusions

The present study found that aortic allografts trigger in recipients not only the cell-mediated rejection, but also donor-specific anti-MHC class I and anti-MHC class II antibody production. Activation of both immune pathways leads to aortic wall destruction with extensive IgG deposition into the tunica media. The low-dose tacrolimus immunosuppression was sufficient to inhibit cell- and antibody-mediated destruction of the donor aortic wall, even by delaying the administration for 1 week after implantation. In clinical practice, we could theoretically delay the use of immunosuppression and decrease the aneurysm and rupture rate of allo-arterial grafts by minimization of immune system suppression of patients with prosthetic graft infection.

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