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Strong Humoral Anti-HLA Immune Response Upon Arbitrarily Chosen Allogeneic Arterial Vessel Grafts

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Abstract

Forty-three patients were grafted with forty-four fresh or cryopreserved allogeneic arterial vessels in order to treat infections of synthetic vascular implants as these often lead to sepsis, amputation and death. All the patients were HLA-typed whereas typing results of the post-mortem donors were inquired or genotyped from residuary vessels' segments. 84% of the patients were cured from the underlying infections with a re-infection rate of only 9% attesting this therapeutic procedure a high success rate for recovering from the bacterial infections. Since the allografts were chosen without considering HLA-histocompatibility between donors and recipients 95% of the patients developed a humoral anti-HLA immune response with 89% of them giving rise to virtually definable donor-specific antibodies. Regarding the clinical outcome 16% of the patients exhibited thromboses as the most frequent complication. In addition to clinical follow up analyses of all patients, tissue excisions of 11 patients' allografts due to various complications but not resulting from persisting infections were analyzed in order to compare these vessels' pre-and post-transplant histological appearance. In contrast to three early excised homografts (after 14 to 45 days) all of the later on explanted vessels (after 8 to 96 months) clearly showed chronic degeneration processes induced by the alloreactive immune response. Although not leading to acute graft dysfunctions comparable to those of solid organs, arbitrarily selected vessels exhibit a high degree of alloimmunization with consequent chronic degeneration processes such as the loss of smooth muscle cells. Additionally, clear signs of fibrosis of the different arterial vessel layers lead to obliterative arteriopathy and thromboses. Thus, apparently the overall practicability of the unmatched allocation of arterial homografts is clearly limited. Further approaches of arterial vessel allo-grafting are required to investigate the hypothesis that the clinical outcome of well HLA-matched homografts may lead to a significantly decreased number of thromboses.

Introduction

Infections of vascular prostheses represent a serious problem in the field of vascular transplantation surgery with an incidence up to 3.5% [1-4]. Thus, the replacements of the infected prostheses by various substitute materials such as i) autologous veins, ii) rifampicin-bonded or silver-coated prostheses or iii) fresh or cryopreserved arterial allografts (homografts) are generally regarded as adequate therapies to overcome this acute life-threatening situation [2,5-7]. The antigenicity of arterial allografts which triggers the humoral immune response in a way similar to processes highly deleterious for solid organ recipients, however, has for years not generally been accepted by vascular surgeons [8]. By contrast it has been known for more than forty years that antibodies which are directed against HLA-antigens of a given donor represent the dominating reason for hyperacute or acute rejections of kidney allografts and allografts of other solid organs [9-11]. Thus, pre-transplant donor-specific anti-HLA antibodies (DSA) are regarded as a contraindication for grafting solid organs according to the transplantation guidelines of most countries and supranational societies (e.g. Eurotransplant) which are responsible for the supervision of the organs' allocations. In order to minimize the upcoming of these harmful DSA and the consequent risk of rejection episodes up to the complete loss of graft function the degree of matched HLA-phenotypes between a recipient and her/his given donor is an essential element of the allocation scheme of post mortem-grafted kidneys as the most frequently transplanted solid organs. With respect to its underlying function as large diameter blood vessel the short-and long term results of arterial allografts, however, have been regarded as excellent already from the beginning of this therapeutic approach. To this day arterial allografts have been regarded as tissue of low antigenicity as rejection phenomena have been thought to have no or only very low impact in terms of the clinical outcome. Consequently arterial allografts have generally been transplanted without considering the matching of AB0-bloodgroups and of HLA-phenotypes between donors and recipients. Furthermore, only a limited number of studies exist which have investigated the allo-specific immune response induced by arterial allografts, and the majority of them is based on animal models [12-16]. There are only few studies mainly of the 1990s in which the cellular and humoral immune responses as a consequence of venous and especially arterial allo-grafting in humans was investigated [8,17-20]. HLA class I and class II antigens, however, well known as pivotal allo-reactive transplantation antigens have been included only in few investigations dealing with arterial allografts [20,21] and are also at present widely neglected among vascular surgeons concerning their influence upon graft function of implanted

arterial vessels. Thus, it was our aim to investigate the patients' immune responses towards these HLA-molecules in order to draw conclusions for their immunogenicity and their relevance as rejection targets in fresh and cryo-preserved arterial allografts.

Materials and Methods

Patients and arterial vessel allografts

43 patients aged between 38 and 82 years (mean=66 years) were grafted using fresh (n=40) or cryo-preserved (n=4) arterial vessels from post-mortem donations at the University Hospital Leipzig between November 2002 and December 2012 in order to treat prostheses infections of various localizations (aorta-aorta, aorta-iliac, aorta-biiliac, aorta-femoral, aorta-bifemoral, aorta-popliteal, femoro-femoral, femoro-popliteal, iliac-iliac, iliac-femoral, iliac-bifemoral, iliac-popliteal, iliac-crural, popliteo-pedal). The patients were sent to the University Hospital at Leipzig from whole Germany as this hospital represented a supra-regional center for the treatment of infected vascular implants. 43 patients were included in our analyses as only for

them and their homografts complete HLA-typing data as well as complete data concerning these recipients' anti-HLA antibody status were finally available (Table 1). As one of the recipients received two allografts from two different donors 44 donations were finally considered for our analyses. The recipients' HLA-phenotypes and those of their respective donors were determined prior to or directly after the transplantation. Both the fresh and cryopreserved donor vessels had been removed during multiorgan extractions. Their HLA-types were taken from the donor data on file at Eurotransplant (Leiden, The Netherlands) or determined by HLA-genotyping using DNA extracted from residual pieces of the grafted vessels if these data were not provided by the transplant center (16/44 homografts). The recipients were investigated for anti-HLA antibodies prior to and 10 to 13 months after allo-grafting. All the vessels' were, with respect to possible allo-immune responses, arbitrarily allocated i.e. the compatibilities of HLA-antigens were no allocation criterion. The recipients' average post-transplantation observation period was 30 months with the longest period of more than eight years. It is noteworthy that no patient deceased while being allo-grafted.

ID	HLA-class I/donor	HLA-class II/donor	HLA-class I/recipient	HLA-class II/recipient
1	A3,**24**; B7,44 (Bw4,6) Cw2,7	DR1, DQ5	A1,2; B8,37 (Bw4,6) Cw6,7	DR3,15; DR51,52 DQ2,6
2	**A**1,2; **B8** (Bw6) Cw7	DR3, DR52; DQ2	A3,33; B14,18 (Bw6) Cw7,8	DR1,13; DR52; DQ5,6
3	A3,28; B14,35 (Bw6) Cw4,8	DR11,13; DR52; DQ3	A3,33; B14,35; (Bw6) Cw4,8	DR1,7; DR53; DQ2,3
4	A3,25; B7,18 (Bw6) Cw7	DR15; DR51; DQ5	A2,11; B35,51 (Bw4,6) Cw1,4	DR1,15; DR51; DQ5,6
5	A3,30; **B18**,51 (Bw4,6) Cw1,5	DR3,4; **DR52**,53; DQ2,3	A3,24; B13,47 (Bw4) Cw6	DR4,7; DR53; DQ2,3
6	A2,33; B14,60 (Bw6) Cw??	**DR1**; **DQ5**	A3,24; B35,60 (Bw6)	DR4,8; DR53; DQ3
7	**A**2,**30**; B13,62 (Bw4,6) Cw3,6	DR1,13; DR52; DQ1	A2; B27,62 (Bw4,6) Cw2,3	DR4,7; DR53; DQ2,3
8 §	**A**3,**24**; B7,35 (Bw6) Cw4,7	DR4,11; **DR52**,53; DQ3	A3;B35,51 (Bw4,6); Cw4	DR1,4; DR53; DQ3,5
9	**A**3,**24**; B7 (Bw6), Cw7	DR1,15; DR51; DQ5	A3,11; B7,37 (Bw4,6) Cw6,7	DR8,15; DR51; DQ4,6
10	A2,3; **B35** (Bw6); Cw4	DR1,11; **DR52**;**DQ5**,7	A2,29; B7,44 (Bw4,6); Cw7	DR7,15; DR51,53; DQ2,6
11	A3; B7 (Bw6); Cw7	DR15; DR51; DQ6	A3,29; B7,18 (Bw6); Cw7	DR11,15; DR51,52; DQ6,7
12	A1,24; **B27**,57 (Bw4); Cw2,6	DR7; DR53; **DQ2**	A1,24; B8,51 (Bw4,6); Cw5,7	DR1,4; DR53; DQ3,5
13	A23,30; **B**13,**44** (Bw4); Cw4,6	**DR7**; DR53; DQ2	A2,26; B27,38 (Bw4)	DR13; DR53; DQ6,7;
14	**A2,11**; B7,13 (Bw4,6); Cw6,7	**DR7**,15; DR51,53; DQ2,6	A1,30; B7,8; Cw7	DR15,17;DR51,52; DQ2,6
15	A1,26; **B8,35** (Bw6); Cw7	**DR1**,17; DR52; **DQ2**,5	A1,26; B45,61 (Bw6); Cw2,6	DR7,13; DR52,53; DQ6,9
16	**A26**; **B**38,**44** (Bw4); Cw4,7	DR7,11; **DR52**,53; **DQ2**,7	A3; B7,40 (Bw6); Cw2,3	DR8,15; DR51; DQ4,6
17	**A3**,25; B60,61 (Bw6); Cw2,3	**DR4**,11; DR52,53; DQ3	A1,2; B55,62 (Bw6); Cw3	DR4,11; DR51,53; DQ7,8
18 §	**A2**,31; B51,60 (Bw4,6); Cw3	**DR4**,13; DR52,53; DQ3,6	A3,32; B7,35; (Bw6); Cw4,7	DR7,15; DR51,53; DQ2,6
19	**A1**,3; B47,57 (Bw4,6); Cw6	DR7,15; DR51,53; DQ2,6	A3,23; B44 (Bw4); Cw4,5	DR7,15; DR51,53; DQ2,6
20	A2,29; B18,57 (Bw4,6); Cw6	**DR11**,15; DR51,52; DQ3,6	A23,24; B7,55 (Bw6); Cw7	DR15,16; DR51; DQ5,6
21	**A1**,3; B7,8 (Bw6); Cw7; Cw7	**DR13**,17; DR52; **DQ2**,**6**	A3,24; B8,13 (Bw4,6); Cw6,7	DR17; DR52; DQ2
22	A1; **B51,57** (Bw4); Cw6	**DR7,10**; DR53; DQ2,5	A1,2; B7,45 (Bw6); Cw6,7	DR4; DR53; DQ7,8

23	A2; **B7** (Bw6); Cw7	**DR8,15**; DR51; DQ5,4	A2; B18,53 (Bw4,6); Cw4,5	DR13,14; DR52; DQ5,6
24	**A1,2**, **B49,51** (Bw4); Cw4,7	DR1,8; DR51; DQ4,5	A25,29; B44,62; (Bw4,6); Cw3	DR7,13; DR52,53; DQ2,6
25	**A3,24**; B35,57 (Bw4,6); Cw4,6	**DR1,7**; DR53; DQ5,9	A3,31; B16,61 (Bw6); Cw3,7	DR11,16; DR51,52; DQ5,7
26	**A2,24**; B7,39 (Bw6), Cw7	**DR8**,15; DR51; DQ4,6	A3,32; B7,27 (Bw4,6); Cw2,7	DR9,15; DR51,53; DQ6,9
27	**A1,11**; B27,35 (Bw4,6); Cw2	**DR4,7**; DR53; **DQ2**,7	A2,28; B44,62 (Bw4,6); Cw3,7	DR11,13; DR52; DQ6,7
28	**A1,3**; **B8**,44 (Bw4,6); Cw5,7	**DR3,4**; DR52,53; DQ2,8	A2,24; B44,57 (Bw4); Cw4,6	DR1,7; DR53; DQ5,9
29	A2; B62 (Bw6); Cw3	DR13; DR52; DQ6	A2,32; B35,39 (Bw6); Cw4,7	DR7,11; DR52,53; DQ2,7
30 §	**A1**,24; B8,39 (Bw6); Cw7	**DR3,11**; DR52; **DQ2**,7	A24,25; B7 (Bw6); Cw7	DR1,15; DR51; DQ5,6
31	**A2,23**; **B44** (Bw4); Cw4,5	DR1,7; DR53; DQ5,9	A1; B8 (Bw6); Cw7	DR17, DR52; DQ2
32	**A25**,28; B18,60 (Bw6); Cw3	DR3,13; DR52; **DQ2**,6	A2,3; B7,39 (Bw6); Cw7	DR13,15; DR51,52; DQ6
33	**A2**,3, B18,57 (Bw4,6); Cw3,6	**DR7**,11; **DR52**,53; DQ7,9	A3; B7,62 (Bw6); Cw3,7	DR1,15; DR51; DQ5,6
34	A2; B51 (Bw4); Cw1	**DR3,11**; DR52; DQ2,7	A2,24; B27,51 (Bw4); Cw1,2	DR1,16; DR51; DQ5
35	A2; **B44,62** (Bw4,6); Cw3,5	**DR4**,13; DR52,53; DQ7,8	A2; B7,13 (Bw4,6); Cw6,7	DR15,17; DR51,52; DQ2,6
36	**A**1,2; B8,38 (Bw4,6); Cw3,7	DR3,13; DR52; **DQ2**,6	A3,32; B35,61 (Bw6); Cw2,4	DR1,13; DR52; DQ5,6
37	**A1**; **B8**,37 (Bw4,6); Cw7	**DR3**,10; DR52; DQ2,5	A2,31; B27,62; Cw2,3	DR4,13; DR52,53; DQ6,7
38	A2, **B8,50** (**Bw6**); Cw6,7	DR3,7; DR52,53; DQ2	A2,28; B51 (Bw4); Cw4	DR1,4; DR53; DQ5,8
39	**A3**; **B35** (Bw6); Cw4	**DR1,11**; DR52; DQ5,7	A2; B7,45 (Bw6); Cw6,7	DR4,14; DR52,53; DQ5,7
40	**A2**,3; B7,62 (Bw6); Cw3,7	**DR**13,**15**; DR51,52; DQ6	A2,11; B55,57 (Bw4,6); Cw3,6	DR7,14; DR52,53; DQ5,9
40 ‡	**A3**,68; B35,56 (Bw6); Cw4,7	**DR1**,11; DR52; DQ5,7	" " " " " "	" " " " " "
41	**A24,32**; B7,39 (Bw6); Cw7,12	**DR15,16**; DR51; DQ5,6	A1,3; B8,35 (Bw6); Cw4,7	DR11,17; DR52; DQ2,7
42 §	**A2,3**; **B7**,**60** (Bw6); Cw3,7	**DR13,15**; DR51,52; DQ6	A1,24; B37,57 (Bw4); Cw6	DR1,7; DR53; DQ5,9
43	**A3,29;B35,51** (Bw4,6); Cw??	**DR8,11**; DR52; DQ4,7	A2; B44,62 (Bw4,6); Cw4,5	DR4,7; DR53; DQ7,9
‡ , second arterial vessel allograft of patient 40; §, cryopreserved vessel				

Table 1: HLA-phenotypes of the patients and their respective donors. The donors' HLA-antigens against which DSA were virtually demonstrable are bold-typed and underlined.

HLA-typing of the recipients and of the donors' residual vessel tissue

All patients/recipients were pheno- and genotyped for HLA class I antigens comprising the loci A, B and C. Phenotyping was performed using Histo Tray ABC 144 plates (BAG, Lich, Germany). Confirmatory genotyping was performed using sequence-specific primer-(SSP-) PCR-based low resolution technique (Innotrain, Kronberg, Germany and Protrans, Ketsch, Germany) both for the HLA class I loci A, B and C and for the HLA class II loci DRB1* (DR), DRB3* (DR52), DRB4* (DR53), DRB5* (DR51) and DQB1* (DQ) resulting in complete low resolution typing results for 43 patients. Typing data of the vessel donors were taken from donor data files which had been provided by the transplant center Leipzig. If these data were not accessible for us (n=16) genotyping of the above mentioned HLA class I and II loci was performed with DNA extracted from residual pieces of the donor vessels through the use of DNeasy kits for the extraction of genomic DNA from tissues (Qiagen, Hilden, Germany).

Detection/Specification of anti-HLA antibodies in order to perform virtual cross-matching

In order to detect anti-HLA antibodies all sera were generally screened for anti-HLA class I antibodies using the QuikScreen ELISA and for anti-HLA class II antibodies using the B-Screen ELISA (both from Biorad, Dreieich, Germany). Patients' sera positive in this first screening step were afterwards investigated in order to specify these antibodies. This was done using the DynaChip HLA antibody analysis technique (Invitrogen/Dynal, Bromborough, UK) until the year 2011 when the DynaChip system was discontinued by the manufacturer for mere commercial reasons. We used the second generation design of

this completely automated system providing 106 positions of a microchip covered with HLA class I and 48 positions covered with HLA class II antigens of different single antigen donors. Thus, apart from a number of single HLA class II DQ-antigens immobilized on some positions this assay did not provide a resolution at the single antigen level but only at the so-called single donor/single ID level. However, the combinations of HLA class I and class II antigens, respectively immobilized by the manufacturer, validly allowed the specifications of the recipients' antibodies in about 70% of these analyses especially when the levels of immunization (so-called panel reactive antibodies/PRA-levels) were not too high. Furthermore, in contrast to the antibody specification systems resolving at the single antigen level, the "single donor"-specification systems allow the determination of the PRA-levels (indicated as percentage of positive reactions) as the groups of the given donors' antigens are immobilized according to their frequencies in the population under analysis. The discontinuation of the DynaChip system by the manufacturer led to the implementation of the Luminex-based anti-HLA antibody specification (Immucor, Rödermark, Germany) in our laboratory both at the single donor level and the single antigen level of resolution in order to validly determine i) the PRA-level and ii) antibody specificities of highly immunized recipients. Both techniques, the DynaChip-and the Luminex-based analyses, were used in order to perform so-called virtual cross-matching i.e. the identification of donor-specific anti-HLA antibodies (DSA) directed against the respective donors' phenotypes.

Histological analyses of allografted vessels excised for various reasons

Excised vessels (n=15) were macroscopically and microscopically investigated at the Institute for Pathology of the University Hospital Leipzig. The resulting pathological findings were consecutively placed at the disposal of the tissue typing laboratory of the University Hospital Halle.

Results

Development of a strong humoral immune response against HLA-antigens of the HLA-antigens of the donors' arterial vessels

Fresh and cryo-preserved donor vessels which had been removed during multiorgan extraction were chosen without considering the HLA-compatibility between the donor vessels and the prospective recipients. The entire set of HLA-class I and class II phenotypes, even if determined by genotyping, of all 43 recipients and 44 donors (as one recipient was allo-grafted two times) is listed in Table 1. At first glance

the typing data demonstrate that the degree of HLA-compatibility between the donor vessels and the respective recipients is, apart from exceptional cases, generally very poor. The donors' HLA-antigens against which the recipients' donor-specific antibodies (DSA) were clearly detectable by means of virtual cross-matching are indicated by bold and underlined lettering. As is visible in Table 1 apart from three recipients (ID4, 11, 29) all of them (93%) clearly developed DSA against donors' HLA-antigens of the vessels transplanted. Patients 11 and 29 (ID11, 29) most probably due to their well-matched donations did not exhibit DSA nor any degree of anti-HLA immunization (Table 2). It is evident that using adequately HLA-matched arterial vessels for allo-grafting the recipients 11 and 29 is due to the fact that both donor vessels were characterized by homozygosities of the HLA phenotypes thus offering only one potential immune target per HLA gene locus or no immune target if both the recipients and their selected donor vessels by chance bore these homozygous phenotypes (Tables 1 and 2). In contrast the lack of demonstrable DSA is not plausible for patient 4 (ID4) who received a poorly HLA-matched allograft which afterwards from a non-immunized state led to a PRA of 50% (Tables 1 and 2). Thus, the lack of demonstrable DSA most probably was the result of the Luminex-based specification assay of the single donor (single ID) resolution used, which only in this particular case did not allow the identification of distinct donor specificities despite a PRA of 50%.

Prior to grafting only three patients (Table 2) were characterized by an immune response against HLA-antigens. This was due to previous transfusion (ID14), pregnancy (ID24) or both transfusion and pregnancy (ID36). Antibody screening as well as antibody specification was accordingly positive resulting in PRA-values of 20%, 48% and 77%, respectively. All other patients included in these investigations did not exhibit any immune response directed against HLA-antigens. This situation, however, was dramatically different as shown by the follow up antibody screening and specification runs which were performed between 10-12 months after allo-grafting (Table 2). Apart from two patients (ID11, 29) all of the originally non-immunized vessel recipients (95%) clearly showed anti-HLA antibodies with PRA-values between 23% and 100%. Also the three patients pre-immunized as shown above exhibited a clear increase in their anti-HLA immune responses from 20% to 95% (ID14), from 48% to 99% (ID24) and from 77% to 86% (ID36). If both groups, comprising pre-immunized (n=3) and non-immunized (n=40) patients, are included 41 out of 43 i.e. 95.4% of all patients exhibited an immune response upon exposure to allo-grafted HLA-antigens. It is noteworthy that 26 out of 43 patients (about 60%) exhibited PRA-values of 80% and higher, thus fulfilling the criteria of being regarded as highly immunized according the guidelines of the Eurotransplant Foundation. This clearly shows that allo-grafted arterial vessels represent a tissue strongly leading to an HLA-mediated allo-immunization.

ID	Screen-ELISA Prä-Tx	PRA % Prä-Tx	HLA A-B-DR MM-scheme	No. MM	Screen-ELISA Post-Tx	PRA % Post-Tx
1	neg.	0	2-2-1	5	pos.	76
2	neg.	0	2-1-1	4	pos.	66
3	neg.	0	1-0-2	3	pos.	73
4	neg.	0	2-2-0	4	pos.	50
5	neg.	0	1-2-1	4	pos.	100

6	neg.	0	2-1-1	4	pos.	68
7	neg.	0	1-1-2	4	pos.	96
8 §	neg.	0	1-1-1	3	pos.	72
9	neg.	0	1-0-1	2	pos.	28
10	neg.	0	1-1-2	4	pos.	94
11	neg.	0	0-0-0	0	neg.	0
12	neg.	0	0-2-1	3	pos.	23
13	neg.	0	2-2-1	5	pos.	53
14 #	pos.	20	2-1-1	4	pos.	95
15	neg.	0	0-2-2	4	pos.	87
16	neg.	0	1-2-2	5	pos.	100
17	neg.	0	2-1-0	3	pos.	100
18 §	neg.	0	1-2-2	5	pos.	80
19	neg.	0	1-2-0	3	pos.	40
20	neg.	0	2-2-1	5	pos.	100
21	neg.	0	1-1-1	3	pos.	47
22	neg.	0	0-2-2	4	pos.	93
23	neg.	0	0-1-2	3	pos.	40
24 #	pos.	48	2-2-2	6	pos.	99
25	neg.	0	1-2-2	5	pos.	73
26	neg.	0	2-1-1	4	pos.	88
27	neg.	0	2-2-2	6	pos.	98
28	neg.	0	2-1-2	5	pos.	82
29	neg.	0	0-1-1	2	neg.	0
30 §	neg.	0	1-2-2	5	pos.	91
31	neg.	0	1-2-2	5	pos.	97
32	neg.	0	2-2-1	5	pos.	20
33	neg.	0	1-2-2	5	pos.	94
34	neg.	0	0-0-2	2	pos.	54
35	neg.	0	0-2-2	4	pos.	88
36 #	pos.	77	2-2-1	5	pos.	86
37	neg.	0	1-2-2	5	pos.	62
38	neg.	0	0-2-2	4	pos.	94
39	neg.	0	1-1-2	4	pos.	83
40	neg.	0	1-2-2	5	pos.	96
	"	"	2-1-2	5	pos.	96

41	neg.	0	2-2-2	6	pos.	98
42 §	neg.	0	2-2-2	6	pos.	98
43	neg.	0	2-2-2	6	pos.	98

ELISA, Enzyme-Linked Immunosorbent Assay; HLA, Human Leukocyte Antigens; MM, Mismatch; PRA%, Panel-Reactive Antibodies in %; Prä-TX, prior to grafting; Post-TX, 10-12 months after grafting; #, Pre-immunized patients as a consequence of transfusions (ID14), pregnancies (ID24) or both pregnancies and transfusions (ID36); §, cryopreserved donor vessel.

Table 2: HLA compatibility of recipients and their arbitrarily chosen vessels, anti-HLA antibody status (PRA%) prior to and 10–12 months after vessel grafting.

The degree of HLA compatibility is further represented by the HLA mismatch scheme only including the HLA-class I gene loci A and B and the HLA-class II gene locus DR indicating up to six HLA-incompatibilities through the use of increasing numbers (up to "2" for each locus). The corresponding schemes are then between 0-0-0 (optimal matching) and 2-2-2 (maximal number of six mismatches). If these mismatch schemes are analyzed the random selection of allografted arterial vessels becomes striking (Table 3). Using the stratification of the transplantation immunologist Gerhard Opelz (Heidelberg, Germany) who initiated the Collaborative Transplant Study (CTS) in 1982 it is evident that 5/44 (11%), 15/44 (34%) and 13/44 (30%) of the transplantations were performed under "immunologically poor" conditions using vessels with six, five and four HLA-mismatches, respectively, given the above mentioned HLA-A-B-DR-mismatch scheme with the highest number of six possible mismatches. Seven out of 44 arterial vessels (16%) were grafted with three HLA-mismatches representing "intermediate conditions" according to this stratification whereas only 3/44 (7%) and 1/44 (2%) of the vessels were allografted using donors with two or with no HLA-mismatch, respectively. There was no patient who received an allograft characterized by one HLA-mismatch. This means according to the CTS-stratification that only 4/44 (9%) of the recipients were allografted under "immunologically privileged" conditions in terms of HLA-compatibility between a recipient and her/his given donor whereas 75% of the patients were allografted insufficiently in this respect. This is strikingly shown in table 3. Of course no other distribution pattern was to be expected for arbitrarily chosen donor vessels due to the high number of HLA-polymorphisms.

No. HLA-Mismatches		No. Graftings per Mismatch/No. of total TX		Percentage of total TX (classification)
6		5/44		11% (poor)
5		15/44		34% (poor) [Σ=75%]
4		13/44		30% (poor)
3		7/44		16% (interm.) [Σ=16%]
2		3/44		7% (good)
1		0/44		0% (good) [Σ=09%]
0		1/44		2% (good)

Table 3: Classification of the allo-grafted vessel transplants according to the number of HLA-incompatibilities (in decreasing order between 6 and 0) which are depicted using the corresponding HLA-mismatch schemes.

Patients' clinical courses and late histopathological degenerations after arterial vessel allo-grafting

As already mentioned nobody of the 43 patients deceased as a direct consequence of the surgery performed. However, in seven out of the 43 patients (16%) the treatment of the infectious disease primarily appearing in the explanted synthetic vascular implants was not successful due to persisting infections which in one case (ID40) after its fast vascular obliteration after only ten days led to the replacement of the first homograft by a second one. In this special case only this second approach of allo-grafting was successful in curing the initial infection as the basal disease. Unfortunately the remaining six patients (14%) were not cured from the primary infection as it persisted and led to the leg's amputation with the homograft implanted before. Taken together the approach of substituting the infected synthetic vascular implants by a secondary homograft in order to cure the patient from the infection was successful in 37 patients (86%) characterizing this surgical approach as highly helpful in this respect. This aspect holds especially true in view of the fact that most of the infections must be regarded as severe concerning the degree of infection and the pathogenic germs involved. Analyses of the explanted synthetic vascular implants of 39 patients (91%) were adequately classified by the highest degree 3 according to Szilagyi [22] or by the degrees 2 or 3 according to Zühlke and Harnoß [23].

These six amputation-derived vessels were not considered in order to investigate possible immunological consequences due to incompatible transplantation antigens regarded as relevant. As expected the invasive inflammatory processes, accompanying the uncured bacterial affections completely covered any possible allogeneic effect potentially observable by histological analyses.

Thus, nine arterial vessels excised for other reasons than ongoing bacterial infections i.e. mainly as a consequence of thromboses were used in order to investigate the excised tissue for potential concomitant allogeneic phenomena. Three out of these nine early excised homografts (ID13, 28 and 39 after 14, 15 and 45 days, respectively) were unremarkable as they did not exhibit any histological abnormalities which may have resulted from allogeneic degenerative processes. The only indications for inflammatory processes without an apparent involvement of the initial bacterial infections were increased

infiltrations of lymphocytes detectable in these three early excised allografted vessels.

This situation, however, demonstrating only marginal pathological findings was completely different for those homografts (ID41, 26, 1, 3, 6, 16, 5, 10) excised later after allo-grafting i.e. after 8, 15, 30, 33, 54, 70, 86 and 96 months, respectively. Histopathological investigations of all those eight vessels which had been excised at later dates revealed clearly definable degenerative alterations in addition to the lymphocytic infiltrations already observed for the early excised vessels. As is demonstrated in Figure 1 all arterial tissue layers were affected by very similar degeneration processes as were in detail described by the respective histopathological reports (not shown). The outer layers (Tunica Adventitia) of all eight excised vessels were characterized by sclerotic alterations through increased proportions of collagenous connective tissue and elastic fibers (Figure 1A). The subsequent layer (Tunica Media/Muscularis) was characterized by a definite loss of smooth muscle cells i.e. by a generally reduced thickness of this muscular layer (Figure 1A). Quite in contrast to the Tunica Media the inner layer (Intima) exhibited a vigorous collagenization in all later

excised homografts accompanied by the phenomenon of hyperplasia i.e. a thickening of this inner layer (Figure 1A). This collagenization-mediated thickening of the Intima led to reductions of these vessels' lumina which was especially evident in three homografts (ID 3, 5 and 10) excised after 33, 86 and 96 months, respectively. Nevertheless, the other five excised vessels were to a minor extent also characterized by reduced homografts' lumina caused by the collagenized and thickened Intima, respectively. Although acute episodes of rejections which sometimes lead to the fast functional loss of a given graft in case of differentiated organs such as kidneys were not observable, chronic pathological degenerative processes which transmurally affected all three arterial vessel layers characterized all of the eight later (i.e. after ≥ eight months) excised homografts. As mentioned above the early excised vessels (up to 45 days) did not exhibit these histopathological phenomena. The data strongly suggest that the phenomena depicted in Figure 1B may be regarded as pathological consequences of HLA-targeted allogeneic immune reactions which due to the reduced function of arterial vessels as solely blood-piping structures become evident in a chronical but not in any acute way.
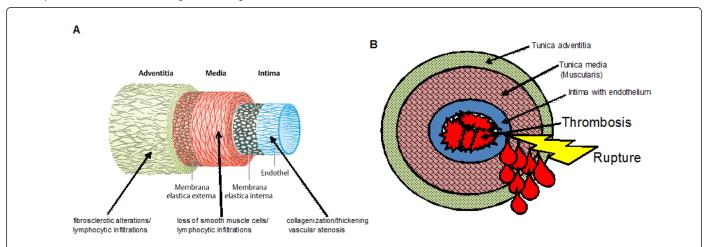

Figure 1: Scheme of the long-term allogeneic degeneration which affects all three tissue layers Tunica adventitia, Tunica media (Muscularis) and Intima of the arterial allografts (A) and possible secondary life-threatening forms of its appearance (B)

It is noteworthy that the excisions and subsequent histopathological investigations of seven out of eight lately excised homografts (ID1, 3, 5, 6, 10, 16, 26) were the consequences of thromboses of the implanted arterial allografts whereas the direct cause for the excision of the homograft of the sixth patient of this group (ID41) was regrettably no more available to be part of these investigations. However, it must be concluded that at least seven out of 43 patients (16.3%) suffered from thromboses which required medical interventions up to amputations in two cases (ID3, 26). In this context it is important to point out that for all these eight patients ongoing infections causing or supporting this pathology were clearly excluded. As mentioned above the same held true for the early excised homografts (ID13, 28 and 39) allowing their disposition as time-variant comparison group without any involvements of infections.

Taken together our data show a high degree of HLA-antigen-mediated allogeneic immunization as a consequence of grafting arbitrarily chosen i.e. HLA-unmatched arterial donor vessels. Due to the reduced complexity of allografted arterial vessels by contrast with functionally differentiated organs this allogeneic immune response does not lead to an acute graft loss but to a chronic vascular

degeneration process. Our data strongly suggest that this immune response leads to a proportion of at least 16% of the patients exhibiting clinically apparent thromboses with subsequent medical interventions up to amputations.

Discussion

Although the procedure of allo-grafting arterial vessels is regarded as gold standard in order to heal infections of synthetic vascular implants the data of the immunogenicity of human arterial allografts are not widely accepted among surgeons supporting this procedure. This is partly due to the lack of current investigations dealing with the long-term development of an allogeneic HLA-targeted immune response. Studies dealing with this aspect were essentially published in the past. Most probably due to their lacking topicality these studies are, in spite of their informative value and their high clinical relevance, no more in the center of investigations characterizing this field of vascular transplantations. At present the success of arterial allo-grafting is more or less solely defined on the basis of the patients' recoveries from life-threatening infections of the vascular implants whereas chronic and

harmful allogeneic effects are widely neglected. However, in contrast to this current tendency several historical investigations are available which in best accordance with our current study provide evidence for harmful allogeneic effects induced by arbitrarily chosen allografts. These investigations date back to the nineties of the last century i.e. they were in parts published more than twenty years ago.

Already in 1991 Plissonier and co-workers [13] reported morphological changes for an animal allograft model using the two rat strains Wistar Kyoto (WKY) and Spontaneously Hypertensive Rats (SHR). In contrast to the Intima of aortic allografts, which exhibited a clear thickening, the Tunica Media was always characterized by a decreased thickness through a reduced density of smooth muscle cells (Figure 1). Furthermore, inflammatory mononuclear infiltrates were described to be predominantly visible in the Tunica Adventitia and the Intima whereas the Tunica Media was affected by this allogeneic inflammatory feature only to a minor extent. Corresponding morphological changes were consequently correlated with genetically determined immune processes mostly affecting both the intimal and the medial layers of the two rat strains used for this experimental approach. It is noteworthy that iso-grafting between syngeneic strains did not show those degenerative changes which the authors referred to as chronic signs of rejection.

In the same year Mennander and co-workers [12] published their data on transplant arteriosclerosis also using aortic allografts of rats. They described an experimental animal model of aortic allografts between two MHC-incompatible rat inbred strains (DA and WF) and promoted their approach of research in order to investigate the phenomenon of transplant arteriosclerosis as being strikingly different from artherosclerotic arteriopathy of humans. In their model the authors described an initial acute inflammation of the Tunica Adventitia which is followed by an immune response of the Intima finally leading to a thickening of this inner vessel layer as a chronic process. The Tunica Media was characterized by chronic signs of necrosis, the subsequent disappearance of smooth muscle cells and their replacement by fibrous tissue. Furthermore, Mennander and co-workers [12] proposed their model to investigate these chronic alterations of the allografts as no acute rejections leading to the transplants' loss were observed. All these harmful effects were largely or completely lacking in syngeneic grafts clearly pointing to the relevance of MHC-antigens as prominent immune targets.

The successive mechanisms of immunological targeting in the course of chronic experimental arterial allografts were similarly depicted for a second time in 1995 by Plissonnier et al. [14] who allografted rat abdominal aortae from Brown-Norway to Lewis rats. In their investigations the authors identified the Tunica Adventitia as the side of major inflammatory cell invasion. This inflammatory infiltration continued in parallel with the persistence of the medial smooth muscle cells. In contrast to the luminal endothelial cells which disappeared very early (as speculated by marginating macrophages), smooth muscle cells of Tunica Media disappeared later as they were specifically targeted by antibodies. As the most delayed phenomenon Plissonnier and co-workers [14] described the proliferation of the Intima characterized by an earlier infiltration of inflammatory cells and the subsequent factually chronicle myofibroblastic proliferation. In 2001 the same group [24] generalized the chronic arterial wall allorejection as a three-stage-process which involves antigens from the Major Histocompatibility Complex and comprises i) a first stage of recognizing histo-incompatibilities of the graft endothelium, ii) a second stage of antibody-dependent injury of medial smooth muscle cells with the inflammatory infiltration of the Tunica Adventitia in parallel, and iii) a third stage of intimal proliferation and adventitial fibrosis. With regard of the first two stages the authors clearly referred to incompatibilities of the MHC-antigens of the grafted arterial vessels as alloreactive immune targets.

The first study investigating the immune response after allo-grafting HLA-unmatched human aortic segments was published by Mirelli and co-workers [20] also about twenty years ago. In accordance with our present study the authors found HLA-mismatch-specific antibodies in all five recipients investigated. These were clearly detectable after 1-3 months and peaked (highest PRA-Value) between 6 and 12 months after the segment's implantation. Already after six months the authors observed a progressive thickening of the aortic wall and an increased absolute number of T-lymphocytes (CD3+, CD4+, CD8+) in the circulation in all five patients. Mirelli and co-workers speculated that this HLA-directed humoral immune response contributed to the implant's degeneration by boosting the narrowing of the vascular lumen. In this context it should be mentioned that until recently this and a consecutive study of Mirelli and co-workers [20,21] were not known to the authors of the present investigations. Thus, although featured by highly concordant results the data of Mirelli and our group's data were as a matter of fact independently collected.

In their later study comprising thirty patients Mirelli et al. [21] grafted both using fresh (n=23) and cryopreserved (n=7) arterial vessels. Furthermore, patients were divided in two groups one of which (10 patients) received immunosuppressive treatment whereas the second group (20 patients) did not receive any immunosuppressive therapy. It is important to note that all homografts were performed between ABO-bloodgroup-compatible post-mortem donors and their recipients in order to exclude this additional allogeneic transplantation barrier. The results were very similar to those of their first study. Three months after the transplantation and reaching a peak after about 12 months there was a significant increase in anti-HLA antibodies leading to a mean PRA of 70% (without immunosuppression) or 30% (cyclosporine treatment), respectively, with most of them definable as donor-specific. As was biologically expectable in terms of antigenicity there was no difference between using cryopreserved donor vessels and fresh homografts. These data are in accordance with our own data although the number of cryopreserved arterial donor vessels (4 out of 44) was only 9% of all. These four donations, however, did not exhibit reduced levels of panel-reactive antibodies nor reduced numbers of donor-specific antibodies (Tables 1 and 2) leading to the conclusion that fresh and cryopreserved arterial homografts are indeed of very similar immunogenicity. Although in that study of Mirelli and co-workers all of the patients were cured from the primary infection one patient died by a rupture of the graft and three patients exhibited stenotic lesions due to chronic rejections during a follow-up period of two years [21]. Interestingly these three patients belonged to the group treated with cyclosporine at a dose of 3-5 mg/kg per day. However, they were treated successfully through conventional stenting. Taken together four patients (13% of the cohort) were clinically affected by allogeneic rejection phenomena observed during a two-year follow-up period with one patient dying from the lethal rupture of the implanted homograft as the worst conceivable consequence of the allogeneic attack.

Also in those days similar humoral and strong cellular immune responses were described by Carpenter and Tomaszewski as a consequence of human saphenous vein bypass allo-grafting [25,26]. In accordance with the study of Mirelli et al. [21] and our investigations

the authors furthermore provided evidence that cryopreservation does not lead to a reduced or completely inhibited antigenicity as all their 40 patients in spite of their immune responses were exclusively allografted using cryopreserved vessels [25,26]. In their first publication the authors speculated that a better degree of HLA-matching between donors and recipients may reduce the poor patency rates of vein allograft bypass and the high degree of vein allograft failure [25]. In their later publication dealing with the same cohort of patients, however, the authors referred to HLA-matching of venous allografts as impossible due to their generally limited numbers [26]. The final refusal to the approach of reducing the vessel's antigenicity through cryopreservation was published in 2007 by Pasquinelli et al. [27]. The authors convincingly described and reviewed the up-to-that-date existing publications that in follow-up investigations both humoral and cellular immune responses basically arise within 30-60 days after surgery although cryopreservation alters the number and viability of endothelial and smooth muscle cells. Apparently the immunogenic properties of the vessels are maintained through the residual cells which are sufficiently capable to induce an immune response in the recipient [28]. In this context the authors pointed to the relevance of HLA class I antigens which were clearly expressed in human aortic and femoral arteries after their adequate cryopreservation using e.g. controlled-rate freezing machines [27,28].

For unknown reasons the aspect of chronical rejection phenomena due to HLA-incompatible vessel allo-grafting has only sporadically but no more systematically been discussed for the last seven years although the discussion concerning a possible benefit of using immunosuppression has continued. This is puzzling in view of the fact that the discussions of patients suffering from the progression of transplant arteriosclerosis nonrelated to the initial infection have as well been ongoing. These problems, however, are in most cases discussed without providing immunological background data regarding HLA-compatibility and specification of the alloantibodies involved [5,6,7,29,30,31,32,33].

Interestingly in their comment on the study of Pupka and co-workers [7] Teebken pointed to the fact that the methods used by Pupka and co-workers were inadequate to distinguish between rejection and infection as both conditions are reflected by their scintigraphic investigations and data on the matching of AB0- and HLA-antigens were completely lacking [34]. Although Pupka and co-workers in their response to that comment without providing evidence speculated that AB0-bloodgroup compatibility is required it must be noted that this highly important immunological aspect has recently been investigated by Della Schiava and co-workers which did not confirm this assumption [35]. In accordance with tentative indications by earlier studies [36,37] the authors provided evidence that (in-)compatibilities of AB0- as well as Rhesus antigens did not lead to altered outcomes regarding death, thrombosis, rupture, stenosis, aneurysmal degeneration and follow up patency over a five years' follow up period thus pointing on a minor relevance of classical blood group antigens in this regard. Della Schiava and co-workers consequently speculated that incompatible HLA-antigens may be the main immune barrier which leads to the observed phenomenon of long term immune-mediated arterial allograft degeneration [35].

Taken together our study in best accordance with other studies provides evidence that randomly selected arterial allografts are highly immunogenic in terms of an HLA-directed immune response. Furthermore, it is highly probable that, apart from cellular immune responses, the HLA-directed humoral alloimmune response leads to a

chronic degeneration with harmful thromboses in about 16% of the patients up to life-threatening vessel ruptures in rare cases. Due to the underlying disease of severely infected vascular prostheses the application of an effective immunosuppressive regimen comparable to that used for solid organ allo-grafting or animal model-based arterial allo-grafting [32,33] in order to avoid consecutive transplant arteriosclerosis is highly limited or even impossible. In this context the low dose immunosuppression slanted toward sirolimus and used in our study did not show any effective outcome. As a consequence of the vast number of studies not entirely mentioned here a comparative study should be initiated in which mid- and long-term degenerative alloimmune effects of arbitrarily chosen versus HLA-matched arterial allograft recipients are compared. This approach may be based on the fact that the vast majority of transplanted vessels are performed using cryopreserved and no more freshly prepared arterial vessels. This holds especially true for Germany where the respective guidelines have accordingly been changed in 2012 leading to the exclusive use of cryopreserved homografts. Thus, the political will and the financial means provided, centralized tissue banks such as the European Homograft Bank (EHB) could be the adequate way to reach the target of comprehensively providing HLA-matched homografts [38].

Although not statistically significant three of the recipients of our study [ID9/PRA=28%, ID11/PRA=0%, ID29/PRA=0%] with two or no HLA-mismatches, respectively, and resulting low or no identifiable anti-HLA antibodies were characterized by functions without any clinical pathological findings over an average follow up period of 42 months (maximum of 78 months) until the end of collecting data for this study. Unfortunately but according to expectations histological data of these patients' homografts have not been available for us. As already phrased by Mirelli and co-workers in 1998 the overall conclusion must be drawn that allografted vessels have rather to be regarded as a biologically active vascular transplant than a tube of mere mechanical function [20].

Conflict of Interest

The authors of this manuscript have no conflicts of interest to be declared.

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