Anti-Alpha-Gal Antibody Response Following Xenogeneic Heart Valve Implantation in Adults

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Background and aim of the study: **The study aim was to investigate the xenoreactive immune response directed at Galα1, 3-Galβ1-4GlcNAc-R (α-Gal) which is known to be a major barrier in xenotransplantation, and to identify factors such as age, gender, ABO group and type of implanted tissue that might affect the anti-α-Gal immune response in adults subjected to bioprosthetic heart valve (BHV) implantation.**

Methods: **A total of 103 early survivors aged >20 years who underwent cardiac surgery using cardiopulmonary bypass was enrolled. Among the patients (45 males, 58 females; mean age 62.8 years), 66 who underwent BHV implantation were assigned as a study group, while the remainder were assigned to a control group. Serum samples were obtained from all patients on three occasions: before surgery (T0); on postoperative day 1 (T1); and on postoperative day 14 or at discharge (T2). A serum sample was also obtained from 31 patients in the study group at the out-patient clinic (T3) at a mean of 38 days after surgery.**

Results: **Anti-α-Gal antibody reactivity at T0 was higher in patients aged <65 years. Anti-α-Gal IgM and IgG reactivity at T2 was higher in the study group when compared to that in controls. In the study group, anti-α-Gal IgM and IgG reactivities were decreased at T1, but then increased at T2 when compared to that at T0. Anti-α-Gal IgG reactivity remained elevated at T3, but the IgM reactivity declined in the study group. None of the factors, including age, gender, ABO group and type of implanted tissue, had any effect on the anti-α-Gal immune response after BHV implantation.**

Conclusion: **BHV implantation in adults elicits an increased formation of anti-α-Gal antibodies, with different patterns for each isotype. Based on the study results, host factors including age, gender and blood type might be less important in the anti-α-Gal immune response following BHV implantation in adults.**

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Heart valve replacement surgery has been performed since the early 1960s. Today, approximately 275,000 prosthetic valves are implanted worldwide each year, about one-half of which are bioprostheses (1). Currently, most commercially available bioprosthetic heart valves (BHVs) are made from porcine aortic valve or bovine pericardium, and are prepared with glutaraldehyde fixation and various anti-calcification treatments. Although the use of BHVs has tended to increase, recent reports have demonstrated their limited durability due to structural valve dysfunction (2-6). The principal pathologic process causing bioprosthetic valve dysfunction is calcification of the xenograft leaflet. The major factors involved in bioprosthetic valve calcification are host-related, and include mechanical stress, chemical treatment and the biochemical composition of the implant (7).

In the field of living organ xenotransplantation, hyperacute rejection mediated by natural anti-α-Gal antibodies and the classically activated complement pathway was known to be a major barrier. In humans and in higher primates, approximately 1% of the circulating antibodies, and at least 80-90% of anti-pig antibodies, are directed towards the α-Gal epitope (8,9). Recently, the presence of the Galα1,

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Table 1: Patient characteristics in each group.

Parameter	Study group (n = 66)	Controls (n = 37)	p-value
Age (years)*	68.7 ± 11.1	52.4 ± 11.2	<0.001
Age >65 years	55 (83.3)	6 (16.2)	<0.001
Gender ratio (M:F)	33:33	12:25	0.100
ABO ratio+	28:38	16:21	1.000
Previous bioprosthesis implantation	3 (4.5)	2 (5.4)	1.000
Chronic liver or kidney disease	0	0	-
Preop. or postop. use of steroids	1	0	1.000
Postoperative significant infection	5 (7.6)	5 (13.5)	0.489
Hospital stay (days)*	22.6 ± 12.5	22.4 ± 11.8	0.939

*Values are mean ± SD.
+ABO ratio = B-containing:non-B-containing.
Values in parentheses are percentages.

3-Galβ1-4GlcNAc-R (α-Gal) epitope was further documented in commercial bioprosthetic valves, notwithstanding the shielding action achieved by glutaraldehyde fixation treatment (10,11). Although hyperacute rejection is not a concern in non-viable xenograft valve implantation, the possible role of an immune system response on tissue calcification has been documented (12-14).

Previously, it has been reported that the implantation of a porcine bioprosthesis elicits the increased formation of anti-α-Gal antibodies in children (15), even though a similar finding was first reported in an adult population by Mangold and colleagues (16). The aim of the present study was to investigate not only the increased formation of anti-α-Gal antibodies following BHV implantation, but also the change with time in anti-α-Gal antibody reactivity, and to identify factors such as age, gender, ABO group and type of implanted tissue that could affect anti-α-Gal antibody reactivity in adults who have undergone BHV implantation.

Clinical material and methods

Patients

A total of 103 early survivors aged >20 years (45 males, 58 females; mean age 62.8 years) who underwent cardiac surgery using cardiopulmonary bypass (CPB) at two cardiovascular centers (Seoul National University Hospital and Sejong General Hospital) were enrolled in the study. Among these patients, 66 who underwent BHV implantation in various positions were assigned as the study group. A bioprosthesis made of bovine pericardium (Carpentier-Edwards Perimount; Edwards Lifesciences) was implanted in 50 patients (75.8%), and a bioprosthesis made from porcine aortic valve and produced by two manufacturers (Epic Supra; St. Jude Medical, Inc.; and Hancock II; Medtronic, Inc.) was implanted in 16 patients (24.2%). In the control group (n = 37), various procedures, including coronary artery bypass grafting, mechanical heart valve implantation, valve repair and septal defect closure, were performed without the use of any xenogeneic materials.

The preoperative and perioperative characteristics of the patients in each group are listed in Table I.

The study was approved by the institutional review board/ethical committee of Seoul National University Hospital (H-0906-054-283), and informed consent was obtained from each of the patients. This study was conducted in accordance with the Declaration of Helsinki, 1975.

Serum sampling

Serum samples were obtained from all patients on three occasions: prior to surgery (T0); at postoperative day 1 (T1); and at postoperative day 14 or at discharge (T2). For 31 patients in the study group, an additional serum sample was obtained for the evaluation of anti-α-Gal antibody reactivity at the out-patient clinic at a mean of 38 days after surgery (T3). All serum samples were analyzed using an enzyme-linked immunosorbent assay (ELISA).

ELISA

The anti-α-Gal antibody reactivity was monitored using ELISA analysis, for which the methodologic details were as used in a previous study (15). Bovine serum albumin (BSA) containing synthesized α-Gal (α-Gal-BSA), prepared by the conjugation of α-Gal linker type 1 (Genkem, Seoul, Korea) and BSA (Armresco, Solon, OH, USA) with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride

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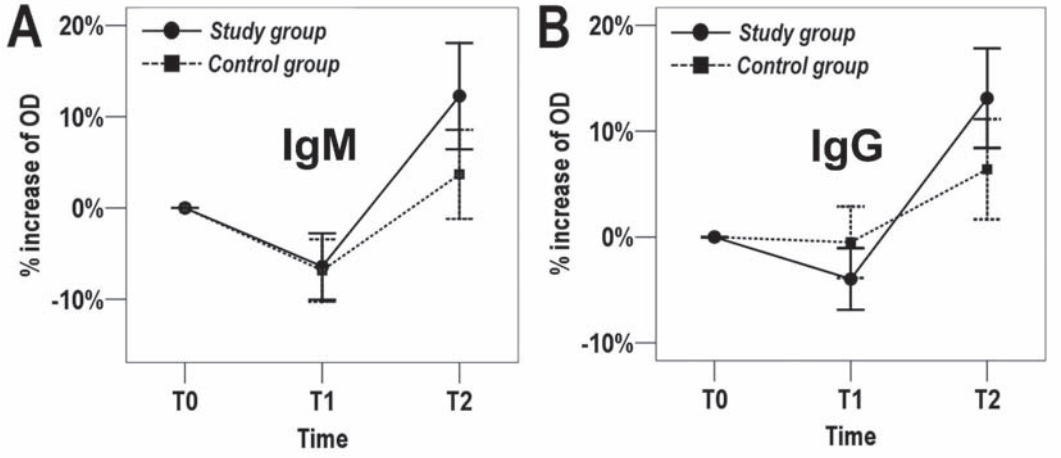

Figure 1: Changes in anti-α-Gal antibody reactivity for each isotype in the two groups. A) IgM. B) IgG. Immediately postoperatively (T1), although the anti-α-Gal antibody reactivity decreased regardless of the isotype in study group, the anti-α-Gal IgG reactivity did not decrease in the control group. At postoperative day 14 or at discharge (T2), the anti-α-Gal antibody reactivity was increased in both groups, though the increase was significantly higher in the study group than in controls.

(Pierce, USA) in conjugation buffer (MES-buffered saline pack; Pierce), was used as a solid-phase antigen. The microtiter plates were coated with 100 µl per well of α-Gal-BSA in phosphate-buffered saline buffer (pH 7.4) (at a concentration of 1 µg/ml for the IgM isotype, or 2.5 µg/ml for the IgG isotype), and this was incubated for 1 h at 37°C. The plates were then washed with deionized water. Aliquots of the patient's serum (100 µl per well) were added to the α-Gal-BSA-immobilized wells at a serial two-fold dilution, from 1:40 to 1: 2,560, in BSA-Triton X-100 (pH 7.4, PBS, 3% BSA, 0.01% Triton X-100). The plates were then incubated for 1 h at 37°C. Donkey anti-human IgG and IgM antibodies (Jackson, Human Research Laboratories Inc.) were used as a secondary antibody at a dilution of 1:15,000 for IgM and 1:10,000 for IgG in BSA-Triton X-100. The optical density (OD) was measured at a wavelength of 450 nm using the Thermo Electron-Lab Systems (Multiskan EX). To obtain the background OD-value, an ELISA was performed as described above but omitting the application of human serum.

Study design

The background OD-values (IgM 0.066 ± 0.008; IgG 0.137 ± 0.012) were subtracted from the test OD-values at a 1:40 dilution. The corrected OD-values at a 1:40 dilution were used for analysis. In order to evaluate the difference of the baseline anti-α-Gal antibody reactivity according to the host factors including age, gender and ABO group, the corrected OD-values of all patients at T0 was used for analysis. The change in anti-α-Gal antibody reactivity with time was evaluated and depicted with the value of the percentage increase in the corrected OD-values from baseline (T0), taking into consideration the individual difference of the anti-α-Gal antibody reactivity. The percentage increase in the corrected OD-values at T1 and T2 was used to compare the anti-α-Gal antibody reactivity between the two groups (study group and controls). In the study group, the change in anti-α-Gal antibody reactivity with time was evaluated and depicted for the 31 patients from whom serum samples at the out-patient clinic (T3) could be obtained. In addition, to evaluate the difference of anti-α-Gal antibody reactivity following the implantation of BHV according to the factors, including age (<65 years versus >65 years), gender (male versus female), ABO group (B-containing versus non-B-containing) and the tissue type of the bioprosthesis (bovine pericardium versus porcine aortic valve), the corrected OD-values at T1, T2, and T3 were analyzed for the 31 patients from whom serum samples were obtained up to the out-patient clinic (T3).

Statistical analysis

All data were presented as mean ± SD. The difference in the anti-α-Gal antibody reactivity between the groups or subgroups was evaluated using an unpaired *t*-test or the Mann-Whitney *U*-test, as appropriate. The percentage increase in anti-α-Gal antibody reactivity at T1, T2, and T3 was compared to that at T0 using a paired t-test. All statistical analyses were performed using SPSS software (SPSS for Windows, v. 13.0; SPSS Inc., Chicago, IL, USA). A p-value <0.05 was considered to be statistically significant.

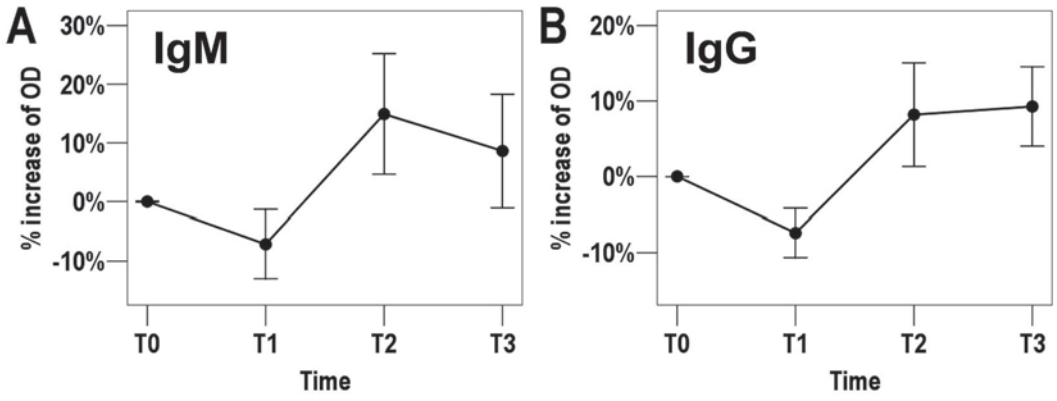

Figure 2: Changes in anti-α-Gal antibody reactivity following BHV implantation (n = 31). A) IgM. B) IgG. The anti-α-Gal IgM and IgG reactivities were decreased at T1 and then increased at T2. The anti-α-Gal IgG reactivity remained elevated at T3, but the IgM reactivity declined.

Results

Baseline anti-α-Gal antibody reactivity

The baseline anti-α-Gal antibody reactivity for each isotype was not different according to gender and ABO blood group, but was higher in patients aged <65 years than in those aged >65 years for each isotype (Table II).

Study group versus control group

Immediately postoperative (T1) anti-α-Gal antibody reactivity

Anti-α-Gal IgM reactivity was significantly decreased, compared to the preoperative value, in both groups (study group, p = 0.001; control group, p <0.001), but there was no statistical difference between the groups (Table III; Fig. 1). The anti-α-Gal IgG reactivity was significantly decreased in the study group but not in controls (study group, p = 0.008; controls, p = 0.768). Again, there was no statistical difference between the groups (Table III; Fig. 1).

Anti-α-Gal antibody reactivity at postoperative day 14 or at discharge (T2)

The anti-α-Gal IgM reactivity was significantly increased, compared to the preoperative value, in the study group, but not in controls (study group, p <0.001; controls, p = 0.133). The percentage increase in anti-α-Gal IgM reactivity in the study group was significantly higher than that in controls (p = 0.05). The anti-α-Gal IgG reactivity was significantly increased in the study group (p <0.001) and, surprisingly, was also increased in controls (p = 0.009). However, the increase in anti-α-Gal IgG reactivity in the study group was significantly higher than that in controls (p = 0.046).

*Table II: Baseline anti-α-Gal antibody reactivity.**

Parameter	IgM	IgG
Age		
<65 years (n = 42)	2.64 ± 0.48	2.08 ± 0.35
>65 years (n = 61)	2.32 ± 0.56	1.82 ± 0.40
p-value	0.003	0.001
Gender		
Male (n = 45)	2.40 ± 0.59	1.95 ± 0.44
Female (n = 58)	2.49 ± 0.51	1.91 ± 0.36
p-value	0.430	0.687
ABO group		
B or AB (n = 44)	2.49 ± 0.55	1.95 ± 0.39
A or O (n = 59)	2.43 ± 0.53	1.91 ± 0.41
p-value	0.606	0.624

Values are mean ± SD.
*Corrected OD-values at 1:40 dilution.

Table III: Percentage increases in corrected OD-values in the two groups.

Immunoglobulin	Sample time	
	T1	T2
IgM		
Study group	-6.4 ± 1.8	12.3 ± 2.9
Controls	-6.8 ± 1.7	3.7 ± 2.4
p-value	0.861	0.05
IgG		
Study group	-4.0 ± 1.5	13.1 ± 2.4
Controls	-0.5 ± 1.7	6.4 ± 2.3
p-value	0.120	0.046

Values are mean differences ± SEM.

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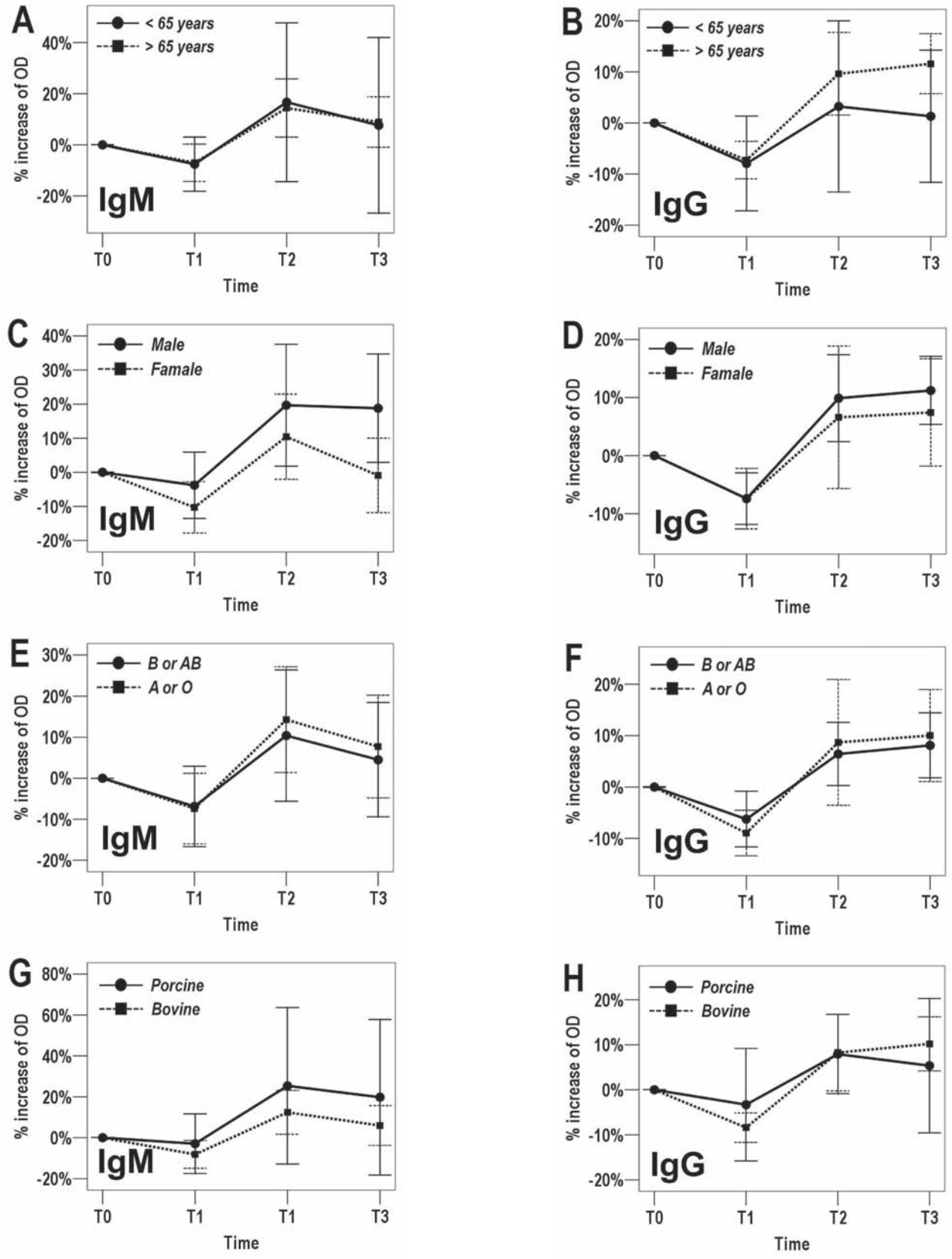

Figure 3: Changes in anti-α-Gal antibody reactivity following BHV implantation according to the factors, including age (A,B), gender (C,D), ABO group (E,F) and type of implanted tissue (G,H). There was no factor affecting the anti-α-Gal immune response after BHV implantation (n = 31).

Table IV: Change in anti-α-Gal antibody reactivity in the study group (n = 31).

	IgM			IgG		
	T1	T2	T3	T1	T2	T3
Mean difference	-7.3	14.9	8.6	-7.4	8.2	9.3
SEM	2.9	5.0	4.7	8.8	3.4	2.6
*p-value	0.018	0.006	0.078	<0.001	0.021	0.001

*, Compared to value at T0, using paired *t*-test.

Anti-α-Gal antibody immune response following BHV implantation

Anti-α-Gal antibody reactivity was decreased at T1, but then increased at T2, when compared to that at T0, regardless of the isotype (Table IV; Fig. 2). Anti-α-Gal IgG reactivity remained elevated at T3, whereas IgM reactivity declined, but the value was not different when compared to that at T0 (Table IV; Fig. 2). On the subgroup analysis, none of the factors, including age (<65 versus >65 years), gender (male versus female), ABO group (B-containing versus non-B-containing) and type of implanted tissue (bovine pericardium versus porcine aortic valve) affected the anti-α-Gal immune response after BHV implantation (Fig. 3).

Discussion

Although non-viable BHV implantation has been performed for more than four decades, the main concern today following bioprosthesis implantation is not catastrophic hyperacute rejection but rather a late degeneration and calcification of the implanted valve, which often requires reoperation. Although the exact role of the immune response in the degeneration and calcification of an implanted bioprosthesis in human is not fully understood, a possible role in tissue calcification has been proposed (12,13). The anti-α-Gal antibody is the major antibody against xenoantigens in humans (8,9), and the presence of the α-Gal epitope has been documented in commercial BHVs with glutaraldehyde crosslinking (10,11). Previously, it has been shown that BHV implantation elicited an increased production of the anti-α-Gal antibody in a clinical setting (11,15-17). In the present study, the baseline anti-α-Gal IgM and IgG reactivities were higher in the younger age group (<65 years). Since young age has been considered to be an important host factor associated with BHV calcification, and the immune mechanism has been considered to play an important role as an initial trigger of the calcific degeneration process in implanted BHVs, the present findings were compatible with those of others and, indeed, strengthened these data.

Previous reports (11,16,17) have indicated that patients who underwent BHV implantation developed significant increases in IgM and IgG directed towards α-Gal after surgery as compared to control patients; this suggests that the implantation of a bioprosthesis in cardiac surgery induces a xenograft-specific immune response. The present findings, that the percentage increase in anti-α-Gal IgM and IgG reactivity was significantly higher in patients who underwent bioprosthesis implantation, as compared to that of the control group, was consistent with the results of previous studies.

Contrary to the results of previous studies in which the anti-α-Gal antibody reactivity was not increased in controls (11,16,17), it was observed that anti-α-Gal IgG reactivity at T2 was significantly increased in controls, although the level was less than that in the study group. Before explaining this finding, it would be better to explain the finding that anti-α-Gal antibody reactivity at T1 was significantly decreased. In previous studies investigating the effect of CPB on immunoglobulin levels in serum, the latter were decreased immediately after CPB (18,19), though the authors considered that this decrease was attributed primarily to hemodilution. Additionally, a similar finding had already been observed with regards to anti-α-Gal immune responses in children, when hemodilution had been considered as the most likely cause (15). The results of the present study also concurred with the explanations of previous studies. In addition, data were obtained with regards to total protein and albumin levels, with globulin level (calculated by subtracting albumin from total protein) being decreased at T1 compared to that at T0 (T0, 2.91 ± 0.46 versus T1, 2.06 ± 0.32; p <0.001). This finding could strengthen the speculation that hemodilution might be the most likely cause of any immediate postoperative decrease in anti-α-Gal antibody reactivity.

With regards to the finding that anti-α-Gal IgG reactivity was increased at T2 in the control group, a possible explanation might be an immunologic rebound - a concept that has often appeared in the field of therapeutic plasmapheresis. The rebound can be explained by a decreased catabolic rate of immunoglobulin according to the decreased serum

concentration of immunoglobulin and the increased synthesis rate of immunoglobulin, which may be due to a depletion of inhibitory proteins (20). In the same context, it might be inferred that anti-α-Gal antibody reactivity could be increased owing to the reduced concentration of immunoglobulin and immune inhibitory proteins caused by hemodilution, and that the increased anti-α-Gal antibody reactivity following BHV implantation might be attributed in part to immunologic rebound. Even though the anti-α-Gal IgG reactivity at T1 was not decreased significantly in the control group, the increase in anti-α-Gal IgG reactivity at T2 could be explained by an immunologic rebound causing a reduction in the concentration of immune inhibitory proteins.

In the present study, the anti-α-Gal IgM response was rapid and transient, whereas the anti-α-Gal IgG response was longer and more delayed. This finding was consistent with that of previous studies (11,15,16), and can be explained by antibody class switching occurring in mature B cells upon exposure to antigens (21).

Possible factors influencing the host immune response include previous exposure to a xenograft, chronic major organ diseases, the use of immunosuppressive agents, and significant infection. In the present study, as the number of patients with a history of bioprosthesis implantation (n = 3), chronic major organ diseases (n = 0), the use of immunosuppressive agents (n = 1) and perioperative significant infection (n = 5) in the study group was small, an analysis of these factors would not have any statistical power.

On comparing the anti-α-Gal immune response following BHV implantation according to age, gender, ABO group and type of implanted tissue, it was observed that although the baseline anti-α-Gal IgM and IgG reactivities were higher in the younger age group (<65 years), the anti-α-Gal immune response did not differ between age groups until the first visit to the out-patient clinic. Since only seven patients aged <65 years were included in this subgroup analysis, this result might be a matter of course. Buonomano and colleagues (22) noted that the anti-α-Gal IgM values in women were higher than those in men, and linked such findings to the fact that autoimmune diseases, which might be mediated in part by anti-α-Gal antibodies, were more prevalent in women, though there was no clear causal relationship between anti-α-Gal antibody reactivity and autoimmune disease. The results of the present study confirmed that an inter-gender difference was not evident. McMorrow and associates (23) observed that anti-α-Gal IgG reactivity in the serum obtained from B antigen-expressing donors (B or AB) was significantly lower than in serum obtained from non-B antigen-expressing donors (A or O), but this was explained by the similarity in structure of α-Gal and the B antigen. Although it was expected that a difference in the anti-α-Gal immune response between the B antigen-expressing group and the non-B antigen-expressing group might be observed, there was in fact no difference. In the present study, the anti-α-Gal immune response was not related to the type of tissue implanted. Although the tissues were obtained from different animals and different organs, the various tissue treatment methods used by various manufacturers might affect the results of immune response, thus limiting any comparison according to the type of tissue.

In conclusion, BHV implantation in an adult population elicits an increased formation of anti-α-Gal antibodies, with different patterns between the two isotypes (IgM and IgG). The increased anti-α-Gal antibody reactivity following BHV implantation might be attributed in part to an immunologic rebound. The host factors, including age, gender and blood type, might be less important in the anti-α-Gal immune response following BHV implantation in an adult population, according to the results of the present study. Although the contribution of immune response directed at the α-Gal epitope to calcification process in xenograft valve implanted in human is not fully understood, and a possible role of an immune response to the xenoantigen other than α-Gal (non-Gal antigen) on the calcification process has been documented (24-26), the anti-α-Gal immune response was evident following BHV implantation in human according to the present results. For these reasons, further studies are mandatory to either eliminate or reduce the α-Gal antigenicity of BHV, including decellularization and enzymatic manipulation. Additionally, it might be preferable to attempt to reduce the cost of the α1,3-galactosyltransferase knock-out pig, which is now available.

Acknowledgements

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