Glutaraldehyde-Fixed Bioprosthetic Heart Valve Conduits Calcify and Fail From Xenograft Rejection

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Background—Glutaraldehyde fixation (G-F) decreases but likely does not eliminate the antigenicity of bioprosthetic heart valves. Rejection (with secondary dystrophic calcification) may be why G-F xenograft valves fail, especially in young patients, who are more immunocompetent than the elderly. Therefore, we sought to determine whether rejection of G-F xenograft occurs and to correlate this with graft calcification.

Methods and Results—Ascending aortas/valves (from rats [syngeneic] or guinea pigs [xenogeneic]) were transplanted (fresh or after 48 hour of G-F) into the infrarenal aortas of young rat recipients for 20 days. A xenogeneic group was also treated with steroids until graft harvest. The valves and media/adventitia were scored blindly for inflammation (0 to 4). Percent graft infiltration by T cells/macrophages was determined (immunohistochemistry), and rat IgG ELISAs were performed. There was >3 times more valve inflammation, >10 times more valve T-cell/macrophage infiltrate, and >3 times antibody rise in the G-F xenogeneic groups compared with the fresh syngeneic or the G-F syngeneic groups (P<0.05). There was >2 times more adventitial inflammation and T-cell/macrophage infiltrate in the xenogeneic groups (P<0.05). Steroid treatment decreased inflammation and antibody rise in the xenogeneic groups (P<0.05). Correlation analysis revealed media/adventitia inflammation (P=0.02) and percent macrophage (P=0.01) infiltration to be predictors of calcification.

Conclusions—G-F xenografts have cellular/humoral rejection and calcify secondarily. (Circulation. 2006;114:318-327.)

Key Words: calcium ■ immune system ■ pathology ■ pediatrics ■ surgery ■ valves ■ xenograft

Heart valve surgery is a challenging problem in pediatric and young adult patients compared with the elderly. In a patient who needs a valve replacement, bioprosthetic valves are probably the best choice because of their excellent function, the technical ease and safety of insertion, and the absence of a requirement for long-term anticoagulation. Bioprosthetic heart valves function well and for long periods of time (>10 years) in patients >70 years of age; however, in young adults and pediatric patients, these valves break down very quickly (<5 years),2,3 making them a poor choice in this patient population, the population that would most benefit from a bioprosthetic valve owing to its advantages.

Bioprosthetic heart valve (porcine/bovine) implantation is a form of xenotransplantation. In live-tissue xenotransplantation, there arises a very aggressive form of humoral and cellular immune system rejection.4 To decrease the immunogenicity of bioprosthetic valves, they are fixed in glutaraldehyde, and this process cross-links and masks the antigens,5,6 supposedly making the valves essentially “immunologically inert.”

Glutaraldehyde-fixed (G-F) valves calcify and fail over time, and it is believed that the G-F process leads to calcification of the valves from various chemical processes.7 However, in medicine, another instance where calcification is often seen occurs in inflammatory states (eg, tuberculosis, silicosis), characterized by inflammation followed by calcification. Young patients likely have a more robust immune system than do older people8; thus, a possible reason why pediatric patients and young adults “destroy” bioprosthetic valves much more quickly than do elderly patients is that they have an aggressive immune system response to the valve. Subsequently, calcification of the valve may occur as a “by-product” of the inflammation.

There is debate in the literature9 as to whether there is or is not an immune system response to bioprosthetic heart valves. Gong et al10 concluded that tissue treatment before implantation was very important in determining the tendency of that
tissue to calcify and that there was no obvious relation between bioprosthetic calcification and immunogenicity. Other groups have used rat models with subcutaneous implants to study rejection or large-animal models, such as juvenile sheep, to study calcification of glutaraldehyde grafts. The problem with these models is either that they do not have blood flowing by the graft (as in the subcutaneous implant models) or that the amount of inflammation that the animal undergoes is not similar to that in primates (as in the sheep model). A better animal model would be one in which blood flows by the graft and in which inflammation can be observed quickly. Numerous experiments have been performed to examine allograft rejection of heart valves in a rat model; however, to the best of our knowledge, no one has studied G-F xenografts to see whether they undergo rejection. If they do, this would be extremely important information in addressing the significant problem that exists in pediatric and young adult heart valve surgery patients.

We hypothesized that G-F xenograft aortic valve conduits undergo xenograft rejection mediated by macrophages and T cells, which can be decreased with steroids, and that calcification can be correlated with the amount of inflammation.

**Methods**

**Experimental Groups**

Inbred Lewis rats, 6 to 8 weeks old and weighing 150 to 200 g, and guinea pigs weighing 160 to 180 g (both from Charles River Labs, St, Constant, Quebec) were used. The young rat recipients were chosen to correlate to young humans. The aortic valve and ascending aorta were harvested from the donor and either immediately transplanted into the infrarenal aorta of the recipient (fresh) or fixed in 10% glutaraldehyde for 48 hours and then thoroughly rinsed in saline before transplantation. The glutaraldehyde concentration and time allotted were similar to those used in the clinical setting for bioprosthetic heart valves for human patients.

The experimental groups were as follows: (1) Fresh Lewis rat (donor) to fresh Lewis rat (recipient): syngeneic control for surgery-induced immune system injury; (2) G-F Lewis rat to Lewis rat: syngeneic glutaraldehyde negative control for the experiments; (3) G-F guinea pig to Lewis rat: xenogeneic glutaraldehyde experimental group; and (4) G-F guinea pig to Lewis rat, wherein the recipient received 0.5 mg/kg of steroid (methylprednisolone; Pfizer, Kirkland, Quebec, Canada) dissolved in water intraperitoneally once a day until graft harvest.

**Surgical Procedure**

A modification of the technique of Yankah et al was used. The surgical success rate was >95%. See the online Data Supplement for further details.

The animals were humanely killed 20 days after transplantation for graft harvest. The aortic valve segment was cut transversely and immersed in 10% formalin for histology and immunohistochemistry. The ascending aorta was cut vertically; half was immersed in 10% formalin for histology and immunohistochemistry, and the other half was frozen at −80°C for calcium determination. At the time of graft harvest, the native aortic valve/aorta of the recipient was harvested and processed. This served as an internal control for assays.

**Histology**

The formalin sections for histology were embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin-eosin (H&E). The slides were scored in a blinded fashion by a pathologist on 2 separate occasions to determine the degree of cellularity/inflammation of the valve, media, and adventitia (and avoiding areas with excessive amounts of muscle or suture material). There was 75% agreement between the scores from the 2 scoring sessions, and the average score from the 2 sessions was used. The scoring scheme was as follows: 0, no inflammation; <1, mild inflammation (consistent with postoperative healing); 2, moderate inflammation; 3, severe inflammation; and 4, very severe inflammation (sheets of inflammatory cells). The scores assigned to the media and adventitia were added together to determine a media/adventitia score. Other findings, such as the presence of eosinophils, thrombus, and infection, were also noted.

**Immunohistochemistry**

The primary antibodies used were anti-rat CD68 (MCA341R; Serotec, Raleigh, NC) at 1:100 dilution (marker for rat macrophages and monocytes) or anti-rat CD3 (MCA7722; Serotec, Raleigh, NC) at 1:200 dilution (marker for rat T cells). The secondary antibody used was biotinylated goat anti-mouse antibody (Jackson Immunoresearch, Westgrove, PA) at 1:200 dilution. A modification of the procedure as outlined in the *Immunology Methods Manual* was used. Please refer to the online Data Supplement for details.

**Rat IgG ELISAs**

All products were from the Bethyl Laboratories Inc (Montgomery, Tex) ELISA kit. The plates were coated with the coating antibody, goat anti-rat IgG-Fc (A110-136A), at 1:100 dilution in carbonate-bicarbonate buffer. Control rat sera (RS10-100) were used to create a standard curve. The secondary antibody used was goat-anti-rabbit IgG-Fc, horseradish peroxidase conjugate (A110-136P), at 1:10 000 dilution. A modification of the procedure as outlined in the *Immunology Methods Manual* was used. Please refer to the online Data Supplement for details.

**TABLE 1. Comparison of Inflammation and Calcification Between Groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Valve Cellularity/Calcification</th>
<th>Media/Adventitia</th>
<th>Calcium Content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inflammation, Median</td>
<td>Score, Median</td>
<td>Protein, Median</td>
</tr>
<tr>
<td></td>
<td>(Minimum–Maximum (n))</td>
<td>(Minimum–Maximum)</td>
<td>(Minimum–Maximum (n))</td>
</tr>
<tr>
<td>Fresh rat to rat (FRR)</td>
<td>0.0 (0.0-0.5) (5)</td>
<td>1.0 (0.0-1.0) (5)</td>
<td>0.8 (0.4-5) (4)</td>
</tr>
<tr>
<td>G-F rat to rat (GRR)</td>
<td>0.0 (0.0-1.5) (6)</td>
<td>1.5 (1.0-2.5) (9)</td>
<td>73.0 (32.4-640.5) (9)</td>
</tr>
<tr>
<td>G-F guinea pig to rat (GGPR)</td>
<td>1.5 (0.0-4.0) (6)</td>
<td>3.0 (2.5-8.0) (8)</td>
<td>89.6 (35.8-134.9) (5)</td>
</tr>
<tr>
<td>G-F guinea pig to rat, with steroids (GGPR-S)</td>
<td>0.5 (0.3-1.0) (7)</td>
<td>2.0 (0.5-2.5) (7)</td>
<td>39.0 (10.8-42.6) (6)</td>
</tr>
</tbody>
</table>

Statistically significant by Kruskal-Wallis test (KW): P<0.01, P=0.01 for GGPR vs FRR, P=0.01 for GGPR vs FRR; P=0.02 for GGPR vs FRR.
P=0.04 for GGPR vs GRR; P<0.01 for GGPR vs GRR; P=0.01 for GGPR vs GRR.
P=0.14 for GGPR vs GGPR-S; P<0.01 for GGPR vs GGPR-S; P=0.05 for GGPR vs GGPR-S.
P=1.00 for FRR vs GRR; P<0.01 for FRR vs GRR; P<0.01 for FRR vs GRR.
P=0.01 for FRR vs GGPR-S; P=0.02 for FRR vs GGPR-S; P=0.01 for FRR vs GGPR-S.
P=0.05 for GRR vs GGPR-S; P=0.54 for GRR vs GGPR-S; P=0.09 for GRR vs GGPR-S.
Calcium Assays

The procedure used was based on the method of Sarkar and Chauhan.16 Protein in tissue was assessed according to the standard method of Lowry et al.17 Please refer to the online Data Supplement for details.

Statistical Analysis

SPSS version 11.5 software (SPSS Inc, Chicago, Ill) was used. All variables were analyzed by nonparametric statistical tests (Kruskal-Wallis test with post hoc testing). A value of \( P \leq 0.05 \) was considered statistically significant. Data are expressed as median (minimum to maximum). Nonparametric correlation analysis was performed to determine the predictors of calcification.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Table 1 numerically demonstrates the inflammation and calcification in the experimental groups and the effect of steroids. The xenogeneic group had significantly more inflammation in the valve and for the media/adventitia compared with the syngeneic groups. There was always more inflammation (in absolute numbers) for the media/adventitia compared with the valve, and this may reflect the fact that intravascular immune system attack may be different from extravascular immune system attack. Steroid treatment decreased inflammation in the valve by \( >50\% \), which was trending toward significance, and the decrease in inflammation was statistically significant for the media/adventitia.

Steroid treatment did not, however, decrease inflammation in the xenogeneic animals to the level of the syngeneic animals, as shown for the xenogeneic steroid-treated animals (G-F guinea pig to rat with steroid, or GGPR-S), which were statistically different from the syngeneic groups (Fresh rat to rat [FRR] and G-F rat to rat [GRR]). There were no apparent serious side effects observed in the animals that received the steroids.

Glutaraldehyde treatment itself did lead to some degree of statistically significant inflammation in the media/adventitia, with some calcification, as shown in the comparison of the FRR versus GRR groups. However, this was not as prominent when the valve was examined. The xenogeneic groups had more inflammation than could be accounted for by G-F alone, as shown in the comparison of the G-F guinea pig to rat (GGPR) and GRR groups.

The mean \( \pm \) SEM for calcification (in \( \mu \)g calcium/mg protein) for the groups was 1.5 \( \pm \) 1.0 for FRR, 149.3 \( \pm \) 64.8 for GRR, and 86.1 \( \pm \) 16.0 for GGPR. Calcification in the glutaraldehyde syngeneic grafts was greater than that in the glutaraldehyde xenogeneic grafts. We thought this might have been a factor related to the different calcification properties of different animal species rather than the type of transplant (xenogeneic versus syngeneic). Thus, to determine whether guinea pig tissues (in general) calcify less than rat...
tissues, we performed G-F syngeneic guinea pig aortic valve conduit–guinea pig transplantations and compared these with the G-F syngeneic rat transplantations. The G-F guinea pig–to–guinea pig syngeneic grafts had 1.8\(\pm\)1.1\(\mu\)g calcium/mg protein (n=3) compared with 149.1\(\pm\)64.8\(\mu\)g calcium/mg protein in the G-F Lewis rat syngeneic transplants. We also performed G-F Lewis rat aortic valve conduit–to–guinea pig transplantations, and this graft had 40.8\(\mu\)g calcium/mg protein, thus showing that rat tissue calcifies more than guinea pig tissue, whether the rat tissue is transplanted into rats or guinea pigs. Thus, guinea pig aortic valve conduits calcify much less than do rat conduits, and this phenomenon is related to the calcification properties of each species and is not a property of the type of transplant (syngeneic versus xenogeneic). The fact that G-F guinea pig valves, which are difficult to calcify, actually had 45 times (86 versus 1.8) more calcification in the xenogeneic combination versus the syngeneic combination and that the xenogeneic combination had significant inflammation suggest that inflammation leads to calcification.

Figure 1 demonstrates H&E stains of (a) native, nontransplanted rat valve and aorta; (b) fresh, syngeneic rat aortic valve graft; (c) G-F syngeneic graft; and (d) G-F xenogeneic guinea pig graft. The native guinea pig valve and aorta (not shown) was similar to the native rat valve and aorta shown in panel a. This figure demonstrates total destruction and cellular infiltration of the G-F xenogeneic valve, with invasion into the media of the aorta and a very severe inflammatory response in the adventitia of the graft. The syngeneic grafts had minimal inflammation. The xenogeneic grafts also had more thrombi around the valves, as well as more eosinophils (shown in Figure 2) in the adventitia compared with the syngeneic animals. Figure 2 demonstrates the significant difference between a xenogeneic graft animal not treated with steroids and one treated with steroids. The area enclosed by the oval in Figure 2a highlights media destruction by the inflammatory process, and the area enclosed by the box highlights eosinophils in the adventitia of the graft. The “G” in Figure 2a is a granuloma. Treating the animal with steroids

<table>
<thead>
<tr>
<th>Group</th>
<th>%CD3 Infiltration, Median (Minimum – Maximum) (n)</th>
<th>%CD68 Infiltration, Median (Minimum – Maximum) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In valve</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRR</td>
<td>1.2 (0.0-2.5) (4)</td>
<td>0.1 (0.1-0.2) (4)</td>
</tr>
<tr>
<td>GRR</td>
<td>2.8 (1.8-3.8) (3)</td>
<td>1.2 (0.6-2.5) (3)</td>
</tr>
<tr>
<td>GGPR</td>
<td>26.1 (10.2-79.8) (4)</td>
<td>18.1 (12.5-42.9) (4)</td>
</tr>
<tr>
<td>GGPR-S</td>
<td>0.2 (0.0-0.2) (5)</td>
<td>3.6 (0.6-5.6) (6)</td>
</tr>
<tr>
<td>In adventitia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRR</td>
<td>0.4 (0.1-3.5) (4)</td>
<td>1.8 (0.8-6.4) (5)</td>
</tr>
<tr>
<td>GRR</td>
<td>10.1 (3.6-13.9) (6)</td>
<td>12.5 (6.7-27.7) (5)</td>
</tr>
<tr>
<td>GGPR</td>
<td>33.9 (12.5-57.9) (4)</td>
<td>33.2 (17.2-50.4) (5)</td>
</tr>
<tr>
<td>GGPR-S</td>
<td>1.5 (0.0-16.6) (5)</td>
<td>18.6 (12.3-26.6) (6)</td>
</tr>
</tbody>
</table>

See the footnote to Table 1 for explanation of experimental groups. Statistically significant by Kruskal-Wallis test: P=0.01. For valve: P=0.03 for GGPR vs FRR; P=0.03 for GGPR vs FRR; P=0.05 for GGPR vs GRR; P=0.05 for GGPR vs GRR. P=0.02 for GGPR vs GGPR-S; P=0.01 for GGPR vs GGPR-S. P=0.22 for FRR vs GRR; P=0.06 for FRR vs GRR. P=0.91 for FRR vs GGPR-S; P=0.01 for FRR vs GGPR-S. P=0.04 for GRR vs GGPR-S; P=0.26 for GRR vs GGPR-S. For adventitia: P=0.03 for GGPR vs FRR; P<0.01 for GGPR vs FRR. P=0.02 for GGPR vs GRR; P=0.05 for GGPR vs GRR. P=0.03 for GGPR vs GGPR-S; P=0.09 for GGPR vs GGPR-S. P=0.01 for FRR vs GRR; P=0.01 for FRR vs GRR. P=1.00 for FRR vs GGPR-S; P=0.01 for FRR vs GGPR-S. P=0.13 for GRR vs GGPR-S; P=0.55 for GRR vs GGPR-S.

Figure 2. H&E stains of tissues from xenogeneic groups. a, G-F guinea pig–to-rat xenogeneic graft, showing an area of media destruction (oval) and of eosinophil infiltration (box). b, Xenogeneic graft in an animal that was treated with steroids from the time of graft implantation to graft harvest. V indicates valve; M, media; A, adventitia; and G, granuloma. Magnification, x200.
beginning at the time of graft implantation essentially yielded a noninflamed graft.

Table 2 numerically describes the amount of T-cell and macrophage infiltrate in the various groups. Approximately one third of the xenogeneic valves and adventitia were infiltrated by T cells, and approximately one fifth of the valve and one third of the xenogeneic adventitia were infiltrated by macrophages; both of these findings were significantly different from those in the syngeneic groups. Macrophages (CD68) were more prominent in the G-F syngeneic groups, whereas in the xenogenic groups, both macrophages and T cells (CD3) were prominent. Treating the xenogeneic group with steroids decreased the infiltrate of both cell types, although the effect on T cells was more pronounced than that on macrophages.

Figures 3 and 4 demonstrate immunohistochemistry staining for rat T cells (Figure 3) and rat macrophages (Figure 4). The samples in both figures are (a) fresh, syngeneic rat valve and aortic graft; (b) G-F syngeneic rat valve and aortic graft; (c) G-F xenogeneic guinea pig valve and aortic graft; and (d) steroid-treated G-F xenogeneic guinea pig valve and aortic graft. There essentially was no T-cell or macrophage staining of the syngeneic valves (fresh or G-F), but there was staining of the xenogeneic valves. The cells appeared to attach to the edges of the valves and then infiltrate toward the middle of the valve, suggesting that these T cells and macrophages were attacking the graft from the intravascular space. Treating a xenogeneic animal with steroids (Figures 3d and 4d) from the time of graft implantation very significantly decreased the infiltration of these cells.

Treating a graft with glutaraldehyde led to T-cell and macrophage infiltration into the adventitia in both syngeneic and xenogeneic grafts, although the xenogeneic grafts had significantly more infiltrate (Figures 3 and 4). Treatment with steroids again led to a reduction in T-cell and macrophage infiltrate in the adventitia of the xenogeneic animals (Figures 3d and 4d). The cellular distribution of T cells and macrophages in the adventitia of these G-F grafts followed the same distribution observed in live xenograft transplantation, with macrophages immediately in contact with the graft and T cells distributed more peripherally. Figure 5 (a and b) demonstrates that in G-F syngeneic grafts, macrophages (CD68) and T cells (CD3) both occurred in the same area, which was in immediate contact with the graft (the media, in this case). However, in the G-F xenograft (Figure 5c and 5d), the macrophages are in contact with the graft, and T cells are located in the periphery.
Table 3 shows an ∼3-fold increase in the amount of rat IgG antibody produced in response to G-F xenografts compared with fresh or G-F syngeneic grafts. Steroid treatment significantly decreased the humoral response to the xenograft. Correlation analysis (Table 4) demonstrates that the media adventitia score and the amount of macrophage infiltrate were significantly correlated with the amount of calcification of the graft (ie, more infiltration meant more calcification of the graft).

**Discussion**

The claim that G-F significantly reduces the antigenicity of bioprosthetic valve xenografts has led some investigators to believe that there is no immunogenicity in G-F tissue. It is generally accepted that calcification of these bioprosthetic valves occurs because of chemical process between free aldehyde groups, phospholipids, and other components with calcium ions in the circulation. With calcification and the mechanical stress from valve opening and closing, the valve eventually deteriorates and fails, necessitating reoperation for valve replacement. This chemically induced calcification by glutaraldehyde likely occurs, as companies that produce such valves have had success with anticalcification treatments. However, there is still a group of patients, namely pediatric and young adult patients, who still develop very early calcification despite these treatments and experience failure of their bioprosthetic valves. One intuitive difference between noninfant pediatric patients and young adults compared with senior citizens (whose bioprosthetic heart valves will work 3 to 4 times longer than in young patients) is the greater immune system competence of the younger groups. Simionescu reviewed articles that ascribed early calcification in pediatric patients to excessive wear and tear and higher calcium turnover secondary to growth; however, even after the adolescent period (young adulthood, when one would not expect high calcium turnover), such patients still develop calcification very soon after transplantation and breakdown of the valves, suggesting that another process is involved, which may be rejection.

We have shown that in an intravascular (as opposed to a subcutaneous) young animal model (to correlate to young humans) there is rejection to G-F xenogeneic grafts. We found a significant rise in anti-rat IgG in the xenogeneic group compared with the syngeneic group, supporting the hypothesis that there is a humoral response to the G-F xenograft. There is also a cellular response, with the cells, including macrophages, T cells, and eosinophils, infiltrating the xenografts. The presence of eosinophilia in transplantation medicine can be suggestive of a more severe form of immune system attack. The distribution of macrophages

**Figure 4.** Immunohistochemistry staining for anti-CD68 (macrophage staining) in tissues from the various groups. a, Fresh rat-to-rat aortic valve conduit; b, G-F rat-to-rat syngeneic graft; c, G-F guinea pig-to-rat xenogeneic graft; and d, xenogeneic graft in an animal that was treated with steroids from the time of graft implantation to graft harvest. V indicates valve; M, media; and A, adventitia. Magnification, ×100.
in intimate contact with the graft and of T cells in more peripheral regions is concordant with what one would expect with live xenograft tissue cellular rejection. Because these xenografts do not have allogenic or syngeneic HLA antigens, macrophages attack the graft first (because they do not require specific antigen recognition) and then, through the release of cytokines and other chemoattractants, T cells are recruited for further attack. In the grafts, we also observed small thrombi more often in the xenograft group than in the syngeneic group. We have also shown that xenograft rejection can be decreased, though not eliminated, by steroid treatment, because the xenogenic steroid group was not statistically the same as the syngeneic groups. The steroids appeared to be more effective at suppressing T cells than macrophages. The presence of glutaraldehyde itself actually causes some degree of inflammation, as was shown in the comparison of the syngeneic fresh group with the glutaraldehyde-treated syngeneic groups. However, the xenogenic groups had significantly more inflammation than could be explained by glutaraldehyde alone. Lastly, we found that calcification was significantly correlated with the amount of inflammation. Osteopontin is a cytokine made by T cells and macrophages that is also involved in calcification. This may be a link between inflammation and calcification. From our review of the literature, the aforementioned findings are novel.

The calcium results that we obtained were interesting in that we expected the xenografts to be more calcified than the syngeneic grafts. Carpentier et al showed that tissues from different animal species calcify in different amounts. We found this to be the case, with guinea pig tissue calcifying much less than rat tissue. However, the fact that these guinea pig valve conduits (which are difficult to calcify) actually calcified in Lewis rats to a high amount, with evidence of T-cell and macrophage infiltration, and

### TABLE 3. Change in Rat IgG Antibody Concentration, Before Graft Implantation to After Graft Harvest

<table>
<thead>
<tr>
<th>Group</th>
<th>Increase in Rat IgG Before Implantation to After Harvest, mg/mL, Median (Minimum–Maximum (n))</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRR</td>
<td>3.10 (1.58–5.23) (3)</td>
</tr>
<tr>
<td>GRR</td>
<td>3.48 (1.26–6.49) (5)</td>
</tr>
<tr>
<td>GGPR</td>
<td>9.31 (7.09–16.54) (3)</td>
</tr>
<tr>
<td>GGPR-S</td>
<td>1.16 (0.36–2.86) (7)</td>
</tr>
</tbody>
</table>

See the footnote to Table 1 for explanation of experimental groups. Statistically significant by Kruskal-Wallis test: *P<0.01.*

*P*=0.05 for GGPR vs FRR.

*P*=0.03 for GGPR vs GRR.

*P*=0.01 for GGPR vs GGPR-S.

*P*=0.79 for FRR vs GRR.

*P*=0.07 for FRR vs GGPR-S.

*P*=0.01 for GRR vs GGPR-S.
that correlation analysis demonstrated that calcification was correlated with inflammation very strongly suggests that the xenograft valves calcify because of immune system injury.

We studied the media and adventitia of the aortic grafts for a number of important reasons. Intravascular immune cells may be unable to adhere to a valve very easily owing to the shear stress of flowing blood. Extravascular immune cells may be able to infiltrate a graft much more easily. Thus, studying the media and adventitia as well as the valve gave us the opportunity to study both systems. Another important reason to study the immune response in the media/adventitia relates to what may happen to grafts that are exposed to the mediastinum (such as occurs with xenograft aortic root replacements and right ventricular–to–pulmonary artery conduits that are performed very frequently for congenital heart surgery). All of these G-F grafts may be destroyed and calcified over time, with the inflammation occurring extravascularly versus intravascularly. It has been well established in the literature that homografts elicit an immune response and calcify. Surgeons in the future may face a similar problem with xenografts that are being placed at present.

Several interesting observations were noted when we compared our histology findings with our immunohistochemistry findings. Considering how cellular and thick the majority of xenogeneic valves were, we would have expected to find more CD3 and CD68 cells, but we did not. This probably means that there are other cells present that we have not looked for yet, such as B cells and natural killer cells (both of which are important in xenograft rejection); in addition, probably more protein was deposited on these xenograft valves (as is known to occur in xenograft bioprostheses implanted into humans).28

One limitation of this study may be that our observations are related not to rejection of the valves per se but simply to a foreign-body reaction to G-F tissue. Although this is possible, we decided that this was less likely, because the cellular distribution of macrophages and T cells was similar to what is seen in live cellular xenograft rejection, and we also observed a significant antibody rise to the xenograft tissue. If the process were simply a foreign-body reaction, then one would expect to see similar findings between G-F syngeneic grafts and xenogeneic grafts because they would both be considered “dead,” foreign-body tissue. Also, some might argue that we did not subject the tissue to G-F long enough. Zilla et al29,30 suggested that longer fixation decreases immunogenicity. We decided that 48 hours was satisfactory, as G-F still does lead to calcification (based on the chemical processes discussed earlier), and that too long of an exposure to glutaraldehyde may yield calcification based on chemical properties.

Some investigators have looked at the role of the immune system in the failure of bioprosthetic valves. Certain groups31–35 have studied animal models, observed the immune system responses in vivo or in vitro, and found evidence suggestive of an immune system response to G-F valves. Schussler et al31 found that glutaraldehyde decreased the humoral response but elicited a cellular xenogeneic response. Human and Zilla32 found strong evidence of a role for circulating graft-specific antibody in the disease of bioprosthetic graft calcification. Mirzaie et al33 found that short-time fixation with glutaraldehyde resulted in marked calcification of porcine cardiac tissue and the destruction of swine leukocyte antigen (SLA)-I epitopes, whereas even after a longer fixation time, epitopes of the SLA-II antigen remained unaffected. Using ELISAs and lymphocyte proliferation tests, Dahm et al34,35 showed that glutaraldehyde-tanned bovine pericardial valves provoked cellular and humoral immunologic reactions. Vincentelli et al36 suggested that the origin of the tissue (autologous versus heterologous) and not glutaraldehyde was responsible for calcification. The aforementioned studies suggest that there is evidence of xenograft rejection, which corroborates our findings. The problem with many of these studies is that the glutaraldehyde-treated tissue was implanted subcutaneously, which is different from an intravascular system wherein blood is constantly flowing by the graft. Meuris et al37,38 have shown that the results in a rat subcutaneous model are very different from the results in a sheep model with the blood in contact with the tissue, suggesting that to mimic the human situation, a system with blood flowing past the tissue is important. Our model mimics the human clinical situation more accurately, because blood flowed by the valves (as in humans) and we used young rats in an attempt to mimic young human patients.

Numerous experiments have been performed in juvenile sheep,37–40 with the valves being placed in the intravascular environment. Those studies have mostly concentrated

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sample Size, n</th>
<th>Spearman Correlation Coefficient (to Amount of Calcification)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valve cellularity/inflammation</td>
<td>15</td>
<td>0.43</td>
<td>0.11</td>
</tr>
<tr>
<td>Media/adventitia score</td>
<td>16</td>
<td>0.58</td>
<td>0.02*</td>
</tr>
<tr>
<td>%CD3 (valve)</td>
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<td>0.40</td>
<td>0.29</td>
</tr>
<tr>
<td>%CD68 (valve)</td>
<td>9</td>
<td>0.58</td>
<td>0.09</td>
</tr>
<tr>
<td>%CD3 (adventitia)</td>
<td>9</td>
<td>0.54</td>
<td>0.14</td>
</tr>
<tr>
<td>%CD68 (adventitia)</td>
<td>9</td>
<td>0.78</td>
<td>0.01*</td>
</tr>
</tbody>
</table>

*Statistically significant.
on calcification of the valves as opposed to inflammation of the valves. There has been some concern that the mild inflammation seen in sheep does not reflect the human situation properly, such that Trantina-Yates et al11 studied the differences in inflammation between G-F xenograft valves in the descending thoracic aorta of sheep and those in nonhuman primates and found that there was much more inflammation in primates (which are closer to humans). Thus, the fact that studies in sheep models have not disclosed much inflammation may not reflect the human situation. Alternatively, the calcification that occurs with such minimal inflammation may imply that the sheep model is actually a more sensitive model for inflammation. Our model found significant inflammation of the xenografts as well as a significant humoral response to the xenografts in a short period of time. Future preclinical studies will need to investigate the situation in larger animal models and test the grafts in different donor/recipient combinations to determine whether a similar process of xenograft rejection occurs.

In conclusion, we found evidence of rejection to G-F xenografts in a young animal model, with calcification that was correlated with the amount of inflammation. A process similar to what we observed may occur in young human patients when they receive a G-F xenograft bioprosthesis. The information gained from this study may help with designing future longer-lasting valves to assist this young patient population.

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Disclosures
None.

References
Clinical Perspective

Cardiologists and cardiac surgeons often encounter a difficult situation when children and young adults need a heart valve replacement. Implanting a mechanical valve gives the patient long-term valve durability and a less likely need for reoperation for valve failure; however, implantation of a mechanical heart valve mandates the use of chronic anticoagulation. Beyond the inconvenience of strictly remembering to and actually taking such medications every day (which young people may not want to do), there also is a cumulative risk of 1% per year of hemorrhage-related complications, as well as a 1% per year cumulative risk of thrombosis-related complications. Thus, over a 25-year period, such patients have an 50% risk of developing a significant complication. Bioprosthetic (xenograft tissue) heart valves are advantageous because they do not require chronic anticoagulation therapy; however, they break down relatively quickly (typically <5 years and sometimes within months) in young patients, necessitating repeat cardiac surgery, with its associated risks. Interestingly enough, elderly patients (>70 years old) do not experience breakdown of their bioprosthetic heart valves, and these valves will last for >15 years in most patients. It is generally accepted that the breakdown of bioprosthetic heart valves is due to calcification of the valve and wear and tear. It is also generally accepted that these valves do not undergo rejection because of glutaraldehyde-fixation, even though the valves are xenograft foreign tissue. There is an age difference in the robustness of the human immune system, and in certain chronic inflammatory conditions, calcification is likely to develop. We have demonstrated in our study using a young animal model that glutaraldehyde fixed xenograft valves undergo rejection and that inflammation is correlated to calcification. We have also shown that the rejection and calcification can be significantly decreased with steroid treatment. This potentially has significant implications for future valve replacement therapy in young patients.
Glutaraldehyde-Fixed Bioprosthetic Heart Valve Conduits Calcify and Fail From Xenograft Rejection

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