A Prospective Analysis of the Immunogenicity of Cryopreserved Nonvalved Allografts Used in Pediatric Heart Surgery

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Background—The purpose of this study was to prospectively determine the immunogenicity of nonvalved allograft tissue used to repair congenital heart defects.

Methods and Results—We prospectively analyzed the immune response of 11 children, 1.4 months to 10 years of age, who required nonvalved allografts to alleviate stenosis during repair of congenital heart defects. In 7 patients, pulmonary arterial grafts were used; in 3 patients, monocusp pulmonary artery grafts were used; and in 1 patient, a section of glutaraldehyde-preserved allograft pericardium was used. We measured the level of HLA panel-reactive antibody (PRA) before surgery, 1 week after, 1 month after, and 3 months after surgery. PRA was determined by the antiglobulin technique and flow cytometry. HLA class I and class II antibodies measured by either technique were negligible before and 1 week after surgery. Nine of 11 patients (82%) exhibited a significant immune response at 1 month after surgery that further increased at 3 months. The measured PRA for class I antibodies with the antiglobulin technique increased to 43±36% at 1 month and to 69±38% at 3 months after surgery. Flow cytometry class I PRA measurements were similar. Class II PRA increased to 26±34% at 1 month and to 41±36% at 3 months. Age negatively correlated with the degree of elevation of PRA, but neither allograft area nor the area indexed to patient body surface area correlated with PRA.

Conclusions—Cryopreserved nonvalved allografts induce a strong HLA antibody response in the majority of children. (Circulation. 2000;102[II]:III-179-III-182.)

Key Words: heart defects, congenital immunology pediatrics rejection restenosis

Nonvalved allograft tissue is frequently used in pulmonary and aortic patch angioplasty procedures during surgery for congenital heart defects. Controversy has persisted for decades regarding the association of valved allograft failure to immunologic injury. We have previously reported a marked HLA alloantibody response to cryopreserved valved allografts used for the repair of congenital heart defects in children. Other investigators have reported similar responses to antibiotic-sterilized and cryopreserved valved allografts, whereas some investigators contend that valved allograft degeneration is not attributable to immunologic injury. Although the reasons are unclear, there is an increased incidence of allograft failure in young children when compared with older children or adults. A recent morphological study demonstrated an increased incidence of T-cell infiltrates in explanted cryopreserved allografts from children compared with adults. Although we and others have demonstrated immunogenicity of valved allografts, little is known about the immunogenicity of nonvalved allografts. We have previously reported preliminary data on the immune response of a subset of patients with valved and nonvalved allografts. The purpose of this study was to expand this analysis to examine prospectively the immune response of a larger group of children receiving nonvalved allografts in the surgical repair of their congenital heart defects and to examine the effects of patient age and allograft size on the measured immune response.

Methods

Patients

We prospectively studied the immune response of 11 children who required a nonvalved allograft patch to alleviate stenosis during repair of a congenital heart defect. The ages of patients ranged from 1.4 months to 10 years (mean age, 1.8±3.2 years; median age, 6.7 months). The diagnoses in these patients were transposition of the great arteries (n=3), coarctation of the aorta (n=2), tetralogy of Fallot (n=2), interrupted aortic arch (n=1), double-outlet right ventricle (n=1), supravalvar aortic stenosis (n=1), and pulmonary atresia (n=1). Nonvalved allografts were required in 4 patients to repair or reconstruct the pulmonary artery, in 4 patients to relieve aortic arch obstruction, and in 3 patients to reconstruct the right
ventricular outflow tract. In 7 patients, pulmonary arterial grafts were used; in 3 patients, monocusp pulmonary artery grafts were used; and in 1 patient, a section of glutaraldehyde-preserved allograft pericardium was used. The size of allograft material inserted was $7.3\pm 6.4$ cm$^2$. The body surface area of our patients was $0.4\pm 0.3$ m$^2$. For purposes of comparison, we indexed the area of each allograft patch to the respective patient’s body surface area. The indexed area of the allografts was $18\pm 10$ cm$^2$ m$^{-2}$ body surface area.

Previous surgery was performed in 10 patients. The types of these previous operations were aortic arch reconstruction ($n=5$), pulmonary artery band placement ($n=3$), arterial switch procedure ($n=2$), ventricular septal defect repair ($n=2$), atrial septal defect repair ($n=1$), repair of pulmonary atresia with an intact ventricular septum ($n=1$), modified Gore-Tex Blalock-Taussig shunt placement ($n=1$), and supravalvular aortic stenosis repair with pericardial autograft ($n=1$). No patient received allograft material in any of these previous operations.

**Study Methods**

Primary Children’s Medical Center Research and Human Subjects Committee Institutional Review Board and the University of Utah Review Committee for Research with Human Subjects Institutional Review Board approved the study protocol. All procedures followed were in accordance with institutional guidelines. Informed consent was obtained from the parent or guardian of each child before entry into the study, and informed assent was obtained from children older than 9 years of age. Blood samples were obtained from each patient at the following times: immediately preceding surgery, 1 week after, 1 month after, and 3 months after allograft placement. Irradiated and leukocyte-filtered blood products were used in all patients to prevent sensitization to allogeneic blood cells. Blood products were filtered with Purecell leukocyte reduction filters (Fall Biomedical Products Co) and irradiated with $^{137}$Cs at 30 Gy. All allografts inserted were obtained from Cryolife, Inc.

We prospectively measured the percent of HLA panel-reactive antibody (PRA) from the blood samples obtained at the indicated intervals. PRA was determined in 2 ways: (1) by use of an antiglobulin cytotoxicity technique against an HLA-select frozen T-lymphocyte panel and (2) by flow cytometry with a pool of HLA class I and II purified antigens coupled to latex beads. HLA A and B serotyping was performed on all patients by use of the standard complement-dependent cytotoxicity (CDC) test and commercial serologic reagents. The antiglobulin technique uses the sensitive anti-human $\kappa$ light-chain immunoglobulin AHG-CDC technique against a frozen T-lymphocyte panel composed of 30 individuals of diverse HLA type and racial background. 12 PRA is expressed as the percentage of lymphocyte panel members against which the patients’ sera react and hence against which the patient has HLA class I antibody.

Using flow cytometry, HLA A, B, and C (class I) and HLA–DR/DQ (class II) antibodies were determined. This technique used affinity-purified, soluble class I and class II antigens from 30 different cell lines that are coupled individually to uniform, fluorescent-positive latex beads and then pooled together to create a panel that represents the majority of serologically recognized HLA class I and class II alloantigens (Flow-PRA I and II Beads; One Lambda). After incubation of the beads with 0.02 mL of the patients’ sera, washing, and staining with saturating IgG goat anti-human IgG, the percent fluorescent-positive beads (% PRA) was calculated after analysis on a Becton Dickinson FACScan flow cytometer. 13

**Statistical Analysis**

Comparisons between continuous data were made with a 1-way repeated-measures ANOVA with Student-Newman-Keuls post hoc analysis. Correlations ($r$) were analyzed by means of Pearson’s correlation. A value of $P<0.05$ was considered significant. All data are expressed as mean±SD except where otherwise indicated.

**Results**

In the 11 patients who received pulmonary allograft tissue, PRA measurements by the antiglobulin cytotoxicity technique were $1.5\pm 3.9$% (range 0% to 13%) before surgery and 2.4±2.4% (range 0% to 23%) at 1 week after surgery, indicating no significant immunologic stimulation (Figure 1A). Flow cytometry yielded class I PRA measurements of 1.8±4.2% before surgery and 2.4±4.9% at 1 week, confirming the insignificant response found by the antiglobulin cytotoxicity technique (Figure 1B). Flow cytometry class II PRA was 0.5±1.5% before surgery and 0.9±2.8% at 1 week, also an insignificant elevation (Figure 1C). Nine of the patients (82%) exhibited a vigorous immune response within 1 month of allograft insertion that further increased at 3 months after surgery. In 2 patients, 1 of whom received the pericardial allograft, there was no significant increase in PRA at 1 or 3 months by either the antiglobulin or flow cytometry techniques. As a group, the measured PRA for class I HLA antibodies with the antiglobulin technique significantly increased to $43\pm 36$% at 1.1±0.2 months after surgery and further significantly increased to $69\pm 38$% at 3.3±0.6 months after surgery ($P<0.05$) (Figure 1A).
Flow cytometry provided unequivocal evidence that the lymphocyte-reactive alloantibodies are HLA specific. The flow cytometry analysis demonstrated significant increases in both HLA class I and class II antibodies. At 1 month after allograft implantation, class I PRA increased to 42 ± 40%, and at 3 months it had increased to 70 ± 38% (Figure 1B). Class II PRA was increased to 26 ± 34% 1 month after surgery, and at 3 months it had increased to 41 ± 36% (Figure 1C). There was a negative correlation between patient age and the degree of elevation in the PRA, although this correlation was confounded by the fact that the oldest patient (who had no PRA response) differed from the other patients in that he received allograft pericardium (Figure 2). We found no significant correlation between allograft area and the PRA (Figure 3). Moreover, there was no correlation between the indexed allograft area to the PRA (Figure 4).

**Discussion**

Cryopreserved nonvalved allograft material is an important component in the repair of many congenital heart defects, particularly to augment luminal diameter of obstructed outflow tracts and large-vessel stenoses. We have previously examined the immune response elicited by valved allografts, with evidence of a brisk, class I HLA IgG antibody response that later broadens in panel reactivity within 3 months. Nonvalved allograft use, however, generally reduces the amount of foreign tissue introduced into the patient as well as eliminates the inclusion of valves and their distinct cellular architecture.

In this study, we prospectively demonstrated that similar to valved allografts, cryopreserved nonvalved allografts induce a strong HLA antibody response in the majority of children that broadens in reactivity within 3 months. This response represents antibody to both HLA class I and class II antigens. We hypothesize that this broad panel reactivity is attributable to the existence of public epitopes and cross-reactive HLA private epitopes in HLA antigens that are prevalent enough to create high-frequency sensitization when encountered by individuals with unique HLA phenotypes. The broad panel reactivity to allograft tissue is confirmed in this study by the nearly identical measured PRA phenotypes in this group of patients with 2 complementary immunologic techniques. We have previously demonstrated that neither open heart surgery with cardiopulmonary bypass nor the administration of leukocyte- and irradiated blood products is sufficient to generate an immune response. In this previous study, we also excluded the possibility that this immune response is due to exposure to blood products because the amount and type of blood products received by both control and study groups did not differ significantly. Thus, this study indicates that even small pieces of cryopreserved nonvalved allograft tissue exhibit sufficient immunogenicity to induce a strong humoral response. The significance of this immunologic response to valved or nonvalved allograft tissue is controversial when considering that freedom from reoperation can approach 90% at 5 years in patients receiving valved allografts. Nevertheless, we postulate that the calcification and luminal stenosis seen in allograft material over time may represent a form of immune-mediated injury similar to the acute rejection seen after solid organ transplantation. This type of immune response has been shown to occur and to result in allograft dysfunction in fresh venous allografts in dogs and aortic valve allografts in rats. Multiple foci of inflammatory T-cell infiltration is also seen in explanted pediatric arterial allografts, valved conduits, and monocusp allografts, consistent with an allogeneic rejection response.

Smaller children have been reported to be at higher risk for allograft dysfunction. Although the cause of this accelerated degeneration is unknown, increased immunologic reactivity in small children has been implicated. Histologic evidence of allogeneic inflammatory infiltration has also been reported in explanted allografts from pediatric patients but not in explanted cryopreserved allografts from adults, again suggesting increased immune response of children to allograft material. We found that there may be an effect of age in our patients with regard to the degree of immune response to allograft tissue. There was a negative correlation between the degree of elevation in PRA and patient age, in that the younger patients had a more vigorous immune response to the nonvalved allograft material. This correlation is confounded by the fact that the oldest patient in this group (10 years old) also was the only patient to have received glutaraldehyde-preserved pericardial material. Thus, further study...
with more patients receiving arterial allograft material will be necessary to confirm this correlation.

There was no correlation between the area of allograft tissue used and the degree of elevation in PRA. This would suggest that the degree of immune response is not directly related to the amount of antigen load (as defined by the size of the allograft) introduced at the time of surgery. The reason that 2 of the 9 children demonstrated no significant immune response is not readily apparent. One of these children received a section of glutaraldehyde-preserved allograft pericardium. It is possible that either the glutaraldehyde affected the immunogenicity of the allograft tissue or that pericardial tissue itself is less immunogenic than the pulmonary arterial tissue used in the other 10 patients. As stated above, it is also possible that this child’s older age also influenced his blunt immunologic response. However, the other child was a 6-month-old boy with complex cyanotic heart disease who received a 4-cm² piece of nonvalved pulmonary allograft as part of a Damus procedure. Since HLA typing on this tissue is generally not available, there is no way of confirming HLA compatibility between the patient and the allograft. Though highly remote, because of this patient’s uncommon HLA profile, this possibly could have accounted for the lack of HLA antibody response. It is also possible that the patients who did not mount an antibody response are genetic nonresponders.2,21

Another possible implication of the HLA antibody response, particularly the broadened humoral reactivity, is the effect it may have on the small subset of patients who may later require heart transplantation after allograft placement. The presence of donor-specific HLA class I antibodies (PRA) has been shown to increase the risk of acute or hyperacute heart transplant rejection and decrease graft survival.22,23 Further ramifications of this are that patients with elevations in PRA to class I or class II antigens may require prospective cross-matching before transplantation. This can result in an increased waiting time for a suitable donor or even failure to find a compatible donor for these children awaiting transplantation.

We conclude that nonvalved allograft tissue used in repair of congenital heart defects in children induce a strong antibody response. The implications of this response warrant further study.

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