Immunogenicity, Antigenicity, and Endothelial Viability of Aortic Valves Preserved at 4°C in a Nutrient Medium

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ABSTRACT **In patients undergoing aortic valve replacement, allograft valves stored at 4°C in a nutrient medium have been associated with excellent immediate and long-term results. The effects of this method of prolonged storage on the antigenic, immunological, and cellular characteristics of these grafts are incompletely understood. This study was designed to study these phenomena in rat aortic valves subjected to antibiotic sterilization and stored for up to 3 weeks in RPMI containing 10% fetal calf serum. Selected valves from Brown Norway rats were implanted heterotopically into the abdominal aorta of Lewis rats. Other valves were studied prior to transplantation. Antigenicity was determined by immunocytochemical staining using monoclonal mouse antibodies directed at Class I and Class II rat antigens. Immunogenicity was determined by duration of second-set skin graft survival following heterotopic aortic valve implant. Endothelial cell viability was determined by flow cytometric analysis of endothelial cells harvested from aortic valve allografts by collagenase digestion. Only fresh valves and valves stored for 1 day were positive for Class I antigens; no valves were positive for Class II antigens. Duration of skin graft survival was prolonged with greater duration of storage, but grafts remained immunogenic after 21 days of storage. Endothelial cell viability declined from 95% in the fresh valves to 64% after 21 days of storage. With prolonged duration of allograft valve storage at 4°C, there is an attenuation of antigenicity, immunogenicity, and endothelial cell viability. Loss of endothelial cells may contribute to the changes in immunological responses to the valve allografts. The expression of antigens on the endothelial surface is not a reliable predictor of immunological response.**

Human valve allografts have been successfully used for a variety of cardiac surgical procedures. One limit on the application of these useful materials is their potential for degeneration. Methods of allograft treatment and storage may exert a powerful influence on ultimate allograft longevity and function. Storage of allografts at 4°C is a time-tested method of preservation that has proven acceptable in achieving reliable clinical results.[1,2] However, other methods of graft treatment and storage have been devised that could improve the quality of the grafts and ex-

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tend the duration of storage that the grafts will tolerate. The effects of the various preservation techniques on specific characteristics of the allografts are not well defined. Among these characteristics that may influence the pathological fate of allografts are the intrinsic antigenicity of the graft, the immunological response of the host to the allograft, the viability of the allograft, the site of origin of the allograft (pulmonary or aortic), and probably other factors not yet defined. The purpose of this investigation was to examine three potential determinants of allograft function: antigenicity, immunological response, and endothelial cell viability, in experimental aortic allografts subjected to various durations of storage at 4°C. One goal of this study was to discern correlations among these factors, with the intent of learning how to predict the in vivo response to allograft insertion from features that could be observed prior to implant.

METHODS

Graft harvest and preparation

Inbred male Brown Norway rats (250-300 g body weight) underwent general anesthesia with 3.6% chloral hydrate solution (1 mL/100 g body weight) administered by intraperitoneal injection. Under clean conditions, the heart and thoracic aorta were excised and rinsed in 0.9% saline. The aortic valve with attached aorta, anterior mitral leaflet, and small portion of left ventricular outflow tract was dissected and placed in chilled, heparinized saline. Some valves were transplanted or studied immediately. Other valves were placed in 4°C RPMI 1640 culture medium (Sigma Chemical Co., St. Louis, MO, USA) with 10% fetal calf serum and the following antibiotics: gentamicin (160 mg/L), piperacillin (1000 mg/L), oxacillin (50 mg/L), metronidazole (200 mg/L), and amphotericin B (100 mg/L). After 24 hours, the tissue was transferred to RPMI 1640 culture medium with 10% fetal calf serum at 4°C. The tissues were maintained in this solution for 1 to 21 days.

The technique of heterotopic aortic valve allograft implantation was originally described by Yankah et al.[3] Recipient Lewis rats (strongly allogeneic relative to the Brown Norway strain, RT1 and non-RT1 incompatible) underwent laparotomy and dissection of the abdominal aorta under a 14 x operating microscope. The recipient aorta was occluded proximally and distally and divided. An end-to-end interposition of the allograft was performed using 8-0 polypropylene suture. The anterior leaflet of the aortic valve was incorporated into the suture line to prevent valve competence, which may predispose to thrombosis. The abdomen was closed and the animals were allowed to recover.

Skin graft

Three weeks following valve implant, full-thickness skin grafts from ears of Brown Norway rats were transplanted to the lateral thorax of the valve recipients. The skin grafts were inspected daily beginning 3 days after transplantation. Rejection was defined by standard criteria.[4]

Flow cytometric studies

Some valved aortic conduits not used for transplantation were analyzed for endothelial cell viability using flow cytometric techniques.[5,6] At the designated time, the grafts, which included the aortic valve and the entire thoracic aorta, were placed in 0.1% Type I collagenase (Sigma Chemical Co., St. Louis, MO, USA) at 38°C for 7 minutes. The endothelial layer was then removed from the graft by gentle rubbing with a glass rod and transferred to a centrifuge tube with 10 mL of phosphate buffer solution. The endothelial cell suspension was centrifuged at 1,500 rpm for 5 minutes. The supernatant was discarded and the sample resuspended in 100 μL of iced Griffonia simplicifolia agglutinin-fluorescein isothiocyanate (GSA-FITC) (Bioproducts for Science, Inc., Indianapolis, IN, USA), 20 μg/mL, for 30 minutes. The cells were washed with phosphate buffer and centrifuged twice to remove any GSA-FITC not bound to the endothelial cell membranes. The cells were resuspended in 0.5 mL of propidium iodide (PI), 50 μg/mL, and analyzed within 3-5 minutes by flow cytometry.

The suspension was placed in a pressurized cell suspension chamber and passed through a 67-μm Millipore filter (Millipore-Waters, Milford,

MA, USA) into the flow chamber. The cells were passed through an argon ion laser (488-nm wavelength at 400-mW power). The emitted light passed through a 525-nm bandpass filter for GSA-FITC analysis and through a 635-nm bandpass filter for PI analysis. Signal intensities were recorded by a MADAD II acquisition system (Coulter Corp., Hialeah, FL, USA). The data was then stored in list mode, gated, and integrated by a Coulter Fluorescence Activated Cell Sorter (Coulter Corp.). Cells that were positive for GSA-FITC and negative for PI were considered to be viable endothelial cells, while those positive for both GSA-FITC and PI were considered nonviable endothelial cells. A minimum of 1,000 endothelial cells was analyzed for each specimen, and the percent endothelial viability was recorded. Nine to 11 allografts of each storage duration were analyzed. The mean and standard error for each duration of storage were calculated.

Immunohistochemical evaluation

Selected aortic allografts not used for transplantation were examined with immunocytochemical techniques for the presence of antigen. Grafts were mounted on cryostat studs with OCT medium (Miles Laboratories, Inc., Naperville, IL, USA) and frozen in liquid nitrogen. Sections 8-μm thick were obtained and mounted for fixation in 100% acetone. The slides were air dried, rehydrated, and immersed in normal horse serum to block nonspecific binding of antibodies. The primary antibody, either OX 18 (Class I) (Accurate Chemical & Scientific Corp., Westburg, NY, USA), OX 6 (Class II), or ascites (control) were added to the specimens. After 1 hour at room temperature, the specimens were washed in phosphate buffered saline and washed in normal horse serum. The secondary antibody, biotinylated antimouse antibody (Vector Laboratories, Burlingame, CA, USA), was then added for 30 minutes. The specimens were again washed in phosphate buffered saline, and then covered in avidin-biotin complex peroxidase reagent (Vector Laboratories) for 30 minutes. The slides were washed in phosphate buffered saline and with tris buffer. The slides were incubated in diaminobenzidine. The specimens were then washed twice in phosphate buffered saline, dehydrated in alcohol, cleared in xylene, and examined with light microscopy. Three allograft specimens from each storage interval were examined. For each group of specimens one fresh thymus was also stained to serve as a control.

Statistical analysis

For statistical analysis of skin graft and endothelial viability results, comparisons were performed using analysis of variance (ANOVA). When significant differences ($p < 0.05$) were found using ANOVA, multiple *t*-tests were performed between fresh controls and each other treatment group using Bonferroni's inequality for multiple comparisons. A p valve < 0.05 was considered statistically significant.

RESULTS

Immunogenicity and endothelial viability results are summarized in Table 1. Duration of skin graft survival increased with increasing duration of storage prior to implantation. Rats that received valves stored for 21 days demonstrated skin graft survival that was significantly longer

TABLE 1

Duration of Storage (Days)	Duration of Skin Graft Survival (Days)	Endothelial Cell Viability (%)
0	4.5 ± 0.9	95 ± 1
3	4.9 ± 0.3	92 ± 2
5		88 ± 5
7	5.2 ± 0.4	86 ± 4**
10		86 ± 2**
14	6.1 ± 0.7*	83 ± 4**
21	6.0 ± 0.6**	64 ± 5**

*$0.05 < p < 0.10$; **$p < 0.05$.

than that of the other groups. The percentage viability for all groups stored 7 days or longer was significantly lower than that of the control group ($p < 0.05$).

At least three allografts of each duration of storage were subjected to immunohistochemical staining. Representative sections are shown in Figures 1 through 3. For all specimens examined, the valve leaflets, aortic wall, and myocardium demonstrated consistent findings, i.e., either all components of the graft were positive for the antigen tested for or all were negative. Fresh grafts and grafts stored for 1 day stained positively for Class I antigens. No graft stored for longer than 1 day stained positively for Class I antigens. No graft stained positively for Class II antigens.

DISCUSSION

The methods of allograft treatment used in this study are similar to those that have been used in some centers for clinical allograft treatment. Storage of human allografts at 4°C has permitted extensive use of these tissues with good results.[1,2] Many of the early efforts to lengthen storage of these grafts instead resulted in rapid graft failure. More recent techniques of cryopreservation appear to offer the hope of permitting even longer storage times without adverse effects on the grafts.[7] However, it will be years before the results of cryopreserved valves can be properly compared to the original preservation techniques. It may take even longer for different cryopreservation techniques to be compared to each other, and to the excellent results of O'Brien and colleagues.[8] Therefore, the study of allografts maintained at 4°C remains a pertinent object of investigation. One of the many points of controversy that remain regarding the storage of allografts is the advantage or disadvantage of using fetal calf serum in the storage medium. Although the use of this agent may enhance fibroblast viability,[9] it may also contribute to increased immunological reactions.[10] However, these investigators did not verify an adverse effect of calf serum on clinical results obtained with allografts stored in this agent.[11]

The major finding in this report is the limited expression of antigens by allograft aortic valves studied prior to implantation. Class I antigens were observed only on untreated grafts and on grafts stored for 1 day. No Class II antigens were demonstrated on grafts either in the fresh state or following any duration of storage. Class II antigens are known to be of considerable importance in solid organ transplants. These antigens may be expressed on the surface of tissues in response to various stimuli.[12] Previous investigations of rat vein allografts demonstrated minimal expression of Class II antigen as much as 24 hours following transplantation. Even after 4 days following transplant, only 40% of endothelial cells stained positively for Class II antigen.[13] Therefore, it is possible that following implantation, the allografts would demonstrate greater antigenic expression.

The importance of endothelial cells to long-term results of allografts is speculative. Endothelial cells are probably the most immunogenic component of transplanted vascular structures.[14] This study has shown aortic valve allografts retain immunogenicity despite marked attenuation of endothelial cell viability, and in the absence of detectable endothelial surface antigens. Endothelial cells may also be important to allografts because they prevent thrombosis. No thrombosis occurred in any allografts implanted in this study, but the observation period was relatively brief. It is possible that thrombosis is a potential problem in grafts implanted for longer durations. Endothelial cells may also play a role in preventing calcification of allografts. This particular variable was not examined in this investigation.

The use of flow cytometric analysis to evaluate the degree of endothelial cell viability in this investigation is based on the observation that Griffonia simplicifolia agglutinin is a specific histochemical marker for vascular endothelium of the rat. Its conjugation to fluorescein permits rapid evaluation of large numbers of cells using automated methods.[5] Exposure of cells to both GSA-FITC, to establish the identity of endothelial cells, and PI, which is excluded by viable cells,[6] thus permits quantitative assessment of endothelial cell viability. It must be recognized that the endothelial cell population evaluated in this study must certainly have been obtained to a

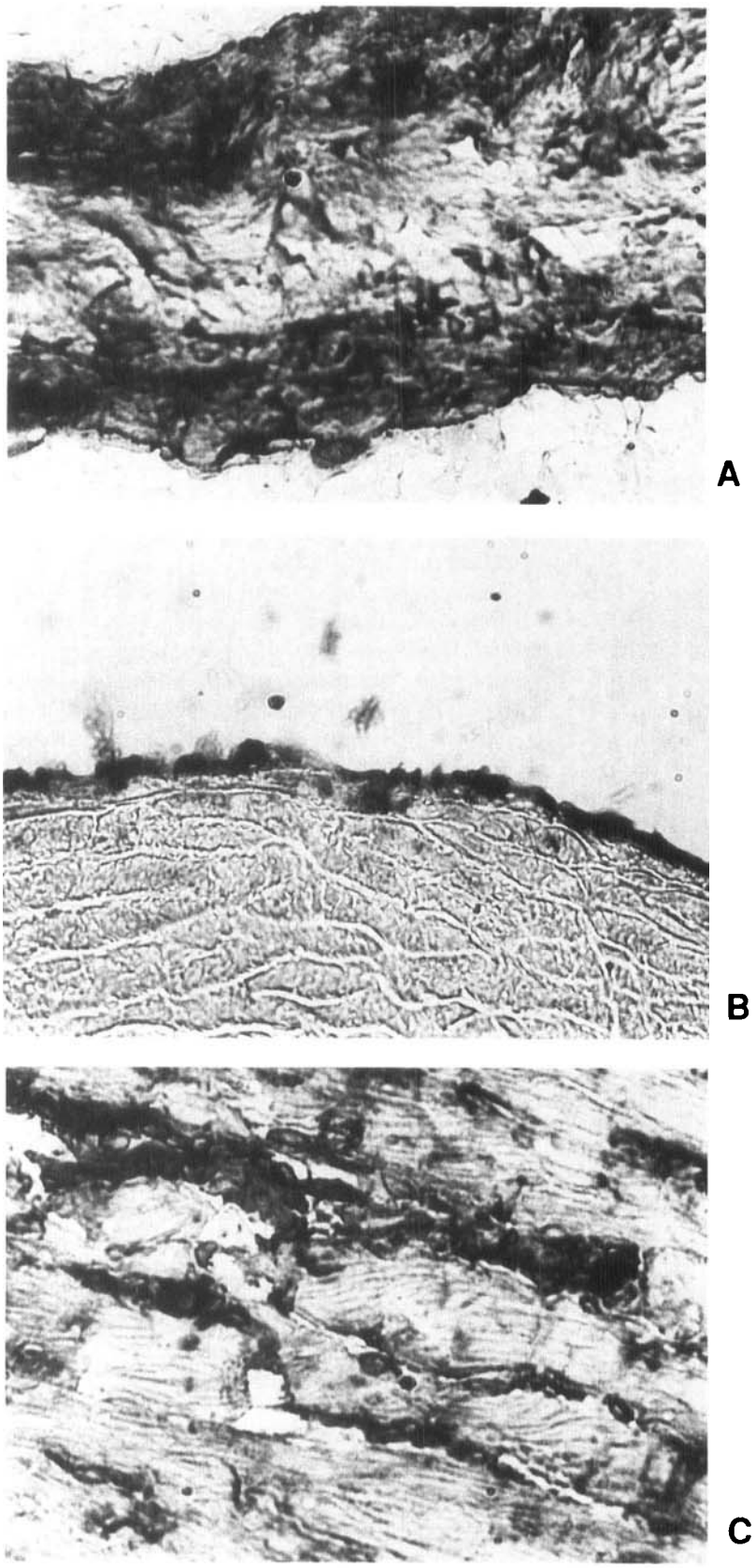

Figure 1. *Untreated aortic valve allograft stained for Class I antigen. Dark precipitate indicates presence of antigen on the aortic valve leaflets (A), aortic wall (B), and myocardium (C).*

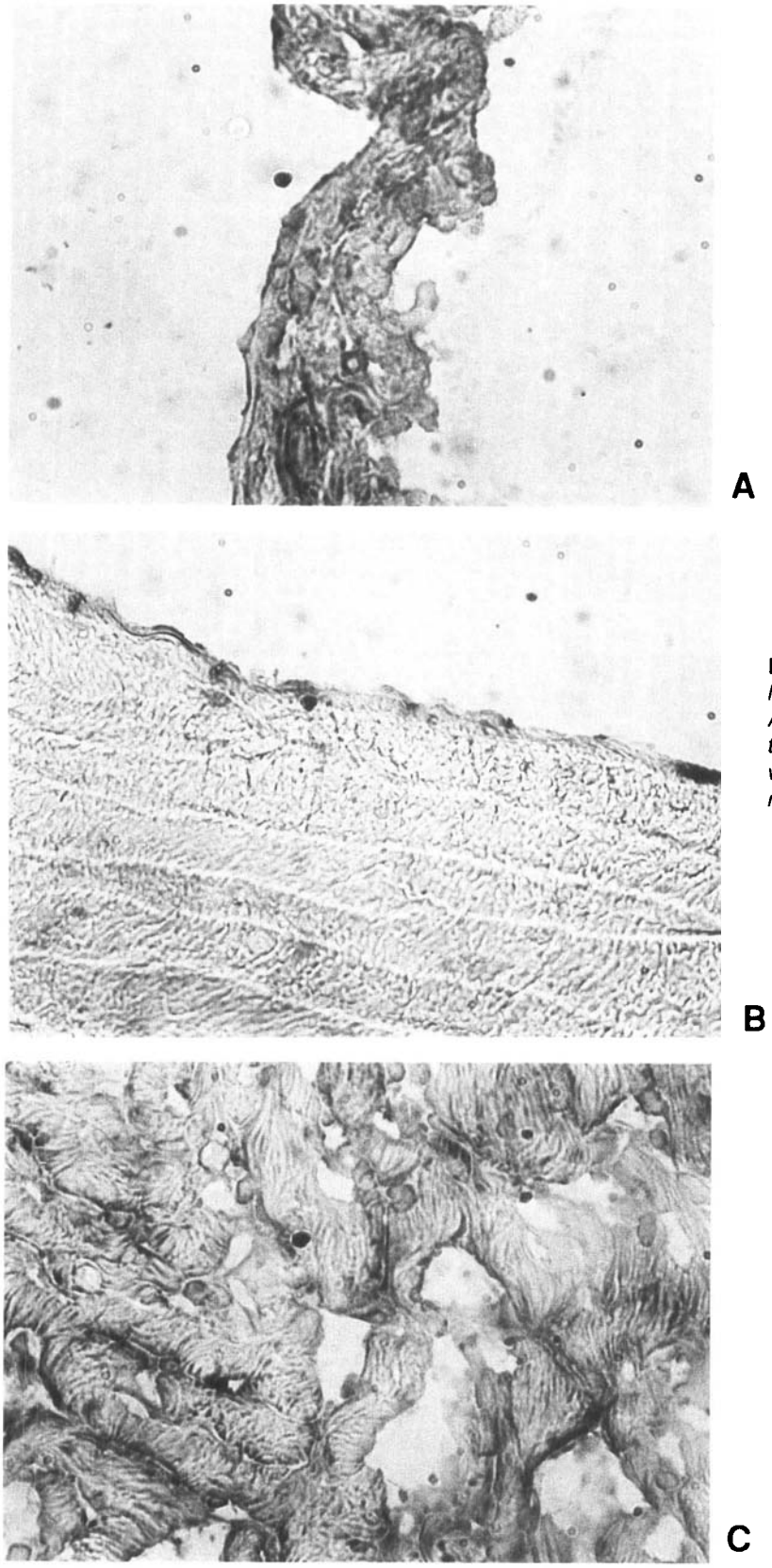

Figure 2. *Untreated aortic valve allograft stained for Class II antigen. Absence of dark precipitate indicates the absence of antigen on the aortic valve leaflets (A), aortic wall (B), and myocardium (C).*

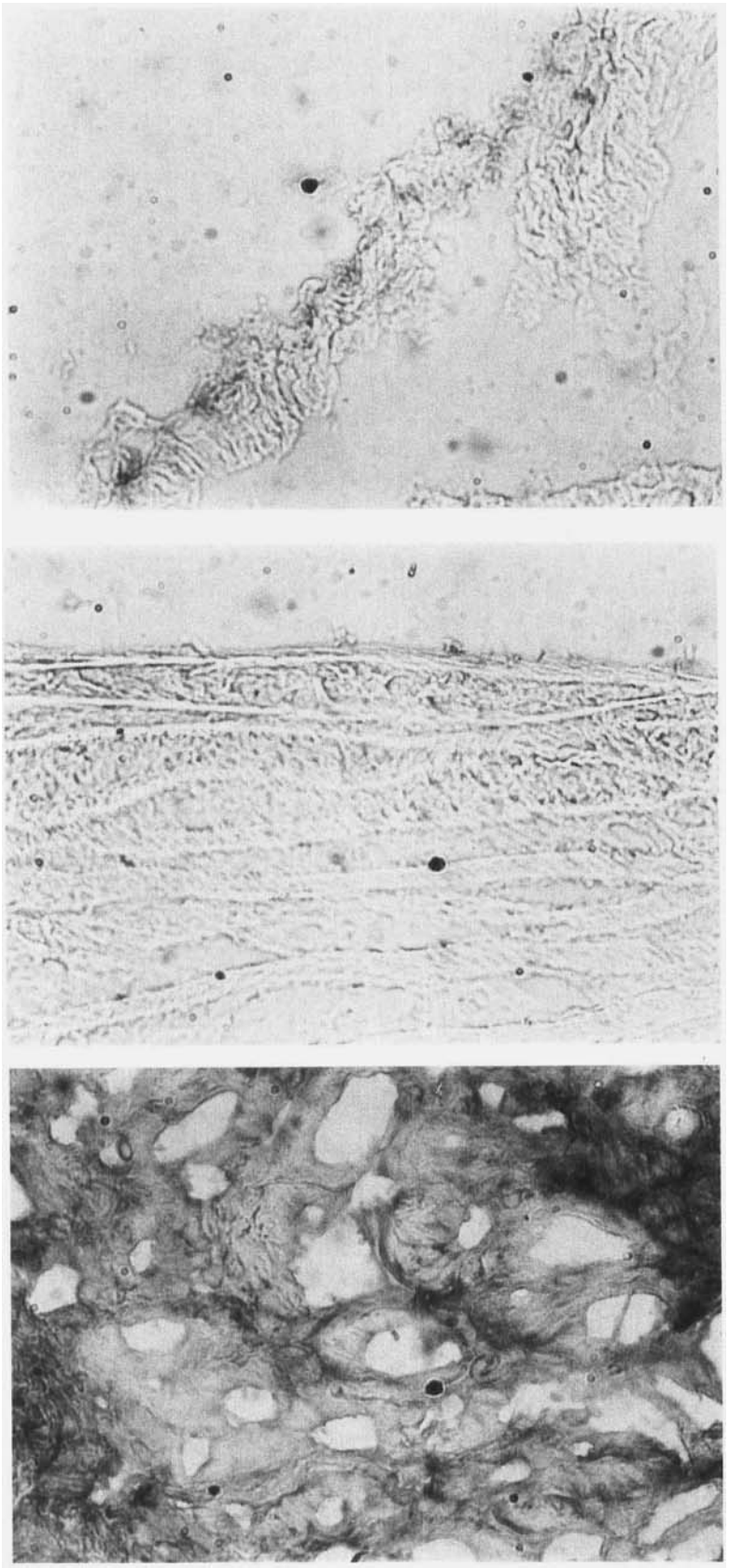

Figure 3. *Aortic valve allograft stored for 3 days at 4°C stained for Class I antigen. Absence of dark precipitate indicates the absence of antigen on the aortic valve leaflets (A), aortic wall (B), and myocardium (C).*

greater degree from the aortic wall than from the valve leaflets. Because our previous observations have shown that endothelial cells may be lost from the leaflets during prolonged storage,[15] it is possible that the endothelial cell population analyzed included few cells from the leaflets.

For the clincial use of allografts, it is not possible to assess antigenic expression nor immunogenic response following implantation. Therefore, it was hoped that information obtained from the study of antigenic expression of allografts prior to implant would provide a parallel estimate of the immunological consequences that followed implantation. However, the results of the present study indicate that immunogenicity cannot be reliably determined from antigenicity. Previous studies have attempted to characterize the antigenic expression of human allografts. One such investigation found that human aortic valves showed positive staining for Class I antigen immediately after harvest, but became negative following 48 hours of storage. The valves were negative for Class II antigen immediately after harvest and after storage.[16] These findings thus correspond closely to our observations in the rat allografts.

In summary, aortic valve allografts stored at 4°C in a nutrient medium demonstrate a gradual decrease in immunogenicity and in endothelial cell viability with increasing durations of storage. Only Class I surface antigens could be detected on any allografts, and these disappeared after a brief duration of storage. Thus, the presence of antigens is not a reliable indicator of the immunological potential of the allograft and cannot be well correlated to the viability of the endothelium. If this same lack of correspondence applies to human allografts, it may be speculated that assessment of human tissues for antigen expression would not be a valuable means of gauging immunological potential.

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