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Immunological Involvement in Porcine Bioprosthetic Valve Degeneration: Preliminary Studies†

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Preliminary animal model studies have indicated that porcine bioprosthetic valves can be recognized by the immune system. This report presents the results of a preliminary investigation on the implanted valves of 27 patients. While none of the 27 demonstrated an immune response to porcine serum proteins, five patients developed antibodies against the implanted valve leaflets. Cells were enzymatically eluted from a degenerated bioprosthetic valve and were classified by means of monoclonal antibodies. Although these cells were not found to be of T-lymphocyte lineage, they did express la determinants and had the morphologic appearance of plasma cells. We therefore conclude that some bioprosthetic valve dysfunction may be immunologically mediated.

The porcine aortic bioprosthetic valve has been widely accepted as a replacement valve in humans. At present, we do not know exactly how the process of valvular dysfunction occurs, but potential causes of valve failure may include autolytic changes before glutaraldehyde fixation, structural fatigue, calcium deposition, and host immune responses.

We have therefore initiated studies using three different approaches to ascertain whether or not the host immune response plays a role in the degeneration process of the porcine bioprosthetic valve in humans. The approaches are designed to determine: 1) the presence and classification of serum antibodies against porcine bioprosthetic valves; 2) the presence and classification of human immunocompetent cells associated with degenerated porcine valves; and 3) the ability of patients with implanted bioprosthetic valves to express an in vitro, cell-mediated immune response against porcine valve extracts.

Methods

Porcine heart valves
Heart valves used in these studies were either fresh frozen porcine valves, newly-processed Hancock porcine bioprosthetic valves, and/or surgically removed, degenerated bioprosthetic valves from patients who required new valves.

Human serum
Serum was obtained from 27 patients who had an implanted porcine valve but showed no clinical signs of valve degeneration. Serum was also obtained from two patients for whom new bioprosthetic valves had to be implanted because the earlier ones had degenerated.

Double immunodiffusion
Standard Ouchterlony double-diffusion tests were performed using double immunodiffusion plates (Hyland Laboratories, pattern D). Peripheral wells contained 7.0 μl of patients’ sera, and the center wells contained pooled porcine serum. The precipitin reaction was allowed to proceed for 72 hours at room temperature in a humid chamber (1).

Indirect and direct immunofluorescence and immunoperoxidase procedures
Frozen tissue sections (4-6 microns thick) of both fresh and glutaraldehyde-treated porcine bioprosthetic valve leaflets were incubated with patients’ sera or control

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Fig. 1

Indirect immunofluorescent staining of frozen sections of a fresh porcine heart valve leaflet. The sections were incubated with patient's serum (Fig. 1-A) or control serum (Fig. 1-B), washed, incubated with FITC-goat F(ab)₂ anti-human IgG, washed, covered with a coverslip, and viewed under a fluorescence microscope at 400X. Arrow in Fig. 1-A points to area of increased fluorescence located primarily on the surface of the valve leaflet when incubated with patient's serum, as compared to background fluorescence when leaflet was incubated with control serum (Fig. 1-B).

Presence and classification of human cells associated with degenerated bioprosthetic valves

A single degenerated porcine valve was placed in cold phosphate-buffered saline immediately after it had been removed from the patient. The valve leaflets were placed in a mixture of 2.5 mg/ml collagenase and 40 μg/ml hyaluronidase (Sigma) in RPMI 1640 media. This mixture was incubated in a shaking water bath at 37°C for one hour (4). The eluted cells were washed, and the mononuclear cell population was isolated by Ficoll-Hypaque gradient centrifugation (5). Cytospin preparations of the mononuclear population were made using a Shandon cytocentrifuge.

The slides were fixed in cold acetone for ten minutes and then washed twice (five minutes per wash) in phosphate-buffered saline (pH 7.2). The excess liquid was drained from the slides, and a 1:20 dilution of a single monoclonal antibody (OKT-3, OKT-4, OKT-6, OKT-8, OKM-1 [Ortho Diagnostic Systems, Inc], or NE-1a [New England Nuclear]) was layered on individual slides. Slides were allowed to incubate for four hours at 4°C in a humid chamber; they were then washed with phosphate-buffered saline (pH 7.2) and drained. A 1:40 dilution of FITC-goat F(ab)₂ anti-mouse IgG was added, and the slides were incubated for another four hours under the same conditions. They were then washed twice with phosphate-buffered saline (pH 7.2) and drained free of excess buffer. Before the slides were covered, a drop of sera for 30 minutes at room temperature in a humid chamber. The sections were thoroughly washed and then incubated with diluted FITC- or peroxidase-labeled anti-human IgG (Cappel Laboratories) for 30 minutes at room temperature (2,3). The slides incubated with the FITC conjugate were covered with a coverslip and examined with a Zeiss fluorescence microscope. The peroxidase-conjugated sections were further treated for ten minutes in Karnovsky solution (3), washed, mounted, and evaluated by routine light microscopy. Normal control sera were used to detect nonspecific staining.

Sections of degenerated porcine valves (4-6 microns thick) were incubated with fluorescein-conjugated goat F(ab)₂ anti-human IgG (Meloy Laboratories) for 30 minutes at room temperature. The slides were washed, covered with a coverslip, and examined by fluorescence microscopy.
Indirect immunoperoxidase staining of frozen sections of a glutaraldehyde-treated porcine heart valve leaflet. The sections were incubated with patient's serum (Fig. 2-A) or control serum (Fig. 2-B), washed, incubated with peroxidase-conjugated goat anti-human IgG, washed, treated with Karnovsky solution, covered with a coverslip, and viewed by light microscopy at 400X. Arrows point to area of increased staining (black reaction product) at the surface of the leaflet when incubated with patient's serum (Fig. 2-A), as compared to background staining when the leaflet section was incubated with control serum (Fig. 2-B).

Results

In the first study, in order to detect the presence of circulating antibodies against porcine sera, we screened the sera of 27 patients who had a porcine valve implanted for more than one year. This study used the standard Ouchterlony double-diffusion technique. None of the sera tested exhibited any reactivity to porcine sera. This negative response indicates an absence of an overwhelming humoral immune response to porcine protein.

Our second study was designed to determine if patients had circulating antibodies against Hancock valves and/or fresh unprocessed valves. Patients' sera were incubated with cryostat sections of both unprocessed fresh porcine valves and glutaraldehyde-processed Hancock valves. The former were incubated with patient sera, washed, and then incubated with fluorescein-conjugated goat F(ab)2 anti-human IgG. Because the glutaraldehyde fixation process causes increased autofluorescence, peroxidase-conjugated goat anti-human IgG was used to detect immunoglobulins to the Hancock valve leaflets.

The sera of 27 patients with functional valves were evaluated for antibodies to the implanted leaflets. The sera from five patients showed increased staining on the leaflet substrate when compared to controls. These five sera showed increased reactivity when incubated with either fresh unprocessed porcine valve sections (Fig. 1) or with Hancock glutaraldehyde-processed valve sections (Fig. 2). In all instances, the increased staining appeared at the surface of the leaflet.

The third study dealt with the isolation of mononuclear cells from a degenerated porcine bioprosthetic valve. Three leaflets from this valve were incubated in a mixture of collagenase/hyaluronidase to obtain a cell suspension. We obtained a total of 10^6 cells, which were 83% viable by dye exclusion. Cytospin preparations of the cells were obtained and treated with monoclonal cell-typing antibodies. The cells were further incubated with fluoresceinated secondary antibody and examined microscopically. Those preparations incubated with anti-la (B-cell/monocyte antibody) contained fluorescent cells (Fig. 3). Their general appearance suggested
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that they were plasma cells. No cell-associated fluores­
cence was found on slides incubated with anti-T-cell or
anti-monocyte antibody.

Discussion

The glutaraldehyde treatment for preparing cardiac
valve heterografts has been postulated to reduce the
antigenicity of the grafts (7) and also to delay the degen­
eration and subsequent calcification of valvular tissue
(8). Frolova, et al (8) found that extracts of glutaraldehyde-
treated valvular tissue remained immunogenic but had
fewer antigenic determinants than extracts of untreated
tissue. Sheikh, et al (9) found that glutaraldehyde-
treated valve tissue retains at least three solubilizable
porcine heart valve determinants.

Animal studies investigating a possible immune response
to bioprosthetic valves have yielded conflicting results.
Studies in which rabbits were injected with a suspension
of glutaraldehyde-processed porcine valve extract indi­
cated that humoral antibodies developed to valvular
components (10). In addition, rabbits grafted subcu­
taneously with glutaraldehyde-treated valve sections
developed a cellular immune response which could be
demonstrated both in vivo and in vitro (11). In contrast, a
murine study demonstrated only a cell-mediated histo­
logical reaction at the site of grafting where non-
murine study demonstrated only a cell-mediated histo­
logical reaction at the site of grafting where non-
glutaraldehyde-treated tissue was implanted (12).

The commercial processing of porcine bioprosthetic
valves removes and/or denatures the endothelial sur­
face and the acid mucopolysaccharides within the con­
nective tissue framework of the valves. Therefore, they
are essentially free of surface lining cells and related
cellular components of donor origin (13). However,
porcine valves implanted in humans for less than two
months showed an influx of plasma proteins, penetra­
tion of erythrocytes, deposition of macrophages and
giant cells, and some deposition of platelets (13). Valves
that had been in place longer than two months demon­
strated similar changes as well as disruption of valvular
collagen and erosion of the valve surface (13). Although
these studies indicate that the host immune response
may play a role in valvular degeneration, the long-term
clinical performance of the implanted valve in adults has
been satisfactory (14). Recent studies have indicated that
porcine bioprosthetic valves deteriorate more rapidly in
children than in adults, which may be due to a more
active immune system in children (15,16).

Of our three preliminary investigations, the first was
designed mainly as a control study to determine if any of
the patients had developed anti-porcine antibodies
from either normal exposure to porcine proteins or
exposure to ubiquitous cross-reactive antigens. Whereas
either situation would have produced false positive
staining in subsequent studies, none of our patients was
shown to have developed anti-porcine antibodies.

In animal models, serum antibodies to porcine valves
have been demonstrated after valvular tissue and tissue
homogenates were injected. Slanczka, et al (11) studied
the immunogenicity of glutaraldehyde-treated versus
untreated porcine valve homogenates. They used immuno­
electrophoretic techniques to determine that serum
antibodies from rabbits immunized with either glu­
araldehyde-treated or untreated porcine valve homo-
genate cross-reacted with the heterologous tissue extract.
The reaction was specific for valve tissue, and no anti­
bodies were found to react with liver or kidney extracts.
Rabbits, which were immunized to the untreated porcine
valves, demonstrated high titers of antibodies,
determined by complement fixation and homologous antigen; and in fact they developed sufficient antibody to demonstrate an anaphylactic response when challenged with antigen.

In a murine study, Villa, et al (12) performed heterografts and intraperitoneal injections with similar valvular tissue. Those mice receiving homogenates of untreated valves demonstrated an immune response both histologically at the site of grafting and by cytotoxicity assay using porcine lymphocytes as target cells. However, mice which received glutaraldehyde-treated valves or homogenates demonstrated minimal cellular infiltration at the site of grafting and produced little or no antibody response by cytotoxicity assay.

In our study, we found that five of 27 patients who had received an implanted valve apparently developed antibody to the residual components of the endothelial layer of the valve leaflet. The antibodies were apparent on tissue sections of unimplanted glutaraldehyde-processed valves and on tissue sections of fresh porcine valve. This suggests that the glutaraldehyde process may not mask all immunogenic sites of the components of the leaflet. However, these results should not be considered definitive because control serum containing proven antibody to porcine leaflets is not available.

Recently, investigators have reported that myocardial fibroblasts possess receptors for the Fc portion of normal human and rabbit IgG immunoglobulins. Danilova, et al (6) reported that human myocardial fibroblasts possess this receptor and that the interstitial connective tissue of human myocardium also exhibits similar binding. In contrast, Gelfand, et al (17) found Fc receptors for immune complexes (sheep erythrocytes + anti-sheep erythrocyte IgG) distributed only in the subendothelial intracellular ground substance of the base, stalk, and tip of human cardiac valves. These studies indicate that binding of immunoglobulins to heart valves may involve Fc receptor binding in addition to normal antigen-antibody binding. No studies have been done to determine if Fc receptors are available on the Hancock bioprosthetic valve or if glutaraldehyde treatment masks the sites.

Spray and Roberts have studied cellular infiltrates found on the surface of implanted valves (13). In all patients whose porcine bioprosthetic valves had been in place longer than two months, they observed infiltration by inflammatory cells (leukocytes, macrophages, and some plasma cells). They also found that histiocytes were deposited, giant cells were formed, and that the fibrocollagen structure of the valve was disrupted. They observed similar changes in 79% (26/33) of the valves in place for less than two months. These authors concluded that the porcine valves do not appear to be biologically inert; however, they observed very few instances of valve failure during their study.

Weesner, et al (18) studied degenerated porcine valves that were implanted in children. Most valves revealed extensive ingrowth of host endothelial cells on the valve surface. Three of nine valves exhibited plasma cells on the valve surface, and three of nine had immunoglobulin deposition detected by immunofluorescence, although the investigators did not state where the deposition was located. They concluded that some porcine valve degeneration may be caused by an immunologic reaction.

In our study, we found that the amount of cellular infiltrate varied. We therefore developed a procedure to elute cells from the leaflet so that the various cell types could be identified. The battery of monoclonal antibodies we used identifies T-cells and T-cell subsets, B-cells, monocytes, and some plasma cells. The greatest reactivity was found on the preparations treated with anti-la antibody. We concluded that because of the size of the cells and the appearance of the nucleus, they were plasma cells. The presence of these cells does indicate that some immune mechanisms may be involved.

In summary, we have found that some patients who received Hancock bioprosthetic heart valves had developed antibodies to components of the valve leaflets. In addition, cells associated with the immune system were recovered from the surface of a degenerated porcine bioprosthetic valve. These results suggest that some patients who receive porcine valve implants may develop an immune response to the bioprosthetic valve.

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