Biocompatibility of a xenogenic elastin-based biomaterial in a murine implantation model: The role of aluminum chloride pretreatment

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Abstract: We have investigated the long-term effect of aluminum chloride ($AlCl_3$) treatment on the calcification and inflammatory reaction of a porcine elastin-derived biomaterial (PEB) in a novel subdermal adult mouse model. Untreated PEB disks and PEB treated with $AlCl_3$ were implanted subdermally in BALB/c mice for 30, 60, and 180 days. The calcification of the elastin disks was examined with histological analysis and atomic absorption analysis of calcium content. The inflammatory reaction was evaluated both with histological analysis of explants and by an enzyme-linked immunosorbent assay of the serum in each mouse to determine the production of antielastin antibodies. Robust calcification was evident in all untreated PEBs with calcium levels of 107.1 ± 11.8, 151.4 ± 14.4, and 227.2 ± 23.8 μg/mg for 30, 60, and 180 days, respectively. $AlCl_3$ treatment only temporarily prevented the calcification of the elastin disks for 30 days. By 60 and 180 days, the $AlCl_3$-treated materials had significant calcification with 88.7 ± 17.4 and 105.3 ± 27.0 μg/mg calcium, respectively. The inflammatory reaction was moderate for both types of implants. The $AlCl_3$-treated implants displayed significantly more macrophage and lymphocyte infiltration at 180 days after implantation, and a trend to higher humoral responses at 30 and 60 days when compared with untreated PEBs. We conclude that PEBs extensively calcify in the adult mice model. $AlCl_3$ treatment of elastin enhances the long-term immunological response to xenogenic elastin implants and merely delays the onset of calcification. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 69A: 55–64, 2004

Key words: elastin; biomaterial; calcification; inflammation; subdermal

INTRODUCTION

We are developing a purified porcine elastin biomaterial (PEB) for the repair of cardiovascular, gastrointestinal, and urological tissues. Elastin, an insoluble hydrophobic extracellular matrix protein, is readily available from porcine elastic arteries and can be rapidly purified with hot alkali digestions.[1,2] With our process, we attain >95% pure elastin as confirmed by amino acid analysis. We have investigated the use of PEBs as stent coverings[3] and gastrointestinal patches in both the duodenum and esophagus.[4,5] These experiments have demonstrated a high degree of biocompatibility and a significant resistance of PEBs to digestive enzymes and acids. Although we observed at most intermittent calcification associated with elastin covered stents, others have reported rapid and extensive calcification in purified elastin implants. Although elastin has many advantages as a biomaterial, its tendency to extensively calcify after implantation may ultimately limit its usefulness as a reconstructive extracellular matrix scaffold.

Recent investigations have highlighted the propensity for purified elastin to calcify when implanted in immature rats.[6–8] The implantation of bovine neck ligament elastin fibers in these models led to rapid

calcium accumulation, with elevated calcium levels evident as soon as 7 days postimplantation. In addition, electron microscopy revealed a tight spatial correlation with calcium crystals and elastin fibers. Pretreatment of the elastin fibers with aluminum chloride (AlCl$_3$) successfully prevented calcification over the 21-day implantation period.[7,9] These results suggest a physiochemical mechanism of elastin-associated calcification mediated by the exposed surface of the elastin fiber. Vyavahare et al.[7] proposed that the AlCl$_3$ treatment permanently bound aluminum to the elastin molecule and altered its spatial conformation, effectively limiting exposure of glycine residues, as indicated by Fourier transform infrared spectroscopy, and thus enhanced the fibers' resistance to calcification. Because numerous splice variants have been identified for elastin[10] and elastin fiber structure can vary greatly between tissues, it is possible that both the process of calcification and propensity for inhibition may be tissue specific. For these reasons, we believe it is necessary to test our porcine-derived elastin in relevant calcification models.

Inflammation and calcification are often synergistic processes, in which ectopic calcium deposits may induce or enhance inflammatory responses and inflammatory cell infiltrates may directly facilitate calcium crystal formation. One component of an inflammatory response is the release of matrix-degrading proteases. Bailey et al.[9] indicated that elastin degradation and matrix metalloproteinases (MMPs) may have a role in elastin calcification. Seven-day studies in immature rats indicated an increase in MMP activity surrounding elastin subdermal implants and a decrease in MMP expression with the treatment of AlCl$_3$ on elastin before implantation. However, later time points were not examined and chronic inflammatory responses, which occur weeks to months postimplantation, may produce a cumulative effect on elastin degradation and thus may lead to markedly enhanced calcification at these later time points.

Both calcification and immune responses may be affected by matrix structure and protein source and for this reason we have undertaken an implant study of our PEB. We have developed an adult mouse model to study the prolonged time course and spatial correlation between immune and calcification responses of untreated PEBs and AlCl$_3$-treated PEBs.

MATERIALS AND METHODS

Elastin biomaterials

Porcine carotid arteries were obtained from swine of approximately 200 lbs. (Carlton Meat Packing, Carlton, Oregon). The arteries were immediately transported in phos-phate-buffered saline (PBS) supplemented with aprotinin (6 mg/100 mL) and 10 mM ethylenediaminetetraacetic acid on ice, the gross fat was dissected away, and the arteries were incubated in 0.5% chlorine for 1 h. Using aseptic techniques, the arteries were then placed in 80% ethanol for a minimum of 72 h at room temperature and subsequently treated with 0.5M NaOH for 60 min with sonication at 60°C, followed by two 30-min room temperature washes in PBS (pH 7.4). The extracted PEBs were then stored in fresh PBS, autoclaved at 121°C for 15 min, and stored at 4°C in PBS.

For testing, the PEBs were labeled and cut longitudinally in half. The treated half of each conduit was incubated in 100 mL of 0.1M AlCl$_3$ in deionized water for 24 h at room temperature on an orbital shaker, whereas the untreated half of PEB was incubated in 100 mL of PBS. The elastin was washed in PBS three times for 30 min each. Disks of 3-mm diameter were cut from both the treated and untreated halves using biopsy punches. The PEB disks were stored in PBS at 4°C until implantation.

Animal study

National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH Publication 85-23 Rev 1985) were observed for all animal experiments. A total of 108 mice were randomized into nine study groups of 12 mice, consisting of three groups [(A) baseline animals of the same age, (B) untreated PEBs, and (C) AlCl$_3$-treated PEBs] at each of three time points (30, 60, or 180 days postimplantation). Each experimental mouse had four subdermal implants of an identical source and treatment, whereas no surgical procedure was performed on the baseline animals.

Six-week-old female BALB/c mice had their hindquarters shaved in preparation for implantation. On the day of implantation, the mice were anesthetized with 1% isoflurane delivered by mask. The right flank surgical area was swabbed with iodine and alcohol and a 16-gauge needle was inserted to create two subdermal pockets. The 3-mm PEB disks were each loaded into an obturator/injector set and a single disk was inserted into each pocket. The animal was repositioned to expose the left flank and the above procedure was repeated.

The animals were returned to their respective cages with food and water *ad libitum*. At the completion of the study, either 30, 60, or 180 days, blood was collected with an ocular bleed and the animal was killed. The experimental disks were exposed and excised. Three disks were trimmed of all extraneous tissue and placed in dry Eppendorf tubes for subsequent calcium and aluminum analysis. The remaining disk was placed in 10% neutral buffered formalin for histological analysis. The baseline animals had dermal tissue from the flank position excised for comparison with the experimental disks.

Elemental analysis

Three disks from each mouse were pooled and prepared for atomic absorption analysis to determine aluminum and

TABLE I
Rank Scores of Calcification and Inflammation of Histology Sections

Score	Calcification	Inflammation
0	No evidence	No evidence
1	Minimal in the outer 1/3 of the implant	Occasionally at the edge
2	Evidence in the middle 1/3 of the implant	Focally outer 1/2 of the implant
3	Partial throughout the implant	Focally inner 1/2 of the implant
4	Complete calcification	Diffuse throughout the implant

calcium contents. The explanted disks and baseline dermal tissues were lyophilized, weighed, and hydrolyzed with $6M$ HCl for 18 h. After evacuation of the HCl with air, the residual was reconstituted with 1 mL of deionized water per dry weight milligram of explanted disk. The aluminum content was determined with graphite furnace atomic absorption with a magnesium nitrate modifier (Pacific Northwest Laboratories, Eugene, OR). The calcium content was determined with flame atomic absorption with 0.9% potassium chloride dilution (1:50, w/w) enabling the cathode tube to detect the calcium (Alexin Analytical Laboratories, Tigard, OR).

The effect of buffers on retention of phosphate during $AlCl_3$ treatment and rinsing was evaluated. PEBs were treated with $AlCl_3$ in PBS or deionized water and subsequently rinsed with either PBS or deionized water. Control PEBs were incubated in either PBS or deionized water alone. The amount of phosphate was determined with inductively coupled plasma atomic emission spectroscopy.

Histological analysis

Histological analysis was performed on one disk from each mouse. Formalin fixed, paraffin-embedded sections (5 μm thick) were stained with Masson's trichrome, Verhoeff van Gieson, hematoxylin & eosin, and von Kossa stains to evaluate matrix preservation, cell infiltration, immunological response, and calcification. Each specimen was blindly ranked for the degree of calcification and inflammatory reaction according to the scores of 0 to 4 in Table I. The number of lymphocytes and macrophages were counted per high-power field (400× magnification). Cell counts were performed in 10 consecutive fields along the graft/tissue interface and were recorded as counts per high-power field. The single averaged value from each animal was then used for statistical analysis.

Macrophage infiltration was confirmed in the 180-day groups by immunostaining with the anti-Mac-3 (BD Biosciences, Franklin Lakes, NJ) primary antibody. Paraffin sections were dewaxed, rehydrated, and treated with 10 mM sodium citrate at 90°C for 10 min for antigen retrieval. Slides were blocked, incubated with anti-Mac-3 (1:100 dilution) for 1 h (37°C), washed, and processed through secondary antibody staining as per a standard avidin-biotin complex kit (Vector Laboratories, Burlingame, CA).

Humoral response

The blood from each mouse was allowed to clot and was centrifuged to extract the serum, which was frozen at −70°C

until used in the enzyme-linked immunosorbent assay (ELISA) for determination of antielastin antibodies. For the ELISA, a 96-well plate was coated with soluble α-elastin (10 mg/mL) for 2 h at room temperature, and subsequently washed and blocked with 30% fetal bovine serum (Gibco, Carlsbad, CA). Serial dilutions of a mouse antielastin serum positive control and experimental serum samples (in triplicate) were incubated for 2 h at room temperature. Plates were then washed six times with PBS/Tween buffer and peroxidase-conjugated goat antimouse immunoglobulin G (Bio-Rad, Hercules CA), diluted 1:1000, was added for 30 min. Plates were washed six times and developed for 5 min with 100 μL of peroxidase substrate per well (tetramethylbenzidine, Microwell; Kirkegaard & Perry Laboratories, Gaithersburg, MD). Coloration was stopped by adding 100 μL of 1M H_3PO_4 per well. The reaction was quantified at 450 nm in an ELISA microplate reader (DiaSorin, Stillwater, MN).

Positive control serum was created by a standard immunization protocol, injecting 100 μL of 1:1 Complete Freund's Adjuvant with porcine α-elastin (547 μg/mL) into the flanks of five BALB/c mice, followed by a peritoneal booster injection at 2 weeks, and serum collection at 5 weeks. Serum from two mice, which developed an antielastin response were used in this study. To normalize the data for each plate, the experimental samples are expressed as percent of positive control.

Because the production of antibodies by BALB/c increases with age, positive and negative antibody reactions were defined. A positive antibody reaction was any ELISA

TABLE II
Amino Acid Analysis for PEBs

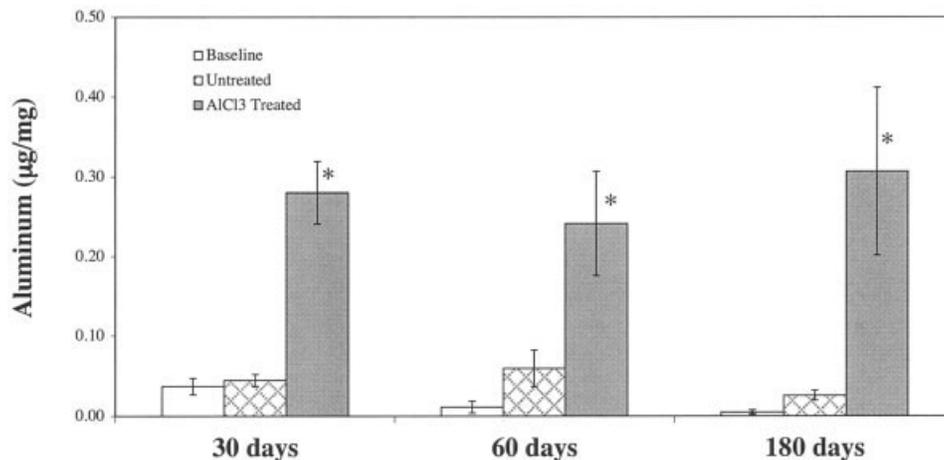| Amino Acid	PEBs	Bovine Neck Ligament Elastin[11]	Porcine Aortic Elastin (Lansing Method)[12]
Hpro	10.7	13.4	11.4
ASP	3.6	6.6	3.5
THR	12.3	7.5	5.2
SER	9.4	8.4	7.6
GLU	18.7	16.1	17.1
PRO	124.7	119.9	122.9
GLY	317.0	344.1	348.5
ALA	235.9	206.7	219.2
VAL	125.0	137.5	130.8
MET	—	2.1	1.0
ILE	21.2	25.5	17.8
LEU	57.5	62.9	53.3
TYR	17.6	8.7	17.6
PHE	33.6	30.2	32.9
HIS	1.0	0.7	0.4
LYS	5.4	2.8	5.5
PECYS	—	0.8	0.2
ARG	6.5	5.9	4.9

Figure 1. The aluminum content of elastin disks at each time point was determined with atomic absorption analysis. At 30, 60, and 180 days, the aluminum amount in the $AlCl_3$-treated elastin disks was significantly larger than the untreated disks and the controls (ANOVA with Tukey B *post hoc*, $p < 0.05$). The amount of aluminum in the implanted $AlCl_3$-treated disks did not change significantly over time (ANOVA with Tukey B *post hoc*, $p < 0.05$).

reading (percent of positive control) that was higher than the average ELISA reading plus two standard deviations of the control mice, which had no implants. Any reading within the two standard deviations of the control average was considered to be negative for production of antiporcine elastin antibodies.

Statistical analysis

Parametric and nonparametric tests were used to analyze all quantitative data as specified in the text below. Alpha

was set at 0.05 and all values are expressed as the average $\pm$ the standard error of the mean.

RESULTS

Amino acid analysis, indicating high levels of glycine, alanine, valine, and proline residues and the lack of methionine and histidine, confirmed the composition of the PEB biomaterial as at least 95% pure elastin and was similar to published values (Table II).[7,11,12]
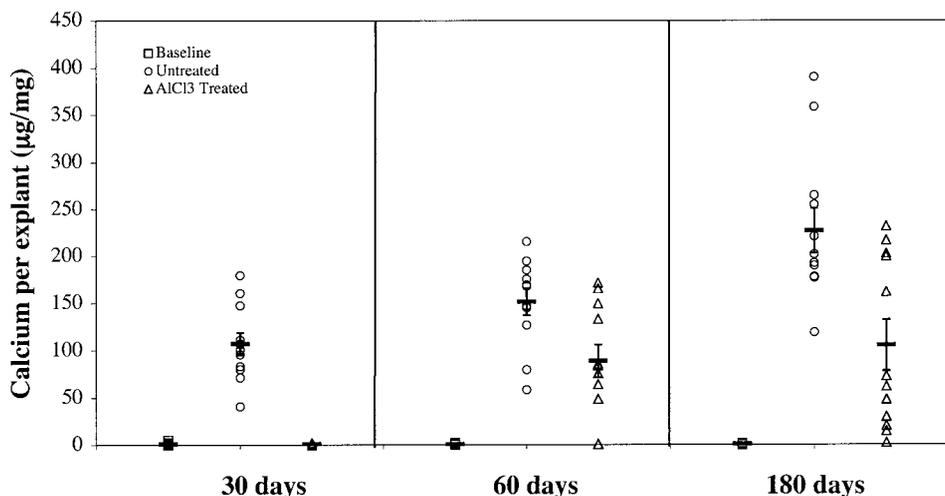

Figure 2. Calcium amounts per dry weight of explanted material were determined with atomic absorption analysis. The untreated disks had significantly more calcium than the baseline dermal tissue and the $AlCl_3$-treated disks (ANOVA, Tamhane *post hoc*, $p < 0.05$). The $AlCl_3$-treated PEBs had significantly more calcium than the baseline dermal tissue at 60 and 180 days (ANOVA, Tamhane *post hoc*, $p < 0.01$). The amount of calcium in the treated PEBs at 60 and 180 days was not significantly different from the untreated PEBs at 30 and 60 days, respectively (*t* test, $p = 0.371, 0.138$), suggesting a delay in the calcification. For the $AlCl_3$-treated PEBs, the calcium content did significantly increase from 30 to 60 days and 30 to 180 days (ANOVA, Tamhane *post hoc*, $p < 0.01$), whereas for the untreated PEBs, the calcium content did significantly increase from 30 to 180 days and 60 to 180 days (ANOVA, Bonferroni *post hoc*, $p < 0.05$).
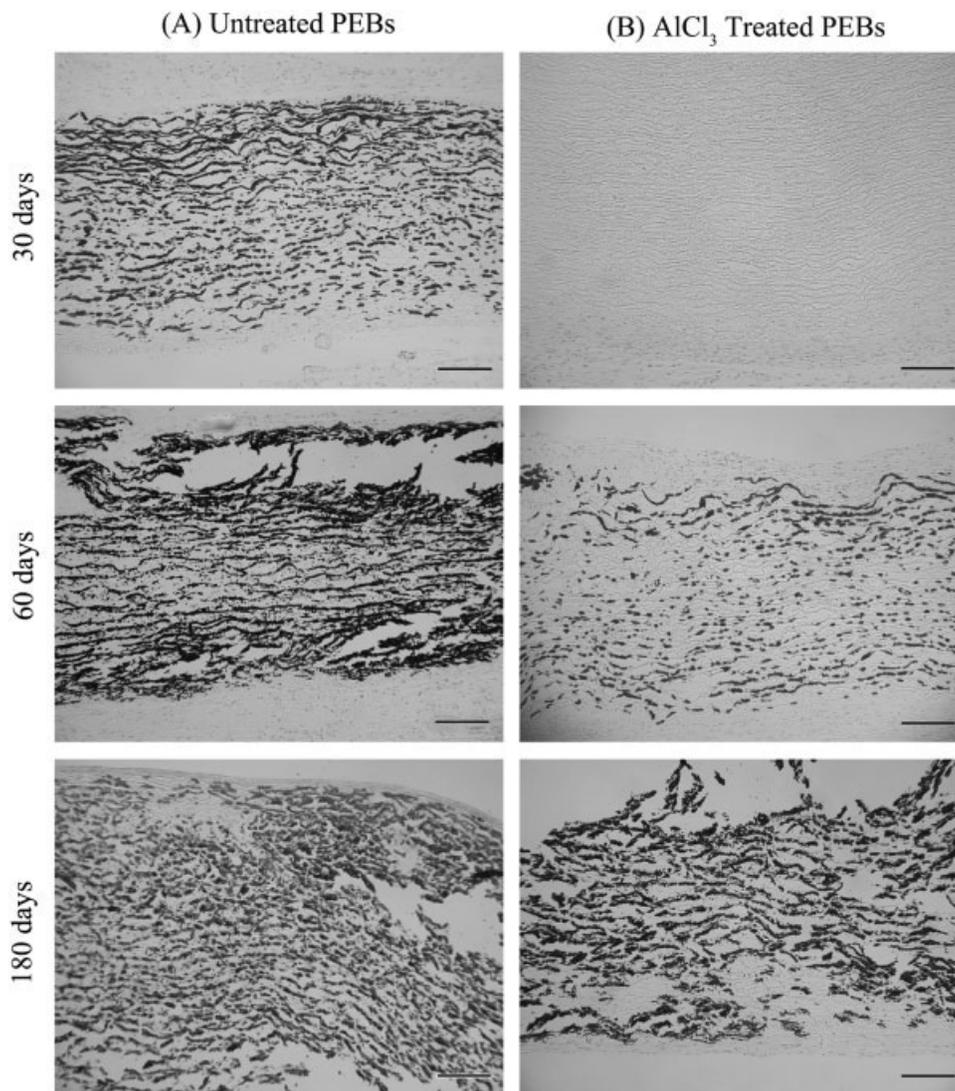
Figure 3. Representative histology images of the calcification of both the (A) untreated PEBs and (B) AlCl$_3$-treated PEBs at 30, 60, and 180 days. The mean scores for the elastin disks were 3.5, 4, and 4 at 30, 60, and 180 days, respectively. The mean scores for the AlCl$_3$-treated elastin disks were 0, 2.5, and 3.5 at 30, 60, and 180 days, respectively (von Kossa stain, original magnification 100×, bar indicates 100 μm). Multiple focal initiation sites of calcification are shown in the untreated PEBs at 30 days and the AlCl$_3$-treated PEBs at 60 days, whereas extensive calcification of the elastin fibers occurred at subsequent time points.

Aluminum analysis

Before implantation, the aluminum amounts were 2.16 ± 0.57 and 0.54 ± 0.18 μg/mg for the AlCl$_3$-treated and untreated materials, respectively. Aluminum content was significantly higher in the AlCl$_3$-treated PEBs compared with the untreated discs at all explantation time points (Fig. 1). The amount of aluminum in the AlCl$_3$-treated PEBs did not change significantly from 30 to 60 to 180 days.

Calcification

The untreated PEB disks had significantly more calcium than the treated disks and age-matched control

dermal tissue samples at each time point (Fig. 2). Calcification was progressive with time, such that untreated disks removed at 180 days had significantly higher calcium values than those removed at 30 or 60 days. The AlCl$_3$-treated PEB disks displayed delayed calcification with virtually no calcium accumulation at 30 days, but substantial accumulation at 60 and 180 days, although the overall accumulation was not as high as untreated specimens. Tissue calcification was both confirmed and localized to the elastin with histology. The median calcification score, according to Table I, for each untreated PEB disk was 3.5, 4.0, and 4.0 at 30, 60, and 180 days, respectively. This was significantly higher than the scores for the AlCl$_3$-treated implants of 0, 2.5, and 3.5 (Wilcoxon's ranked sum test, $W = 79$, 102, 118 and $p = 0.001$, 0.003, 0.021,
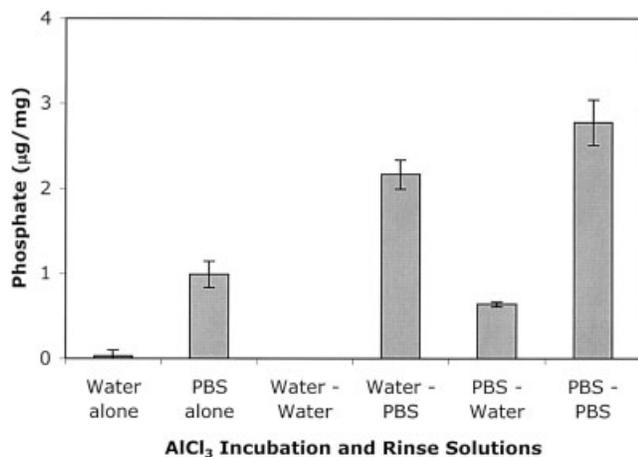
Figure 4. The amount of phosphate in PEBs, which were incubated in either deionized water alone, PBS alone, or 0.1M AlCl$_3$ with deionized water or PBS and then rinsed in either deionized water or PBS. The PBS AlCl$_3$–water rinse treatment group had significantly less phosphate than both the water–PBS and PBS–PBS group, indicating that the PBS was not tightly bound to the PEBs (ANOVA, Bonferroni *post hoc*, $p < 0.05$).

respectively). Representative histology images of the median ranks are shown with von Kossa staining illustrated in Figure 3. The calcification of the AlCl$_3$-treated disks appears at 60 days and is significant by 180 days. Treated samples with minimal calcification show a similar pattern of calcification observed in earlier time points for untreated specimens with multiple focal initiation sites, which with time extend along the entire fiber, but not into the surrounding tissue. Extensively calcified treated samples are virtually identical to the untreated samples at the same time points with the calcification tightly associated with the elastin lamella.

To evaluate the effect of phosphate on the calcification of PEBs, we measured the amount of phosphate given several treatments: untreated PEBs in water and PBS alone, PEBs treated with AlCl$_3$ in deionized water or PBS and then rinsed in deionized water or PBS (Fig. 4). Although significantly more phosphate was present in the treated condition (AlCl$_3$ treatment in deionized water with a PBS rinse), the phosphate was easily disassociated from the PEBs with the deionized water rinse and thus was not tightly bound to the PEBs.

Inflammatory reaction

The median inflammatory reaction rank of the untreated PEB disks was 2 on a scale of 0 to 4 (Table I) for each time point (Table III). The AlCl$_3$-treated disks had a higher initial score of 3 at 30 days, which decreased to 1 at 60 days, and was 2 at 180 days, whereas the untreated PEB disks had consistent median scores of 2 for all time points. There was no significant difference between the inflammation scores of the untreated and treated disks (Wilcoxon's ranked sum test). Overall there was a mild reaction to all of the implants (Fig. 5) with the cellular reaction limited to the edge of the implant. There was no evidence of increased cellularity surrounding the implants and minimal cells within the PEB implants. There were only isolated incidences of a single giant cell at the cut edge of the PEB. Most of the implants were surrounded by a thin collagen layer.

The number of lymphocytes and macrophages per high-powered field of view was counted for each implant (Table IV). The AlCl$_3$-treated PEBs had significantly more lymphocytes (t test, $p < 0.05$) and a trend toward more macrophages after 180 days of implantation compared with the untreated disks, otherwise the cellular reaction was equivalent. The number of lymphocytes surrounding the AlCl$_3$-treated disks significantly increased from 60 to 180 days [analysis of variance (ANOVA) with Tukey HSD *post hoc*, $p < 0.05$]. The number of macrophages did not significantly change over time for either of the groups. The cellular reaction to the untreated PEBs remained at the periphery of the implant, whereas the AlCl$_3$-treated elastin had deeper infiltration of cells into the disk (Fig. 5).

Humoral response

Characterization of the elastin-specific humoral response by ELISA revealed 10/36 responders in the untreated and 11/36 in the treated group (combined values for all time points). Using defined criteria, each mouse was described as having either a positive or negative antibody reaction. The percent of positive reactions for each group and time point are indicated in Figure 6. There was no significant difference in the number of positive reactions between the untreated and treated groups at any time point.

DISCUSSION

Ectopic calcification occurs in many pathological diseases, such as atherosclerosis,[13] and can be a signif-

TABLE III
Median Values of Inflammation Rank Scores With Statistics

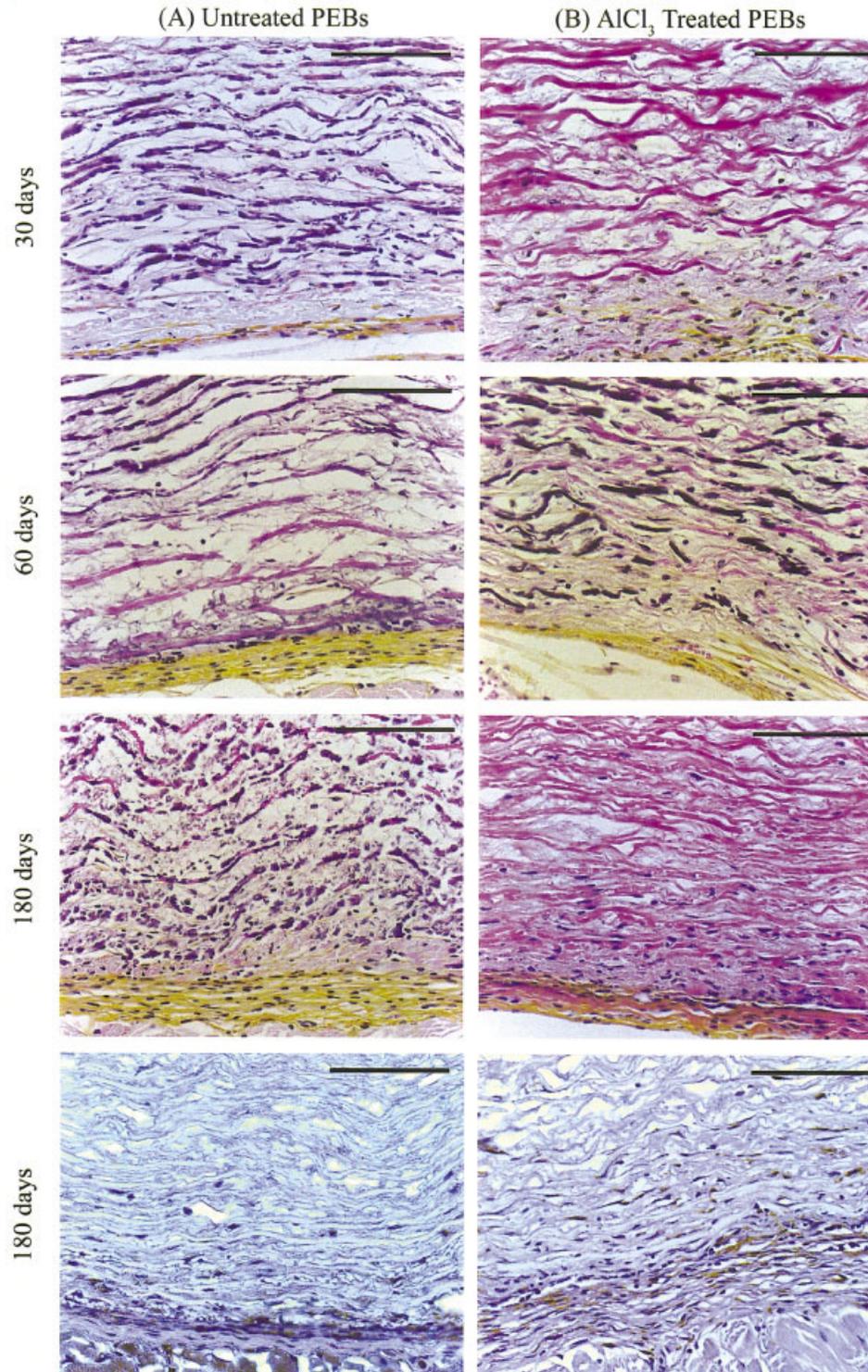| Time (Days)	Untreated PEB	AlCl$_3$ Treated	Wilcoxon W	p Values
30	2	3	135	0.373
60	2	1	135	0.371
180	2	2	138	0.442

Figure 5. Representative histology images of the cellular reaction to the untreated and AlCl$_3$-treated elastin disks at 30, 60, and 180 days. The mean scores for the elastin disks were 2, 2, and 2 at 30, 60, and 180 days, respectively. The mean scores for the AlCl$_3$-treated disks were 3, 1, and 2 at 30, 60, and 180 days, respectively. The cell infiltration was limited to the edges of the implants with no increase in cellularity surrounding the implants. Macrophages remained at the periphery of the untreated PEBs, but penetrated deeper into the AlCl$_3$-treated PEB disks [hematoxylin phloxine saffron (HPS), original magnification 400×, bar indicates 100 μm].

TABLE IV
Number of Inflammatory Cells per High-Magnification Field of View

Time (Days)	Macrophages			Lymphocytes		
	Untreated	AlCl₃ Treated	p Values	Untreated	AlCl₃ Treated	p Values
30	11.42 ± 2.41	13.83 ± 2.84	0.654	10.75 ± 3.04	9.17 ± 2.00	0.505
60	16.00 ± 4.38	7.33 ± 2.98	0.493	9.75 ± 3.65	6.75 ± 2.62	0.103
180	8.58 ± 1.82	19.67 ± 3.94	0.045	6.08 ± 1.00	13.58 ± 3.38	0.014

icant complication of bioprosthetic implants.[14] Although elastin is a primary constituent of cardiovascular tissues, its precise role in pathological vascular calcification remains speculative, perhaps because of the multifactorial nature of the disease processes involved.[13,15,16] Purified elastin, however, calcifies very rapidly in animal models. Our PEB implants, which are at least 95% pure elastin, were heavily calcified as early as 30 days (107.1 ± 11.8 μg/mg) after subdermal implantation in the adult mouse model. These results are consistent with previous work on bovine elastin implanted in weanling rat models, where implant calcium levels reached 89.73 ± 9.84 μg/mg after 21 days.[7] The morphology of the mineralization was similar in both studies with multiple initiation sites leading to calcium crystal formation, which was spatially associated with elastin fibers. Thus, our results suggest that purified porcine arterial elastin calcifies at a similar rate and by a similar mechanism as does bovine nuchal elastin. The relative lack of calcification in the normal vasculature suggests that the calcification of elastin implants may result from immune reactions elicited in response to the xenogenic purified elastin or alternatively from the lack or extraction of endogenous inhibitors of vascular calcification. The latter hypothesis is supported by work in a number of knockout mouse models and in models of warfarin inhibition. These studies display extensive vascular calcification indicating the presence of potent endogenous inhibitors within the normal artery wall.[17–19] However, inflammatory responses are a characteristic of all implants and may modify the elastin's susceptibility to calcification. Although the PEBs produced a modest humoral response (approximately 30% of all animals showing a positive antibody titer), recruitment of inflammatory cells to the subdermal PEB implants was evident at all time points. No degradation of the elastin fibers was histologically apparent; however, it remains possible that inflammatory proteases may have produced more minor alteration to the fiber surface, and thus the precise role of inflammation in PEB calcification remains speculative.

There is a building impetus to use mouse models to study implant biocompatibility because this species allows for the use of knockout or null animals[20] and thus may aide in the design of more mechanistic stud-
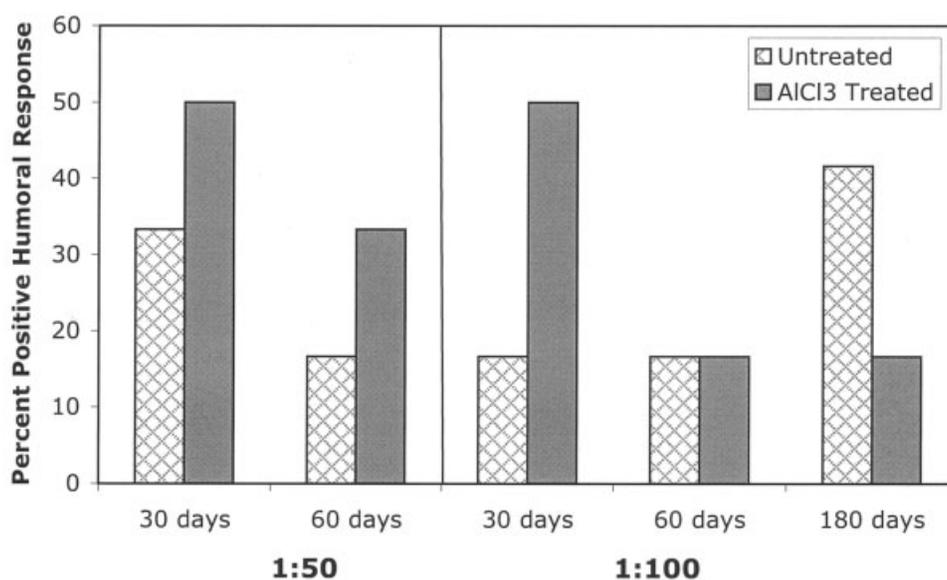

Figure 6. Percent of positive humoral reactions determined with the ELISA assay. There was no significant difference between the humoral response to the untreated and treated elastin at any time point (Pearson χ^2 test). Yet, mice with the AlCl₃-treated PEBs tended to have more positive reactions at 30 and 60 days, whereas the mice with untreated PEBs had higher antibody response at 180 days.

ies. It is clear that the species, implant site, and animal age all influence the biological response to an implanted material.[21–23] Khouw et al. compared the foreign body reaction to subcutaneously implanted hexamethylenediisocyanate crosslinked dermal sheep collagen between several strains of adult rats and mice demonstrating that mice produced more calcification than the rat strains, yet displayed a less-severe cell-mediated immune response (fewer giant cells and less phagocytosis of the implanted material).[23] The age of the recipient animal can also have a significant role. Young animals such as the weanling rats have been used for their robust calcification responses, yet cell-mediated immune responses in such young animals have been demonstrated to be more transient in nature.[21] The adult BALB/c mouse used in the current study produced robust calcification and prolonged cell-mediated immunity and thus appears to be an acceptable model for biocompatibility testing.

Webb et al. developed the use of $AlCl_3$ pretreatment to prevent calcification of bioprosthetic materials. When applied to glutaraldehyde crosslinked bovine pericardium[24,25] or fresh aortic wall allografts,[6,26] $AlCl_3$ treatment significantly reduced calcification for up to 120 days in the weanling rat subdermal model, although it did not prevent it. The morphology of calcification was similar in both materials with the initial calcification of cells and followed by the extracellular matrix. The aluminum, localized to the devitalized cells and not the collagen and elastin, had a concentration of at least 13.4 ± 1.8 nmol/mg (or 1.79 ± 0.24 µg/mg) in the implanted tissue to prevent calcification. Our results do not have this threshold effect of aluminum concentration with aluminum levels unchanged from 30 to 180 days, while calcification occurring at 60 and 180 days. Vyavahare et al.,[7] applied $AlCl_3$ treatment to purified bovine nuchal elastin and showed complete inhibition of calcification for 21 days in a weanling rat subdermal implantation model. Our data are consistent with these results, in that the $AlCl_3$ treatment inhibited the calcification of PEBs at 30 days; however, at longer time points, 60 and 180 days, we discovered a dramatic increase in the calcification of the $AlCl_3$-treated PEBs. The amount of calcium in our treated PEB at 60 days is similar to that previously reported for untreated elastin implanted 21 days (89.73 ± 9.84 µg/mg) in the weanling rat model.[7] The $AlCl_3$ treatment used in this study was very similar to those previously reported; however, we did use a PBS wash before implantation, which resulted in a modest reversible binding of phosphate to the PEB (Fig. 4). Although phosphate is strongly associated with *in vivo* calcification,[27,28] significant phosphate levels exist in normal animals,[29] thus long-term implantation of the PEBs would produce substantial phosphate exposure and would very likely outweigh any brief *in vitro* exposure. Taken together, these results suggest that

$AlCl_3$ treatment simply produces a modest delay (approximately 1 month) in the onset of calcification; however, once initiated, the rate of calcification, at least over the first 30-day period, is similar in treated and untreated elastin (with calcium accumulating at 3.6 µg/mg/day from 0 to 30 days in untreated and 2.9 µg/mg/day from 30 to 60 days in treated samples).

$AlCl_3$ treatment produced a more pronounced cell-mediated immune response. This was particularly evident at 180 days, where significantly more macrophages and lymphocytes had infiltrated the treated PEBs. At 60 and 180 days, there was a negative correlation ($r = -0.625$, -0.659 and $p = 0.03$, 0.02, respectively) between macrophage infiltration and calcification in these $AlCl_3$-treated implants suggesting that macrophages may be involved in modifying the implants' susceptibility to calcification.

CONCLUSIONS

PEBs calcify within 30 days in the subdermal adult mouse model, yet display minimal cellular and humoral reactions after prolonged implantation. Treatment of these biomaterials with $AlCl_3$ delays but does not inhibit calcification. Treated PEBs also develop a significant cell-mediated immune response after prolonged implantation. We conclude that $AlCl_3$ is not the optimal treatment for inhibition of calcification in elastin-based biomaterials

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