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Inhibition of Mineralization of Glutaraldehyde-pretreated Bovine Pericardium by $AlCl_3$

Mechanisms and Comparisons with $FeCl_3$, $LaCl_3$, and $Ga(NO_3)_3$ in Rat Subdermal Model Studies

Catherine L. Webb,* Frederick J. Schoen,† William E. Flowers,* Allen C. Alfrey,‡ Cynthia Horton,* and Robert J. Levy*

From the Section of Pediatric Cardiology, Department of Pediatrics, University of Michigan Medical Center, Ann Arbor, Michigan; the Department of Pathology,† Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts; and the Renal Section,‡ Denver Veterans Administration Hospital, and the Division of Renal Medicine, University of Colorado Medical Center, Denver, Colorado*

In the present study, the authors investigated the mechanism by which Al^{3+} preincubations inhibited the pathologic calcification of glutaraldehyde-pretreated bovine pericardium (GPBP) implanted subdermally in rats. The concentration dependency of the Al^{3+} anticalcification effect was compared with that of other trivalent metal ions (Fe^{3+}, Ga^{3+}, La^{3+}) known to interact with calcium phosphates. In vitro ***incubations of GPBP were carried out in $AlCl_3$ (10^{-3} mol/l [molar] to 10^{-1} mol/l) to ascertain both the optimal conditions for uptake of Al^{3+} and the time course of Al^{3+} dissociation. Al^{3+} uptake by GPBP was concentration dependent and occurred rapidly, with tissue levels after 1 hour not differing significantly from those after 72 hours of incubation. Analyses of GPBP samples preincubated in $AlCl_3$ (0.1 mol/l, 24 hours) showed that more than 75% of the Al^{3+} remained tightly bound after 60 days'*** in vitro ***release at 37°C, pH 7.4. Preincubations of GPBP in $AlCl_3$ significantly inhibited calcification after subdermal implantation in rats for 60 days (Ca^{++} = 5.1 ± 0.9 μg/mg, 11.5 ± 4.6 μg/mg, 70.3 ± 23.0 μg/mg, mean ± standard error [SE], for 10^{-1} mol/l, 10^{-2} mol/l, 10^{-3} mol/l $AlCl_3$, respectively), compared with controls (Ca^{++} = 110.0 ± 9.3 μg/mg). All animals were free of Al^{3+}-mediated adverse effects on bone, as determined by light microscopic evaluation of femoral epiphyseal growth plates. Transmission electron microscopy coupled with electron energy loss spectroscopy (EELS) of GPBP incubated in 10^{-1} mol/l $AlCl_3$ for 24 hours demonstrated discrete Al^{3+} localization in the sarcolemma and cytoplasmic and nuclear membranes of devitalized pericardial connective tissue cells at intracellular sites coincident with phosphorus loci. Similar intracellular localization remained prominent in explants removed after 60 days; no calcific deposits were noted in these specimens. Preincubations in Fe^{3+} but not Ga^{3+} and La^{3+} solutions yielded significant inhibition of GPBP calcification, which did not differ significantly from that provided by Al^{3+} and had a comparable concentration dependency. Light microscopic examination (Prussian blue staining) and EELS of $FeCl_3$-preincubated explants demonstrated Fe^{3+} localization within devitalized GPBP connective tissue cells. The authors conclude that Al^{3+} and Fe^{3+} significantly inhibit the pathologic mineralization of glutaraldehyde-pretreated bovine pericardium by mechanisms that are likely related to the high affinity of these cations for membrane-associated and other intracellular phosphorus loci. (Am J Pathol 1991, 138:971–981)***

Dystrophic calcification frequently causes the clinical failure of bioprosthetic heart valves fabricated from glutaral-

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Address reprints requests to Robert J. Levy, MD R-5014 Kresge II, Pediatric Cardiology Research Laboratory, University of Michigan Medical Center, Ann Arbor, MI 48109-0576.

dehyde-pretreated porcine aortic valves or bovine pericardium.[1–3] Recent work from our laboratory has demonstrated that the dystrophic calcification of subdermal implants of pericardial bioprosthetic tissue (GPBP) may be inhibited by preincubating the tissue in dilute solutions of $AlCl_3$.[4] The use of Al^{3+} was stimulated by observations that some patients undergoing chronic hemodialysis for renal failure developed osteomalacia due to skeletal deposition of Al^{3+},[5–7] mainly caused by trace level Al^{3+} contamination of dialysate or intravenous tubing.[7]

The present study was carried out to understand the mechanisms of Al^{3+}-mediated inhibition of the dystrophic calcification of rat subdermal implants of glutaraldehyde-pretreated bovine pericardium. The time course for uptake of Al^{3+} by GPBP was characterized, as was the *in vitro* dissociation of Al^{3+} from GPBP at pH 7.4 at 37°C. In addition, the tissue localization of Al^{3+} was assessed by light and electron microscopic techniques. The concentration dependency of Al^{3+} inhibition of GPBP calcification in the rat subdermal model was studied in comparison with that of other metallic cations known to interfere with calcium-phosphate formation, including Fe^{3+}, Ga^{3+}, and La^{3+}.[8–10] The possibility of adverse skeletal effects in rats with implants due to metallic ion exposure was also evaluated.

Methods

Materials and In Vitro *Incubations*

Fresh mature bovine parietal pericardium was cross-linked for 24 hours in 0.6% glutaraldehyde (Polysciences, Warrington, PA) at pH 7.4 (0.05 mol/l [molar] HEPES buffer) at 4°C, then stored in 0.2% glutaraldehyde in the same buffer at 4°C.[11–13] Using a #7 cork borer, the glutaraldehyde-pretreated bovine pericardium was cut into 1-cm diameter discs, rinsed free of glutaraldehyde with sterile saline, and preincubated in solutions of $AlCl_3$ (Analytic Reagent Grade, ACS, Mallinckrodt, St. Louis, MO) at concentrations of 10^{-1} mol/l, 10^{-2} mol/l, and 10^{-3} mol/l (pH 3.9–5.6) for 24 hours at 25°C.

Control specimens of GPBP were preincubated in either 0.05 mol/l HEPES buffer (Sigma Chemical Co., St. Louis, MO) for 24 hours at 25°C, pH 7.4. or in 10^{-3} mol/l HCl, pH 3.6, to approximate the pH of the $AlCl_3$ solutions. GPBP preincubated in $AlCl_3$ or control solutions for durations ranging from 1 hour to 3 days were retrieved and analyzed for Al^{3+} (see below). To study the dissociation of Al^{3+}, samples of preincubated GPBP were placed in either 0.05 mol/l potassium phosphate or 0.05 mol/l HEPES (pH 7.4, 37°C) under perfect sink conditions. GPBP samples were removed and periodically assayed (see below). GPBP specimens were also preincubated in other metallic salt solutions for use in comparative subdermal implant studies, including $FeCl_3$ (Sigma) (10^{-1} mol/l, 10^{-2} mol/l, 10^{-3} mol/l), $FeCl_3$ (10^{-1} mol/l) plus sodium citrate (Mallinkrodt) (10^{-1} mol/l), $LaCl_3$ (10^{-1} mol/l), and $Ga(NO_3)_3$ (Sigma) (10^{-1} mol/l).

Implantation and Retrieval Techniques

Using ketamine (Park-Davis, Morris Plains, NJ) (0.057 mg/g) and xylazine (Haver, Shawnee, KA) (0.008 mg/g) anesthesia, as previously described,[11–13] GPBP were implanted in male, weanling rats (50 to 60 g, CD, Sprague Dawley, Charles River Laboratories, Burlington, Massachusetts) in subcutaneous pouches dissected in the ventral abdominal wall. Each animal received two separate subdermal implants. To examine the skeletal effects of toxic aluminum doses, one group of experimental animals received GPBP implants that had not been pretreated with $AlCl_3$, but each animal was administered aqueous $AlCl_3$ (15 mg/kg/day) by a daily subcutaneous injection for 21 days.

After 21 and 60 days, subgroups of rats were euthanized with an overdose of pentobarbital (80 mg/kg) and the GPBP samples explanted. A representative thin cross-sectioned strip of each specimen was fixed for morphologic studies (see below). Approximately one half of each explant was exhaustively washed with 0.9% NaCl and deionized water, freeze dried, minced, and prepared for calcium analysis by atomic absorption spectroscopy.[11,12] The remainder was immediately lyophylized, minced, and prepared for Al^{3+} analysis using flameless atomic absorption spectroscopy[14] for explanted specimens, or neutron activation analysis[15] for GPBP samples derived from *in vitro* studies.

Additional control GPBP specimens for transmission electron microscopy-electron energy loss spectroscopy studies (see below) were implanted subdermally in the rat, and removed after 24 and 48 hours.

Morphologic Methods

Light Microscopy

Representative samples of GPBP were placed in Karnovsky's fixative (cacodylate buffered 2.5% glutaraldehyde, 2% paraformaldehyde at pH 7.2).[11,12,16] Femurs obtained at death from each group were dissected free of attached muscle and fixed in 10% neutral buffered formalin. Undecalcified specimens of both GPBP and

bone were embedded in JB-4 glycolmethacrylate medium (Polysciences) and sectioned to 2 μ.

Sections 2 to 3 μ thick were stained with hematoxylin and eosin (H&E) for overall morphology and von Kossa stain for calcium phosphates, as previously described[11–13]; staining for aluminum was done using the aluminon procedure.[17–19] Briefly aluminon buffer was prepared by mixing equal volumes of 5 mol/l NH_4Cl and 5 mol/l NH_4HCO_3, adjusting the pH to 5.2 with 6 N HCl. A 2% solution of Aluminon was made by first dissolving the ammonium salt of aurine tricarboxcylic acid in a small volume of Aluminon buffer by heating to 75°C for 15 minutes to bring the partially dissolved reagent into complete solution, which was filtered immediately while hot. The differentiating solution was prepared by adjusting the Aluminon buffer to pH 7.2 with 1.6 mol/l NH_4HCO_3. Cut sections were stained in buffered Aluminon heated in a water bath to 45 to 48°C for 20 to 30 minutes. Stained sections were briefly rinsed in distilled water, differentiated by a brief rinse for 3 to 5 seconds in the NH_4HCO_3 solution, rinsed in distilled water for 1 minute, and counterstained with dilute aniline blue for 2 minutes. The sections were then further rinsed in distilled water and 1% acetic acid for 1 minute each, dehydrated, and mounted in Permount (Fisher Scientific, New York, NY). In sections stained by this method, aluminum hydroxide appeared bright red, whereas cytoplasm and nuclei appeared blue. Bone from a patient with aluminum-related hemodialysis osteopathy was provided as a positive control by Peter A. Dervan, MD (Dublin, Ireland).

Electron Microscopy

For transmission electron microscopy with electron energy loss spectroscopy (EELS), Karnovsky's solution-fixed GPBP specimens were dehydrated after 24 hours in graded concentrations of ethanol to absolute, and stored in this solution.[13] After solvent exchange to propylene oxide, they were embedded in Araldite and sectioned with an ultramicrotome. Ultrathin sections (30 to 40 nm) exhibiting a dark gray to transparent interference color in distilled water were retrieved immediately after sectioning and placed on 600-mesh grids. Unstained sections were examined in a Zeiss EM 902 transmission electron microscope (Oberkochen, FRG) containing a spectrometer that analyzes electrons for energy-selected imaging. Other applications have been described.[20,21]

Briefly, electrons traversing the specimen were dispersed by the magnetic field of the analyzer into a spectrum of energies. The spectrum generally consisted of an electron distribution in which the intensity decreases continuously with increasing energy loss. Superimposed on this decreasing intensity were characteristic stepwise increases in intensity that correspond to electron absorption of ionization edges of elements in the specimen. A particular energy region in the spectrum was selected by a slit system in the column of the microscope below the analyzer. The spatial distribution of the element of interest superimposed on the information in the continuum of the spectrum was contained in an electron spectroscopic image (ESI) taken with an energy loss just greater than a particular absorption edge. This was compared with a reference ESI taken at an energy loss just below the selected absorption edge. The difference between the two images represented the two-dimensional map of the element. The specific energy loss levels (Δe) used in this study were 55 to 75 eV for aluminum, 110 to 140 eV (L2, 3 = 132.2) for phosphorus, and 320 to 360 eV (L2, 3 = 346.4) for calcium.

Results

Al^{3+} Binding to GPBP: Uptake and Dissociation

Al^{3+} uptake was rapid and dependent on the concentration of the incubation solution (Table 1). After incubation in a 10^{-1} mol/l $AlCl_3$ solution, uptake was significantly greater than that noted in either 10^{-2} mol/l or 10^{-3} mol/l $AlCl_3$, but Al^{3+} uptake after 1 hour was not significantly different from that after 3 days. Increased uptake with increasing concentration was observed for all incubation durations studied, indicating rapid diffusion of Al^{3+} throughout the GPBP tissue, and with a relatively high binding affinity.

Al^{3+} was relatively tightly bound in an apparently poorly soluble form. Dissociation of Al^{3+} from GPBP was studied comparing incubations in both HEPES and potassium phosphate buffers (pH 7.4). Potassium phosphate incubation might have been expected to have retarded dissociation due to the formation of aluminum phosphates. However, Figure 1 demonstrates that minimal dissociation of Al^{3+} took place after 60 days of *in vitro* incubation in either buffer. Furthermore, because phosphate incubations did not further enhance the high level of Al^{3+} binding, the poorly soluble aluminum salts

Table 1. *Al^{3+} Uptake by Pericardial Bioprosthetic Tissue In Vitro (25°C)*

Duration of preincubation	Preincubation $AlCl_3$ Concentration		
	10^{-1} mol/l	10^{-2} mol/l	10^{-3} mol/l
	[Al^{3+} Content (nmol/l/mg)]*		
1 hour	351.4 ± 15.8	96.4 ± 17.4	24.6 ± 4.9
24 hours	334.6 ± 56.3	98.0 ± 14.7	52.5 ± 3.3
3 days	394.5 ± 48.0	105.3 ± 2.3	40.0 ± 8.8

* Mean ± Standard Error.

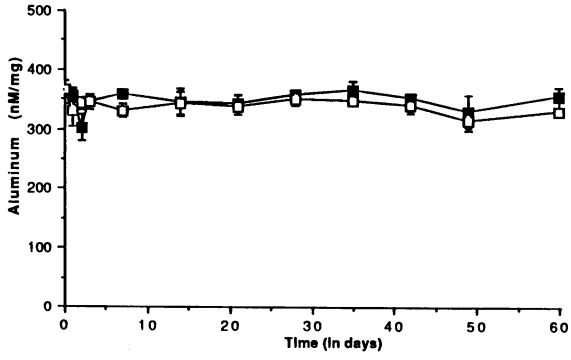

Figure 1. In vitro *dissociation of Al^{3+} from GPBP expressed as residual Al determined by neutron activation. Incubations at 37°C, pH 7.4, 0.05 mol/l HEPES (■) or 0.05 mol/l KH_2PO4 (□) resulted in comparatively little loss of Al after 60 days.*

formed could either be aluminum phosphates or other less soluble compounds.

Inhibition of Dystrophic Calcification by $AlCl_3$

Pretreatment by $AlCl_3$ in aqueous solutions of 10^{-1} mol/l and 10^{-2} mol/l significantly inhibited GPBP calcification compared with controls after both 21 and 60 days (Figure 2). After 21 days' implantation, all three pretreatment concentrations (10^{-1} mol/l, 10^{-2} mol/l, and 10^{-3} mol/l $AlCl_3$) significantly inhibited GPBP calcification compared with controls. Although explanted GPBP exposed to the higher Al^{3+} pretreatment concentrations had virtual complete inhibition of GPBP calcification (Figure 2) after 60 days implantation', GPBP pretreated with 10^{-3} mol/l $AlCl_3$ contained less than 70% of control Ca^{++} (Figure 2). Morphologic studies confirmed the reduction in calcification due to the $AlCl_3$ preincubations (Figure 3). Furthermore, calcified 60-day implants that had been pretreated with 10^{-3} mol/l $AlCl_3$ had Al^{3+} levels of 3.01 ± 0.01 nm/mg (Figure 4), in comparison with the implants pretreated with 10^{-2} mol/l $AlCl_3$, which were not calcified and had Al^{3+} levels of 13.2 ± 0.2 nm/mg.

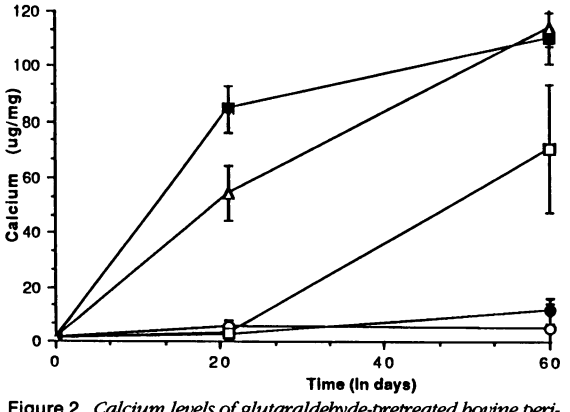

Figure 2. *Calcium levels of glutaraldehyde-pretreated bovine pericardium before and after 21- and 60-day rat subcutaneous implants. Experimental groups were preincubated in $AlCl_3$, 10^{-1} mol/l (○), 10^{-2} mol/l (●), 10^{-3} mol/l (□) for 24 hours at 25 °C. The control groups were preincubated in either 0.05 mol/l HEPES buffer (■) or 10^{-3} mol/l HCl (△) for 24 hours at 25 °C. Significant inhibition of calcification occurred in the groups pretreated with 10^{-1} mol/l and 10^{-2} mol/l $AlCl_3$ after both 21- and 60-day implants.*

In the $AlCl_3$ preincubation groups, a reduction in GPBP Al^{3+} content occurred during the interval between implantation and retrieval at 21 and 60 days (Figure 4). Preimplant GPBP Al^{3+} levels were 334.5 ± 25.1 nm/mg, 100 ± 64.1 nm/mg, and 73.5 ± 8.6 after preincubation in 10^{-1} mol/l, 10^{-2} mol/l, and 10^{-3} mol/l $AlCl_3$ solutions, respectively. The relative decrease in Al^{3+} content (70% to 77% of unimplanted levels) in all three experimental groups, after 21 days, implantation was comparatively greater than the decrease in tissue Al^{3+} content that occurred in the interval between 21 and 60 days' implantation (Figure 4), in which only an additional 8% to 16% of the original Al^{3+} content was lost. The greatest relative loss of GPBP Al^{3+} after a 60-day implant (93%) occurred in the experimental group pretreated with 10^{-3} mol/l $AlCl_3$ (Figure 3).

GPBP implanted and removed after 21 days from four animals treated systemically with 15 mg/kg/day $AlCl_3$ calcified comparably with control explants (Ca^{2+} data: 57.2 ± 17.7 μg/mg *versus* 84.6 ± 0.8 μg/mg [$P > 0.1$], control, preincubated at pH 7.4; 54.4 ± 10.2 μg/mg, control, preincubated at pH 3.6). Furthermore this systemic dose of Al^{3+} caused severe bone toxicity (See below and Figure 9), despite the fact that it was not effective for preventing GPBP implant calcification. Thus these results emphasize the localized mechanism of action of Al^{3+} preincubations of GPBP, which inhibited calcification without adverse effects on bone (see below).

Localization of Aluminum After In Vitro *Incubations*

Light and electron microcopy studies demonstrated that Al^{3+} was localized to cells. Aluminon histologic staining[17–19] of sections from each of the groups of unimplanted $AlCl_3$-preincubated GPBP showed Al^{3+} associated with devitalized fibroblasts and vascular wall cells of the GPBP (Figure 5). Electron energy loss spectroscopy of unimplanted GPBP pretreated with 10^{-1} mol/l $AlCl_3$ showed an intrinsic intracellular localization of aluminum ions (Figure 6) superimposed on cytoplasmic and nuclear phosphorus loci, probably mainly associated

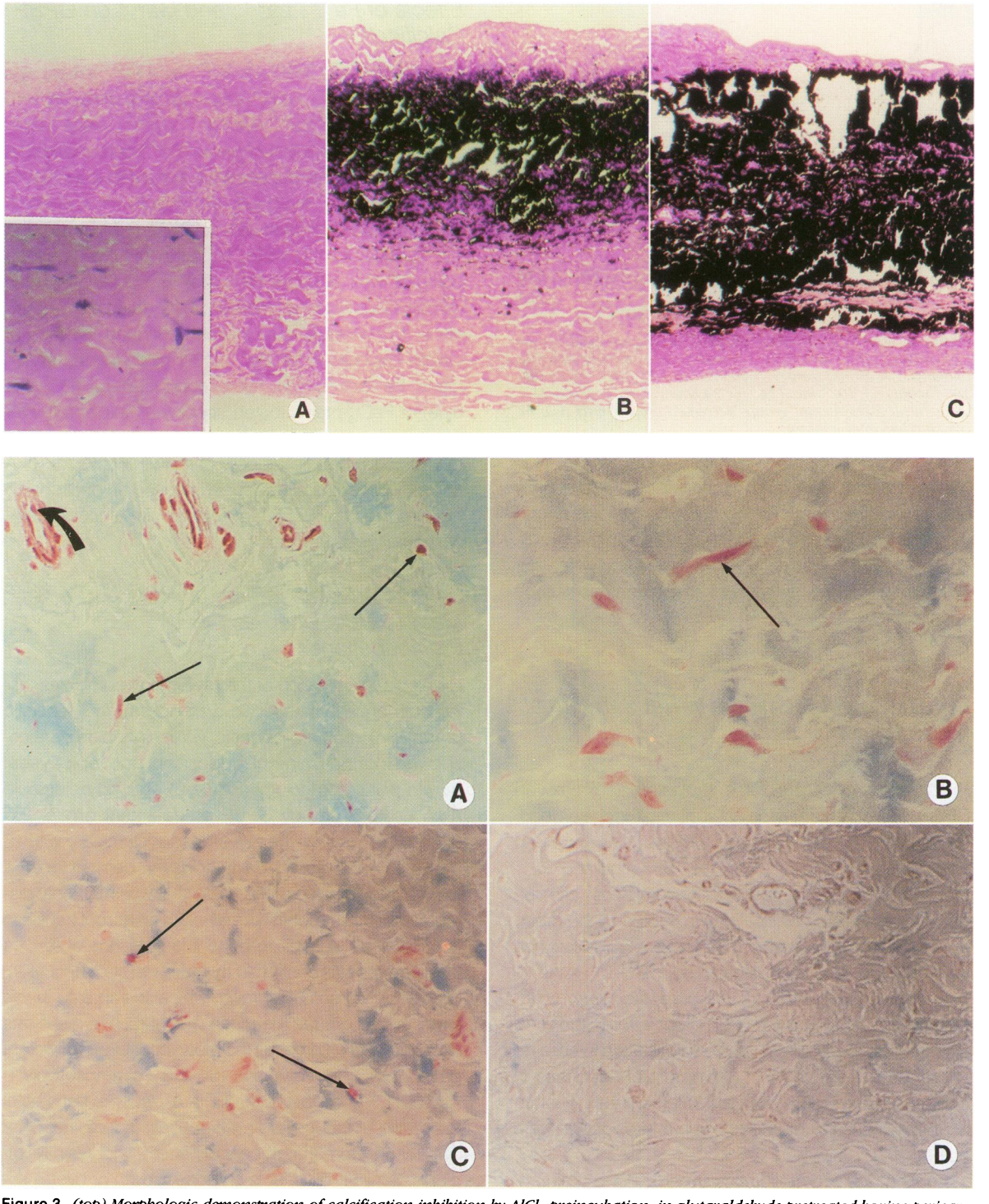

Figure 3. *(top) Morphologic demonstration of calcification inhibition by $AlCl_3$ preincubation, in glutaraldehyde-pretreated bovine pericardium following 60-day subcutaneous implantation in rats.* **A**: *Absence of calcification in specimen preincubated in 10^{-2} mol/l $AlCl_3$. Inset demonstrates normal appearance of pericardial cells and collagen bundles after implantation.* **B**: *Incubation in 10^{-3} mol/l $AlCl_3$; moderate calcification is present.* **C**: *Control implant without $AlCl_3$ incubation; severe mineralization is evident.* **A, B,** *and* **C**: *von Kossa stain (calcium phosphates black); magnification, ×150. Inset in* **A** *H&E; magnification, ×375.*

Figure 5. *(bottom) Light histologic demonstration of aluminum localization in glutaraldehyde-pretreated bovine pericardium, incubated in 10^{-2} mol/l $AlCl_3$, unimplanted and implanted.* **A** *and* **B**: *Unimplanted pretreated pericardium, illustrating cellular localization of aluminum (red) in both interstitial connective tissue cells (arrows) and vascular wall cells, including endothelium (curved arrow).* **C**: *Incubated tissue after 60 days implantation, demonstrating persistent but diminished aluminum staining (red) in connective tissue cells (arrows). Vascular wall cells also retained stain.* **D**: *Control unimplanted pericardium not pretreated with $AlCl_3$, demonstrating lack of staining for aluminum. All stained using the Aluminon technique. Magnification:* **A, C,** *and* **D**, *×375,* **B**, *×600.*

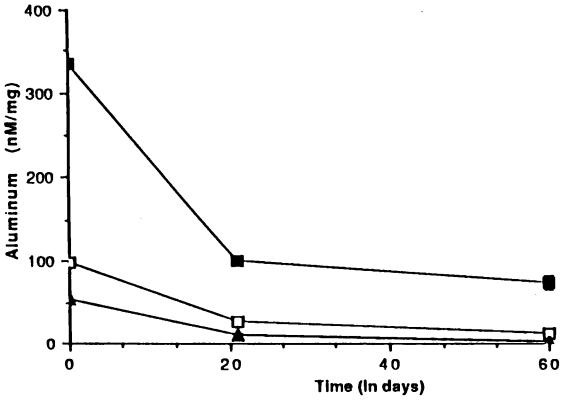

Figure 4. *Aluminum levels of glutaraldehyde-pretreated bovine pericardium before and after 21- and 60-day rat subcutaneous implants. The groups were preincubated in $AlCl_3$, 10^{-1} mol/l (■), 10^{-2} mol/l (□), 10^{-3} mol/l (▲) for 24 hours at 25 °C. Tissue Al^{3+} levels in all experimental groups decreased progressively with increasing length of implant.*

with membranes. Aluminum was localized predominantly within devitalized cells, in the nucleus, cytoplasm, and membranes, in virtually the identical sites as phosphorus. Furthermore Al^{3+} was not noted in association with either collagen or elastin in either light or electron microscopy specimens.

Localization of Aluminum in Rat Subdermal GPBP Explants

Histologic examination of the implanted and removed GPBP showed an absence of calcium phosphate deposition in the Al^{3+}-preincubated GPBP samples explanted after 21 days (Figure 6), and in the 10^{-2} mol/l and 10^{-1} mol/l $AlCl_3$ groups at 60 days. Nevertheless, after 60 days' implantation, calcification was noted in the group that had been pretreated with 10^{-3} mol/l $AlCl_3$. Aluminon staining suggested a relative reduction in Al^{3+} in $AlCl_3$-preincubated explanted tissue compared with unimplanted tissue (Figure 5). EELS results from studies of explanted tissue, however, also demonstrated intense residual localization of Al^{3+} within cellular structures in close opposition to phosphorus loci (Figure 6). Furthermore minimal calcium and no mineral precipitates were noted.

In contrast, control (unimplanted, not aluminum-pretreated) specimens examined by EELS had identical high phosphorus concentrations at membranes and in the nuclei of cells, and demonstrated the formation of early deposits containing coincident loci of calcium and phosphorus after as little as 48 hours' implantation, an interval after which deposits are first noted by conventional transmission electron microscopy[11,12] (Figure 7). No aluminum was noted in these specimens.

Effects of Fe^{3+}, La^{3+}, and Ga^{3+} on GPBP Calcification

Preincubation of GPBP with Fe^{3+} and Fe^{3+}-citrate significantly inhibited GPBP calcification compared with controls (Table 2). La^{3+} and Ga^{3+} did not inhibit calcification of GPBP implants, however. Fe^{3+} demonstrated a concentration-dose dependency comparable to that of Al^{3+}. Fe^{3+} (10^{-1} mol/l) was significantly more effective than were less concentrated solutions (10^{-2} mol/l and 10^{-3} mol/l). Furthermore, although the Fe^{3+}–citrate complex was effective for inhibiting calcification, this combinations was not more inhibitory than Fe^{3+} used alone. Morphologic examination confirmed the lack of calcific deposits in the Fe^{3+} (10^{-1}mol/l)-treated specimens. Fe-specific light microscopic staining (Prussian blue) of $FeCl_3$-preincubated specimens explanted after 21 days demonstrated Fe^{3+} localization within the devitalized connective tissue cells of the GPBP (Figure 8). A cellular localization of iron superimposed on phosphorus loci was noted by EELS (not shown), similar to that demonstrated with aluminum.

Lack of Adverse Effects of $AlCl_3$ and other Metallic Salts Used as GPBP Preincubations

Histologic examination of the rat femoral epiphyses, using both phosphate-specific (von Kossa), Al^{3+}-specific (Aluminon), and Fe-specific (Prussian blue) stains, showed normal mineral deposition and epiphyseal growth plate morphology (Figure 9), with no detectable Al^{3+} or Fe^{3+} in any GPBP pretreatment group. Explanted femurs from the rats treated systemically with 15 mg/kg/day $AlCl_3$ for 21 days, however, demonstrated a femoral growth plate morphology that was markedly disordered compared with normals, with an abrupt transition from cartilage to calcified bone; the presence of Al^{3+} in these femurs was confirmed by Aluminon stain (Figure 9). In addition, rat somatic growth was also impaired in this experimental group. The mean weight gain of these rats was only 79% as great as the mean control group weight gain (130.5 ± 14.4 g *versus* 164 ± 6.2 g, respectively; $P < 0.001$). In contrast, animals in all $AlCl_3$ experimental pretreatment groups had a weight gain that did not differ significantly from that of controls after 21- or 60-day implants. Similarly no effects on bone growth or overall somatic growth were noted to be associated with any of the other metallic preincubation solutions.

Discussion

Mechanisms of Calcification Inhibition

Al^{3+} inhibited pathologic calcification of GPBP in the rat subdermal model after preincubations in dilute solutions

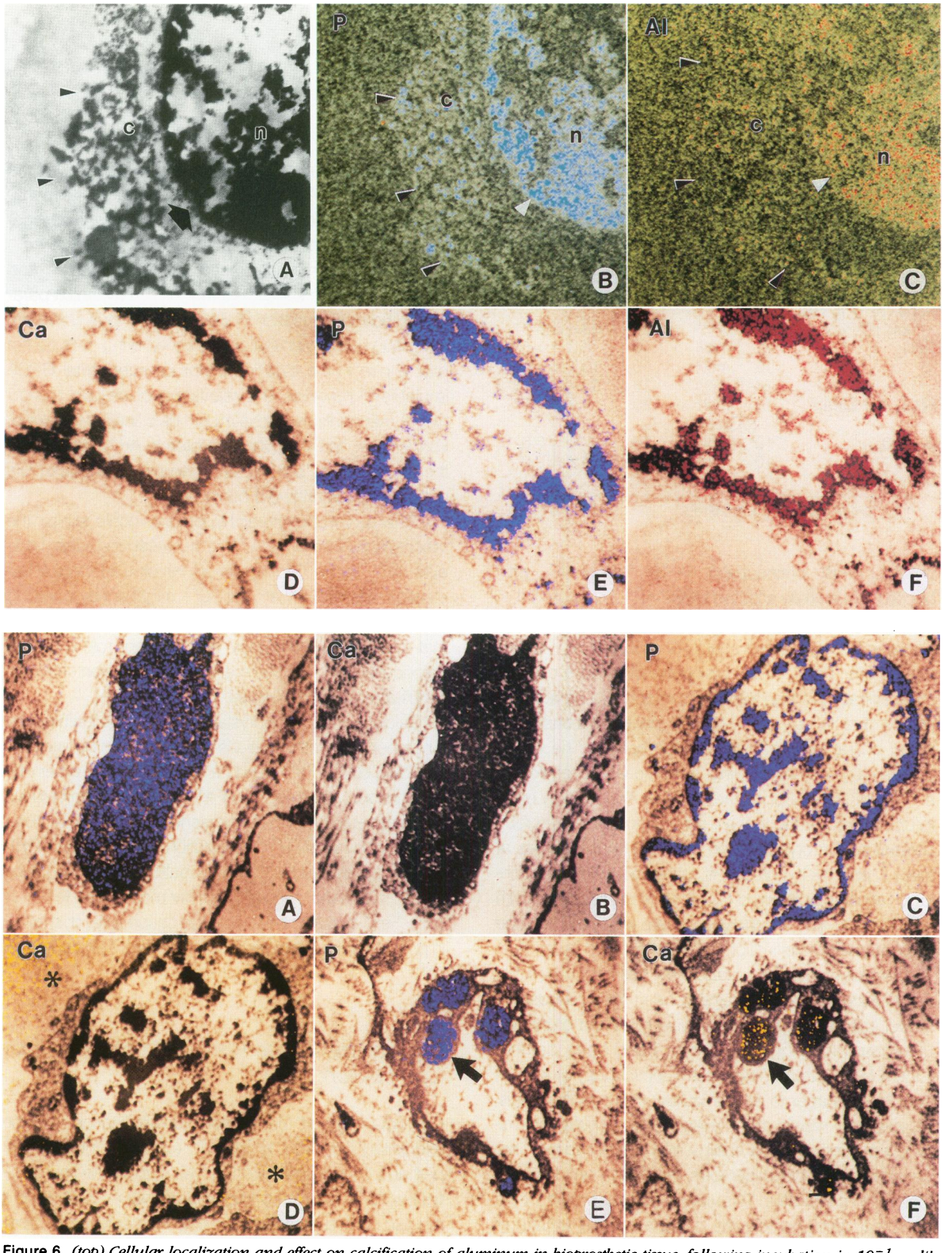

Figure 6. *(top) Cellular localization and effect on calcification of aluminum in bioprosthetic tissue, following incubation in 10^{-1} mol/l $AlCl_3$, in both unimplanted tissue and tissue implanted for 21 days, using transmission electron microscopy with electron energy loss spectroscopy (EELS).* **A**, **B**, *and* **C** *are ultrastructural morphology, map of phosphorus (blue), and map of aluminum (red/orange), respectively, of a cell in the unimplanted tissue. The nucleus (Nu), cytoplasm (Cy), and limiting cell membrane (open arrows) are demonstrated in* **A**. **D**, **E**, *and* **F** *show calcium, phosphorus, and aluminum maps, respectively, of a cell from tissue that was incubated in 10^{-1} mol/l $AlCl_3$ and subsequently implanted subdermally in the rat for 21 days. Unimplanted tissue shows that phosphorus and aluminum are localized to identical sites in the cell, predominantly the nucleus and cytoplasmic membranes. After implantation, aluminum not only remains localized in the tissue to the same sites as phosphorus but the accumulation of calcium (yellow) at these sites is minimal. Magnification, ×10,000.*

Figure 7. *(bottom) Localization of initial precipitation of apatitic mineral in experimental bioprosthetic valve calcification, by electron energy loss spectroscopy (EELS). Elemental maps (phosphorus [blue] and calcium [yellow]) produced using the Zeiss EM 902, of glutaraldehyde-pretreated porcine aortic valve;* **A** *and* **B**: *unimplanted tissue;* **C** *and* **D**, *tissue implanted subdermally in the rat for 24 hours; and* **E** *and* **F**, *tissue implanted for 48 hours. A single cell is shown in each set of photographs. Phosphorus, but not calcium, can be demonstrated in the unimplanted tissue (***A** *and* **B**), *primarily in the nucleus and focally in the cell membrane. After 24 hours' implantation (***C** *and* **D**), *there is accumulation of calcium, focally associated with the nucleus and cytoplasm, as well as diffusely in the extracellular space (asterisks). After 48 hours' implantation (***E** *and* **F**), *there is accumulation of calcium in association with the localized phosphorus to form electron-dense, well-defined precipitates (arrows).*

Table 2. *Effects of Preincubations on GPBP Rat Subdermal Implant Calcification (21 days)*

Salt	Concentration	n	Explant Ca^{2+} (μg/mg) [Mean ± Standard Error]
None (pH 7.4, 0.05 mol/l HEPES)	—	27	63.6 ± 5.7
$FeCl_3$	10^{-1} mol/l	10	6.0 ± 1.5*
	10^{-2} mol/l	10	10.1 ± 4.2*
	10^{-3} mol/l	10	36.4 ± 12.3
$FeCl_3$, Na-Citrate	10^{-1} mol/l (of each)	10	5.9 ± 1.7*
$Ga(NO_3)_3$	10^{-1} mol/l	10	87.4 ± 8.6
$LaCl_3$	10^{-1} mol/l	7	50.2 ± 9.6
Unimplanted	—	10	1.2 ± 0.1

* $P < 0.001$ compared with HEPES.

of $AlCl_3$. GPBP preincubation in 10^{-2} mol/l $AlCl_3$ produced the minimally effective GPBP Al^{3+} levels of 13.4 ± 1.8 nm/mg required for complete inhibition of GPBP calcification throughout the course of a 60-day rat subdermal implant. $FeCl_3$ GPBP preincubations had a comparable efficacy to those with $AlCl_3$. Importantly no adverse effects due to any of the GPBP preincubations were noted on rat growth or bone morphology. The rapid-uptake, high-affinity binding, and slow dissociation of Al^{3+} from GPBP support the view that Al^{3+} acts by binding to membrane loci that are rich in phosphorus, thereby preventing the crystallization of calcium phosphates at these sites. This hypothesis is supported by the morphologic results that showed that Al^{3+} was associated with GPBP cells. The morphologic data suggest that the mechanism of calcification inhibition by Fe^{3+} is similar.

Previous work from our laboratory has established that calcification in the bioprosthetic heart valve subdermal model is initially noted at devitalized cell and organellar membrane locations,[11,12] which have been demonstrated by electron energy loss spectroscopy studies in the present paper to be particularly rich in phosphorus. Morphologic results of the present study (both light microscopy and electron energy loss spectroscopy), are consistent with the hypothesis that aluminum binds to these very same sites. This view is also supported by the chemical data, namely the observed rapid uptake of Al^{3+} by the GPBP tissue, as well as high-affinity binding, and the slow dissociation of bound aluminum during the course of *in vitro* incubations under physiologic conditions. The measured *in vivo* loss of Al^{3+} (Figure 3), however, was far greater than that noted *in vitro* (Figure 1), suggesting removal by either protein binding or phagocytosis or both of Al^{3+}. This might impair the sustained inhibition of GPBP calcification by Al^{3+} preincubation in the clinical setting.

Alternative hypothetical mechanisms have been advanced for the Al^{3+}-mediated (of Fe^{3+}) inhibition of physiologic (bone) mineralization. Investigations of others have shown that Al^{3+} inhibits both hydroxyapatite formation and dissolution *in vitro*.[22,23] Furthermore, investigations of bone metabolism have demonstrated that Al^{3+} may inhibit the normal function of osteoblasts,[24] and other results have suggested that Al^{3+} may inhibit parathyroid hormone function.[25] Because previous work from our group has shown that host-derived living cells do not play a role in the calcification of rat subdermal GPBP implants,[26] however, the relevance of cellular or hormonal mechanisms to dystrophic calcification is limited. Furthermore, the insignificant systemic Al^{3+} exposure in the animals in the present study would also seem to rule out a role for parathyroid hormone.

Other as yet unexplored hypotheses could explain the mechanism of action of Al^{3+} in the present studies, however. Al^{3+} (or Fe^{3+}) may simply inhibit Ca-P crystal nucleation. Alternatively Al^{3+} or Fe^{3+} might act by inhibiting alkaline phosphatase. Work by our group[27] suggested that this enzyme may play an important role in GPBP calcification, and various metal ions have been shown to inhibit alkaline phosphatase.[28]

Calcium phosphate crystallization can be inhibited by a number of the other cations investigated in our studies, including Ga^{3+}, Fe^{3+}, and La^{3+}.[8–10] Of these other metallic cations, however, only Fe^{3+} inhibited bioprosthetic tissue mineralization in the present studies. Furthermore, our observation of Fe^{3+} localization to devitalized GPBP cells suggests that Fe^{3+} preincubation may act to inhibit GPBP calcification through a similar mechanism to that of Al^{3+}. Fe^{3+} has also been noted to cause osteodystrophy in patients with severe renal compromise,[29] analogous to Al^{3+}.

Implications for the Prevention of Pathologic Calcification

Our data suggest that pathologic calcification may be exquisitely sensitive to metallic (cationic) interactions, perhaps through interference with membrane-linked initial events in calcium-phosphate formation. Furthermore the use of metallic cations as pretreatments, or incorporated into biomaterials, may offer a viable solution to the clinical problem of pathologic calcification. The use of biomaterial implants containing metallic cations as calcification inhibitors would appear to be especially advantageous in view of the absence of adverse effects in our studies. Clearly the effective dose administered to an animal or human subject by way of incorporation into a biomaterial implant, such as a bioprosthetic heart valve, is exceedingly small compared with the systemic dose required to achieve the same level of inhibition of dystrophic calcification. For example, it can be calculated that

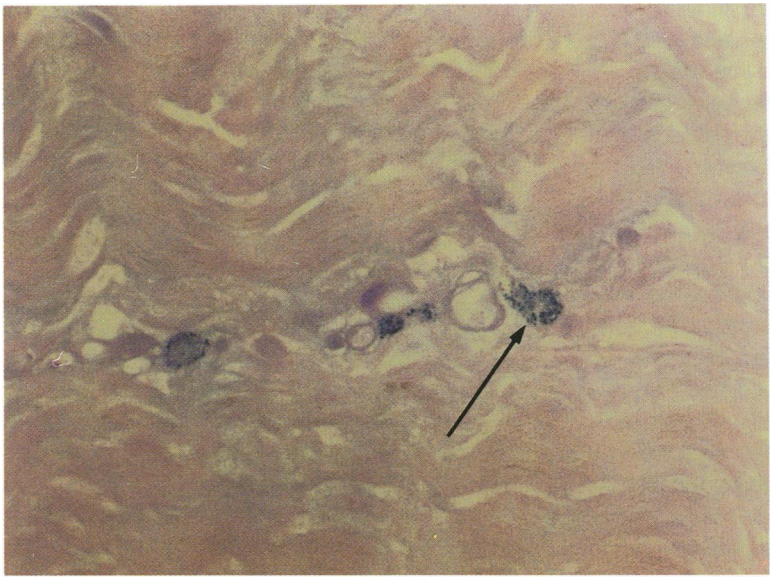

Figure 8. *(At left) Light histologic demonstration of Fe^{3+} localization in GPBP incubated in 10^{-1} mol/l $FeCl_3$ and 10^{-1} mol/l sodium citrate for 24 hours, and implanted for 21 days subdermally in the rat. Explanted tissue has Fe^{3+} in the connective tissue cells as fine, punctate, cell-oriented deposits (arrow). The tissue had minimal calcification compared with control, untreated, but implanted tissue. Stained using the Prussian blue technique (iron blue); magnification, ×600.*

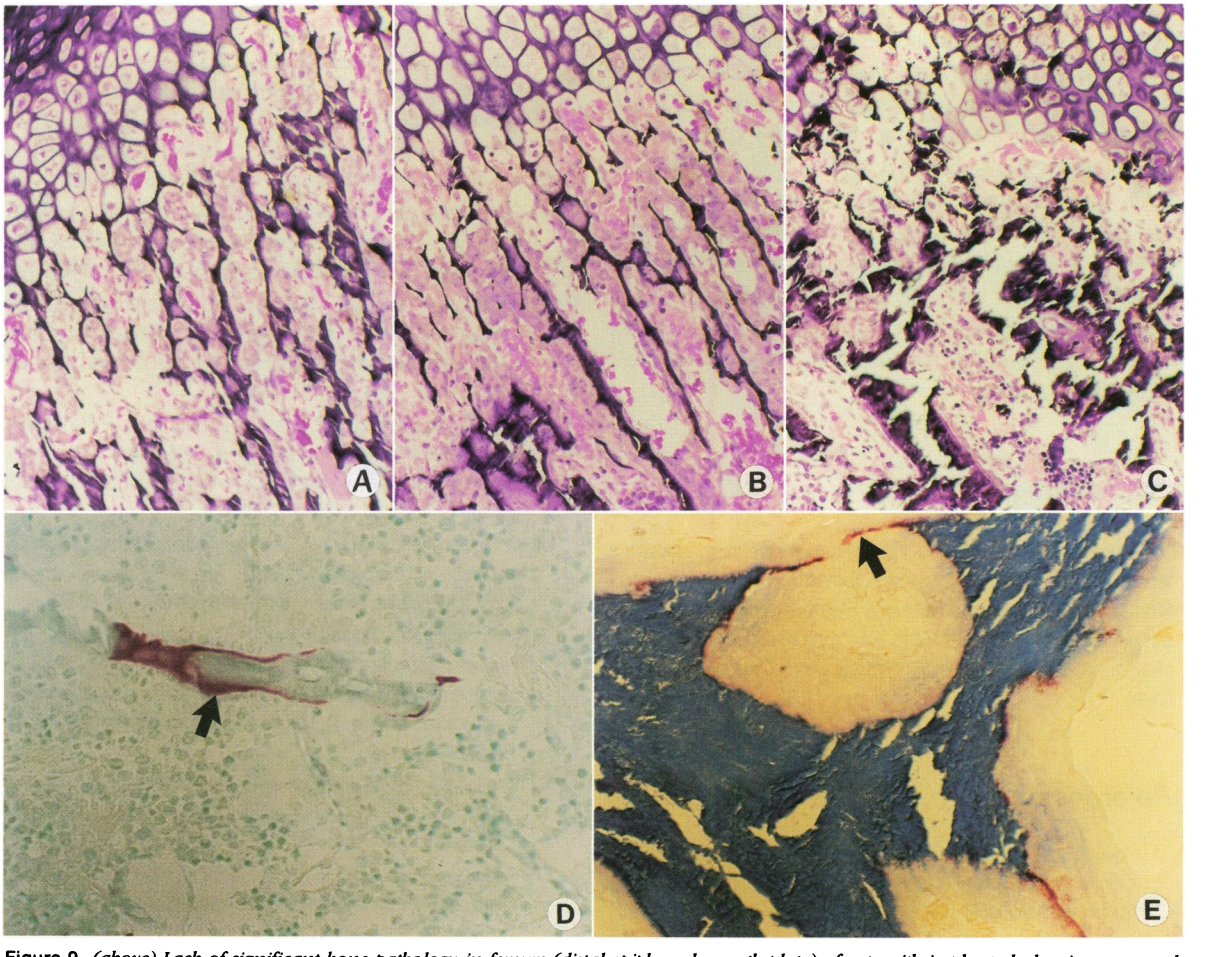

Figure 9. *(above) Lack of significant bone pathology in femurs (distal epiphyseal growth plate) of rats with implanted aluminum-treated pericardium (21-day implants).* **A**: *Femur from a rat implanted with pericardium incubated in 10^{-1} mol/l $AlCl_3$, demonstrating normal morphology of skeletal maturation. Compare with* **(B)** *a femur from a control animal, and* **(C)** *a severely disrupted femoral epiphyseal growth plate from a rat treated with 15 mg/kg/day $AlCl_3$ for 21 days.* **D**: *Aluminum staining at the edges of bony trabeculae (arrow) in femur shown in* **(C)** *from the aluminum-overloaded rat.* **E**: *Bone from patient with dialysis-induced osteodystrophy, demonstrating staining for aluminum (red) at the edges of mineralized bone (arrow). Specimen courtesy of Dr. P. Dervan, Dublin, Ireland.* **A**: *through* **C**: *H&E magnification, ×150.* **D** *and* **E**: *aluminon stain (aluminum red); magnification, ×375.*

the highest Al^{3+} loading used in the present study (10^{-1} mol/l preincubation, see Table 1), which resulted in 334 nm/mg cuspal tissue, could have resulted in a total aluminum dose of 33,400 nmoles in a trileaflet valve prosthesis containing 100 mg (dry weight) of valve tissue. This would equate to a total of 901.8 μg of aluminum—12.8 μg/kg for a 70-kg adult or 90.2 μg/kg for a 10-kg child—well below toxic dosages for aluminum salts.[14]

Although efficacy of Fe^{3+} and Al^{3+} for preventing GPBP calcification in the subdermal model has been demonstrated, both effectiveness and safety in the circulatory system remains to be shown. Certain Al^{3+} salts, such as kaolin, are known to be thrombogenic,[30] and thus a propensity to Al^{3+} pretreatment-induced platelet adhesion or other thromboembolic potential must be excluded. Furthermore, although no morphologic evidence of tissue deterioration was noted in this study, metallic salt exposure could conceivably damage the bioprosthetic tissue, promoting premature mechanical failure. Another potential problem could be limited long-term efficacy, although adjunctive controlled release implants may be efficacious. Controlled release drug delivery systems, composed of polymeric matrices with dispersions of anticalcification agents, are sustained release preparations that can provide low-dosage regional therapy.[31–33] $AlCl_3$ and $FeCl_3$ have recently been incorporated into polymeric controlled release matrices, which significantly inhibited GPBP calcification in the rat subdermal model without adverse systemic side effects.[34] This approach could help to avoid depletion of Fe^{3+} or Al^{3+} from preincubated GPBP, as well as minimize GPBP exposure to preincubation solutions.

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