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Effects of orthodontic tooth movement on the alcian blue staining patterns of rat alveolar bone: an histochemical study

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Summary. There is little information available concerning the effects of orthodontic forces on glycosaminoglycans (GAG) of alveolar bone. The present study identifies changes in Alcian blue staining intensity in rat alveolar bone undergoing resorption resulting from a heavy (25 g) tipping force aplied to the adjacent teeth by a separating spring. One day after force application, bone from treated animals (internal control and experimental sides) demonstrated more intense staining with Alcian blue, pH 1.0 ($p<0.005$) and pH 2.5 ($p<0.05$) than external controls (untreated animals). By day 3, the intensity of Alcian blue staining of treated alveolar bone was similar to untreated. Chondroitinase AC, ABC and testicular hyaluronidase predigestion did not completely block the staining reaction, suggesting that both GAG and noncollagenous proteins were demonstrated. Mean cross-sectional areas of the interdental septum of the experimental side were nearly 44% less than that of the internal control side after 3 days and nearly 62% less after 5 days. The study suggested that alterations in bone GAG levels occurred prior to tooth movement as histochemical changes occurred after force application but before initiation of significant septal resorption. A precise appraisal of the types of macromolecules effected awaits future biochemical analysis. The results of the present work strongly suggest the use of an external control group for future studies, as Alcian blue staining reactions of the internal control side of treated animals were not similar to those of external controls.

Key words: Orthodontic force - Tooth movement - Alveolar bone - Glycosaminoglycans - Histochemistry

Introduction

When an orthodontic force is applied to a tooth, the adjacent periodontal ligament and alveolar bone is remodeled. Alveolar bone adjacent to pressure zones is resorbed and new bone is deposited in tension zones. Recent reviews of this process are available (Mostafa et al., 1983; Rygh, 1984). Bone remodeling coincident to orthodontic forces is likely to be a result of either bone bending (Baumrind, 1969; Grimm, 1972; Heller and Nanda, 1979) or an inflammatory response to tissue injury (Baumrind, 1969; Grimm, 1972; Heller and Nanda, 1979; Roberts et al., 1981; Rygh, 1984). Compression of alveolar bone results in increased levels of acid phosphatase and prostaglandin synthetase and decreased levels of lactate dehydrogenase (Lilja et al., 1983) and cyclic AMP (Davidovitch and Shanfeld, 1975). There is little information available concerning the effects of orthodontic forces on noncollagenous proteins and proteoglycans of bone.

Alcian blue is a frequently used histological stain for demonstration of glycosaminoglycans (GAG) (Lev and Spicer, 1964). It is recently shown that Alcian blue also demonstrates bone sialoproteins (Fisher and Termine, 1985). There is a evidence that bone proteoglycans contain mostly chondroitin-4-sulfate with lesser amounts of chondroitin-6sulfate and iduronic acid-containing polymers (Prince et al., 1983). Recent studies report the isolation of two types of bone proteoglycans containing either: 1) one chondroitin sulfate chain attached to a glu-gln-rich protein, or 2) two chondroitin sulfate chains attached to a leu-rich core (Fisher and Termine, 1985); both components of osteoid at the mineralizing front. During mineralization, proteoglycan cores are degraded, the one-chain form being degraded more slowly than the two-chain form. The chondroitin sulfate persists within the mineral compartment. The function of GAG of the mineralized compartment is currently unknown (Fisher and Termine, 1985).

There is contradictory information concerning a relationship between orthodontic forces and levels of bone GAG. Moskowitz and Kronman (1969) report that orhodontic force has no noticable effect on Alcian blue

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or PAS staining reactions of bone. However, preliminary work in our laboratory suggests that Alcian blue staining of bone was altered during early phases of orthodontic tooth movement. Thus, the present study seeks to document these changes and relate them to resorption patterns of the stressed alveolar bone.

Materials and methods

Twenty-four female Sprague-Dawley rats, age six weeks, were studied. Twelve animals were weighed, anesthetized with ether, and a spring of the design of Hadj-Salem (1971) placed between the left first and second maxillary molar teeth. These animals were termed «treated» herein. The spring was constructed of stainless steel orthodontic wire, 0.012 inches in diameter, and was calibrated to place an initial heavy (25 g) separating force on the molar teeth. The right side served as an internal control. In addition, twelve animals were weighed, anesthetized with ether, sham operated, and served as external (untreated) controls. At 1, 3 and 5 days after treatments, animals were weighed, anesthetized, killed, maxillae hemisected and then removed by blunt dissection. Tissues were fixed by immersion in 10% neutral buffered formalin containing 0.5% cetylpyridinium chloride to preserve GAG (Engfeldt and Hjertquist, 1968), demineralized in 4.13% EDTA at pH 7.0 (Warshawsky and Moore, 1967), dehydrated in ethanols, embedded in paraffin wax, and sectioned serially at 6 μm in a sagittal plane. Alternate sections were mounted on slides, six sections per slide, and alternate slides stained with Alcian blue 8GX (pH 1.0) to demonstrate sulfated GAG (Lev and Spicer, 1964), or with Alcian blue 8GX (pH 2.5) to demonstrate carboxylated GAG (Luna, 1968). Slides from all groups were stained at the same time. Quantitative measurements were then made of the septal area and Alcian blue staining reactions.

Enzyme digestion. One slide from each animal was chosen for enzyme digestion. Alternate sections were treated with 5 μl drops of enzyme solution or buffer alone. Chondroitinase AC (from *Arthrobacter aurescens)* and ABC (from *Proteus vulgaris*) (Sigma) were prepared at a concentration of 2.0 mg/ml in 0.1 M tris-HCL buffer at pH 8.0 (Yamada, 1974). Chondroitinase AC removes chondroitin 4 and 6 sulfates; chondroitinase ABC additionally removes dermatan sulfate (Yamada, 1974). Bovine testicular hyaluronidase (Sigma) was prepared at a concentration of 0.5 mg/ml in 0.1 M phosphate buffer at pH 5.0 (Luna, 1968). This enzyme removes all GAG from tissues (Luna, 1968). Sections were incubated for 4 hours at 37^0 in 100% humidity, rinsed with distilled water, and stained with Alcian blue (pH 1.0). The staining intensity of chondroitinase ABC and hyaluronidase treated sections were compared with unstained ones.

Calculation of septal area. Sections demonstrating complete coronal and radicular pulps were chosen for analysis, as these represented the best possible mesio-distal sections through the midline of the tooth and of the interdental septum. Tracings were made using a camera lucida apparatus and areas were calculated using a digitizing tablet and microcomputer. The mean area was calculated for each of four experimental animals and, for each group, means compared by analysis of variance and by Duncan's New Multiple Range Test.

Image analysis. Quantification of the Alcian blue staining intensity was performed using a Cambridge Quantimet Q10 image analysis system interfaced with a video camera attached to an Olympus photomicroscope. Histological images were digitized into grey values and background corrections made to remove shading effects. Three readings were made over the interdental septum in each section: in the crestal, middle and apical thirds. Each area of analysis was 100 μm^2. Readings were averaged for each section and means calculated for each animal and for each group. Data was transformed to a percentage of external control (external control=100% staining reaction), converted to radians, and compared by factorial analysis of variance and by Duncan's New Multiple Range Test.

Results

The interdental septum of untreated rats (external controls) demonstrated varied staining patterns with Alcian blue. The depository surfaces and lacunar walls were intensely stained by pH 1.0 (AB–1) (Fig. 1) and at pH 2.5 (AB–2.5) (Fig. 2). Bone matrix and periodontal ligament were moderately stained, AB–2.5 more intensely (Figs. 1, 2). In the following discussion, three tissue groups will be compared: treated (internal control and experimental sides) and untreated (external controls).

Separating springs produced displacement of the crowns and approximation of the roots of the first and second molar teeth (Fig. 3), resulting in pressure on alveolar bone of the apical and lower middle thirds and tension on the crestal and upper middle thirds of the septum. In general, teeth were maximally separated after 5 days of treatment. There was no significant weight change between treated and untreated groups during the study.

Orthodontic forces produced resorption of the interdental septum, resulting in a smaller cross-sectional area. Mean areas of internal controls were significantly greater than those of experimentals after 3 days (44% less than internal control) ($p<0.01$ and after 5 days (62% less than internal control) ($p<0.001$) of treatment. Mean areas of 1 day experimentals were greater than those of 3 days (36% less than 1 day) ($p<0.005$) or 5 days (62% less than 1 day) ($p<0.001$) (Table 1, Fig. 12).

After one day, AB–1 and AB–2.5 staining reactions were more intense in internal controls than in external controls (Figs. 4.5). Mean AB–1 staining intensity was 20% greater ($p<0.005$) and mean AB–2.5 was 22% greater ($p<0.05$) than in external controls (Table 2). In

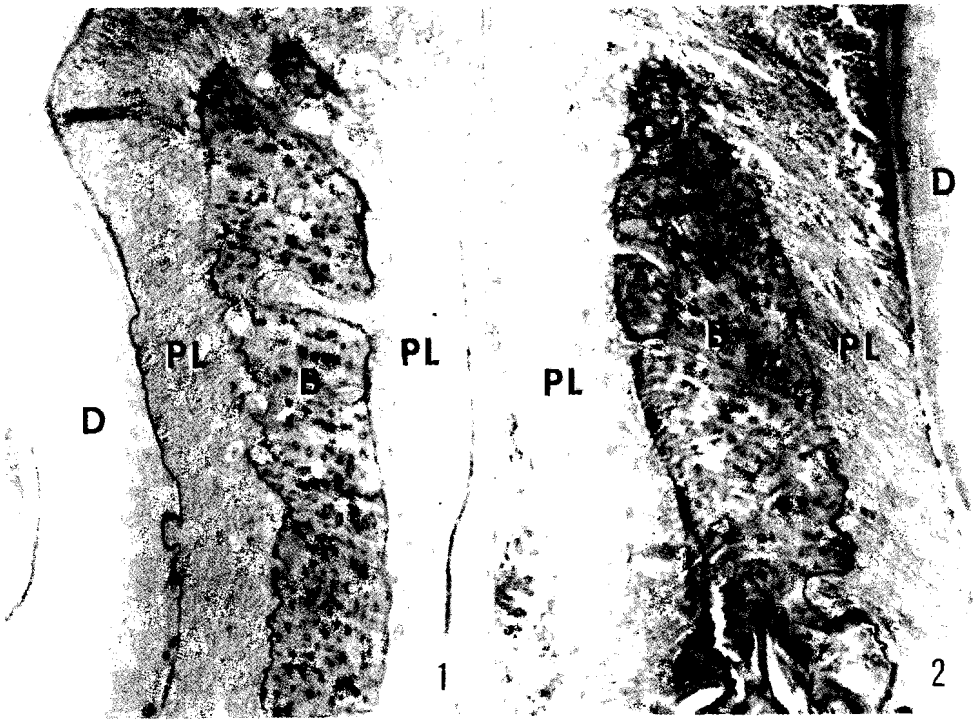

Fig. 1. Interdental septum, AB–1, external control. The periodontal ligament (PL) on the resorptive side (left) and the matrix of the interdental septum (B) are slightly AB–1(+). The periodontal ligament (PL) on the deposition side (right) is AB–1(–). Walls of osteocyte lacunae and osteoid are AB–1(+). D dentin. × 160

Fig. 2. Interdental septum, AB–2.5, external control. The periodontal ligament (PL) on the resorptive side (right) and the matrix of the interdental septum (B) are AB–2.5(+). Stain density is greater than in AB–1 tissues. Walls of osteocyte lacunae and osteoid are AB–2.5(+). The periodontal ligament (PL) on the depository side (left) is slightly AB+. D, dentin. × 160

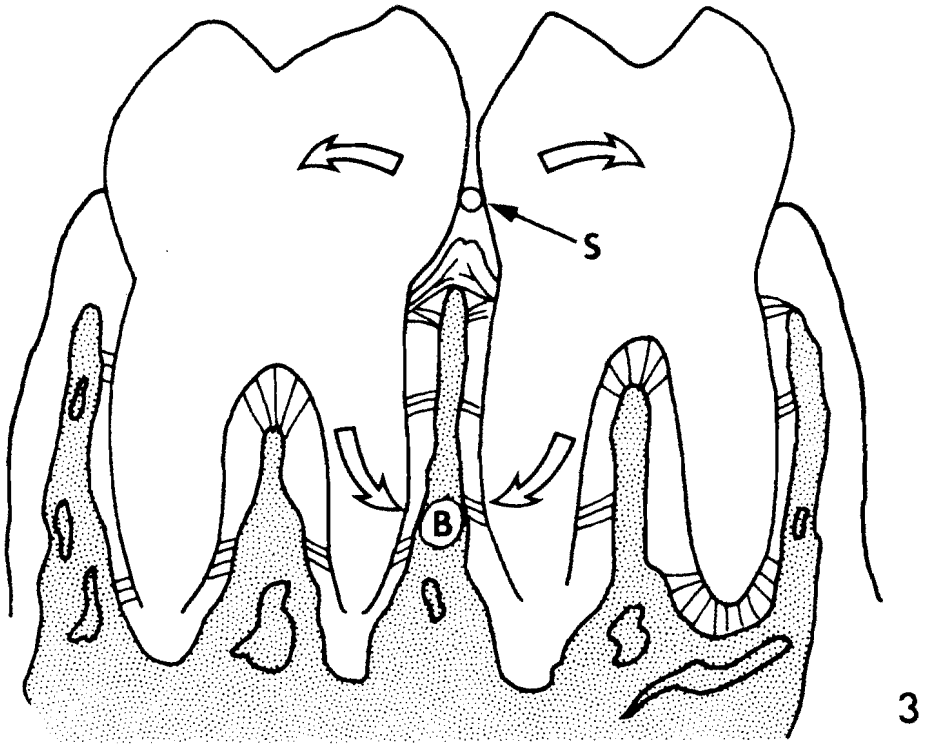

Fig. 3. Diagram illustrating tooth movement (arrows) resulting from insertion of a separating spring (S). Adjacent teeth are tipped, placing a pressure force on the apical and middle thirds of the periodontal ligament and interdental septum (B). A tensile force is placed on the transseptal fibers, the crestal fibers of the periodontal ligament, and the bone of the alveolar crest.

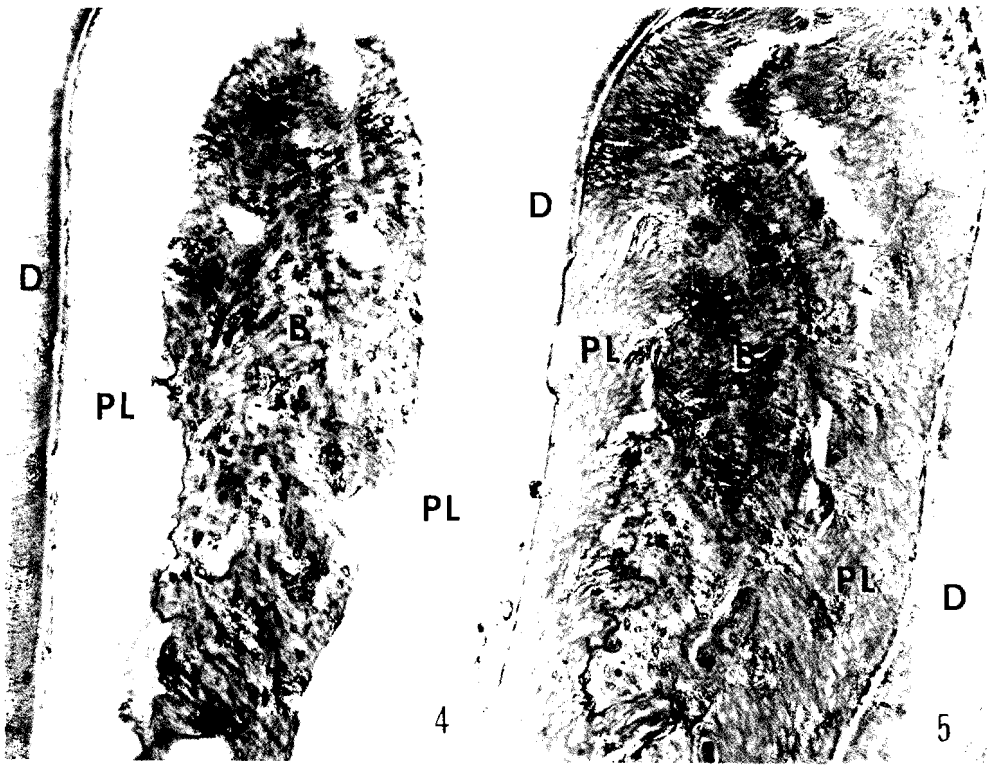

Fig. 4. Interdental septum, AB–1, one day internal control. The matrix of the interdental septum (B) demonstrates more intense AB–1 staining than in external controls. Walls of osteocyte lacunae and osteoid are AB–1(+). The periodontal ligament (PL) on the resorptive side (left) is more intensely stained than that (PL) on the deposition side (right). D, dentin × 160

Fig. 5. Interdental septum, AB–2.5, one day internal control. Interdental septum (B) stains more intensely than either external control AB–2.5 or AB–1 tissues. In the crestal third, walls of osteocyte lacunae, and osteoid stain intensely with AB–2.5. The periodontal ligament (PL) stains with more intensity in internal controls than in external controls. D, dentin. × 160

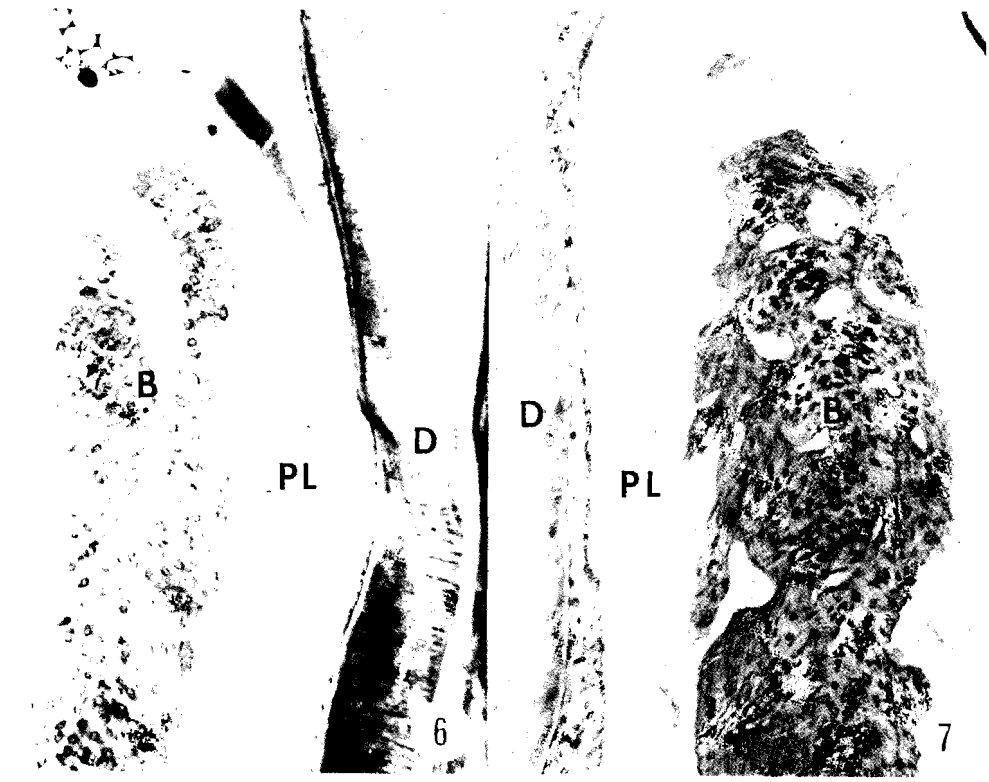

Fig. 6. Interdental septum, AB–1, one day experimental. The interdental septum (B) stains more intensely than external controls, but slightly less intensely than internal controls. The heaviest reaction is in the crestal and apical thirds of the septum. Walls of osteocyte lacunae are also stained. The periodontal ligament (PL) is AB–1(-). D, dentin. × 160

Fig. 7. Interdental septum, AB–2.5, one day experimental. The interdental septum (B) stains with slightly less intensity and the periodontal ligament (PL) stains with much less intensity than internal control. Osteocyte lacunar walls are demonstrated by the stain. D, dentin. × 160

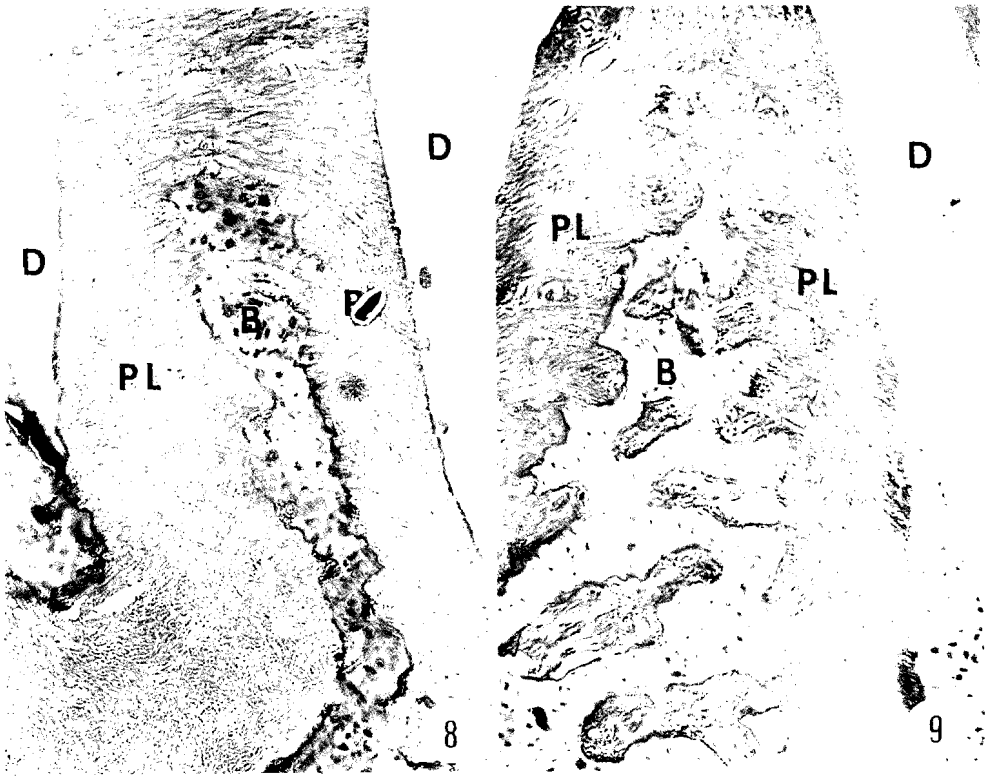

Fig. 8. Interdental septum, AB–1, five day experimental. The interdental septum (B) is narrowed in its middle and apical thirds and blunted at the alveolar crest. Bone stains with a slightly greater intensity than external controls. Walls of osteocyte lacunae are AB-(+) in the crestal third. The periodontal ligament (PL) stains more intensely than in one day animals. D, dentin. × 160

Fig. 9. Interdental septum, AB+2.5, five day experimental. The interdental septum (B) demonstrates much internal resorption. Bone matrix stains less intensely than in one day animals. Osteocyte lacunar walls are slightly AB–2.5(+). The periodontal ligament (PL) stains more intensely than in one day animals. D, dentin. × 160

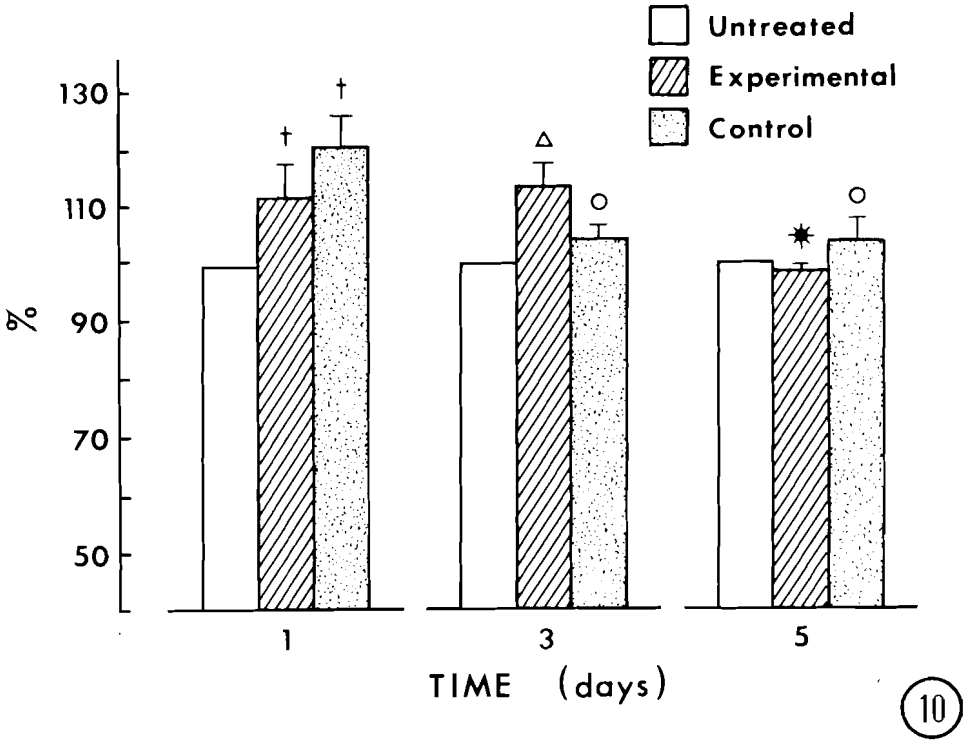

Fig. 10. Comparison of AB–1 stain intensities [(expressed as a percentage of external controls (100%)] †significantly greater than external control, $p<0.001$; △significantly greater than external control, $p<0.005$; ○significantly less than one day internal control, $p<0.05$; ✸significantly less than three day experimental, $p<0.05$.

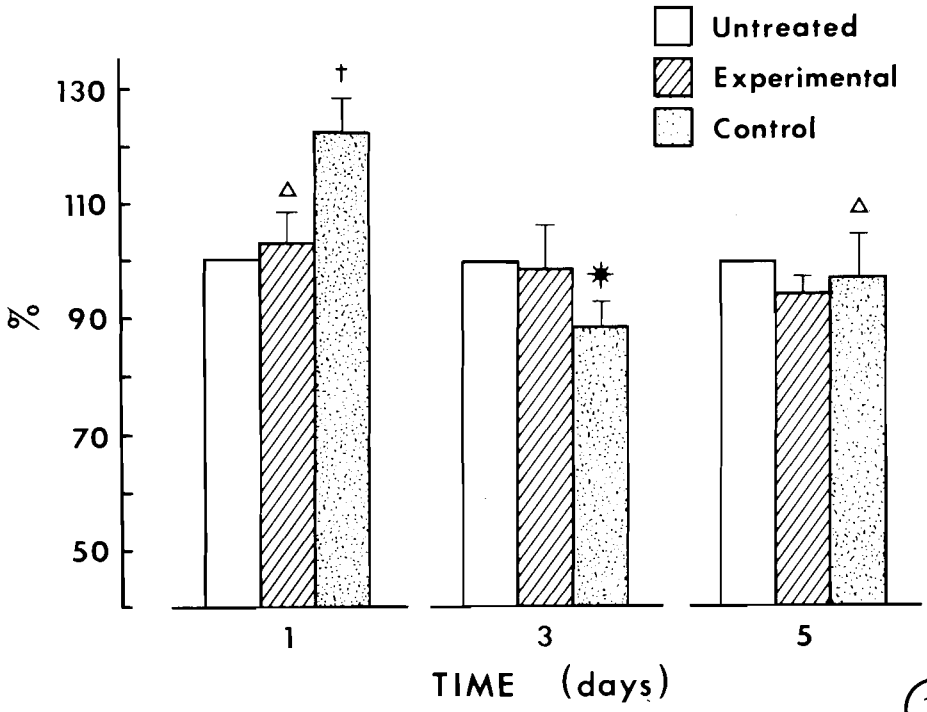

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Fig. 11. Comparison of AB–2.5 stain intensities [expressed as a percentage of external control (100%)]. ⁻significantly less than one day internal control, $p<0.05$; ᵗsignificantly greater than external control, $p<0.05$; *significantly less than one day internal control, $p<0.05$

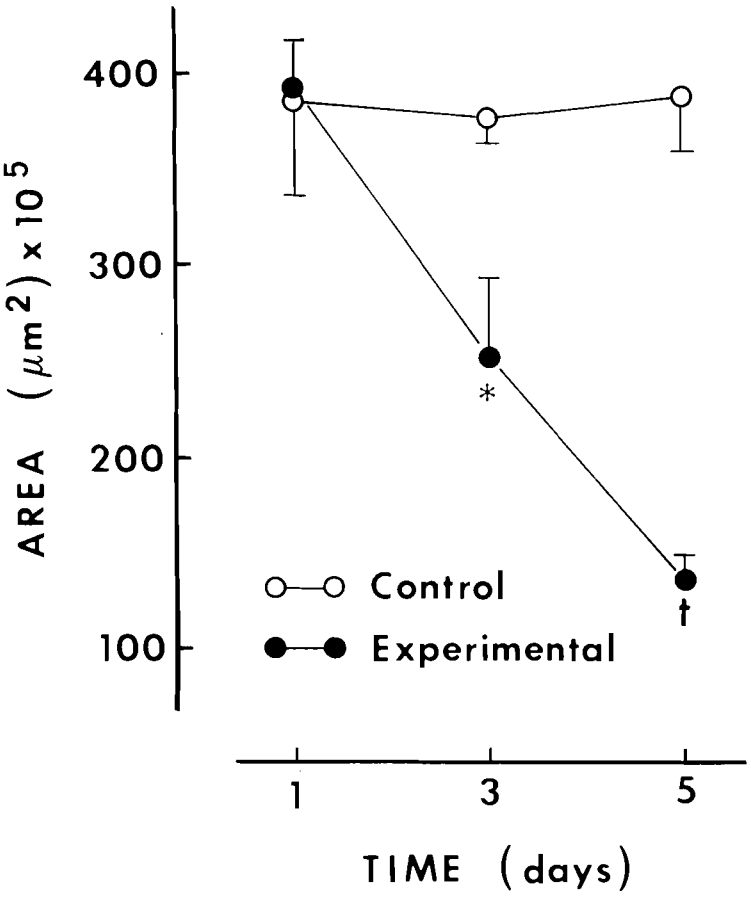

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Fig. 12. Comparison of mean areas of the interdental septum of experimental and internal control sides of treated animals. *Significantly less than internal control, $p<0.01$; ᵗsignificantly less than internal control, $p<0.001$

Table 1. Mean area of the interdental septum following insertion of a separating spring. Four animals comprised each group. Numbers in parentheses indicate number of measurements per group.

DAY	AREA (μm^2) GROUP	
	CONTROL	EXPERIMENTAL
1	386,162 ± 39,639 (16)	390,725 ± 32,231 (14)
3	377,931 ± 8,849 (26)	250,427 ± 38,779 (15)*,z
5	391,989 ± 28,704 (12)	149,272 ± 6,141 (22)t,x,o

t significantly different from 3 day experimental, $p < 0.05$.
x significantly different from 1 day experimental, $p < 0.001$.
o significantly different from 5 day control, $p < 0.001$.
* significantly different from 3 day control, $p < 0.01$.
z significantly different from 1 day experimental, $p < 0.005$.

Table 2. Comparison of mean densities of tissues of external control (untreated), experimental (E) and internal control (C) samples stained with Alcian blue.

Treatment	N	Stain pH	% External control
1 Day (C)	4	1	120.87 ± 5.55*
1 Day (C)	4	2.5	122.40 ± 6.61t
1 Day (E)	4	1	111.55 ± 5.99*
1 Day (E)	4	2.5	103.17 ± 5.29x
3 Day (C)	4	1	104.35 ± 2.85o
3 Day (C)	4	2.5	89.84 ± 4.54x
3 Day (E)	4	1	108.28 ± 4.49t
3 Day (E)	4	2.5	99.36 ± 6.91
5 Day (C)	4	1	104.51 ± 4.37o
5 Day (C)	4	2.5	96.86 ± 7.08o
5 Day (E)	4	1	98.72 ± 0.43#
5 Day (E)	4	2.5	94.83 ± 2.21
External control	4	1	100 ± 0.56
External control	4	2.5	100 ± 0.48

* significantly different from external control, $p < 0.005$.
t significantly different from external control, $p < 0.05$.
o significantly different from 1 Day control, $p < 0.05$.
x significantly different from 1 Day control, $p < 0.005$.
#significantly different from 3 day experimental, $p < 0.05$.

Table 3. Analysis of variance of the relative densities of treated (experimental and control) and external control (untreated) tissues stained with Alcian blue, pH 1.0.

SOURCE	DF	SS	MS	F
A (treatment)	2	0.055	0.027	4.98*
B (Time)	2	0.090	0.045	8.18+
AB	4	0.121	0.030	5.50+
Total	35	0.415		

* $p < 0.05$
+ $p < 0.005$

Table 4. Analysis of variance of the relative densities of treated (experimental and control) and external control (untreated) tissues stained with Alcian blue, pH 2.5.

SOURCE	DF	SS	MS	F
A (treatment)	2	0.190	0.095	4.61*
B (Time)	2	0.065	0.032	1.57
AB	4	0.391	0.098	4.76+
Total	35	1.200		

* $p < 0.05$
+ $p < 0.005$

AB–2.5 tissues, the crestal and upper middle thirds of the septum were intensely stained (Fig. 5). In comparison to external controls, the periodontal ligament stained intensely with AB–2.5 (Fig. 5). On experimental sides, the bone matrix of the crestal and upper middle thirds of the septum was intensely stained by AB–1 (Fig. 6) but not significantly greater than internal controls. Mean stain intensity was 11% greater than external controls ($p < 0.005$) (Table 2). Mean AB–2.5 intensity was significantly less than internal controls (19%, $p < 0.005$), but not significantly different from external controls (Table 2). AB–2.5 evenly stained the majority of the bone matrix, except for very intense areas surrounding osteocyte lacunae (Fig. 7). The crestal third of the septum and the periodontal ligament were not as intensely stained as internal controls. The apical third and lower middle third of the septum stained more intensely with AB–2.5 than with AB–1

After three days, mean treated AB–1 staining reactions were slightly greater than external controls (4% greater in internal controls, 8% greater in experimentals ($p<0,05$) and treated AB–2,5 slightly less than external controls (10% less in internal Controls, 1% less in experimentals (Table 2). After 5 days some osteocyte lacunae near the alveolar crest of experimental tissues were intensely Alcian blue positive (Fig. 8). Treated AB–2.5 tissues stained with slightly less intensity than external controls (3% less in internal controls, 5% less in experimentals) (Figs. 9, 11, Table 2). The periodontal ligament of treated animals was slightly AB–1 positive (Fig. 10) and intensely AB–2.5 positive (Fig. 11).

Analysis of variance suggested significant changes in

mean AB–1 and AB–2.5 intensity as a result of treatment (AB–1 and AB–2.5, $p < 0.05$) and time (AB–1, $p < 0.005$). There was a significant interaction between treatment and time in both AB–1 and AB–2.5 ($p < 0.005$) tissues (Tables 3 and 4). Predigestion of tissue sections with chondroitinase AC, ABC or testicular hyaluronidase did not completely abolish the AB–1 staining reaction [(significantly greater than unstained, $p < 0.001$ (AC), $p < 0.005$ (ABC), $p < 0.05$ (H)].

Discussion

The present study reports changes in the distribution and intensity of Alcian blue stain within alveolar bone of rats experiencing experimental tooth movement. One day following spring insertion, the intensity of Alcian blue staining significantly increased on both internal control and experimental sides. By day 3, the staining intensity returned to nearly that of untreated (external control) tissues. Coincidently, by 3 days, there was a significant loss of alveolar bone on the experimental side. These observations contradict those of Moskowitz and Kronman (1969) who report that Alcian blue and PAS staining intensity is not altered by orthodontic forces. These different interpretations may be a result of differences in fixation techniques. Loss of GAG during histologic tissue processing represents a problem of fixation. Addition of 0.5% cetylpyridinium chloride to an aldehyde fixative results in a loss of only about 7% of the acid GAG (Engfeldt and Hjertquist, 1968), most occurring during initial fixation. Acid GAG and cetylpyridinium ions form cetylpyridinium-GAG complexes which are insoluble in water and other aqueous solutions of low ionic strength (Engfeldt and Hjertquist, 1968). The use of this compound in the present study likely resulted in minimal GAG loss and allowed precise study of changes in this distribution during tooth movement.

The initial increase in Alcian blue staining of alveolar bone of one day experimental animals is difficult to explain. Alcian blue, at pH 1.0, predominately stains sulfated-GAG and Alcian blue, at pH 2.5 stains predominately carboxylated, but also some sulfated-GAG (Lev and Spicer, 1964). Thus, our staining patterns suggest that levels of both sulfated-GAG and hyaluronic acid may be initially effected by tooth movement. Chondroitinase digestions confirm that some of the Alcian blue positive component is chondroitin sulfate. Alcian blue can also stain sialoproteins of bone (Fisher and Termine, 1985). The residual AB–1 stain following testicular hyaluronidase digestion, as reported herein, demonstrates non-proteoglycan components of bone. It is possible that the large, rapid increase in staining of alveolar bone in treated animals is related to increased *de novo* synthesis of GAG as staining appears to increase around osteocytes. However placement of a heavy orthodontic force, as herein, results in degradative, not synthetic, enzymes within bone (Lilja et al., 1983). Release of plasma proteins into the bone by a vascular injury could also result in increased staining intensities. Plasma alpha$_2$–HS–glycoprotein, alpha–1–acid glycoprotein, and albumin are concentrated within bone matrix (Ashton et al., 1976; Triffitt et al., 1976, 1978; Triffitt and Owen, 1977; Herring, 1977; Mbuyi et al., 1982). In diseased bone matrix, increased levels of various plasma proteins are apparent; in particular, alpha$_2$–HS–glycoprotein (Quelch, Cole and Melick, 1984), which, because of its chemical nature, could be stained with Alcian blue. These proteins could possibly diffuse through the abundant canalicular network of alveolar bone (Johnson and Highison, 1983). It is reported that GAG levels decrease in resorbing cementum (Alexander and Swerdloff, 1980). This situation also occurs in bone and could explain the decrease in Alcian blue staining coincident to resorption of the interdental septum.

The present study illustrates histochemical changes in alveolar bone coincident to high orthodontic forces, and suggests further biochemical studies for the precise determination of these chemical changes. Whether these histochemical changes are a response to tissue injury or a prelude to bone remodeling is an important question requiring further study. The results of our study strongly suggest the use of an untreated control group for future studies as histochemical differences suggest that bone proteoglycans and noncollagenous proteins of the control side of the treated animal during the early stages of tooth movements, are not of similar concentration or distribution to those of untreated animals.

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