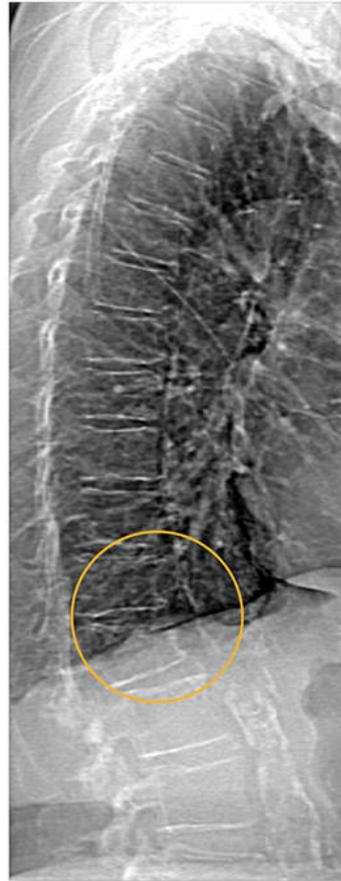


# Powerful images. Clear answers.



Manage Patient's concerns about  
Atypical Femur Fracture\*



Vertebral Fracture Assessment –  
a critical part of a complete  
fracture risk assessment



Advanced Body Composition<sup>®</sup>  
Assessment – the power to  
see what's inside

Contact your Hologic rep today at [insidesales@hologic.com](mailto:insidesales@hologic.com)

\*Incomplete Atypical Femur Fractures imaged with a Hologic densitometer, courtesy of Prof. Cheung, University of Toronto

ADS-02018 Rev 001 (9/17) Hologic Inc. ©2017 All rights reserved. Hologic, Advanced Body Composition, The Science of Sure and associated logos are trademarks and/or registered trademarks of Hologic, Inc., and/or its subsidiaries in the United States and/or other countries. This information is intended for medical professionals in the U.S. and other markets and is not intended as a product solicitation or promotion where such activities are prohibited. Because Hologic materials are distributed through websites, eBroadcasts and tradeshows, it is not always possible to control where such materials appear. For specific information on what products are available for sale in a particular country, please contact your local Hologic representative.

## Repetitive, Negligible Force Reaching in Rats Induces Pathological Overloading of Upper Extremity Bones\*

ANN E BARR,<sup>1,2</sup> FAYEZ F SAFADI,<sup>3</sup> IRENE GORZELANY,<sup>1</sup> MAMTA AMIN,<sup>1</sup>  
STEVEN N POPOFF,<sup>3</sup> and MARY F BARBE<sup>1-3</sup>

### ABSTRACT

**Work-related repetitive motion disorders are costly. Immunohistochemical changes in bones resulting from repetitive reaching and grasping in 17 rats were examined. After 3–6 weeks, numbers of ED1<sup>+</sup> macrophages and osteoclasts increased at periosteal surfaces of sites of muscle and interosseous membrane attachment and metaphyses of reach and nonreach forelimbs. These findings indicate pathological overloading leading to inflammation and subsequent bone resorption.**

**Introduction:** Sixty-five percent of all occupational illnesses in U.S. private industry are attributed to musculoskeletal disorders arising from the performance of repeated motion, yet the precise mechanisms of tissue pathophysiology have yet to be determined for work-related musculoskeletal disorders. This study investigates changes in upper extremity bone tissues resulting from performance of a voluntary highly repetitive, negligible force reaching and grasping task in rats.

**Materials and Methods:** Seventeen rats reached an average of 8.3 times/minute for 45-mg food pellets for 2 h/day, 3 days/week for up to 12 weeks. Seven rats served as normal or trained controls. Radius, ulna, humerus, and scapula were collected bilaterally as follows: radius and ulna at 0, 3, 4, 5, 6, and 12 weeks and humerus and scapula at 0, 4, and 6 weeks. Bones were examined for ED1-immunoreactive mononuclear cells and osteoclasts. Double-labeling immunohistochemistry was performed for ED1 (monocyte/macrophage lineage cell marker) and TRACP (osteoclast marker) to confirm that ED1<sup>+</sup> multinucleated cells were osteoclasts. Differences in the number of ED1<sup>+</sup> cells over time were analyzed by ANOVA.

**Results:** Between 3 and 6 weeks of task performance, the number of ED1<sup>+</sup> mononuclear cells and osteoclasts increased significantly at the periosteal surfaces of the distal radius and ulna of the reach and nonreach limbs compared with control rats. These cells also increased at periosteal surfaces of humerus and scapula of both forelimbs by 4–6 weeks. These cellular increases were greatest at muscle attachments and metaphyseal regions, but they were also present at some interosseous membrane attachments. The number of ED1<sup>+</sup> cells decreased to control levels in radius and ulna by 12 weeks.

**Conclusions:** Increases in ED1<sup>+</sup> mononuclear cells and osteoclasts indicate that highly repetitive, negligible force reaching causes pathological overloading of bone leading to inflammation and osteolysis of periosteal bone tissues.

**J Bone Miner Res 2003;18:2023–2032**

**Key words:** osteoclast, macrophage, inflammation, repetitive motion, work-related musculoskeletal disorder

### INTRODUCTION

WORK-RELATED MUSCULOSKELETAL DISORDERS (WMSD) account for approximately 65% of all occupational illnesses in U.S. private industry<sup>(1)</sup> and cost from \$20 to \$55

billion annually.<sup>(2)</sup> Epidemiologic and field studies suggest a positive relationship between the severity of WMSD and the performance of highly repetitive and/or forceful work tasks for periods of months or years.<sup>(3-5)</sup> Diagnoses related to WMSD include tendonitis, myositis, carpal tunnel syndrome, stress fracture, and osteoarthritis.<sup>(6,7)</sup> The types of pathophysiological changes that occur with submaximal repeated trauma include inflammation, tissue microdamage,<sup>(8,9)</sup> and muscle atrophy and fiber type changes.<sup>(10-12)</sup>

\*This work was presented in part as an abstract at the 39th Annual Meeting of the American Society for Cell Biology, Washington, DC, December 11–15, 1999.

The authors have no conflict of interest.

<sup>1</sup>Department of Physical Therapy, College of Health Professions, Temple University, Philadelphia, Pennsylvania, USA.

<sup>2</sup>These authors contributed equally.

<sup>3</sup>Department of Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, Pennsylvania, USA.

We have developed a rat model of repetitive reaching and grasping that permits the examination of both tissue and behavioral responses. Thus far, we have reported evidence for localized inflammation of muscle, tendon, and connective tissues of the reach forelimb that peaks at 4–6 weeks of performance of a highly repetitive, negligible force task. This inflammatory response coincides with degradation of motor behavior and task avoidance. We have also reported evidence of a widespread and systemic inflammatory response as noted by the presence of inflammatory macrophages in the nonreach forelimb and pro-inflammatory cytokines in serum of animals at 6–8 weeks of task performance.<sup>(8,9)</sup> In the course of these experiments, we noticed that areas of localized inflammation predominated at bone-tendon interfaces of muscles participating in the reaching task. We also found phagocytic cells in the periosteum of such bony sites. This finding suggested to us a reactive response of bone tissue to repetitive movements. We were interested in further exploring this response.

When exposed to dynamic, cyclical loading *in vivo*, bone may respond along a continuum ranging from anabolism, with low magnitude, brief cyclical strains,<sup>(13–15)</sup> to catabolism and even microfracture, with high magnitude, cyclical strains of long duration.<sup>(16–18)</sup> Sites of osteogenesis in cyclically loaded bone have been related to areas of high strain gradients or strain rates,<sup>(19,20)</sup> increasing loading frequency,<sup>(14)</sup> and increasing peak strains.<sup>(21)</sup> Studies of rats running on treadmills<sup>(22,23)</sup> and performing repetitive jumping<sup>(24)</sup> have shown that increasing the intensity of weight-bearing exercise is associated with diminishing returns in biomechanical competence, mass, and bone morphology of vertebral and limb bones. More intensive treadmill running in rats resulted in decreased mechanical competence and appositional bone formation of the tibia, while the femur exhibited increased appositional bone formation.<sup>(25)</sup> Periosteal and marrow edema as well as tibial stress fractures have been reported in runner athletes with shin splints.<sup>(26)</sup> Clearly, there are limits to the adaptability of bone to applied loads that are well below a magnitude required for traumatic injury when such loads are persistently applied. In addition, the response of bone to repetitive loading *in vivo* is site-specific. Yet unclear are the load exposure thresholds and the mechanism(s) of pathophysiological bone responses. One study suggests that stress fracture results from bone weakening caused by the simultaneous occurrence of adaptive remodeling and continued cyclical loading.<sup>(18)</sup> Another possibility is the suppression of osteogenesis, which has been shown in bone tissue cultures under static loads,<sup>(27)</sup> high force resistance training,<sup>(28)</sup> and strenuous running.<sup>(16,25)</sup> In the majority of *in vivo* models, cyclical bone loading is accomplished through repetitive weight-bearing activities. This makes it impossible to distinguish between bones' responses to externally applied loads versus those applied by muscle contraction. Our model in the rat of highly repetitive reaching permits the observation of tissues that have been exposed to non-weight-bearing muscular loads known to produce soft tissue and systemic inflammation consistent with WMSD.<sup>(8,9)</sup> This model provides an opportunity to study the effects of both non-weight-bearing

repetitive loading and mechanisms of naturally occurring inflammation on upper limb bone tissues *in vivo*.

During inflammation, pro-inflammatory cytokines, such as interleukin-1 (IL-1), are released by injured cells and inflammatory cells, such as macrophages. Studies have shown that IL-1 is a potent stimulator of bone resorption through osteoclast stimulation.<sup>(29–31)</sup> The presence of IL-1-producing tissue macrophages and IL-1 in blood serum in our model of WMSD may also be associated with bone resorption.<sup>(8)</sup> We hypothesize that any adaptive remodeling of bone tissue undergoing repetitive loading in our model would be modulated, and possibly counteracted, by the superimposed inflammatory response.

The purpose of this study was to determine the effects of a highly repetitive, negligible force reaching and grasping task on the magnitude and morphology of the response of forelimb bone tissue in a rat model of WMSD. We used immunohistochemical techniques to identify cells involved in inflammatory and resorptive processes in bone (macrophages, osteoclasts, and their precursors). We analyzed regions of bone adjacent to inflamed musculotendinous attachments of the forearm, humerus, and scapula as well as regions of these bones at some distance from locally inflamed musculotendinous tissues.

## MATERIALS AND METHODS

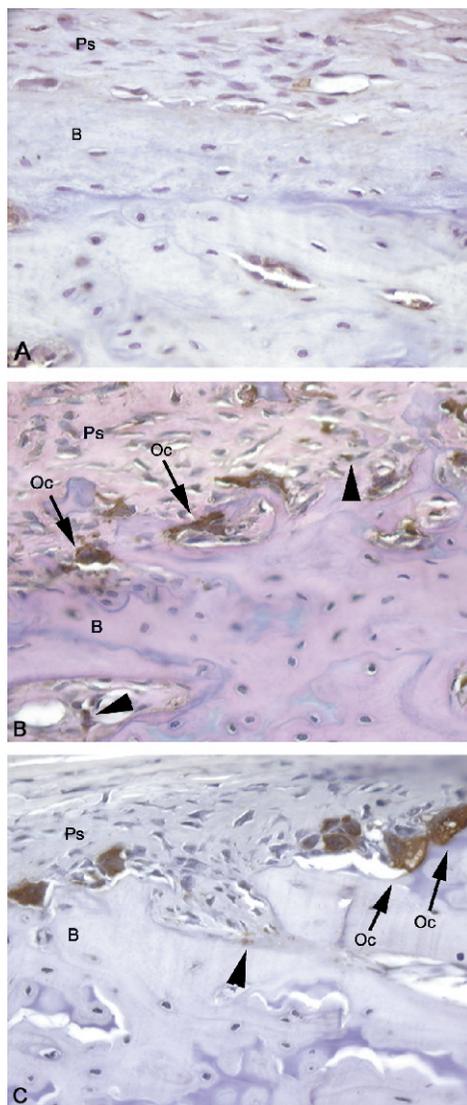
### *Description of subjects*

Twenty-four adult female Sprague-Dawley rats (age 12–14 weeks at onset of experiment) were used. Seventeen rats were trained to perform a highly repetitive forelimb reaching and grasping task with negligible force for up to 12 weeks. Four rats served as shaped-only controls to rule out the effects of training and were killed after the initial training period. Three rats served as age-matched, normal controls (no shaping). The experimental and shaped-only animals were food deprived so that they maintained 80–90% of full body weight as defined by weights of age-matched, normal controls. Experiments were approved by the Temple University IACUC in compliance with National Institutes of Health Guidelines for the Humane Care and Use of Laboratory Animals.

### *Task regimen*

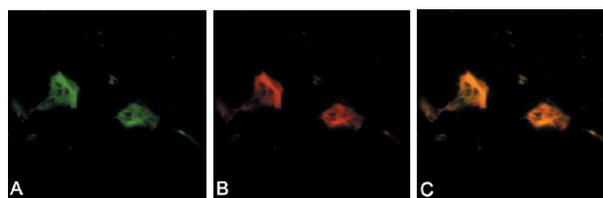
Rats performed a repetitive reaching and grasping food retrieval task as described fully in Barbe et al.<sup>(8)</sup> Briefly, the rats were placed in operant test chambers for rodents (MED Associates Inc., Georgia, VT, USA) with a portal located in one end. The portal was fitted with a 1.5-cm-wide tube that sloped downward 10° with respect to the chamber floor and was located at the animal's shoulder height. The tube was 2.5 cm in length so the elbow had to be fully extended for the animal to reach pellets of food. Food pellets (45 mg; Bioserv, Frenchtown, NJ, USA) were dispensed (pellet dispenser; MED Associates Inc.) every 15 s during the reach task. An auditory indicator (stimulus clicker; MED Associates Inc.) provided a cue that a pellet had been dispensed, thereby cueing the animal to attempt a reach.

During an initial 7- to 10-day shaping period, experimental and shaped-only control rats were first encouraged to



**FIG. 1.** ED1-immunoreactive cells in the periosteum of the distal radius in reach and nonreach limbs of trained rats. (A) Control rat. (B) Reach limb of a 6-week trained rat. (C) Nonreach limb of a 6-week trained rat. Sections are counterstained with hematoxylin. B, bone; Oc, osteoclast (also indicated by arrows); Ps, periosteum. Arrowheads indicate ED1<sup>+</sup> mononuclear cells (macrophages or precursors to macrophages or osteoclasts).

reach through open bars for food pellets placed on an elevated platform for 5 minutes/day. When they began to reach freely for the food, they were transferred to the test chamber until they could reach into the tube dispenser with no specified reach rate for 10–20 minutes per day. When they were able to perform the task consistently, experimental rats ( $n = 17$ ) were begun on the task regimen at the defined target rate of 4 reaches/minute for 2 h/day, 3 days/week for 3–12 weeks. The side used to reach and the number of reaches performed per minute were recorded in each session. Tissues of ambidextrous animals were not included in this study. Seventeen experimental animals initiated the task regimen, but beyond 3 weeks, groups of



**FIG. 2.** Large multinucleated ED1<sup>+</sup> cells in the periosteum of the distal radius also express TRACP. (A) ED1<sup>+</sup> multinucleated cells labeled with Cy3-conjugated anti-mouse secondary antibody (green). (B) TRACP immunoreactivity of the same cells as shown in A labeled with Cy2-conjugated anti-mouse secondary antibody (red). (C) Colocalization of ED1 and TRACP (yellow), indicating that the multinucleated ED1<sup>+</sup> cells are osteoclasts.

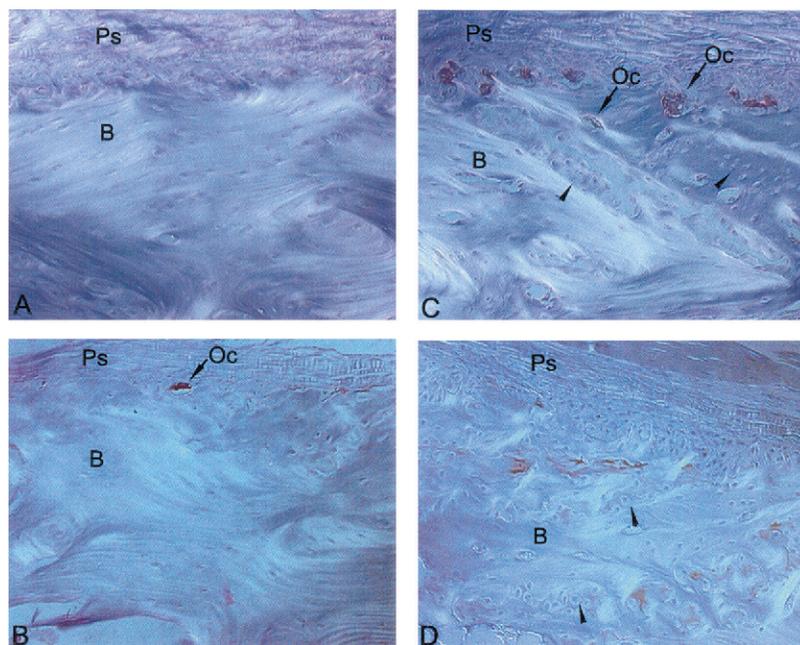
animals were periodically killed for histological tissue analysis. Shaped-only control rats were killed immediately after the shaping period.

*Histological analyses*

The numbers of rats whose tissues were examined for histological changes at various time-points were as follows:  $n = 4$  for week 3;  $n = 3$  each for weeks 4, 5, and 6;  $n = 4$  for week 12;  $n = 4$  for shaped-only controls; and  $n = 3$  for normal controls. The latter two control groups were combined and considered to be exposed to the task regimen for 0 weeks because their tissues were histologically and immunohistochemically indistinguishable. After death by lethal overdose (Nembutol; 120 mg/kg body weight), animals were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and postfixed for 2 h in situ. Bones were collected, decalcified, and paraffin embedded. Five-micrometer sections of longitudinally cut bones on coated slides (Fisher plus) were de-paraffinized and hydrated.

Two primary antibodies were used in this study. Mouse anti-rat monocyte/macrophage monoclonal antibody (catalog no. MAB1435, clone ED1; Chemicon, Temecula, CA, USA) was used as a marker of monocyte lineage cells including tissue macrophages, osteoclasts, and their precursors.<sup>(32–34)</sup> Anti-ED1 recognizes a single chain glycoprotein of 90–100 kDa expressed on lysosomal membranes of myeloid cells. Weak cell surface expression may also occur with anti-ED1. Monoclonal mouse anti-TRACP (Clone Zy-9C5; Zymed Laboratories Inc., South San Francisco, CA, USA) was used as another marker of osteoclasts and macrophages.<sup>(35–38)</sup> Anti-TRACP reacts specifically with the 16-kDa subunit of human TRACP.

Tissue sections (on slides) were treated with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 minutes (omitted for immunofluorescence), washed, treated with 1% pepsin in 0.01N HCL for 20 minutes, washed, and blocked with 4% goat serum for 20 minutes. Monoclonal antibody staining for ED1 was performed as described in Barbe et al.<sup>(8)</sup> Sections were counterstained with hematoxylin, unless used for differential interference contrast microscopy, and mounted with DPX mountant (BDH Laboratory Supplies, Poole, UK). Negative immunocontrols were provided by slides to which primary antibody had not been added.



**FIG. 3.** Differential interference contrast microscopy (20 $\times$  magnification) showing ED1<sup>+</sup> cells and periosteal-bone matrix organization of the distal radius. (A) Control, (B) reach limb of a 3-week trained rat, (C) reach limb of a 6-week trained rat, and (D) reach limb of a 12-week trained rat. B, bone; Oc, osteoclast (also indicated by arrows); Ps, periosteum. Arrowheads indicate areas of closely packed osteocytes.

Double-labeling immunohistochemistry was performed sequentially with anti-ED1 and secondary antibody conjugated to Cy2 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) steps first, and anti-TRACP second. Tissues stained for TRACP were processed with a modification of the procedure recommended for the Zymed Histostain-TRACP Kit. Briefly, sections were incubated with undiluted TRACP antibody overnight at 4°C and incubated with Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) for 2 h at room temperature. Fluorescent-stained sections were coverslipped with 80% glycerol-PBS. Negative immuncontrols were provided by slides to which neither primary antibody had been added.

#### Bioquantification

The number of ED1<sup>+</sup> cells was counted in both reach and nonreach forelimbs using a bioquantification system (Bioquant TCW 98; R & M Biometrics, Nashville, TN, USA) attached to a Nikon microscope. Cells with a defined threshold of peroxidase staining were counted in a 0.0643-mm<sup>2</sup> area using a 40 $\times$  objective or in a 0.3173-mm<sup>2</sup> area using a 20 $\times$  objective. For each site analyzed, ED1<sup>+</sup> mononuclear cells and ED1<sup>+</sup> osteoclasts cells were counted separately using Bioquant data filters that limited the size of cells counted (one set of counts excluded the larger, multinucleated osteoclasts; the other set excluded smaller, mononuclear cells). Three adjacent fields were measured per site analyzed. Four bones were examined in the upper extremities: radius, ulna, humerus, and scapula. Cell counts were obtained at the periosteum-bone interface of the radius and ulna in rats that had performed the task for either 0, 3, 4, 5, 6, or 12 weeks as follows: in both the reach and nonreach forelimbs, in proximal and distal regions, in two sites (sites of muscle attachment on the lateral radius and medial ulna and sites of interosseous membrane attachment on the me-

dial radius and lateral ulna), and by cell type (ED1<sup>+</sup> mononuclear cells and ED1<sup>+</sup> osteoclasts). Cell counts were obtained at the metaphyseal region of the radius and ulna in rats that had performed the task for either 0, 3, 4, 5, 6, or 12 weeks in both forelimbs and by cell type. Cell counts were obtained at the periosteum-bone interface of the humerus in rats that had performed the task for either 0, 4, or 6 weeks as follows: in both the reach and nonreach limbs, in three regions (distal, middle, and proximal), and by cell type. Cell counts were obtained at the periosteum-bone interface of the scapula in rats that had performed the task for either 0, 4, or 6 weeks as follows: in both the reach and nonreach forelimbs, in three regions (glenoid cavity and adjacent sites of muscular attachments, spine, and the body of the scapula), and by cell type. TRACP immunostaining was used in combination with morphological characteristics to confirm that the large, multinucleated ED1<sup>+</sup> cells were osteoclasts.

#### Data analysis

For all statistical analyses, microscopic field (three fields/tissue) was used as a blocking factor, and  $p \leq 0.05$  was considered significant. Post hoc analyses were carried out using the Bonferroni method for multiple comparisons, and adjusted  $p$  values are reported.

Counts from radius and ulna were combined for statistical analysis. For the periosteal-bone interface of the radius and ulna, differences in the number of ED1<sup>+</sup> cells were analyzed using a four-way ANOVA with the factors week (0, 3, 4, 5, 6, and 12), limb (reach and nonreach), region (proximal and distal), and site (attachments of muscle and of interosseous membrane). For the metaphyseal regions of the distal radius and ulna, differences in the number of ED1<sup>+</sup> cells were analyzed using a three-way ANOVA with the factors week (0, 3, 4, 5, 6, and 12), limb, and cell type. Counts from the different regions analyzed in the scapula and the hu-

merus were combined for statistical analysis. For the scapula and humerus, differences in the number of ED1<sup>+</sup> cells were analyzed using three-way ANOVAs with the factors week (0, 4, and 6), limb, and cell type. We chose fewer time points to analyze for the humerus and scapula to increase statistical power while capturing the peak response indicated at the muscle attachment sites of the distal forearm bones.

RESULTS

After 4–6 weeks of task performance, ED1<sup>+</sup> mononuclear cells and osteoclasts were apparent in the reach limb along the periosteal-bone interface (Fig. 1B), particularly at sites of muscle attachment, compared with controls (Fig. 1A). Increased numbers of ED1<sup>+</sup> cells were also visible in similar sites in the nonreach forelimb bones (Fig. 1C). The large, multinucleated cells that were ED1<sup>+</sup> and associated with resorptive lacunae (Figs. 1B and 1C) were also immunopositive for TRACP (Fig. 2), indicating that these cells were osteoclasts. The bone matrix of the distal radius showed signs of progressive reorganization (Fig. 3) over time. At 3 weeks (Fig. 3B), the periosteum contained numerous ED1<sup>+</sup> mononuclear cells compared with control tissue (Fig. 3A). The collagen fibers of the periosteum had begun to lose their closely packed, parallel alignment, which continued to worsen through week 6 (Fig. 3C). By 6 weeks, the bone matrix showed increased lamellar disorganization and the presence of ED1<sup>+</sup> cells (osteoclasts, macrophages, and their precursors) in large lacunae that invaded the cortical bone (Fig. 3C). Osteocytes were also more numerous and closely packed at 6 and 12 weeks (Figs. 3C and 3D), and collagen fibers were more randomly arranged in reach limb bone compared with the control bone (Fig. 3A). The collagen fibers of the periosteum had begun to regain their closely packed, parallel alignment at 12 weeks (Fig. 3D), and there were fewer ED1<sup>+</sup> cells at this time-point. However, even at 12 weeks, the bone matrix was hypercellular and did not appear to be as well-organized and lamellar as control bone.

The ANOVAs yielded numerous significant main effects and interactions. Figures 4–7 illustrate the significant ANOVA results.

Figure 4 shows that the mean number of ED1<sup>+</sup> mononuclear cells and osteoclasts in the distal radius and ulna at sites of muscle attachment (Figs. 4A and 4C) increased significantly above controls in weeks 4–6 in both the reach and nonreach limbs. At sites of interosseous membrane attachments in the distal radius and ulna (Figs. 4B and 4D), the number of ED1<sup>+</sup> mononuclear cells increased significantly above controls in the reach limb and in week 4 only. By week 12, the number of ED1<sup>+</sup> cells at the periosteal-bone interface had returned to control levels despite continued task performance. Figure 5 shows that the cell counts for the proximal radius and ulna at muscle (Figs. 5A and 5B) and interosseous membrane (Figs. 5C and 5D) attachments did not increase significantly above controls.

Figure 6A shows that the number of ED1<sup>+</sup> mononuclear cells increased significantly above controls in the distal forearm metaphyses of the reach limb in weeks 3–5 and of

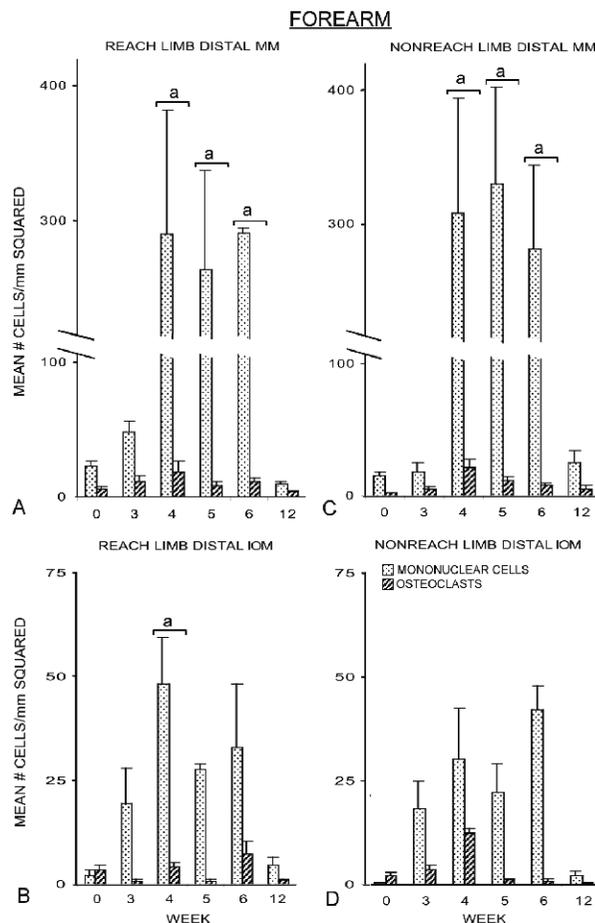
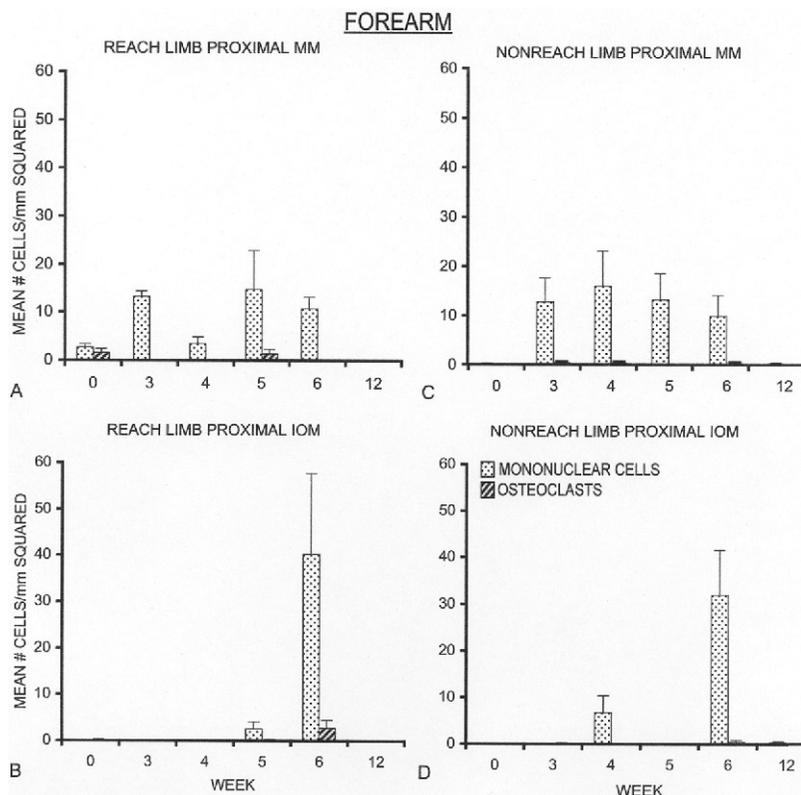


FIG. 4. Bioquantitation of ED1-expressing cells in the distal radius and ulna at the bone-periosteal border shows an increase in ED1<sup>+</sup> mononuclear cells and osteoclasts in the reach and nonreach limbs by 4–6 weeks of task performance. ED1<sup>+</sup> mononuclear cells and osteoclasts in the distal radius and ulna at sites of muscle (MM) attachments of the (A) reach and (C) nonreach limbs. ED1<sup>+</sup> mononuclear cells and osteoclasts in the distal radius and ulna at sites of attachment of the interosseous membrane (IOM) of the (B) reach and (D) nonreach limbs. Data are expressed as mean cell number ± SE. <sup>a</sup>*p* < 0.05 compared with 0-week controls.

the nonreach limb in weeks 3 and 4. Figure 6B shows that the number of ED1<sup>+</sup> osteoclasts increased significantly above controls in the distal forearm metaphyses of the reach limb in weeks 4 and 5 and of the nonreach limb in weeks 3 and 5. In weeks 6 and 12, the number of ED1<sup>+</sup> cells in the distal forearm metaphyses had returned to control levels despite continued task performance.

Figure 7A shows that the mean number of ED1<sup>+</sup> mononuclear cells increased significantly above controls in the scapula of the reach limb in weeks 4 and 6 and of the nonreach limb in week 6. Figure 7B shows that the number of ED1<sup>+</sup> osteoclasts increased significantly above controls in the scapula of the reach limb in week 6 only. Figure 7C shows that the number of ED1<sup>+</sup> mononuclear cells increased significantly above controls in the humerus of the reach and nonreach limbs in weeks 4 and 6. The number of ED1<sup>+</sup> osteoclasts (Fig. 7D) increased significantly above



**FIG. 5.** Bioquantitation of ED1-expressing cells in the proximal radius and ulna at the bone-periosteal border shows no increases in ED1<sup>+</sup> mononuclear cells and osteoclasts in the reach and nonreach limbs across weeks of task performance. ED1<sup>+</sup> mononuclear cells and osteoclasts in the proximal radius and ulna at sites of muscle (MM) attachments of the (A) reach and (C) nonreach limbs. ED1<sup>+</sup> mononuclear cells and osteoclasts in the proximal radius and ulna at sites of attachment of the interosseous membrane (IOM) of the (B) reach and (D) nonreach limbs. Data are expressed as mean cell number  $\pm$  SE.

controls in the humerus of the reach limb in week 4 only and returned to control levels by week 6.

## DISCUSSION

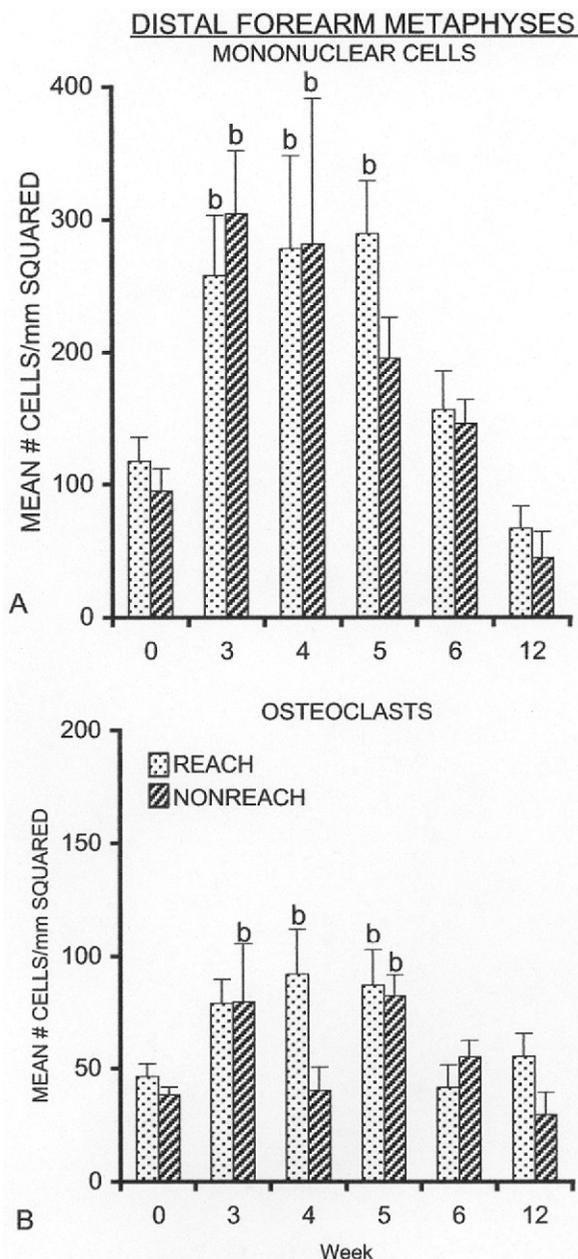
We have previously shown that this high repetition, negligible force task is associated with decrements in motor performance across time.<sup>(8)</sup> In addition, inflammation of loose connective tissues, muscle, and tendon is observed to peak by 4–6 weeks of task performance in the reach and nonreach limbs. This soft tissue inflammatory response is accompanied by the elevation of IL-1 $\alpha$  above control levels in serum. In the present study, ED1-expressing mononuclear cells and osteoclasts also increased dramatically by 3–6 weeks of task performance in the distal radius and ulna of both the reach and nonreach limbs and in the humerus and scapula.

In this study, we used a monoclonal antibody (ED1) that recognizes a lysosomal and cell surface membrane protein present in monocytes in peripheral blood, subsets of macrophages, osteoclasts, and precursors of macrophages and osteoclasts.<sup>(32–34)</sup> The ED1<sup>+</sup> mononuclear cells observed in the periosteum and metaphyseal bone are most likely exudate macrophages engaged in bone resorption and remodeling<sup>(39,40)</sup> or osteoclast precursors.<sup>(34,35,39)</sup> The ED1<sup>+</sup> multinucleated cells located in resorptive lacunae had the morphological features of osteoclasts and also stained positive for TRACP reactivity. TRACP expression has been shown to be restricted to bone-resorbing osteoclasts, specific subsets of activated macrophages, and their precursors in skeletal tissues.<sup>(35–38)</sup>

This increase of macrophages, osteoclasts, and their precursors in bone suggests a mechanism whereby bone resorption is induced by task-related inflammation of the soft tissues, such as tendon, adjacent to the bone matrix microenvironment. The infiltration of macrophages and subsequent upregulation of proinflammatory mediators, such as IL-1 $\alpha$ , may have stimulated osteoclastic activity through paracrine mechanisms.<sup>(29–31)</sup>

A second possible mechanism underlying bone remodeling in our model is the tensile overloading of the bone at the attachments of tendons and ligaments. Turner et al.<sup>(41)</sup> reported the presence of woven bone at the periosteal surface of rat tibia subjected to mechanical peak bending moments of 40N at a frequency of 2 Hz for 18 s/day in only 12 days. Interestingly, when sham-loaded controls were exposed to the same peak loading force in the absence of bending moments, periosteal woven bone formation was also observed. The authors concluded that the woven bone formation was a response to direct trauma by the loading apparatus on the periosteal and more superficial tissues and was, therefore, a pathological rather than an adaptive response. This same observation has been made in subsequent studies.<sup>(42–44)</sup> The disorganized appearance of our periosteal bone tissues by 6 weeks of task performance is consistent with that of woven, or immature, bone.<sup>(45)</sup>

The location of woven bone formation in our specimens was at sites of musculotendinous, and to a lesser extent, ligamentous attachments. It seems likely, therefore, that the observed reactive response was to tensile loads resulting from muscle contraction and from ligament stretching with



**FIG. 6.** Bioquantitation of ED1-expressing cells in metaphyseal bone of the distal radius and ulna shows an increase in ED1<sup>+</sup> mononuclear cells and osteoclasts in the reach and nonreach limbs by 3–5 weeks of task performance. (A) ED1<sup>+</sup> mononuclear cells and (B) ED1<sup>+</sup> osteoclasts in distal radius and ulna metaphyseal bone. Data are expressed as mean cell number ± SE. <sup>b</sup>*p* < 0.01 compared with 0-week controls.

extreme joint range of motion (e.g., full forearm pronation would cause tension to develop in the interosseous membrane). The presence of inflammatory and bone resorbing cells at the disorganized periosteal-bone junction is also consistent with stress fracture<sup>(45)</sup> and the subsequent formation of woven bone.<sup>(17)</sup> Therefore, our results are strongly suggestive of pathological overloading whereby insufficient recovery between task exposures does not allow healing, thereby compromising the biomechanical competence of the

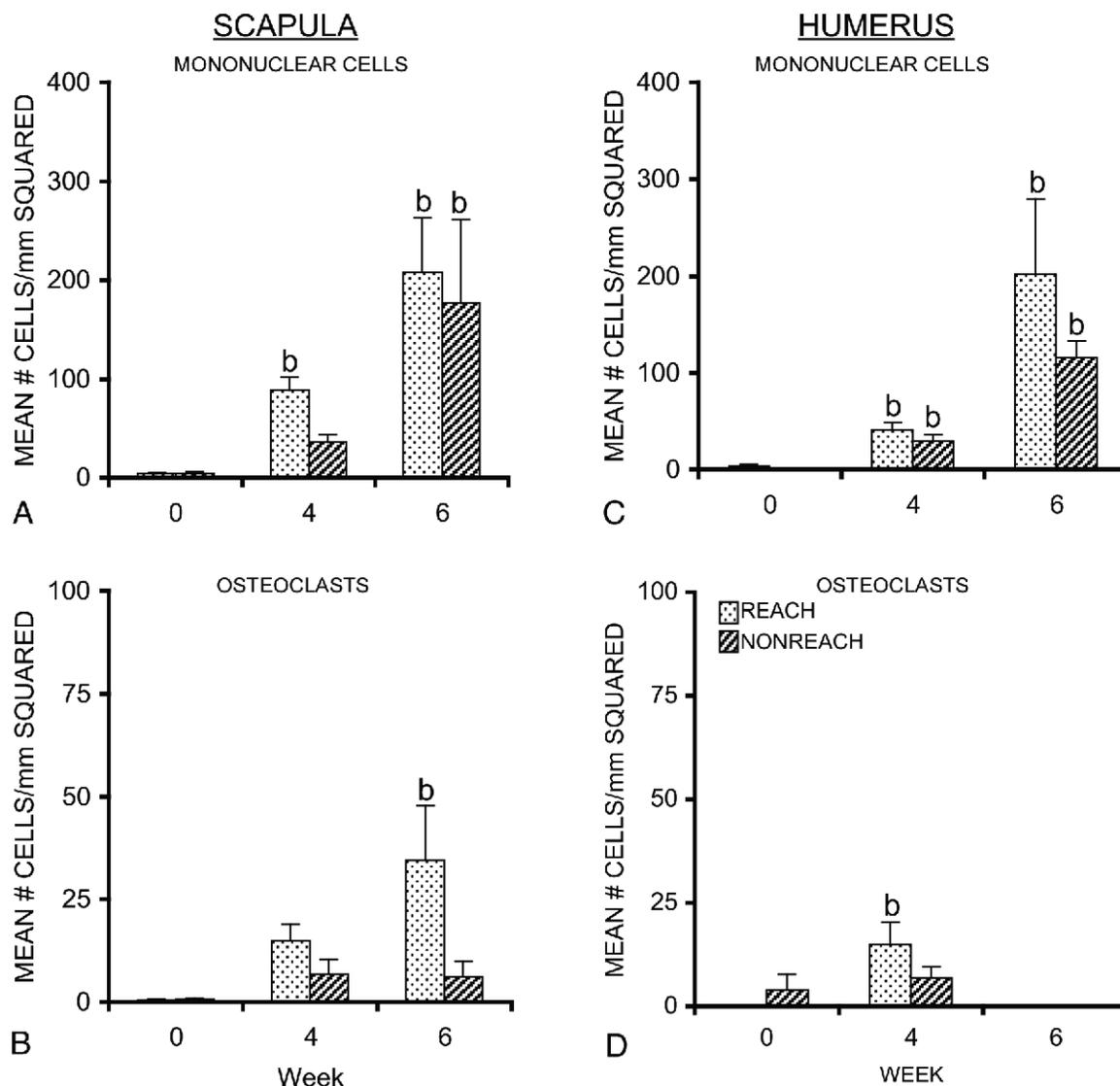
bone with regard to the task demands. The area in which we observe woven bone formation is also consistent with the development of WMSD.

A comparison between the cell count results for the distal and proximal regions of the forelimb further supports this load-induced inflammation mechanism. Most muscles that control wrist and forepaw flexion and extension insert over small areas of the distal radius and ulna, whereas they originate on the distal humerus. In addition, more proximal muscle attachments on the humerus and the scapula control the elbow flexion and extension and glenohumeral elevation required for the reaching task. Tension at the attachment sites of these muscles would induce the highest localized increases in bone strain at the distal forearm, along the entire humerus and at the scapular sites examined. These localized increases in bone strain could have stimulated an inflammatory reaction. In the case of the metaphyseal bone in the distal radius and ulna, the inflammatory response that was observed to occur earlier at 3 weeks may have resulted from increased compressive loads generated across the metaphyses by the combined contraction of both wrist and forepaw flexors and extensors. The combined effects of these muscles would expose these bone regions to even higher localized bone strains, which explains the relatively high response magnitude observed in the distal forearm metaphyses.

The increase in ED1<sup>+</sup> cells in the nonreach limb is also consistent with a load-induced inflammation mechanism. We noted that during task performance, rats typically placed the open forepaw of the nonreach limb on the chamber wall along side the portal while the reach limb was inserted into the food dispensing tube. The resulting elevation, pronation, and wrist extension of the nonreach upper limb seems to have produced forces and postures extreme enough to affect both musculotendinous attachment sites and distal forearm metaphyses.

We did not look for increases in bone apposition in our bone specimens, but such adaptive bone remodeling may occur with repetitive reaching. Recent studies examining adaptive bone formation suggest that brief daily periods of physiological loads can be strongly osteogenic.<sup>(13,46)</sup> Furthermore, this anabolic effect is enhanced when load cycles are separated by rest periods of 10–14 s.<sup>(15,42)</sup> In our protocol, animals reached at a rate of 8.3 reaches/minute, or 1 reach every 7–8 s.<sup>(8)</sup> We would expect these regular rest periods to favor bone apposition, which would eventually improve the fitness of the bone tissues to withstand the loads needed to perform the repetitive task. Such adaptive bone formation was reported in rats that performed a repetitive tower climbing exercise for distances of 0.12–0.16 m in 30 minutes/day for 4 or 8 weeks.<sup>(47)</sup> Our observed decrease of ED1<sup>+</sup> cells by 12 weeks of task performance may indicate an adaptation to the task leading to a resolution of both inflammation and bone resorption. We hope to explore this possibility in future studies.

Although we did not explore the role of systemic inflammation in this study, we suspect that the serum increases in IL-1 $\alpha$  seen in our earlier study<sup>(8)</sup> helped to drive the osteolytic response. However, further research is needed to elucidate this hypothesized systemic inflam-



**FIG. 7.** Bioquantitation of ED1-expressing cells in the scapula and humerus at the bone-periosteal border shows an increase in ED1<sup>+</sup> mononuclear cells and osteoclasts in the reach and nonreach limbs by 4–6 weeks of task performance. (A) ED1<sup>+</sup> mononuclear cells and (B) ED1<sup>+</sup> osteoclasts in the scapula at sites of muscle attachments. (C) ED1<sup>+</sup> mononuclear cells and (D) ED1<sup>+</sup> osteoclasts in the humerus at sites of muscle attachments. Data are expressed as mean cell number  $\pm$  SE. <sup>a</sup> $p < 0.01$  compared with 0-week controls.

matory response. One possible approach to testing this hypothesis is to examine bones, such as frontal or parietal bones of the calvaria, that are not directly involved in task performance.

In conclusion, this study suggests two possible mechanisms for reactive bone remodeling resulting from the performance of a highly repetitive, negligible force reaching and grasping task in a rat model: (1) paracrine stimulation of osteoclast activity by inflamed soft tissues near the injury site and (2) pathological overloading leading to inflammation and subsequent autocrine stimulation of osteoclast activity by inflamed periosteal bone tissues at the injury site. Regardless of the mechanism(s) involved, this response peaked by 3–6 weeks of task performance and was observed at sites of musculotendinous and ligamentous attach-

ments as well as distal forearm metaphyses. Thus, even negligible force tasks, when performed at a high rate, can cause at least temporary periods in which newly remodeled bone may lack the maturity and biomechanical competence to withstand task demands. The findings of other investigators regarding such reactive bone responses to repetitive loading indicate that the response can be modified or prevented by controlling both load magnitude as well as loading frequency such that adaptive remodeling is eventually favored over chronic and disabling tissue damage. Such an approach is in keeping with current ergonomic principles of work modification to prevent WMSD. Future experiments with animal models such as ours need to be conducted to help to define acceptable parameters for repetitive occupational tasks.

## ACKNOWLEDGMENTS

This study was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR46426 (to AEB); National Institute of Occupational Safety and Health Grant OH03970 (to MFB); and the Temple University Department of Anatomy and Cell Biology.

## REFERENCES

- Bureau of Labor Statistics 2002 Workplace injuries and illnesses in 2001. Available online at <http://www.bls.gov/iif/home.htm>. Accessed on January 23, 2003.
- National Research Council and Institute of Medicine 2001 Musculoskeletal Disorders and the Workplace. National Academy Press, Washington DC, USA, pp. 57–59.
- Bernard BP (ed.) 1997 Musculoskeletal Disorders (MSDs) and Workplace Factors: A Critical Review of Epidemiologic Evidence for Work-related Musculoskeletal Disorders of the Neck, Upper Extremity, and Low Back. Department of Health and Human Services, National Institute of Occupational Safety and Health Cincinnati, OH, USA.
- Latko WA, Armstrong TJ, Franzblau A, Ulin SS, Werner RA, Albers JW 1999 Cross-sectional study of the relationship between repetitive work and the prevalence of upper limb musculoskeletal disorders. *Am J Ind Med* **36**:248–259.
- Moore JS, Garg A 1994 Upper extremity disorders in a pork processing plant: Relationships between job risk factors and morbidity. *Am Ind Hyg Assoc J* **55**:703–715.
- Feuerstein M, Miller VL, Burrell LM, Berger R 1998 Occupational upper extremity disorders in the federal workforce. *J Occup Environ Med* **40**:546–555.
- Hales TR, Bertsche PK 1992 Management of upper extremity cumulative trauma disorders. *Am Assoc Occupation Health Nurs J* **40**:118–128.
- Barbe MF, Barr AE, Amin A, Gaughan J, Gorzelany I, Safadi F 2003 Repetitive motion causes local injury, systemic inflammation and reach pattern decrements in rats. *J Orthop Res* **21**:7–16.
- Barr AE, Barbe MF 2002 Pathophysiological tissue changes associated with repetitive movement: A review of the evidence. *Phys Ther* **82**:173–187.
- Dennet X, Fry HJH 1998 Overuse syndrome: A muscle biopsy study. *Lancet* **23**:905–908.
- Stauber WT, Knack KK, Miller GR, Grimmitt JG 1996 Fibrosis and intercellular collagen connections from four weeks of muscle strains. *Muscle Nerve* **19**:423–430.
- Stauber WT, Smith CA, Miller GR, Stauber FD 2000 Recovery from 6 weeks of repeated strain injury to rat soleus muscles. *Muscle Nerve* **23**:1819–1825.
- Rubin C, Turner AS, Bain S, Mallinckrodt C, McLeod K 2001 Anabolism: Low mechanical signals strengthen long bones. *Nature* **412**:603–604.
- Rubin C, Turner AS, Muller R, Mitra E, McLeod K, Lin W, Qin Y 2002 Quantity and quality of trabecular bone in the femur are enhanced by a strongly anabolic, noninvasive mechanical intervention. *J Bone Miner Res* **17**:349–357.
- Srinivasan S, Weimer DA, Agans SC, Bain SD, Gross TS 2002 Low-magnitude mechanical loading becomes osteogenic when rest is inserted between each load cycle. *J Bone Miner Res* **17**:1613–1620.
- Bourrin S, Genty C, Palle S, Gharib C, Alexandre C 1994 Adverse effects of strenuous exercise: A densitometric and histomorphometric study in the rat. *J Appl Physiol* **76**:1999–2005.
- Li G, Zhang S, Chen G, Chen H, Wang A 1985 Radiographic and histologic analyses of stress fracture in rabbit tibias. *Am J Sports Med* **13**:285–294.
- Muir P, Johnson KA, Ruaux-Mason CP 1999 In vivo matrix microdamage in a naturally occurring canine fatigue fracture. *Bone* **25**:571–576.
- Judex S, Gross TS, Zernicke RF 1997 Strain gradients correlate with sites of exercise-induced bone-forming surfaces in the adult skeleton. *J Bone Miner Res* **12**:1737–1745.
- Lanyon LE 1992 Control of bone architecture by functional load bearing. *J Bone Miner Res* **7**:S369–S375.
- Hsieh Y, Turner CH 2001 Effects of loading frequency on mechanically induced bone formation. *J Bone Miner Res* **16**:918–924.
- Newhall KM, Rodnick KJ, van der Meulen MC, Cater DR, Marcus R 1991 Effects of voluntary exercise on bone mineral content in rats. *J Bone Miner Res* **6**:289–296.
- Raab DM, Smith EL, Crenshaw TD, Thomas DP 1990 Bone mechanical properties after exercise training in young and old rats. *J Appl Physiol* **68**:130–134.
- Umemura Y, Ishiko T, Yamauchi T, Kuronon M, Mashiko S 1997 Five jumps per day increase bone mass and breaking force in rats. *J Bone Miner Res* **12**:1480–1485.
- Forwood MR, Parker AW 1991 Repetitive loading, in vivo, of the tibiae and femora of rats: Effects of repeated bouts of treadmill running. *Bone Miner* **13**:35–46.
- Fredericson M, Bergman A, Hoffman KL, Dillingham MS 1995 Tibial stress reaction in runners: Correlation of clinical symptoms and scintigraphy with a new magnetic resonance imaging grading system. *Am J Sports Med* **23**:472–481.
- Burger EH, Klein-Nulend J, Veldhuijzen JP 1992 Mechanical stress and osteogenesis in vitro. *J Bone Miner Res* **7**:S397–S401.
- Bennell K, Page C, Khan K, Warrington S, Plant D, Thomas D, Palamara J, Williams D, Wark JD 2000 Effects of resistance training on bone parameters in young and mature rats. *Clin Exp Pharmacol Physiol* **27**:88–94.
- Gowen M, Mundy G 1986 Actions of recombinant interleukin 1, interleukin, and interferon-gamma on bone resorption in vitro. *J Immunol* **136**:2478–2482.
- Kimble RB, Vannice JL, Bloedow DC, Thompson RC, Hopfer W, Kung VT, Brownfield C, Pacifici R 1994 Interleukin-1 receptor antagonist decreases bone loss and bone resorption in ovariectomized rats. *J Clin Invest* **93**:1959–1967.
- Nakamura H, Yoshino S, Shiga H, Tanaka H, Katsumata S 1997 A case of spontaneous femoral neck fracture associated with multicentric reticulohistiocytosis: Oversecretion of interleukin-1beta, interleukin-6, and tumor necrosis factor alpha by affected synovial cells. *Arthritis Rheum* **40**:2266–2270.
- Damoiseaux JG, Döpp EA, Calame W, Chao D, Macpherson GG, Dijkstra CD 1994 Rat macrophage lysosomal membrane antigen recognized by monoclonal antibody ED1. *Immunology* **83**:140–147.
- Dijkstra CD, Döpp EA, Joling P, Kraal G 1985 The heterogeneity of mononuclear phagocytes in lymphoid organs: Distinct macrophage subpopulations in rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology* **54**:589–599.
- Siminia R, Dijkstra CD 1986 The origin of osteoclasts: An immunohistochemical study on macrophages and osteoclasts in embryonic rat bone. *Calcif Tissue Int* **39**:263–266.
- Miao D, Scutt A 2002 Recruitment, augmentation and apoptosis of rat osteoclasts in response to short-term treatment with 1, 25-dihydroxyvitamin D3 in vivo. *BMC Musculoskel Dis* **3**:16 (online).
- Hallen JM, Kaija H, Stepan JJ, Vihko P, Vaananen HK 1998 Studies on the protein tyrosine phosphatase activity of tartrate-resistant acid phosphatase. *Arch Biochem Biophys* **352**:97–102.
- Janckila AJ, Cardwell EM, Yam LT, Li CY 1995 Hairy cell identification by immunohistochemistry of tartrate-resistant acid phosphatase. *Blood* **85**:2839–2844.
- Miyamoto A, Kunisada T, Hemmi H, Yamane T, Yasuda K, Yamazaki H, Hayashi SI 1998 Establishment and characterization of an immortal macrophage-like cell line inducible to differentiate to osteoclasts. *Biochem Biophys Res Commun* **242**:703–709.
- Dreyer CW, Pierce AM 2001 The localization of ED1 antibody in the resorbing hard tissues of the periodontium. *Aust Orthod J* **17**:35–40.
- Nakamura K, Sahara N, Deguchi T 2000 Temporal changes in the distribution and number of macrophage-lineage cells in the periodontal membrane of the rat molar in response to experimental tooth movement. *Arch Oral Biol* **46**:593–607.
- Turner CH, Forwood MR, Rho J-Y, Yoshikawa T 1994 Mechanical loading thresholds for lamellar and woven bone formation. *J Bone Miner Res* **9**:87–97.
- Robling AG, Burr DB, Turner CH 2001 Recovery periods restore mechanosensitivity to dynamically loaded bone. *J Exp Biol* **204**:3389–3399.
- Tate MLK, Steck R, Forwood MR, Niederer P 2000 In vivo demonstration of load-induced fluid flow in the rat tibia and its

- potential implications for processes associated with functional adaptation. *J Exp Biol* **203**:2737–2745.
44. Turner CH, Takano Y, Owan I 1995 Aging changes mechanical loading thresholds for bone formation in rats. *J Bone Miner Res* **10**:1544–1549.
  45. Athanasou NA 1999 Normal skeletal structure and development. In: *Colour Atlas of Bone, Joint, and Soft Tissue Pathology*. Oxford University Press, Oxford, UK, pp. 3–4.
  46. Robling AG, Burr DB, Turner CH 2000 Partitioning a daily mechanical stimulus into discrete loading bouts improves the osteogenic response to loading. *J Bone Miner Res* **15**:1596–1602.
  47. Notomi T, Ukimoto N, Okazaki Y, Tanaka Y, Nakamura T, Suzuki M 2001 Effects of tower climbing exercise on bone mass, strength, and turnover in growing rats. *J Bone Miner Res* **16**:166–174.

Address reprint requests to:  
*Ann E Barr, PT, PhD*  
*Department of Physical Therapy*  
*College of Allied Health Professions*  
*Temple University*  
*3307 North Broad Street*  
*Philadelphia, PA 19140, USA*  
*E-mail: aebarr@temple.edu*

Received in original form January 24, 2003; in revised form May 13, 2003; accepted June 10, 2003.