

Electromagnetic Fields Can Affect Osteogenesis by Increasing the Rate of Differentiation

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Electromagnetic fields of various kinds can alter osteogenesis in animals with osteotomies and patients with nonunions, but the underlying cellular mechanisms are unknown. The aims of this study were to determine whether 1 gauss at 60 Hz affected periosteal proliferation and differentiation in either the normal rat tibia or 1 to 14 days after a surgically induced defect. In the injured rats, using histologic study, autoradiography, and morphometry, it was found that exposure for 1 or 3 days had no effect on proliferation but that it produced an increase in osteoblasts 3 days after the injury. Proliferation and differentiation were unaffected by exposure in the absence of injury. The results suggest that the primary effect of the fields was to promote differentiation but not proliferation. Because fields can stimulate proliferation of osteoblastlike cells *in vitro*, the results of this study may indicate the presence

of an *in vivo* factor that antagonizes the tendency of fields to increase mitotic activity.

Animal studies have shown the phenomenon of electrically induced osteogenesis, as assessed on the basis of histologic, biomechanical, and cellular endpoints.^{4,9,13,24,26,29,31} Although not every study reports the effect, and not all positive reports describe a beneficial effect, it generally has been observed that there exists a threshold and upper limit within which stimulatory effects of electromagnetic fields on bone growth are produced.^{6,14,16} The mechanisms leading to observed effects have not been established, and various theories have been proposed.^{5,12,17,18,21,22,25,30}

At least 3 processes could account for the effects of fields on bone. Fields could increase proliferation of osteoprogenitor cells, the most differentiated osteoblast precursors retaining the ability to proliferate. They could modulate the fraction of osteoprogenitor cells destined to differentiate into osteoblasts. Increased proliferation and differentiation are capable independently of increasing the osteoblast population, thereby increasing the net amount of synthesized matrix without altering mean matrix production per cell. Finally, fields could serve as an agonist for 1 or more

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of the cytokines that control matrix synthesis; osteoblast metabolism would then be upregulated, leading to more matrix synthesis per cell. Various *in vitro* studies provide support for all 3 possibilities^{10,11,18,19,23,27}; thus, *in vivo* studies are needed to facilitate evaluation of the relative importance of the various possibilities.

If fields affected proliferation in animal or human studies, it would be plausible to expect that the effect might escape regulation in some cases, leading to oncogenesis. But despite many animal studies and a diverse clinical experience,^{3,8,16} no instances of field induced oncogenesis have been documented. This is some evidence that exposure does not increase mitosis. In addition, in human studies the field induced bone growth typically was confined to the fracture gap and adjacent bone and did not occur in more distant areas, even though such areas experienced a stimulus that was comparable with that experienced by cells in the fracture gap.^{3,8} This observation also suggests that fields primarily affect differentiation, not proliferation, because an effect on proliferation would be expected to result in callus formation on cortical bone in regions distant from the fracture gap, events that are not observed. The effect of field exposure on resting and injury induced proliferation and differentiation was measured in an animal model as a means of assessing the mechanism that mediates the effect of electromagnetic fields on bone.

SUBJECTS AND METHODS

Animals

Mature male Fischer rats (Harlan Sprague-Dawley, Indianapolis, TN) were used in all studies; they were caged individually with a light to dark cycle of 12:12 (light commencing at 6:00 am.), and fed and watered on demand. The animals were not used until they reached a body weight of 200 grams, which required a minimum of 1 week after they were received.

A unilateral defect was made in the anteromedial region of the tibia, inferior to the saphenous artery bifurcation; the data reported here pertain to the response within this region of interest, which was 6 x 2 mm (located 7—3 mm distal to the tibial crest notch). After the animals were anesthetized (sodium

pentobarbital, intraperitoneal, 50 mg/kg), the limb was shaved, and a 1.5-cm incision was made through the skin directly over the tibial crest, with care not to injure the underlying bone and adjacent muscle. The superficial fascia was separated from the overlying skin on all sides of the incision, and the skin was retracted to expose the tibia. In 1 group of rats, a defect 1.1 mm in diameter and approximately 0.5 mm deep was placed in the center of the region of interest using a hand held jeweler's burr. To eliminate an influence of the endosteum and marrow on the healing response, care was taken to ensure that the defect did not penetrate the medullary cavity.

After the operation, the rats were assigned randomly to 1 of 3 groups (30 rats/group). The first group was exposed to the fields for 24 hours commencing at recovery from the anesthesia. The rats in the second group were exposed for 3 days (from anesthesia recovery), and the third group received no exposure (sham field group). To control for the effect of fields on bone in the absence of the surgically induced defect, the entire procedure was repeated using rats that had not undergone surgery; the animals in this part of the study were exposed for 1 or 3 days or were sham exposed.

The rats were sacrificed 1, 2, 3, 5, 7, and 14 days after commencement of exposure or sham exposure (5 surgically treated rats and 5 rats not surgically treated in each exposure group at each time interval); 5 to 7 animals were treated at the same time per pair of coils. All rats were given tritiated thymidine 1 hour before sacrifice (intraperitoneal, 1 μ Ci/gram of body weight, diluted with sterile water to a final volume of 0.5 ml, specific activity 2 Ci/mmol, ICN Biochemicals, Irvine, CA).

All animal procedures, including operations, radioisotope injections, and sacrifices (carbon dioxide suffocation) were done between 10:00 am. and 2:00 p.m. to minimize potential effects related to circadian rhythms.

Apparatus

A magnetic field was generated using a pair of coils with radii of 65 cm. Each coil (250 turns of 21-gauge magnet wire) was wound around a wooden core and held in place using plywood sheets glued together to form a sandwich; the intercoil distance was 65 cm. The coil current was obtained from a wave generator (Model 182 A, Wavetek, San Diego, CA) that was amplified (Model 7500, Krohn-Hite, Avon, MA) and

then applied to the coils. Coil current was monitored continuously (Model 175, Keitliley, Cleveland, OH), and the resulting magnetic field in the intercoil region was measured with a flux probe (Model 1846, Magnalab, Boulder, CO). The animals were housed in plastic cages with plastic tops and water bottles, resulting in a completely nonmetallic environment. The applied field was 1 gauss (rms), 60 Hz, and was homogeneous to $\pm 5\%$. It was chosen for convenience because a wide array of electromagnetic fields (including 1 gauss, 60 Hz) have been used in animal and clinical studies. As a consequence of the comparability of the applied field to the fields used in other studies, the induced current densities also are comparable; their precise characteristics would depend on the tissue model used for their calculation.

A second unit, identical to the exposure unit in all respects except for the absence of the coils inside the plywood sheets, was used to provide sham exposure. The exposure and sham exposure units were located in the same room, separated by approximately 2.5 meters. The fringing fields at the site of the sham exposed animals was less than 1% of that experienced by the animals in the exposure unit. The geomagnetic field in the animal room (measured using a fluxgate magnetometer, MAG-OI, 6MW, Redwood City, CA) was 0.54 gauss (vertical) and 0.20 gauss (horizontal).

Tissue Processing

The region of interest was recovered, processed, embedded in glycol methacrylate, and sectioned completely at 4 μm in the longitudinal plane. For each animal, sets of 3 sections were selected from the middle of the defect, from halfway between the middle of the defect and its medial edge, and from halfway between the middle and lateral edge. In the rats that did not receive the bone defect, sections were selected as if the defect were present.

One section in each trio was processed for autoradiography and counterstained with van Gieson's, the second was stained with methyl green and thionin, and the third with toluidine blue and basic fuchsin.^{1,28} Additional details regarding the histologic methodology and the criteria for identification of pertinent cell types are given elsewhere.¹⁵

Measurements

Quantitative determinations of proliferation, osteoblastic concentration, and callus formation were made within ± 3 mm from the center of the defect; in the tibias that lacked the defect, the 6-mm length was

anatomically comparable with that studied in the animals with bone injury. Proliferation was assessed in the autoradiographs by counting the number of labeled cells (≤ 5 grains/nucleus) in the cambium and within the defect; the counts were normalized by the length of the conical bone surface along which the labeled cells were located, and the value used in all subsequent calculations was the mean of the 3 representative sections, expressed as cell count per millimeter of bone. The cell counts were expressed as linear densities, rather than ratios (mitotic index, for example), to ensure that the measurements characterized only 1 dependent variable, not a confounded pair of variables. The distance of each labeled cell in the cambium from the intact cortical bone surface and the distance of each cell in the defect from the location of the original cortical surface were measured.

Differentiation was assessed by counting the number of osteoblasts (methyl green and thionin sections) in the cambium and within the defect. The osteoblast count also was normalized by the length of the bone surface and expressed as the number of osteoblasts per millimeter of bone. The results were averaged for 3 sections, and the mean was used in all statistical evaluations.

Periosteal callus thickness was measured (toluidine sections) from the original bone surface to the superficial edge of the callus at 200- μm intervals along the cortical surface. All morphometric measurements were made using a computer based system (Bioquant System IV, R&M Biometrics, Nashville, TN). The data were evaluated using the unpaired *t* test, analysis of variance, and analyses for statistical power ($\beta = 0.8$) and linear trend; the significance level for all tests was 0.05.

RESULTS

Animals Not Surgically Treated

Baseline cambial proliferation in the region of interest was not altered by either 1 or 3 days' exposure (Fig 1). The number of osteoblasts (activated bone lining cells) normally present on the cortical surface in the resting periosteum was significantly lower in the 3-day (but not 1-day) exposed animals (Fig 2)

The Cambium After Injury

Osteoid formation on the cortical surface adjacent to the defect began on Day 1; attachment of the fibrous periosteum at the defect

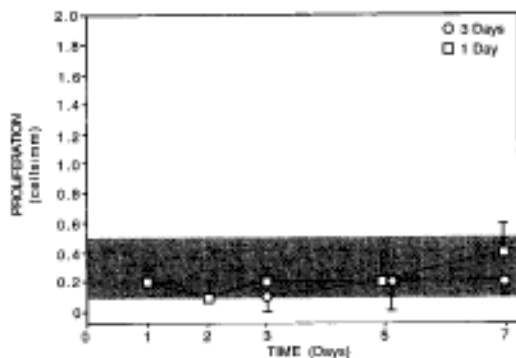


Fig 1. Proliferation in the cambium in rats not surgically treated but exposed to electromagnetic fields (1 gauss, 60 Hz, commencing at time = 0) for 1 or 3 days (mean \pm standard error). Stippled region indicates mean \pm standard error of control (unexposed) rats. N = 5 at each time point.

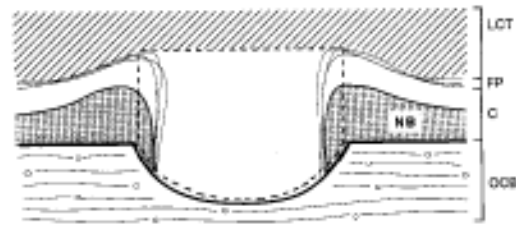


Fig 3. Tissue compartments within the region of interest (6 x 2 mm) at 7 days after operation. OCB: original cortical bone; C: cambium; FP: fibrous periosteum; LCT: loose connective tissue. The stippled region in the cambium is the new bone (NB) deposited on the original cortical surface. The injury site (dashed line) is the cylindrical region centered on the bone defect and bounded inferiorly by the original cortical bone and superiorly by the loose connective tissue. The bone defect was 1.1 mm in diameter and 0.5 mm at its deepest point.

margin occurred during Day 2 and was followed by centripetally directed bone growth into the defect. Thus, dynamic assessment of the cellular changes required consideration of events in the cambium and the defect (Fig 3).

Proliferation in the cambium adjacent to the defect apparently was not altered by either 1 or 3 days of field exposure; in both

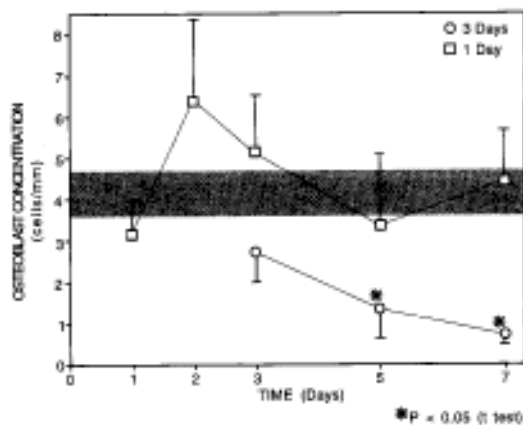


Fig 2. Osteoblast concentration in the cambium in rats not surgically treated but exposed to electromagnetic fields (1 gauss, 60 Hz, commencing at time = 0) for 1 or 3 days (mean \pm standard error). Stippled region indicates mean \pm standard error of control (unexposed) rats. N = 5 at each time point.

groups, peak proliferation occurred 2 days after injury and was 150 times greater than baseline (Fig 4). The perpendicular distance between the proliferating cells and the cortical bone surface increased progressively in all 3 surgically treated groups during the week after operation (Fig 5) because of systematic displacement of cambial cells by callus formation on the original bone surface. Fourteen days after injury, the mean distance of the proliferating cells from the cortical surface in the 3-day exposed group was sig

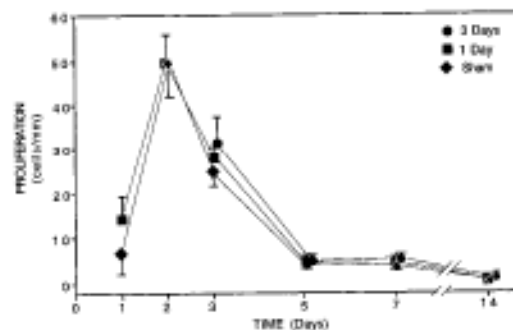


Fig 4. Proliferation in the cambium in rats with surgically induced defects exposed to electromagnetic fields (1 gauss, 60 Hz) for 1 or 3 days (mean \pm standard error); operation and commencement of exposure at time = 0. N = 5 at each time point.

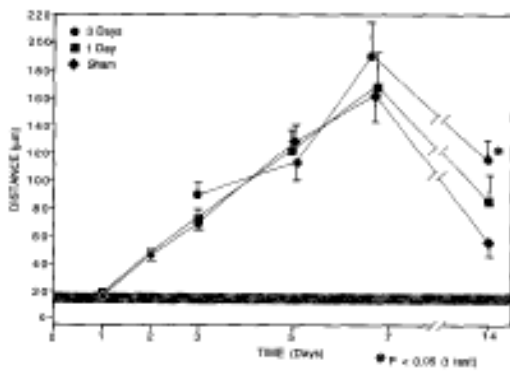


Fig 5. Distance between proliferating cells in the cambium and original bone surface in surgically treated rats exposed to electromagnetic fields (1 gauss, 60 Hz, commencing at time = 0) for 1 or 3 days (mean \pm standard error). Stippled region is the mean distance \pm standard error for unoperated rats (N = 5).

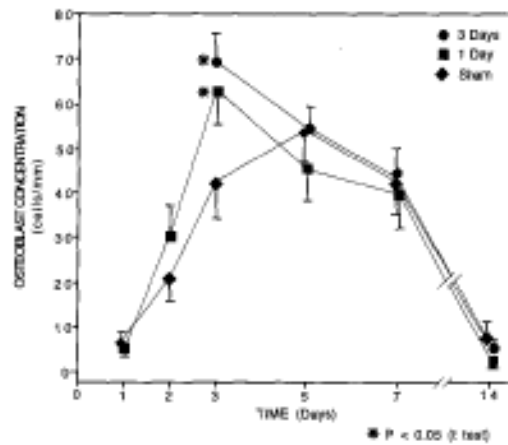


Fig 6. Osteoblast concentration in the cambium in rats with surgically induced defects exposed to electromagnetic fields (1 gauss, 60 Hz) for 1 or 3 days (mean \pm standard error); operation and commencement of exposure at time = 0. N = 5 at each time point.

nificantly greater than that of the sham exposed group.

The osteoblasts observed on Day 1 were derived solely from activation of the bone lining cells, as evidenced by the absence of labeled osteoblasts in animals recovered 24 hours after labeling (data not reported); by Day 2, the cortical surface was covered completely with osteoblasts, indicating that the activation process was complete. In both exposed groups, osteoblast concentration was significantly greater than that in the sham exposed rats at 3 days after injury (Fig 6). Because activation of bone lining cells was complete by Day 2 and there was no field induced increase in proliferation (Fig 4), the field induced increase in osteoblasts (Fig 6) probably was attributable to enhanced differentiation from the parent population of activated osteoprogenitor cells. The osteoblast concentration at 3 days was proportional to the time of exposure (linear trend analysis, $p < 0.05$).

With the possible exception of the group exposed for 3 days and evaluated 7 days after operation, the field induced increase in osteoblasts was not reflected in the amount of callus measured on the cortical surface adjacent to the defect (Fig 7).

The Bone Defect

Clot initially filled the defect and was replaced by granulation tissue beginning on Day 3, as evidenced by the initial appearance of proliferating cells (Fig 8). The proliferating cells in the cambium (Fig 4) were alka

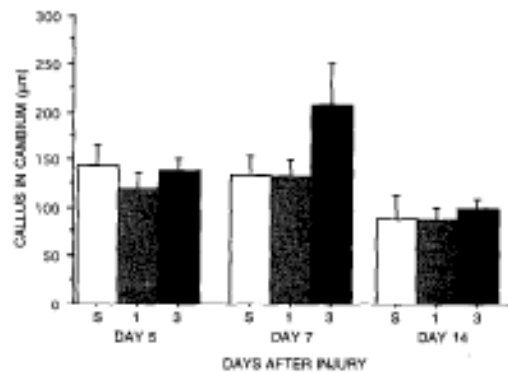


Fig 7. Callus formed on the cortical surface adjacent to the defect in rats exposed to electromagnetic fields (1 gauss, 60 Hz) for 1 or 3 days, or sham exposed (5) (mean \pm standard error); operation and commencement of exposure at time = 0. N = 5 in each group at each time point. Results of all intergroup comparisons lacked statistical significance.

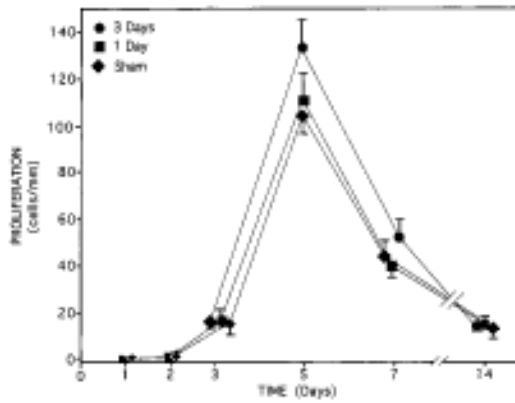


Fig 8. Proliferation in the defect in surgically treated rats exposed to electromagnetic fields (1 gauss, 60 Hz) for 1 or 3 days (mean \pm standard error); operation and commencement of exposure at time = 0. N = 5 at each time point.

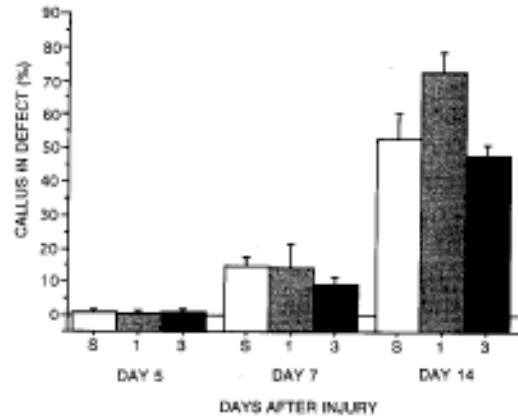


Fig 10. Callus formed in the defect in rats exposed to electromagnetic fields (1 gauss, 60 Hz) for 1 or 3 days or sham exposed (S) (mean \pm standard error); operation and commencement of exposure at time = 0. N = 5 in each group at each time point. Results of all intergroup comparisons lacked statistical significance, except 1 versus 3 at Day 14 ($p < 0.05$).

line phosphatase positive, but those in the defect consisted of positive and negative cells¹⁵ (osteoprogenitors and fibroblasts, respectively). Osteoblasts appeared in the defect between Days 5 and 7 (Fig 9), and by Day 14 the defect was approximately 50% filled with bony callus in the sham exposed

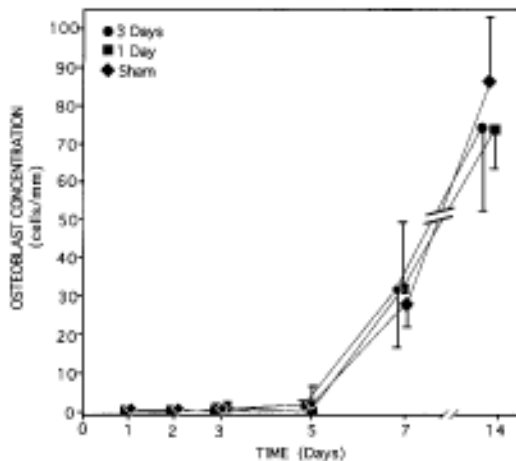


Fig 9. Osteoblastic concentration in the defect in surgically treated rats exposed to electromagnetic fields (1 gauss, 60 Hz) for 1 or 3 days (mean \pm standard error); operation and commencement of exposure at time = 0. N = 5 at each time point.

and 3-day exposed animals, but almost 73% filled in the 1-day exposed group (Fig 10).

DISCUSSION

Mitotic activity in the cambium increased significantly after bone injury, but field exposure for 1 and 3 days had no significant effect on baseline or injury induced proliferation (Figs 1 and 4), at least as assessed using the pulse labeling technique. Proliferation within the bone defect similarly was unaffected by exposure, although the possibility of an effect around Day 5 could not be discounted (Fig 8). When contrasted with the *in vitro* findings that fields increased proliferation,^{2,7,10,11,18,19} the results may suggest that an inhibitory factor prevents field induced proliferation *in vivo*. Alternatively, the osteoblastlike cells typically studied in culture may not be an appropriate model for normal osteoblasts, at least with regard to regulatory control of the cell cycle, because of the presence of stimulatory factors. There is no evidence indicating that any particular biologic response is uniquely associated with a specific electro

magnetic field characteristic such as frequency or wave shape. However, there also is no evidence tending to exclude the possibility that particular electromagnetic field parameters might account for interstudy differences, such as the difference between in vitro studies and the results reported here regarding the effect on proliferation.

In contrast to the apparent absence of a field effect on injury induced proliferation (Figs 4 and 8), exposure for 1 and 3 days transiently increased the osteoblast concentration in the periosteum adjacent to the injury site (Fig 6). The increase can be reliably interpreted as increased differentiation of cambial osteoprogenitor cells because all bone lining cells were activated by Day 2, and that presumably is the only other process by which the number of osteoblasts can be augmented significantly. The proportionality between the time of exposure and the number of osteoblasts by Day 3 could mean that the primary transduction event involved structures that mediate differentiation, but the better view probably is that the effect on differentiation observed on Day 3 is strongly and nonlinearly dependent on the timing and duration of the preceding field exposure. A field effect on osteoblasts was not revealed in the defect (Fig 9), but too few measurements were made within the pertinent time interval (5 to 14 days) to provide a fair test of that possibility.

In rats not surgically treated, bone lining cells appeared as osteoblasts or fibroblasts, depending on whether they were activated or resting, respectively. The periosteal osteoblasts did not divide, and osteoblasts were not seen above the layer of bone lining cells; thus, it follows that either differentiation did not occur in the noninjured periosteum or that it occurred with such rarity that it was unobservable. Either alternative could explain the absence of a field induced increase in differentiation in the periosteum of uninjured rats (Fig 2). Good evidence was found that the fields downregulated the other process by which osteoblasts appeared in the

periosteum, activation of bone lining cells (Fig 2). The physiologic consequences of the effect seem dubious because it constitutes a change in a relatively rare process. If it occurred in typical human studies,³ it probably would not have been observed or had clinical consequences. Because activation of bone lining cells probably is at least partly under hormonal control, the observation that 3-day, but not 1-day, exposure affected activation may implicate central nervous system processes in mediating the effect of fields. There is evidence that the neuroendocrine system mediates the effects of chronic field exposure on fracture healing.²⁰ If signal transduction involved the central nervous system, the effect would be irrelevant to clinical studies because they typically involve local application of fields.

An overall unambiguous effect of duration of exposure on callus formation was not observed (Figs 7 and 10), for several possible reasons. First, despite efforts to standardize the injury and eliminate factors not essential in the bone injury response (such as a contribution from marrow cells), the inherent variability in callus formation limited the precision with which any field induced effects could be observed. Consequently, a larger number of rats would have been needed to provide a realistic chance (statistical power of 80%) of observing significant differences attributable to field exposure. For example, in the 3-day exposed group sacrificed after 7 days and the 1-day exposed group sacrificed after 14 days (Figs 7 and 10), 17 and 12 rats would have been required, respectively.

Second, there is no simple relationship between the amount of callus present and the effect of exposure because the appearance of callus varied nonlinearly with time and position in the region of interest. In the cambium, osteoid was present as early as 1 day after operation and reached a maximum in 7 days, after which it was modeled. In contrast, callus within the defect increased (from 0) consistently with time until the defect was filled

with new bone. One possibility is that 3-day exposure retarded bone healing by reducing or delaying the amount of osteoclastic activity in the cambium that would otherwise have occurred (Fig 7) and 1-day exposure increased osteoblastic activity in the defect (Fig 10). Because cambial proliferation occurred mostly in the superior region of the cambium, the evidence that exposure increased the mean distance of the proliferating cells from the cortical surface (Fig 5) could indicate either that the fields increased bone formation or retarded modeling.

Third, callus formation in the defect probably is the most reliable indicator of the effect of any treatment because healing is essentially complete when the defect is filled with new bone. Callus formation in the defect began at about Day 5 (Fig 10) and was complete by Day 21, but the amount of callus was measured at only 2 times within this interval (Fig 10, Days 7 and 14). Thus, the frequency of the data obtained was not sufficient to ascertain the time course of bone growth with the precision needed to assess the effect of field exposure.

The results indicated that the fields affected differentiation but had no material effect on proliferation. Most reported studies used specialized field parameters, and generally available clinical devices normally are used 3 to 8 hours per day. In this study, in contrast, a single frequency field was applied continuously for 1 to 3 days. Because the spectral composition of the applied fields and the duration of exposure could independently influence the nature of the cellular response, the extent to which the result reported in this study can be generalized remains to be determined.

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