12. E. Robbins and P. I. Marcus, *Science* **144**, 1152 (1964).
13. J. J. Decosse and N. Aiello, *J. Histochem. Cytochem.* **14**, 601 (1966).
14. W. J. Dixon and F. J. Massey, Jr., *Introduction to Statistical Analysis* (McGraw-Hill, New York, 1969), p. 308.
15. B. L. Welch, *Biometrika* **38**, 330 (1951).
16. F. M. Kendall, G. Loffredo, C. A. Nicolini, Internal Report 7/75 (Biophysics Division, Temple University Health Sciences Center, Philadelphia, 1975).
17. G. Moser, R. Muller, E. Robins, *Exp. Cell Res.* **91**, 73 (1975).
18. C. Nicolini, *Biophys. Biochim. Acta* **458**, 243 (1976).
19. Supported by National Cancer Institute grants CA-20034, CA-18258, CA-12923, and CA-11463. T.B. is a recipient of research career development award CA-00088.
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22 November 1976

Direct Resorption of Bone by Human Monocytes

Abstract. *Cultured human peripheral blood monocytes stimulate the release of bone mineral and matrix from killed long bones of fetal rats. These effects were inhibited by cortisol but were not altered by hormones that normally stimulate osteoclastic bone resorption. There was no evidence of morphologic differentiation of the monocytes into osteoclasts during bone resorption.*

Monocytes and macrophages are often seen adjacent to bone-resorbing surfaces in patients with chronic inflammatory diseases, such as rheumatoid arthritis, periodontal disease, and cholesteatoma, and also in those with tumors that metastasize to bone. Monocytes and macrophages are also found in the normal bone-marrow cavity adjacent to remodeling endosteal bone surfaces. However, although there is in vivo experimental evidence that osteoclasts may be derived from circulating mononuclear cells (*1*), there has been no evidence that these cells themselves are capable of direct bone resorption. We have now shown that circulating human monocytes can stimulate bone mineral and matrix resorption in vitro.

Human peripheral blood monocytes were obtained from normal blood donors at the Connecticut Red Cross, Farmington, Connecticut. Human leukocytes were obtained from plateletpheresis donations from normal donors. After the platelets had been removed from the other blood cells in the plateletpheresis apparatus, red cells were separated by dextran sedimentation to leave a leukocyte buffy coat. The neutrophils in the leukocyte buffy coat were removed by Ficoll-Hypaque (Pharmacia) density sedimentation. When cells were suspended for 90 minutes in medium containing 20 percent fetal calf serum in plastic petri dishes at 37°C in an atmosphere of 5 percent CO_2 and air, monocytes adhered to the plastic surfaces (*2*). The nonadherent lymphocytes were removed by gently rinsing the plastic petri dishes with fresh, warmed medium. Macrophages were grown as colonies from human bone-marrow aspirates cultured on semisolid mediums (*3*) (McCoy's 5A in methylcellulose—4000 count sec^{-1}) (Fisher) with 10 percent fetal calf serum and 5 percent horse serum. Macrophage colonies were clearly discernible between the 10th and 12th days of culture. The nature of these colonies was confirmed by Wright's staining of smeared cells.

The assay for bone resorption used in these experiments has been described (*4*). Pregnant rats at the 18th day of gestation were subcutaneously injected with 0.2 mc of ^{45}Ca or 1 mc of [^{3}H]proline. The next day, the fetal rat radius, ulna, and tibia were removed, and the mineralized shafts were dissected free from the cartilaginous ends and adjacent soft tissue. The bones were killed by freeze-thawing three times and were then exposed to ultraviolet light for several hours. The bones were either split or left intact and were then cultured with the cell preparations for 5 to 8 days. The cells were cultured in BGJ medium (Gibco) supplemented with either bovine serum albumin (4 mg per milliliter of medium) or 10 percent fetal calf serum inactivated by heat. Bone resorption was measured as the release of ^{45}Ca from the cultured bones into the medium. Values were expressed as means ± standard errors for four pairs of bone cultures. Statistical differences were analyzed with *t*-tests. In some experiments the rat bones were labeled with [^{3}H]proline to measure matrix resorption, expressed as the percent of total radioactivity released into the medium during the period of culture (*5*).

Table 1. Bone mineral release in the presence of cultured human mononuclear cells from paired killed bones previously incorporated with ^{45}Ca. Cells were cultured for 5 days in BGJ medium supplemented with 4 mg of bovine serum albumin per milliliter of medium at a density of 1×10^6 cells per milliliter. Results are expressed as ratios of ^{45}Ca released from killed bones cultured with the indicated cells to that released from paired bones cultured without the cells. Values are means ± standard errors for four pairs of bone cultures.

Culture	Ratio (treated-to-control)
Mononuclear cells	1.37 ± .14*
Adherent mononuclear cells	1.30 ± .03*
Nonadherent mononuclear cells	1.13 ± .03*

*Significantly greater than 1.0 ($P < .05$, *t*-test).

When human mononuclear cells, from which neutrophils were separated by Ficoll-Hypaque sedimentation, were cultured with killed bones for 5 to 8 days, there was an increase in both ^{45}Ca release (Table 1) and [^{3}H]proline release from the bones compared with the comparison (paired) bones cultured without cells. When the cells were cultured for 8 days with four pairs of bones previously labeled with [^{3}H]proline, the treated-to-control ratios for radioactivity released into the medium were 1.52 ± 0.13 ($P < .01$; Table 1). The greatest increase in mineral release from the killed bones occurred between the 4th and the 8th days of culture (Table 2). The mononuclear cells were much more effective in resorbing killed bone than the nonadherent population ($P < .01$; Table 1).

Monocytes are not the only cells we have found to cause bone resorption directly. Fetal rat calvarial cells also cause matrix resorption and mineral release from dead bones, although their effects are usually much greater than those of monocytes (*6*). During an 8-day period, calvarial cells cause a 40 to 200 percent increase in mineral release compared with paired bones cultured without cells, whereas monocytes usually cause a 30 to 60 percent increase in mineral release. Marrow macrophages are also capable of resorbing killed bone. When colonies of macrophages obtained from normal human bone marrow aspirate were cultured on semisolid mediums, adjacent but noncontiguous killed long bones of fetal rats were visibly resorbed during a 48-hour period between the 10th and 12th days of culture. None of the other cells that have been tested under these conditions [including nonadherent human lymphocytes (Table 1), rat fibroblasts, and nonadherent rat spleen cells] have caused mineral to be released from killed bones.

Hormones that normally stimulate osteoclastic bone resorption and mineral release from live bones in organ culture —such as parathyroid hormone (PTH), osteoclast activating factor (OAF), 1_{α},25-dihydroxycholecalciferol [1_{α},25-$(OH)_2D$], and prostaglandins-E_2 (PGE_2) —did not increase mineral release from killed bones stimulated by mononuclear cells during an 8-day period (Table 2). In a separate experiment (experiment 2), salmon calcitonin (SCT) (100 milliunits per milliliter of medium) did not inhibit

Table 2. Effects of hormones (*13*) on the release of ^{45}Ca from paired, killed, long bones of fetal rat. The bones were cultured with mononuclear cells in the presence (treated group) or absence (control group) of the indicated hormone. Cells were cultured for 8 days in BGJ medium with 10 percent fetal calf serum inactivated by heat. Fifty percent of the medium was replaced by fresh medium on day 4. The cell density was 2×10^6 cells per milliliter of medium. Values are expressed as mean percent change ± standard errors (compared with the paired culture) for four pairs of bone cultures.

Hormone treatment	Percent change during days		
	0 to 4	4 to 8	0 to 8
	Experiment 1		
No hormone	12 ± 11	35 ± 14*	27 ± 10*
PTH	3 ± 4	4 ± 1	3 ± 1
OAF	10 ± 8	−1 ± 1	−2 ± 1
$1_\alpha,25(OH)_2D$	23 ± 14	−11 ± 13	1 ± 5
PGE_2	3 ± 15	1 ± 6	2 ± 7
	Experiment 2		
No hormone	20 ± 3*	81 ± 5*	61 ± 5*
SCT	2 ± 4	18 ± 18	2 ± 9
Cortisol	−10 ± 5	−33 ± 4*	−28 ± 4*

*Significantly different from 0.0 ($P < .05$, t-test).

bone resorption stimulated by mononuclear cells (Table 2). However, cortisol at $10^{-6}M$ significantly inhibited ^{45}Ca release during 8 days of culture. The effect of cortisol may have resulted from inhibition of lysosomal enzyme release from the mononuclear cells. Lysosomal enzymes may be responsible for bone mineral and matrix resorption (*7*), and cortisol inhibits the release of lysosomal enzymes from live bones (*8*) and peritoneal monocytes (*9*) in culture.

The effect of mononuclear cells on bone resorption was not a result of changes in the pH of the medium produced by the cultured cells. Parallel treatment groups of monocytes and killed bone were cultured for 8 days at adjusted pH and without modification of pH. The pH was maintained in one group by adding 0.01 ml of $0.15M$ $NaHCO_3$ daily. In the other group, $0.01M$ isotonic NaCl was added daily. In the group treated with $NaHCO_3$, the pH at the beginning of culture was 7.28 and at the end was 7.45. The monocytes increased ^{45}Ca in the medium by 21 ± 3 percent, compared with paired control bones to which $NaHCO_3$ was also added. In the cultures treated with NaCl, the pH at the beginning of the experiment was 7.28 and at the end was 6.80. The increase in ^{45}Ca in the medium compared with the corresponding paired bones was 33 ± 4 percent. Thus, in this experiment, mononuclear cells stimulated bone resorption irrespective of substantial changes in pH.

To test whether mononuclear cells need to grow directly on bone to produce resorption, monocytes were cultured in liquid mediums with bones suspended on wire meshes at the interface between the air and the medium, floating free in the medium, or on the plastic floor of the culture well with the mononuclear cells in direct contact. Under these conditions in two separate experiments, there were no significant differences in the increase in ^{45}Ca release from the killed bones. The results suggest that bone resorption stimulated by mononuclear cells does not require direct apposition of the mononuclear cells on the endosteal bone surface, as the bones suspended on screens were resorbed to the same degree as the bones in direct contact with the cells. This hypothesis was confirmed by culturing six pairs of killed bones (i) in cell-free medium previously conditioned by the presence of monocytes and (ii) in control medium. Adherent mononuclear cells were cultured for 8 days without bone. Cell-free medium from this culture was then added to killed fetal rat bones for 8 days, and control medium that had not been incubated with cells was added to paired killed bones from the same litter. There was a significant increase in ^{45}Ca release from the bones cultured with the conditioned medium (treated-to-control ratio, 1.31 ± 0.06; $P < .05$).

There was no evidence during the period in which we cultured human mononuclear cells that there was any differentiation or transformation of the mononuclear cells cultured for as long as 10 days on endosteal bone surfaces. At the end of this period, the bones were fixed and decalcified in Bouin's fixative, stained with hematoxylin and eosin, and examined by light microscopy. All the cells seen in these sections were either mononuclear or binuclear cells with the morphologic appearances of normal monocytes or macrophages.

These experiments show that circulating human mononuclear cells and macrophages are capable of stimulating mineral and matrix resorption from dead bone in organ culture. These cells or their progeny may play a role in normal endosteal bone resorption. They may also be of importance in the pathogenesis of bone resorption that occurs in a number of pathological states. In these situations, the release of local humoural agents such as PGE_2 and OAF by the chronic inflammatory cells may stimulate osteoclasts to resorb the adjacent bone (*10*). This possibility is supported by the observation that prostaglandins are present in increased concentrations in tissues invaded by chronic inflammatory cells (*11*). The OAF might also be released by antigen-stimulated lymphocytes adjacent to areas of bone resorption in chronic inflammatory disease.

Our experiments suggest another mechanism for bone resorption adjacent to areas of chronic inflammation, namely, that monocytes and macrophages directly cause resorption independent of osteoclast mediation. It is also possible that monocytes and macrophages could cause part of the bone resorption that occurs in some patients with malignant diseases. Monocytes and macrophages are often found adjacent to tumor cells (*12*), and they may contribute to the local bone resorption that occurs at the site of osteolytic bone metastases.

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References and Notes

1. D. A. Fischman and E. D. Hay, *Anat. Rec.* **143**, 329 (1962); D. G. Walker, *Endocrinology* **91**, 916 (1972); D. G. Walker, *Science* **190**, 784 and 785 (1975); G. Göthlin and J. L. E. Ericsson, *Virchows Arch. B.* **12**, 318 (1973); K. Buring, *Clinical Orthopaedics and Related Research* **110**, 293 (1975).
2. Z. A. Cohn, *Methods Enzymol.* **32**, 758 (1974).
3. D. L. Pike and W. A. Robinson, *J. Cell Physiol.* **76**, 77 (1970).
4. L. G. Raisz, *J. Clin. Invest.* **44**, 103 (1965); C. L. Trummel, G. R. Mundy, L. G. Raisz, *J. Lab. Clin. Med.* **85**, 1001 (1975).
5. J. S. Brand and L. G. Raisz, *Endocrinology* **90**, 479 (1972).
6. G. R. Mundy, J. G. Bandelin, A. J. Altman, unpublished observations.
7. G. Vaes, in *Lysosomes in Biology and Pathology*, J. T. Dingle and H. B. Fell, Eds. (North-Holland, London, 1969), p. 217.
8. H. B. Fell and L. Thomas, *J. Exp. Med.* **114**, 343 (1961); H. B. Fell and L. Weiss, *ibid.* **121**, 551 (1965).
9. E. Wiener and Y. Marmary, *Lab. Invest.* **21**, 505 (1969).
10. L. G. Raisz, C. L. Trummel, G. R. Mundy, R. A. Luben, in *Calcium Regulating Hormones*, R. V. Talmage, M. Owen, J. A. Parsons, Eds. (Excerpta Medica, Amsterdam, 1975), p. 150.
11. J. M. Goodson, F. Dewhirst, A. Brunetti, *J.*

Dent. Res. **52**, 182 (19 → D. R. Robinson, A. J. Tashjian, Jr., L. Levin, *J. Clin. Invest.* **56**, 1181 (1975).
12. P. Alexander, *Annu. Rev. Med.* **27**, 207 (1976).
13. The PTH (400 μg/ml) was provided by G. Aurbach; OAF was obtained from normal activated human leukocytes (*10*); $1_{\alpha},25(OH)_2D$ ($10^{-9}M$) was provided by H. DeLuca; PGE_2 ($10^{-5}M$) was from Upjohn; SCT (100 milliunits per milliliter) was from Armour; and cortisol ($10^{-6}M$) was from Sigma.
14. G.R.M. is the recipient of an American Cancer Society Faculty Research Award, FRA-148. Supported by grant DT-49 from the American Cancer Society and by contract NO1-CB-6-3980 from the National Cancer Institute.

30 September 1976; revised 23 November 1976

Apical Dichotomy Demonstrated in the Angiosperm *Flagellaria*

Abstract. *An equal dichotomy of an angiosperm vegetative apex, previously suspected on morphological grounds, is demonstrated both externally in whole apices and in histological preparations. Dichotomy is a normal developmental feature with complete continuity in growth from the simple to the bifurcated state. The accessibility of this rare phenomenon in this plant makes further, possibly experimental, approaches feasible.*

Recent collections from New Guinea have provided what is, to our knowledge, the first indisputable demonstration of equal dichotomy of the vegetative shoot apex as a normal developmental feature in an angiosperm, the monocotyledonous liane *Flagellaria indica* L. (Flagellariaceae). Branching in existing seed plants (gymnosperms and angiosperms) has been thought to occur exclusively by axillary branching, with a lateral meristem produced as an obvious appendage to the parent shoot apex, usually in the axil of a leaf. This lateral meristem is usually initiated relatively late and on the side of the parent apex, so disturbing its organization minimally. The converse of this, apical dichotomy, has been thought to be a primitive feature of vascular plants, although developmental details are lacking (*1*, *2*).

A number of recent reports, however, have produced circumstantial evidence for apical dichotomy of the vegetative axis in angiosperms, mostly in the monocotyledons—as in the Palmae (*Chamaedorea*) (*3*), (*Hyphaene*) (*4*), (*Nypa*) (*5*), Strelitziaceae (*Strelitzia*) (*6*), and Flagellariaceae (*Flagellaria*) (*7*)—but also in one dicotyledonous family, Cactaceae (*Mamillaria*) (*8*). Other examples relate to specialized lateral appendages, for example, *Asclepias* (*9*) and certain Alismatideae (*10*). In the examples of vegetative dichotomy, convincing demonstration of the process of dichotomy has always been difficult either because the apex is inaccessible, or because it is so small that dichotomous branching is not easily distinguished from early stages of leaf inception, or for both of these reasons. In some examples, material is difficult to obtain. This has meant also that it has not been possible to study changes in cytohistological zonation of apices during the process of bifurcation.

Flagellaria indica is an exception. It is a sprawling vine of wet and often disturbed sites common throughout the Old World tropics (*11*). Aerial shoots arise from a sympodial rhizome system and are supported by leaf-tip tendrils. Leaf arrangement is distichous, so that precise planes for sectioning are available and the shoot apex is conical and stands well above the youngest leaf primordium (Fig. 1A). Axillary branching is restricted to the underground rhizome that generates the aerial shoots, and to the paniculate inflorescence, which terminates each branch of the aerial system. Branching of the vegetative aerial shoots is by regular bifurcation at distant intervals. However, there are no axillary meristems in the aerial shoots. An earlier study (*7*) of limited material produced good evidence that bifurcation was the result of equal dichotomy of the vegetative apex, but it was deficient in convincing photographic documentation. A more recent extensive collection from a locality 4 miles east of Port Moresby, Papua, New Guinea, produced abundant material, which made good this deficiency. Material was fixed in formalin–acetic acid–alcohol, subsequently dissected in absolute ethyl alcohol and stained in acid fuchsin. A series of apices showing progressive stages in dichotomy were obtained and photographed by an epi-illumination technique (*12*). The same specimens were then processed by routine histological methods and sectioned in a median longitudinal plane. This allowed surface and sectional views of the same apex to be compared (Fig. 1, B–E). As well, some apices were pre-

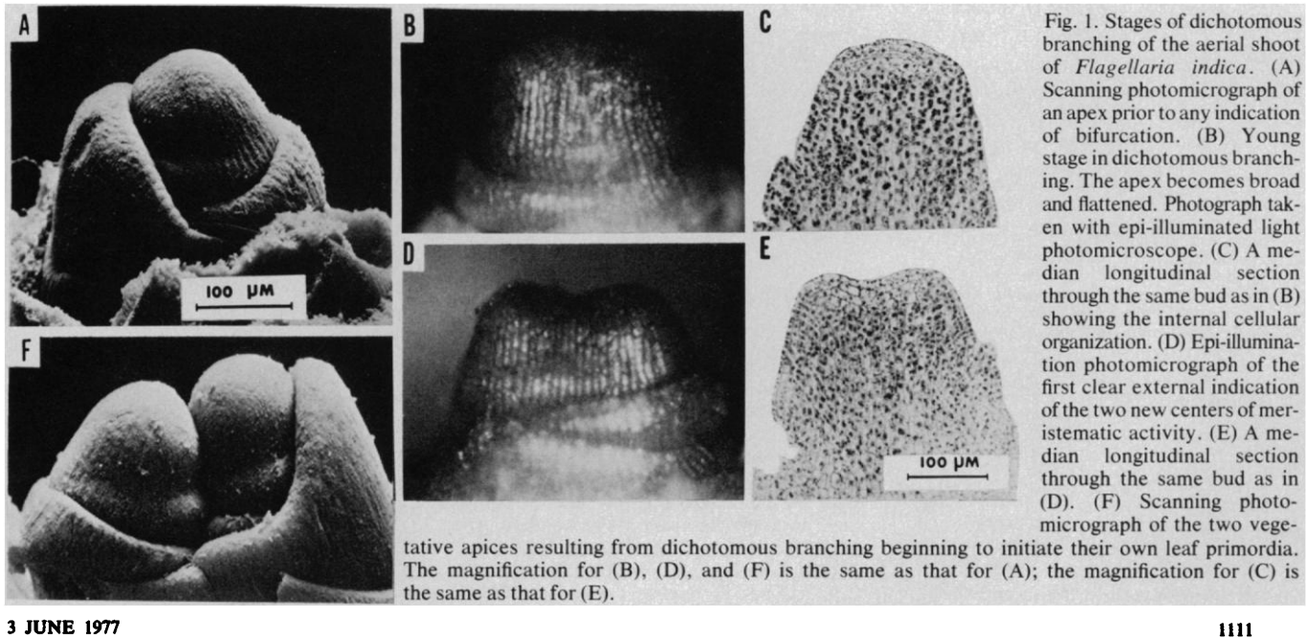

Fig. 1. Stages of dichotomous branching of the aerial shoot of *Flagellaria indica*. (A) Scanning photomicrograph of an apex prior to any indication of bifurcation. (B) Young stage in dichotomous branching. The apex becomes broad and flattened. Photograph taken with epi-illuminated light photomicroscope. (C) A median longitudinal section through the same bud as in (B) showing the internal cellular organization. (D) Epi-illumination photomicrograph of the first clear external indication of the two new centers of meristematic activity. (E) A median longitudinal section through the same bud as in (D). (F) Scanning photomicrograph of the two vegetative apices resulting from dichotomous branching beginning to initiate their own leaf primordia. The magnification for (B), (D), and (F) is the same as that for (A); the magnification for (C) is the same as that for (E).