ditions of confinement as well as by the cold water. However, reports of leatherback turtles in temperate waters do not suggest that these animals are usually lethargic at sea (*1*, *2*). Although it remains possible that *Dermochelys* seen off Nova Scotia are carried out of their normal areas by the Gulf Stream (*13*), the smaller marine turtles are reported less frequently in northern waters (*1*, *14*). The thick, fibrous, oil-saturated layer below the epidermis might put the leatherback at some advantage over other marine turtles with respect to insulation. Thick peripheral layers are important in the thermal equilibrium of mammals such as seals and polar bears (*15*). These various considerations, taken together with the great temperature difference found here, suggest that large *Dermochelys* may be adapted thermally for existence in reasonably cold water.

Bogert (*13*) has pointed out that there is an upper limit in bulk beyond which behavioral means, such as moving into the sun, are inadequate for the regulation of body temperature because it will take too long for the animals to warm up. This limitation may have restricted the dinosaurs to uniformly warm climates and may be responsible for the fact that most large reptiles today are tropical (*13*). However, if regulation is achieved by muscular activity and some heat retention, rather than by behavioral means, then large bulk becomes an advantage. Our data show that the largest living turtle, *Dermochelys*, is indeed capable of maintaining a considerable differential between its body temperature and the ambient level in a situation where behavioral regulation is not possible. This finding is relevant to speculations about the thermal existence of large extinct reptiles (*16*) and the present distribution of *Dermochelys*. Now it would be interesting to know how long the leatherback turtle, with its periodic appearances in temperate waters, is capable of maintaining such differentials.

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Bone Resorbing Activity in Supernatant Fluid from Cultured Human Peripheral Blood Leukocytes

Abstract. *A new soluble mediator was found in supernatant fluid from cultures of human peripheral blood leukocytes that were stimulated by phytohemagglutinin, or by antigenic material present in human dental plaque deposits. This soluble factor produced bone resorption in organ cultures of fetal rat bones as measured by increased release of calcium-45, and also increased the number of active osteoclasts.*

The supernatants of stimulated leukocyte cultures contain soluble mediators with a variety of biological activities, which are secreted by activated lymphocytes (*1*). Antigens present in bacterial culture filtrates of organisms common to the oral cavity stimulate lymphocytes, from subjects with periodontal disease, to proliferate in vitro (*2*). We have established a direct correlation between the degree of in vitro reactivity of lymphocytes to antigens in dental plaque deposits (Ag) and the severity of periodontal disease. We have also shown that supernatants from human peripheral blood leukocyte cultures stimulated with Ag contain a mediator, lymphotoxin, which is toxic to human gingival fibroblasts (*3*). In periodontal disease, as in other types of chronic inflammation, bone is frequently resorbed in areas adjacent to inflammatory sites. We have now found that supernatants of leukocyte cultures stimulated by Ag or by phytohemagglutinin (PHA) induce osteoclastic resorption of fetal rat bone in organ cultures.

Replicate cultures of 2×10^6 peripheral blood leukocytes from four human subjects were established in a volume of 1 ml of Roswell Park Memorial Institute medium (RPMI) 1640 with 20 percent plasma, as previously described (*3*). Subjects A and D had destructive periodontal disease; subjects B and C had asymptomatic minimal gingivitis, as do most adults. Cultures were either unstimulated, or stimulated with an ultrasonicated solubilized fraction of pooled Ag or PHA (*4*) for 6 days at 37°C in a humidified atmosphere of 5 percent CO_2 in air. After the cultures were sedimented (200*g* at 22°C), the supernatants were aspirated, appropriately pooled, filtered (0.45-μm Millipore filter), and stored for up to 30 days at −20°C. The sedimented leukocytes were exposed to tritiated thymidine ([^{3}H]TdR) in order to measure the increase in DNA synthesis (*5*).

The technique for measuring bone resorption in organ culture has been described (*6*). Paired shafts of the radius or ulna of 19-day rat fetuses, labeled by injection of the mother with ^{45}Ca on the previous day, were incubated in 0.5 ml of medium at 37°C in an atmosphere of 5 percent CO_2 in air. One shaft was incubated in control medium (*7*); the other was incubated in test medium consisting of the super-

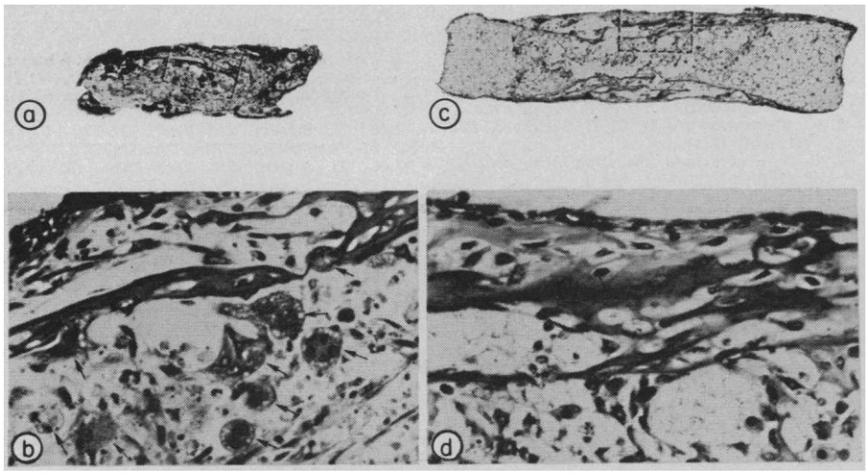

Fig. 1. Representative example of histologic appearance of bones cultured for 96 hours with supernatants from leukocyte cultures stimulated with Ag or with control medium (10 percent plasma, 40 percent RPMI 1640, and 50 percent modified BGJ). (a) and (b) Treated sections at ×50 and ×300 magnification, respectively; (c) and (d) control sections at ×50 and ×300 magnification, respectively. Numerous osteoclasts and loss of matrix can be seen in (b). Specimens were fixed in Bouin's solution, embedded in paraffin, and stained with hematoxylin and eosin.

natant of leukocyte cultures or of one or more of its components mixed 1 : 1 with modified Biggers, Gwatkin, Judah medium (BGJ) (*8*). The cultures were maintained for 4 to 6 days, and the medium was changed every 2 days. The ratio of ^{45}Ca released into the medium from treated and control bone cultures was used as a measure of bone resorption.

Supernatants from unstimulated cultures from two subjects (A and B) caused bone resorption, but supernatants stimulated with Ag resulted in greater and more rapid bone resorption (Table 1). In two other subjects (C and D) only supernatants from stimulated cultures resulted in bone-resorbing activity. Although the amount of periodontal disease correlated with the degree of lymphocyte stimulation, even leukocyte supernatants from patients with minimal gingivitis produced significant bone-resorbing activity. Furthermore, the mean incorporation of [^{3}H]TdR by leukocyte cultures stimulated by PHA was much larger than by those stimulated with Ag, but culture supernatants from each produced similar degrees of bone resorption by 96 hours after addition of supernatants to cultures. The increased release of ^{45}Ca from cultured bone exposed to supernatant of stimulated leukocyte cultures was also associated with the appearance of increased numbers of osteoclasts and the loss of bone matrix (Fig. 1). In contrast with the few osteoclasts present in normal bone, these osteoclasts were large and showed intense vacuolization; many were located on bone spicules. Bone-resorbing activity was no longer detectable when supernatants were diluted with BGJ medium (*8*) in a ratio of 1 : 10.

Control experiments indicated that the bone-resorbing activity was produced by cultured leukocytes. There was no such activity in medium incubated at 4°C for 4 hours, with or without leukocytes. Culture medium, with or without PHA or Ag, incubated at 37°C for 6 days caused a small inhibition of ^{45}Ca release, but this inhibition was completely overcome by the bone-resorbing activity released by stimulated lymphocytes.

Bacterial endotoxin has been implicated in the pathogenesis of alveolar bone loss in periodontal disease because it stimulates bone resorption in vitro (*9*). Endotoxin was not responsible for the changes seen in our studies, however, because both Ag and PHA were ineffective in directly stimulating resorption in the absence of lymphocytes.

The bone resorption produced by supernatants of stimulated leukocyte cultures is not only comparable in amount to that produced by the maximally effective doses of parathyroid hormone in culture, but also results in the appearance of increased numbers of active osteoclasts (*6*). Therefore, our hypothesis is that stimulated lymphocytes produce a factor or factors which induce osteoclast formation and activity. We have tentatively named this new activity osteoclast-activating factor.

Alveolar bone resorption in periodontal disease is probably the major factor in tooth loss. Bone resorption also contributes to disability in rheumatoid arthritis and chronic osteomyelitis. The ability of lymphocytes to secrete a factor which activates osteoclasts to resorb

Table 1. Effects of additions and supernatants from cultures of peripheral blood leukocytes from four human subjects (A to D) on bone resorption in organ culture. Supernatants were from 6-day leukocyte cultures, except where indicated. Autologous plasma was used except in the cultures containing D leukocytes, where plasma from subject C was substituted. Cumulative ^{45}Ca release is expressed as the mean ± standard error for four pairs of bone cultures. Results in italics are significantly different from 1.0 ($P < .01$).

Additions	Mean ratio of [^{3}H]TdR incorporation in stimulated to unstimulated leukocytes	Cumulative ^{45}Ca release (ratio of treated to control mixtures)	
		0 to 48 hours	48 to 96 hours
Culture medium			
None		0.93 ± 0.02	0.84 ± 0.04
PHA		.88 ± .03	*.80 ± .03*
Ag		.99 ± .04	*.81 ± .02*
None (4°C, 4 hours)		1.06 ± .02	1.05 ± .01
Supernatant from A leukocytes			
None		*1.30 ± .02*	*2.23 ± .31*
PHA	297	*1.48 ± .03**	*2.25 ± .11*
Ag	52	*2.97 ± .17**	*3.63 ± .29**
Supernatant from B leukocytes			
None		*2.18 ± .12*	*2.73 ± .31*
PHA	137	*2.82 ± .20**	*3.25 ± .24*
Ag	6	*2.69 ± .19*	*3.67 ± .30**
Supernatant from C leukocytes			
None		0.94 ± .02	0.99 ± .02
PHA	289	*1.43 ± .05**	*1.86 ± .06**
Ag	5	1.21 ± .11	*1.67 ± .16**
Supernatant from D leukocytes			
None		0.99 ± .03	0.90 ± .05
PHA	109	*1.48 ± .08**	*1.77 ± .08**
Ag	24	1.42 ± .21	*1.65 ± .11**
None (4°C, 4 hours)		1.10 ± .08	1.09 ± .06

* Differs significantly from effect of respective supernatant of unstimulated leukocyte culture ($P < .05$).

bone may play an important role in the pathogenesis of bone loss near areas of chronic inflammation. Identification of the factor may lead to new approaches in treatment or prevention of this bone loss.

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5. Tritiated thymidine (1 μc) (specific activity, 6.0 μc/mmole; Schwarz Biochemical) was added to each residual 0.15-ml sedimented cell culture, incubated for 4.5 hours, and harvested by trichloroacetic acid precipitation. The counts per minute of radioactivity were determined in a Packard Tri-Carb scintillation spectrometer.
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Histochemical Phosphorylase Activity in Regenerating Muscle Fibers from Myophosphorylase-Deficient Patients

Abstract. *Fresh frozen sections of mature skeletal muscle fibers from patients with genetically determined "absence" of skeletal muscle phosphorylase (McArdle's disease) have no histochemical phosphorylase activity. That regenerating muscle fibers, in vitro and in vivo, from such patients do have histochemical phosphorylase activity present suggests a loss of enzyme activity with fiber maturity.*

Complete absence of skeletal muscle phosphorylase (McArdle's disease) (*1*) has been considered a genetically determined defect of glycogen metabolism, which results in the absence of biochemically and histochemically determined myophosphorylase activity in skeletal muscle (*2*). Attempts at demonstrating the enzyme immunologically in affected patients have also failed (*3*), suggesting that the enzyme is absent or inactivated both as an antigen and in its enzymatic activity.

Using collagen-coated cover slips (*4*) with the Maximow chamber, we have explanted small fragments of muscle (*5*) obtained by open or needle biopsy from three adult patients with verified myophosphorylase deficiency (*6*) and ten patients with histologically normal muscle. The muscle developed in vitro from the mononuclear myoblast stage through the early multinucleated stage to form very long, cross-striated fibers containing multiple large nuclei with one or two large round nucleoli. This growth occurred outside the original explant. No differences were noted in the overall growth pattern and cellular morphology of muscle derived from affected patients as compared to normal controls. At varying developmental stages, whole mounts of the cultures were rinsed in a balanced salt solution, dried in air for 1 hour, and stained for phosphorylase by the Takeuchi technique (*7*). This technique depends on the development of a purple color when newly formed long glycogen branches are reacted with dilute iodine.

Although the fresh frozen sections of the skeletal fibers from muscle biopsies of the three patients with phosphorylase deficiency showed complete absence of phosphorylase histochemically (Fig. 1a), the early multinucleated fibers and striated myofibers newly grown in vitro from those patients' biopsies had definite phosphorylase activity (Fig. 2, c to e). The distribution and amount of deep purple stained material was similar to that in cultured muscle from histologically normal biopsies (Fig. 2, a and b). In other cultures of similar developmental stages from the patients with phosphorylase deficiency, "preformed" cellular glycogen stained with the periodic acid–Schiff reaction was abundant in the newly formed early multinucleated muscle fibers and striated myofibers. It was diastase-sensitive and similar in distribution to the phosphorylase staining, but somewhat more abundant; cultures from the control patients were similar.

We used four types of histochemical controls. (i) A frozen section of the muscle biopsy from a phosphorylase-deficient patient and from a control patient with normal phosphorylase activity, and the tissue culture of muscle from the phosphorylase-deficient patients were stained simultaneously in the same solution. Only the biopsy from the phosphorylase-deficient patient failed to show staining of skeletal muscle fibers (although smooth muscle fibers in arterial vessels were stained), a procedure which ruled out possible technical errors. (ii) Sister cultures of similar developmental stages from the phosphorylase-deficient patients were dried in air for 1 hour and dipped into the dilute iodine without prior (histochemical) incubation; they did not show staining. (iii) Other sister cultures grown from the muscle of phosphorylase-deficient patients were incubated in the histochemical medium for phosphorylase, from which the substrate glucose-1-phosphate was omitted and then processed as usual; these cultures failed to show staining (Fig. 2f). (iv) Fresh frozen sections of muscle biopsies from a normal patient, a phosphorylase-deficient patient, and a phosphorylase-deficient patient with regenerating muscle fibers were dried in air for 1 hour, and handled as in (ii) and (iii) above; again, no staining occurred.

We considered the possibility that fibers from phosphorylase-deficient pa-