the organism via direct stimulation of the phagocytic mechanisms. Lysozyme, therefore, may function as a self-stimulating secretory product of the macrophage.

These composite studies denote that the glucan-induced hyperfunctional state of the reticuloendothelium is associated with a significant increase in serum lysozyme activity. This increased activity may contribute to bactericidal properties of glucan by providing the host with a nonspecific mechanism whereby certain invading microorganisms may be reduced to less virulent forms. Since infectious complications are common in malignant conditions (*22*) and glucan possesses marked antitumor activity (*5, 6*), the antibacterial properties of glucan that we have now demonstrated make this compound an attractive immunotherapeutic agent.

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Inhibition of Bone Resorption in vitro by a Cartilage-Derived Anticollagenase Factor

Abstract. *A cartilage-derived factor containing a specific collagenous inhibitor was found to block reversibly parathyroid hormone–stimulated ^{45}Ca release from fetal rat bone in vitro. Morphologic and quantitative histometric examination revealed that this factor modulates osteoclastic activities.*

Elucidation of the relation between osteoclasts and bone resorption has been investigated in systematically accelerated bone resorptive states induced by parathyroid hormone (PTH) stimulation both in vivo and in vitro (*1*). However, with the exception of the effects of specific circulating hormones on osteoclastic activities, little attention has been directed toward local regulatory mechanisms on these cells.

Proteoglycan-free cartilage extract which contains protease inhibitors prevents the proliferation of endothelial cells in culture (*2*). High activity of protease inhibitor also occurs in other poorly vascularized tissues, such as blood vessel walls, cornea, and dentin (*3*). Since endothelial cells penetrate extracellular matrices, presumably by enzymatic mechanisms, their control may be due to inhibition of proteolysis. The protease inhibitors from cartilage and aortic wall have been isolated and found to be low-molecular-weight cationic proteins capable of specifically inhibiting collagenolytic activity isolated from normal and pathological tissues (*2*). Since collagenase and vascular elements have been implicated in resorptive events of bone matrix (*4*), we have now evaluated the effect of this cartilage-derived material containing a specific collagenase-inhibitor and found that it reversibly inhibits osteoclastic activity in fetal rat bone in organ culture.

The organ culture technique which measures the resorption of bone has been described (*5*). Paired shafts of the radius and ulna from 19-day-old rat fetuses were radioactively labeled by injection of the mother with ^{45}Ca on the previous day. These shafts were cultured either in BGJ_b medium (Gibco) supplemented with 1 mg of bovine serum albumin (Pentex) per milliliter or in such medium containing additions of the cartilage-derived inhibitor (*6*). Bone resorption was stimulated in one of the shafts by the addition of 2.8 international

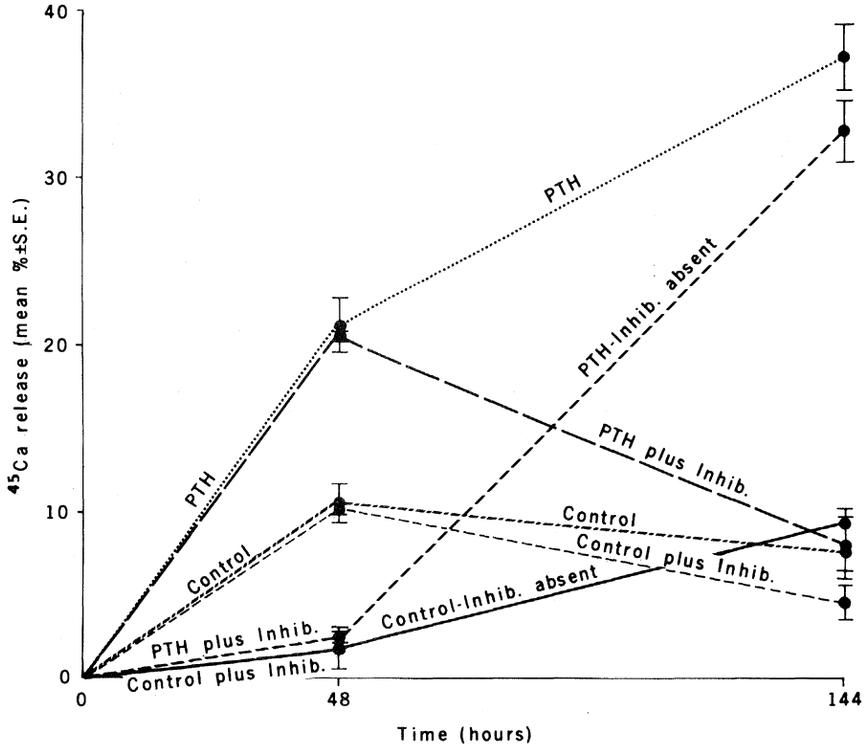

Fig. 1. Effects of the presence in and absence from the culture medium of the cartilage-derived inhibitor (*Inhib.*) on PTH-stimulated bone resorption in organ culture. Values are expressed as the mean percentage (± standard error) of ^{45}Ca released from four pairs of cultured bones after 48 and 144 hours of incubation.

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units of PTH (Inolex) per milliliter every 48 hours to the culture medium while its contralateral member served as the control by receiving no hormone. The cultures were maintained for up to 144 hours, and the medium with or without fresh additives was changed every other day. The percentage of ^{45}Ca released from each bone into the culture medium was used as a measure of bone resorption. The degree of bone resorption was determined by liquid scintillation spectrometry from the counts per minute of ^{45}Ca radioactivity present in culture medium at each 48-hour interval and the

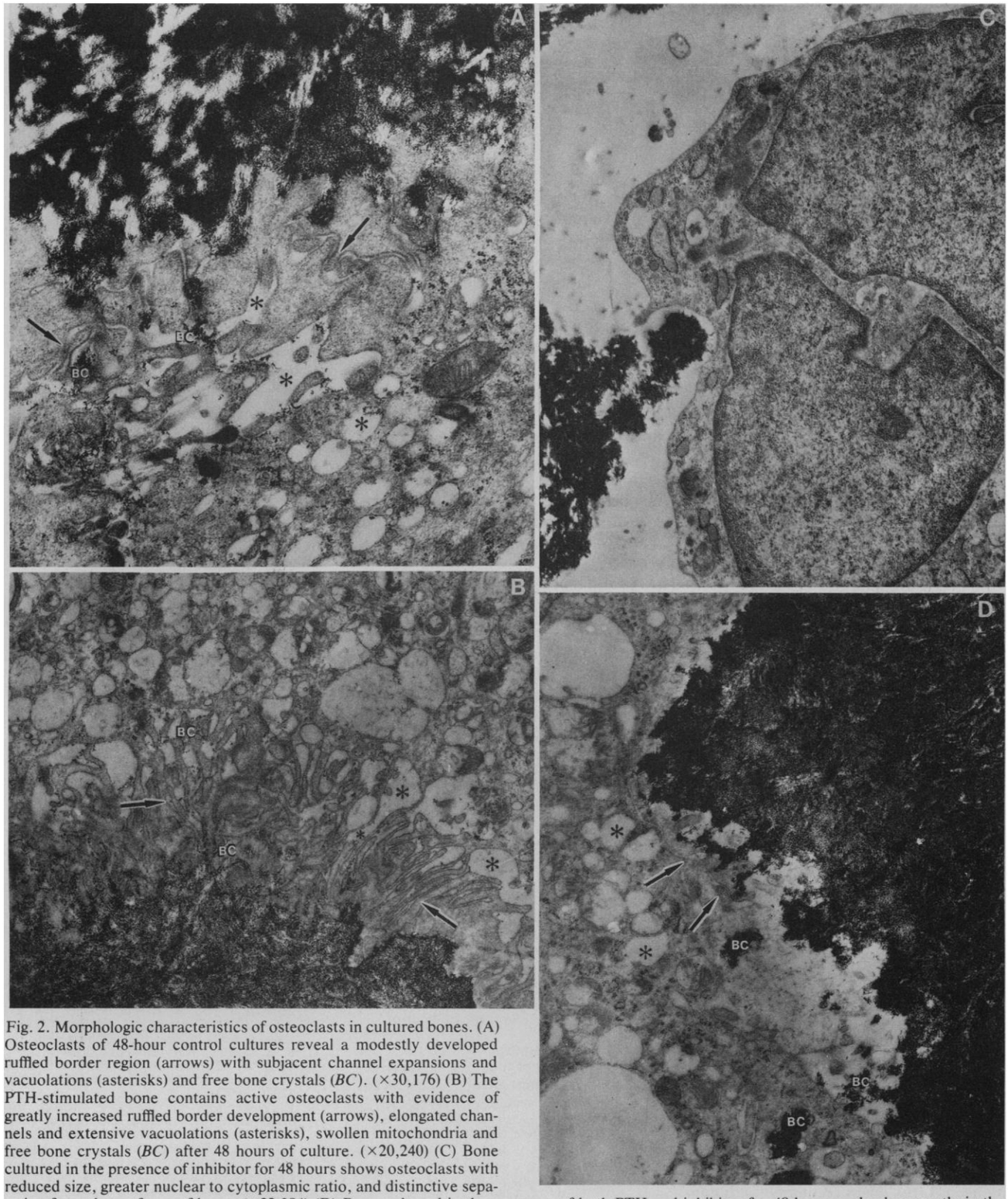

Fig. 2. Morphologic characteristics of osteoclasts in cultured bones. (A) Osteoclasts of 48-hour control cultures reveal a modestly developed ruffled border region (arrows) with subjacent channel expansions and vacuolations (asterisks) and free bone crystals (*BC*). (×30,176) (B) The PTH-stimulated bone contains active osteoclasts with evidence of greatly increased ruffled border development (arrows), elongated channels and extensive vacuolations (asterisks), swollen mitochondria and free bone crystals (*BC*) after 48 hours of culture. (×20,240) (C) Bone cultured in the presence of inhibitor for 48 hours shows osteoclasts with reduced size, greater nuclear to cytoplasmic ratio, and distinctive separation from the surfaces of bone. (×22,384) (D) Bone cultured in the presence of both PTH and inhibitor for 48 hours and subsequently in the presence of PTH alone for the following 48 hours shows osteoclasts reassociated with the bone surface and evidence of the development of a ruffled border (arrows), vacuolations (asterisks), and the release of free bone crystals (*BC*) from the bone surfaces. (×22,384) All sections were stained with uranyl acetate and lead citrate.

counts per minute of ^{45}Ca radioactivity remaining in the cultured bone shafts after 144 hours of culture. Statistical differences were analyzed by means of Student's *t*-test.

Specific bone resorption occurred in cultures to which PTH was added, whereas resorption failed to occur in bones cultured in the presence of the cartilage-derived inhibitor (Table 1). However, recovery from the inhibition of PTH produced by the presence of the cartilage-derived inhibitor could be achieved by transferring the bones after 48 hours of culture to medium which only received PTH (Fig. 1). In fact, the percentage of ^{45}Ca released from these explants during the subsequent 96 hours closely approximated values obtained from bones cultured for a total of 144 hours in the presence of PTH only. Furthermore, the effect of PTH on bones resorbing during the first 48 hours could be significantly diminished by substituting their medium with medium containing the cartilage-derived inhibitor, even though fresh PTH also was added at both 48 and 96 hours. The percentage of ^{45}Ca released at the end of the culture period from these bones was not significantly different from the values obtained from control bones cultured for a total of 144 hours in medium not containing the cartilage-derived inhibitor.

These experiments indicate that the cartilage-derived inhibitor significantly suppressed PTH-stimulated bone resorption which could not be overcome with repeated additions of PTH to the explants. However, the action of this cartilage-derived inhibitor is reversible, since bones cultured for 48 hours in its presence were found equally capable of resuming PTH-stimulated resorption when transferred to medium devoid of the cartilage-derived inhibitor. The biologic potency of this cartilage-derived inhibitor was reflected in its ability to completely inhibit PTH-stimulated bone resorption which had already progressed for 48 hours.

Since the role of the osteoclast in bone resorption has been shown to require its intimate relationship with the bone surface (*7*), undecalcified explants from each experimental period were processed for electron microscopy and photographed with light and electron microscopy (*8*). The PTH-stimulated bones revealed significantly increased numbers of active osteoclasts (Table 2) lying on bone surfaces when compared with control explants (Fig. 2). In contrast, bones cultured in the presence of the cartilage-derived inhibitor contained osteoclasts

Table 1. Effect of the cartilage-derived inhibitor on PTH-stimulated bone resorption in vitro. The ^{45}Ca release is expressed as the mean ratio ± standard error of PTH-treated to untreated cultures for four pairs of fetal rat bone shafts during 144 hours of culture.

Inhibitor	^{45}Ca release (ratio of treated to control cultures)
Absent	2.79 ± 0.19*
Present	1.07 ± 0.03

*Differs significantly from the value of 1.0, $P < .01$.

of which more than 50 percent were physically separated from the bone surfaces, and their numbers were significantly reduced. In bones initially cultured in the presence of the cartilage-derived inhibitor and subsequently cultured in its absence, more than 90 percent of the osteoclasts were observed to be reassociated to the bone surfaces.

Uncalcified cartilage resists invasion by endothelial elements, multinucleated cells, and tumor cells (*2*). Invasion is defined as the interpenetration of a cell or tissue into adjacent tissues combined with the destruction of the matrix of the invaded tissue. Such proliferative invasive cells elaborate collagenase which degrades collagen, the major organic component of connective tissue matrices (*2, 9*). As suggested previously (*2, 9, 10*), the resistance by uncalcified cartilage to such cellular invasion may well be due to material present within the matrix which selectively inhibits the action of collagenase. Since active osteoclasts share the characteristics of collagen degradation, these cells could also be interpreted as being "invasive." We have now shown that the collagenase inhibitor-enriched material isolated from cartilage inhibits osteoclastic resorption in addition to the above-mentioned processes. We have therefore tentatively named it "anti-invasion factor" (AIF).

Table 2. Quantitative histometric observations of the effect of PTH with and without the presence of the cartilage-derived inhibitor on osteoclasts in cultured fetal rat bones. Values were collected and averaged from the total number of osteoclasts and their nuclei in six complete midsagittal sections of each 48-hour cultured bone, and are expressed as the means ± standard error of the counts.

Additives	Average number of	
	Osteoclasts per total midsagittal section	Nuclei per osteoclast
Control	8.5 ± 0.16*	4.3 ± 0.12*
PTH	12.5 ± 0.27†	5.9 ± 0.22†
Inhibitor	5.3 ± 0.03*†	2.4 ± 0.05*†
PTH plus inhibitor	5.5 ± 0.09*†	3.1 ± 0.11*†

*Significantly different from PTH-stimulated bones, $P < .01$. †Significantly different from control bones, $P < .01$.

Osteoclastic regulation during bone turnover is exemplified in the growth and development of long bones, fracture repair, periodontal disease, and rheumatoid arthritis. Bone resorption associated with such events may involve mediators such as the lymphokine osteoclast-activating factor (OAF) and prostaglandins (PGE) (*11*). We have also recently tested these mediators in our culture system and found reversible inhibition of both OAF- and PGE_2-stimulated bone resorption with AIF. Modulation of bone cell activities may therefore be controlled locally in part by tissue-specific enzyme regulators. Since neither Trasylol (bovine basic pancreatic trypsin inhibitor; Bayer) nor soybean trypsin inhibitor act as collagenase inhibitors (*2*) and also show no effects when added to our system, it can be concluded that the specific inhibition of collagenase modulates osteoclastic function.

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6. Slices of fresh bovine nasal septa cartilage were extracted with 1*M* NaCl (*p*H 5.8; 48 hours at 4°C). After decantation, the NaCl concentration was raised to 3*M* by adding solid NaCl. The ex-

tract was then ultrafiltered (4°C) on an Amicon XM-50 membrane [molecular weight (M.W.) cutoff 50,000] and afterward concentrated and dialyzed into physiologic saline with an Amicon UM-2 membrane (M.W. cutoff 2,000) to yield a protein concentration of 5 mg/ml. This cartilage-derived material with a molecular weight between 2,000 and 50,000 is essentially free of uronic acid and hydroxyproline and is enriched in the collagenase inhibitor. After being passed through a Millipore filter (0.22 μm pore size) for sterilization, portions were stored frozen at −70°C until tested. For bone cultures, the cartilage-derived inhibitor was diluted with medium to yield a concentration of 300 μg of protein per milliliter.

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Nonhistone Proteins HMG_1 and HMG_2 Change the DNA Helical Structure

Abstract. *Two chromatin nonhistone proteins (from calf thymus) of the high mobility group, HMG_1 and HMG_2, reduce the linking number (topological winding number) of a circular DNA if the covalent closure of the DNA is carried out in their presence. This indicates that these proteins can either unwind the double helix, or induce a supercoiling of the DNA.*

Extraction of calf thymus chromatin with 0.35*M* NaCl yields two groups of nonhistone proteins. On the basis of their electrophoretic mobilities on gel, the two groups have been referred to as the low mobility group and the high mobility group (HMG) (*1*). The HMG group consists of four proteins designated HMG_1, HMG_2, HMG_{14}, and HMG_{17}. All four proteins have been purified to homogeneity, and the amino acid sequence of one of them (HMG_{17}) has been determined (*2*).

Physicochemical studies of HMG_1, HMG_2, and HMG_{17} have been undertaken (*3*, *4*). HMG_1 and HMG_2 are similar to each other, each containing about equal molar amounts of acidic and basic amino acids (*1*). Their molecular weights are approximately 26,000 (*5*, *6*), and both have globular structures with approximately 45 percent of the residues in the α-helix configuration (*7*, *8*). The two proteins interact with DNA nonspecifically and form soluble complexes (*5*). The proteins destabilize the DNA double helix, as indicated by a lowering of the melting temperature of DNA in the presence of the proteins (*3*).

We now report that both HMG_1 and HMG_2 proteins alter the configuration of the DNA double helix. Circular PM2 DNA containing one single-chain scission per molecule was converted to the covalently closed form by *Escherichia coli* DNA ligase in the presence of increasing amounts of either HMG_1 or HMG_2. On removal of the bound protein, the electrophoretic mobility of the DNA in agarose gel was examined. The electrophoretic pattern of the control DNA sample ligated at 24°C in the absence of HMG_1 and HMG_2 is shown in Fig. 1a. The electrophoresis condition was such that the group of covalently closed DNA bands were all negatively supercoiled. This can be achieved by carrying out electrophoresis at 4°C in an Mg^{2+}-containing buffer (*9*). As was interpreted previously, a group of covalently closed DNA bands differing in linking numbers (topological winding numbers) are formed because of thermal fluctuation of the DNA helix configuration (*10*). When increasing amounts of HMG_1 were present in samples during treatment with ligase, the covalently closed DNA formed showed a progressive increase in electrophoretic mobility (Fig. 1, b to g). This increase in mobility results from an

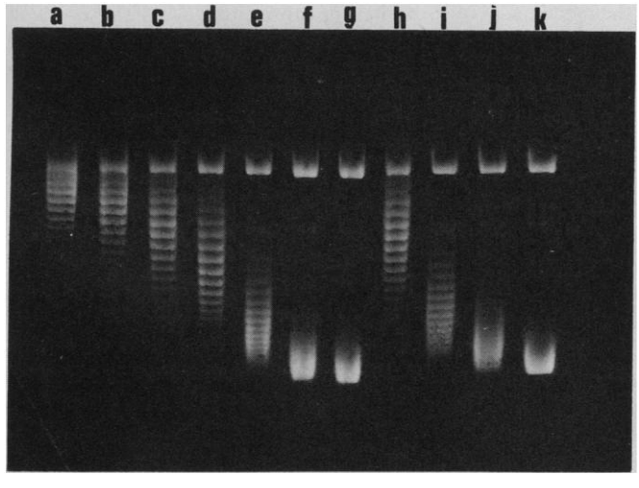

Fig. 1. Reaction mixtures (86 μl each) contained 18 m*M* tris-HCl, *p*H 8.0, 2.7 m*M* $MgCl_2$, 2.3 m*M* potassium phosphate, 44 μg of bovine plasma albumin per milliliter, 29 μg of NAD per milliliter, 0.9 m*M* EDTA, 0.5 m*M* dithiothreitol, 6 percent (by volume) glycerol, 13 μg of PM2 DNA (containing one single-chain scission per molecule) per milliliter, and varying amounts of HMG_1 and HMG_2 proteins. The reaction mixtures were incubated at 24°C for 10 minutes, and 4 μl of an *E. coli* ligase stock (in the same reaction medium) was added to each tube, and incubation was continued for another hour. The reaction was stopped by the addition of 15 μl of solution containing 25 percent (by volume) glycerol, 5 percent sodium dodecyl sulfate, and 0.25 mg of bromophenol blue per milliliter. Half of the volume of each stopped reaction mixture was placed on an agarose (0.7 percent) slab gel for electrophoresis in buffer containing 5 m*M* magnesium acetate, 40 m*M* tris-HCl, *p*H 8.0, 1 m*M* EDTA (trisodium salt). Electrophoresis was carried out at 4°C and at 3 V/cm for 39 hours. The numbers of HMG_1 molecules per DNA molecule during the ligase treatment were: (a) 0, (b) 40, (c) 80, (d) 120, (e) 160, (f) 240, and (g) 320. The numbers of HMG_2 molecules per DNA molecule were (h) 50, (i) 100, (j) 150, and (k) 200. The HMG proteins were prepared by the method of Johns *et al.* (*1*). Concentrations of HMG_1 and HMG_2 were determined spectrophotometrically taking $E^{280}_{1\%} = 82$. A value of 6.5×10^6 was used for the molecular weight of PM2 DNA.

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