INFECTION AND IMMUNITY, Mar. 1996, p. 959–965 Vol. 64, No. 3
0019-9567/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Pasteurella multocida Toxin Is a Mitogen for Bone Cells in Primary Culture

PAUL B. MULLAN AND ALISTAIR J. LAX*

Institute for Animal Health, Compton, Newbury, Berkshire, RG20 7NN, United Kingdom

Received 17 May 1995/Returned for modification 26 July 1995/Accepted 15 December 1995

The effect of recombinant *Pasteurella multocida* toxin (PMT) on primary cultures of embryonic chick bone-derived osteoblastic cells was investigated. It was found that PMT was a potent mitogen for primary derived chicken osteoblasts. The toxin stimulated DNA synthesis and cell proliferation in quiescent osteoblasts at the first passage and accelerated cell growth in subconfluent cultures. Cell viability was not affected by PMT, even at relatively high concentrations. Osteoblast numbers increased in a dose-dependent manner in response to PMT. Intracellular inositol phosphates were elevated in response to PMT, but no elevation in cyclic AMP (cAMP) levels was evident. Indeed, PMT inhibited cAMP elevation in osteoblasts in response to cholera toxin at a stage before other PMT-mediated events take place. In addition to increased cell turnover, PMT down-regulated the expression of several markers of osteoblast differentiation. Both alkaline phosphatase and type I collagen were reduced, but osteonectin was not affected. The in vitro deposition of mineral in cultures of primary osteoblasts and osteoblast-like osteosarcoma cells was also inhibited by the presence of PMT. This suggests that PMT interferes with differentiation at a preosteoblastic stage.

Bacterial toxins have provided a significant contribution to our understanding of the biochemical events involved in the regulation of cell growth and differentiation. The targets of many of these toxins are key components of the signalling pathways regulating such processes. *Pasteurella multocida* toxin (PMT) is the most potent mitogen yet identified for cultured fibroblasts, inducing DNA synthesis and cell division in the picomolar range (18, 42). It is mitogenic for several cell lines (42) and induces anchorage independence in Rat-1 cells (18). Furthermore, the toxin has been shown to promote growth of tertiary mouse embryonic cells (42) and increase proliferation of T cells (50). PMT consists of a single polypeptide of 146 kDa in molecular mass. There is indirect evidence that the toxin is taken up by cells and processed via an acidic compartment (42), though details of this process are currently unclear. The toxin exerts these effects via stimulation of phosphoinositol breakdown (46), possibly by interaction with a G protein (30). PMT activates increased phosphorylation of the MARCKS protein by protein kinase C and also mediates other phosphorylation events (46).

The gene for PMT has been cloned and sequenced by several groups (5, 25, 38). A motif found in several toxins which ADP-ribosylate their target (25) is not functional in PMT (52), and there is no evidence in vivo for ADP-ribosylation activity (46). Recently the protein sequence of PMT has been shown to have some similarities with the cytotoxic necrotizing factors (CNF1 and -2) found in *Escherichia coli* (9, 34). These toxins induce DNA synthesis in HeLa cells (8) but produce multinucleated cells (12, 35). CNF induces membrane ruffling which is known to be mediated by the G protein, Rho, and there is some evidence that CNF2 interacts directly with Rho (35). Another toxin, the dermonecrotic toxin of *Bordetella* species, has been sequenced recently and shown to have some homology to the CNF toxins (51). These toxins therefore appear to form a new family of toxins which affect signal transduction pathways involved in the control of cellular growth.

P. multocida is the causative agent of atrophic rhinitis (AR) (10), a nonfatal respiratory disease of pigs in which the growth of the nasal turbinate bones is affected (22). PMT by itself can reproduce these symptoms (20), even when purified recombinant PMT is administered by the intraperitoneal route (24). PMT has been shown to induce resorption of bone cultured in vitro (14, 22). It was reported that PMT did not significantly modify osteoblasts (as assessed by bone formation and cell necrosis) but induced an increase in osteoclasts and osteoclast precursors (22). However, because of the complex signalling interactions which take place between osteoblasts and osteoclasts (39), the primary target(s) of PMT in vivo is unclear from this work. We have sought to investigate the effects of PMT on cultures of bone derived cells and show here that PMT is mitogenic for primary derived osteoblasts.

MATERIALS AND METHODS

Other materials not listed below were obtained as follows. Cholera toxin, alzarin red stain, phosphate-buffered saline tablets (PBS), dexamethasone, β-glycerophosphate, and ascorbic acid were all obtained from Sigma; [^{3}H]thymidine (248 GBq/mmol) and [^{3}H]inositol (370 to 740 GBq/mmol) were obtained from NEN (DuPont). Fetal calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO. Rabbit anti-chicken bone osteonectin was a kind gift from Larry Fisher (National Institutes of Health, Bethesda, Md.). Highly purified recombinant PMT was kindly provided by Phil Ward (Institute for Animal Health, Compton, Newbury, United Kingdom).

Cell culture of osteoblasts. Rat osteoblast-like osteosarcoma cell lines ROS 17/2.8, ROS 25/1, and UMR 106 were obtained from John Heath (University of Oxford) and were cultured in DMEM supplemented with 10% FCS, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Osteoblasts were isolated according to the method of Nijweide et al. (33). Briefly, the calvariae from 17-day-old chicken embryos were stripped of both the periosteum and endosteum. The calvariae were digested in a solution of 1 mg of crude collagenase per ml (Worthington Enzymes, Freehold, N.J.) in Ca^{2+}-Mg^{2+}-free PBS at 37°C in a shaking water bath. The calvariae were digested for 10-, 45-, and 60-min intervals. Cells from the first digestion period were discarded (since this fraction contained large numbers of contaminating fibroblastic cells). Cells liberated in the last two fractions were pooled, washed in DMEM (containing 10% FCS), counted in a hemocytometer, and plated in 35-mm-diameter dishes (NUNCLON, Roskilde, Denmark) at a concentration of 5×10^5 cells per dish. Osteoblasts were usually left to grow to confluence (about 5 to 7 days).

* Corresponding author. Phone: (44) (1635) 578411. Fax: (44) (1635) 577263.

Measurement of DNA synthesis. This was performed by the incorporation of [^{3}H]thymidine into confluent, quiescent osteoblast cells by the method of Dicker and Rozengurt (13). Difficulties in obtaining quiescent osteoblast preparations were overcome by removing growth medium and replacing it with DMEM (with 0.5% FCS) for up to 48 h before the addition of toxin. Different assays employed different cell concentrations. Stimulation of DNA synthesis in osteosarcoma cells was performed in 35-mm dishes with cells initially seeded at 10^5 cells per well. Osteoblasts were seeded at 5×10^5 per 35-mm well. Both cell types were grown to confluence (7 days) in DMEM (+10% FCS). The medium was replaced with 2 ml of serum-free DMEM-Waymouth's medium 1:1 (vol/vol) containing 1 μCi (37 kBq) of [^{3}H]thymidine per ml, in the presence or absence of various concentrations of PMT. After 40 h, DNA synthesis was assessed by measuring the level of [^{3}H]thymidine incorporated into half the acid-precipitable material. In thymidine pulsing experiments, osteosarcoma cells were plated in a similar manner, but after 7 days, the medium was replaced with DMEM-Waymouth's medium for 72 h before the addition of toxin and label (40 h). Again the level of [^{3}H]thymidine incorporated into half the acid-precipitable material was used as a measure of DNA synthesis.

Measurement of cell number. This was performed as described elsewhere (43). Briefly, osteoblasts were plated in 10% FCS–DMEM at low density (2×10^4 per well in 24-well dishes) for the measurement of cell proliferation in subconfluent cells. PMT (20 ng/ml) was added, and at 48-h intervals, the cells were removed from the culture wells with 0.05% trypsin–0.53 mM EDTA, pelleted at $350 \times g$ for 5 min, and washed twice in PBS, before being counted with a hemocytometer (cell counts were performed in triplicate). Confluent osteoblasts were counted in a similar manner except that the cells were plated at 2×10^5 per well on a 24-well dish, cultured for 5 days in 10% FCS–DMEM, and incubated for 24 h in 0.1% FCS–DMEM, before the addition of a range of PMT concentrations for 72 h.

Measurement of cell viability. This was performed by the trypan blue dye exclusion method. Osteoblasts were seeded at 5×10^4 per well in 24-well dishes, and nonadherent cells were removed after 24 h by washing the wells several times with 10% FCS–DMEM. PMT (20 ng/ml) was added to the experimental wells. After 48-h intervals, cells were removed from the culture well surface as detailed above and pooled with supernatants (in case dead cells had detached from the well surface). After pelleting ($350 \times g$, 5 min), the cells were washed in PBS, repelleted, and resuspended in a small volume of 0.2% filtered trypan blue in PBS (5 min). The suspension was then placed on a microscope slide, and osteoblast viabilities were determined (500 cells per well were counted). Dead or damaged cells appeared dark blue, while intact healthy cells appeared colorless.

Alkaline phosphatase (ALP) measurement. ALP assay was performed by a modification of the Sigma kit assay (no. 86). Briefly, osteoblasts were plated in 96-well tissue culture plates at a concentration of 2.5×10^4 to 5×10^4 per well in 10% FCS–DMEM. PMT was added in serial dilutions in triplicate at day 4, and the osteoblasts were cultured for a further 72 h. After 7 days, the plates were washed in prewarmed PBS (37°C), fixed (with a citrate-acetone-formaldehyde fixative [18 mM citric acid, 9 mM sodium citrate (pH 3.6)-acetone-formaldehyde (37%, vol/vol), in a 2.5:6.5:0.8 ratio]), washed in deionized water, and stained for ALP activity. The A_{540} values were read in a microplate reader (the wavelength of maximum absorbance for the naphthol AS-BI diazonium salt product). ALP concentrations were then calculated from a standard curve. Corresponding cell counts were performed for the determination of the ALP concentration per cell.

Measurement of mineralization. Mineralization studies were performed on 96-well multidishes. Osteoblasts and osteosarcoma cells were plated at 2×10^4 per well and grown for 4 days in 10% FCS–DMEM. Toxin was added on day 4 as before (serial dilutions in triplicate) and cultured for a further 72 h. At day 7, the cells were washed 3× with prewarmed PBS (37°C), fixed (with the same fixative), and stained with 2% alzarin red dye (pH 4.2) for 5 min (27). The wells were then rinsed with distilled water (three times), dishes were air dried, and the A_{492} was measured in a microplate reader. Alzarin red deposits a red lake in the presence of calcium and phosphate.

Osteoblasts were also cultured in the presence of mineral supplements. These included ascorbate (250 μM), β-glycerophosphate (10 mM), and glucocorticoids such as dexamethasone (1 μM). The presence of ascorbate and glucocorticoids has been cited as essential for nodule mineralization of calvarially derived cells whilst β-glycerophosphate enhances this effect (1, 36). The term supplemented medium refers to the use of these additives in the culture media.

Immunodetection of chicken type I collagen and osteonectin. Confluent osteoblasts were treated with a range of PMT concentrations (0 to 100 ng/ml) for 48 h. The osteoblast monolayers were lysed in sodium dodecyl sulfate (SDS) loading buffer (consisting of 0.4 ml of 1.25 M Tris-Cl [pH 6.8], 4.4 ml of distilled water, 0.8 ml of glycerol, 1.6 ml of 10% SDS, 0.4 ml of β-mercaptoethanol, and 0.4 ml of 0.1% aqueous bromophenol blue), and protein determinations were performed. Standardized protein concentrations (approximately 5 to 10 μg per well) were loaded and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (12% gels) on the Bio-Rad Mini-Protean system (23). Following Western blotting (immunoblotting) (with 0.025 M Tris, 0.2 M glycine, and 0.1% SDS), the nitrocellulose filters (Hybond Extra-C, Amersham) were probed with rabbit anti-chicken type I collagen (Serotec) at a 1:2,000 dilution, after blocking with 1% Marvel in PBS containing 0.1% Tween 20 (BDH, Merck). The blots were then treated with a 1:1,000 dilution of goat anti-rabbit immunoglobulin G (horseradish peroxidase conjugated). Chicken type I collagen was detected by autoradiography with ECL (Amersham). Similarly, nitrocellulose blots of osteoblast lysates were probed with a 1:2,000 dilution of a rabbit anti-chicken bone osteonectin antibody.

Cyclic AMP (cAMP) assay. Osteoblasts were plated at 2×10^5 cells per well in 24-well dishes. On reaching confluence (approximately 5 days), the medium was replaced with 0.1% FCS–DMEM and left for 24 h. This was replaced with serum-free DMEM-Waymouth's medium (1:1 [vol/vol]) containing (i) no addition (control), (ii) PMT (20 ng/ml), (iii) cholera toxin (100 ng/ml), or (iv) a mixture of both PMT (20 ng/ml) and cholera toxin (100 ng/ml) (added to the wells in triplicate at hourly intervals up to 4 h). On completion, the medium was removed and the intracellular cAMP was extracted in ice-cold, dilute acid (0.1 M HCl, 10 min). Extracts were then immediately stored at −20°C until assay. This was performed with the radioimmunoassay kit from Amersham (TRK 432).

Measurement of inositol phosphates. This was done according to the method of Nanberg and Rozengurt (31). Briefly, 2×10^5 osteoblasts were plated in 35-mm dishes and grown to confluence (7 days). The medium was replaced with 0.1% FCS in DMEM for 24 h, followed by serum-free DMEM for 24 h. The label myo-[^{3}H]inositol was added 16 to 24 h before the addition of PMT. The osteoblast monolayer was exposed to PMT for a minimum of 6 h. Inositol phosphates were extracted in ice-cold 3% perchloric acid, neutralized with 1 M potassium hydroxide, and added to ion-exchange columns containing 1 ml of Dowex AG 1-X8 resin (Bio-Rad). Elution of total inositol phosphates was performed by the addition of 1 M ammonium formate–0.1 M formic acid. A 1-ml aliquot of the eluent was added to liquid scintillant and counted for 10 min.

Statistical analyses. Analysis of data was performed by one-way analysis of variance. Significance values were calculated from the differences between control and experimental samples. Control values for each experiment are stated in the figure legends.

RESULTS

PMT induces DNA synthesis in osteoblast and osteoblast-like cells. It was decided to look initially at the effect of PMT on synthesis in osteosarcoma cells, which are known to possess several osteoblast-like characteristics such as responsiveness to parathyroid hormone (PTH) and expression of osteoblast markers (37). However, these cells displayed high background levels of incorporated [^{3}H]thymidine uptake (5.5×10^5 to 8×10^5 cpm), even in serum-free medium (presumably because of their transformed nature). Of the three osteosarcoma cell lines tested, only ROS 17/2.8 cells responded significantly to PMT (Fig. 1A); neither ROS 25/1 nor UMR 106 cells showed a significant increase in DNA synthesis under these conditions. However, PMT did stimulate DNA synthesis in ROS 25/1 cells when the monolayers were quiesced for longer and pulsed briefly with [^{3}H]thymidine near the end of this period (Fig. 1B). Incubation for up to 72 h in serum-free media was needed to visualize this effect.

Osteoblasts from primary culture proved much easier to quiesce than the osteosarcoma cells. Osteoblasts derived in this manner are heterogeneous in nature, consisting of osteoblasts, preosteoblasts, and other cells at varying degrees of differentiation (33). However, populations comprising a high percentage of ALP-positive cells could routinely be obtained. PMT induced a dose-dependent increase in thymidine uptake in response to PMT over a 40-h period, significant at 0.5 ng/ml and approximately threefold above the untreated control values at maximal stimulation (Fig. 2). A PMT concentration in the region of 20 ng/ml appeared to cause maximal stimulation of DNA synthesis which was similar to the value for Swiss 3T3 fibroblasts of around 10 to 20 ng/ml (42).

It was important to obtain osteoblasts from the first passage since subsequent passaging led to the loss of a number of osteoblast characteristics such as responsiveness to PTH and staining for ALP. In addition, continued passaging yielded populations of bilobular, fibroblastic cells with much increased background thymidine uptake. PMT did stimulate DNA synthesis in quiescent cultures of these cells, but the higher background and lower response to PMT made the effect significant only at quite high PMT concentrations (primary culture: controls, [29.4 ± 3.9]%; 20 ng of PMT per ml, [95.4 ± 4.4]%; after first passage: controls, [47.8 ± 3.8]%; 20 ng of PMT per ml,

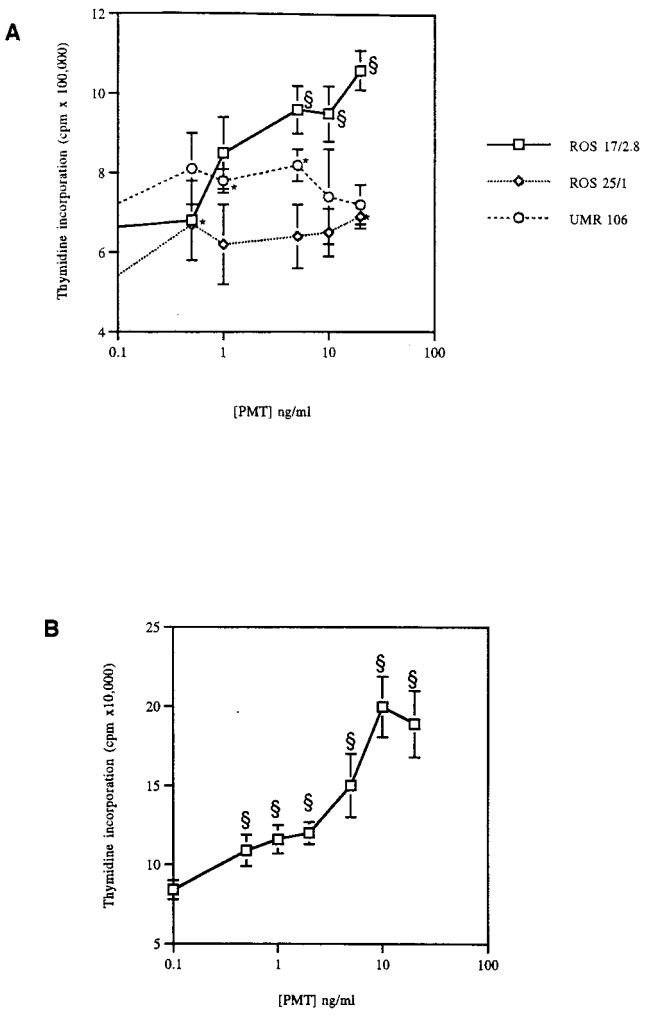

FIG. 1. (A) Stimulation of DNA synthesis in three osteoblast-like osteosarcoma cell lines. Cells were plated onto 35-mm culture wells at a density of 10^5 cells per well and grown to confluence (7 days) in 2.5 ml of DMEM–10% FCS. This was replaced with serum-free DMEM-Waymouth medium, 1:1 (vol/vol), containing 1 μl (0.037 MBq/μl) of [^{3}H]thymidine per ml and a range of PMT concentrations. After 40 h, DNA synthesis was assessed by measuring the level of [^{3}H]thymidine incorporated into half of the acid-precipitable material. Control values were 6.6 ± 0.9, 5.1 ± 0.9, and 7.0 ± 0.3 (10^5 cpm) for ROS 17/2.8, ROS 25/1, and UMR 106 cells, respectively. (B) Stimulation of DNA synthesis in an osteosarcoma cell line by thymidine pulsing. ROS 25/1 cells were plated as described for Fig. 1A. Cells were grown to confluence (7 days), and then serum-free DMEM was added for 72 h. The medium was replaced with 2 ml of serum-free DMEM-Waymouth's medium 1:1 (vol/vol) containing a range of PMT concentrations. After 40 h, cells were pulsed with 2 μl of [^{3}H]thymidine (0.037 MBq/μl) per well and cultured for a further 8 h. DNA synthesis was assessed as described above. Control values were 5.9 ± 0.7 (10^4 cpm). Each datum point is the mean of determinations of three independent experiments ± standard deviation. Asterisk, $P < 0.05$; section sign, $P < 0.01$.

[71.2 ± 5.9]%; all values expressed as percent relative mitogenicity compared with 10% FCS).

PMT stimulates osteoblast proliferation. When osteoblasts were plated at low density and grown to confluence, PMT accelerated osteoblast growth significantly at every time point over an 8-day period (Fig. 3A). This was also the case in confluent or nearly confluent populations which became quiescent after 24 h of incubation in serum-free medium. After 72 h of exposure to 5 to 10 ng of PMT per ml, the osteoblast count had almost doubled (Fig. 3B). After 96 h of proliferation, osteoblast growth appeared to have reached completion and the cells had consequently lifted from the surface of the culture wells. The viability of osteoblasts treated with PMT was assessed by dye exclusion. PMT did not cause necrosis in osteoblasts even at PMT concentrations as high as 100 ng/ml (data not shown). Indeed, over an 8-day period, 20 ng of PMT per ml maintained the viability of subconfluent osteoblasts slightly better than in control conditions.

PMT inhibits osteoblast differentiation. The effect of PMT on osteoblast differentiation was assessed by examining the expression of various markers. Treatment of confluent or nearly confluent primary osteoblasts with toxin for up to 48 h induced a 20 to 25% decrease in ALP activities (Fig. 4). When the increase in cell numbers was taken into account, it became clear that ALP activity per cell was much reduced upon toxin addition (Fig. 4). Even the addition of 1.95 ng of PMT per ml resulted in a 40 to 50% reduction of ALP expression per osteoblast.

Another biochemical marker of osteoblast differentiation is the ability to form mineralized bone nodules in vitro (15). From observation, it appeared that nodule formation and initial seeding density had a linear relationship (data not shown). UMR 106 cells, reported to show a number of osteoblast characteristics (29), showed a dramatic reduction in the levels of mineralization when PMT was present (Fig. 5A). Both in normal and supplemented medium, the amount of staining was reduced in response to PMT in a dose-dependent manner, reaching approximately 60% and 40% of controls, respectively, with 11.1 ng of PMT per ml over a 4-day incubation period. Primary osteoblasts also showed this effect, though to a lesser degree than UMR cells (Fig. 5B). Over the same time period, primary osteoblasts incubated with 11.1 ng of PMT per ml stained approximately 70% and 75% of controls in normal and supplemented media, respectively.

The expression of the most abundant protein in bone, namely, type I collagen (45), was also down regulated in re-

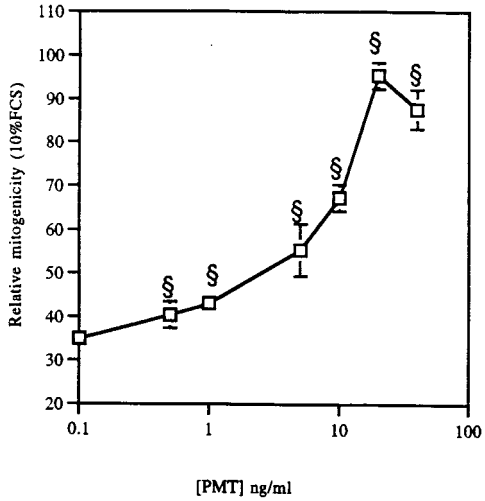

FIG. 2. Stimulation of DNA synthesis in primary osteoblasts. Primary derived chicken osteoblasts were isolated from embryonic calvariae as described in Materials and Methods. Cells were grown to confluence in 10% FCS–DMEM (5 to 7 days) or 0.5% FCS–DMEM (48 h). The medium was replaced with 2 ml of DMEM-Waymouth medium 1:1 (vol/vol) containing 2 μl of [^{3}H]thymidine (0.037 MBq/μl) and various concentrations of PMT. The osteoblasts were cultured for a further 40 h, and the incorporation of label was measured in the acid-precipitable material as before. Mitogenicity is expressed as the percentage of 10% FCS. Control values were (29.4 ± 2.5)%. Each datum point is the mean of determinations of three independent experiments ± standard deviation. Section sign, $P < 0.01$.

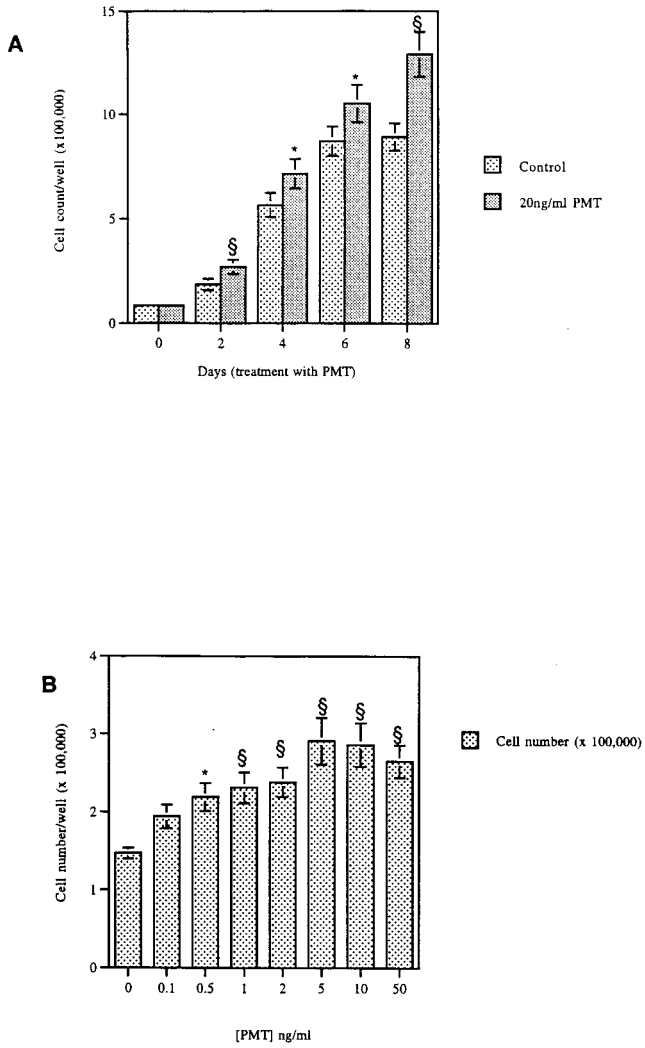

FIG. 3. (A) Stimulation of cell proliferation of subconfluent primary osteoblasts. Osteoblasts were plated at low density in 24-well culture dishes (see Materials and Methods) in 10% FCS–DMEM in the presence or absence of 20 ng of PMT per ml. Cell counts were performed every 48 h. (B) Stimulation of cell proliferation in confluent primary osteoblasts. Osteoblasts were plated at high density in 24-well culture dishes and grown to confluence (5 to 7 days), in serum-free DMEM (24 h), and finally in the presence or absence of various concentrations of PMT in 0.1% FCS–DMEM (72 h). In both graphs, each datum point represents the means of three independent cell counts ± standard deviation. Asterisk, $P < 0.05$; section sign, $P < 0.01$.

sponse to PMT. As Fig. 6 shows, the amount of protein recognized on the blot as type I collagen (approximately 120 kDa) declined in response to increasing concentrations of the toxin. However, the expression of osteonectin was not affected by PMT (Fig. 6). Osteonectin is expressed in a variety of different cell types but is thought to play a prominent role in the attachment of mineral to collagen in bone (48).

Effect of PMT on cAMP levels and inositol phosphate production. Intracellular cAMP is an important secondary messenger in signal transduction systems (41). However, PMT did not affect the intracellular concentration of cAMP in quiescent osteoblasts (Fig. 7A). Cholera toxin (CT), an agent known to increase intracellular cAMP in a variety of cell types (47), did increase levels in primary osteoblasts. However, the elevation of cAMP induced by 100 ng of CT per ml was reduced by the presence of PMT.

The release of inositol phosphates (notably inositol triphosphate, IP_3) from intracellular stores is another common secondary messenger often elevated in response to mitogens. PMT induced a biphasic release of IP_3 in primary osteoblasts with a maximal response in the region of 5 to 10 ng of PMT per ml (Fig. 7B). IP_3 releases were lower as the concentration of PMT was raised above 10 ng/ml.

DISCUSSION

PMT as a mitogen. We have investigated the interaction of PMT with primary bone cells, which are potentially a natural target for PMT. PMT has been shown previously to be a mitogen for a number of established cell lines (18, 42, 50), but it was not known whether it would fulfill a similar mitogenic potential in cultures of primary cells. Osteosarcoma cells have been useful in previous studies of osteoblasts (37), but they do not possess all the characteristics of osteoblasts (44). PMT was mitogenic for these cells, but in view of their transformed phenotype, the difficulty in obtaining quiescent cells, and the doubts about their osteoblast characteristics, it was decided to use primary bone cells. The chicken was chosen as a model for the preparation of bone cells since (i) it is a widely used source for the derivation of both osteoblasts and osteoclasts and protocols are freely available in the scientific literature (40), (ii) the cells are of embryonic origin and are relatively easy to maintain, and (iii) the calvariae of the embryonic chicks possess a thicker layer of potential osteoprogenitor cells than sources in other species (33).

The results show that PMT stimulated DNA synthesis in primary osteoblasts at extremely low concentrations in a dose-dependent manner and led to cell proliferation. The toxin was also capable of stimulating cells which had been quiesced in serum-depleted medium. As many of these osteoblasts would probably be entering interphase before toxin addition, PMT appeared to stimulate cells to reenter the cell cycle.

Whilst PMT elicits many of the properties of a tumor promoter (18, 42, 46), there is no evidence that the stimulated cells are transformed. The toxin stimulates prolonged cell turnover in Swiss 3T3 cells for several passages after its removal from the culture medium, but the effect is finite (42). Similarly,

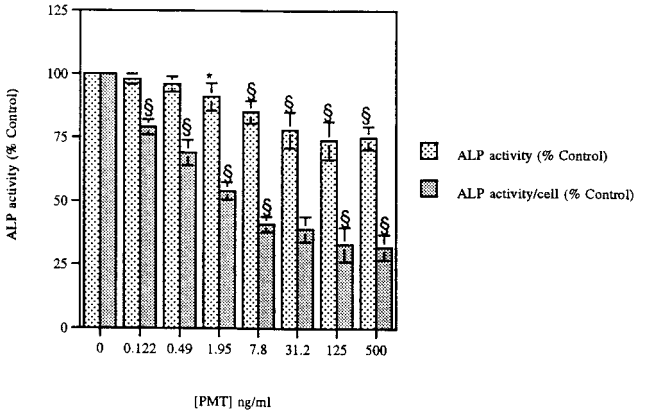

FIG. 4. Effect of PMT on osteoblast expression of ALP. Osteoblasts were plated in 96-well dishes and cultured for up to 7 days. At day 4, PMT was added in serial dilutions in triplicate. On day 7, analyses of ALP activities per well were made. ALP data were presented as a percentage of control. Cell numbers at the end of the incubation period were counted and used to calculate the concentration of ALP per cell. Control values were 0.081 ± 0.012 DEA units per well. Each bar represents the mean of three independent experiments ± standard deviation. Asterisk, $P < 0.05$; section sign, $P < 0.01$.

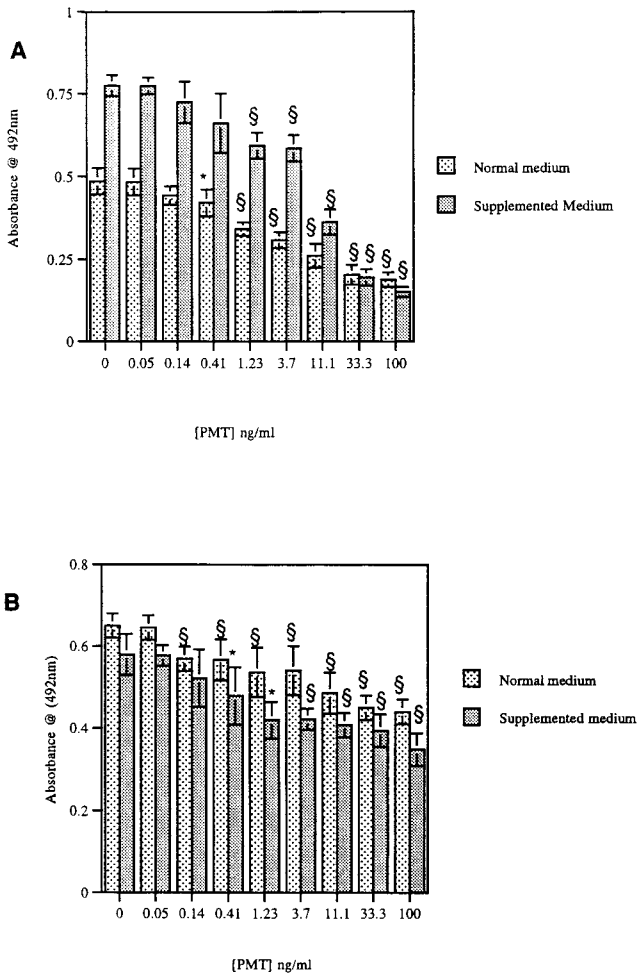

FIG. 5. (A) Effect of PMT on the mineralization of UMR 106 osteosarcoma cells. UMR 106 cells were plated in 96-well dishes in 10% FCS–DMEM. PMT was added in triplicate at day 4. At day 7, the monolayers were fixed and stained with 2% alzarin red (pH 4.2) for 5 min. The degree of mineralization was assessed by reading A_{492}. (B) Effect of PMT on the mineralization of primary osteoblasts. Osteoblasts were plated in 96-well dishes in 10% FCS–DMEM, and PMT was added in triplicate at day 4. After fixation, the osteoblasts were stained with alzarin red and mineralization was assessed as described above. Each bar represents the mean of three independent experiments ± standard deviation. Asterisk, $P < 0.05$; section sign, $P < 0.01$.

chicken osteoblasts reached a stage after confluence when they ceased to proliferate and lifted off the culture surface (data not shown) even in the presence of serum or toxin. The toxin did not appear to cause cell necrosis even at very high concentrations. Indeed, prolonged exposure to the optimum stimulatory concentration of PMT (20 ng/ml) actually helped maintain osteoblasts in a more viable state compared with control cultures.

PMT caused the release of inositols from intracellular stores in osteoblasts and, as in Swiss 3T3 cells, appeared to act via a cAMP-independent pathway (42). The toxin may be unique in its mitogenic action, since most other well-known mitogens except transforming growth factor β (TGF-β) (i.e., platelet-derived growth factor [PDGF], FGF, bombesin, etc.) cause the elevation of intracellular cAMP levels (41). TGF-β, like PMT, is known to cause a mitogenic effect (accompanied by down-regulation of osteoblast markers [26]), on responsive osteoblasts (53) without any apparent rise in intracellular cAMP levels (21). PMT actively inhibited the elevation of cAMP by cholera toxin within 1 h. This effect is puzzling since time course studies of toxin action show that the shortest time between toxin addition and detection of the first biochemical event (namely, the rise in inositol phosphates) is 2 h, presumably for binding, internalization, and processing to take place (42).

PMT and differentiation. PMT inhibited the surface expression of ALP, an enzyme which is abundant in the mature osteoblast phenotype and which is thought to be very important in the formation of calcified bone. Although not exclusively confined to osteoblasts, ALP is more abundant in this cell type than any other (2).

Another late differentiation parameter of osteoblast development is the ability to form calcified nodules in vitro. It has been estimated that only about 0.3% of isolated calvarial cells are osteoprogenitor cells, each of which develops into a nodule without need for the cooperativity of surrounding cells (44). One of the problems of using primary bone cells in this manner is the heterogeneity of the resultant culture. Nodules represent groups of fully mature, active bone cells (about 100 per nodule) and are surrounded by cells of less well defined phenotypes (2). Nodules consist of cells rich in ALP which constitutively deposit mineral in the form of poorly crystallized hydroxylapatite. The deposition of mineral in vitro was also inhibited by PMT, and the number and size of actively mineralizing nodules found in PMT-treated wells were significantly reduced, despite having greater cell numbers. The expression of type I collagen, which constitutes >95% of total bone collagen, was also down-regulated in response to PMT. This protein is usually optimally expressed in the early proliferative phase of osteoblast maturation (44). Thus PMT simultaneously inhibits the secretion of the protein which comprises the major structural components of the bone matrix and inhibits the deposition of mineral in vitro. These effects are likely to have a very negative effect on the ability of osteoblasts to form fully functional bone.

The protein osteonectin and/or its mRNA are detected in several nonbone tissues, but osteonectin only accumulates in bone in appreciable quantities (48), though its function is not fully understood (4). The expression of osteonectin mRNA occurs in both proliferating and nonproliferating bone cells and is not unduly affected by growth factor-like activity (11). For example, stimulation of quiescent Swiss 3T3 fibroblasts by FGF does not affect osteonectin expression (3). Osteonectin expression was not altered in response to the mitogenic stimulation of the osteoblasts with PMT.

These results indicate that PMT, while promoting osteoblast

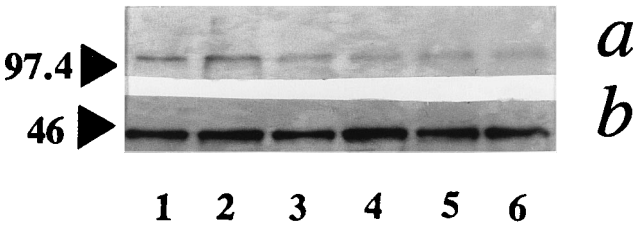

FIG. 6. Effect of PMT on expression of type I collagen and osteonectin. Osteoblasts were plated at 2×10^5 cells per well in 24-well dishes and grown to confluence (5 days). The medium was replaced with 0.1% FCS–DMEM (24 h) before the addition of 0 (lane 1), 1 (lane 2), 5 (lane 3), 10 (lane 4), 50 (lane 5), and 100 (lane 6) ng of PMT per ml for 3 days. Following SDS-PAGE and Western blotting, the expression of type I collagen (*a*) and osteonectin (*b*) was assessed (see Materials and Methods). Numbers on left indicate relative molecular mass (in kilodaltons).

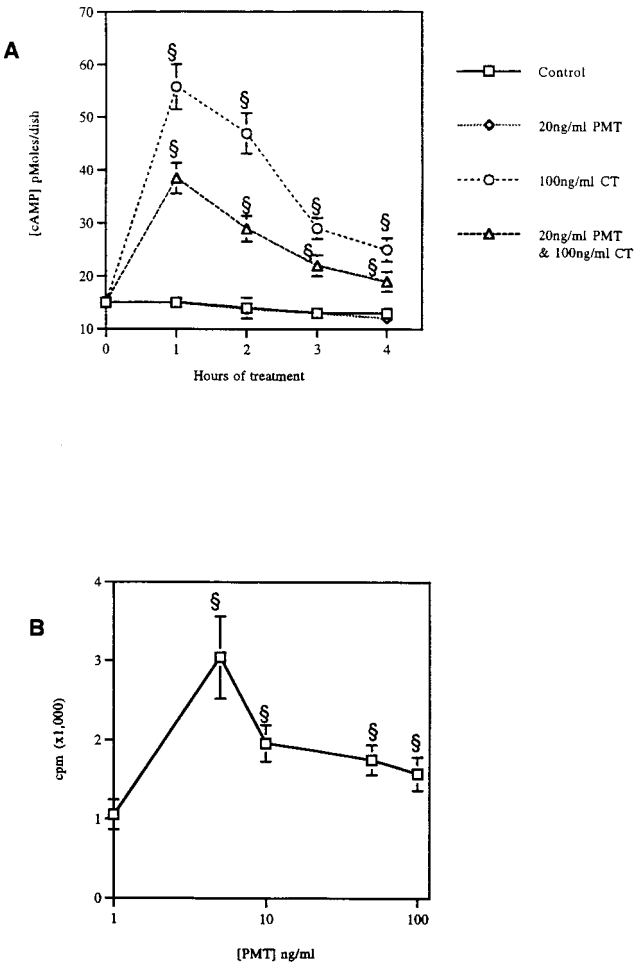

FIG. 7. (A) Effect of PMT on cAMP levels in primary osteoblasts. Osteoblasts were plated at 2×10^5 cells per well in 24-well dishes in 10% FCS–DMEM (5 days) and then in 0.1% FCS–DMEM (24 h). The medium was replaced with serum-free DMEM containing (i) no addition, (ii) PMT (20 ng/ml), (iii) CT (100 ng/ml), or (iv) a mixture of both. Intracellular cAMP levels were measured according to the Sigma kit format. (B) Effect of PMT on inositol phosphates in primary osteoblasts. Osteoblasts were plated at 2.5×10^5 per 35-mm dish and grown to confluence (7 days), in 0.1% FCS–DMEM (24 h), and then in serum-free DMEM (12 to 24 h). [^{3}H]inositol was added 16 to 24 h before the addition of PMT. The monolayers were exposed to PMT for a minimum of 6 h. Control values were 0.58 ± 0.09 (10^3 cpm). In both graphs, each datum point represents the mean of three independent determinations $\pm$ standard deviation (section sign, $P < 0.01$).

proliferation, halts osteoblast differentiation at a preosteoblastic stage and thereby inhibits terminal differentiation to functional osteoblasts. In many ways, its effect on cell differentiation is typical of a potent mitogen. Osteoblasts possess receptors for several well-known mitogens such as PDGF, FGF, and epidermal growth factor (EGF). Both aFGF and bFGF stimulate osteoblast proliferation but strongly inhibit ALP activity and collagen production (6, 16). Similarly, EGF stimulates osteoblast proliferation, inhibits ALP activity and collagen synthesis, and promotes expression of collagenase (17, 32). PDGF increases osteoblast DNA synthesis, but the synthesis of type I collagen is also increased (7). In general, potent growth factors promote osteoblast proliferation but strongly inhibit differentiation. Our results show that PMT is another tool with which to study differentiation in this system.

PMT and bone resorption. Bone loss is a feature of several diseases, most notably osteoporosis (19). In theory, bone loss could be due to the failure of osteoblasts to lay down bone, increased resorption by osteoclasts, or both. Many of the better-known osteotrophic molecules, notably PTH, appear to cause bone resorption by the initial dedifferentiation of osteoblasts followed by the modulation of osteoclast activity. PTH, for example, also inhibits ALP expression and nodule formation and promotes production of collagenase and other proteases not normally expressed by mature osteoblasts (28). This has the effect of eroding the protective osteoid layer surrounding bone, allowing access for osteoclasts at the bone surface. Such dedifferentiated cells also produce prostaglandins, prostacyclins, and a number of unknown protein factors reported to be responsible for the activation of osteoclast resorption (54).

Previous studies with PMT and bone have shown that the toxin stimulates bone resorption, with an increase in osteoclast activity, but did not show a role for osteoblasts and were unable to exclude a potential role for other cell types in the nasal cavity or identify the target for PMT (14, 22). We have shown that PMT directly affects osteoblast function, where, as with Swiss 3T3 fibroblasts (42), the principal action of PMT appears to be the stimulation of cell growth. Indeed the interconversion of the osteoblast and fibroblast phenotypes has been postulated in several papers (26, 33), and the formation of fibrous tissue instead of bone has also been reported in AR (19). It may be that PMT causes an inhibition of normal osteoblast development, promoting a preosteoblast-fibroblast population incapable of forming a normal bone matrix. This may play a role in the development of AR, but it is reasonable to assume that there will be other contributory factors, notably the control of osteoclast behavior (and therefore resorption) by osteoblasts. The production of second messengers by osteoblasts, the stimulation of mature osteoclasts, or the recruitment of osteoclast precursors may all be routes by which PMT can effect resorption (22). Growth factors such as PDGF and EGF are known to stimulate resorption indirectly via osteoblasts by a prostaglandin-mediated mechanism (7, 49). Whether PMT has a similar effect on osteoblast-osteoclast interactions remains to be elucidated.

ACKNOWLEDGMENTS

We are grateful to Phil Ward (IAH, Compton, Newbury, Berkshire, United Kingdom) for providing purified recombinant PMT and Theresa Higgins, Enrique Rozengurt, and Nadira Ali (ICRF, Lincoln's Inn Fields, London, United Kingdom) for advice on cell assays.

This work was supported by a BBSRC grant (SCMB 721).

REFERENCES

1. **Bellows, C. G., J. E. Aubin, J. N. M. Heersche, and M. E. Antosz.** 1986. Mineralized bone nodules formed *in vitro* from enzymatically released rat calvarial cell populations. Calcif. Tissue Int. **38:**143–154.
2. **Beresford, J. N., S. E. Graves, and C. A. Smoothy.** 1993. Formation of mineralized nodules by bone derived cells *in vitro*: a model of bone formation. Am. Med. Genet. **45:**163–178.
3. **Blanchard, J. M., M. Pfechaczyk, C. Dani, J. C. Chambard, A. Franchi, J. Pouyssegur, and P. Jeantur.** 1985. *C-myc* gene is transcribed at high rate in G_0-arrested fibroblasts and is post-transcriptionally regulated in response to growth factors. Nature (London) **317:**443–445.
4. **Bolander, M. E., P. Gehron-Robey, L. W. Fisher, K. M. Conn, B. S. Prabhaker, and J. D. Termine.** 1989. Monoclonal antibodies against osteonectin show conservation of epitopes across species. Calcif. Tissue Int. **45:**74–80.
5. **Buys, W. E. C. M., H. E. Smith, A. M. I. E. Kamps, E. M. Kamp, and M. A. Smits.** 1990. Sequence of the dermonecrotic toxin of *Pasteurella multocida* ssp. *multocida*. Nucleic Acids Res. **18:**2815–2816.
6. **Canalis, E.** 1985. Effect of growth factors on bone cell replication and differentiation. Clin. Orthop. Relat. Res. **193:**246–263.
7. **Cantrella, M., T. L. McCarthy, and E. Canalis.** 1989. Platelet derived growth factor enhances deoxyribonucleic acid and collagen synthesis in osteoblast-enriched cultures from fetal rat bone. Endocrinology **125:**13–19.
8. **Caprioli, A., G. Donelli, V. Falbo, R. Possenti, L. G. Roda, G. Roscetti, and**

F. M. Ruggeri. 1984. A cell division-active protein from E. coli. Biochem. Biophys. Res. Commun. **118:**587–593.
9. **Caprioli, A., V. Falbo, L. G. Roda, F. M. Ruggeri, and C. Zona.** 1983. Partial purification and characterization of an *Escherichia coli* toxic factor that induces morphological cell alterations. Infect. Immun. **39:**1300–1306.
10. **Chanter, N.** 1990. Advances in atrophic rhinitis and toxigenic *Pasteurella multocida* research. Pig News Info. **11:**503–506.
11. **Cochran, B. H., J. Zullo, I. M. Verma, and C. D. Stiles.** 1984. Expression of a *c-fos* gene and a *fos*-related gene is stimulated by platelet-derived growth factor. Science (Washington, D.C.) **226:**1080–1083.
12. **De Rycke, J., E. A. Gonzalez, J. Blanco, E. Oswald, M. Blanco, and R. Boivin.** 1990. Evidence for two types of cytotoxic necrotizing factor in human and animal clinical isolates of *Escherichia coli*. J. Clin. Microbiol. **28:**694–699.
13. **Dicker, P., and E. Rozengurt.** 1980. Phorbol esters and vasopressin stimulate DNA synthesis by a common mechanism. Nature (London) **287:**607–612.
14. **Felix, R., H. Fleisch, and P. L. Frandsen.** 1992. Effect of *Pasteurella multocida* toxin on bone resorption in vitro. Infect. Immun. **60:**4984–4988.
15. **Gerstenfeld, L. C., S. D. Chipman, C. M. Kelly, K. J. Hodjens, P. Lee, and W. Landis.** 1988. Collagen expression, ultra-structural assembly and mineralization in cultures of chicken embryo osteoblasts. J. Cell Biol. **106:**979–989.
16. **Globus, R., J. Plonet, and D. Gospodarowicz.** 1988. Cultured bovine cells synthesize fibroblast growth factor and store it in their extracellular matrix. Endocrinology **124:**1539–1547.
17. **Hata, R., H. Hori, Y. Nagay, S. Tanaka, M. Kondo, M. Hiramatsu, N. Utsumi, and M. Kumegawa.** 1984. Selective inhibition of type I collagen synthesis in osteoblastic cells by epidermal growth factor. Endocrinology **115:** 867–876.
18. **Higgins, T. E., A. C. Murphy, J. M. Staddon, and A. J. Lax.** 1992. *Pasteurella multocida* toxin is a potent inducer of anchorage-independent cell growth. Proc. Natl. Acad. Sci. USA **89:**4240–4244.
19. **Horowitz, M. C.** 1993. Cytokines and estrogen in bone: anti-osteoporotic effects. Science (Washington, D.C.) **260:**626–627.
20. **Il'ina, Z. M., and M. I. Zasukhin.** 1975. Role of *Pasteurella* toxins in the pathogenesis of infectious atrophic rhinitis. Sb. Nauchn. Rab. Sib. Nauchno-Issled. Vet. Inst. **25:**76–86.
21. **Jongen, J. W. J. M., E. C. Willemsteinvanhove, J. M. Vandermeer, M. P. Bos, H. Juppner, G. V. Serge, A. B. Abousamra, J. H. M. Feyen, and M. P. M. Herrmannerlee.** 1995. Down regulation of the receptor for PTH and PTH-rP by TGF-β in primary foetal-rat osteoblasts. Endocrinology **136:**3260–3266.
22. **Kimman, T. G., C. W. G. M. Lowik, L. J. A. Van De Wee-Pals, C. W. Thesingh, P. Defize, E. M. Kamp, and O. L. M. Bijvoet.** 1987. Stimulation of bone resorption by inflamed nasal mucosa, dermonecrotic toxin-containing conditioned medium from *Pasteurella multocida* and purified dermonecrotic toxin from *P. multocida*. Infect. Immun. **55:**2110–2116.
23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
24. **Lax, A. J., and N. Chanter.** 1990. Cloning of the toxin gene from *Pasteurella multocida* and its role in atrophic rhinitis. J. Gen. Microbiol. **136:**81–87.
25. **Lax, A. J., N. Chanter, G. D. Pullinger, T. Higgins, J. M. Staddon, and E. Rozengurt.** 1990. Sequence analysis of the potent mitogenic toxin of *Pasteurella multocida*. FEBS Lett. **277:**59–64.
26. **McCauley, L. K., A. J. Koh, C. A. Beecher, Y. Q. Cui, J. D. Decker, and R. T. Francheschi.** 1995. Effects of differentiation and transforming growth-factor beta (1) on PTH/PTHRP receptor messenger RNA levels in MC3T3-E1 cells. J. Bone Miner. Res. **10:**1243–1255.
27. **McGee-Russell, S. M.** 1958. Histochemical methods for calcium. J. Histochem. Cytochem. **6:**22.
28. **Meikle, M. C., S. Bord, R. M. Hembry, J. Compston, P. I. Croucher, and J. J. Reynolds.** 1992. Human osteoblasts in culture synthesize collagenase and other matrix metalloproteinases in response to osteotropic hormones and cytokines. J. Cell Sci. **103:**1093–1099.
29. **Michaelangeli, V. P., D. M. Findlay, A. Fletcher, and T. J. Martin.** 1986. Calcitonin gene-related peptide (cGRP) acts independently of calcitonin on cAMP formation in clonal osteogenic-sarcoma cells (UMR-106-01). Calcif. Tissue Int. **39:**44–48.
30. **Murphy, A. C., and E. Rozengurt.** 1992. *Pasteurella multocida* toxin selectively facilitates phosphatidylinositol 4,5-bisphosphate hydrolysis by bombesin, vasopressin and endothelin. J. Biol. Chem. **267:**25296–25303.
31. **Nanberg, E., and E. Rozengurt.** 1988. Temporal relationship between inositol polyphosphate formation and increases in cytosolic calcium in quiescent 3T3 cells stimulated by PDGF, bombesin and vasopressin. EMBO J. **7:** 2741–2748.
32. **Nicolas, V., J. R. Nefussi, P. Colln, and N. Forest.** 1990. Effects of acidic fibroblast growth factor and epidermal growth factor on subconfluent fetal rat calvarial cell cultures: DNA synthesis and alkaline phosphatase activity. Bone Miner. **8:**145–156.
33. **Nijweide, P. J., A. Van der Plas, and J. P. Scherft.** 1981. Biochemical and histochemical studies on various bone cell preparations. Calcif. Tissue Int. **33:**529–540.
34. **Oswald, E., and J. De Rycke.** 1990. A single protein of 110kDa is associated with the multinucleating and necrotizing activity coded by the Vir plasmid of *Escherichia coli*. FEMS Microbiol. Lett. **68:**279–284.
35. **Oswald, E., M. Sugai, A. Labigne, H. C. Wu, C. Fiorentini, P. Boquet, and A. D. O'Brien.** 1994. Cytotoxic necrotizing factor type 2 produced by virulent E. coli modifies the small GTP-binding proteins Rho involved in assembly of actin stress fibres. Proc. Natl. Acad. Sci. USA **91:**3814–3818.
36. **Page, K. M.** 1982. Bone and the preparation of bone sections, p. 324–325. *In* J. D. Bancroft and A. Stevens (ed.), *In* Theory and practice of histological techniques, 2nd ed. Churchill Livingstone Inc., New York.
37. **Perry, H. M., W. Skogen, J. Chappel, A. J. Kahn, G. Wilner, and S. L. Teitelbaum.** 1989. Partial characterisation of a parathyroid hormone-stimulated resorption factor(s) from osteoblast-like cells. Endocrinology **125:** 2076–2079.
38. **Petersen, S. K.** 1990. The complete nucleotide sequence of the *Pasteurella multocida* toxin gene and evidence for a transcriptional repressor, *TxaR*. Mol. Microbiol. **4:**821–830.
39. **Raisz, L. G.** 1988. Hormonal-regulation of bone growth and remodeling. Ciba Found. Symp. **136:**226–238.
40. **Rodan, G. A., and S. B. Rodan.** 1984. Expression of the osteoblast phenotype, p. 244–285. *In* W. A. Peck (ed.), Bone and mineral research 2. Elsevier Science Publishing, Amsterdam.
41. **Rozengurt, E.** 1986. Early signals in the mitogenic response. Science **234:** 161–166.
42. **Rozengurt, E., T. Higgins, N. Chanter, A. J. Lax, and J. M. Staddon.** 1990. *Pasteurella multocida* toxin: potent mitogen for cultured fibroblasts. Proc. Natl. Acad. Sci. USA **87:**123–127.
43. **Rozengurt, E., and J. Sinnett-Smith.** 1983. Bombesin stimulation of DNA synthesis and cell division in cultures of Swiss 3T3 fibroblasts. Proc. Natl. Acad. Sci. USA **80:**2936–2940.
44. **Scutt, A., H. Mayer, and E. Wingender.** 1992. New perspectives in the differentiation of bone-forming cells. Biofactors **4:**1–13.
45. **Simmons, P. J., G. N. Kent, R. L. Jilka, P. M. Scott, M. Fallon, and P. V. Kohn.** 1982. Formation of bone by isolated, cultured osteoblasts in millipore diffusion chambers. Calcif. Tissue Int. **34:**291–294.
46. **Staddon, J. M., N. Chanter, A. J. Lax, T. E. Higgins, and E. Rozengurt.** 1990. *Pasteurella multocida* toxin, a potent mitogen, stimulates protein kinase C-dependent and -independent protein phosphorylation in Swiss 3T3 cells. J. Biol. Chem. **265:**11841–11848.
47. **Stavric, S., J. I. Spiers, J. Konowalchuk, and D. Jeffrey.** 1978. Stimulation of cyclic AMP secretion in Vero cells by enterotoxins of *Escherichia coli* and *Vibrio cholerae*. Infect. Immun. **21:**514–517.
48. **Termine, J. D., H. K. Kleinman, S. W. Whitson, K. M. Conn, M. L. McGarvey, and G. R. Martin.** 1981. Osteonectin, a bone-specific protein linking mineral to collagen. Cell **26:**99–105.
49. **Toriyama, T., I. Morita, and S.-I. Murota.** 1992. The existence of distinct classes of prostaglandin E_2 receptors mediating adenylate cyclase and phospholipase C pathways in osteoblastic clone MC3T3-E1. Prostaglandins Leukot. Essent. Fatty Acids **46:**15–20.
50. **Van Diemen, P. M., G. de Vries Reilingh, and H. K. Parmentier.** 1994. Immune responses of piglets to *Pasteurella multocida* toxin and toxoid. Vet. Immunol. Immunopathol. **41:**307–321.
51. **Walker, K. E., and A. A. Weiss.** 1994. Characterization of the dermonecrotic toxin in members of the genus *Bordetella*. Infect. Immun. **62:**3817–3828.
52. **Ward, P. N., T. E. Higgins, A. C. Murphy, P. B. Mullan, E. Rozengurt, and A. J. Lax.** 1994. Mutation of a putative ADP-ribosylation motif in the *Pasteurella multocida* toxin does not affect mitogenic activity. FEBS Lett. **342:** 81–84.
53. **Wong, G. L., and M. C. Ng.** 1992. Maturation associated changes in the cellular composition of mouse calvariae and in the biochemical characteristics of calvarial cells separated into subclasses on percoll density gradients. J. Bone Miner. Res. **7:**701–708.
54. **Yoshikawa, T., and T. Hanada.** 1981. Histopathological studies on pigs with atrophic rhinitis showing retarded growth. Jpn. J. Vet. Sci. **43:**221–231.

Editor: S. H. E. Kaufmann