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DEPRESSIVE EFFECTS OF ACRYLIC CEMENT COMPONENTS ON BONE METABOLISM

Isotope Release and Phosphatase Production Studied in Vitro

JOHN GREGOR PEDERSEN, BJARNE LUND & INGE REIMANN

Department of Orthopaedic Surgery U, Calcified Tissue Research Lab., Rigshospitalet, Denmark

In vitro methyl methacrylate bone cement components were found to depress the release of radioactive calcium and proline as well as the activity of both alkaline and acid phosphatases. These effects were dose dependent and reached levels observed for dead bone. These observations may reflect part of the pathogenesis of loosening of joint replacements involving the use of bone cement.

Key words: acrylic cement; bone; enzymes; histochemistry; isotopes.

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The widespread use of bone cement in joint replacement and the more frequent use of this substance to stabilize pathological fractures (Harrington et al. 1972), even ordinary Colles' fractures (Charnley 1970), increases the demand for more knowledge about acrylic cement-bone interactions. Observations of increasing loosening of joint replacements and non-union of fractures treated with bone cement poses the question whether bone cement has an inhibitory effect on bone turnover.

The effects of monomer on human polymorphonuclear leukocytes are depressed chemotaxic activity and impaired killing of bacteria (Petty 1978a,b). Monomer from bone cement has further been shown to be highly toxic to tissue in the rabbit's ear, leading in high concentrations to fulminant necrosis (Linder 1976). In an animal model the presence of residual monomer in polymethyl methacrylate was shown to cause a considerable inhibition of bone formation (Garcia et al. 1981).

To examine the effects of monomer and polymer on bone, we have used radioactive calcium and radioactive proline release and semiquantitative histochemical estimation of alkaline and acid phosphatase activity, parameters previously used to evaluate changes in bone turnover (Pedersen et al. 1982).

MATERIAL AND METHODS

Test substances

We used CMW® cement as commercially available for clinical use. The monomer was added to the medium directly from the ampoule. To 40 g polymer 100 ml sterile water was added and allowed to stand for 1 week with a magnetic stirrer. After precipitation of residual particle-polymer, the supernatant was added to the medium. Neither the monomer nor the polymer caused any change in the pH value of the medium.

Isolation and culture

Calvaria from 4-day-old mice injected subcutaneously 2 days previously with Ca-45 or H-3 proline were removed and cultured for 48 h, according to the technique of Reynolds & Dingle (1970). The parietal bones were cut into two pieces, one for control and the other for experiments. The isolated bone pieces were

cultured in test tubes containing 2 ml Medium 199 (Gibco 115 EE), to which bovine serum albumin fraction V (5 g/l) and Ampicillin (40 mg/l) had been added. The culture was incubated in an atmosphere of 5% CO_2, 75% N_2 and 20% O_2 at 37°C and regassed after 24 h. Each calvarium was decalcified in 1 ml 1 N hydrochloric acid at 90°C. Half a ml from this solution and from the medium were then processed by standard liquid scintillation counting techniques.

Release was expressed as the percentage of total isotope found in the medium. The values were then calculated as a ratio between the treated and the control bones.

In order to study the reversibility of the effect of monomer on calcium release, we removed both the treated and the control bone after 1 day of culture in medium containing 1 μl monomer per ml medium. Both bones were then transferred to fresh control media and cultured for another 2 days. The experiment was stopped as described above.

Histochemical analysis

Non-labelled calvaria grown for 48 h were used for semiquantitative histochemical examinations. The specimens were frozen at –20°C immediately after removal from the medium and sections 6 μm thick were cut in a cryostat. Twelve sections were prepared from each specimen, six for determination of alkaline phosphatase and six for acid phosphatase activity. For histochemical demonstration of non-specific alkaline phosphatase activity the simultaneous coupling method described by Burstone (1959) was used with naphtol-AS-BI-phosphate (Sigma N 22508) as substrate (0.2 mg/ml) and Fast Red Violet LB (Difco 8194-12) as indicator (1.4 mg/ml). Acid phosphatase activity was demonstrated by Burstone's modification of the simultaneous coupling method described by Barka & Anderson (1963) with naphtol-AS-BI-phosphate (Sigma N 22509) as substrate (0.5 mg/ml) and hexazotized pararosaniline (Sigma P 37507) as indicator.

The examinations were performed by light microscopy at magnification 100×, at temperature 22°C and at pH 8.3 for alkaline phosphatase and pH 5.0 for acid phosphatase. In order to obtain a semiquantitative estimation of the enzyme activity the initial time was recorded, i.e. the interval between incubation and occurrence of the first staining reaction. For alkaline phosphatase the reaction was first seen as a red colour at the surface of the bone located to osteoblasts and preosteoblasts (Figure 1). For acid phosphatase, the first colour was observed in the osteoclasts corresponding to the lysosomes (punctate reaction) (Figure 1). For control purposes, incubations without substrate were carried out simultaneously. To ensure that the substrate concentration was sufficient to achieve zero order kinetics, the initial time was measured on serial sections with different concentrations of the substrate.

As a control for enzyme diffusion in the incubation medium, serial sections were incubated in pairs with and without preincubations for varying periods of time with the respective buffer. The control for enzyme diffusion showed that, within the times obtained in this study, there was no, or inconsiderably decreased, activity for both alkaline and acid phosphatase.

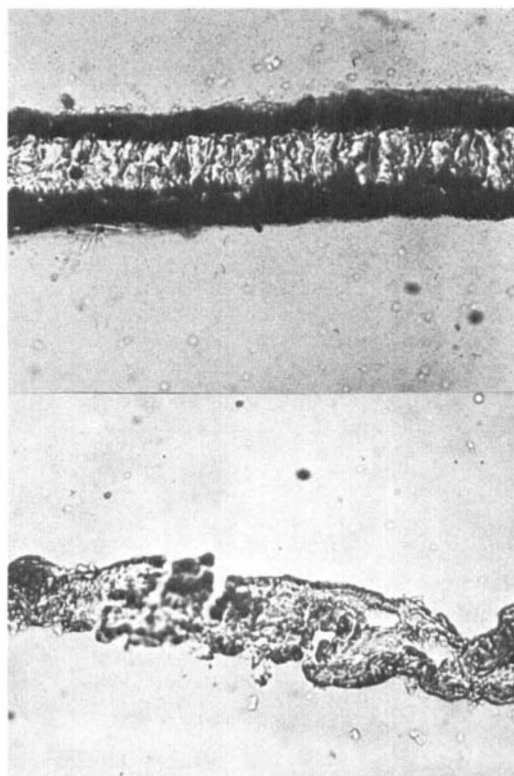

Figure 1. Upper. Photograph of frozen section, 6 μm thick, from a mouse calvarium stained for alkaline phosphatase. The reaction (black colour) is seen in the osteoblasts and preosteoblasts. Magnification ×100. Lower. Frozen section, 6 μm thick, stained for acid phosphatase. The reaction (black colour) is seen in the osteoclasts. Magnification ×100.

Statistical analyses were performed using the Wilcoxon rank-sum test for paired and unpaired data.

RESULTS

Isotope release

A highly significant depression of resorption was found for the isotopes with a ratio of 0.59 for both when exposed to 1 μl/ml monomer (Table

Table 1. Ratios for calvaria exposed to 1 μl monomer per ml medium and for controls

Ca-45 release T_1/C	0.59±0.09
H-3 proline release T_1/C	0.59±0.07
Ca-45 release C/C	1.02±0.10
Ca-45 release D/C	0.45±0.06
H-3 proline release C/C	1.02±0.06
H-3 proline release D/C	0.43±0.05

C: control bone. D: dead bone. T_1: bone exposed to 1 μl monomer per ml medium. Releases expressed in ratios. n = 12. Value for T_1/C was significantly different from controls at $P < 0.001$. Each value represents mean ± S.D. of 12 incubations.

1). The inhibition of calcium release was dose dependent (Figure 2), and the reduction by monomer in calcium release was significant at concentrations of 0.25 μl monomer or more per ml medium. At the highest concentration, the ratio 0.48 was equal to the ratio found when dead bone was compared to control (Table 1).

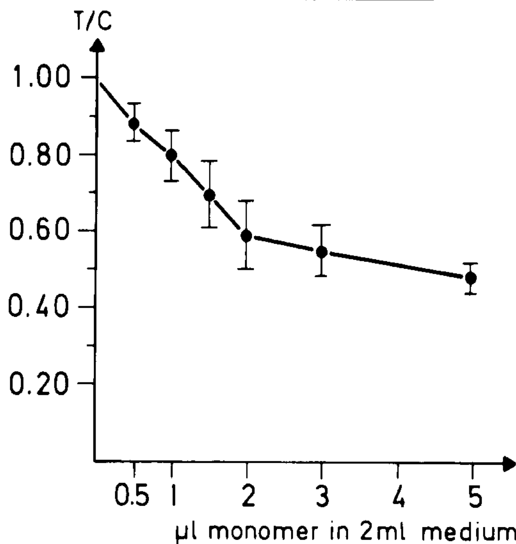

Figure 2. Dose-dependent release curve of radioactive calcium from calvaria cultured for 2 days and exposed to monomer. T/C: ratio between exposed and control bones. Each value represents mean ± S.D. of 12 incubations. All values were significantly different from control group at $P < 0.001$.

Table 2. Ratios for calvaria exposed to 1 μl monomer per ml medium with subsequent transfer to control medium, dead bone exposed to monomer, and dilution of medium

Ca-45 release T_{1a}/C	0.56±0.04
Ca-45 release T_{1b}/C	0.58±0.08
Ca-45 release T_1/C	0.59±0.09
Ca-45 release D_{100}/D	0.96±0.05
Ca-45 release C_{100}/C	1.00±0.12

C: control bone. T_{1a}: bone exposed to monomer 1 μl per ml medium for 1 day. T_{1b}: bone primarily exposed to monomer and transferred to control medium and cultured for a further 2 days. T_1: bone exposed to 1 μl monomer per ml medium. D: dead bone. D_{100}: dead bone exposed to 100 μl monomer per ml medium. C_{100}: bone exposed to 100 μl sterile water per ml medium. Each value represents mean ± S.D. of 12 incubations. All values for T/C were significantly different from control group at $P < 0.001$.

In the reversibility experiment no significant difference was found between the ratio from the first day and the following 2 days. The depressive effect of monomer on calcium release was thus not reversible in this short time. Monomer did not have any significant effect on the resorption from dead bone (Table 2).

The release of H-3 proline followed a significant inhibition curve (Figure 3), with increasing concentration of monomer giving increased depression of release down to a ratio which equalled the ratio found for dead bone to control, 0.43 (Table 1).

The depression of calcium release by aqueous polymer extract also showed dose dependency and inhibition to the level found for dead bone to control (Figure 4).

The dilution of the medium by the addition of 25–50 μl per ml medium did not *per se* influence the results (Table 2); when 100 μl sterile water was added per ml medium to the test bones resorption was unchanged.

Phosphatase activity

Table 3 shows the initial times for alkaline and acid phosphatase activity when the test bones were exposed to 1 μl monomer per ml medium. There was a highly significant increase in initial

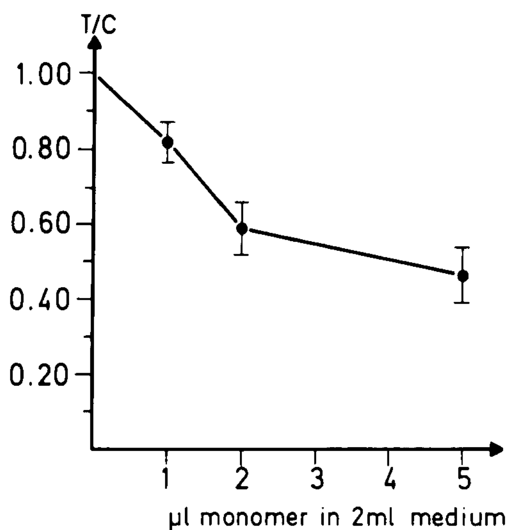

Figure 3. Inhibition curve of H-3 marked proline release by monomer from calvaria cultured for 2 days. T/C: ratio between exposed and control bones. Each value represents mean ± S.D. of 12 incubations. All values significantly different from control group at $P < 0.001$.

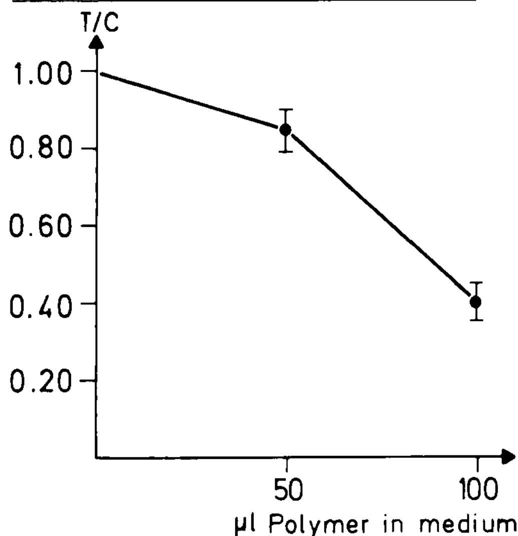

Figure 4. Dose-dependent release curve of radioactive calcium from calvaria cultured for 2 days and exposed to aqueous extract of polymer. T/C: ratio between exposed and control bones. Each value represents mean ± S.D. of 12 incubations. All values were significantly different from control group at $P < 0.001$.

Table 3. Relationship between initial time in seconds for alkaline and acid phosphatase activity when mouse calvaria, cultured for 2 days, were exposed to 1 µl monomer per ml medium. Initial time evaluated by histochemistry

Alkaline phosphatase	C	19.4± 1.8
	T_1	27.8± 3.1
Acid phosphatase	C	86.1± 5.5
	T_1	209.0±29.5

C: control bone. T_1: bone exposed to 1 µl monomer per ml medium. Each value represents mean ± S.D. of 12 incubations for alkaline and six incubations for acid phosphatase activity. T_1 for both alkaline and acid phosphatase activity was significantly different from C at $P < 0.001$.

time for alkaline phosphatase activity, from 19 to 28 s. For acid phosphatase activity the increase was even more pronounced, from 86 to 209 s.

DISCUSSION

The inhibition of calcium and proline release indicates an effect of the cement components on both bone mineral and bone matrix. Alkaline and acid phosphatase activity are regarded as indicators of bone formation and bone resorption, respectively (Bourne 1972, Vaes 1968, Reimann & Christensen 1979). Monomer and aqueous extract of polymer thus seem to affect the function of both osteoclasts and osteoblasts.

These findings are in agreement with the results from a recent study of Garcia et al. (1981), who demonstrated depressed bone formation and resorption after exposure to methylmethacrylate. Monomer has been shown to be highly toxic to different living tissues in several experimental systems (Linder 1976, Petty 1978a,b Hulliger 1962); even a general systemic effect on the cardiovascular system has been observed in humans (Charnley 1970, Feith 1975).

The effect of the aqueous extract of polymer in this study is surprising, since the polymer is generally regarded as being inert. The powder of the CMW®bone cement package contains 97% polymethyl methacrylate and 3% residual benzoyl peroxide. The radio-opaque agent was not

added. The toxic agent in this aqueous extract has not been determined.

The depressive effect of bone cement components on bone turnover indicates that acrylic cement may play a role in the pathogenesis of loosening of implants by inhibiting bone formation at the site of cement bone interface. The late local result of the use of acrylic cement in artificial joint replacement with loosening is a well-known clinical problem (Griffith et al. 1978). *In vivo,* the depressed bone turnover by bone cement may initially be augmented by thermal damage from the temperature rise in the polymerizing cement, although the latter probably is of minor importance (Jefferiss et al. 1975). *In vivo,* Albrektsson & Linder (1981), using a chamber technique, found that bone cement within the first 24 days caused a decrease in the amount of bone. Clinical evidence of an inhibitory effect on bone by acrylic cement has been reported in postmortem studies (Charnley 1970).

In this study, monomer exhibited an effect on bone in 1–2 days. Linder et al. (1976) found *in vitro* a rise and fall in monomer leakage from polymerizing cement within minutes, but monomer has been found circulating in the blood long after cement implantation in bone (Feith 1975); a long time effect is thus possible. To the monomer effect should be added the possible *in vivo* effect of polymer. The impaired healing of fractured bone treated with acrylic cement interposition indicates a long-term inhibitory effect on bone formation (Lindwer & van den Hooff 1975, Rhinelander et al. 1979).

The monomer is metabolised to non-toxic chemical components in the body (Bratt & Hathway 1977). The effect of circulating monomer is thus of minor interest, while locally the highest concentration of unmetabolised monomer must be found, giving the strongest toxic effect at the cement bone interface. We have demonstrated a dose-dependent depression of monomer on bone turnover *in vitro* at very low concentrations; our test concentration was only 20% of that in the human blood during joint replacement (Pahuja et al. 1974), and much lower than the concentration of monomer leaking, when bone cement is setting *in vitro* (Bechtel et al. 1973).

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Correspondence to: John Gregor Pedersen, Afd. D, Rigshospitalet, Copenhagen, Denmark