Cement particles containing radio-opacifiers stimulate pro-osteolytic cytokine production from a human monocytic cell line

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Proponents of the biological theory of aseptic loosening have in recent years tended to concentrate on the production and distribution of particulate ultra-high-molecular-weight polyethylene (UHMWPE) debris around the potential joint space. However, mechanical loading of cemented implants with the differing elastic moduli of metal stems, polymethylmethacrylate (PMMA) cement and bone can result in relative micromotion, implying the potential for production of metal and PMMA particles from the stem-cement interface by fretting wear.

In order to investigate the production and biological reactivity of debris from this interface, PMMA and metal particulate debris was produced by sliding wear of PMMA pins containing barium sulphate and zirconium dioxide against a Vaquasheened stainless steel counterface. This debris was characterised by SEM, energy-dispersive analysis by X-ray (EDAX) and image analysis, then added to cell cultures of a human monocytic cell line, U937, and stimulation of pro-osteolytic cytokines measured by ELISA.

Large quantities of PMMA cement debris were generated by the sliding wear of PMMA pins against Vaquasheened stainless steel plates in the method developed for this study. Both cements stimulated the release of pro-osteolytic TNF α from the U937 monocytic cell line, in a dose-dependent fashion. There was a trend towards greater TNF α release with Palacos cement than CMW cement at the same dose. Palacos particles also

caused significant release of IL-6, another pro-osteolytic cytokine, while CMW did not. The particulate cement debris produced did not stimulate the release of GM-CSF or IL1 β from the U937 cells. These results may explain the cytokine pathway responsible for bone resorption caused by particulate PMMA debris.

Radio-opaque additives are of value in surgical practice and clinical studies to quantify the relevance of these in vitro findings are required before the use of cement containing radio-opacifier is constrained.

J Bone Joint Surg [Br] 2003;85-B:900-5.

Received 12 September 2001; Accepted after revision 12 March 2003

Biological studies of aseptic loosening of total joint replacements have concentrated on the production of ultra-high-molecular-weight polyethylene (UHMWPE) debris from the main sliding articulation. The cement-stem interface, however, represents a potential source of metal and polymethylmethacrylate (PMMA) particulate debris. Compared with the main articulation, the importance of this mechanism of generation of debris may have been underestimated. Identification of factors which can reduce the potential generation of debris at this interface may influence the long-term survival of total joint arthroplasties.

Numerous studies¹⁻⁹ have found damage to the stem of a total hip replacement (THR) in vivo, and metal and PMMA debris in the periprosthetic tissues. The seminal work on the histology of periprosthetic tissues by Willert et al¹ recorded the presence of PMMA and cement opacifiers in local tissues and clearly showed polishing of metal implant stems, although no metallic debris was identified. The authors speculated on "the long-term effects of abraded methylmethacrylate".

In their comprehensive review of particulate debris, Howie et al² described the presence of metal, PMMA, opacifier and polyethylene debris in periprosthetic tissues. There have been several papers which have analysed the failure of cemented THRs, mainly with titanium alloy stems.³⁻⁵ All of these described titanium debris in periprosthetic tissues, attributing it mainly to wear of the femoral head. Anthony et al,⁶ describing four cases of revision for localised osteolysis, drew attention to defects in the cement mantles of all the implants, which corresponded to the

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doi:10.1302/0301-620X.85B6.12813 \$2.00

radiographic lytic sites. They stated that this allowed the egress of fluid under pressure from the joint space to the cement-bone interface where it would cause osteolysis. Polyethylene, cement and metal debris were found in tissue from all four lytic lesions. A mechanism was suggested for the production of metal and cement debris with enlargement of the internal dimension of the cement mantle. Explanted matt-surfaced Exeter prostheses were shown which had consistent polishing along the anterolateral and posteromedial borders, consistent with retroversion torque. The authors stated that little could be done to reduce the production of wear debris, while contrasting the surface finish of the matt and polished Exeter prostheses. They emphasised the importance of obtaining a complete mantle of cement around the femoral stem in order to prevent focal osteolysis. In a later publication,⁸ the same authors described and quantified the anterolateral and posteromedial polishing pattern on over 40 explanted stems of various types, some of which were not loose at the time of revision. They concluded that they had "identified an important long term mechanism for the production of fine particulate metallic and PMMA debris".

After the experience with the Exeter THR, when a change from a polished to a matt surface finish on the stainless-steel stem led to a much increased rate of early failure,⁶ the cement-stem interface has received attention as a potential source of additional wear debris.⁷⁻⁹

Our aim was to generate particulate wear debris from a simulated stem-cement interface under sterile conditions. This debris was then co-cultured with a human monocytic cell line and the response measured by immunoassay of four of the cytokines implicated in the pathogenesis of periprosthetic osteolysis.

Materials and methods

Generation of PMMA particles. We used a reciprocating pin-on-plate wear testing rig housed in a class-2 safety cabinet (Gelaire BSB4), in a dedicated tissue culture room. The apparatus and components were sterilised before use and cleaned to remove bacterial endotoxin. Cylindrical PMMA cement pins of 22 mm length and 7 mm diameter with a bevelled end were manufactured by DePuy International (Blackpool). Pins of cement containing different radiopaque additives, 9.2% barium sulphate (CMW 1; DePuy) and 15.6% zirconium dioxide (Palacos R; Schering-Plough, Welwyn, UK), were tested in sliding wear tests against a stainless-steel counterface with the Vaquasheen surface finish. The lubricant was filtered endotoxin-free distilled water (Baxter, Thetford, UK) and the stroke length was 25 mm. The test materials and test conditions were as follows: CMW1RO, 4 hours at 0.18 Hz, 2640 cycles; Palacos R, 4 hours at 0.18 Hz, 2640 cycles; CMW1RO, 6 hours at 1 Hz, 21600 cycles; Palacos R, 6 hours at 1 Hz, 21600 cycles; CMW1RO, 5 hours at 1 Hz, 18000 cycles; Palacos R, 6.5 hours at 1 Hz, 23400 cycles.

At the end of each test the lubricant was collected and the cement debris produced was recovered by filtration on to polycarbonate membranes (Whitman International Ltd, Maidstone, UK), with a pore size of 0.1 μm . The filters were then dried and weighed. Subtraction of the postfiltration from the prefiltration weight allowed calculation of the mass of debris produced during each of the tests.

Samples of each filter were then cut out, mounted and sputter-coated with gold for SEM (CamScan Electron Optics, Cambridge, UK) or flash-coated with carbon for energy dispersive analysis by X-ray (EDAX). The latter allowed identification of cement by its radiopaque additive, and when combined with back-scattered SEM, the identification of metal debris originating from the counterface.

Preparation of particles. The polycarbonate filters were ultrasonically agitated in RPMI 1640 cell-culture medium supplemented with 10% (v/v) fetal calf serum (FCS), 2mM L-glutamine, 20mM HEPES, 60 $\mu\text{g.l}^{-1}$ penicillin and 100 $\mu\text{g.l}^{-1}$ of streptomycin, in order to transfer the cement debris from the surface of the filter into the liquid medium. The filters were then dried and reweighed as previously described to determine the mass of debris transferred into the medium. The mass of debris transferred, the volume of culture medium used, and the mean particle volume from image analysis were used to calculate the concentration of particles which would provide, in a 200 microlitre aliquot, a 50:1 volumetric (μm)³ ratio of particles to cell numbers. A further 10:1 dilution step was then performed to provide a solution with a 5:1 ratio.

Co-culture of U937 cells and debris. We used 48-well plates (Becton Dickinson, New Jersey), with five replicates of each test condition, and appropriate positive and negative controls. U937 cells in culture medium (800 μl) were added to each well to provide 3.0×10^5 cells.well⁻¹. We then added 200 μl of cement debris solution (test wells), cell culture medium (negative controls), or one of three concentrations of lipopolysaccharal (LPS) (Sigma-Aldrich, Poole, UK) (positive controls) to give a final volume of 1 ml.well⁻¹. Cultures were incubated at 37°C for 24 hours in 5% (v/v) CO₂ in air. Three 200 μl aliquots were then aspirated from each well and subsequently used for the estimation of cytokines.

Viability assays. The viability of the cells at the beginning of the experiment and at 24 hours was estimated using replicate co-cultures by the (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide method (MTT method).¹⁰

Cytokine assays and statistical analysis. Assays were performed for four cytokines which have been implicated in the mediation of osteolysis in aseptic loosening. Commercial ELISA kits (Genzyme Diagnostics, Cambridge, Massachusetts) were used to assay the concentrations of tumour-necrosis factor alpha (TNF α), interleukin (Ih) IL6, IL1 β and granulocyte monocyte colony stimulating factor (GM-CSF) according to the manufacturers' instructions. The results were expressed as ng cytokine/OD 560 nm. Thirty wells were used (five replicates each of negative control, positive

Table I. Amount of cement debris generated (μg) in pin-on-plate tests

Test cement	Duration (hrs)	Frequency (Hz)	Cycles	Mass of debris (μg)
CMW1 RO	4.0	0.183	2640	407
CMW1 RO	5.0	1.0	18 000	30 746
CMW1 RO	6.0	1.0	21 600	35 982
Palacos R	4.0	0.183	2640	402
Palacos R	6.0	1.0	21 600	179
Palacos R	6.5	1.0	23 400	175

control, Palacos at 5 and 50 $\mu\text{m}^3/\text{cell}$, CMW at 5 and 50 $\mu\text{m}^3/\text{cell}$. Each replicate was tested with ELISA for the concentration of each of the four cytokines, and the mean of the five replicates was calculated for each cytokine assayed.

The data were analysed by one-way analysis of variance and subsequent calculation of the least significant difference (LSD) for each set of means.¹¹

Results

Production of clean debris. Using the new abrasive wear method, significant amounts of particulate cement debris were generated. Table I gives the details of the test materials and duration together with the masses of debris produced.

In the two short initial tests, the mass of debris produced was almost identical for the two pins of different cements. The only visible changes to the pins were linear scratch marks which developed on the sliding surface of both pins. There were no macroscopic changes to the plates. For the four subsequent experiments, the duration of the test was increased by approximately 50% and cycle frequency by a factor of six. This increased the cycle number by almost an order of magnitude (Table I). The six-fold alteration in sliding speed also changed the tribological conditions at the

Fig. 1

Scanning electron micrograph of a filter membrane loaded with cement debris.

contact. Under these conditions, the CMW pins produced much more debris than the Palacos pins ($6066 \mu\text{g.h}^{-1}$ compared with $28 \mu\text{g.h}^{-1}$). Examination of the cement pins and Vaqueheened steel plates also revealed marked differences. The Palacos pins bore the same linear scratch marks on the sliding surface as in the short tests. The plates from the two long Palacos tests had palpable and visible wear scars corresponding to the area of sliding contact. This was consistent with our findings in explanted prostheses.¹² Three-dimensional profilometry demonstrated plateau-like areas produced on the Vaqueheened finish by abrasive wear of the

Fig. 2

EDAX spectrum from a CMW test showing the peak for barium (same wavelength as titanium).

Fig. 3

EDAX spectrum from a Palacos test showing the peak for zirconium.

metal counterface. In the longer CMW tests the plates were macroscopically undamaged. The pins, however, were very worn. The whole of the bevelled area was worn away, leading to an increased contact area.

Imaging of clean debris. SEM revealed that the polycarbonate filter membranes were heavily loaded with particulate cement debris (Fig. 1). Particles ranged in size from 20 μm to or below 1 μm (common). Backscattered SEM, which detects particles of high density (in this context, metal), gave a qualitative impression that there was more metal debris on filters containing Palacos cement debris. This was consistent with the clear wear scars on plates from Palacos tests.

The EDAX spectra on large areas clearly distinguished between CMW and Palacos cement debris. The spectrum from a CMW test with a peak for barium (same wavelength as titanium) is shown in Figure 2. The spectrum from a Palacos test with a peak for zirconium is shown in Figure 3.

Production of cytokines by U937 cells in response to cement debris. The results for the specific activity for each of the four cytokines produced by U937 cells in response to cement debris are presented in Figures 4 to 7. The LSD was used for the statistical comparison of means, according to the method of Sokal and Rohlf.¹¹ The mean specific activities of TNF α for CMW 50 μm^3 per cell, Palacos 50 μm^3 per cell and the positive control were all significantly greater than the negative control at the 1% level. CMW at 50 μm^3 per cell had higher activity than CMW at 5 μm^3 per cell and similarly 50 μm^3 Palacos stimulated significantly more TNF α release than 5 μm^3 Palacos, both at the 1% level of significance.

Palacos stimulated more TNF α release from the monocytic cell line than CMW when tested at 50 μm^3 per cell. This difference was significant at the 5% level.

Although both cements in a 50 μm^3 per cell ratio stimulated IL6 release, only that due to Palacos was statistically significantly greater than the negative control (1% level). The positive control was significantly higher than the negative control (1% level) lending the experiment internal validity.

Only the LPS (positive control) stimulated measurable amounts of IL1 β .

None of the cement particle tests stimulated more GM-CSF release than negative controls. The positive control gave significantly higher levels of GM-CSF than the negative control (1% level) lending the experiment internal validity.

Discussion

In previous studies on particulate cement debris, such as that of Sabokbar et al,¹³ the debris was made by crushing and milling polymerised bone cement. In the method developed for our study, copious quantities of PMMA cement debris were generated by the sliding wear of PMMA pins against Vaquasheened stainless steel plates. This debris was

largely of phagocytosable dimensions (0.5 to 10 μm). It was believed that two different wear processes were occurring with the different cements. The Palacos pins with the very abrasive zirconium dioxide opacifier rapidly wore off the peaks of the Vaquasheen surface finish, resulting in a smooth surface and lower wear. The less abrasive CMW cement did not alter the surface and was worn away more by the textured metal surface. However, since this type of wear (sliding wear of relatively long amplitude) probably only occurs at very advanced stages of aseptic loosening in vivo, it is not directly applicable to the fretting type of wear predicted at the cement-stem interface and will not be further discussed.

The method described for producing debris was relatively straightforward and produced useful quantities of debris which were eminently suitable for use in cell-culture studies. It is our belief that this technique is more representative of debris generated in vivo than that generated by grinding and milling polymerised PMMA cement.

The study of Sabokbar et al¹³ on the effects of particulate cement debris on macrophages in vitro used the presence of an osteoclastic enzyme together with resorption pits on human bone slices as outcome measures. Much greater osteoclastic activity was found with zirconium-containing cement than with that containing barium sulphate.

The particulate cement debris in our study did not stimulate the release of GM-CSF or IL1 β from the U937 monocytic cell line. Two other cytokines strongly implicated in osteolysis were stimulated. Both cements stimulated the release of TNF α , in a dose-dependent fashion. Although not reaching statistical significance, Palacos cement seemed to stimulate more TNF α release than CMW cement at the same dose. Also Palacos was the only cement to cause significant release of IL6, another pro-osteolytic cytokine.

The results of our study were therefore at variance with those of Sabokbar et al, with a trend towards more cytokine release with Palacos cement, albeit with a different outcome measure from that used in their study.

Factors which may explain the difference included the method of production of the cement. Palacos debris was seen on back-scattered SEM to contain more metal debris than the CMW debris. Although this difference may well occur in vivo, as evidenced by the polishing observed on explants, it does introduce another variable into the experiment. The outcome measure of Sabokbar et al of bone-resorption pits was semiquantitative, although it may be argued to represent a final common pathway beyond the release of cytokine and cell transformation. With the small numbers in their study a statistical re-evaluation was necessary to find a difference between the two types of opacifier.

Wimhurst, Brooks and Rushton¹⁴ recently published a study which compared production of cytokine (IL6, IL1 β , TNF α) from human primary macrophages when exposed to different cement particles. The cement debris was produced by diamond grinding of cubes of polymerised cement. The study compared Palacos cement with no opacifier, Palacos

Fig. 4

Mean production (ng/ml/OD + 95% CI) of TNF α by cement-debris-stimulated U937 cells (SA, cytokine activity corrected for viable numbers of cells; negative control, cells in culture medium alone; CMW and Palacos, cement particles added in 5 and 50 μm^3 per cell ratios; positive control, LPS added to cells).

Fig. 5

Mean production (ng/ml/OD + 95% CI) of IL6 by cement-debris-stimulated U937 cells (for abbreviations and explanation see legend to Fig. 4).

Fig. 6

Mean production (ng/ml/OD + 95% CI) of IL1 β by cement-debris-stimulated U937 cells (for abbreviations and explanation see legend to Fig. 4).

Fig. 7

Mean production of GM-CSF (ng/ml/OD + 95% CI) by cement-debris-stimulated U937 cells.

with barium sulphate, Palacos with zirconium dioxide and CMW cement with barium sulphate. Considerable care was taken to exclude contamination. Although variation between the response of macrophages from different buffy coat donors was observed, no significant difference was found between the differing preparations of cement. In vitro studies, such as ours and those of Sabokbar et al and Wimhurst et al, have yet to explain the variation in clinical survivorship shown in the Swedish Arthroplasty study.¹⁵

Many arthroplasty surgeons find the use of radiopaque bone cement to be of value in the assessment of postoperative radiographs and hence in quality control for variations in surgical technique. Radiopaque additives are of use in the assessment of the quality of the cement mantle and prediction of, and clinical observation for, failure by aseptic loosening. Excellent clinical results have been obtained with cemented total joint prostheses using radiopaque cement.¹⁶

The call for the consideration of use of cement without a radiopaque additive in the study by Sabokbar et al may be premature. Further experimental work including clinical studies to quantify the relevance of these in vitro findings is needed before the use of cement containing radiopacifier is discontinued.

We wish to thank the post-doctoral fellows in the Bioengineering Research Group at the University of Leeds, including Bridget Matthews, Joanne Tipper, Tim Green and Jenny Pickard, for their invaluable help in the preparation of this paper.

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