Inflammatory responses of human primary macrophages to particulate bone cements in vitro

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We have investigated whether the particle-stimulated release of inflammatory cytokines from human primary macrophages in vitro was dependent upon the type of bone cement used. Particles of clinically relevant size were produced from Palacos R without radio-opacifier, Palacos R with BaSO₄, Palacos R with ZrO₂ and from CMW3 which contains BaSO₄. All four preparations produced significantly greater release of tumour necrosis factor alpha, interleukin-6 and interleukin-1 beta than a negative control but there were no significant differences between them. The differences in the ability to stimulate bone resorption and in clinical performance between proprietary bone cements previously recorded are not explained by the release of the cytokines most commonly implicated in osteolysis.

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Aseptic loosening remains the most important cause of failure of total hip arthroplasty. The aetiology of this process appears to be multifactorial, with the surgical technique, joint fluid pressure, micromovement of the implant and wear debris all being involved. In the Swedish Hip Registry, Malchau and Herberts stated that “the type of cement has a drastic association to risk for revision”. The lowest risks were associated with Palacos, Gentamicin, Palacos R and Simplex. CMW had slightly worse results and the highest risks were associated with Sulfix. Proprietary bone cements differ in their basic components. Palacos is a methylmethacrylate copolymer, CMW is a powder poly(methylmethacrylate) (PMMA) polymer, Simplex is a mixture of PMMA and methylmethacrylate-styrene copolymers and Sulfix is a copolymer of PMMA and polybutylmethacrylate. CMW and Simplex contain barium sulphate (BaSO₄) to make them visible on radiography while Palacos and Sulfix have zirconium dioxide (ZrO₂). The commercially available bone cements also differ in their physical properties, namely viscosity, porosity and fatigue strengths. These inconsistencies in material and physical properties may be responsible for the differences in outcome associated with their use.

Particles of ultra-high-molecular-weight polyethylene, metals/alloys and bone cement have all been found in the fibrous pseudomembrane surrounding aseptically loose implants. The number of particles present correlates with the size of osteolytic defects and retrieval studies have characterised the nature, size, shape and number of the particles. Iwaki et al. found that the mean equivalent circle diameter (ECD; the diameter of a circle having the same area as that of the measured feature) of cement particles was between 0.65 and 1.51 μm and the load of cement particles was between 2.2 × 10⁷ and 2.2 × 10⁸ per gram of retrieved membrane.

The role of particulate debris in the pathogenesis of aseptic loosening has been linked to the presence of tissue macroparticles in the fibrous pseudomembrane around the implant. These cells are believed to interact with wear particles and release inflammatory cytokines which have been detected in the pseudomembrane in retrieval studies. Tumour necrosis factor alpha (TNF-α), interleukin-one beta (IL-1β) and interleukin-6 (IL-6) have all been found in such tissue and have been shown to be released by macrophages in response to particulate debris. IL-1β and TNF-α are believed to stimulate the activation and differentiation of osteoclasts and IL-6 to reduce the proliferation of osteoblasts. Release of these cytokines from monocytes and macrophage cell lines in response to particulate debris has been used to assess differences in the inflammatory potential of materials such as alloys, bone cements, polyethylene and ceramics.

We have investigated the effect of particles of bone cement on the release of inflammatory cytokines from primary human macrophages in vitro. To examine whether any of the differences detected were due to radio-opacifiers, one bone cement, Palacos (Schering-Plough, Welwyn, UK), was used without a radio-opacifier, with BaSO₄ and with ZrO₂, both at 15.6% weight/weight. To investigate differ-
ences between proprietary bone cements, CMW3 (CMW Laboratories, Blackpool, UK), which contains 10% BaSO₄, was also tested.

Materials and Methods

Production of particles. The bone cements were polymerised according to the manufacturers’ instructions, cut into blocks of 2 cm², mounted on a Rotoforce-3 specimen mover (Struers, Rodovre, Denmark) and counter-rotated at 300 rpm and 30 N against a 20 μm diamond grinding disc on a Rotopol-21 grinding/polishing machine (Struers) for four hours with constant irrigation of glycerol as lubricant.

The particles thus produced, suspended in glycerol, were collected in sterile containers, vortex-mixed with endotoxin-free water and centrifuged at 3500 × g for five minutes. This wash process was repeated six times for each sample. The particles were suspended in as minimal a volume of endotoxin-free water as possible (approximately 3 ml) and dried for one hour at 40°C. Approximately 1 g of particles of each type of bone cement was produced by this method. Suspensions of each type of particle were subjected to microbiological culture for 72 hours. None produced bacterial growth. Endotoxin screening was performed by Bio*Whittaker (Wokingham, UK) on endotoxin-free water which had been in contact with particles for four hours at 37°C, using a quantitative, kinetic assay for the detection of Gram-negative bacterial endotoxin. The level of endotoxin contamination was found to be less than 0.015 endotoxin units/ml. Particles were sterilised by Isotronic plc (Swindon, UK) with gamma irradiation at 25 kiloGray/2.5 MRads.

Particles from each of the cements were sized using light microscopy at a magnification of ×250 and image-analysis software (PC Image; Synoptics, Cambridge, UK). Approximately 2000 particles of each type were analysed and expressed in terms of ECD. Sizing was confirmed by SEM and contamination from the manufacturing process excluded by energy-dispersive x-ray analysis.

Isolation of macrophages. We used a modification of the method described by Denholm and Wolber¹⁹ to isolate monocytes from human blood. A mean of 7 × 10⁶ monocytes with a purity of 83% and viability of 97% can be obtained from 30 to 40 ml of whole blood. A brief summary of the modified procedure is given below.

Instead of whole blood, 40 ml of a buffy coat of blood cells from a single, healthy human volunteer with no evidence of inflammatory arthropathies was obtained from the Blood Transfusion Service. All work was carried out in a class-II tissue-culture hood and all solutions, medium and chemicals were endotoxin-free as per testing by the manufacturers.

The buffy coat was diluted with an equal volume of phosphate-buffered saline (PBS, produced with endotoxin-free water and autoclaved at 121°C) at 37°C and layered onto Ficoll (‘Histopaque’, Sigma-Aldrich Co Ltd, Poole, UK) in a ratio of three parts of diluted blood to one part Ficoll and centrifuged at 1100 × g for 30 minutes in an IEC Centra MP4R (International Equipment Company, Dunstable, UK). The mononuclear layer was removed, diluted in PBS and centrifuged for five minutes at 1100 × g. The pellet from this stage was diluted in PBS and added to a solution of Hanks’ Buffered Salt Solution and Percoll (both Sigma-Aldrich Co Ltd) at pH 7.0, mixed and centrifuged for 30 minutes at 475 × g in an IEC Centra SR, which has a fixed angle, allowing a gradient to form.

The creamy yellow layer thus produced was removed, diluted in PBS and centrifuged at 600 × g in the IEC Centra MP4R for five minutes. The final pellet was suspended in 1 ml of Dulbecco’s Modified Eagle Medium (DMEM) + 10% human male AB serum (heat-inactivated and sterile-filtered) and 1% glutamine (Sigma-Aldrich Co Ltd). A sample of this suspension was stained with Trypan Blue (Sigma-Aldrich Co Ltd) and viable cells (impermeable to Trypan Blue) counted under an Improved Neubauer Haemocytometer (Western Laboratory Services Ltd, Aldershot, UK).

The cells were diluted in DMEM + 10% AB serum and 1% glutamine to produce a seeding concentration of 0.5 × 10⁶/well in a volume of 0.5 ml; 24-well plates were placed in an incubator at 37°C and 5% CO₂ for 45 minutes to allow adherence, washed with DMEM + 10% AB serum and 1% glutamine and fresh medium added.

The plates were cultured for seven days during which the cell morphology changed from that of rounded cells typical of blood monocytes to cells showing flattened cytoplasm spread out on the substrate. The ability to degrade acetylated low-density lipoprotein, previously used as a marker of macrophage differentiation, has been shown to occur between days 5 and 9 of culture.²⁰ On the eighth day suspensions of bone-cement particles in 0.5 ml of serum-free medium were added to the cells.

In order to produce a dose-response curve and to allow an appropriate concentration of particles to be chosen, an initial study was performed on macrophages isolated from three separate donors. Concentrations ranged from 10⁷ to 10⁹ particles per well, with a total of 18 wells of each concentration per experiment.

In the definitive experiment, macrophages were obtained from six new donors and eight wells were used to test each material for each donor. Medium alone acted as a negative control and a suspension of 3 × 10⁷ particles/0.5 ml zymosan (Sigma Aldrich Co Ltd) as a positive control. After 24 hours the wells were harvested, each 0.5 ml sample being centrifuged for one minute at 12000 × g to remove particles and cells before being divided into 100 μl aliquots for analysis.

Measurement of toxicity and cytokine release. Toxicity was recorded by lactate dehydrogenase (LDH) assay (Promega, Southampton, UK). The amount of TNF-α, IL-1β and IL-6 in each sample was recorded using commer-
cially available kits (Endogen, Woburn, Massachusetts). Each sample was tested in duplicate.

**Statistical analysis.** The results were analysed using a linear mixed effect model with the type of particle as the fixed effect and subject as the random effect so that differences between individuals as well as between particles could be detected. This generates a univariate analysis of variance and post-hoc testing was performed using the Tukey method. All statistical analyses were performed using SPSS Base 8.0 for Windows (SPSS Inc, Chicago, Illinois).

**Results**

**Size of the particles.** The mean (±SD) sizes (μm) of the particles were as follows: Palacos without radio-opacifier, 1.30 ± 0.76; Palacos with BaSO₄, 1.32 ± 0.72; Palacos with ZrO₂, 1.24 ± 0.88; and CMW3, 1.24 ± 0.70.

**Cell counts.** In total, nine isolations of macrophages from buffy coats were performed. The mean number of mononuclear cells per experiment was 9.2 × 10⁷ (SD 1.27 × 10⁷), i.e. ten times as many cells as can be obtained from the same volume of whole blood.

**Concentration of particles.** The dose-response curve to the Palacos-based particles was sigmoidal in shape, with maximal release of all three cytokines at 5 × 10⁸ particles/well which declined after this concentration (Fig. 1). Toxicity was seen to increase with concentration (Fig. 2). A concentration of 3 × 10⁸ particles per well was used to obtain an optimal response of cytokines but to avoid high toxicity.

**Toxicity.** There was no significant difference in the toxicity of the four bone cements tested as shown by release of LDH (Fig. 3). All were, however, significantly more toxic than the negative control (p < 0.001) and less toxic than zymosan (p < 0.001).
Inflammatory cytokines. There was no significant difference in the release of TNF-α, IL-1β and IL-6 between Palacos with radio-opacifier (BaSO₄ or ZrO₂) and Palacos without radio-opacifier (p > 0.8). No significant difference between the BaSO₄- and ZrO₂-containing Palacos cements was seen (p > 0.9) (Figs 4 to 6).

There was no significantly different release of cytokines with CMW when compared with any of the Palacos cements (p = 0.1) (Figs 4 to 6).

There were significant differences between individuals’ magnitude of response to the particles for LDH (p < 0.001) and TNF-α (p = 0.03) but not for IL-1β or IL-6.

Discussion

Our study examined the response of inflammatory cytokines of human primary macrophages to particles of bone cement, of clinically relevant size, in vitro. In contrast to previous studies, three of the particle types were manufactured from a single base cement so that the only difference between them was the presence or absence of radio-opacifier, and in two the type of radio-opacifier. The opportunity to compare two commonly used proprietary bone cements was also taken. Care was taken to avoid endotoxin contamination of the particles during their manufacture and the level of endotoxin, by a highly sensitive assay, was found to be less than 0.015 endotoxin units/ml.

The size of the particles produced corresponds well to that seen in the retrieval study of Iwaki et al.⁵ The concentration of $3 \times 10^8$ particles per well of $0.5 \times 10^8$ cells corresponds to the $\times10$ surface area ratio used by Shanbhag et al.²²,²³ in their experiments but is sevenfold higher than the ratio of 100:1 of particle volume to U937 cells used by Ingham et al.¹⁷

Significantly greater release of all three cytokines was observed compared with the negative control. There were also differences between individuals in the magnitude of the response of LDH and TNF-α to particles. No significant difference, however, was found between bone cement which contained radio-opacifiers and that without, nor was there any difference between cements containing BaSO₄ or ZrO₂. There was also no difference in the release of cytokines between CMW and Palacos.

Several studies investigating the reasons for the differences in clinical performance between commercially available bone cements have focused on the radio-opacifiers which they contain. Rae,²⁴ in experiments on mouse macrophages, concluded that the BaSO₄ contained within bone cement was probably not harmful. Lazarus et al.²⁵ found that the presence of BaSO₄ in bone cement intensified the inflammatory response in a rat subcutaneous pouch model as measured by the leukocyte count and release of TNF-α and prostaglandin E₂, and increased the amount of bone resorption in an implant model first described by Howie et al.²⁶ when compared with bone cement without BaSO₄. In their assay in vitro, Sabokbar et al.²⁷ found that cement with radio-opacifiers caused more bone resorption than that without. They also showed that CMW caused 50% more bone resorption than Palacos and ascribed this difference to the fact that CMW contains BaSO₄ and Palacos ZrO₂. Ingham et al.¹⁷ found a significant difference between bone cements with radio-opacifiers and those without in their ability to stimulate bone resorption in vitro, but did not find a difference between BaSO₄- and ZrO₂-containing cements. There was also no significant difference in the release of IL-1β, IL-6 or TNF-α between any of the proprietary bone cements which they studied using the human monocytic cell line, U937. Caravia et al.²⁸ in their sliding wear tests, found that bone cements with radio-opacifiers produced significantly more surface damage than those without and that the cement with ZrO₂ was significantly worse than that with BaSO₄.
Results from a recent in vivo murine model suggest that TNF-α is essential to the development of particle-induced osteolysis since mice failing to express TNF-α receptors are protected from this process. The release of TNF-α, IL-1β and IL-6 in response to particulate bone cements, however, does not explain the differences in bone resorption demonstrated by Sabokbar et al and Ingham et al in vitro and of Lazarus et al in vivo, nor the differences in clinical performance seen in the Swedish Hip Registry. While cement particles do stimulate release of inflammatory cytokines in vitro, the three most commonly studied cytokines do not appear to be responsible for differences in performance between proprietary bone cements. Possible alternative routes by which these effects could be mediated include signalling pathways in bone involving the amino acid, glutamate, and the recently described osteoprotegerin ligand. 

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References


