Endotoxin contamination of particles produces misleading inflammatory cytokine responses from macrophages in vitro

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Particulate prosthetic materials are often studied by adding them to monocytic cells in vitro and measuring the release of cytokines as an indicator of their inflammatory potential. Endotoxin is known to be a contaminant of particle preparations and also stimulates the release of cytokines. It is usual to use a proprietary endotoxin test to avoid erroneous results.

Four different formulations of cement were found to be free from endotoxin using standard, gel-clot tests but stimulated different levels of release of cytokines from macrophages. These differences were explained when a more sensitive, kinetic endotoxin assay showed that release correlated with minor contamination with endotoxin. In a repeat experiment using cement particles with low or undetectable levels of endotoxin by kinetic assay, differences in the ability of the formulations to stimulate the release of cytokines were not seen.

Endotoxin is adsorbed on to the surface of particles and it is this combination which stimulates increased release of cytokines. In both the above methods for determination of endotoxin, the water in which the particles had been soaked was examined rather than the particles directly. Further investigations showed that a kinetic assay directly on a particle suspension is the most sensitive method to measure contamination with endotoxin.

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The role of particulate debris in the pathogenesis of aseptic loosening is well documented and it has been linked to the presence of tissue macrophages in the synovial-like membrane around the implant. These cells phagocytose wear particles and release inflammatory cytokines which can be detected in the membrane in retrieval studies.

Tumour necrosis factor α (TNFα), interleukin-1β (IL-1β) and interleukin-6 (IL-6) have all been found in periprosthetic tissue, and have been shown to be released by monocytes and macrophages in response to particulate material.

Endotoxin is the lipopolysaccharide component of Gram-negative bacterial cell walls. The ability of endotoxin to elicit a pyrogenic response in vivo requires that all parenteral products be tested for its presence. Ragab et al first suggested a possible role for endotoxin in the response of cells to particulate wear debris. They showed that most of the cytokines released from mouse marrow cells in response to commercially available titanium particles were due to contamination with endotoxin. There is also evidence that adsorbed endotoxin can synergise with particles in stimulating release of TNFα.

There are several methods available for the detection of endotoxin. One involves direct measurement of lipopolysaccharide fatty acids by gas chromatography-mass spectrometry while the others are based on the action of an enzyme present in Limulus amoebocyte lysate (LAL) on endotoxin, first described by Bang.

These last methods use a variety of endpoints to determine the presence of endotoxin, namely the production of a gel clot, an increase in turbidity or the release of a chromogenic substrate.

We have carried out a series of experiments to investigate the ability of bone-cement particles containing different radio-opacifiers to stimulate the release of cytokines from human macrophages. To confirm the absence of endotoxin in our particle preparations we used a gel-clot-based LAL assay which is easily carried out in a laboratory without the need for expensive equipment. Subsequently, a more sensitive kinetic assay was used. The results indicated that levels of endotoxin in particle preparations, not detected by gel-clot assay, could stimulate the release of cytokines from macrophages and give misleading results. We have thus investigated the best method for determining levels of contamination with endotoxin.

Materials and Methods

Preparation of particles. These were prepared from...
blocks of polymerised cement as previously described. Briefly, blocks of cement were ground against a diamond grinding disc using glycerol as a lubricant. After extensive washing in endotoxin-free water the particles were dried, tested for endotoxin and sterilised by exposure to 25 kGy of \( \gamma \)-irradiation (Isotron, Swindon, UK). We prepared particles of four different formulations of cement for comparison. Palacos (Schering-Plough, Welwyn, UK) was used without radio-opacifier and after the addition of either 15.6% w/w \( \text{BaSO}_4 \) or the same concentration of \( \text{ZrO}_2 \). CMW3 (CMW Laboratories, Blackpool, UK) containing 10% w/w \( \text{BaSO}_4 \) was also employed. Four preparations of cement, two contaminated with (1 and 2) and two free from endotoxin (3 and 4), were subsequently used to investigate the most suitable method for the detection of endotoxin.

**Testing for endotoxin.** All reagents and glassware used for the determination of endotoxin were obtained from Bio-whittaker, Wokingham, UK. For initial testing, particles of cement were suspended in endotoxin-free water by sonication and diluted with phosphate-buffered saline (PBS), layered in situ they were suspended at a concentration of 0.85 mgml\(^{-1}\), which is the concentration used for their biological evaluation. They were then soaked for one hour as described in the USA Pharmacopoeia Bacterial Endotoxins test (USP method), centrifuged to remove particles and the supernatant used in the assay. Subsequently, two further methods were used for testing particles. In the first, they were suspended as above and incubated at 37°C for four hours, followed by a further round of sonication to remove endotoxin. For testing particles in situ they were suspended at a concentration of 0.85 mgml\(^{-1}\) and diluted 1/10 in endotoxin-free water to give a concentration which had previously been shown not to interfere with the kinetic LAL assay by measuring the recovery of a known concentration of endotoxin added to the sample.

**Gel-clot assay.** The kit used was the Pyrogent plus (Bio-whittaker) with a labelled sensitivity of 0.125 enzyme units (EU) per ml. A series of dilutions of reference endotoxin was carried out in quadruplicate to determine the actual sensitivity of the test and the water samples prepared from the cement particles were assayed in duplicate. No inhibition of the reaction by water prepared in this way was shown by spike recovery experiments.

**Kinetic assay.** The assay used was the Kinetic-QCL and was carried out by Biowhittaker. The sample to be tested is mixed with LAL reagent so that any endotoxin present activates coagulase. This acts on a colourless, peptide-based substrate to release p-nitroanaline. The release of this yellow reaction product is monitored over time and provides very sensitive detection of endotoxin down to 0.005 EU.ml\(^{-1}\).

**Isolation of macrophages.** Monocytes were isolated from a buffy coat of blood cells obtained from a single healthy human volunteer (Blood Transfusion Service, Brentwood, UK) using a modification of the method of Denholm and Wolber as previously described. Briefly, the buffy coat was diluted with phosphate-buffered saline (PBS), layered on to Ficoll (Histopaque; Sigma-Aldrich Co Ltd, Poole, UK) and centrifuged at 1100 \( \times g \) for 30 minutes. The mononuclear cell layer was washed in PBS, mixed with Hank’s buffered salt solution and Percoll (Sigma-Aldrich Co Ltd) and centrifuged at 475 \( \times g \) for 30 minutes in a fixed angle rotor. The resulting monocyte layer was washed in PBS, resuspended in Dulbecco’s modified eagle medium (DMEM) containing 10% heat-inactivated human male AB serum and 1% glutamine (Sigma-Aldrich Co Ltd) and seeded at 0.5 \( \times 10^6 \) cells per well in 24-well cell-culture plates. After 30 minutes the culture medium was replaced with fresh medium to remove non-adherent cells.

The plates were cultured for seven days during which the monocytes developed a macrophage-like cell phenotype. We had previously determined that these cells provide more reproducible responses to the addition of particles. On the eighth day, suspensions of \( 3 \times 10^5 \) bone-cement particles in 0.5 ml serum-free medium were added to the cells. Medium alone acted as a negative control and a suspension of \( 6 \times 10^7 \) particles per ml of zymosan (Sigma Aldrich Co Ltd) as a positive control. After 24 hours, culture medium from the wells was harvested and centrifuged for one minute at 12000 \( \times g \) to remove particles and cells before being divided into 100 \( \mu l \) aliquots for analysis. Samples for assay of lactate dehydrogenase (LDH) were stored for 24 hours at 4°C before use. Samples for assay of TNF\( \alpha \) were stored at -70°C.

**Measurement of toxicity and release of cytokines.** A commercial LDH assay (CytoTox 96; Promega, Southampton, UK) was used to determine cell toxicity. The amount of TNF\( \alpha \) was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Endogen; Woburn, USA). Each sample was tested in duplicate.

**Statistical analysis.** We analysed the results using a linear mixed-effect model with type of particle as the fixed effect. This generates a univariate analysis of variance and post-hoc testing was performed by the Tukey method. All statistical analysis was performed using SPSS Base 8.0 for Windows (SPSS Inc, Chicago, Illinois).

**Results**

The mean diameter of the particles was approximately 1.3 \( \mu m \). In experiments using the first batch of cement particles univariate analysis of variance revealed a significant difference in the release of TNF\( \alpha \) between particles (p < 0.001) (Fig. 1a). Post-hoc testing showed that all four types of bone cement caused more release of TNF\( \alpha \) than medium alone (p < 0.001). The three cements containing radio-opacifier stimulated higher levels of TNF\( \alpha \) than plain cement (p < 0.001). There was no significant difference between the \( \text{BaSO}_4 \) and \( \text{ZrO}_2 \)-containing Palacos cements (p = 0.267) but the CMW3 particles produced significantly higher levels of TNF\( \alpha \) than the Palacos cements (p < 0.001). All four bone cements caused more toxicity...
than medium alone (p < 0.001) and less than zymosan (p < 0.001), but there was no significant difference between the cements (p > 0.8) (Fig. 2a). The concentration of endotoxin as determined by gel-clot assay was less than the sensitivity of the assay (< 0.125 EU/ml) for each of the particles tested.

The second batch of particles produced results which were remarkably different from the first. All four cements caused significantly more release of TNFα than medium alone (p < 0.02), but the magnitude of release was dramatically lower than was seen with the first batch of particles, except for the plain particles and the positive control (zymosan) which remained at the same level (Fig. 1b).

There was no significant difference in the release of

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Table I. Concentrations of endotoxin (EU.ml\(^{-1}\)) measured using the kinetic LAL assay in two separately prepared batches of particles

<table>
<thead>
<tr>
<th>Batch</th>
<th>Plain</th>
<th>Barium</th>
<th>Zirconium</th>
<th>CMW</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.065</td>
<td>0.11</td>
<td>0.039</td>
<td>0.191</td>
</tr>
<tr>
<td>2</td>
<td>0.013</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

Table II. Concentrations of endotoxin (EU.ml\(^{-1}\)) measured by three different detection methods in four particle preparations

<table>
<thead>
<tr>
<th>Preparation method</th>
<th>Cement 1</th>
<th>Cement 2</th>
<th>Cement 3</th>
<th>Cement 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>USP</td>
<td>0.079</td>
<td>0.043</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Sonication (\times 2)</td>
<td>0.161</td>
<td>0.066</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Cement in situ</td>
<td>0.283</td>
<td>0.108</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
TNFα between Palacos with and without radio-opacifier (p > 0.8) and between the BaSO₄- and ZrO₂-containing Palacos cements (p > 0.9). There was also no significantly different release of cytokines with CMW when compared with any of the Palacos cements (p = 1.0).

There was no significant difference in the toxicity of the four bone cements tested as shown by release of LDH (p > 0.5) (Fig. 2b). All were, however, significantly more toxic than the negative control (p < 0.001) and less toxic than zymosan (p < 0.001).

Testing of the two batches of particles for endotoxin using the sensitive kinetic method showed differences in the measured levels of endotoxin between the batches (Table I). The values were higher for all the particle preparations produced in the first batch with the highest in the CMW particles which also produced the greatest release of TNFα.

When endotoxin was present, as in cements 1 and 2, the method of preparing the particles for kinetic assay made a difference to the concentration of endotoxin measured. A rise in the measured concentration of endotoxin in the supernatant in which the particles had been soaked was seen between particles soaked for one hour (USP method) and those soaked for four hours with a further round of sonication (Table II). There was a further increase in measured endotoxin when the assay was carried out with the particles in situ. When the particles were free from endotoxin as in cements 3 and 4, none was detected on the particles whichever method of preparation was used.

**Discussion**

The results from the first batch of cements showed a significant difference in the release of TNFα between the four bone cements studied but no differences in their toxicity, CMW promoted a greater response than any of the Palacos-based cements. Of the three cements made from the same base, those containing radio-opacifiers were more proinflammatory than the plain cement. These results appeared to correlate with the clinical performance of bone cements in the Swedish Hip Registry and with the in vitro bone resorption assay of Sabokbar et al, in which CMW caused more bone resorption than Palacos and both stimulated more resorption than a plain cement. We were, however, cautious about accepting these results because of studies which have shown that very low levels of endotoxin could synergise with particles in stimulating release of TNFα from a mouse macrophage cell line. Using the highly sensitive quantitative kinetic assay on endotoxin-free water in which the particles had been soaked produced levels of endotoxin which related to the level of the cytokine release that the particles produced. The highest levels of cytokine release were detected using the CMW particles from batch 1, which also had the highest contamination with endotoxin. These results indicated that endotoxin was present on the particles but had not been detected by the gel-clot LAL assay. We hypothesised that the source of this endotoxin was contamination during production of the particles. We therefore produced a second batch of each of the four types of particle taking care at each stage of production to minimise contamination. This was achieved by ensuring that the water used during the preparation of particles was free from endotoxin, tested by the kinetic endotoxin assay, and by reducing the drying time of the particles after washing away the glycerol. The kinetic assay confirmed that the levels present in water in which these particles had been soaked was below 0.01 EU/ml. These particles produced significantly greater release of TNFα compared with the negative control. No significant difference, however, was found between bone cements which contained radio-opacifiers and those which did not, nor between cements containing BaSO₄ or ZrO₂. There was also no difference in the release of cytokines between CMW and Palacos.

Our study clearly indicated that the method used for ensuring that preparations of particles do not contain endotoxin is critically important. The gel-clot assay, which produced negative values for all particles in batch 1, did not detect the lower amounts of endotoxin which were discovered by the kinetic assay. The method of testing these particles involved soaking at 37°C as recommended in the USP method and then assaying the resulting water. It has been reported recently that this method of evaluation of endotoxin significantly underestimates the amount present on commercial titanium particles. Therefore a negative endotoxin result for the cement particles in our study may similarly have underestimated the true amount of endotoxin present even when using the sensitive kinetic assay. The results showed that increasing the length of particle soaking and including a further sonication step improved the detection of endotoxin, but the highest levels were found when the particles were tested in situ. The levels were, however, less than fourfold greater than those measured using the USP method whereas the increases seen by Ragab et al were of the order of 100-fold. This discrepancy could reflect differences in the adherence of endotoxin to specific types of particle or to our use of sonication to disperse the particles during soaking. Importantly, when the particles were prepared in such a way as to avoid contamination with endotoxin none was detected regardless of the method used for detection.

The gel-clot LAL test used in our study was not sensitive enough to detect small amounts of endotoxin which had a profound effect on particle-stimulated release of cytokines. The ability of endotoxin to adsorb to the surface of the particles makes the detection method used critical in confirming the absence of endotoxin in particle preparations and preventing the production of potentially misleading results. We suggest that quantitative kinetic testing, on a particle suspension that has been shown not to interfere with the detection of added endotoxin, is the most sensitive method.
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References


