Bone-resorptive effects of endotoxin-contaminated high-density polyethylene particles spontaneously eliminated in vivo

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Wear particles commonly used for experiments may carry adherent endotoxin on their surfaces, which may be responsible for the observed effects. In this study, we attached titanium plates to the tibiae of 20 rats. After osseointegration, endotoxin-contaminated or uncontaminated high-density-polyethylene (HDPE) particles were applied. Contaminated specimens showed a dramatic resorption of bone after seven days but new bone filled the site again at 21 days. Uncontaminated specimens showed no resorption.

In 18 rats we implanted intramuscularly discs of ultra-high-molecular-weight polyethylene (UHMWPE) with baseline or excess contamination of endotoxin. Excess endotoxin disappeared within 24 hours and the amount of endotoxin remained at baseline level (contamination from production). Uncontaminated titanium discs did not adsorb endotoxin in vivo. The endotoxin was measured by analytical chemistry.

Locally-applied endotoxin stimulated bone resorption similarly to that in experiments with wear particles. Endotoxin on the surface of implants and particles appeared to be inactivated in situ. A clean implant surface did not adsorb endotoxin. Our results suggest that endotoxin adhering to orthopaedic implants is not a major cause for concern.

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A commonly accepted theory is that wear particles generated by attrition or abrasion of components within an implant are responsible for late aseptic loosening and bone resorption. It has been reported that particles for experimental use may carry adherent endotoxin which is not revealed by routine analysis, for example, by the Limulus amoebocyte lysate (LAL) assay, and this may be responsible for the observed effects of wear particles in vitro. In addition, measurable amounts of endotoxin have been found on the surface of commercially available prostheses (unpublished data) which suggests that contamination by endotoxin may be the de facto culprit in wear-particle-mediated osteolysis.

If this is so, a number of questions arise. First, can endotoxin-contaminated particles be responsible for the osteolysis seen in various experiments in vitro and vivo? Secondly, since the body has a number of mechanisms for countering the effects of endotoxins, will the supposed reaction be short- or long-lasting? Thirdly, since endotoxins are strongly adherent to implant materials, will they be dealt with in situ or will they be transported away by certain, yet to be identified, carrier proteins into the circulation to the spleen and lymph nodes? In addition, anti-endotoxin antibodies have been detected in human blood, probably as a result of enteric leakage, and preliminary results have indicated that small amounts of endotoxin are present in the blood of rats (unpublished data). Therefore, the fourth question is whether an endotoxin-free implant surface could act as ‘fly-paper’, attracting and binding endotoxins, possibly resulting in unfavourable reactions around the implant.

In an attempt to answer the first and second questions, we used a rat model to determine the effects of endotoxin-contaminated and endotoxin-uncontaminated high-density polyethylene (HDPE) particles at two different time points.

To clarify the third and fourth questions, we implanted highly endotoxin-contaminated discs of ultra-high-molecular-weight polyethylene (UHMWPE), not deliberately contaminated UHMWPE discs and decontaminated titanium (Ti) discs intramuscularly into rats and measured the levels of adherent endotoxins at different time intervals. Endotoxin was measured by the determination of certain specific constituents of the lipid portion of the endotoxin.
molecule which indicated endotoxin adhering to particles and discs irrespective of its biological activity.

Materials and Methods

Analysis of endotoxin. After hydrolysis of particles or discs, 3-hydroxy (3-OH) fatty acids with carbon chain lengths of 10, 12, 14, 16 and 18 were measured by gas chromatography-tandem mass spectrometry (GC-MS-MS) according to a procedure previously applied to determine endotoxin in environmental samples. These acids serve as chemical markers for endotoxins and are quantified by using pentadeuterated 3-OH C 14:0 as an internal standard. The number of moles of endotoxin is calculated assuming that four moles of 3-OH fatty acids (FAs) correspond to one mole of endotoxin. This allows the measurement of endotoxin which is adhering to surfaces to levels of 1 to 10 ng in a studied sample.

Particles at the bone-implant interface. The particles were produced by Shamrock Technologies (Newark, New Jersey) using a proprietary method. They were reported to be of 100% HDPE and to have a specific gravity of 0.95 g/ml and a median size of 4.6 μm with 10% under 0.9 and 90% under 8.6 μm in diameter according to reported Coulter analysis by the manufacturer. The endotoxins were in the form of lyophilised powder, prepared by phenol extraction from Escherichia coli of serotype 055:B5 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The particles were suspended in an endotoxin solution (1 mg/ml water), 1 mg of particles per ml of solution. The suspension was then freeze-dried, washed five times in 70% ethanol and dried at room temperature. Control particles underwent the same treatment but with water instead of endotoxin solution.

We used male Sprague-Dawley rats (Møllegård, Køge, Denmark) with a mean body-weight of 350 g. Institutional guidelines for the care and treatment of laboratory animals were adhered to.

The rats were anaesthetised with an intraperitoneal injection containing diazepam (2 mg) and pentobarbital (11 mg). Each rat also received an intramuscular injection of 5 μg of streptomycin (Streptocillin; Boehringer-Ingelheim, Copenhagen, Denmark). All the implant components and surgical equipment were sterilised. Sterile gowns, gloves, surgical masks and theatre caps were used. The leg on the operated side was shaved and the entire rat was placed in a sterile surgical glove in which a hole was then cut and the leg pulled through the hole using tweezers. The foot was then clothed in sterile adhesive plastic (Steri-Drape; 3M Health Care, St Paul, Minnesota) and the leg washed with chlorhexidine.

Titanium plates (4 × 13 mm) were fixed to the medial aspect of the tibia, with cortical screws at each end measuring 1.5 mm in length and a central screw which could be removed without disturbing the position and stability of the plate (Fig. 1). The central screw protruded beyond the surface of the plate by 0.5 mm and had a diameter of 2.5 mm. A depression was milled out in the tibia to correspond to its protrusion. Beneath this central screw lay the circular test surface with an area of 4.9 mm². After insertion of the plate, a period of four weeks ensued during which bone was given time to grow towards the central screw and osseointegrate, producing a tight fit.

After four weeks, the rats were anaesthetised and prepared again, as described above, and a longitudinal incision was made along the side of the plate. The central screw was removed and either endotoxin-contaminated or endotoxin-free HDPE particles were applied to the test surface. As a way of standardising the amount of particles applied, a specially crafted pellet-making device was used. Each pel-
let was 0.06 mm$^3$ in volume and a total of five pellets was placed on the test surface of each rat, using sterile scalpel blades. The levels of the studied 3-OH fatty acids corresponded to concentrations of endotoxin of 13 pmol/mg for the contaminated particles and 0.1 pmol/mg for the uncontaminated particles. The total local dose of endotoxin was approximately 3.9 pmol per rat. This corresponded to an estimated contamination of 0.5 pmol/mm$^2$ of surface area.

The rats were divided into two main groups: 15 were given endotoxin-free particles and 15 contaminated particles. Eight animals from each group were killed on day 7 and the remainder on day 21 (Table I). After extraction of the tibia and removal of the plate, the section beneath the test surface was decalcified with EDTA and prepared using standard histological techniques. Sections were cut at right angles through the middle of the test surface and stained with haematoxylin and eosin. For purposes of blinding, the specimen identification numbers were covered by paper fastened with Scotch tape and a random number assigned to the glass slide by a third person. We evaluated the resorption areas, defined as an absence of bone at the interface and drawn manually on a computer screen using a computerised video system attached to the microscope (Videoplan; Kontron Bildanalyse GmbH, Esching, Germany) at a screen magnification of 125. When the experiment began, there was bone contact with the titanium plate. In the specimens in which bone had been replaced by soft tissue, the area of bone loss could be estimated.

Three specimens were excluded because of poor histological quality, wrong embedding and failure to osseointegrate. All the exclusions were made while the specimens were blinded. One rat died three days after the second operation and was also excluded.

For analysis, non-parametric statistics were used. The Kruskal-Wallis test was followed by the Mann-Whitney U test for relevant comparisons.

**Intramuscular discs.** We used UHMWPE and Ti discs taken from total hip replacement components. The UHMWPE discs had a diameter of 10 mm and a height of 2 mm and the Ti discs a diameter of 10 mm and a height of 1 mm. The UHMWPE discs were divided into two groups, one of which was purposefully contaminated with endotoxin, while the other served as a control. The contamination was carried out by immersing each disc in a solution of endotoxin (1 mg/ml, 2 ml) in a test-tube for 20 minutes. The control discs were immersed in sterile water for 20 minutes six times, changing the water between suspensions. Analysis of six extra discs showed the contaminated discs to have a content of endotoxin of approximately 0.82 nmol, and the uncontaminated of 0.015 nmol at the time of insertion. This corresponded to an estimated amount of endotoxin of 3.7 pmol/mm$^2$ of surface area for the contaminated discs. The Ti discs were heated in an oven at 400°C for seven hours. Subsequent analysis of four extra discs which had been immersed in a solution of endotoxin (1 mg/ml, 2 ml) confirmed that the discs were still able to pick up endotoxin. Titanium discs treated with endotoxin after heat preparation showed levels of endotoxin similar to those of UHMWPE discs treated with endotoxin. The endotoxin used was the same as described above for the particle experiment.

The discs were then inserted intramuscularly into the abdominal wall of 18 female Sprague-Dawley rats. One disc of each type (uncontaminated UHMWPE, contaminated UHMWPE and titanium) was inserted into each rat. The two UHMWPE discs were placed on one side of the abdomen and the titanium discs on the other at an approximate distance of 1 cm from each other or more. The surgery was carried out with stringent attention to asepsis. All the instruments were autoclaved and held over a Bunsen-burner flame between the handling of the discs. Additionally, different sets of instruments were used for the three types of disc.

The discs were then extracted at eight different time points between five minutes and 21 days and the content of endotoxin analysed. One set of discs each was extracted at five minutes and one hour and two or three sets at the other time points. The extractions were performed with the same attention to sterility and the discs were placed into baked test-tubes. Discs with the same treatment and time in the rat were placed in one test-tube, so that the results were pooled for each time point. The level of endotoxin was determined by GC-MS-MS as described above for the UHMWPE particles.

The results were evaluated by regression analysis over log time for the concentration of 3-hydroxymyristic acid (3-OH 14:0-marker of the specific endotoxin used for contamination) compared with the sum of the other 3-OH fatty acids studied.

**Results**

The rats recovered quickly from surgery and had no sign of discomfort from the implants.

**Particles at the bone-implant interface.** Under histological evaluation, the HDPE particles could be clearly seen both on the surface of the bone and in the marrow cavities. At day seven, the specimens which had received endotoxin showed large areas of resorption and apparent signs of inflammatory activity. The uncontaminated specimens were relatively unaffected by the presence of particles (Fig. 2). In specimens from the 21-day groups, generally no resorption was seen and the histological appearance of the endotoxin-contaminated and endotoxin-free specimens was
similar. In the endotoxin-contaminated specimens, however, the particles were more often found deep within the bone as if new bone had filled a previous resorption cavity and enveloped them (Fig. 3). There were two exceptions, one contaminated and one uncontaminated specimen at 21 days. Both showed a high degree of resorption and inflammation and also showed neutrophils and an abundance of round cells.

The areas of resorption differed between the groups (p = 0.016). Those in the seven-day endotoxin group were larger than in the seven-day group control (p = 0.002), but the 21-day endotoxin group was similar to the 21-day
control and showed less resorption than the seven-day endotoxin group (p = 0.04). The control groups at seven and 21 days did not differ (Table II, Fig. 4)

**Intramuscular discs.** The contaminated UHMWPE discs lost the added excess endotoxin (14 carbon length 3-OH fatty acid) within 24 hours in the rats. The remaining baseline amount of endotoxin (including 3-OH 14:0) remained constant at between 0.02 and 0.03 nmol thereafter at all time points and was the same for contaminated as for uncontaminated discs. Regression analysis showed that there was a decrease in the amount of 3-OH 14:0 over log time in the contaminated discs ($r^2 = 0.62$, p = 0.02), but not in the control group and not for the other fatty acids. The titanium discs did not adsorb any endotoxin and the measured amounts on the discs ranged from 0.005 nmol to unmeasurable (Table III, Fig. 5). The discs were even seen to lose the minute amounts of endotoxin measured at the early time points.

**Discussion**

The question of contamination of the surface of orthopaedic implants with endotoxin has been of some concern as a possible cause of prosthetic loosening. Endotoxins, or lipopolysaccharides (LPS), are a component of the outer membrane of Gram-negative bacterial cells and have a complex structure which is unique in nature. They consist of three components: lipid A, a hydrophobic phosphorylated disaccharide with short fatty-acid chains which anchor the molecule in the bacterial membrane; the core portion which is made up of a short series of sugars; and the O antigen, a long carbohydrate chain which protrudes above the outer membrane and covers the bacteria with a hydrophilic layer. Of these lipid A is responsible for the greatest part of host responses to an infection with Gram-negative bacteria and then only when the bacterial outer membrane is broken up.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Median difference</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endotoxin 7 days - control 7 days</td>
<td>0.82</td>
<td>0.002</td>
</tr>
<tr>
<td>Endotoxin 7 days - endotoxin 21 days</td>
<td>0.67</td>
<td>0.04</td>
</tr>
<tr>
<td>Endotoxin 21 days - control 21 days</td>
<td>-0.02</td>
<td>0.6</td>
</tr>
<tr>
<td>Control 7 days - control 21 days</td>
<td>-0.08</td>
<td>0.9</td>
</tr>
</tbody>
</table>

**Table II.** Differences in measured resorption areas (mm$^2$) with particles with or without endotoxin at 7 and at 21 days

**Table III.** Regression analysis of the content of 3-OH fatty acid on intramuscular discs over log time

<table>
<thead>
<tr>
<th></th>
<th>14 length 3-OH fatty acid</th>
<th>10, 12, 16 and 18 length 3-OH fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r$^2$</td>
<td>p value</td>
</tr>
<tr>
<td>UHMWPE-endotoxin contaminated</td>
<td>0.62</td>
<td>0.02</td>
</tr>
<tr>
<td>UHMWPE-endotoxin uncontaminated</td>
<td>0.04</td>
<td>0.65</td>
</tr>
<tr>
<td>Titanium</td>
<td>0.18</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Resorption areas with particles with or without endotoxin at seven and 21 days. At day seven, the endotoxin-contaminated particles had given rise to substantial resorption. This reaction had subsided at 21 days, but both specimens still displayed massive resorption as seen by the two outlying values. The histological appearance suggested that these represented infection. They were, however, included in the statistical analysis.

3-OH fatty acid on endotoxin-contaminated UHMWPE discs retrieved from intramuscular implantation after 5 minutes, 1 hour, 5 hours, 24 hours and 4, 7, 14 and 21 days. By 24 hours, the added 3-OH-14 fatty acids had decreased to equal the other fatty acids and thereafter the amount of endotoxin remained at this level.
and the molecule is exposed and bound to carrier molecules. At low concentrations, this response is characterised by fever, release of inflammatory agents such as interleukin-1 and tumour necrosis factor alpha leading to the acute-phase response, as well as activation of complement by way of the alternative pathway. At higher concentrations, the outcome is shock and intravascular coagulation leading to multiple-organ-system failure and death. Hence reaction to the contaminated particles was expected.

After a period of three weeks, however, the difference between contaminated and uncontaminated groups had vanished in respect of both the histological appearance and measured resorption areas, and there was no discernible reaction to the particles at all at this point. Other studies have also shown signs of relatively rapid neutralisation of endotoxin at a bone interface. There are two possible explanations for this, acting in conjunction or separately. First, as described above, the host response to the exposure of endotoxin is swift and powerful. Endotoxin-reactive proteins, such as complement products, opsonise the molecules as well as activate synergistic systems which work together to break down and remove the harmful substance quickly. For our experiment, this means that once the initial effect had subsided, the HDPE particles returned to being innocuous and did not prevent the formation of new bone and the filling of the resorption cavity. The second explanation is that the endotoxins are simply ‘washed away’, or picked up by carrier molecules, and taken care of at other locations in the body. This appears to be plausible since the endotoxin molecule is water-soluble, antibodies have been shown to be present in the blood, and the strength of the bond with polyethylene with respect to endogenous carrier molecules is unknown although endotoxin is reported to be strongly adherent to many implant materials.

The second part of our study, however, would seem not to substantiate this ‘wash-away’ hypothesis since considerable levels of adherent endotoxins were measured after three weeks of intramuscular implantation, while the response to endotoxin-contaminated particles was seen to have subsided within two to three weeks. The initial decrease in the levels of endotoxin cannot explain the cessation of the effect by endotoxin in the particle experiment since the levels after this decrease still remained comparable to the starting load in the particle experiment. Thereafter, the endotoxin levels did not decrease, indicating that inactivation of endotoxin must have occurred in situ.

Since the surface area of ground-up powder is much larger than that of the surface of implants and thus presents a larger area to which endotoxin molecules could adhere, the relative amounts of endotoxin possibly implanted with orthopaedic implants should be much less than with the particles in our model. This would indicate that, although endotoxin may be shown to adhere to and be present on orthopaedic implants, its clinical effect, if any, would be slight in respect of late aseptic loosening of the prosthesis.

Our model does not directly address the issue of sub-clinical Gram-negative infections. As mentioned above, small titres of antibodies against endotoxins are present in most healthy individuals with no clinical signs of infection, possibly as a result of enteric leakage. Theoretically, this could provide a small, continuous exposure to endotoxin and a low-grade reaction around the prosthesis and its eventual loosening. None of our results can positively disprove this theory, but since the titanium discs in our second experiment did not pick up endotoxin, the theory of implant materials acting as ‘fly-paper’ did not find support. Apparently, either the endotoxin shown to be present in blood is strongly bound to carrier molecules or the titanium had become coated with proteins which did not allow attachment of the endotoxin molecules. Also, it does not seem possible that such a low-grade reaction would lead to the dramatic results which are seen in loosening of the prosthesis, especially since our results show a rapid and efficient disposal of large amounts of endotoxin. Furthermore, studies have indicated that a careful cementing technique, as well as the correct positioning and the absence of early micromovement of the prosthesis, is crucial in order to avoid late loosening. This suggests that later changes may be of less importance.

Our results raise concerns about previous experiments with particles. It has been reported that measurement of the levels of endotoxin by standard methods such as by measuring supernatants with the amoeba lysate assay, are unsatisfactory in many cases. Therefore, undetected particle-adhered endotoxins may be responsible for some findings in vitro and also in vivo in experiments over a short time period. Of course, the observation that particles are harmless in our model does not preclude the possibility that they may be an important agent in the process of loosening of the prosthesis, when combined with other factors, such as instability, fluctuation of pressure, or the presence of synovial fluid. Concerning the two rats with marked osteolysis in the 21-day group, previous studies using a similar model have shown the HDPE particles to be by themselves practically innocuous. This, combined with the fact that the other specimens of the same groups showed such marked difference to these in terms of histological appearance and resorption areas, would indicate that the most likely explanation was infection. Nonetheless, they were included in the statistical analysis.

Our method of measuring endotoxin in clinical samples, i.e., by determining the 3-OH FAs, was suggested in the 1970s but has since gained little attention for this type of sample. This approach has a number of advantages. It is not dependent upon having a liquid medium for the reaction to take place. This means that adhered endotoxin, which may go undetected in the amoeba lysate assay, does not present a problem. Furthermore, it does not discriminate between active and inactivated endotoxin, presenting the possibility of measuring the absolute amount of endotoxin at the site. Further research is needed to establish the relationship
between absolute amounts of endotoxin and its measurable biological effect.

The uncontaminated UHMWPE particles did not cause resorption areas, whereas contaminated particles did. Therefore the model which we have used is capable of detecting resorptive processes and thus increases the validity of earlier results with respect to the harmless nature of PE particles in this model.

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References