MACROPHAGES STIMULATE BONE RESORPTION WHEN THEY PHAGOCYTOSE PARTICLES

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We investigated in vitro a mechanism by which particulate debris may induce bone resorption and cause implant loosening. We first studied two standard particles: latex, which is considered to be inert, and zymosan, which is inflammatory.

Macrophages that phagocytosed either particle became activated, and stimulated 15 times as much bone resorption as did control macrophages. For activation to occur, 100 times more latex than zymosan had to be phagocytosed.

We also found that bone cement and polyethylene particles activated macrophages in a similar manner, and that the necessary amounts of these were intermediate between those of latex and zymosan. None of the particles were toxic.

It was concluded that implant loosening may result from bone resorption stimulated by mediators released by macrophages that have phagocytosed particles of bone cement or polyethylene.

The most frequent long-term complication of joint replacement is loosening. This occurs if the supporting bone is resorbed. Large amounts of particulate debris, be it Teflon, high density polyethylene (HDP), polymethylmethacrylate (PMMA) or metal have often been associated with bone resorption (Charnley 1963; Vernon-Roberts and Freeman 1977; Revell et al 1978; Maguire, Coscia and Lynch 1987). This bone resorption is thought to be a manifestation of the foreign-body reaction, and it is likely that macrophages and giant cells are responsible (Goldring et al 1986). Although these cells cannot resorb viable bone, they do release mediators, like prostaglandin E₂ (PGE₂), that stimulate osteoclastic bone resorption (Chambers and Horton 1984; Nathan 1987; Vaes 1988).

PMMA and HDP particles are found at bone–implant interfaces, the latter presumably having reached there from the joint cavity, by arthrographically demonstrated clefts (Murray and Rodrigo 1975; Malcolm 1988). If macrophages, which have phagocytosed these interfacial particles, release mediators that stimulate bone resorption, then the implant will loosen as its bony support will be lost.

Little is known about the effect on macrophages of orthopaedic particles; however, phagocytosis of latex and zymosan has been studied in some detail. Latex consists of polystyrene spheres and is considered to be inert, non-inflammatory and non-toxic; whereas zymosan, obtained from yeast cell walls, is inflammatory but non-toxic. Phagocytosis of zymosan causes activation and increased PGE₂ release, whereas latex does not (Humes et al 1977; Baggiiolini, Schnyder and Dewald 1982). Release of the cytoplasmic enzyme lactate dehydrogenase (LDH), a marker of cell damage, is not affected significantly by latex or zymosan, but is increased by toxic particles like silica (Schorlemmer et al 1977; Rae 1986). Toxic particles cause a series of morphological changes in macrophages, consisting of retraction of pseudopodia, blebbing and smoothing of the surface, and finally cell destruction (Waters et al 1975).

The aim of this study was first to determine whether macrophages phagocytosing foreign particles release mediators which stimulate bone resorption, and secondly to determine if there was a relationship between the amount of resorption and the type of particle. Latex and zymosan were investigated first and the results compared with the effect of HDP and PMMA particles.

METHODS

Macrophages were cultured with various particles and the culture medium, into which they had released mediators, was assayed for bone resorption, PGE₂ and LDH. The macrophages were stained with May–Gruenwald and Geimsa stain and examined microscopically.
Methods not previously described (Rae 1975; Murray, Rae and Rushton 1989) are described below. Student's t-test was used for statistical analysis.

Macrophages. Mouse peritoneal macrophages were cultured (10⁶ cells/ml) in Medium 199 (M119) with 10% Foetal Bovine Serum (FBS) for 24 hours on glass (10⁶ cells/cm²). Non-adherent contaminating cells were then washed off and M199 containing ultrasonically dispersed particles was added. After a further 24 hours culture the conditioned medium was collected, centrifuged (1000 G for 10 minutes) and assayed.

Particles. Latex polystyrene spheres, of average diameter 1.101 μm (Sigma Ltd, Poole, UK) were washed three times by ultrasonic dispersion in M199 and centrifuged (1000 G for 20 minutes), before being diluted and added to the macrophages.

Bone cement (CMW Labs Ltd. Blackpool, UK) is formed by mixing particles, consisting primarily of methylmethacrylate polymer, with methylmethacrylate monomer. These PMMA particles, without added monomer, were washed twice in M119, by ultrasonic dispersion and centrifugation (1000 G for 20 minutes), and then diluted.

Bone resorption assay. Neonatal mouse calvaria, prelabelled with ⁴⁵Ca, were cultured in sample medium. The percentage of the ⁴⁵Ca released was used as a measure of bone resorption.

So that paired t-tests could be used, each calvarium was halved; one half was cultured in test medium and the other in control medium. The macrophage-conditioned medium was serially diluted before being assayed. Sigmoid dose response curves were generated as the ⁴⁵Ca release depended on the dose of conditioned medium (mean and standard errors (s.e.m.) were plotted). The magnitude of the bone resorption stimulus was determined from the shift of the dose response curve. The significance of the increase in bone resorption was determined by comparing test and control ⁴⁵Ca release at doses in the intermediate, sloping part of the dose response curves.

RESULTS

Phagocytosis of latex. Macrophages cultured with a large dose of latex (10⁶ particles/ml) caused significantly more ⁴⁵Ca release than control macrophages (p < 0.001, p < 0.001, p < 0.05), and the bone resorption increased by a factor of 13 (Fig. 1). In a series of similar experiments, doses from 10⁶ to 10⁸ particles/ml were used, at each dose the experiment was repeated (Table I). (The conditioned media were assayed at 0.4 × 10⁵ cells.days/ml so that phagocytosis would cause a large increase in ⁴⁵Ca release (Fig. 1).) At each dose an estimate of the increased bone resorption was made from the average increased ⁴⁵Ca

![Graph](image)

Table I. Bone resorption stimulated by phagocytosis of various doses of latex

<table>
<thead>
<tr>
<th>Latex dose (x10⁶)</th>
<th>Control Mean ± SE</th>
<th>Latex Mean ± SE</th>
<th>Increase Mean ± SE</th>
<th>n</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁴</td>
<td>15.28 ± 0.76</td>
<td>27.06 ± 0.77</td>
<td>11.78 ± 0.67</td>
<td>10</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>10⁵</td>
<td>19.74 ± 1.49</td>
<td>29.58 ± 0.60</td>
<td>9.84 ± 1.61</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>10⁶</td>
<td>21.77 ± 2.03</td>
<td>22.42 ± 1.78</td>
<td>0.65 ± 0.69</td>
<td>12</td>
<td>NS</td>
</tr>
<tr>
<td>10⁷</td>
<td>19.74 ± 1.49</td>
<td>23.53 ± 1.23</td>
<td>3.79 ± 3.73</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>10⁸</td>
<td>20.24 ± 1.22</td>
<td>19.90 ± 1.07</td>
<td>0.33 ± 1.03</td>
<td>12</td>
<td>NS</td>
</tr>
</tbody>
</table>

*paired calvaria

Zymosan particles (Sigma Ltd) were dispersed in distilled water (10 mg/10 ml), boiled and centrifuged (1000 G for 10 minutes) three times. They were then dispersed in M199 and diluted.

HDP particles were generated from an acetabular prosthesis (Charles F. Thackray, Leeds, UK). An HDP cylinder, sterilised in 70% ethanol, was rotated (60 rpm) whilst being forced (10 N) against 1200 grit silicon carbide paper. This was carried out in FBS under sterile conditions. The wetting agents in FBS prevented the particles from clumping and sticking to the walls of the containing vessel. The particle suspension was centrifuged (1000 G for 10 minutes). HDP has a density slightly less than FBS so the small HDP particles remained in suspension, the large HDP particles floated, and contaminating silicon carbide sank. The resulting suspension containing small HDP particles (about 1 μm in size) was removed, diluted with M119, and added to the macrophages. Polarising-light microscopy of these small HDP particles demonstrated no contaminating silicon carbide.

![Diagram](image)
release using a standard dose response curve (Fig. 2). Only at the largest dose (10^6 particles/ml) was there a significant or appreciable increase in resorption; at this dose activation was considered to have occurred.

In a separate series of experiments the PGE_2 and LDH released by macrophages, cultured with various doses of latex, was determined (Tables II and III). At each dose the average increased release was calculated (Fig. 2). Only at the largest dose (10^6 particles/ml) was there a significant increase in PGE_2 release (p < 0.001, p < 0.05, p < 0.05). At this dose it increased by a factor of five. At no dose was there a consistently significant increase in LDH release.

There were no microscopic morphological changes suggesting that latex was toxic. At the activating dose (10^6 particles/ml), almost all the macrophages were completely full of latex, and on average there were 165 particles within each cell (Table V).

**Phagocytosis of zymosan.** Phagocytosis of zymosan (100 µg/ml) caused a significant increase in ^45^Ca release (p < 0.02, p < 0.001, p < 0.001), and increased the bone resorption by a factor of 18 (Fig. 2). The increased PGE_2 and LDH release, caused by various doses of zymosan, was determined in two experiments and averaged (Fig. 2). At doses of 10 µg/ml and above the PGE_2 release increased by a factor of five increases that were significant (p < 0.001) in both experiments. Only at a dose of 100 µg/ml was there a consistently significant (p < 0.001) increase in LDH release, and at this dose it doubled.

There were no microscopic morphological changes suggestive of a toxic effect, although each phagocytosed zymosan particle was associated with a vacuole. At a dose of 100 µg/ml the macrophages appeared to be full of zymosan. At 10 µg/ml, the activating dose, the mean number of particles per cell was 2.1 (Table V).

A comparison was made between the effect of maximally stimulating doses of latex and of zymosan (Table IV). Different particles did not cause significantly different bone resorption or PGE_2 release.

**Phagocytosis of high density polyethylene.** As the HDP suspension contained serum, controls with the same amount of serum but no HDP were used. These were prepared by filtering the suspension, and were called 'filtered controls' so as to distinguish them from 'standard controls', which contained neither serum nor particles. The increased release of PGE_2 and LDH, caused by various dilutions of the HDP suspension, was calculated by dividing the test release by the filtered control (Fig. 3). At a dilution of 100 the PGE_2 release increased by a factor of five (a significant increase as the test release was significantly (p < 0.001) more than the standard control, and as the filtered control was significantly (p < 0.01) less at this dose than at lower doses). At this dose there was also a small but significant increase in LDH release (significant as the test release was significantly (p < 0.001) more than the standard control, and as there was no change in the filtered control). Lower doses of
HDP did not affect the release of PGE₂ or LDH (as test releases were similar to the standard controls).

It was impossible, even with polarised-light microscopy, to determine how many HDP particles each macrophage had phagocytosed, as the particles were ragged, overlapped one another, and were of variable size. At a dilution of 10, the macrophages were full of HDP. At a dilution of 100, the activating dose, 60% of the macrophages contained HDP, and the cytoplasm of these cells was not as stretched as at a dilution of 10 (Table V). There were no morphological changes to suggest that HDP was toxic.

**Phagocytosis of polymethylmethacrylate.** The increased PGE₂ and LDH release caused by various doses of PMMA particles was determined (Fig. 3). At a dose of 100 μg/ml, significantly (p < 0.001) more PGE₂ was released, but there was no increase in LDH released. At 1000 μg/ml, the PGE₂ release increased further and was approximately six times the control release. At this dose the LDH release also increased significantly (p < 0.001), by a factor of 1.6.

Most of the PMMA particles were small (a few microns) and irregular, but some were large (up to 50 μm) and spherical. It was impossible to estimate how many particles each macrophage had phagocytosed because of their irregularity, and because some macrophages were spread on the surface of the larger particles. At a dose of 1000 μg/ml, almost all the macrophages were full of PMMA. At 100 μg/ml, the activating dose, 70% of the macrophages contained PMMA (Table V). There were no morphological changes to suggest that PMMA was toxic.

**DISCUSSION**

Macrophages that phagocytosed zymosan or a large amount of latex became activated in a similar manner: they both stimulated about 15 times as much bone resorption, and released five times as much PGE₂ as control macrophages. Surprisingly, Humes et al. (1977) and Baggiolini et al. (1982) found that latex neither caused activation nor an increased release of PGE₂, but this is probably because they used lower doses of latex. As latex is one of the least inflammatory particles known and zymosan one of the most, it is likely that phagocytosis of any foreign particle will result in activation, provided there are enough particles. As activation results in a greater increase in bone resorption than in PGE₂ release, the macrophages must release other, more important, stimulators of bone resorption than PGE₂. However, as the increase in PGE₂ release occurred at the same dose of latex as did the increase in bone resorption, the PGE₂ release can be used as an indicator of activation and bone resorption. Phagocytosis of HDP, PMMA, latex and zymosan all caused approximately a five-fold increase in PGE₂ release, so it is likely that all these particles activate macrophages in a similar manner.
For macrophages to be activated and cause bone resorption, they have to phagocytose different amounts of different particles. About 100 times more latex than zymosan particles are necessary for activation, so zymosan can be considered to be 100 times more damaging than latex (Table V). Unfortunately, it was impossible to count the number of HDP or PMMA particles phagocytosed, but the amount necessary to cause activation can be quantified in other ways (Table V). Zymosan was the most harmful as the smallest weight was necessary to cause activation, and as it was always associated with vacuoles. Latex was the least harmful as it was the only particle that did not necessarily cause activation when phagocytosed by most macrophages. At the activating dose of latex, the macrophages were full and there was a large excess of particles, whereas for the other particles this state was not reached until 10 times the activating dose. Therefore HDP and PMMA are more harmful than latex and less harmful than zymosan.

Latex and zymosan are considered to be non-toxic, and neither caused morphological changes suggestive of toxicity. It was therefore surprising that the highest dose of zymosan consistently caused a significant increase in release of the LDH from macrophages. However, the release only doubled, which is a relatively small increase compared with the tenfold rise caused by silica (Rae 1986). Small increases in LDH release caused by latex and zymosan have been recorded by other workers (Schorlemmer et al 1977; Rae 1986). It seems likely that a small increase in LDH release is not associated with toxicity and severe damage. It may represent leakage of cytoplasmic enzymes resulting from membrane perturbations caused by phagocytosis. Large doses of HDP and PMMA caused only a 1.5 times increase in LDH release, and no morphological changes suggestive of toxicity, so it is unlikely that HDP or PMMA were toxic. For all particles the increased LDH release only occurred at 10 times the activating dose. It can therefore be concluded that phagocytosis can cause activation and stimulation of bone resorption without there being a toxic effect.

Most orthopaedic implants are associated with foreign particles. These particles are phagocytosed by macrophages. If there are enough particles then the macrophages become activated and release soluble factors which stimulate bone resorption. Whatever the nature of the particles, even if they are supposedly inert like latex, a large amount of bone resorption will be stimulated if there are a large number of particles. However, some particles, for example HDP and PMMA, are likely to stimulate considerable amounts of bone resorption in smaller quantities. The HDP and PMMA particles used experimentally are similar to HDP and PMMA particles which occur at the bone–implant interface of cemented total joint replacements. HDP and PMMA particles may therefore cause resorption of the bone supporting these implants, and this will result in loosening.

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REFERENCES


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