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BIOLOGICAL RESPONSE TO PARTICULATE DEBRIS: *IN VITRO* AND *IN VIVO* STUDIES

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Abstract

In this study, we compared the results of a human monocyte *in vitro* model and a canine *in vivo* model, to evaluate the response to different types of particulate wear debris. Both the *in vitro* and *in vivo* studies demonstrated that titanium-6 aluminum-4 vanadium (TiAlV), ultrahigh molecular weight polyethylene (UHMWPE) and other particulate wear debris are stimulatory to macrophages and elicit release of several identifiable mediators involved in periprosthetic inflammation and bone resorption. Further, in the *in vivo* studies, these same particles initiated the formation of interfacial membranes which were histologically and biochemically similar to those seen in clinically failed joint arthroplasties. *In vitro* models using monocytes are thus useful for studying individual species response to different types of particles. *In vivo* models have the advantage of accounting for multiple cell types and paracrine effects.

Key Words: Total hip replacements, aseptic loosening, wear debris, polyethylene debris, titanium-alloy, interleukin-1, osteolysis, prostaglandin E_2, interleukin-6, canine model.

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Introduction

While significant advances have been made in the manufacture of components, instrumentation, and surgical techniques for total hip replacements (THR), the occurrence of osteolysis, with or without aseptic loosening remains the leading cause of failure of these procedures [4, 24, 26, 28, 36]. Goldring *et al.* [12] demonstrated that when fibrous interfacial membranes from failed cemented arthroplasties were placed in organ culture, they released various inflammatory mediators such as prostaglandin E_2, collagenase and gelatinase. These mediators are responsible for osteoclast maturation, stimulation and degradation of the extracellular matrix. Numerous investigators have since demonstrated that interfacial membranes additionally release interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α), also key components of the bone resorptive cascade [5, 7, 35, 37]. Histologically, interfacial membranes consist of macrophages with intracellular wear debris within a loose connective tissue matrix. *In vitro* monocytes/macrophages phagocytize prosthetic wear debris and are stimulated to release the same mediators associated with bone resorption [2, 11, 14, 15, 32]. In the clinical setting, macrophages are thus stimulated by wear debris generated from the prosthetic components and release inflammatory mediators. These mediators in turn stimulate osteoclasts to resorb bone, eventually resulting in the bone resorption associated with osteolysis and aseptic loosening [9, 10, 11, 19, 38].

There are numerous particle related variables, such as composition and concentration, which can affect the cellular response, and we have used *in vitro* and *in vivo* models to study these variables. Due to the predominance of macrophages in periprosthetic tissues, human peripheral blood monocytes were used as the target cells in the *in vitro* model to compare the cellular response following challenge with submicron titanium-6 aluminum-4 vanadium (TiAlV) and ultrahigh molecular weight polyethylene particles (UHMWPE) particles [31, 34]. In the *in vivo* model, a canine THR was performed and particles were introduced at the bone-implant interface

to study the host response to TiAlV and other debris [8].

Materials and Methods

In vitro model: Monocyte response to particulate debris

In this model, human monocytes were challenged with submicron sized, clinically relevant titanium or UHMWPE particles [8]. The titanium particles used were TiAlV (0.77 $\pm$ 0.4 μm, fabricated in-house using a custom designed TiAlV ball mill) and commercially pure Ti (CpTi, 0.81 $\pm$ 0.4 μm, Johnson and Mathey, Danvers, MA). UHMWPE particles were either fabricated from the GUR 415 base resin [23] (fabricated PE, 0.66 $\pm$ 0.2 μm) or retrieved from interfacial membranes during revision THR [33] (retrieved PE, 0.47 $\pm$ 0.2 μm). Since we were comparing particles of different densities, the traditional approaches of dosing cells by weight percent, or volume percent introduce errors. Thus, to reduce these errors and standardize particle dosing, monocytes were challenged with particles based upon the surface area ratio (SAR) of the particles and cells [32, 34] A 1 X SAR results in roughly equal particle to cell surface areas, whereas a 10 X SAR would constitute particles with surface area, ten times that of the cells [31, 32]. For all particle species, the surface area was calculated using the formula for spherical particles. Particles were sterilized by gamma-ray irradiation, and coated with proteins from pooled human type AB serum (Sigma, St. Louis, MO).

Monocytes were separated from peripheral blood from healthy volunteers by sequential discontinuous Percoll gradients [1]. After an overnight adherence to commercially available 24-well tissue culture plates (Corning Glass, Corning, NY), approximately 1 x 10^6 viable, monocytic cells were cultured with particles at final particle concentrations of 0.1 X, 1.0 X and 10 X SAR in 1 ml Macrophage-serum free medium (M-SFM; GibcoBRL, Grand Island, NY). Controls consisted of non-phagocytizable 22 μm, Ti particles (Ti-22; Cerac, Milwaukee, WI), opsonized yeast cell wall zymosan (0.38 mg; Sigma), and phorbol myristate acetate (PMA; 1 μg; Sigma). Within four hours of culture, monocytes were observed using a tissue culture microscope to have initiated particle phagocytosis. Cells were cultured with particles or controls for 24 hours at 37°C in 5% CO_2. Supernatant from the cell-particle co-cultures, referred to as monocyte conditioned medium (MCM) were collected and the concentration of IL-1ß and IL-6 were determined by ELISA (R & D Systems, Minneapolis, MN) and PGE_2 levels were measured using a radio-immunoassay (Amersham, UK). Cytokine and PGE_2 studies were performed with monocytes from eleven volunteers separately.

While monocyte viability at the start of the culture was measured by trypan blue dye exclusion, it was not possible to use this technique due to obscuring of the cell profile by the phagocytized particles. We thus used the N-acetyl glucosamine assay for hexosaminidase, for quantitating cell viability after culture with particles [22]. Briefly, at the end of the culture period, adherent monocytes were washed and the substrate(p-nitrophenol-ß-d-glucoseaminide) for the enzyme hexosaminidase was added and cultured at 37°C for 4 hours. Further enzyme activity was blocked by glycine buffer, the supernatant was harvested, filtered and the hydrolytically cleaved nitrophenol measured by absorption spectrophotometry [30, 31].

In vivo model: Canine response to particulate debris

Uncemented right THR was performed on adult male canines (30-35 kg) randomized into control or experimental groups [8]. Controls received a TiAlV femoral prosthesis (distal stem, diameter 10 mm) with a proximal fiber-metal pad; a 29 mm fiber-metal coated acetabulum and an UHMWPE acetabular liner (Zimmer, Warsaw, IN). Animals in the experimental groups received a femoral prosthesis wherein the proximal porous coated region was circumferentially recessed by 2 mm during manufacture of the implant (Zimmer). Intraoperatively, different types of particles mixed in clotted blood were introduced in the resulting gap. The particles used were cobalt-chrome-alloy (CoCr; 100 mg, 10.2 $\pm$ 1.5 μm; Zimmer), TiAlV (100 mg, 3.1 $\pm$ 0.5 μm; Zimmer) or high density polyethylene (HDPE; 30 mg, 4.1 $\pm$ 2 μm; Shamrock Technologies, Newark, NJ). Animals were sacrificed after 12 weeks and the periprosthetic tissues were harvested and analyzed histologically and biochemically.

Tissues were stained with hematoxylin and eosin (H&E) and graded for macrophage response and general cellularity [27]. Tissue samples were also placed in organ culture for 72 hours and the supernatants were assayed for IL-1 (D10.G4.1 T-helper cell bioassay), PGE_2, collagenase and gelatinase [21]. Assay measurements were standardized to the contralateral joint capsule and reported as "units of activity." Group means were determined and compared using a multivariate analysis of variance and unpaired Student's t-test.

Results

In vitro model

Monocytes had begun to phagocytize particles within an hour of cell-particle coculture and within 24 hours nearly all particles were phagocytized, especially at the 0.1 X and 1 X SAR. However, at the 10 X SAR, many particles remained extracellular (Fig. 1). During culture

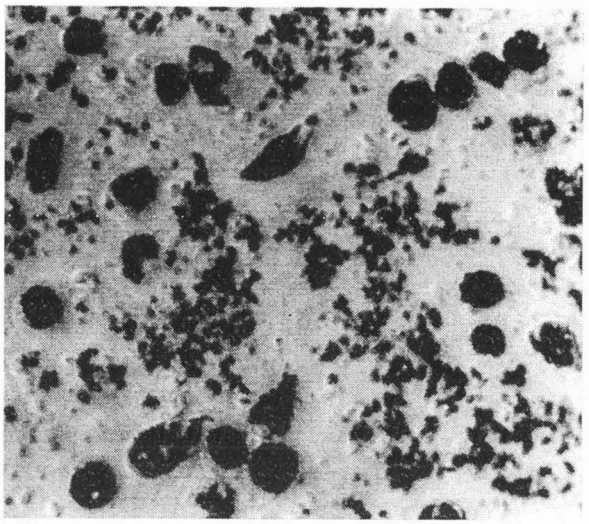

Figure 1. Monocytes in culture with a 10 X SAR dose of TiAlV particles. The monocytes have clearly internalized the particles. Photo width is approximately 60 μm.

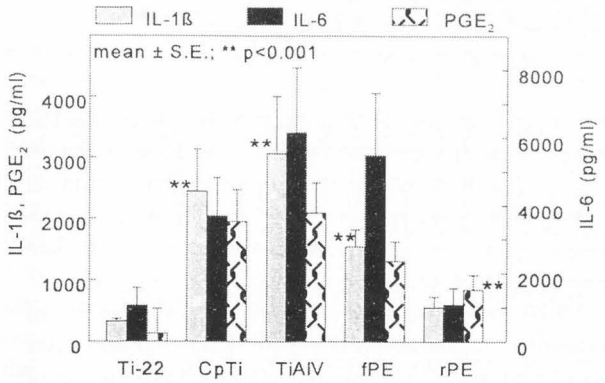

Figure 2. Interleukin-1, Interleukin-6 and Prostaglandin E_2 released by human monocytes after a 24 hour culture with different types of particles at the 10 X SAR.

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with particles, monocytes released different amounts of cytokines and mediators depending upon the composition and dose of the challenging particles. In general, the cells released very low levels of cytokines at the 0.1 X SAR, which increased marginally at the 1 X SAR and significantly at the 10 X SAR. At the 10 X SAR, monocytes demonstrated the most discernible differences in cytokine and mediator release, depending upon the composition of the particles.

TiAlV particles were the most stimulatory at all SARs tested and elicited a 13-fold increase in IL-1ß release compared to non-stimulated cells (Fig. 2). While both the UHMWPE fractions were significantly less stimulatory, the retrieved PE particles were essentially non-stimulatory. Similar trends were seen with release of IL-6 and PGE_2 (Fig. 2), with TiAlV particles being the most stimulatory followed by CpTi, fabricated PE and retrieved PE. Non-phagocytizable Ti-22 particles were essentially unable to elicit significant cytokine or mediator release at any concentration. PMA elicited a eleven-fold increase in IL-1ß, a four-fold increase in PGE_2 release, but was ineffective in eliciting IL-6. Zymosan elicited sixteen-fold and eight-fold increases in IL-6 and PGE_2, compared to non-stimulated cells respectively.

Cellular hexosaminidase levels provided an indication of particle cytotoxicity. The number of particles per cell were estimated based on overall cell and particle count estimates and represent the particle density in the culture milieu. At the 0.1 X SAR level, we found that monocytes tolerated culture at a density of 11-12 particles of TiAlV, CpTi or fabricated PE and 35 particles of retrieved PE per monocyte. At higher particle concentrations, cell viability decreased significantly (Fig. 3). At the highest concentration tested, only 25% of monocytes survived the 24-hour culture with TiAlV or CpTi (density of 1120-1190 particles of TiAlV or CpTi per cell) whereas 50% of cells survived UHMWPE culture (density of 1130 fabricated PE or 3520 retrieved PE particles per cell).

In vivo model

All control total hip replacements were stable. and a thin fibrous membrane surrounded the non-porous portion of the stem. Generally, control membranes were comprised of a few macrophages amidst organized fibrous stroma. Membranes around the gap controls were less organized and more cellular than controls. Implants with added particles were grossly loose and associated with thick fibrous pseudocapsules. Metal staining of the inner pseudocapsule was observed in the CoCr and TiAlV groups.

Interfacial membranous tissues around implants in the CoCr, TiAlV and HDPE groups had abundant macrophages and foreign body giant cells with particles present in their cytoplasm. Histologically, the concentration of debris in the tissue sections varied and a heightened cellular reaction was noted around areas where debris had collected. CoCr specimens were the most cellular and in areas of CoCr particle agglomeration, nuclear degeneration and cellular swelling resembling tissue necrosis had occurred. Tissues associated with TiAlV debris were histologically similar to tissues around CoCr debris, but nuclear degeneration or necrosis was not observed. Tissues with HDPE particle debris had decreased cellularity and increased fibrous stroma. The membranes also contained more giant

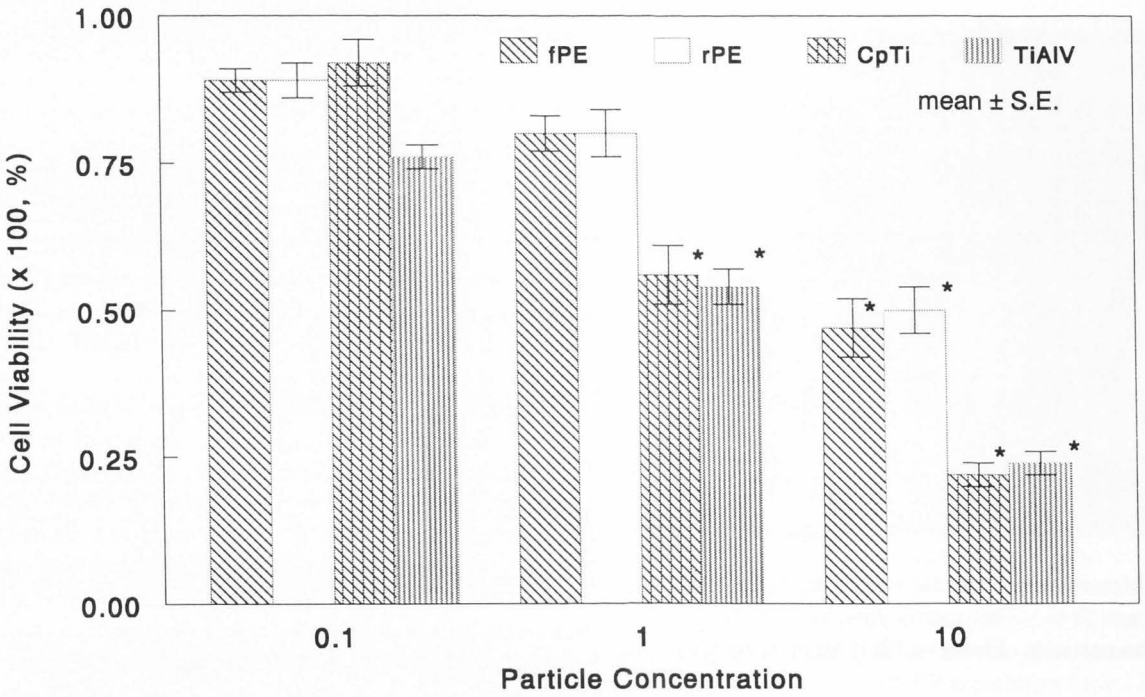

Figure 3. Viability of human monocytes after a 24 hour culture with different types of particles.

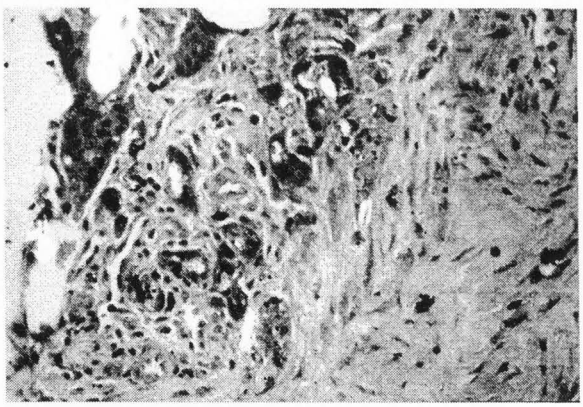

Figure 4. Histomicrograph of interfacial tissues around a gap prosthesis in polarized light, wherein TiAlV particles were introduced. Numerous TiAlV and birefringent UHMWPE particles are seen, often surrounded by giant cells. Photo width is approximately 85 μm.

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cells, but showed no areas of cytotoxic cell changes.

When tissue sections from CoCr and TiAlV particle groups were examined using polarized light microscopy, varying amounts of birefringent UHMWPE particles, presumably from the acetabular component were clearly seen (Fig. 4). These UHMWPE debris resembled wear debris present in human interfacial membranes with a combination of small (1-10 μm) irregular spherical particles and larger filaments and shards 20-30 μm in length [24, 27, 33]. Particularly striking was the observation that the *in vivo* generated UHMWPE particles were surrounded by an intense cellular reaction with abundant macrophages and foreign body giant cells (Fig. 4).

All periprosthetic tissues released varying levels of collagenase, gelatinase, PGE_2 and IL-1. Membranes from control groups were associated with lower levels of mediators than the experimental groups (Table 1). Gap-only implants were generally associated with intermediate levels of mediator release. CoCr debris associated membranes released the highest levels of collagenase and gelatinase among the experimental groups (Table 1). TiAlV debris associated membranes released the highest levels of PGE_2. CoCr and HDPE associated membranes released very high levels of IL-1 in the supernatant, but were not statistically significant. HDPE particles were associated with the lowest levels of collagenase and PGE_2.

Discussion

Osteolysis and aseptic loosening are currently the leading causes of failure of THR. The prevailing hypothesis is that prosthetic wear debris stimulates monocytes and macrophages to release several biochemical mediators, which in turn stimulate resorption of the sur-

Table 1. Cytokine and mediator levels in periprosthetic tissues around canine THR.

Group	Collagenase (mean ± SE)	Gelatinase (mean ± SE)	IL-1[1] (mean ± SE)	PGE_2 (mean ± SE)
Control (n = 5)	0.55 ± 0.2	5.83 ± 3.7	1.08 ± 0.3	0.49 ± 0.2
Gap only (n = 5)	1.91 ± 1.0*	14.67 ± 3.9*	2.41 ± 1.4	4.78 ± 2.9*
CoCr (n = 7)	9.16 ± 3.5*	5.9 ± 2.0	45.87 ± 29.2	3.37 ± 1.58*
TiAlV (n = 7)	3.96 ± 1.6*	1.71 ± 0.5	15.4 ± 7.3	10.88 ± 9.7
HDPE (n = 5)	1.6 ± 0.5*	4.76 ± 1.8	46.0 ± 35.3	0.62 ± 0.3

All values are compared to contralateral capsular tissues [8].
[1]Only n = 3 determinations were possible for IL-1. *Significantly greater than controls, $p < 0.05$.

rounding bone [11, 12, 19, 20, 24, 38]. In this report, we have summarized our *in vitro* and *in vivo* studies, conducted to test various aspects of wear debris-induced osteolysis [8, 34].

In vitro, we observed that different particles types were cytotoxic depending upon their composition and concentration. While human monocytes were tolerant of lower particle densities (10-35 particles per cell), higher particle concentrations (> 1000 per cell) resulted in significant cell death. TiAlV and CpTi particles were both significantly more cytotoxic in culture with monocytes than either the fabricated or retrieved UHMWPE fractions. This parallels the cellular necrosis associated with aggregates of TiAlV and CoCr particles observed in the canine model [8]. TiAlV particles consistently elicited the highest levels of cytokines and PGE_2, followed closely by CpTi particles. Cytokines elicited by UHMWPE particles were significantly lower than those elicited by titanium particles, albeit higher than controls. The biochemical findings also paralleled those from the canine model. *In vivo*, TiAlV or CoCr debris were associated with the highest levels of all mediators tested. Also consistent with the *in vitro* studies, HDPE particles were not as stimulatory in the canine model. Even though the HDPE and UHMWPE particles were similarly non-stimulatory, there are differences between them that are noteworthy. While the HDPE has an average molecular weight of between 200,000-600,000, the molecular weight of the UHMWPE is generally 4-6 x 10^6. The UHMWPE particles used in the *in vitro* model were also of a smaller size (0.4-0.6 μm) compared to the 4.1 μm mean size of the HDPE particles used in the canine model. Submicron UHMWPE particles were not available when the canine study was initiated. Despite these differences, the lower stimulatory capacity of the polyethylene particles suggests that other properties of the material, such as surface energy, particle shape and metal ion release by metal particles may also play an important role in stimulating an enhanced biological response.

The TiAlV, CpTi and CoCr particles used in these experiments represent material used in total hip replacements and total knee replacements. Regarding the size characteristics of metal debris, there is a paucity of data compared to polyethylene particles. Several investigators have reported that metallic debris (TiAlV, CpTi and CoCr) are "fine" and range in size from 0.1-25 μm and irregular in shape [3, 25, 33]. The metal particles used in both of our studies were generated by different fretting mechanisms and were also irregular in shape. In the cell culture studies, the mean sizes of the TiAlV and CpTi particles were 0.77 μm and 0.81 μm respectively. Since the canine study was initiated much earlier, finer metal debris were not available and the mean sizes of the TiAlV and CoCr particles used were 3.1 μm and 10.2 μm, respectively. Despite this comparatively larger mean size, the majority of the metal particles were phagocytizable and within the range described as present in patients around failed joint replacements [3, 25].

To prevent endotoxin contamination, all particles used in the cell culture and the animal model, except for the retrieved polyethylene particles, were prepared in a very clean environment. This involved the presence of distilled water, antibiotics and in the later stages 95% ethyl alcohol. The technique to extract the retrieved polyethylene particles [33] involved extensive sequential digestions with 4 M potassium hydroxide to clean the particles of any organic detritus. These precautions were taken to eliminate not just endotoxin, but any other organic contaminant which affect scanning electron microscopy and compositional characterization of the particles. Scanning microscopy of the particles accompa-

nied by energy dispersive x-ray analysis and when appropriate, Fourier transform infrared spectroscopy also confirmed pure particle preparations without other organic contamination. Since we had taken stringent precautions, and our analyses suggested no organic contaminants, we did not separately test for endotoxin.

The increased stimulatory nature of TiAlV particles to human monocytes is in agreement with previous studies [2, 11, 14]. The increased cellularity and necrosis around CoCr particles was also reported by Cohen in 1959 [6] and Howie and Vernon-Roberts [16, 17]. Intra-articular injection of UHMWPE particles is known to induce loosening of previously implanted poly (methylmethacrylate) plugs [18]; while in the rabbit tibia, a histiocytic and giant cell response was observed [13]. There are however, no previous *in vitro* studies to provide the comparison for the decreased stimulatory response to submicron UHMWPE particles.

It was observed in both our *in vitro* and *in vivo* studies that the metal particles (TiAlV, CoCr and CpTi) stimulated a more cellular response, were more cytotoxic and stimulated release of higher levels of cytokines and PGE_2. The fact that our study shows TiAlV and CpTi to be greater stimulators of mediator release than UHMWPE, does not refute previous assertions that macrophage-mediated osteolysis and aseptic loosening is a primary reaction to UHMWPE wear debris [24, 29]. It merely suggests that titanium and its alloys are more potent stimulators of mediator release, but are typically less abundantly seen than UHMWPE debris around revised human THR tissues. Therefore, because of the overwhelmingly greater number of UHMWPE particles present, UHMWPE remains the focal particle in precipitating aseptic loosening. Determining the actual number of particles present around aseptically loose prostheses is very difficult and depends on several factors such as prosthesis design, quality of the materials and patient activity to name a few. Based on histological studies of tissues around failed joint replacements, we believe that a range of particle concentrations from a SAR of 1 X to 10 X adequately models the *in vivo* environment.

The detection of birefringent UHMWPE particles in the membranes in which only metal particles were introduced in the gap prosthesis represents a serendipitous observation of third-body UHMWPE wear. Metal particles probably advanced into the metal-UHMWPE articulation, resulting in abrasion of the liner. This suggests that we may be unable to study the *in vivo* effects of metallic debris without the confounding effects of third-body UHMWPE. Despite these limitations, the generation of third-body UHMWPE remains a clinically relevant complication. There are other notable differences between the *in vivo* and *in vitro* models. In our *in vitro* model, only macrophages were involved. However, in an *in vivo* situation, several other cell types are involved for longer periods of time. Different cell types also release several additional cytokines and mediators. Together these cells and mediators may provide a biological response similar to the clinical pathological situation. This highlights the shortcomings of the *in vitro* system and also the importance of performing *in vivo* models. Conversely also, *in vitro* studies allow a systematic study of individual cell species effects without the confounding effects of multiple cell types, mediators and third-body wear.

Our *in vitro* and *in vivo* models, have demonstrated that TiAlV, CpTi, CoCr and UHMWPE wear debris are stimulatory to different extents and precipitate the release of many biochemical mediators. These mediators appear to be released by monocytes, tissue macrophages and foreign-body giant cells in response to interaction with particulate debris. Bone resorption is stimulated by these biochemical mediators which can lead to osteolysis and aseptic loosening of total joint replacements.

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Discussion with Reviewers

T.W. Bauer: What was the rationale for measuring IL-1 and IL-6? Are there other cytokines that might be important (e.g., TNF-α)?

Authors: While TNF-α and PGE_2 are important mediators participating in the inflammatory cascade, in our previous studies we found that IL-1, IL-6 and PGE_2 were more sensitive to particle composition and thus elected to measure these mediators. Collagenase and gelatinase are also important enzymes and these were assayed in the canine study.

T.W. Bauer: The available figures do not convincingly demonstrate the suggested toxicity of Co-Cr particles. Have the authors attempted to quantify differences in necrosis in any way?

Authors: After harvesting interfacial tissues, a majority of the sample was placed in organ culture for analysis of biochemical mediators and a small sample was prepared for histological analysis. In these samples, due to the extensive tissue necrosis, it was difficult to get very clear histological sections and thus, we could not quantify the necrosis.

T.W. Bauer: Were the particles uniformly distributed in the gaps, or were they more concentrated distally? Did histologic changes correlate with particle concentration variations within each femur?

Authors: Intra-operatively the particles were mixed in clotted blood and placed on the porous coatings in the proximal aspect of the component. After 12 weeks post-operative, at the time of harvest, the particles appeared to have been distributed within the gap, along the distal stem and also in the joint capsule where they caused third body wear of the UHMWPE liner. Histologically, within the same section, there was a disparity in the concentrations of particles and a heightened cellular reaction was noted around areas with more particles.

T.W. Bauer: In **Results**, if IL-1 levels were not statistically significant, then they must not have been "very high."

Authors: Even though high levels of IL-1 were released, we only had 3 determinations for IL-1 and thus, statistical significance could not be determined.

T.W. Bauer: In Table 1, the gap only control seemed to produce more PGE_2 and much more gelatinase than the "no-gap" control, PE or Co-Cr group. Might motion be important?

Authors: We agree with the reviewer that implant motion might be an important determinant in levels of mediators released. In gap implants with the presence of wear debris, complete bone healing also did not occur in 12 weeks and an inflammatory milieu was maintained.