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ORIGINAL ARTICLE

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Enhanced cytokine production from human macrophages stimulated by polyethylene particles retrieved from interface membranes after failed total hip arthroplasty

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Abstract Little is known about the specific effects of the ultra-high molecular weight polyethylene (UHMWP) debris that is obtained from human interface membranes after revision for a failed total hip arthroplasty. This paper reports the effects of retrieved polyethylene particles on human monocyte/macrophages (M/M). Macrophages were prepared from normal human peripheral blood by the conventional Ficoll–Hypaque method. Polyethylene wear debris was obtained from human interface membranes and prepared by the method of papain digestion. Human M/M were dispensed at 1.0×10^6 cells/well in a 24-well culture plate. UHMWP and latex particles were added immediately after plating the cells and directly onto the cells (1×10^6 cells/well) at final particle concentrations of 100 μg/well, 200 μg/well, and 500 μg/well. At the end of 24 h incubation, the culture supernatant was removed and assayed for IL-1β, IL-6, and TNF-α activities by ELISA. Cellular morphology and architecture were studied using light and electron microscopy. Human M/M cultured with retrieved UHMWP particles caused significantly more IL-1β, IL-6, and TNF-α release than macrophages cultured with latex ($P < 0.05$). The addition of latex and polyethylene particles to human M/M resulted in a dose-dependent increase in IL-1β, IL-6, and TNF-α release. Electron microscopy revealed that 90% of the UHMWP particles were less than 1 μm diameter. The average particle size was approximately 0.7 μm diameter (range 0.1–15 μm). Human M/M exposed to PE particles demonstrated extensive filopodia formation as compared with the cells exposed to latex particles. In summary, we have demonstrated that polyethylene particles isolated from interfacial membranes obtained at revision surgery are potent stimulators of human M/M.

Key words Human monocyte/macrophages · Ultra-high molecular weight polyethylene (UHMWP) · Interface membrane tissue · Total hip arthroplasty (THA)

Introduction

It has become increasingly more apparent that aseptic failure of total hip arthroplasty (THA) is associated with the biological response to particulate wear debris.[1–7] It has been suggested that small wear particles are phagocytized by macrophages, fibroblasts, and foreign body giant cells, resulting in cell activation and the release of chemical mediators such as IL-1β, IL-6, and TNF-α. These substances have been implicated in osteoclast activation and bone resorption, and are likely to play a role in aseptic loosening.[8–12] Stimulation of a variety of cell types by synthetic particles has been reported by several investigators.[12–21] Rae[13] reported that cobalt–chrome alloy had a toxic effect on murine macrophages, while Horowitz et al.[17] reported that polymethylmethacrylate (PMMA) particles were toxic to macrophages, causing cell death. In a study of the response of macrophages in culture to zymosan, latex, and polyethylene, Murray and Rushton[16] demonstrated the potential for a differential cellular response to particulate material. Using metallic particles in the size range seen in failed arthroplasties, Maloney et al.[20] have shown that there is a differential response of fibroblasts in monolayer culture to titanium, cobalt, chromium, and titanium–aluminum alloy.

These studies and others suggest that fine particulate material has a direct effect on human cells.[12–21] However, these studies have been carried out using particles that were manufactured and not generated in vivo. In addition, most reports have focused on the metabolic response of cells in culture to metallic debris, in part because metallic particulates are relatively easy to manufacture and are readily available from commercial sources. The cellular response to ultra-high molecular weight polyethylene (UHMWP) particles is of even greater interest owing to the large quantities of UHMWP particles detected in interface membranes in

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vivo.[1–7] In the past, however, it has been difficult to evaluate the effect on cells of exposure to particulate polyethylene because of the difficulties in manufacturing polyethylene debris in the appropriate size range.

In this study, we were able to circumvent that problem by using polyethylene particles isolated from interface membranes harvested at the time of revision surgery of failed uncemented total hip arthroplasties. The purpose of the study was to evaluate the interaction of these retrieved particles with human macrophages in cell culture using light and electron microscopy, and to study their effects on human macrophage metabolism.

Materials and methods

Preparation of human polyethylene particles and latex particles

Tissue samples were taken from the implant–bone interface of 17 patients who were undergoing revision surgery for failed uncemented total hip arthroplasties; there were ten women and seven men. The mean age was 63 years (range 48–79 years). The average time between the initial arthroplasty and the revision was 56 months (range 27–74 months). The primary diagnosis that led to the first total hip arthroplasty was rheumatoid arthritis in all patients. All of the failed cementless femoral components were made of titanium alloy; nine were Harris–Galante (Zimmer, Warsaw, IN, USA), and eight were Omnifit stems (Osteonics, Allendale, NJ, USA). The indication for revision surgery was pain and radiographic evidence of loosening in all cases.

Papain digestion was carried out as previously reported.[6] The formalin-fixed membrane was cut into 5 × 5-mm sections and weighed. Papain suspension at a concentration of 7 μg/ml in 0.05 M sodium phosphate, pH 6.5, containing 0.02 M N-acetyl cysteine was added to the tissue samples at a ratio of enzyme to tissue of 47 μg : 1 g. The enzymatic hydrolysis was carried out at 65°C in 2 ml enzyme suspension for 12–24 h. If the sample was not completely hydrolyzed after 24 h, an additional aliquot of papain (10 μl) was added to the mixture. After the specimen was dissolved, the supernatant was removed and an equal volume of 95% ethanol was added. This mixture was then centrifuged at 1000 g for 10 min. UHMWP debris, distributed in the two layers, was washed and resuspended in ethanol. Any large floating PE particles were removed. After evaporation of ethanol, the small UHMWP debris remained. The size and shape of the particles were analyzed by a method which involved the use of scanning electron microscopy. Digests of particles were spread on graphite and gold-coated with a sputter-coater. At a magnification of 4000–5000 times, the size of the particle was measured to the nearest tenth of a micrometer. For each specimen, 100 particles were measured from a representative sample in each digest, and a mean particle size was calculated.

Latex particles, considered to be relatively inert, were used for comparison. Two sizes of latex particles were tested; 5 μm and 0.81 μm (University of Yamagata, Yamagata, Japan). UHMWP and latex particles were prepared in sterile polypropylene tubes and exposed to gamma-ray irradiation (2.2 Mrad ^{137}Cesium source, model 143; J.L. Shepperd Irradiator, San Fernando, CA, USA).

Cell separation

Mononuclear cells from 17 healthy volunteers were obtained from heparinized peripheral blood by the conventional Ficoll–Hypaque method.[22] The mononuclear cell population was enriched for human M/M by adherence to plastic . Approximately 90% of the adherent cells were monocytes, as detected by esterase staining.[23] These cells were cultured in RPMI 1640 and 10% fetal bovine serum (Gibco, Grand Island, NY, USA) supplemented with penicillin (50 μg/ml) and streptomycin (50 μg/ml) for 24 h, and then incubated at 37°C in 5% CO_2. The cells were harvested, washed with Gey's balanced salt solution to leave a thin film of fluid on the cells, counted, and dispensed at 1.0×10^6 cells/well in a 24-well culture plate (Costar, Cambridge, MA, USA).

To ensure intimate contact with the cells, UHMWP and latex particles were added immediately after plating the cells and directly onto the cells (1×10^6 cells/well) at final particle concentrations of 100 μg/well, 200 μg/well, and 500 μg/well. Cultures were grown in this minimal-medium "dryout" period for 3 h to induce contact between the PE and the cells. After incubation for 3 h, 500 μl serum-free Neuman–Tytell medium were added. 500 μl of medium was then added to each well every 6 h. Finally, the volume of each well was adjusted to 1 ml. After 24 h, the supernatants were recovered for assay. Seventeen separate human macrophage cultures were established, and each was then assayed twice for biochemical analysis. Negative control cultures were also exposed to a similar dryout period, but without the particles.

Microscopic analysis

Light microscopy was used to evaluate morphological changes in the M/M that resulted from particle exposure. The number of cells in a visual field of a phase contrast microscope were counted over a specific location of the dish every 6 h. After 24 h, cells were fixed in 2% glutaraldehyde and stained with Giemsa solution. Electron microscopy was also used to analyze the ultrastructural changes in M/M following exposure to particulate materials. Cells were fixed for 1 h at 4°C with glutaraldehyde (2–2.5%) in 0.067 M PBS (pH 7.4), washed with PBS, and fixed with osmium (1%) for 1 h at 4°C for electron microscopic analysis. Dehydration was done with alcohol (50%, 70%, 80%, 90%, 95%, 100%) for 10 min at each concentration. Cells were put into a mixture of Epon and ethanol (1 : 1) for 30 min, embedded in pure Epon for 12 h at 50°C, and finally embedded in pure Epon for 12 h at 60°C. Morphology of the human M/M was observed by a JEM 1200 EX electron microscope (JEM, Tokyo, Japan).

Assay for cytokine production

At the end of 24h incubation, the culture supernatant was removed and assayed for IL-1, IL-6, and TNF-α activities by an enzyme-linked immunosorbent assay (ELISA) (Quantikine, R&D Systems). Briefly, 200μl of the culture medium was added to 96-well microplates coated with mouse anti-human IL-1, IL-6, and TNF-α monoclonal antibodies (first antibody). After 2h incubation at room temperature (IL-1, IL-6), or at 37°C (TNF-α), the wells were washed, and 200μl horseradish peroxidase conjugated goat anti-human IL-1, IL-6, and TNF-α polyclonal antibodies were added. Following an additional 2h incubation at room temperature (IL-1, IL-6), or at 37°C (TNF-α), the wells were washed again and incubated at room temperature for 20min with chromogen (a mixture of H_2O_2 and tetramethylbenzidine). Then, 50μl $2NH_2SO_4$ was added to stop the reaction, and the optical density at 450nm was measured using a microtiter reader. Each assay was performed twice. Statistical analysis was performed using Student's *t*-test, and $P < 0.05$ was considered to be statistically significant.

Results

Particle characterization

Scanning electron microscopy (SEM) showed that 90% of the UHMWP particles were less than 1μm diameter. The average particle size was approximately 0.7μm (range 0.1–15μm). The small PE particles appeared spherical or globular (Fig. 1A). Characterization of the latex particles revealed that they were round, with sizes of 0.81μm and 5μm, confirming the specifications given by the manufacturer (Fig. 1B).

Microscopic analysis

When looking at the numbers of cells on the dishes, the latex and UHMWP dishes showed no differences in the number of cells compared with the control. This suggests that there was no release of a cytotoxic substance from the material. Approximately 90% of the latex particles were phagocytosed by human M/M. The majority of polyethylene particles were also phagocytized by the macrophages, but because of their irregular shape, the percentage phagocytized was difficult to quantify.

Electron microscopic analysis

Electron microscopy revealed that most of the polyethylene particles were phagocytized as several clumps of PE particles. This suggests that clumps of PE particles may have been added to the cells rather than discrete particles. In contrast, latex particles were phagocytized as discrete particles.

The numbers of vesicles, lysosomes, and rough endoplasmic reticulum (RER) were not significantly different between the human M/M exposed to latex particles and those cells without the particles. In contrast, human M/M exposed to PE particles demonstrated extensive filopodia formation (Fig. 2A) as compared with the cells exposed to latex particles (Fig. 2B). Mitoses of macrophages were not observed in this experimental period.

Analysis of chemical mediators

Effect of UHMWP particles and latex on IL-1β, IL-6, and TNF-α released by human M/M

Human M/M exposed to retrieved polyethylene particles at all three concentrations resulted in a significant increase in

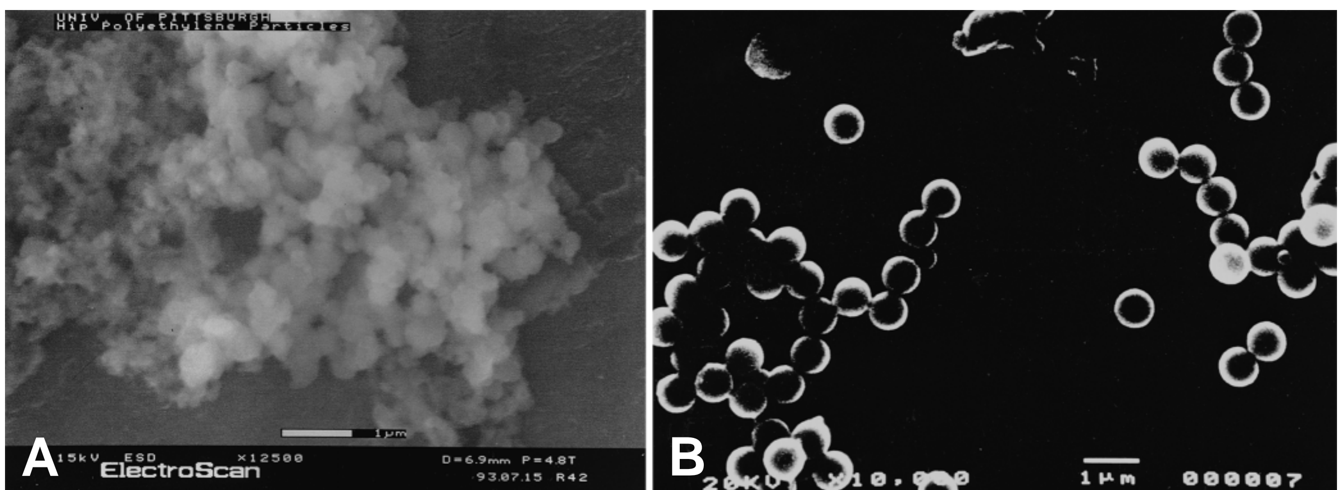


Fig. 1. Scanning electron micrograph of retrieved and synthetic particles. **A** Retrieved ultra-high molecular weight polyethylene (UHMWP) particles. **B** Synthetic latex particles

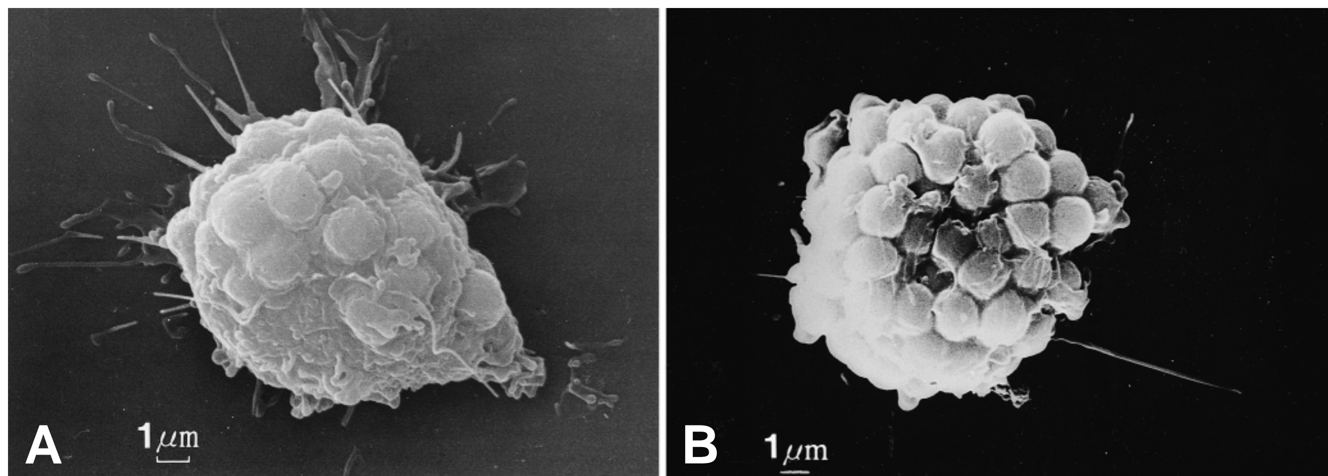


Fig. 2. **A** Electron micrograph of the cultured macrophage 6 h after incubation with UHMWP particles. UHMWP particles were found to have been phagocytosed by the macrophage. Extensive filopodia can be observed in the macrophage. **B** Electron micrograph of the cultured macrophage 6 h after exposure to latex particles (0.81 μm). Latex particles were also found to have been phagocytosed by the macrophage

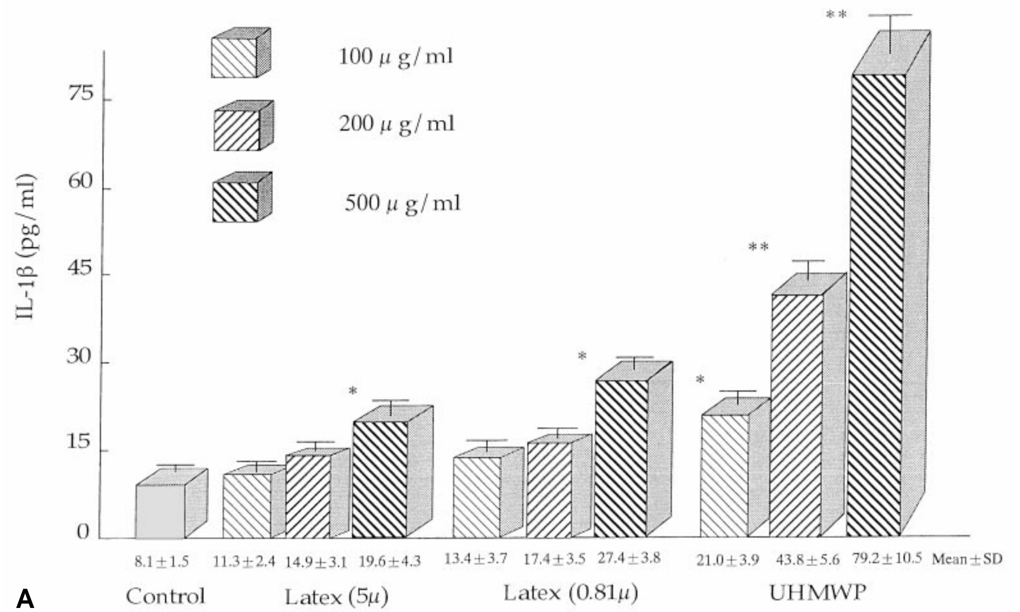
the levels of IL-1β, IL-6, and TNF-α in the culture medium compared with negative controls ($P < 0.05$) (Fig. 3). This response was dose-dependent. When compared with latex particles, exposure to UHMWP particles also led to a significant elevation of cytokine release at the two higher concentrations (200 μg/ml and 500 μg/ml). At the highest concentration of retrieved polyethylene particles (500 μg/ml), there was a greater than 10-fold increase in TNF-α release compared with negative controls. The effects of particles of different composition on TNF-α release from human M/M were qualitatively similar to the effects on IL-1 levels, although the concentration of IL-1 was much lower than that of TNF-α. In addition, both IL-1 and TNF-α were increased approximately 3-fold in a similar concentration of latex (0.81 μm). Retrieved polyethylene particles stimulated mononuclear cells in vitro, and at the highest particle concentration demonstrated a 5-fold increase in IL-6 production compared with controls. A similar concentration of latex particles (0.81 μm) produced a two-fold increase in IL-6 compared with controls. The level of cytokines in the medium after exposure to small (0.81 μm) latex particles was slightly higher than those in human M/M cultured with larger latex particles (5 μm). These differences were not statistically significant. Exposure of both sizes of latex particles resulted in a significant elevation of IL-1, IL-6, and TNF-α compared with negative controls.

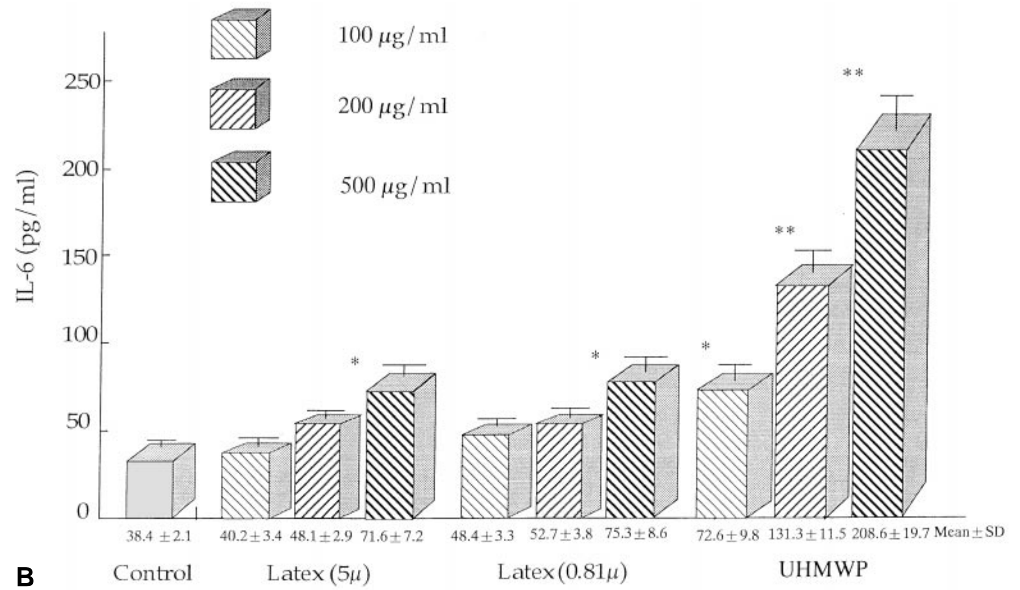
Discussion

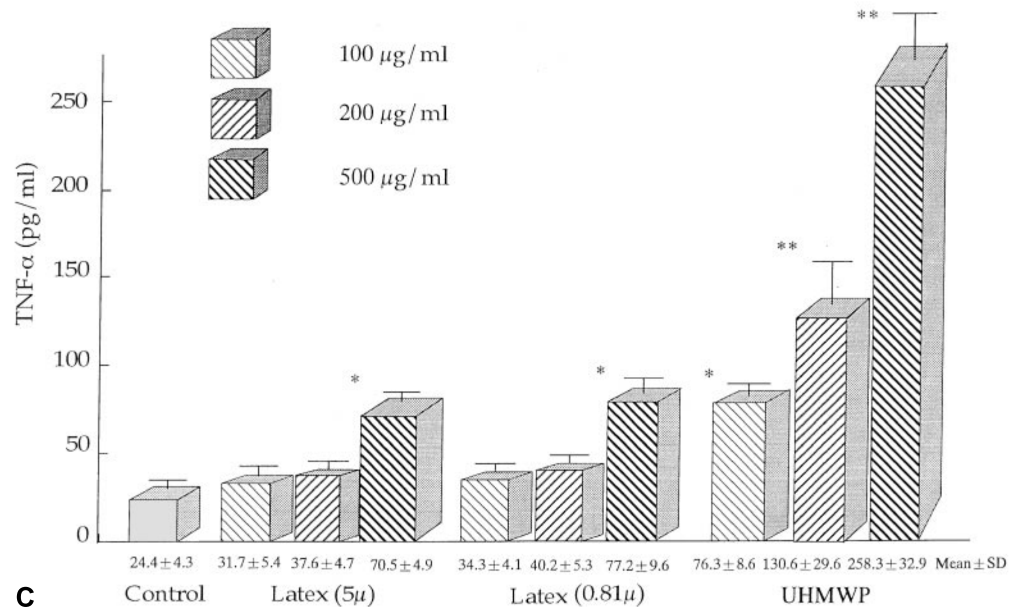
Aseptic loosening and osteolysis remain the most important long-term problems in total hip arthroplasty. Early work on the biochemistry of the soft-tissue membrane that develops between implant and bone during loosening demonstrated that this tissue was capable of producing prostaglandin E2 and collagenase.[1] Histologically, it has been shown that the "loosening membrane" had large amounts of polyethylene debris and a nonspecific chronic inflammatory reaction, including macrophage infiltration and a variable number of giant cells and fibroblasts. More recently, immunohistochemical studies have been done to identify the cell types in the interfacial tissue.[2–4] These studies have shown that the predominant cell type present in the interfacial membrane is the macrophage. Newer techniques, including in situ hybridization, have shown that the tissue between implant and bone in aseptic loosening and osteolysis produces a variety of cytokines, including IL-1, IL-6, and TNF-α.[2–4] These are substances of particular interest because of their potential role in pathologic bone resorption and fibrosis. Although there is controversy over the role of macrophages and their ability to resorb bone directly, they secrete a variety of substances that can either stimulate or inhibit bone resorption by modulating osteoclast activity. Cytokines such as IL-1β, IL-6, and TNF-α have been identified as mediators capable of modulating osteoclast activity.[8–11] IL-1 and TNF-α both stimulate osteoclastic bone resorption and induce IL-1, IL-6, and TNF-α. Thus, IL-1 and TNF-α may contribute directly as well as indirectly, to osteoclastic bone resorption through the induction of IL-6 production by macrophages.

Polyethylene debris, as a potential stimulus for cell activation, is of interest because of its ubiquitous nature. Analyses of membranes from failed, cementless total hip arthroplasties have shown that polyethylene particles are present in large numbers.[1–6] In vitro wear studies have estimated that millions of polyethylene particles may be generated with each gait cycle.[7] Since polyethylene particles float and aggregate in any culture fluid, a suspension is difficult to prepare and process. In addition, it is difficult to control the mixing concentration and the size of the particles. Chang et al.[24] embedded the polyethylene particles and macrophages in agarose gel so that the particles would not float. Horowitz and Gonzales[25] developed a culture method by combining

Fig. 3. IL-1β (**A**), IL-6 (**B**), and TNF-α (**C**) production from human macrophages activated by various particles. The results are represented as the mean value and standard deviation (mean ± SD). * $P < 0.05$ (versus control); ** $P < 0.05$ (versus latex)







two Petri dishes. The macrophages were allowed to adhere to the bottom of a small Petri dish which was then floated in a large Petri dish containing a polyethylene particle suspension. Because latex particles are considered to be relatively inert to cells, we chose them for comparison.

In this study, we avoided the problem of manufacturing submicron PE particles by examining the effect of retrieved polyethylene particles on the most common cell types found in the granulomatous tissue around failed hip implants. However, there are some limitations to this study. Latex particles are phagocytized as discrete particles, whereas retrieved PE particles are phagocytized as clumps. Further studies are necessary to determine if the method of PE particle internalization affects the cellular response.

Macrophages were stimulated to secrete IL-1, IL-6, and TNF-α when challenged with both latex particles and retrieved polyethylene particles in a dose-dependent manner. In the macrophage cultures, the two highest concentrations of polyethylene (200 μg/ml, 500 μg/ml) were more stimulatory than the highest concentration of latex particles (500 μg/ml). At the highest concentration of polyethylene particles (500 μg/ml), there was a greater than 10-fold increase in TNF-α release compared with negative controls. In contrast, there was only a greater than 5-fold increase in IL-6 release compared with negative controls. The reason for this is unknown.

Interestingly, macrophages that phagocytized small (0.81 μm) latex particles released slightly higher IL-1, IL-6, and TNF-α than macrophages that phagocytized larger (5 μm) latex particles, although the difference is slight and not statistically significant. Since the morphology of these particles was similar, the reported differences may be due to particle size, total surface area, or mode of particle internalization. Particles greater than 0.5 μm in size are internalized through "phagocytosis", while particles much smaller than this are internalized through "pinocytosis".[26] The mechanism by which cells internalize particles may be an important determinant of particle effect in terms of induction of protein synthesis and cellular metabolism. The total surface area of the small latex particles (0.81 μm) was greater than that of the larger latex particles (5 μm). Increasing the surface area appears to stimulate increased enzyme release.[27,28]

Macrophages that phagocytized UHMWP paticles obtained from human interface membranes released three times as much IL-1β and TNF-α as macrophages that phagocytized latex. This may be due to the different elemental compositions of polyethylene and latex, or to the differences in size, shape, total surface area, oxide film, and protein coating. It seems likely that a protein coating may be an important factor. Polyethylene particles extracted from tissue obtained during revision surgery might be limited to particles with proteins or other biological substances adhering to them even after repeated strict sterilization and washing, and there is a risk of denaturation of the polyethylene. This means that while it is possible to reproduce the shape and size of the polyethylene particles, the effects of other factors on the experiment are unpredictable.

The microscopic analysis performed in this study shows that macrophages exposed to polyethylene particles undergo a morphological change consistent with cell activation. This change includes the formation of pseudopodia. An increase in the RER may be an important finding, as this is the site of synthesis of proteins such as IL-1β, IL-6, and TNF-α.

In summary, we have demonstrated that polyethylene particles isolated from interfacial membranes obtained at revision surgery are potent stimulators of macrophages. This stimulation was dose-dependent. Although the potential for an activated macrophage to directly resorb bone remains controversial, the cytokines measured in this study are capable of inducing osteoclast activation, thus leading to bone resorption.[8–11,29] These data support previous work postulating the importance of polyethylene wear particles in aseptic loosening.

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