A comparison of the inflammatory potential of particulates derived from two composite materials

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In order to develop total joint prostheses with moduli of elasticity close to bone while retaining excellent strength characteristics, composite materials are being developed. Composites consist of graphite fibers embedded in a polymer matrix. We studied the inflammatory potential of particulates derived from two composites with different matrix components, polysulfone (PFS) and polyetherketoneketone (PEKK), in the rat subcutaneous air pouch model. Neat components of the composites were studied separately in the air pouch. Particulates also were studied in culture using the macrophage cell line RAW 264.7, adherent synovial cells (ASC), and human polymorphonuclear neutrophils (PMNs). Particles derived from the PEKK-containing composite material consistently were less inflammatory than the PFS composite-derived particles, as measured by PMN infiltration, neutral metalloprotease activity, tumor necrosis factor (TNF) activity, and prostaglandin E_2 (PGE_2) accumulation. Results from the neat materials confirmed the findings in the composite-derived material. PEKK composite-derived material produced less TNF from macrophage cultures, but there were no significant differences noted in PGE_2 production from ASC or in superoxide anion generation from PMNs. Particles from both PSF and PEKK produced minimal inflammatory responses in the rat subcutaneous air pouch. PEKK elicited a response virtually the same as the saline control and significantly less than that produced by particles of PSF.

INTRODUCTION

The advent of total joint arthroplasty has revolutionized the treatment of many patients with endstage arthritis. Over 30 years ago, Charnley and Kettlewel[1] engineered what has become the modern arthroplasty using metal and plastic components attached to bone with acrylic cement. Since then many refinements in technique, design, and materials have taken place that have improved the function and prolonged the survival of total joint arthroplasties. Now more than 500,000 total joint arthroplasties are performed each year.

A composite material is one that has been formed by combining two or more materials so that their performance together is significantly better than either component alone.[2] Polymer composites, usually using carbon fibers embedded in a polymer matrix, offer many advantages over the current alloys used for prostheses. They have excellent corrosive resistance, they elicit minimal inflammatory responses in bulk form, and they are radiolucent. In addition, the strength and modulus of the prosthesis can be controlled by changing the relative fiber content and orientation. Thus polymer-containing composites can be made with a modulus closer to bone, causing minimal stress shielding and promoting bone growth and prosthesis stability.

Several polymers have been or are being studied as possible matrix components. Polysulfone (PSF) has been considered and presently is in use. Newer polymers, polyetherketoneketone (PEKK) and polyetheretherketone (PEEK), have shown promising properties as well and are in the experimental stage. Biocompatibility studies of matrix components are important. Studies of bulk material have been performed and, in general, PSF and PEKK are well tolerated.[3] Few data are available regarding the effect of particulate forms of PSF or PEKK. Since wear is inevitable in the setting

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of total joint arthroplasty, knowledge of the compatibility of wear particles is essential to being able to predict the ultimate success of joint implants.

We compared in the rat subcutaneous air-pouch model of the periprosthetic space the relative inflammatory potential of particles derived from composites of graphite fibers embedded in PSF and particles of PEKK-containing composites. *In vitro* studies with monocyte/macrophage, polymorphonuclear cells, and human rheumatoid synovial cells were performed to validate our results. In addition to particles derived from composite materials, we also compared particles derived from pure, or neat, materials, i.e. carbon fibers, PSF, and PEKK.

MATERIALS AND METHODS

Composite-derived particulate production

Wear debris was produced by mechanically grinding the composite material with one of two methods that resulted in rough-surfaced, irregularly shaped particles. A high-speed steel rotary cutter (MSC Industrial Supply Co., model 0439216) was used at slow speed (under 300 rpm) to grind composites for the production of mechanical particles under twenty microns in greatest dimension. A Sears Craftsman hobby rasp (Sears model 931286) and ten-inch flat file (Sears model 931257) were used to generate wear particles between fifty and 350 microns in greatest dimension. Before final filtering to obtain the desired size range, as described below, the particles were ground to even smaller diameters in a porcelain mortar and pestle in a suspension of deionized water while being exposed to a magnetic stir bar (Fischer Scientific).

Neat particulate production

Wear debris was produced by grinding the neat material by hand using a glass mortar and pestle in a suspension of deionized water.

Particle sizing

Using a series of nylon and polyethylene meshes of varying sizes fit to modified plastic Buchner funnels,

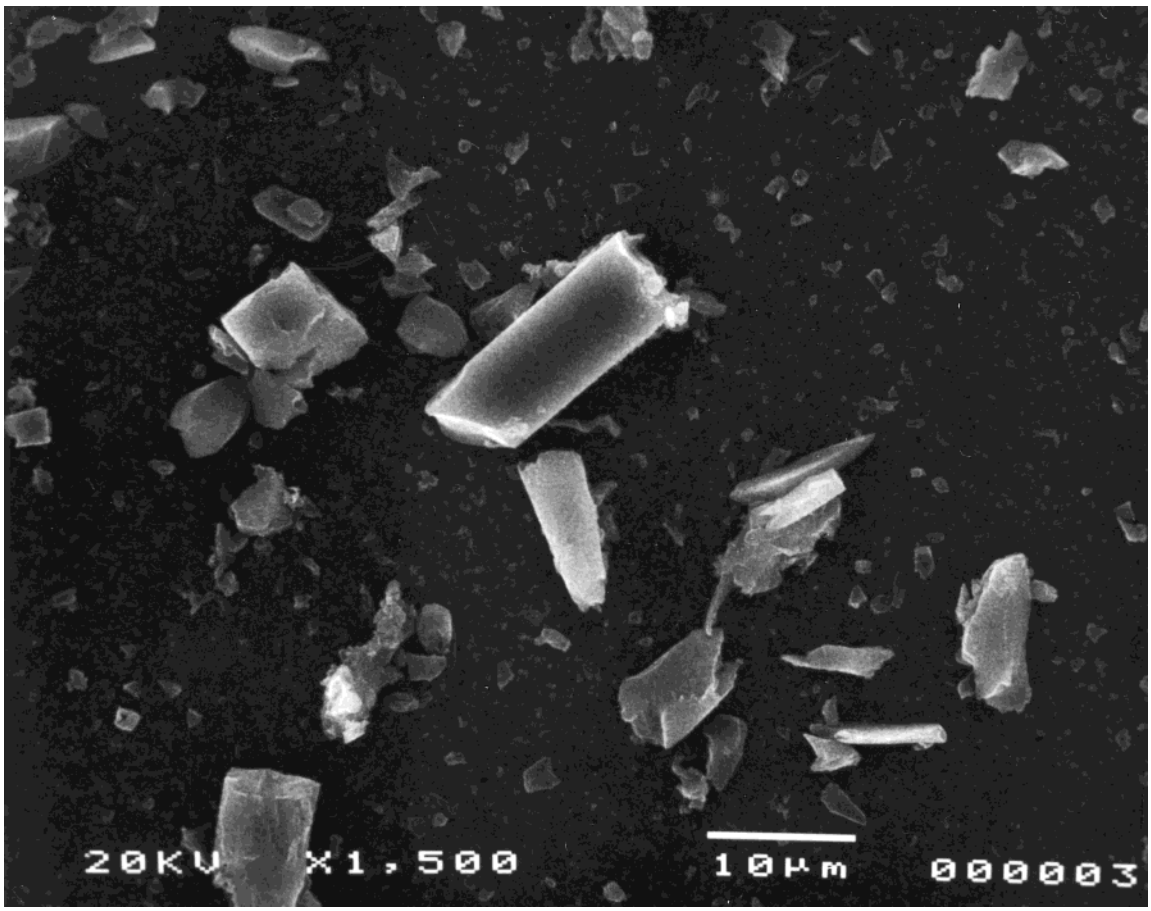


Figure 1. Scanning electronmicrographs of particles derived from PSF composite (original magnification ×1500). The rod-shaped structures are the graphite (carbon) fibers and the irregularly shaped particles are the matrix components.

the mechanical wear debris were filtered to the appropriate size range of less than twenty microns (Spectra mesh, Fischer Scientific, Malvern, PA).

Particles were dried using a Fischer microfiltration system and a 0.22 μm filter (Fischer Scientific, Malvern, PA) and then placing in a dessication chamber for 3 days. The particles were counted and then suspended in the appropriate amount of sterile saline (0.9% NaCl) to achieve the desired concentration of 10^8 particles/mL. Once the particles were suspended in the saline, the suspension was analyzed using the scanning electron microscope (Jeol JSM-T330A) for characterization of the particle morphology and size. Absence of particle contamination by metals or silicon was confirmed by electron dispersive X-ray analysis (EDXA) using a Kevex instrument (Kevex International Corp., Foster City, CA). Polaroid photographs of the particles were analyzed using computerized image analysis (Apple Computers Inc, Cupertino, CA) to determine the average aspect ratio (maximum/minimum dimensions) of the mechanically produced particles. The particles were sterilized by gamma irradiation (2.5 Mrad). Five mL of each of the various suspensions of particles in saline were drawn into ten-mL syringes using large (sixteen- or eighteen-gauge) needles in preparation for injection into the rat subcutaneous air pouch.

Particles were sterilized by gamma irradiation (2.5 Mrad). The limulus test was used to ensure the absence of endotoxin from the sterilized particle suspensions. All particles and filtrates were found to be endotoxin free. All particles were found to be free of metallic debris by EDXA (electron dispersive X-ray analysis).

Rat subcutaneous air pouch procedure

NIH guidelines for the care and use of laboratory animals (NIH publication #85-23, Rev. 1985) were observed. Air pouches were prepared according to the method of Edwards et al. Male Sprague-Dawley rats (175–200 g) were anesthetized using a ketamine/xylazine intramuscular injection. Rats were then shaved and prepped with alcohol over the dorsal skin. A subcutaneous injection of twenty mL of air was made using a twenty-five gauge needle attached to a

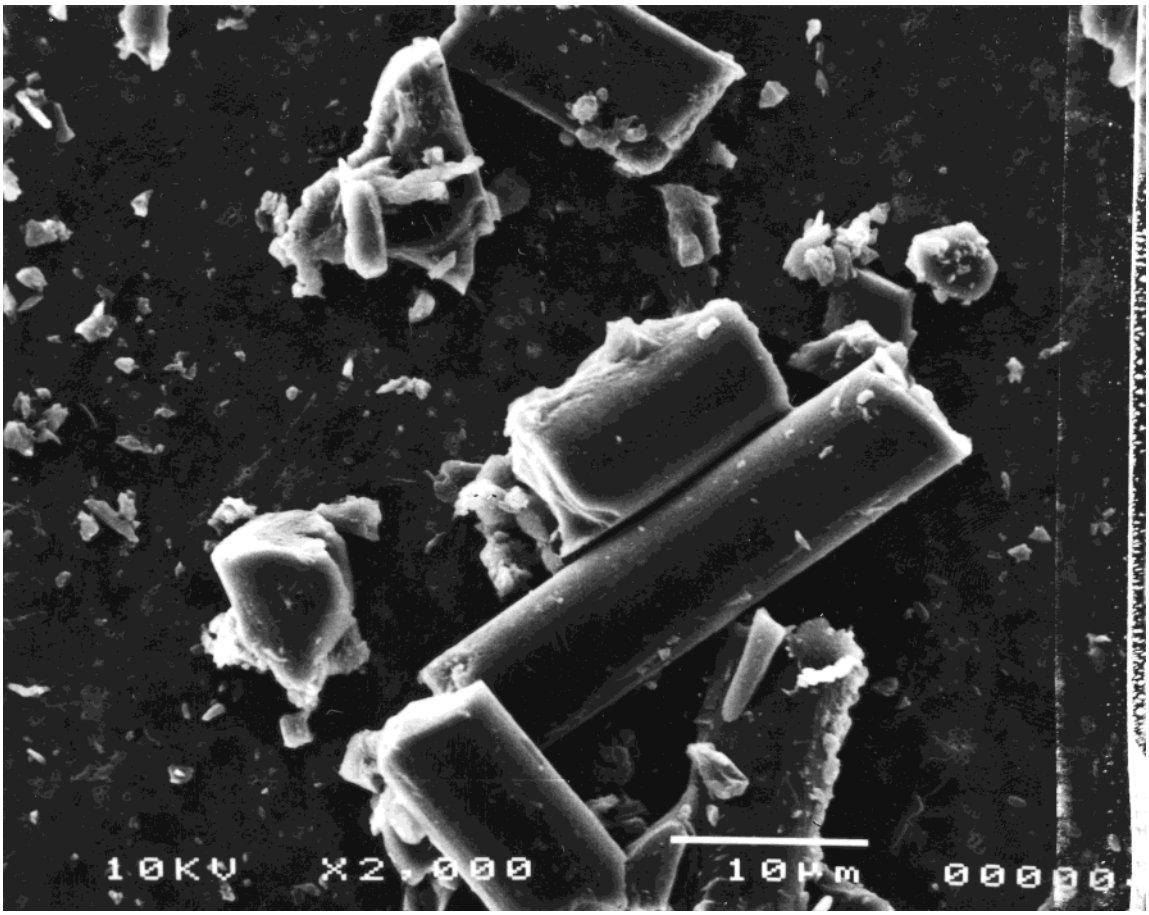


Figure 2. Scanning electronmicrographs of particles derived from PEKK composite (original magnification ×2000). The matrix particles in this photograph of the composites appear to have very similar morphologies to those shown in Figure 1.

0.22 μm filter and a twenty mL syringe. After 4 days the pouches were reinjected with ten cc of air to keep them inflated. On day 6 (2 days after reinflation), 5 cc of particulate suspension, filtrate, or saline were injected using eighteen- or twenty-gauge angiocaths. At various time points following the injection (1, 6, 12, 24, or 72 h) the pouch was irrigated with 5 mL of sterile saline, and the fluid was withdrawn for analysis. Leukocyte counts (WBC) were performed on fresh aspirates. The remaining fluid was centrifuged and the resultant supernatant stored at −70°C for later assays: tumor necrosis factor alpha (TNFα), matrix metalloprotease (MMP), and prostaglandin E_2 (PGE_2).

Rats were sacrificed following the pouch aspiration

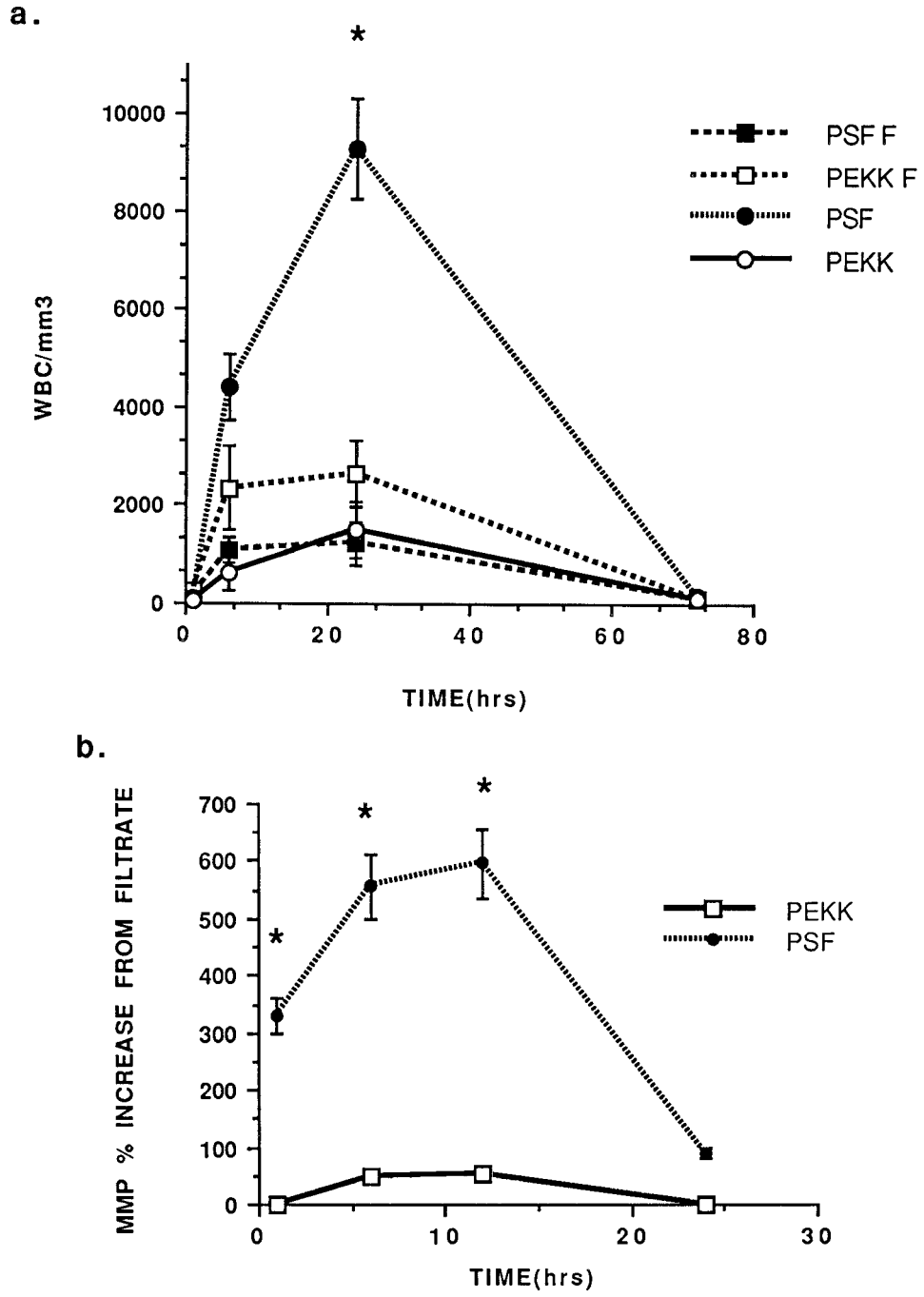


Figure 3. Results of injection into the rat subcutaneous air pouch of particles derived from composite materials: (a) leukocyte counts; (b) matrix metalloprotease activity; (c) prostaglandin E_2 levels; (d) tumor necrosis factor activity. PSF F indicates PSF filtrate PEKK F indicates PEKK filtrate and PSF and PEKK indicate particulates derived from respective composites. Values = ±S.E.M., $n = 6$. *signifies values significantly different from all other values, $p \leq 0.01$.

in a standard carbon dioxide chamber. The entire subcutaneous pouch was harvested using a longitudinal incision over the dorsum and sharp dissection with an iris scissor. Pouch tissue was preserved in formalin for histology (light microscopy).

Assays

Leukocytes (WBC) were counted using a standard counting chamber. Aliquots were smeared on a glass slice and stained with Wright's stain and examined

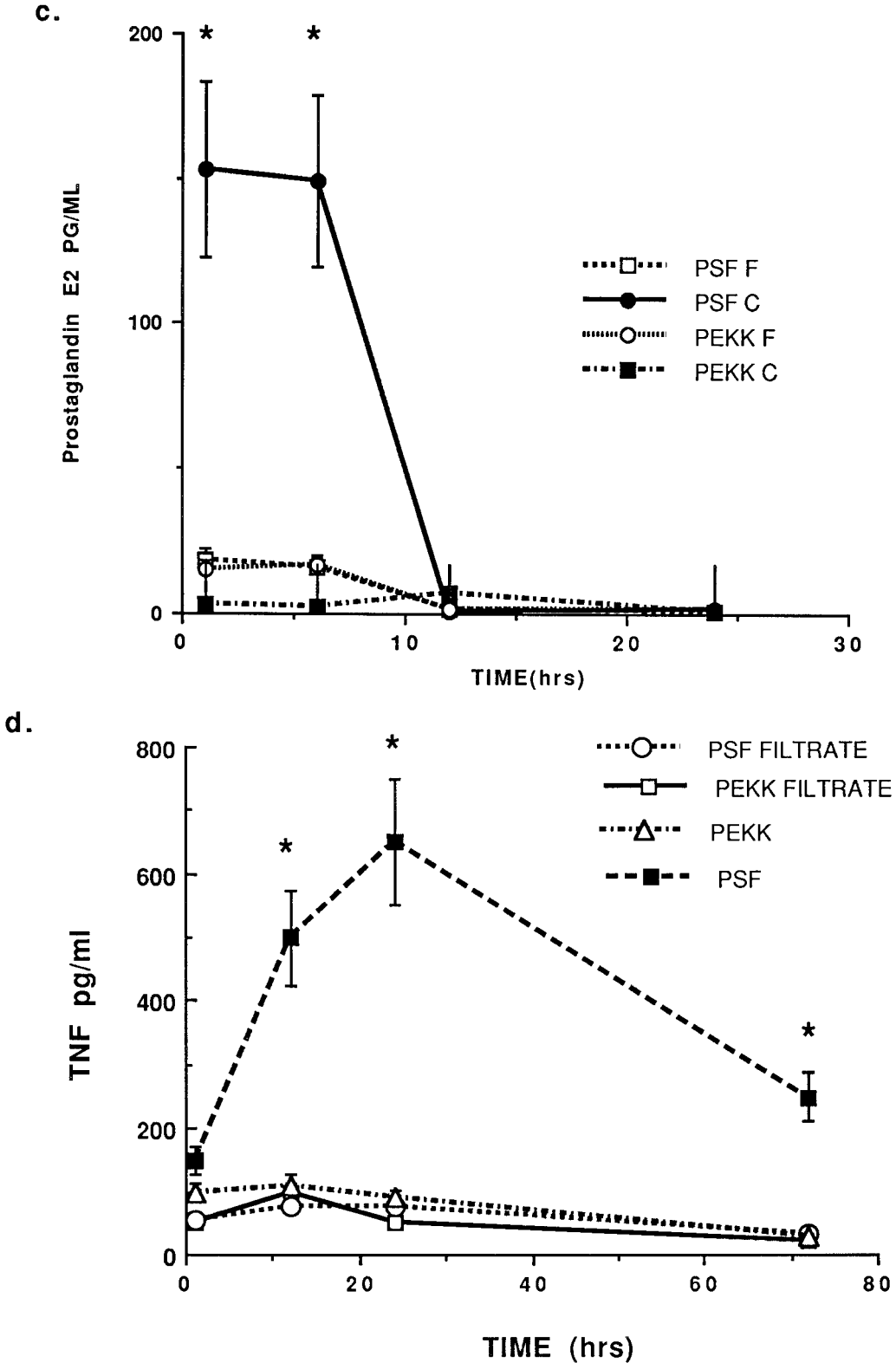


Figure 3. *Continued.*

microscopically for leukocyte differential when feasible. PGE_2 levels were determined using a PGE_2-specific monoclonal antibody with an enzyme-linked immunosorbent assay (ELISA). Matrix metalloprotease (MMP) levels were determined by a fluorescent assay using Suc-GPLGP-MCA as the substrate.[4] TNFα activity was measured by a described cell lytic assay.[5] Briefly, WEHI-164 CI.3F cells were grown in 96-well flat-bottomed trays (Flow Laboratories, Inc.) with 3500 cells in 50 mL per well. Plates were incubated at 37°C with 5% CO_2 for 24 h, 20 mL MTT stock per well was added and incubated 4 h at 37°C with 5% CO_2. One hundred ml of 10% SDS in 0.01 M HCL per well was added and incubated overnight at 37°C with 5% CO_2. On the third day cells were read using a plate reader with a wavelength of 595 nm. Wells containing cells and medium without any added samples gave the maximum absorbance. Cell lytic assays were used because no antibodies to rat TNFα were available and cross reactivity with mouse did not provide adequate sensitivity.

Macrophage cultures

The transformed rat monocyte/macrophage cell line RAW 264.7 was used to study particulate cell interactions. RAW 264.7 cells were grown in Dulbecco's modified eagles medium (DMEM) with 10% fetal calf serum (FCS) in 24-well culture dishes at a concentration of 10^6 cells/well. Particles were added and incubated at a concentration of 10^7 particles per mL, with 400 μl of medium added to each well. Incubations were carried out for 24 h, at which time the supernatants were centrifuged and assayed for TNFα.

Synovial cell cultures

ASC were obtained as described previously.[9] Fresh samples of synovium were obtained at synovectomy from patients with clinically defined rheumatoid arthritis or osteoarthritis. The superficial layer of synovium was washed with calcium- and magnesium-free phosphate-buffered saline (PBS). This layer then was dissected and serially exposed to both collagenase and trypsin digestion to release the cells. The resulting cell suspension was washed vigorously and plated in Dulbecco's modified eagles medium (DMEM) with 10% fetal calf serum (FCS) in plastic petri dishes. After overnight incubation, the floating cell population was removed and the adherent cells were vigorously washed with PBS and then cultured in DMEM 10% FCS.

PMN isolation

Separation of cells was performed from blood obtained from healthy donors. After ficoll–hypaque density centrifugation, the mononuclear cells were removed, the remaining cells sedimented using 6% dextran, and hypotonic lysis of the remaining RBC then was performed. After being washed two times, the cells were counted and cell viability was determined by trypan blue dye exclusion.

Superoxide assay

Superoxide anion production was measured by the reduction of cytochrome C. PMN were suspended in Hanks balanced salt solution (HBSS) at a concentration of 3×10^6 cells/mL. Cytochrome C was added at a final concentration of 1.2 mg/mL in the presence of 0.5 μg/mL cytochalasin B. After the experimental conditions were met, the final volume was brought to 1 mL. After incubation at room temperature for 10 min, the reaction was stopped by placing the tubes on ice

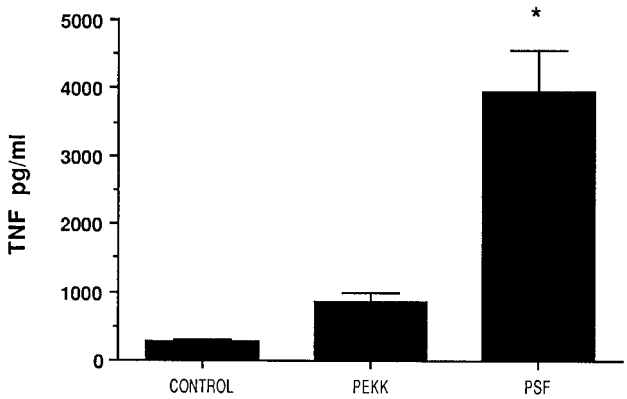


Figure 4. Tumor necrosis factor activity from supernatant of RAW 264.7 cells exposed to particulates derived from the composite materials. PSF-derived particles result in significantly more TNF activity. Values are ± S.E.M., $n = 6$. *signifies values significantly different from controls and PEKK, $p \leq 0.01$.

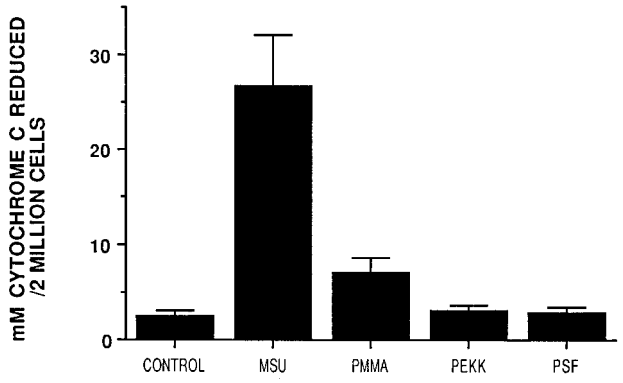


Figure 5. Superoxide anion production by polymorphonuclear cells exposed to a variety of particles. MSU indicates monosodium urate, PMMA indicates polymethylmethacrylate, PSF indicates polysulfone, and PEKK indicates polyetherketoneketone. Neither PSF composite-derived nor PEKK composite-derived particles generated significant amounts of superoxide anion. Values are ± S.E.M., $n = 6$.

and then centrifuging. The supernatants were assayed for cytochrome C reduction at 550 nm in a Beckman spectrophotometer. Results are expressed as moles of reduced cytochrome C per 2×10^6 cells.

RESULTS

Figures 1 and 2 show scanning electronmicrographs of particles produced from the composite materials. The rod-like particles are the carbon graphite fibers. Image analysis measurements of the particles derived from the two different composites show them to have an equal size distribution (data not shown). The size ranges of both are very similar, with the majority of the particulates in the 5 micron range. The matrix particles, i.e. the PEKK and the PSF, are visually indistinguishable. Manufacturer's specifications state that the per cent matrix in both the PSF and PEKK composite is approximately 60%.

Composite materials

These materials contained a mixture of matrix component (either PSF or PEKK) and carbon graphite particles. AS shown in Figure 3, particles derived from composites containing PSF as the matrix component produced significantly more inflammation as measured by leukocyte influx [Fig. 3(a)], PGE_2 production [Fig. 3(b)], neutral metalloprotease activity [Fig. 3(c)], and TNF production [Fig. 3(d)]. Please note that in this series of experiments, filtrates (saline exposed to particles) of each polymer were used as controls. In fact, MMP levels are expressed as per cent increase over filtrate. PEKK composite is almost identical to filtrates alone. Also note that although PSF composite-derived particles are more inflammatory than PEKK composite-derived particles, they still produce little inflammation compared to other particles, such as polyethylene or monosodium urate (MSU) (data not shown).

Figure 4 shows the results of *in vitro* exposure of particulates derived from PEKK and PSF composites to the murine macrophage cell line RAW 264.7. These

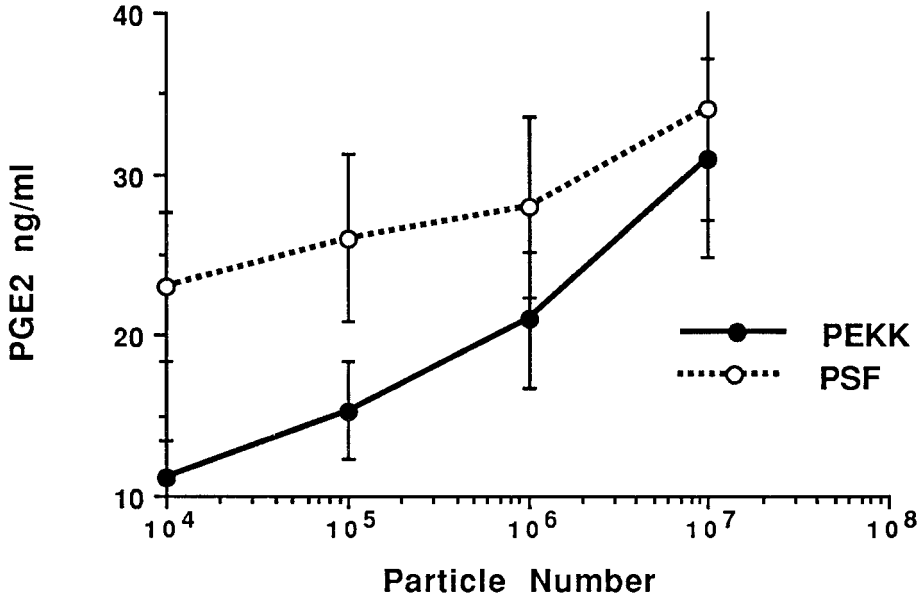


Figure 6. Prostaglandin E_2 production by adherent synovial cells exposed to particulates derived from the composite materials. Both particles produced significant amounts of PGE_2. Although at each point PSF resulted in more PGE_2, there was no significant difference between the particles at any time point. Values are ± S.E.M., $n = 6$.

cells produced TNFα in response to a variety of stimuli and in general responded in the same fashion as human monocytes. PSF composite-derived particles stimulated greater amounts of TNFα as compared to PEKK-derived particles of the same size. Our results reinforce the *in vivo* studies in the air pouch.

In addition to exposing PSF and PEKK composite to macrophages in culture, we incubated both with human polymorphonuclear particles (PMNs). As a measure of activation we measured the release of superoxide anions after 30 min of exposure. As shown in Figure 5, there was no significant release of anion in response to particulates derived from either PSF or PEKK composites whereas MSU crystals produced large amounts of superoxide anions.

Figure 6 shows PGE levels obtained when the composites were exposed to human adherent synovial cells in culture. The baseline levels of PGE_2 were high

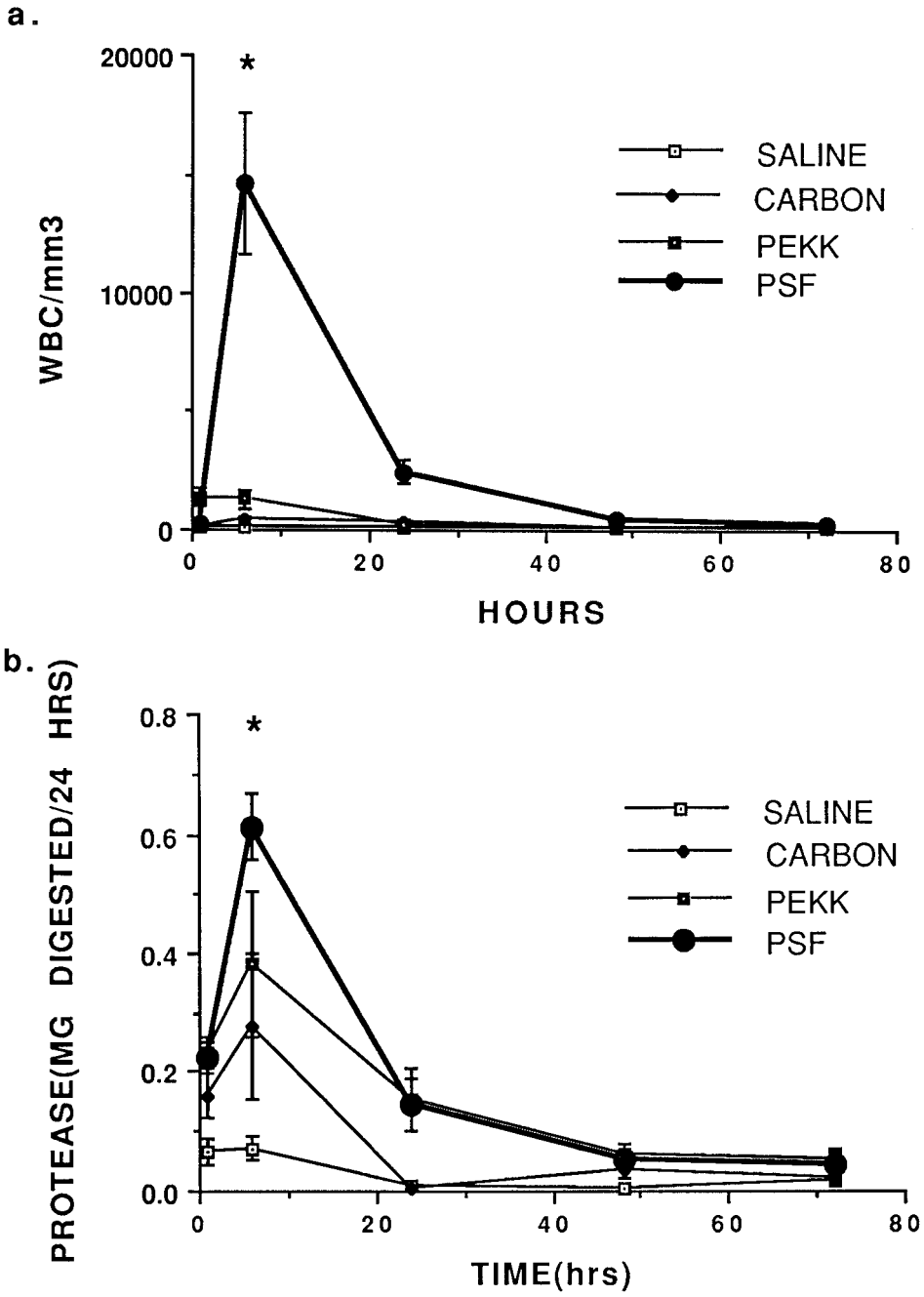
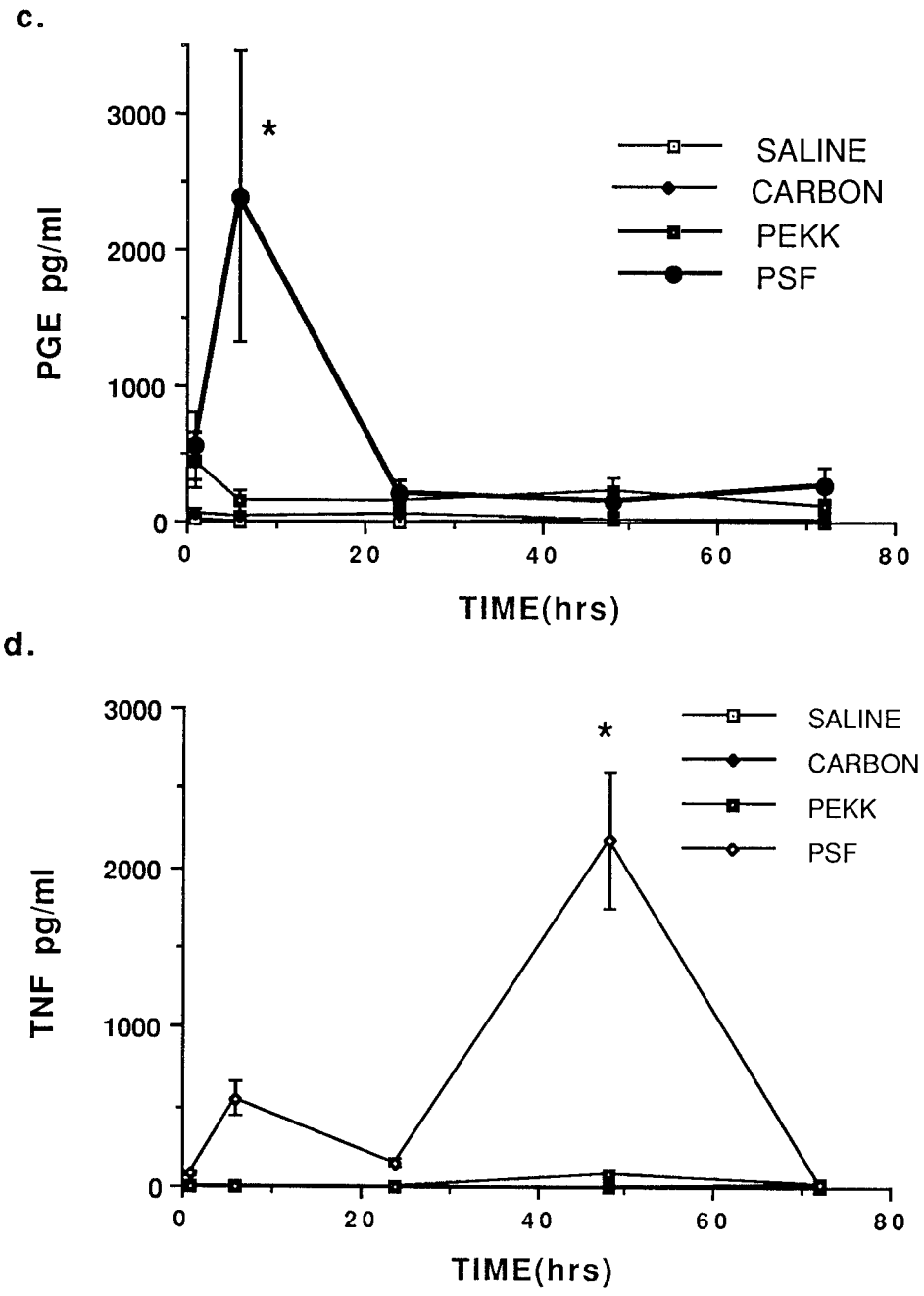


Figure 7. Results of injection of particles derived from neat materials into the rat subcutaneous air pouch: (a) leukocyte counts; (b) neutral metalloprotease activity; (c) prostaglandin E_2 levels; (d) tumor necrosis factor activity. These studies were performed with particles derived from pure materials, not from composites. Saline indicates pouches injected with saline alone and used as controls. In each mediator measured, PSF particles show significant inflammatory responses compared to PEKK and carbon particles. Values = ±S.E.M., $n = 6$. * signifies values significantly different from all other values, $p \leq 0.01$.

in these cultures. Although there was a consistent trend suggesting a difference between the two materials, there is no significant difference at any time point. Both stimulated PGE_2 over control levels.

Neat materials

These studies were performed with pure materials so that each preparation contained either only PEKK, PSF, or carbon graphite particles. Results are shown in Figures 7(a–d). Figure 7(a) shows the results of the leukocyte influx into the pouch, and consistent with findings using composite-derived particles, the PSF particles produced significantly more leukocyte influx at the 6- and 24-h time points [Fig. 7(a)]. Matrix metalloprotease activity [Fig. 7(b)], PGE_2 levels [Fig. 7(c)], and TNFα activity [Fig. 7(d)] were all greater in the pouches injected with particles of PSF. Please note that the values for each of the mediators in the PSF group tended to be greater with the neat materials since the actual number of PSF particles was greater, i.e., there were no carbon particles.



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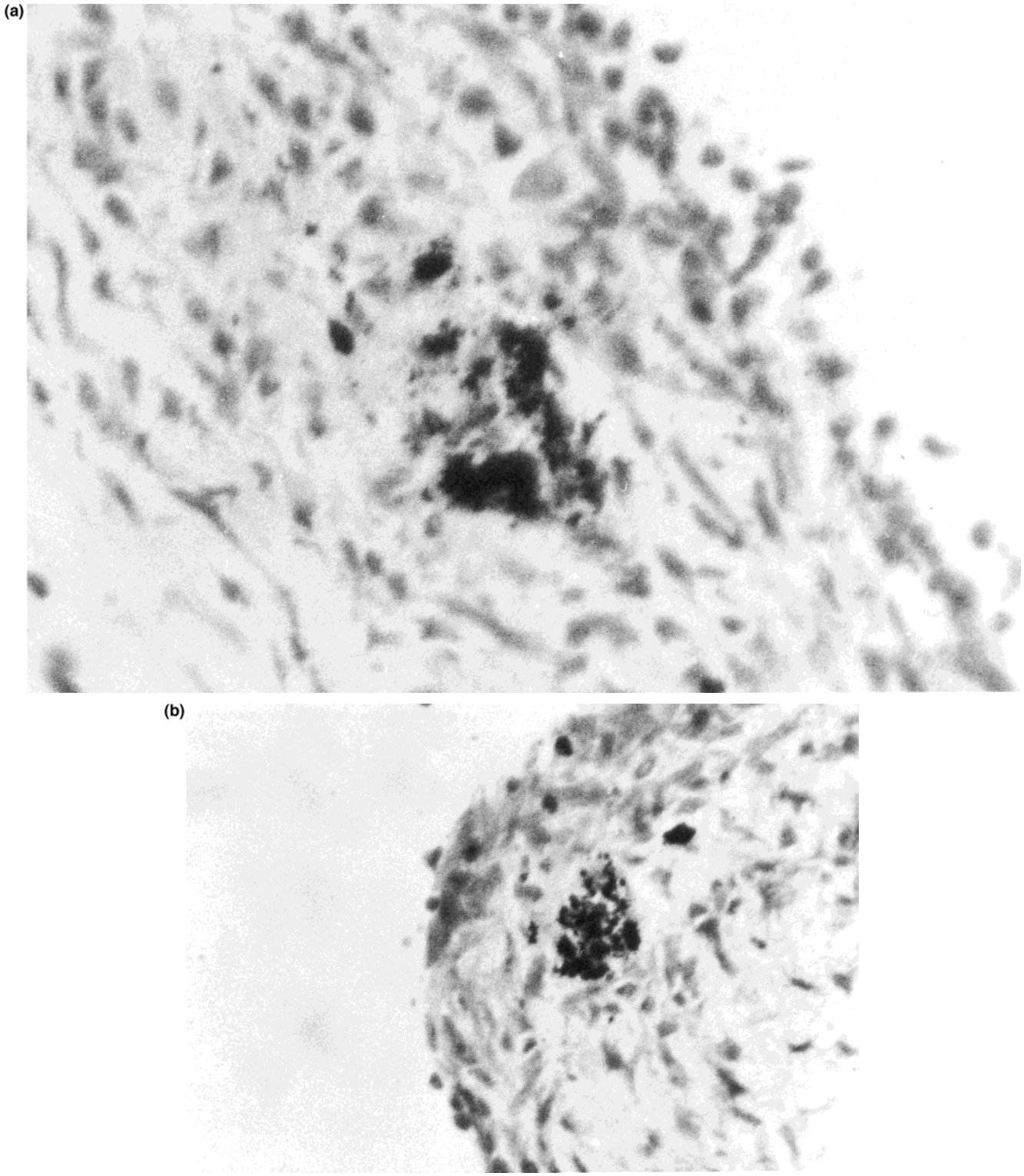

Figure 8. Hematoxylyn and eosin stain of pouch lining. Figure 8(a) shows lining containing PSF particles and 8(b) shows PEKK particles. The two types of particles are indistinguishable by light microscopy. The histology of the pouches showed a similar histology with thickening of the lining and some mononuclear cell infiltration.

Histology

Figure 8(a) shows pouch-lining tissue taken from pouches at 24 h. Particles of PSF can be seen in the superficial lining layers. A similar area taken from a PEKK-injected pouch is shown in Figure 8(b). Although particles of each polymer are shown in these photomicrographs, the actual number of particles remaining in the tissue appeared to be small.

DISCUSSION

The subcutaneous air pouch in the rat has been used extensively in our laboratory to evaluate the inflammatory potential of a variety of particulates. The distinct advantages of this model are its approximation of the tissues surrounding implants, the ability to actually quantitate mediators of inflammation and bone resorption, and the ability to carefully control the experimental conditions. In previous publications,[6–8] we have found this model to provide reliable values.

This study clearly suggests that particles derived from composites containing PEKK as the matrix components are acutely less inflammatory than composites containing PSF. Studies with neat materials confirm that the PSF particles appear to be the source of the difference in phlogistic potential.

Several limitations should be noted. First, our model is not strictly a model of chronic inflammation. The subcutaneous air pouch procedures used in this investigation measures acute inflammation and is more analogous to what might occur with each episode of recurrent particle shedding. The inflammation subsides rapidly and is virtually gone by 72 h. In Addition, we have done no studies to determine rates of clearance of the particles from the pouch. The prolonged presence of one particle compared to another could affect adversely longterm implant survival.

We also should point out that although PSF appears to be more inflammatory than PEKK, both polymers are only minimally phlogistic compared to polymethylmethacrylate, monosodium urate, or polyethylene.[6–9] This is especially illustrated by the number of particles necessary to produce an inflammatory response (10^8/mL for the polymers versus 10^7/mL for PMMA or MSU). The differences shown emphasize the relatively insignificant inflammatory nature of PEKK rather than the inflammatory nature of PSF.

In conclusion, composite materials show great promise for use as components of joint prostheses. Wear of implants is inevitable in any joint replacement, making wear particle development a possible complication in total joint arthroplasties. We provide evidence in this investigation that particles of PEKK and carbon fibers cause minimal inflammatory responses in the rat subcutaneous air pouch model of inflammation. PSF does cause an inflammatory response although it, too, is minimal and short-lived in the rat subcutaneous air pouch.

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