Phagocytosis of Different Particulate Dermal Filler Substances by Human Macrophages and Skin Cells

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BACKGROUND. **Foreign substances have been introduced into the human body with varying degrees of success. Polymethylmethacrylate (PMMA) microspheres of different sizes recently have been manufactured for use as a filler substances in the skin and other organs.**

OBJECTIVE. **To establish whether the size of PMMA microspheres determines whether various cell types initiate phagocytosis.**

METHODS. **The capacity of three different cell lines—U-937 cells, XS 106 and XS 52 Langerhans cells, and HaCaT keratinocytes—to phagocytose microspheres of varying sizes was examined using light and confocal microscopy as well as fluorescence-activated cell sorter (FACS) analysis. Tumor necrosis factor (TNF)-α secretion was also determined.**

RESULTS. **The U-937 cells, keratinocytes, and Langerhans cells could phagocytose PMMA particles of 20 μm or smaller. Microspheres larger than 20 μm were not ingested by any of the cells.**

CONCLUSION. **Microspheres larger than 20 μm have a lower likelihood of being phagocytosed. Thus this study suggests that microspheres 40–50 μm in diameter are less likely to initiate an inflammatory reaction when injected into the dermis and subdermis as a filler substance. On the other hand, microparticles made of silicone and polymethacrylate were phagocytosed, possibly because of their different structure.**

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THE CORRECTION of acne scars or other dermal defects and treatment of the aging face require soft tissue filler materials. Furthermore, patients who suffer from urinary incontinence or gastroesophageal reflux can now be treated with filler materials.[1,2] The ideal filler substance has a number of characteristics, including permanence and lack of immunogenicity.[3] Unfortunately all biologic fillers produced from collagen, fat, and even cartilage will be resorbed over time when injected into the body. Therefore nonresorbable substances such as silicone particles, polytetrafluoroethylene (PTFE), and polymethylmethacrylate (PMMA) microspheres, as well as slowly resorbable substances such as dextran, polylactic acids (PLAs), and hydroxyapatite have been used as injectable filler materials for the above indications. According to their chemical composition, size, shape, surface structure, and surface charge, these materials demonstrate different biocompatibility. Thus smooth-walled particles are encapsulated with fibrous tissue, while particles with an irregular surface may initiate an inflammatory response.[4]

Particles of varying sizes may induce a foreign body reaction when introduced into the body.[5] This reaction is mediated by macrophages and results in a cytokine cascade characterized by the production of tumor necrosis factor (TNF)-α and interleukin (IL)-1 and IL-6.[6] In the orthopedic arena, aseptic loosening of a joint with concomitant debris-induced osteolysis is an important cause of total hip arthroplasty failure.[7] These observations have stimulated a number of *in vitro* studies to predict the chemical characteristics of the particles that evoke the response of macrophages in human joints.[8] Although these particles induce proinflammatory cytokines in the macrophage, the response of native skin cells has not been tested.[9]

Since the size and shape of the injected particles appear to be critical in inducing a foreign body response, we compared the capacity of various-size PMMA microspheres to stimulate phagocytosis and TNF-α production by human macrophage-like cells (U-937) *in vitro*. Furthermore, we compared the capacity of U-937 cells to phagocytose chemical particles of varying sizes to that of cultured Langerhans cell lines and a keratinocyte cell line. We found that Langerhans cells as well as the keratinocyte cell line were able to phagocytose the smaller microspheres.

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Materials and Methods

Particles

Sterile PMMA microspheres of different sizes (4.3 μm, 8.3 μm, 20 μm, 47.8 μm, and 72 μm) were purchased from European Medical Contract Manufacturing (EMCM B.V., Nijmegen, The Netherlands). In addition, sterile PMMA microspheres 32–40.2 μm in diameter, charge no. C-319/1993 used in the former Arteplast, and absolutely smooth microspheres 40.2 μm in diameter, charge no. I-203/2000 used in Artecoll, were received from Mediplant GmbH (Frankfurt/Main, Germany). Sterile PLA D,L-PLA microspheres, MG 100 200, with a mean diameter of 40 μm (range 22–49 μm), made from resomer R 208, and collagen-coated PLA RJ73 100-B, with a mean diameter of 37.7 μm (range 0.6–60 μm), made from R 207 PLA resomer were purchased from Oakwood Laboratories, LLC (Oakwood, OH). Fluoresbrite 0.5 μm fluoresceinated microspheres, made using the latex method, were purchased from Polysciences (Warrington, PA). Rhodamine-labeled PMMA microspheres 4.9 and 30–50 μm in diameter were obtained from Microparticles GmbH (Berlin, Germany).

Because of nonspecific adherence of small microspheres to each other, the exact concentration of the microspheres added was difficult to determine. However, smaller sizes of PMMA microspheres (0.5, 4.3, 8.3 μm) were used at a minimum concentration of 10^5 microspheres/dish. Three other dermal filler materials currently on the European market were tested for phagocytic activity in the macrophage cultures: Macroplastique MPQ-2500 (Uroplasty, Geleen, The Netherlands) consists of polydimethylsiloxane particles, mean diameter 171 μm (range 16–409 μm) suspended in polyvinylpyrrolidone; Dermalive (DermaTech, Paris, France) consists of PMMA particles, mean diameter 55 μm (range 10–135 μm) suspended in hyaluronic acid; and New Fill (Pharmabiotech, Beaufour, France) consists of PLA microspheres, mean diameter 46 μm (range 20–100 μm) suspended in methyl cellulose.

Cell Culture

The human macrophage-like cell line, U-937, was obtained from the American Tissue Culture Collection (ATCC) (Rockville, MD). The cells were grown in RPMI 1640 (ATCC) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. A total of 4×10^5 cells/well in 24 well plates were incubated for 3 days with 10^{-7} M phorbol myristate acetate to induce differentiation before addition of the microspheres. The human keratinocyte cell line, HaCaT, a generous gift of Dr. N. Fusenig, Heidelberg, Germany, was cultured in DMEM plus 10% fetal calf serum and 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. The murine Langerhans cell line, XS 106, a generous gift of Dr. A. Takashima, was cultured in RPMI that contained 10 mM Hepes, 1% nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M β-mercaptoethanol, 1 mM sodium pyruvate, and 10% heat inactivated fetal calf serum (cRPMI) plus 5% conditioned medium from NS-47 fibroblasts (NS supernatant) and 0.5 ng/ml mouse recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) exactly as described.[10] The murine Langerhans cell line, XS 52, a generous gift of Dr. Takashima, was cultured in cRPMI plus 10% NS supernatant plus 2 ng/ml mouse GM-CSF as described.[11]

Light Microscopy

Cells incubated with microspheres were examined using an inverted tissue culture microscope and photographed using a digital camera.

Coulter Elite Flow Cytometer Fluorescence-Activated Cell Sorter (FACS) Analysis

For flow cytometry experiments, the cells were incubated with the microspheres for 1 day before the supernatants containing the microspheres were removed. The viable cells still attached to the Petri dishes were then washed once with Hepes buffered saline plus 0.02% ethylenediaminetetraacetic acid (EDTA) and incubated in 0.25% trypsin for 10 minutes. The detached cells were transferred into tubes and the trypsin was inactivated by the addition of medium-containing serum. The cells were immediately placed on ice and analyzed with the Coulter Elite flow cytometer. Analysis was performed as described.[7] Cells were analyzed under the direction of J. Nordberg at the VA Core Flow Facility. Approximately 5000 cells were evaluated for forward and side scatter characteristics in order to determine the percentages of cells that had ingested microspheres. Since phagocytosis reaches a plateau independent of concentration for larger particles, exact particle counts were not performed.[7] As a control for nonspecific adherence of microspheres to the macrophages, cells in replicate wells that had not been incubated with microspheres were also trypsinized and placed on ice. To these cells, microspheres were added immediately before FACS analysis. On FACS analysis, the more granular the cells, that is, the more microspheres the cells contain, the more side scatter the cells demonstrate. To determine the percentage of dead cells in the cultures, some samples were stained with 0.5 μg/ml propidium iodide before analysis.

When fluoresceinated microspheres were added to the cells in culture, trypan blue (0.4%) was added to the cells immediately before analysis using FACS to obscure the fluorescence of the microspheres present on the outside of the cells.[12]

Confocal Laser Microscopy

Rhodamine-labeled 4.9 and 30–50 μm microspheres were added to HaCaT cells and incubated for 1 day. The cells plus microspheres were then visualized using a laser scanning confocal microscope.

TNF-α Determination

U-937 cells were incubated with various microspheres for 3 hours. For TNF-α assays, the supernatants were centrifuged

to remove cellular debris and particles. These supernatants were frozen at −80°C (see below). As a positive control, replicate cultures were incubated with 1 μg/ml lipopolysaccharide derived from *Escherichia coli*, serotype 026 iB6 (lot no. 46H4024). After the incubation, supernatants of the cells were harvested and centrifuged to remove the cellular debris and frozen at −80°C. The media were transported to Quest Diagnostics (San Juan Capistrano, CA) where an enzyme-linked immunosorbent assay (ELISA) for TNF-α was performed using good laboratory practice.

Results

U-937 Cells

Assessment Using Light Microscopy. When examined with light microscopy, U-937 cells appeared to have phagocytosed 4.3, 8.3, and 20 μm microspheres (Figure 1), but not 40.2 μm PMMA microspheres (Artecoll). These cells also ingested PLA microspheres (New-Fill) (10–130 μm) and silicone particles (Macroplastique) (16–409 μm). By light microscopy and manual cell count, 20% of U-937 cells ingested New-Fill and 32% of cells ingested Macroplastique (not shown). When U-937 was incubated with PMMA particles (Dermalive) (10–130 μm), less than 1% of the cells ingested the particles.

U-937 cells failed to ingest particles larger than 40.2 μm. The larger microspheres included New-Fill and PMMA microspheres of 47.8 and 72 μm, as well as microparticles in Dermalive and Macropore. None of the PMMA microspheres with a diameter larger than 20 μm were phagocytosed. However, microspheres composed of collagen-coated PLA, which contained particles of 0.6–60 μm, as well as the smaller particles of the other three dermal fillers, appeared to be phagocytosed by U-937 cells.

Phagocytosis Determined by FACS Analysis. To more accurately evaluate phagocytosis of microspheres, quantitative measurements by FACS analysis were used to determine the percentage of U-937 cells that could phagocytose PMMA particles of varying sizes (4.3–40.2 μm) after culture for 24 hours (Table 1). An increase in side scatter correlates with an increasing number of microspheres ingested by the cell.[6] The smaller microspheres (4.3, 8.3, 20 μm) were phagocytosed by the cells, whereas the 40.2 μm microspheres were not phagocytosed after incubation for 24 hours. Particles larger than 40.2 μm could not be assayed with FACS because the aperture of the instrument was too small to allow entry of the larger particles.

In a separate series of experiments, the U-937 cells were incubated with various size microspheres for 3 hours. The supernatant media from these cultures were analyzed for the presence of TNF-α (see below). The attached cells were trypsinized and the side scatter of the cells was determined using FACS analysis as before (Table 2). As shown in Table 2, U-937 cells also phagocytosed 4.3, 8.3, and 20 μm PMMA microspheres after 3 hours, but not microspheres of larger diameter.

Since it has been reported that cells that phagocytose particles can become nonviable, the viability of the cultures containing PMMA microspheres of varying sizes was determined in the same experiments using propidium iodide exclusion as a marker for viability (Table 3).[7] The cultures that contained relatively small microspheres (4.3–20 μm) contained 5–16% nonviable cells. In contrast, cell cultures that contained I-203, that is, the larger-size microspheres (40.2 μm), showed no dead cells.

TNF-α Secretion. To determine the effect of particle size on TNF-α release from cultured U-937, the cells were incubated for 3 hours with microspheres of various sizes and chemical composition (Table 4). Lipopolysaccharide (1 μg/ml) served as the positive control for TNF-α release. Only the media from lipopolysaccharide-stimulated cells as well as the cells that had been incubated with 10–60 μm collagen-coated PLA microspheres contained detectable amounts of TNF-α. As a control to determine whether the collagen-coated PLA microsphere suspension was contaminated by bacteria and whether this led to TNF-α secretion, these microspheres were aliquoted into separate plates and cultured for 72 hours. No bacterial contamination was observed.

Keratinocytes: HaCaT Cells

Assessment Using Light and Confocal Microscopy. HaCaT cells were incubated with PMMA microspheres of varying sizes and examined using a light microscope. The 4.3, 8.3, and 20 μm microspheres but not the 40.2 μm microspheres were phagocytosed (not shown). To determine whether the microspheres were internalized by the HaCaT cells, confocal microscopy was performed. HaCaT cells incubated with rhodamine-labeled 4.3 and 40.2 μm microspheres showed that the 4.3 μm but not the 40.2 μm microspheres were phagocytosed (Figure 2).

Phagocytosis Determined by FACS Analysis. We also determined whether microspheres of a smaller dimension (0.5 μm) could be internalized by the HaCaT cells. FACS analysis of HaCaT cells incubated with these fluoresceinated microspheres demonstrated phagocytosis by 69 ± 2.1% of the cells (Table 5), while 77 ± 2.8% of the U-937 cells ingested microspheres of the same size.

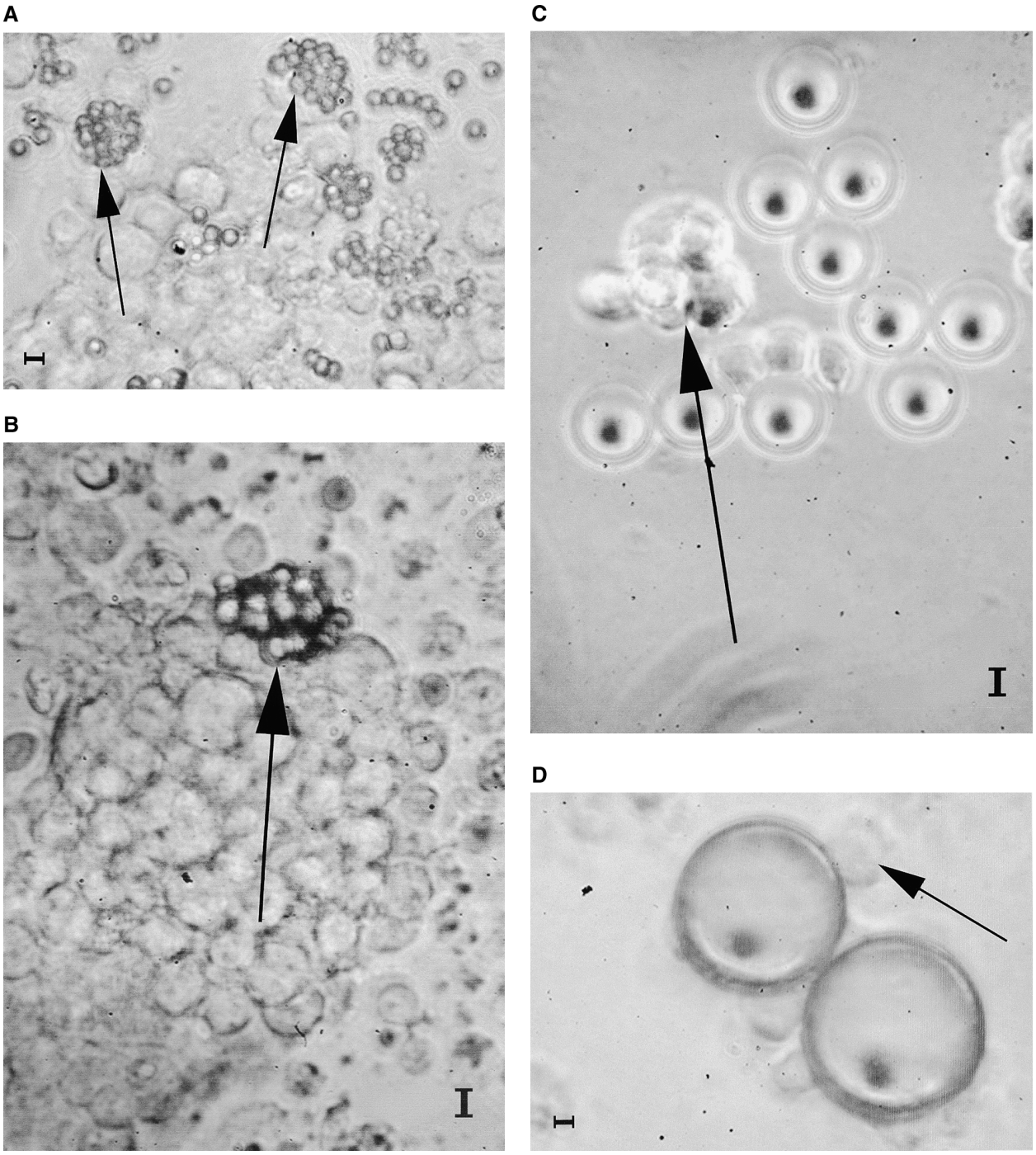

Figure 1. The U-937 cell line was incubated with microspheres of varying sizes for 1 day. The cells and microspheres were photographed as described in Methods: A) 4.3 μm microspheres, B) 8.3 μm microspheres, C) 20 μm microspheres, D) 40.2 μm microspheres. Arrows point to cells that have ingested microspheres in A–C and to cells attached to the outer surface of microspheres in D. The bar measures 4 μm.

Langerhans Cells

Assessment Using Light Microscopy. Visual assessment of Langerhans cells XS 106 incubated with either 4.3 or 40.2 μm microspheres showed phagocytosis only in the cultures containing 4.3 μm microspheres (not shown). Langerhans cells XS 52 incubated with 4.3 and 40.2 μm microspheres also demonstrated ingestion of only the 4.3 μm microspheres (not shown).

Phagocytosis Determined by FACS Analysis. FACS analysis demonstrated phagocytosis of the 0.5 and 4.3 μm

Table 1. The Capacity of U-937 Cells to Phagocytose PMMA Microspheres

Mean diameter of microshperes (μm)	*Side scatter (% of cells above control)*
None	Less than 1
4.3	15
8.3	24
20	19
38 (C-319)	Less than 1
40.2 (I-203)	Less than 1

Microspheres of varying sizes were incubated with U-937 cells for 1 day, the cells trypsinized, placed on ice, and submitted for FACS analysis. For each sample a total of 5000 cells was counted. The values for the side scatter for the cells incubated or not incubated with microspheres are shown. The values for the side scatter of the control cells that were incubated with the microspheres on ice have been subtracted from the values obtained for the cells that had been incubated with microspheres at 37°C for 1 day. Similar results were found in two separate experiments.

Table 3. Comparison of the Number of Nonviable U-937 Cells in the Culture and the Size of the Particles With Which the Cells Were Incubated

Size of microspheres (μm)	*Nonviable cells (%)*
None	0
4.3	13 ± 6.5
8.3	16 ± 2.2
20	5 ± 3.1
38 (C-319)	2.5 ± 1.1
40.2 (I-203)	0

After trypsinization to remove the cells from the Petri dish, the cells were labeled with propidium iodide as described in Methods. Subsequent FACS analysis of the cells that had been incubated with microspheres of varying sizes for 1 day determined the number of nonviable cells in the cultures. For each sample, a total of 5000 cells was counted. Values represent the average of triplicate samples ± standard deviation. The nonviable cells are those positively stained with propidium iodide.

microspheres but not the 40.2 μm microspheres by the XS 106 cells (Tables 3 and 5). Of note was that a higher percentage of epithelioid-type cells (HaCaT) phagocytosed the 0.5 μm microspheres (69%) than did the Langerhans cells XS 106 (37%).

Discussion

We have examined the capacity of three different cell types to phagocytose particles of varying sizes and found that microspheres of 0.5 and 4.3 μm are phagocytosed by the macrophage, Langerhans, and keratinocyte cell lines. However, PMMA microspheres of 40.2 μm were not ingested by any of the cells tested nor were larger microspheres of the same or different material. Thus the capacity of cells to phagocytose microspheres appears to depend on the diameter of the microspheres.

It has been demonstrated recently that HaCaT cells can ingest 1 μm microspheres and that this activity is regulated by protease-activated receptor-2, a protein involved in cell proliferation and regulation of the acute inflammatory response.[13] We examined whether HaCaT cells can ingest larger microspheres and have found that they are capable of ingesting PMMA microspheres up to 20 μm in diameter. In contrast to a previous report that freshly isolated murine Langerhans cells are much more phagocytic than cultured Langerhans cells that demonstrated virtually no phagocytosis, our results show that cultured murine Langerhans cells are capable of phagocytosing 0.5 μm microspheres.[9] The chemical nature of the microspheres and the type of Langerhans cells cultures were different and may explain the differing results. Other investigators have reported that Langerhans cells phagocytose *Leishmania major* in vivo, suggesting that Langerhans cells indeed are capable of internalizing particles as part of their antigen-presenting function.[14] In the skin, the size of the

Table 2. Side Scatter of U-937 and XS 106 Langerhans Cells Incubated With Microspheres of Varying Sizes

Cell type	*Diameter of microspheres (μm)*	*Side scatter (%)*
U-937	None	2.1 ± 0.8
	4.3	12.1 ± 1.7
	8.3	10.9 ± 4.7
	20	10.1 ± 2.4
	40.2 (I-203)	1.3 ± 0.8
XS 106	None	0.9 ± 0.2
	4.3	11 ± 0.8
	40.2	0

U-937 cells were incubated with microspheres of varying sizes for 3 hours. The cells were then trypsinized and the side scatter determined using FACS analysis. XS 106 cells were incubated with microspheres for 24 hours and the side scatter determined using FACS analysis. For both cell types the values for controls tested on ice have been subtracted. For each sample, a total of 5000 cells was counted. Values represent the average of triplicate samples ± standard deviation.

Table 4. Secretion of TNF-α by U-937 Cells Incubated With Microspheres of Varying Sizes

Diameter of microspheres (μm)	*TNF-α (pg/ml)*
None	Undetectable
PMMA 38 μm (C-319) (Arteplast)	Undetectable
PMMA 40.2 μm (I-203) (Artecoll)	Undetectable
Polylactic acid 40 μm powder (208)	Undetectable
Collagen-coated polylactic acid 36.7 μm (207)	390 ± 189.3
PMMA 4.3 μm	Undetectable
PMMA 8.3 μm	Undetectable
PMMA 20 μm	Undetectable
Silicone oil medical grade	Undetectable
Dextran (Reviderm)	Undetectable
Lipopolysaccharide (1 μg/ml)	1678 ± 359.1

U-937 cells were incubated with various microspheres for 3 hours. The supernatant media were collected and the presence of TNF-α was determined. Values represent the average of triplicate samples ± standard deviation. This experiment was repeated two times and showed similar results.

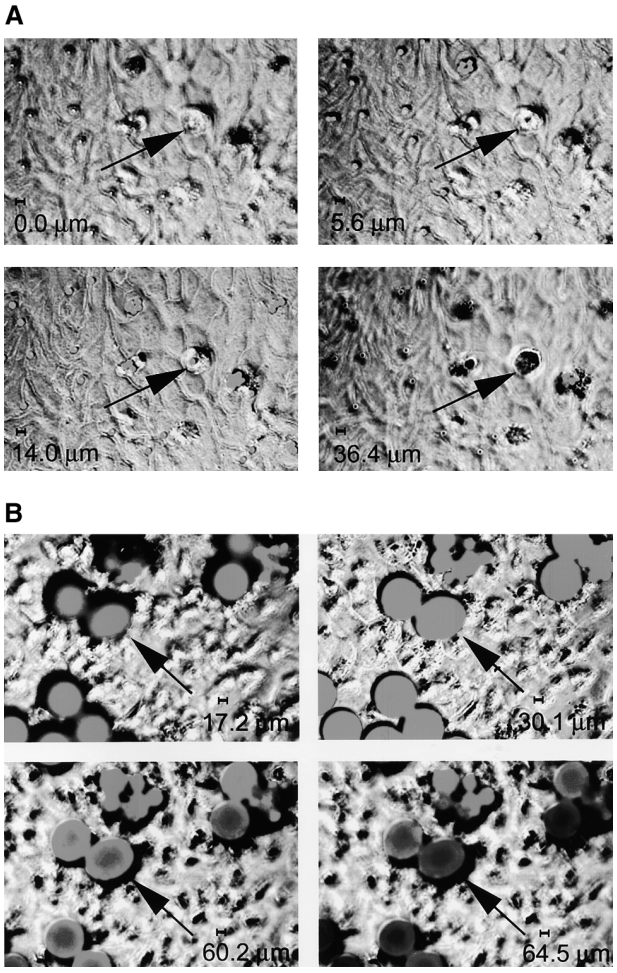

Figure 2. Confocal microscopy of optical sectioning at various levels. HaCaT cells were incubated with either A) 4.9 µm microspheres or B) 30–50 µm microspheres for 1 day and images were obtained using a confocal microscope. The arrows follow the progression of a single cell at different levels of imaging. The bar measures 4 µm.

foreign particle introduced may determine, in part, which cell type ingests the foreign object.

PMMA microspheres of any diameter (4.3–40.2 µm) used in these experiments did not induce TNF-α production. This differs from previous reports that showed TNF-α release from macrophages incubated with either 4.5 µm Al_2O_3 and ZrO_2 microspheres or 0.325, 5.5, and 200 µm PMMA microspheres.[7,8] The reason for the divergent results are unclear, but could relate to differences in manufacture of the microspheres or the different cell types used.

Less inflammation usually results in a longer-lasting tissue filler.[3] Inflammation can be triggered by a number of factors. These include recognition by the host of a peptide as nonself that can result in the production of antibodies by plasma cells. Also, particles can be recognized as foreign and this can result in initiation

Table 5. Comparison of the Capacity of Various Cell Types to Ingest 0.5 µm Microspheres

	Fluorescent cells (%)
Macrophage (U-937)	77 ± 2.8
Keratinocytes (HaCaT)	69 ± 2.1
Langerhans cells (XS 106)	37 ± 1.5

U-937 macrophage, HaCaT cells, and Langerhans cells were incubated with fluoresceinated 0.5 µm microspheres for 1 day. The cells were trypsinized and analyzed using the FACS. The percentage of green cells ± standard deviation is shown.

of a macrophage-mediated inflammatory response that starts with phagocytosis and proceeds to the release of inflammatory mediators and finally cell death.[7]

The search for a soft tissue filler substance that does not evoke an inflammatory response has led to the injection of a variety of biomaterials including microparticles and microspheres into wrinkles, dermal defects, the urethra of patients with urinary incontinence, and in patients with gastroesophageal reflux disease.[15–17] These substances differ in chemical composition, surface structure, surface charge, and particle size and have been shown to evoke different host reactions.

Many hard materials such as metals, ceramics, and polymers, as well as soft materials such as silicone gel, PTFE, and hydrogels, and biological materials such as ivory, coral, soybean oil, silk, and catgut, have been used as alloplastic substitutes for human tissue or as suture materials. Although the chemical composition of the implant seems to be of primary importance, its physical form is equally critical in determining biocompatibility.[18,19] A variety of physicochemical factors affect phagocytosis, including particle size, shape, contact angles, collision factors, surface tension, and surface charge. A simple experiment with rods of 1 mm in diameter but different shapes implanted into rats showed that triangular polymer implants with sharp edges caused significantly higher cellular responses and acid phosphatase enzyme activity than square or round implants.[20] Thus it appears that particles with irregular surfaces initiate a foreign body reaction that can lead to granuloma formation.[18]

One of the questions that prompted this investigation was whether early Artecoll implants contained impurities and could induce a phagocytic response *in vivo*.[21] Since impurities from PMMA nanoparticles attached to some microspheres can induce a foreign body reaction, it is conceivable that the phagocytosis found by McClelland et al.[21] was due to these impurities.

We have demonstrated that whereas small microspheres (0.5–20 µm) are phagocytosed by a variety of cells found in the skin, PMMA microspheres 40.2 µm or larger are not phagocytosed by these cells, nor do they stimulate TNF-α production. This would suggest that microspheres 40.2 µm or larger are less likely to

initiate an inflammatory response when implanted into human tissues than smaller diameter microspheres or filler materials made of noninert materials. Thus smooth-surfaced PMMA microspheres of about 40 μm appear to be ideal filler substances for use in augmenting soft tissues in cosmetic and reconstructive applications.

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