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Synergistic Effect of *Staphylococcus aureus* and LPS on Silica-Induced Tumor Necrosis Factor Production in Macrophage Cell Line J774A.1

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Abstract In this study, we investigated the synergistic effects of *Staphylococcus aureus* extracts (membranes and walls) and lipopolysaccharide (LPS) derived from *Escherichia coli* on tumor necrosis factor (TNF) production in the pathogenesis of silica-induced inflammation. The synergistic induction of TNF by silica particles (<20 μm) in combination with either *S. aureus* extracts or LPS was examined in J774A.1 cell cultures. Media from the treated and untreated cell cultures were assayed for TNF, using the mouse WEHI 164 cell cytotoxicity assay and enzyme immunoassay. Cells exposed simultaneously to silica and 0.5 μg/ml *S. aureus* extracts (or 0.5 ng/ml of LPS) produced a significantly higher level of TNF than those produced by the inducer alone. Our results indicate that device-associated infections (or pyrogen contamination) could enhance inflammatory responses, because of particles produced by the wear of medical implants or particulate biomaterials used for clinical purposes.

Key words: Synergistic effect, *Staphylococcus aureus*, lipopolysaccharide, tumor necrosis factor, silica, macrophage, phagocytosis

Silica (SiO_2) is a material that may produce particle-related inflammatory responses, such as chronic inflammation, granuloma formation, and lung fibrosis. Hazardous exposure to silica may occur in mining, quarrying, glass manufacture, the production of pottery, porcelain and lining bricks, or during boiler descaling and enameling. Other significant exposures may occur from wear particles emanating from medical devices such as dental resin composites, bone cement, and silica xerogel. [2, 13, 14, 17, 21, 28, 32].

Several investigators have measured cytokine production in mononuclear cells exposed to various sources of silica. Claudio *et al.* [6] showed a significant production of tumor necrosis factor (TNF) in RAW 264.7 cells exposed to silica particles *in vitro*, and Perkins *et al.* [25] measured cellular release of interleukin (IL)-1β, TNF-α, IL-6, granulocyte macrophage colony stimulating factor, and prostaglandin E_2 during *in vitro* culture, as indicators of asbestos-induced macrophage activation.

The effects of silica exposure on the macrophage secretion of proinflammatory cytokines and on macrophage responses to lipopolysaccharide (LPS) have not been fully characterized. It has been suggested that silica particles, due to synergistic effects between the particles and LPS, stimulate macrophage response to LPS treatment, resulting in the increased secretion of TNF-α.

Studies of clinical retrievals indicate that a few species seem to dominate biomaterial-centered infections. *Staphylococcus aureus* and *S. epidermidis* have most frequently been isolated from infected biomaterial surfaces, but *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, β-hemolytic streptococci, and enterococci have also been isolated. Therefore, we investigated the ability of Gram-positive *S. aureus* and LPS derived from *E. coli* to induce TNF-α in macrophage cells *in vitro*.

Silica particles (Sigma; MO, U.S.A.) used in this study had a maximal diameter <20 μm, which can be phagocytosed by macrophages. Macrophage cells (J774A.1) and *S. aureus* were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). J774A.1 cells were grown at 37°C in a humidified atmosphere (5% CO_2/95%

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air) in a macrophage culture medium, which consisted of RPMI1640 with L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. LPS (strain: *E. coli*) was purchased from Sigma Chem., Co. (St. Louis, MO, U.S.A.). LPS products were lyophilized powders prepared by phenol extraction and contained 10,000 endotoxin units per mg LPS. Bacterial cultures (Nutrient broth) of *S. aureus* were grown from a single colony picked from freshly streaked plates using a similar method as previously described [24]. *Staphylococcus aureus* was incubated for 12–16 h at 37°C with vigorous shaking, and the bacterial cells were harvested by centrifugation at 6,000 ×*g* for 10 min. Immediately after harvesting, the bacteria were suspended in 0.1 M sodium citrate (pH 3.0) to give a concentration of 4 g of wet cells/10 ml. The suspension of cell pellets was ultrasonicated at 20 kC using a sonicator (XL-2020; Misonix; NY, U.S.A.) for 15 min at 4°C, and then dialyzed against distilled water (DW) for 24 h at 4°C. The suspension of broken cells was decanted, and the pH was adjusted to 4.7 with 1 M $NaHCO_3$. An equal volume of 80% (w/v) aqueous phenol was added, and the mixture was stirred for 1 h at 65°C. After cooling, the emulsion was centrifuged at 1,500 ×*g* for 10 min, the aqueous layer was removed, and the phenol layer was washed with 1 volume of DW. Both the aqueous and phenol layers were dialyzed against DW, and both fractions were then lyophilized.

Before incubation with macrophages, silica particles were washed with 70% ethanol and evaluated for the possibility of LPS-contamination, using the Limulus Amebocyte Lysate (LAL) test with a sensitivity of 10 pg/ml. The silica particles were then coated with FBS, essentially by published procedure [12].

TNF-α levels in cultured media were measured by using the WEHI 164 cell-cytotoxicity assay [11]. When cell cultures were 90% confluent, they were washed with PBS and digested with trypsin-EDTA (0.25% porcine trypsin, 0.02% EDTA in Hank's balanced salt solution) for 10 min and plated at an initial density of 2×10^5 cells/well in 96-well plates. After a 12 h incubation period, the medium was removed and confluent cell monolayers were further incubated for 24 h with 200 μl of the conditioned media from silica and/or *S. aureus* extracts (or LPS) treated macrophage cells. After 24 h of incubation, the medium was removed, and adherent viable WEHI 164 cells were stained with 0.2% crystal violet in 2% ethanol for 10 min at room temperature. After extensive lysis of the stained cells with 0.5% SDS in 50% ethanol, absorbance was measured at 595 nm using an automated plate reader. Percent cytotoxicity was calculated from the following equation: (1−OD of test/OD of control)×100. Cytotoxic titers (U/ml) were expressed as reciprocal values of the dilution that showed 50% cytotoxicity of target cells, as described previously [1]. The units of TNF were calculated based on the cytotoxicity of known concentrations of murine recombinant (mr)TNF-α (Serotec, U.K.) in the standard curve of the same assay.

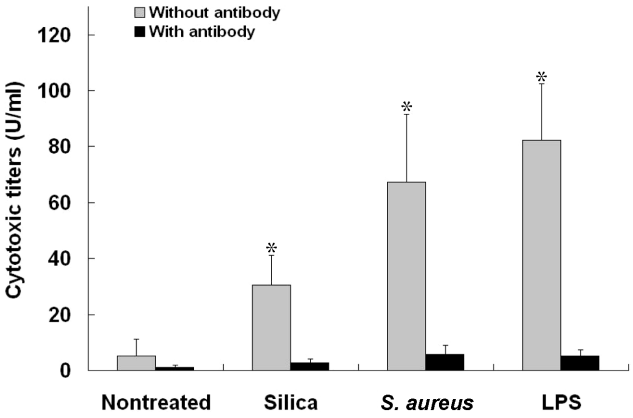

Fig. 1. Inhibition of the cytolytic activity of silica, *S. aureus* (membranes and walls), and LPS-treated macrophage culture media on WEHI 164 sarcoma cells by anti-TNF-α antibodies. Silica particles were added at a concentration of 4 mg/well. Membranes and walls of *S. aureus* were prepared from ultrasonicated *S. aureus* cell suspensions, as described in the text. *Staphylococcus aureus* (membranes and walls) extracts and LPS were used at concentrations of 10 μg/ml and 10 ng/ml, respectively. Each point represents average± SEM of four determinations. *Statistically different from the nontreated control group, $p<0.05$.

TNF-α was also measured in an enzyme-linked immunoadsorbent assay (Endogen, MA, U.S.A.) according to package insert directions. This assay is specific for the measurement of natural and recombinant murine TNF-α, and detects a minimum level of 10 pg/ml of biologically active TNF-α.

In order to confirm the TNF-α activity of conditioned media, the neutralization effect of anti-TNF-α antibody on conditioned media was investigated. As shown in Fig. 1, anti-mouse TNF-α antibodies (IgG concentration, 10 μg/ml) caused a significant inhibition (approximately 90%) of the cytotoxicity of WEHI 164 cells mediated by silica, *S. aureus* extracts, or LPS-treated culture media. This is consistent with the previous reports [7, 23, 34] that TNF-α or TNF-like molecules mediate the cytolysis of WEHI 164 cells during monocyte/macrophage-supernatant treatment. These results indicate that most of the cytotoxic activity would be due to TNF-α.

Supernatants of silica-treated macrophage cultivation demonstrated a dose-dependent increase of specific lysis against WEHI 164 cells in the range from 0.5 to 8 mg (silica)/well (Fig. 2A). The intensity of cytotoxic responses demonstrated by the conditioned media from *S. aureus* extracts (0.4 to 50 μg/ml) and LPS (0.4 to 50 ng/ml)-treated J774A.1 cells showed a similar pattern in terms of TNF titers against WEHI 164 cells (Fig. 2B, 2C). Although Claudio *et al.* [6] reported that J774A.1 cells showed a small increase of TNF-α production when treated with

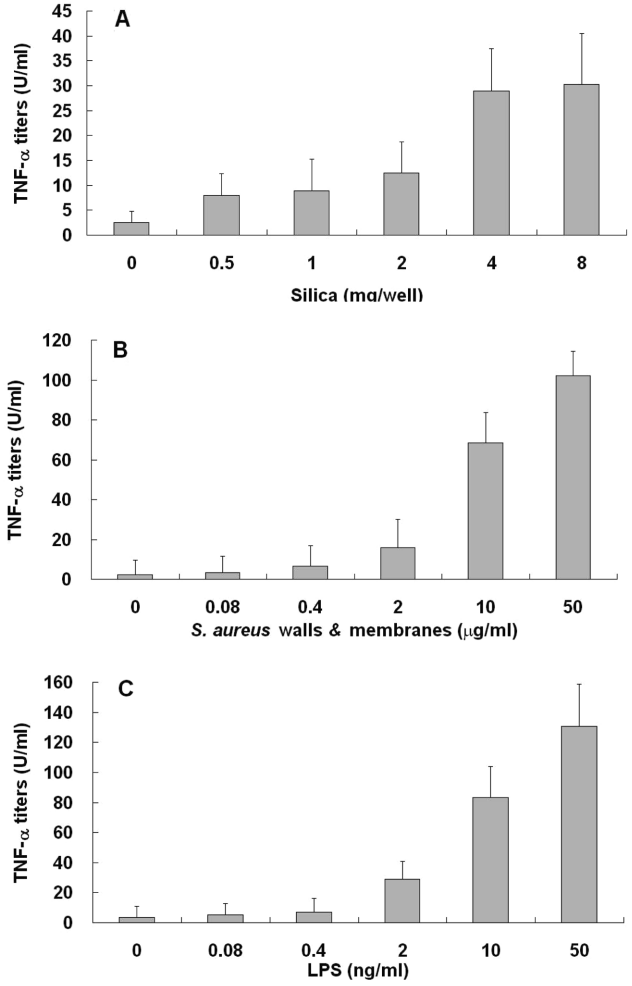

Fig. 2. Production of supernatant TNF-α by silica (A), *S. aureus* extracts (membranes and walls, B), and LPS-treated J774A.1 (C).
J774A.1 cells (4×10^5 cells/well) were cultured as described in the text for 24 h in 24-well plates, and silica, *S. aureus* extracts, and LPS were added at the indicated concentrations. After 24 h of incubation, the supernatants were collected to determine the specific lysis of WEHI 164 sarcoma cells. The units of TNF-α were calculated based on the cytotoxicity of known concentrations of murine recombinant (mr)TNF-α in the standard curve of the same assay. Each point represents average±SEM of four determinations.

LPS, J774A.1 cells showed 35-fold increase of TNF-α secretion in response to LPS in the present assay system (Fig. 2C).

When J774A.1 macrophages were exposed to 0.5–4 mg/well silica particles for 24 h, a dose-dependent increase of specific lysis against WEHI 164 cells was observed over the levels observed in the nontreated control macrophages (Fig. 2C). The macrophage-derived cytokine TNF-α has been implicated in the inflammatory response to a host of biomaterials, and TNF-α is considered to be the initiator protein of particle disease, leading to aseptic loosening of endoprostheses [9, 15, 31]. Boynton *et al.* [4] observed a significant elevation of TNF-α secretion by human monocyte-derived macrophage in response to high-density polyethylene particles, and Rader *et al.* [26] demonstrated *in vitro* induction of TNF-α by cobalt, polyethylene, alumina ceramic (Al_2O_3), and zirconium dioxide in a human monocytic cell line. Several investigators have suggested that biological molecules adsorbed onto particles may play a key role in determining macrophage response [3, 27, 33]. Adsorbed endotoxins may be of particular importance, since they are either exogenously or endogenously present widely, and strongly adhere to many biomaterials. It is widely accepted that the main proinflammatory mediators induced by bacteria and their cell wall components are cytokines, primarily TNF-α, IL-1, and IL-6 [16, 22]. The main bacterial components responsible for the induction of these clinical manifestations are endotoxin (LPS) in Gram-negative bacteria [5, 20], and peptidoglycan (PepG) and lipoteichoic acid (LTA) in Gram-positive bacteria [8, 10]. The main target cells activated by these bacterial components are monocytes and macrophages, which are activated through two pattern recognition receptors, CD14 and Toll-like receptors (TLRs) [8, 10]. In the present study, we observed synergistic TNF-α induction in J774A.1 cells exposed to combinations of low concentrations of either *S. aureus* extracts or LPS and silica particles. Compared with the nonstimulated control group, cells cultured with 0.5 mg/well of silica, 0.5 μg/ml of *S. aureus* extracts, or 0.5 ng/ml of LPS produced an identical and minimum TNF-α release (Fig. 3). When 0.5 mg/well of silica was added together with either 0.5 μg/ml of *S. aureus* extracts or 0.5 ng/ml of LPS, TNF-α production was significantly higher after treatment than that observed after the addition of inducer alone (Fig. 3, $P<0.05$).

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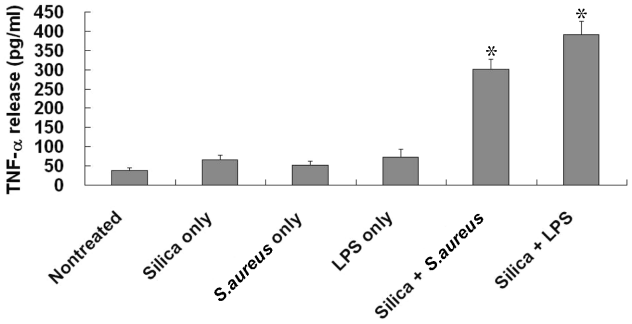

Fig. 3. Synergistic TNF-α induction in J774A.1 cells exposed to a combination of low concentration of *S. aureus* extracts (or LPS) and silica particles.
Cells were cultured and treated with 0.5 mg of silica particles and 0.5 μg/ml of *S. aureus* extracts (or 0.5 ng/ml of LPS) for 24 h. The amounts of TNF-α in the treated and control samples were measured in an enzyme-linked immunoadsorbent assay according to package insert directions. Each point represents average±SEM of four determinations. *Statistically different from silica, *S. aureus* extracts, LPS, and nontreated cells, $p<0.05$.

isolated from infected biomaterial surfaces. Therefore, this work has shown that implant-associated infection and corrosion of implanted medical devices may produce acute and chronic inflammatory responses in the host. In addition to the Gram-positive bacterial infection (such as *S. aureus* and *S. epidermidis*) at the implantation site, pyrogenic contamination (Gram-positive organisms related) of implanted biomaterials may cause inflammatory stimulation. LAL test (pyrogenicity testing) has been performed extensively in the medical device and pharmaceutical industries. However, the limitation of the test is that it detects only the pyrogens of Gram-negative bacterial endotoxin. Consequently, even though the biomedical materials were contaminated with Gram-positive bacteria, the specimen could be judged to comply with the bacterial endotoxin limit for medical devices.

How Gram-positive organisms initiate an inflammatory response is not yet clear. It is difficult to pinpoint one component (such as LPS for Gram-negative organisms) that is responsible for initiating Gram-positive inflammation. Previous studies [8, 30] have shown that the cellular components of Gram-positive organisms, such as LTA and PepG, can produce an inflammatory response. In our study, the membranes of *S. aureus* were extracted with a phenol-water mixture, in order to exclude the possible involvement of teichoic acid in the membrane of *S. aureus* for the stimulation of TNF-α. The activity of TNF-α induction was mainly found in the phenol layer, but also in the aqueous layer to a lesser extent, where lipoglycan of membranes was generally thought to be accumulated (Table 1). This is in agreement with the currently held opinion that the two bacterial wall components, PepG and LTA, work together to cause systemic inflammation associated with Gram-positive organisms.

Our present findings showed synergistic induction of the inflammatory cytokine TNF-α by combinations of LPS with silica particles in the murine macrophage cell line J774A.1. We previously reported that, when silica particles (0.5 mg/well) were added together with 5 ng/ml of LPS, cyclooxygenase-II activity, a useful marker of inflammatory responses, was increased several-fold, compared with that caused by the inducer alone [18, 19]. These results suggest that the intake of silica particles together with a minute amount of LPS could enhance a significant release of inflammatory mediators by macrophages in the presence of implantation-associated infections (or pyrogen contamination). In addition to the bacterial infection at the implantation site, pyrogenicity of implanted biomaterials may also cause trouble. The bacterial endotoxin limit set by the Food and Drug Administration (FDA) for medical devices is 0.5 EU (the LAL reactivity of 0.1 ng of US Pharmacopeia Reference Standard Endotoxin/ml, based on a 40 ml rinse [29]). Significantly, the concentration of LPS used in our assay system showed a synergistic induction of TNF, which is lower than the bacterial endotoxin limit set by the FDA for medical devices. However, the susceptibilities of cells to LPS and the biological activities of LPS extracted from different Gram-negative bacteria vary. It remains to be determined as to what extent the concentration of LPS preparations can produce synergistic effects in inducing a significant release of inflammatory mediators by macrophages. Further *in vitro* studies will be necessary to confirm the diverse sensitivity of LPS on different macrophage cell lines and different kinds of bacteria.

Table 1. TNF-α production in phenol extracts of *S. aureus* walls and membranes.

Material	TNF-α release[a] (pg/ml) stimulated with following amounts (μg/ml) of materials		
	20	5	1
Membranes and walls	2,570	554	96
Phenol layer	6,500	3,240	965
Aqueous layer	4,200	1,188	470

[a]Each material at the indicated doses was incubated with J774A.1 cells (2×10^6 cells) for 24 h at 37°C. Each value of TNF-α production represents the mean of triplicate determinations.

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