Direct Suppression of Phagocytosis by Amphipathic Polymeric Surfactants

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Recent studies have demonstrated that phagocytosis of colloidal particles by the mononuclear phagocytes of the liver and spleen can be controlled by either coating or stabilizing particulate carriers with the amphipathic polymeric surfactants, F108 and T908. These surfactants consist of copolymers of polypropylene oxide (PPO) and polyethylene oxide (PEO) and, when adsorbed to particulate surfaces, significantly decrease sequestration of particulates by the mononuclear phagocytes (MPS) of the liver. To evaluate these observations further, murine peritoneal macrophages were incubated for varying periods with surfactant-coated and noncoated polystyrene particles (PSPs). Phagocytosis was monitored using gamma counting and quantitative fluorescence microscopy. The data show that phagocytosis is decreased when PSPs are coated with F108 and T908. In addition, suppression of phagocytic activity was observed when cells were pretreated with the surfactant and then challenged with noncoated particles. The data confirm previous observations that polymeric surfactants consisting of PEO and PPO protect particulate carriers from rapid uptake by the MPS of the liver. Further, F108 and T908 suppress phagocytosis directly without affecting the integrity, viability, or functional state of the cell.

KEY WORDS: particulate carriers; phagocytosis; suppression; polymeric surfactants.

INTRODUCTION

The potential usefulness of particulate systems as drug carriers has been the subject of numerous investigations (1–5). Particulate carriers afford protection for labile drugs, facilitate the solubility of insoluble therapeutics, and provide a vehicle for controlled-directed drug delivery. However, a major concern that must be addressed before this type of technology can be realized is the activity of the mononuclear phagocytic system (MPS). Following intravenous (iv) administration, particulate carriers are recognized as foreign and are rapidly removed from blood by the resident population of macrophages in the liver and spleen (6–10). In a number of instances, this systemic response to opsonized particulates has been fortuitously used to target therapeutics and diagnostic agents to the liver or to the MPS in general (11–14). However, the efficiency and fidelity of this process make it difficult to deliver drugs via particulate carriers to anatomical sites other than those having a rich population of macrophages.

Recent studies have shown that phagocytosis of colloidal particles by the MPS of the liver can be modulated by surfactant coatings (15–22), e.g., F108 and T908, which are triblock copolymers of polyethylene oxide (PEO) and polypropylene oxide (PPO). After iv injection, polystyrene and poly(methyl methacrylate) nanoparticles coated with F108 and T908 are not rapidly sequestered by the liver. In comparison to their noncoated counterparts, these surface-modified particulates experience prolonged circulating half-lives and altered tissue distributions.

When F108 and T908 are adsorbed to PSPs (polystyrene particles), they are believed to orient such that the hydrophobic domains (PPO) of the surfactant bind the surface of the particulate and the hydrophilic regions (PEO) extend freely into the external milieu (19). These coatings are known to alter the charge and surface characteristics of the particulate, but precisely how these physicochemical alterations affect the biological behavior of the particulate is still a matter of conjecture. Studies have shown that the inability of coated particles to be recognized and phagocytosed by the MPS is related to the observation that, once coated, particulates such as polystyrene particles (PSP) do not bind significant amounts of serum proteins (23,24). In the absence of opsonization, particulates avoid the MPS and circulate freely.

The effects of amphipathic coatings on phagocytosis of particulate carriers have been viewed as being dependent solely on surface modifications of the coated particulate. However, recent studies have shown that low concentrations of the pluronic surfactant F-68 directly affect the pinocytic uptake by cultured fibroblast (26). In addition, previous studies have documented the effects of amphiphilic surfactants on cell surface morphology (14,27). It is reasonable, therefore, to suggest that F108 and T908 could block phagocytosis by exerting a direct effect on the activity of the macrophage.

The present study was performed to elucidate the mechanism by which F108 and T908 inhibit phagocytosis and to explore the possibility that these amphipathic coatings directly reduce the phagocytic activity of macrophages. For these experiments, peritoneal macrophages were used as a cell model for the MPS, and phagocytosis was studied using PSPs either devoid of or coated with F108 and T908. The data confirm earlier results regarding the suppressive effects of polymeric coatings on phagocytosis of PSPs (15–22). Further, the present study shows that surfactant coatings can also suppress phagocytosis by a direct interaction with the cell.

MATERIALS AND METHODS

Materials

Poloxamer F108 and poloxamine T908 were purchased from BASF Corporation (Albany, NY) and polystyrene particles were a product of Polysciences, Inc. (Warrington, PA). Tissue culture medium was obtained from Hazelton, Inc. (Lenexia, KS). Unless otherwise specified, other bio-

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chemicals and reagents used in this study were a product of Sigma Biochemicals (St. Louis, MO).

Iodination of PSP

Polystyrene particles (100 nm) were diluted 2.2×10^{11} particles in 100 μl in PBS (phosphate buffered saline) containing 10 mCi ^{125}I-sodium iodide (NEN-Dupont, Boston, MA). After the addition of 10 *N* NaOH, the solution was placed in a gamma radiation field receiving a total exposure of 1×10^8 rad. The irradiated particles were exhaustively dialyzed to remove free label. The specific activity of the ^{125}I-labeled nanoparticles was approximately 600 μCi/mg.

PSP Coating with F108 and T908

Polystyrene particles, either radioiodinated (100 nm) or fluorescenated (1 μm), were washed in PBS and then incubated at room temperature for at least 1 hr with PBS containing 4% surfactant. For experiments addressing the effects of F108 and T908 on cell activity, cells were pretreated with freshly prepared 0.04% surfactant for 1 hr at 37°C prior to the addition of noncoated PSPs.

Blood Clearance and Liver Uptake of ^{125}I-PSP

After an overnight fast, male Sprague Dawley rats, weighing ~250 g (Taconic Laboratory Animals and Services, Germantown, NY), were anesthetized with nembutal (50 mg/kg). A catheter was placed in the jugular vein of each animal, and the experiment was initiated by injecting ~1 μCi of ^{125}I-labeled PSPs (1.0×10^{11} particles). At various times after injection, blood samples were drawn and placed in a heparinized tube. The experiment was terminated 1 hr postinjection. Radioactivity associated with blood and tissue samples was determined using a gamma counter (Beckman Inst. Inc., Somerset, NJ).

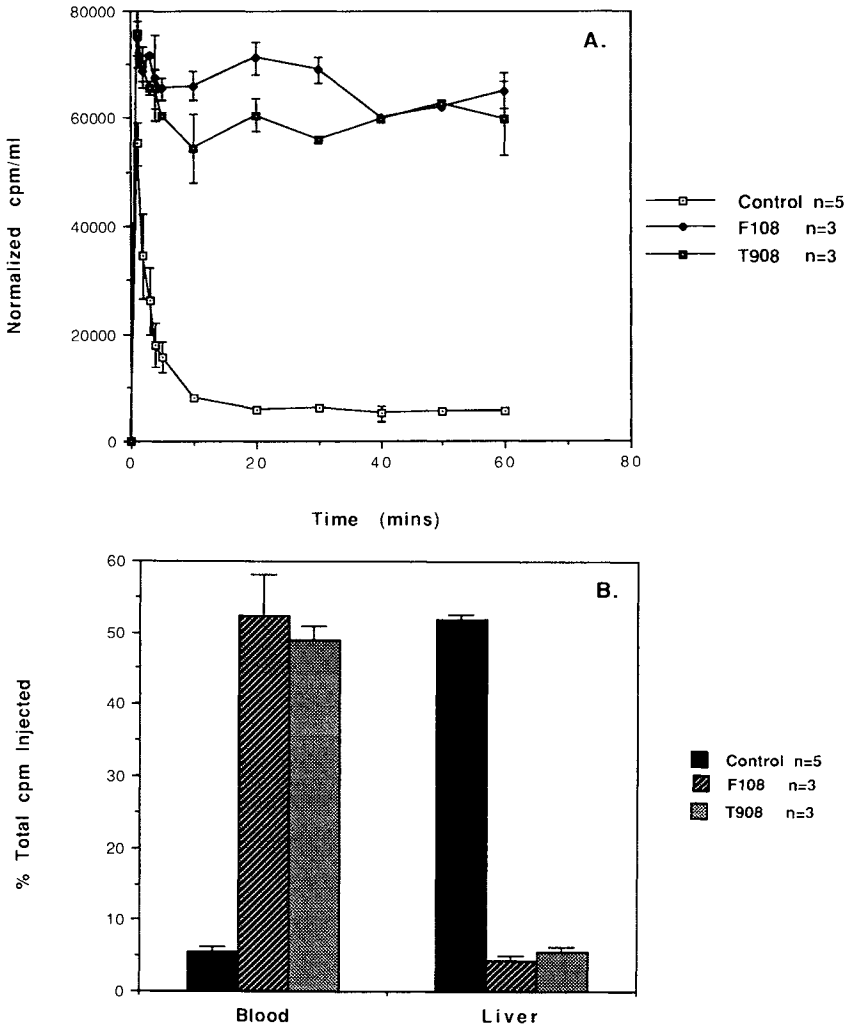

Fig. 1. Biodistribution of F108-and T908-coated polystyrene particles. In A and B, the effects of F108 and T908 on the blood clearance and liver deposition of PSPs are shown. PSPs were precoated with 4% surfactant as described under Materials and Methods. The presence of the surfactant coating altered the circulating half-life of PSPs. In comparison to controls (noncoated PSPs), PSPs coated with either F108 and T908 were not rapidly cleared from the circulating pool of blood and they were not sequestered by the liver. At 60 min postinjection (B) greater than 50% of the dosage was detected in the circulating pool of blood.

Murine Peritoneal Macrophages

Murine peritoneal macrophages were harvested by lavage using pathogen-free male mice weighing ~30 g (Taconic Laboratory Animals and Services, Germantown, NY). Macrophages were collected by centrifugation (500*g* for 10 min) and plated in RPMI medium (M.A. Bioproducts, Walkersville, MD) containing 5% fetal bovine serum (Hyclone, Logan, UT) and 50 μg/ml gentamicin (Tri Bio Laboratories, Inc., State College, PA). In order to remove nonadherent cell contaminants, cells were washed 1 hr after plating with fresh medium. Routinely the preparation of macrophages was approximately 95% pure.

Phagocytosis of Polystyrene Particles

Murine peritoneal macrophages were harvested as described above and assayed for phagocytosis using either a fluorescence or a radioisotope assay. For the fluorescence assay, cells were plated at a density of 2×10^6 cells on Lab-Tek two-chamber slides (Nunc, Inc., Naperville, IL). After a 24-hr incubation at 37°C, fluorescenated polystyrene particles, 1.0×10^9 particles per well, were added to each sample and the cells were left to incubate for an additional 2 hr. Afterward the samples were rinsed in medium minus serum and fixed with 4% formaldehyde in PBS for 2 hr. The samples were rinsed with PBS, mounted in buffered glycerol, and analyzed by light microscopy. Quantitation of images was performed using Image I (Universal Imaging Corp., Media, PA).

In addition to a fluorescence assay, phagocytosis was also monitored using ^{125}I-labeled polystyrene particles. Cells were plated in 48-well tissue culture dishes at a density of 5.0×10^5 cells per well. For this assay cells were incubated for various periods of time with radiolabeled polystyrene particles (5.0×10^8 particles per well). At the end of the incubation, cells were washed three times with PBS, then solubilized with 1.0% sodium dodecyl sulfate (SDS), and total radioactivity associated with the cell lyzsate was determined using a gamma counter.

Other Assays

Cell viability was routinely monitored by dye exclusion assay, and to monitor cell activation, prostaglandin (PGE_2) release was quantified in cell culture supernatants by radioimmunoassay (duPont-NEN, Boston, MA).

RESULTS

Biodistribution of PSPs

To determine the effects of F108 and T908 on the blood clearance and the liver deposition of coated and noncoated polystyrene particles, PSPs were administered to rats as described above. As demonstrated in Fig. 1, the surfactant coatings dramatically alter the behavior of the PSPs in the circulating pool of blood and effectively suppress liver uptake. Figure 1A shows that the surfactant coatings significantly prolonged the circulating half-life of PSPs. For animals injected with noncoated PSPs, at the termination of the experiment, only 10.0% of the dosage was detected in the circulating pool of blood. In contrast, data obtained with coated particulates showed that greater than 60% of the injected dosage was present, 1 hr post injection, in the circulating blood pool. Significant loss of particulates from the blood pool was not observed until ~6 hr. At this time, desorption of the surfactant coating from the particle is believed to occur (data not shown).

The presence of a circulating pool of PSPs in animals given a bolus injection of surfactant coated-particles correlates with our inability to detect a high percentage of the labeled particulates in the liver. As illustrated in Fig. 1B, the total percentage of radioactivity detected in the liver of animals injected with noncoated PSPs was 60%. In comparison, only 5.0% of the dosage was detected in the liver of animals injected with surfactant coated PSP's.

Interaction of Macrophages with PSPs

Differences in the behavior of coated and noncoated PSPs *in vivo* are related to the interaction of PSPs with macrophages. To substantiate these claims, murine peritoneal macrophages were incubated for various periods of time with radiolabeled PSPs (~100 nm in diameter) that were either coated or noncoated with surfactant. Figure 2 illustrates the effect of F108 and T908 on cellular uptake of PSPs. The rate of particle uptake (Fig. 2) was significantly suppressed when cells were challenged with surfactant-coated PSPs. The surfactant coating decreased uptake approximately fivefold.

The morphological data shown in Fig. 3, and quantified in Fig. 4, demonstrate the inhibitory effect of surfactant coatings on the phagocytosis of PSPs that were ~1.0 μm in diameter. Though the particles were an order of magnitude larger than those described in Fig. 2, the effect of the surfactant coating on particle uptake was identical. Cells incubated with noncoated PSPs phagocytosed a significantly greater number of particulates (Fig. 3a) than those exposed

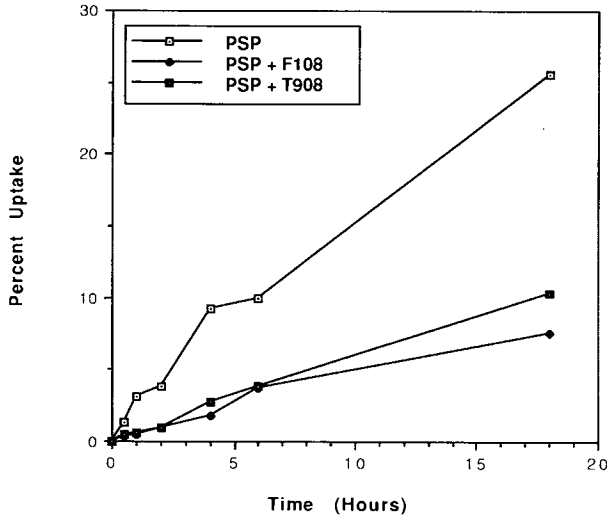

Fig. 2. Cellular uptake of F108- and T908-coated polystyrene particles. Murine peritoneal macrophages were incubated for various times with ^{125}I-labeled PSPs (~100 nm) that were either noncoated (control) or coated with 4.0% F108 and T908. The surfactant coating effectively suppressed the rate and extent of PSP uptake. The values represent averages of triplicate readings.

to surfactant-coated PSPs (Figs. 3b and c). When cells were challenged with the surfactant-coated particles, very few particles were associated with cell surface and/or cell interior (Figs. 3b and c). This behavior was not restricted to a specific population of cells. The majority of cells assayed appeared to respond in a similar fashion. Noncoated PSPs were rapidly phagocytosed. However, when cells were challenged with coated particulates, PSP uptake was significantly suppressed (Fig. 4). Whether expressed in terms of particles per cell or area percentage of fluorescence per cell, the data clearly showed the inhibitory effect of surfactant coatings on PSP uptake. The micrographs also attest to the general integrity of the cells. Though unable to phagocytose, cells exposed to surfactant-coated particles appear to be morphologically intact, and when assayed for viability using trypan blue, greater than 95% of the cells excluded the dye.

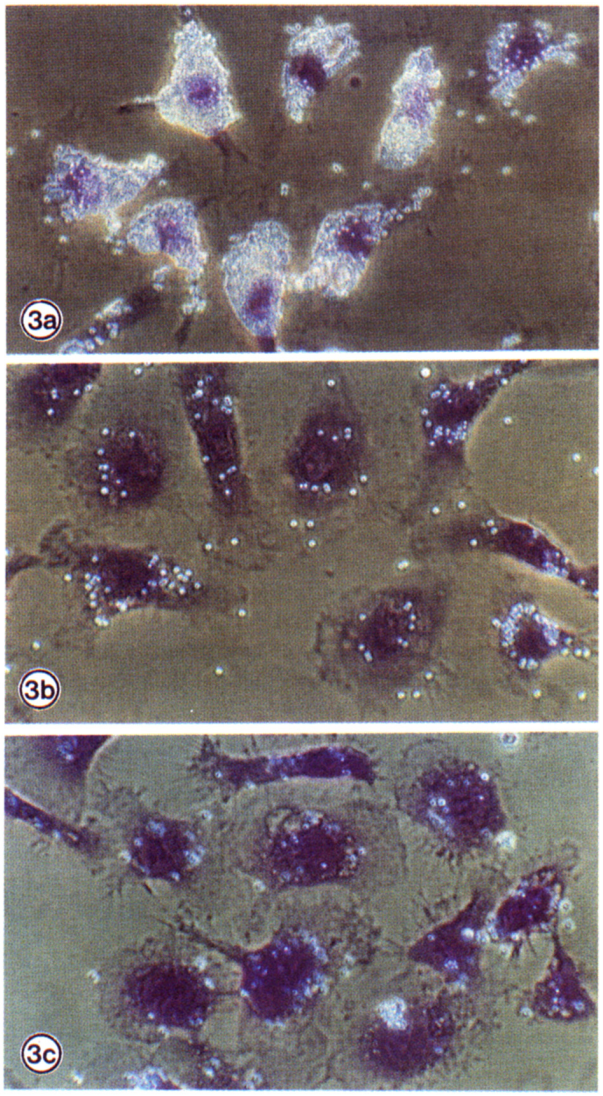

Fig. 3. Interaction of coated and noncoated PSPs with macrophages. The micrographs show the effects of surfactant coatings on the uptake of fluorescenated PSPs. Cells were incubated for 2 hr with PSP (a) or PSPs coated with 4.0% F108 (b) and T908 (c) and then processed for microscopy. The micrographs clearly illustrate the suppressive effect of polymeric surfactants on phagocytic uptake of fluorescenated PSPs. The presence of the surfactant coating did not affect the morphological integrity of the cells. However, particles coated with 4.0% surfactant appear to lack the adsorptive properties of noncoated PSPs. The surfactant appeared to interfere with the interaction of particles with the cell surface. ×1500; reduced to 65% for reproduction.

Effects of Polymeric Surfactants on the Functional State of the Macrophage

In addition to the effects that amphipathic surfactants have on phagocytosis when adsorbed to the surface of PSPs, we tested whether these reagents were capable of directly affecting cell function. To study the effects of F108 and T908 on macrophage activation, cells incubated with and without F108 and T908 were assayed for their ability to secrete prostaglandins (PGE_2) (Fig. 5). Since these studies were designed to determine the effects of F108 and T908 on cell function, cells were incubated with a concentration of surfactant that produced no adverse side effects. In the presence or absence of a macrophage activator, such as lipopolysaccharide (LPS), the release of PGE_2 by cells incubated with 0.04% F108 and T908 was not significantly different from controls. These results were independent of the presence or absence

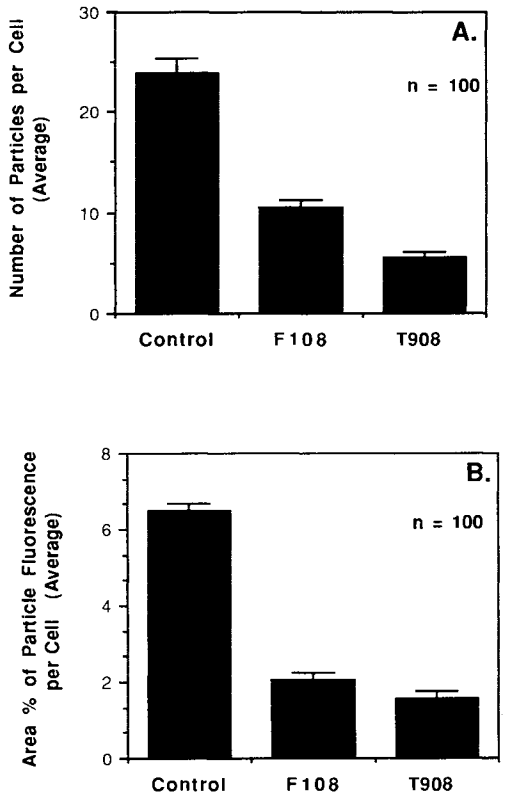

Fig. 4. The effects of F108 and T908 on PSP uptake. To quantify the effects of surfactant coatings on phagocytosis of fluorescenated PSPs, cells from the experiment described in the legend to Fig. 3 were analyzed for the number of particles per cell and area percentage of fluorescence per cell. Both methods of analysis demonstrate the suppressive effects of polymeric surfactants on the phagocytosis of PSPs. During the 2-hr incubation period uptake of surfactant-coated PSPs were suppressed by ~3-fold. For each experimental condition $n = 100$.

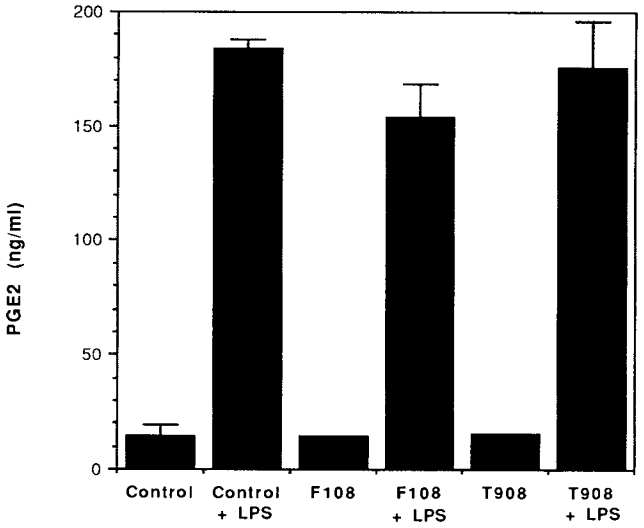

Fig. 5. The effects of surfactant on PGE_2 release by macrophages. To determine the effects of F108 and T908 on macrophage activation, the concentration of PGE_2 in tissue culture medium was determined after incubating peritoneal macrophages with either F108 or T908 in the presence or absence of LPS. The data show that surfactant-treated cells, whether in an activated or a nonactivated state, released an amount of PGE_2 comparable to controls. The amount of PGE_2 released in activated and nonactivated cells was, respectively, 175 and 10 ng/ml.

of PSPs in the culture medium (data not shown). In addition to PGE_2 release, cells were also monitored for the release of IL1 (interleukin-1) and the presence of cell surface antigens. Results from these studies were consistent with the data obtained for PGE_2 and further demonstrated that the concentration of surfactants used in this study which was 0.04% did not adversely affect cell function.

Interaction of Macrophages with Polymeric Surfactants

To determine if F108 and T908 are capable of directly affecting phagocytosis, studies were performed in which surfactant-treated macrophages were challenged with noncoated PSPs. For this study macrophages were preincubated with medium containing surfactant at a concentration that does not affect cell function. It should be noted that this low concentration, 0.04%, when used to coat PSPs, was not effective in suppressing PSP uptake (Fig. 6B). However, when macrophages were preincubated with F108 and T908 and subsequently challenged with noncoated PSP, phagocytic activity was suppressed (Fig. 6A). The degree of suppression ranged from 2.5- to 5-fold and was comparable to that observed in Fig. 4. Furthermore, these effects were reversible (Fig. 6C). If surfactant-treated cells were rinsed, noncoated PSPs were internalized to a greater extent than a control population of cells which had not been directly exposed to either F108 and T908.

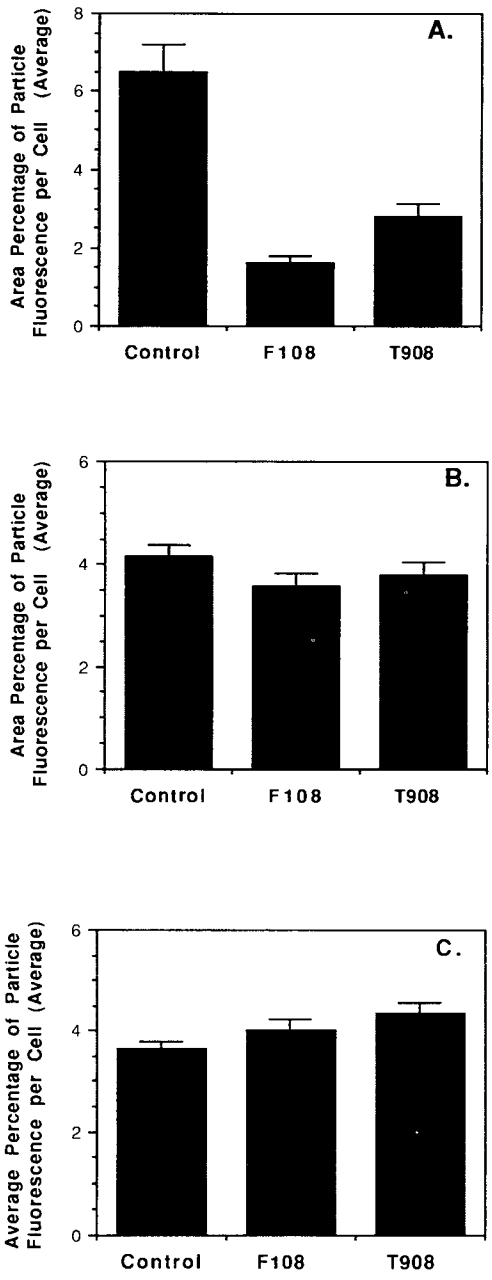

Fig. 6. Direct effects of surfactant on phagocytosis. In A, cells were pretreated with 0.04% surfactant and then incubated with noncoated PSPs. Preexposing the cells rather than the PSPs to low concentrations of surfactant also suppressed phagocytosis. This suppression was readily reversed when the surfactant-treated cells were placed in fresh medium which did not contain surfactant and then challenged with noncoated PSPs—C. To ensure that the suppression observed in A was independent of particle–surfactant interaction, cells were incubated with PSPs that were coated with 0.04% F108 or T908. B shows that phagocytosis was not suppressed when this low concentration of surfactant was used as a coating for PSPs.

DISCUSSION

Delivery of drug-laden particulates to diseased sites that are non-MPS in origin has proven to be a difficult task to achieve. Advances in this area have been primarily directed at altering the composition and/or the surface properties of the particulate so as to discourage interaction of the particulate with cells of the MPS (4,6,9,15,19). The data presented substantiate the observation that polymeric surfactants composed of block copolymers of PPO and PEO effectively in-

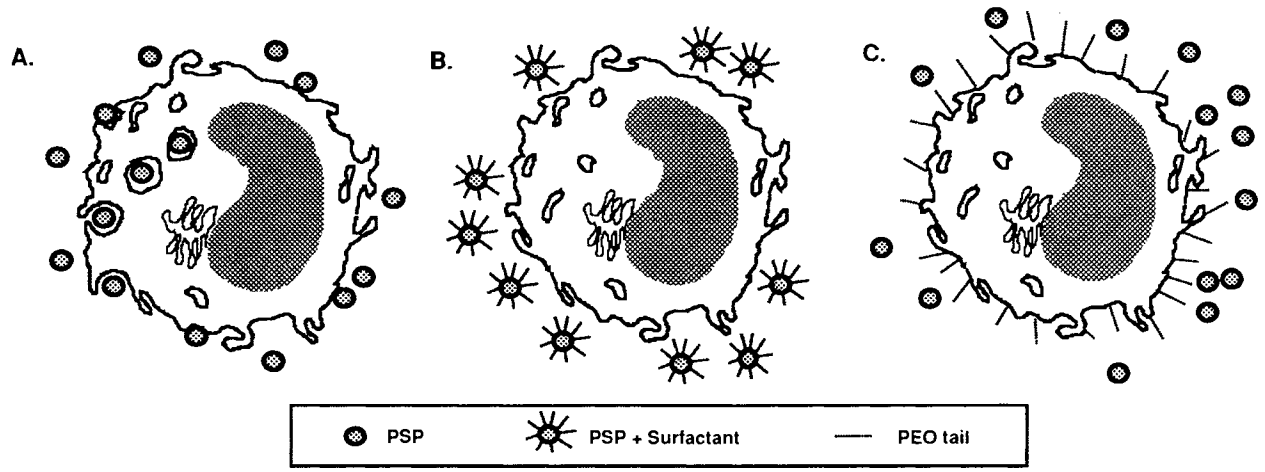

Fig. 7. Mechanism of action: surfactant-induced suppression of phagocytosis. The diagram illustrates the mechanism whereby polymeric amphipathic surfactants directly suppress phagocytosis. In A, a control macrophage is depicted in the process of adsorbing, engulfing, and sequestering PSPs. Since phagocytosis is a two-step process, involving adsorption of the particulate to the cell surface followed by its uptake, interference with either of these steps suppresses the process. When PSPs are coated with 4.0% surfactant, the particles are not readily adsorbed to the cell surface and therefore cannot be phagocytosed—B. As depicted in C, phagocytosis is also suppressed when cells are pretreated with a low concentration of surfactant, 0.04%. This type of suppression may be due to either the adsorptive or the uptake phase of phagocytosis.

terfere with the interaction of particulates with the MPS. The data show that PSPs coated with F108 and T908 have a prolonged circulating half-life and are not rapidly sequestered by the liver. Though this technology has been applied to various types of colloidal systems, e.g., polycyanoacrylate particles and emulsions, the rationale for using PSPs coated with either F108 and T908 was used on previous studies carried out by Illum *et al.* (15–20). Also, PSPs are conveniently available in various size ranges and modifications that are easily assayable. Finally, PSPs coated with F108 and T908 have been used to demonstrate that physicochemical properties of particulates play a role in dictating the biological fate of the particulate. Particulates either coated or stabilized with F108 and T908, as we and others have demonstrated (17–22), are not rapidly phagocytosed.

The intriguing and novel observation that we report is that amphipathic surfactants, F108 and T908, have a direct effect on cell function. As shown in Fig. 6A, macrophages pretreated with the surfactants do not have a membrane surface that readily binds PSPs. Since phagocytosis of particulates is dependent on adherence to the cell surface followed by internalization, the suppression we observed could conceivably be the result of surfactant affecting either of these steps. Though our experiments were not designed to differentiate between factors affecting adherence vs internalization, we speculate that the surfactant interacts with the cell membrane in a manner that mimics the interaction of hydrophilic surfaces with particulates in general. In Fig. 7, we have illustrated our concept on how hydrophilic surfactants interact with the macrophage cell surface. Similar to the proposed mechanism of interaction between block copolymers and particulates, it is reasonable to predict that the block copolymer at the cell surface orients itself such that the hydrophobic PPO units adhere to the hydrophobic domains of the plasmalemma or become sequestered in the bilayer. Unless adsorbed to other cell surface components, the hydrophilic PEO tail, more than likely, extends extracellularly. Particles, though opsonizable since they were added to culture medium containing serum, fail to interact with the surfactant treated cells. Steric constraints resulting from either repulsive charge interactions or failure of the particle to interact with surfactant-masked domains of the cell surface are plausible explanations for our results.

There are two other observations from this study which deserve comment. First, as shown in Fig. 6, the suppression of phagocytosis by macrophages pretreated with F108 and T908 was reversible. When cells were placed in medium minus surfactant, PSPs were readily phagocytosed. These data suggest that, in the time frame studied, the surfactant was no longer effective, indicative of rapid degradation or retrieval of the cell surface membrane via endocytic processes. Finally, neither of the surfactants, at a concentration of 0.04%, had an adverse effect on the integrity or functional state of the macrophage, suggesting that polymeric surfactants consisting of PPO/PEO may have little or no effect on cell activation. This observation is most important when considering the practicalities involved in developing nanoparticulate carrier systems for therapeutic and diagnostic applications.

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