Different Fates of Phagocytosed Particles after Delivery into Macrophage Lysosomes

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Abstract. Phagocytosis in macrophages is often studied using inert polymer microspheres. An implicit assumption in these studies is that such particles contain little or no specific information in their structure that affects their intracellular fate. We tested that assumption by examining macrophage phagosomes containing different kinds of particles and found that although all particles progressed directly to lysosomes, their subsequent fates varied. Within 15 min of phagocytosis, >90% of phagosomes containing opsonized sheep erythrocytes, poly-e-caprolactone microspheres, polystyrene microspheres (PS), or polyethylene glycol–conjugated PS merged with the lysosomal compartment. After that point, however, the characteristics of phagolysosomes changed in several ways that indicated differing degrees of continued interaction with the lysosomal compartment. Sheep erythrocyte phagolysosomes merged together and degraded their contents quickly, poly-e-caprolactone phagolysosomes showed intermediate levels of interaction, and PS phagolysosomes became isolated within the cytoplasm. PS were relatively inaccessible to an endocytic tracer, Texas red dextran, added after phagocytosis. Moreover, immunofluorescent staining for the lysosomal protease cathepsin L decreased in PS phagolysosomes to 23% by 4 h after phagocytosis, indicating degradation of the enzyme without replacement. Finally, PS surface labeled with fluorescein-labeled albumin showed a markedly reduced rate of protein degradation in phagolysosomes, when compared to rates measured for proteins in or on other particles. Thus, particle chemistry affected both the degree of postlysosomal interactions with other organelles and, consequently, the intracellular half-life of particle-associated proteins. Such properties may affect the ability of particles to deliver macromolecules into the major histocompatibility complex class I and II antigen presentation pathways.

MACROPHAGES take up particles and microorganisms by phagocytosis, producing membrane-bounded organelles of plasma membrane origin called phagosomes. Phagosomes usually fuse with lysosomes, making phagolysosomes, wherein the ingested materials are degraded (Hart and Young, 1975; Knapp and Swanson, 1990). The early models of phagosome–lysosome fusion, in which a single kind of phagosome fuses with lysosomes to become a phagolysosome, have been supplanted by models in which phagosomes change progressively before fusing with lysosomes (Beron et al., 1995; Desjardins, 1995). Using fixed *Staphylococcus aureus* as a model particle, Pitt et al. (1992) showed that within 15 min of phagocytosis, phagosomes lose certain membrane proteins such as mannose receptor and Fc receptor and gain proteins such as mannose 6-phosphate receptor (MPR),[1] lysosome-associated membrane proteins (LAMP), and cathepsin D. Desjardins et al. (1994*a*) reported that phagosomes containing latex beads acquire markers of late endocytic organelles and lose markers of early endocytic organelles (Rab 5) during their transformation to phagolysosomes. These studies indicate that the phagosome may undergo multiple interactions with other endocytic compartments before finally meeting the lysosome.

Pathogenic microorganisms that can alter their course inside macrophages are thought to do so by producing molecules that specifically inhibit phagosome–lysosome fusion (Armstrong and Hart, 1971; Joiner et al., 1990; Goren et al., 1976). Given the recent indications that phagosomes undergo some remodeling before fusing with lysosomes, it is possible that there are points before phagosome–lysosome fusion where pathogenic microorganisms could interrupt membrane traffic and prevent delivery into the lysosomal compartment. To date, however, no such early interruptions have been documented.

Although the phagolysosome is generally considered the end point of the phagocytic pathway (Kornfeld and

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1. *Abbreviations used in this paper*: F-BSA, fluorescein-conjugated BSA; FDx, fluorescein dextran; LAMP, lysosome-associated membrane protein; MHC, major histocompatibility complex; MPR, mannose 6-phosphate receptor; PCL, poly-e-caprolactone microspheres; PS, polystyrene microspheres; amine-PS, PS derivatized with amino groups; PEG-PS, polyethylene glycol–conjugated PS; SE, sheep erythrocytes; TRDx, Texas red dextran.

Mellman, 1989; Beron et al., 1995), there are indications that phagolysosomes may continue to change after phagosome–lysosome fusion. Using polystyrene latex beads, Desjardins et al. (1994*b*) reported that annexin VI was more abundant in 24-h-old phagosomes than in 1-h-old phagosomes. Storrie and colleagues showed that lysosomes continually mix and exchange contents by fusion (Ferris et al., 1987). Berthiaume et al. (1995) reported that macromolecules inside phagolysosomes can be separated by size, indicating that lysosomes and phagolysosomes continue to fuse and fragment long after their first encounter.

Macrophages are capable of presenting antigenic peptides to thymocytes (T cells) via both the major histocompatibility complex (MHC) class I and II pathways. Proteins internalized in association with phagocytosed particles generally are degraded to antigenic peptides in endosomes or lysosomes. These peptides associate with MHC class II molecules in endosomes or in special loading compartments (Harding, 1995; Amigorena et al., 1994; Tulp et al., 1994), and the peptide–MHC II complex recycles to the cell surface for presentation to T cells (Germain, 1994). For this processing, proteins or peptides never need to cross membrane bilayers. However, recent studies have shown that some proteins internalized in association with particles can be presented by the MHC class I pathway, which in some cases requires that those proteins first cross a membrane into the cytosolic space before degradation and delivery into the ER for association with MHC class I molecules (Harding, 1995; Kovacsovics-Bankowski and Rock, 1995). It is not yet known to what degree particles contribute to the course or efficiency of protein delivery into either antigen presentation pathway.

Previous studies described the intracellular behavior of phagosomes and phagolysosomes using inert model particles and assumed that such particles contain little information in their structure that would affect their intracellular destination. However, there is wide variation in the reported rates of maturation and phagosome–lysosome fusion (Hart and Young, 1975; Rabinowitz et al., 1992; Wang and Goren, 1987; Desjardins et al., 1994*a*). This could be due to differences between the macrophages studied or between the dynamics of different kinds of particles after phagocytosis. It is still not known whether all phagosomes traffic similarly or change with similar kinetics.

Here, we tested the hypothesis that the particle itself can influence the intracellular trafficking of its phagosome inside a macrophage. Our studies indicate that particle chemistry does not affect the progression of phagosomes to lysosomes, but influences later events. Properties of those phagolysosomes, such as the content of cathepsin L, the accessibility to endocytic tracer molecules, and the degradation of associated proteins, differ between different phagosomes. Our observations indicate that the dynamics of phagolysosomes could be modulated to affect the intracellular processing and retention of antigens.

Materials and Methods

Cell Culture

Macrophages were derived from bone marrow extruded from the femurs of female C3H/He mice (The Jackson Laboratory, Bar Harbor, ME) and cultured as previously described (Racoosin and Swanson, 1989). Cells were harvested after 6 d of culture and plated in wells of 24-well dishes onto 12-mm glass coverslips at a density of 0.8–2.7 × 10^5 cells per well. Plated cells were cultured overnight in DME + 10% heat-inactivated FBS + 100 U/ml pen-strep (DME-10F; Gibco BRL, Gaithersburg, MD).

Preparation of Opsonized Particles

Polystyrene microspheres (PS) (1 μm, diam), PS derivatized with amino groups (amine-PS), and poly-e-caprolactone microspheres (PCL) (0.5–3 μm, diam) were purchased from Polysciences, Inc. (Warrington, PA). To opsonize particles with anti-BSA antibody, particles were initially associated with BSA by nonspecific adsorption or by covalent coupling. To allow nonspecific adsorption of BSA onto particles, PS and PCL were incubated with 10 mg/ml BSA for 1 h at 37°C and washed by centrifugation. To conjugate BSA to PS, amine-PS were first activated by incubating with 0.5% glutaraldehyde overnight, then washed by centrifugation, incubated with BSA (10 mg/ml) for 2 h, and washed again by centrifugation. In some experiments, as indicated in the text, human serum albumin was substituted for BSA. To conjugate albumin and polyethylene glycol to PS, aldehyde-activated amine-PS were incubated with methoxypolyoxyethylene amine (50 mM in PBS; Sigma Chemical Co., St. Louis, MO) for 2 h, washed, then incubated with albumin (10 mg/ml) for 2 h, and washed. Sheep erythrocytes (SE) (Cappel Organon Teknika Corp., Durham, NC) were loaded with fluorescein-conjugated BSA (F-BSA) by suspending washed erythrocytes in hypotonic phosphate buffer (5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 5 mM $MgCl_2$, pH 7.4) containing 3 mg/ml F-BSA at 4°C for 10 min. F-BSA–loaded erythrocyte ghosts were resealed by adding 4.5% NaCl to restore isotonicity (0.9% final concentration). Then they were incubated at 37°C for 30 min and washed free of extracellular F-BSA using PBS and centrifugation.

Particles with associated albumin were opsonized for 30 min with rabbit anti-BSA antibodies or goat anti-human serum albumin antibodies (Cappel Organon Teknika Corp.) in PBS with a dilution ratio of 1:25. Sheep erythrocytes were opsonized with rabbit anti–sheep erythrocyte IgG (Cappel Organon Teknika Corp.) diluted 1:200 in PBS. Particles opsonized with IgG were used in all experiments.

Quantitation of Late Endosome and Lysosome Markers in Phagosomes

To label lysosomes with fluorescein dextran (FDx), average mol wt 10,000, macrophages were incubated with FDx (5 mg/ml; Molecular Probes, Inc., Eugene, OR) in medium for 60 min at 37°C, washed three times with PBS, and incubated in warm PBS without FDx for 60 min. Opsonized particles were added to the FDx-loaded macrophages in cold PBS (1–1.5 × 10^6 per well) and centrifuged at 700 rpm for 5 min to increase binding to cells. Wells were then aspirated to remove the unbound particles, replaced with PBS, and incubated for 2 min at 37°C to allow phagocytosis. To identify particles phagocytosed during the 2-min pulse, the remaining extracellular particles were marked with fluorophore by incubating cells with fixable Texas red dextran (TRDx), average mol wt 10,000 (0.5 mg/ml; Molecular Probes, Inc.), for 1 min at 4°C. TRDx adsorbed rapidly onto any extracellular particles. Wells were then washed with warm PBS and incubated for various times before fixation (20 min in 3.7% formaldehyde, 0.05% glutaraldehyde, 0.25 M sucrose, 1 mM EGTA, 0.5 mM EDTA, and 20 mM Hepes, pH 7.4) and observation by fluorescence microscopy. In the microscope, phase-bright particles not stained with rims of fixable TRDx were counted as intracellular particles phagocytosed during the pulse. In all experiments, the total number of particles per cell was in the range of 5–30, and 20–30% of cell-associated particles were internalized during the pulse (i.e., contained no TRDx). Of these intracellular particles, those with rims of FDx were counted as the phagosomes that fused with lysosomes (see Fig. 1 *A*).

For immunolabeling of lysosomes, macrophages were pulsed with particles for 2 min at 37°C, incubated with fixable TRDx for 1 min at 4°C, and chased in PBS at 37°C (see Fig. 1 *B*). At various times, cells were fixed at room temperature for 20 min using a modified paraformaldehyde-lysine-periodate fixative (McLean and Nakane, 1974) (final concentration: 70 mM NaCl, 5 mM KCl, 70 mM lysine-HCl, 5 mM $MgCl_2$, 2 mM EGTA, 10 mM $NaIO_4$, 3.7% paraformaldehyde, 4.5% sucrose, 20 mM MES, pH 7.5). Next, cells were washed three times with TBS and permeabilized for 20 s in −20°C methanol. Cells were then washed three times with TBS + 2% heat-inactivated goat serum and incubated for 1–3 h with affinity-purified rabbit antibody against cation-independent mannose 6-phosphate receptor (anti-MPR) (MacDonald et al., 1989), with rat anti–LAMP-1 mAb

(No. 1D4B; Developmental Studies Hybridoma Bank, Dept. of Biology, Univ. of Iowa, Iowa City, IA) (Chen et al., 1985*a*), with rabbit polyclonal anti-cathepsin L antibody (No. MEP-11-3) (Dong et al., 1989), a gift from Dr. Gary Sahagian (Tufts University, Boston, MA), or with rabbit polyclonal anti–cathepsin D antibody, a gift from Dr. Sadaki Yokota (Yamanashi Medical School, Japan). Macrophages were then washed three times with TBS + 2% heat-inactivated goat serum and incubated for 1 h with fluorescein-conjugated secondary antibodies (Vector Laboratories, Inc., Burlingame, CA). Finally, cells were washed with several changes of TBS + 2% heat-inactivated goat serum, mounted in a photostabilizer (90% glycerol, 10% phosphate buffer, 1 mg/ml phenylenediamine), and observed using a fluorescence microscope (photoskop III; Carl Zeiss, Inc., Thornwood, NY). Particles without associated TRDx were scored as intracellular particles phagocytosed during the pulse. Of these intracellular particles, those with rims of fluorescein-conjugated secondary antibodies were counted as phagosomes positive for LAMP-1, cathepsin L, cathepsin D, or MPR (see Figs. 1 *B* and 2). For each condition, at least 50 TRDx-negative particles were scored for the presence of fluorescein. Data from two to four separate experiments were pooled and expressed as mean ± SEM. Micrographs were taken using TMAX 400 or P3200 film (Eastman Kodak Co., Rochester, NY) at ASA 1600.

Delivery of Subsequently Endocytosed Contents into Phagosomes

Delivery of subsequently endocytosed fluorophore into various phagosomes was measured microscopically. Macrophages on coverslips were incubated for 10–20 min with opsonized particles. Cells were then washed and chased for variable periods, pulsed 5 min with TRDx (1 mg/ml), washed, and incubated for another variable interval (I_{TRDx}) in DME-10F (see protocol in Fig. 6 *A*). At 120 min after phagocytosis, cells were washed, fixed with 3.7% formaldehyde, and prepared for microscopy. For each coverslip, at least 100 phagosomes were scored for the presence of TRDx.

Measurement of F-BSA Degradation

To prepare F-BSA, 25 μl of 26 mM fluorescein 5-isothiocyanate (Molecular Probes, Inc.) in dimethyl formamide (final concentration: 640 μM) was added to BSA (10 mg in 1 ml) in 50 mM carbonate buffer, pH 9.2, and then stirred for 2 h. Next, 10 mg/ml glycine was added to the mixture to stop the reaction (final concentration: 1 mg/ml), and incubation continued for 15 min. The F-BSA conjugates were then separated from other reactants using a Sephadex G-25 column (Sigma Chemical Co.) equilibrated with PBS. The resulting F-BSA had a fluorescein/BSA molar ratio of ~2, as indicated by the absorbance of BSA and fluorescein at 280 nm and 495 nm, respectively.

Cells plated into 24-well culture dishes at 2.5–2.7 × 10^5 per well were incubated with free F-BSA (8.5 mg/ml), or with various particles containing associated F-BSA. PS and PCL both contained 5–8 pmol F-BSA per 10^6 particles. After 10 to 20 min, cells were washed and incubated further in DME-10F. F-BSA degradation was measured by the method of Twining (1984), with modifications. At various times, duplicate samples were washed three times with PBS and then lysed with 0.2 ml of 0.25% sodium dodecyl sulfate. To obtain fluorescence of degraded F-BSA, one sample was mixed with 0.2 ml of cold TCA (20%) and placed in an ice bath for 1 h. To obtain total fluorescence, the other sample was mixed with 0.2 ml of 2% trypsin and incubated at 37°C for 1–4 h. Particles and insoluble proteins of both samples were sedimented by centrifugation. To measure the fluorescence of each lysate at the same pH , the supernatants (350 μl) of trypsin- and TCA-treated lysates were diluted with 1.6 ml of 0.5 M carbonate buffer, adjusted to pH 9.6 and 10.1, respectively. Fluorescein fluorescence was measured with a spectrofluorometer (SPF-500C; SLM/AMINCO, Urbana, IL) at an excitation wavelength of 495 nm and an emission wavelength of 514 nm. The percentage of degraded protein was calculated as TCA-soluble fluorescence released from lysed cells divided by total fluorescence released in the trypsin-treated samples × 100.

Results

Phagosomes Progressed to Lysosomes at Similar Rates

To determine whether particle chemistry affects the progression of phagosomes to lysosomes, we measured the kinetics of phagosome–lysosome fusion for phagosomes containing various particles opsonized with IgG. These included SE, biodegradable PCL (Pitt, 1990), or nonbiodegradable PS. Resolution of these kinetics was enhanced by a method that identified only those particles internalized within a 2-min period (Figs. 1 and 2).

All phagosomes containing opsonized particles acquired lysosomal markers at similar rates. Staining for MPR increased shortly after phagocytosis and then decreased rapidly to a low level (Fig. 3 *A*), consistent with a maturation process in which phagosomes transiently acquire late endosome-like properties. The lysosomal membrane glycoprotein LAMP-1 (Chen et al., 1985*b*) was absent from phagosomes after short chase periods, with fewer than 6% staining positively. After a 15-min chase, however, >90%

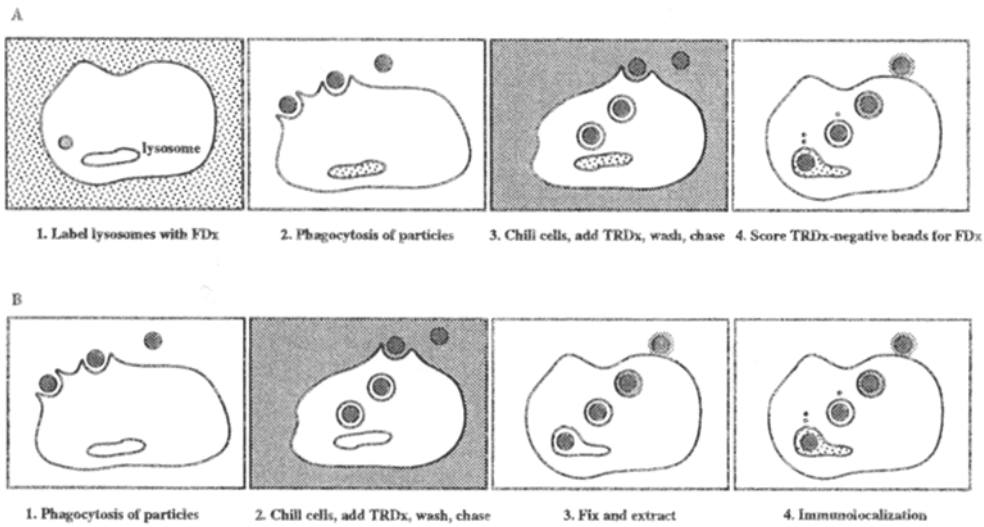


Figure 1. Identification of a cohort of phagosomes progressing toward lysosomes. (*A*) Macrophage lysosomes were loaded with FDx by pinocytosis. FDx-loaded cells were pulsed for 2 min with particles, incubated briefly in the cold with fixable TRDx, and then incubated at 37°C before fixation. Fixed cells were observed by phase-contrast microscopy and by fluorescence microscopy using fluorescein and Texas red filters. Phase-bright but Texas red–negative particles (*, **) were counted as intracellular particles internalized during the pulse. Phagosomes containing Texas red–negative but fluorescein-positive particles (**) were scored as ones that had fused with lysosomes. (*B*) Macrophages were pulsed with particles, then with fixable TRDx, and then incubated at 37°C before fixation. Fixed and extracted cells were stained with primary antibody and then with fluorescein-conjugated secondary antibody. Phagosomes were scored by the same criteria as in *A*.

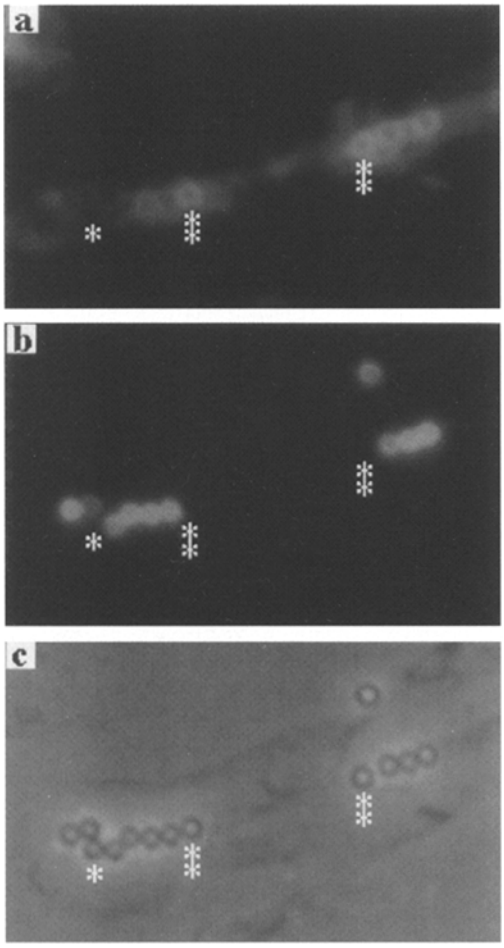


Figure 2. Representative images of phagosomes fused with lysosomes. Macrophages were pulsed 2 min with PS, chilled and incubated with fixable TRDx, and then washed and incubated for 10 min at 37°C before fixation. Fixed cells were stained with rabbit anti-cathepsin L antibody and then with fluorescein-conjugated goat anti-rabbit IgG-antibody. (*a*) Fluorescein image, indicating cathepsin L; (*b*) Texas red image, indicating excluded beads; (*c*) phase-contrast image. Phase-bright but Texas red–negative particles (*, **) were counted as intracellular particles. Of these intracellular particles, colocalization with cathepsin L was indicated by the presence of fluorescein (**).

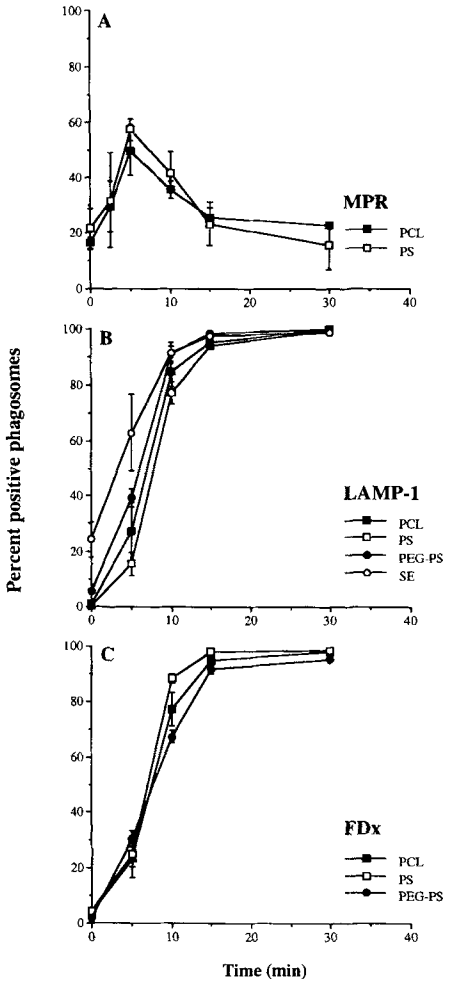


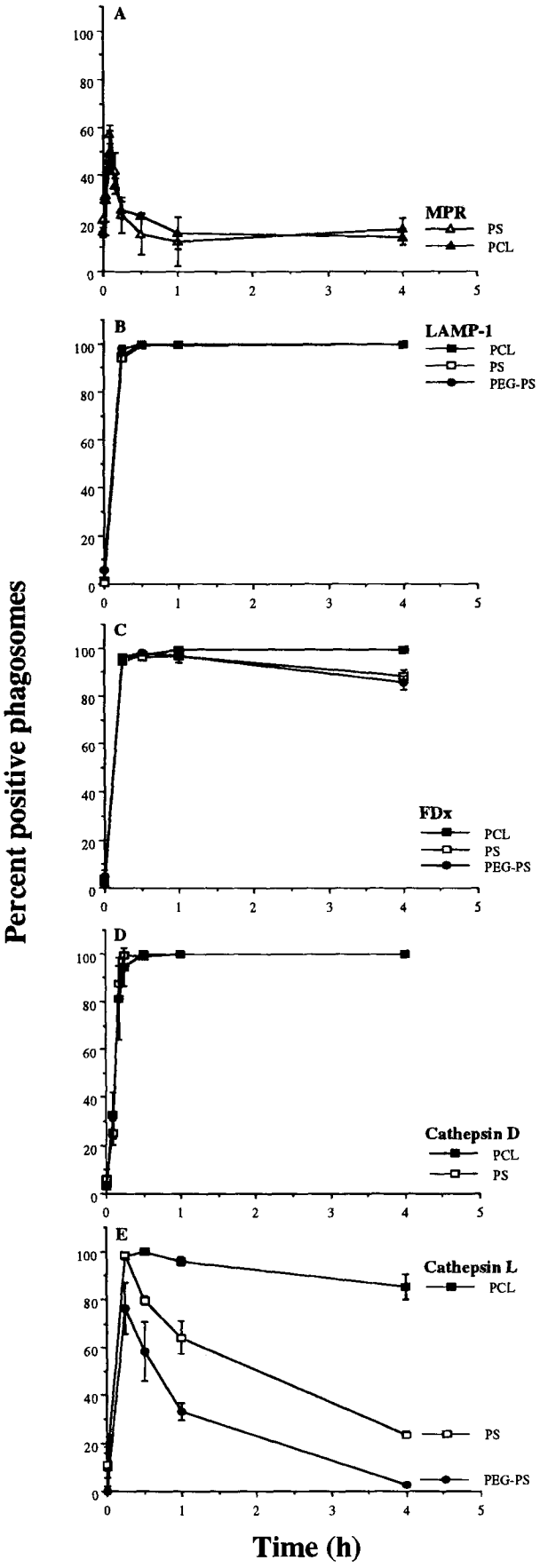
Figure 3. Phagosomes progressed to lysosomes at similar rates. Macrophages were examined using phase-contrast and epifluorescence microscopy. Phagosomes of the same age were identified as described in Figs. 1 and 2, and the results are shown as the percentage of Texas Red–negative phagosomes that stained positively for MPR (*A*), LAMP-1 (*B*), or FDx that had been preloaded into lysosomes by endocytosis (*C*). Each point represents the average of three to four separate experiments ± SEM.

of the phagosomes had acquired LAMP-1, and by 30 min, all of the particles were in LAMP-1–positive compartments (Fig. 3 *B*). Similarly, when FDx was preloaded into lysosomes by endocytosis, by 15 min after phagocytosis, >90% of phagosomes contained FDx (Fig. 3 *C*). Thus, using LAMP-1 and FDx as lysosome markers, phagosome–lysosome fusion was similar in rate and extent for all particles tested. The rates of phagosome delivery into the lysosomes were similar to the rates measured for macropinosomes (Racoosin and Swanson, 1993). The principal difference between the maturation of phagosomes and macropinosomes was in the dynamics of MPR labeling.

Different Retention of Cathepsin L in Phagolysosomes

Although early postphagocytic events were much the same for each particle, later characteristics varied considerably. SE were difficult to monitor after early time points, as they first merged together into larger phagolysosomes and then disintegrated into tubules and smaller vesicles (see also Knapp and Swanson, 1990; Berthiaume et al., 1995). The synthetic particles were easier to trace for long periods and allowed quantitative study of later events. Five markers were localized: LAMP-1, FDx preloaded into lysosomes by endocytosis, MPR, and the lysosomal proteases cathepsin L (Kirschke et al., 1977) and cathepsin D. Staining for MPR remained low after the initial transient increase (Fig. 4 *A*). Phagosomes containing solid particles all retained LAMP-1 until 4 h after phagocytosis (Fig. 4 *B*). For PCL phagolysosomes, FDx that entered the phagosome from preloaded lysosomes remained associated with the particles at 4 h after phagocytosis. For PS phagolysosomes, this FDx staining decreased slightly, with ~88–90% retaining FDx at 4 h (Fig. 4 *C*). Cathepsin D labeling remained high in all phagolysosomes (Fig. 4 *D*).

More striking changes were observed for cathepsin L.



All phagosomes reached maximal labeling for cathepsin L at 15 min after phagocytosis (Fig. 4 *E*). Thereafter, PCL phagosomes continued to stain for cathepsin L, whereas PS and polyethylene glycol–conjugated PS (PEG-PS) phagosomes lost the enzyme. At 4 h after phagocytosis, 85% of PCL phagolysosomes retained cathepsin L, but only 23% of PS phagolysosomes and 3% of PEG-PS phagolysosomes stained positively. Fig. 5 shows this selective loss of cathepsin L staining in representative PS phagolysosomes. The phagosomes were without cathepsin D (Fig. 5 *A*) or cathepsin L (Fig. 5 *D*) initially after phagocytosis. At 15 min after phagocytosis, PS phagosomes showed rims of cathepsin D (Fig. 5 *B*) and cathepsin L (Fig. 5 *E*), but by 4 h after phagocytosis, they retained cathepsin D (Fig. 5 *C*) but had lost cathepsin L (Fig. 5 *F*). The fact that cathepsin D staining remained high indicated that the loss of cathepsin L was not due to an MPR-dependent sorting mechanism.

Different Accessibility of Phagolysosomes to Endocytic Tracers

Endocytic compartments are usually accessible to subsequently added tracer molecules (Ferris et al., 1987; Knapp and Swanson, 1990; Berthiaume et al., 1995). To test whether phagolysosomes containing various particles traffic differently, we measured the accessibility of phagolysosomes to subsequently added endocytic tracer TRDx (Fig. 6 *A*). We used a protocol in which all phagosomes were 120 min old, and TRDx was allowed variable periods within that 120 min to reach phagosomes. PCL phagolysosomes showed a gradual increase in the TRDx-positive fraction with increasing TRDx incubation times (Fig. 6 *B*). However, PS phagolysosomes showed little colocalization with TRDx even after the longest chase interval. PEG-PS phagolysosomes were least accessible, showing <3% colocalization throughout the period tested.

We next considered if PS phagolysosomes inhibited vesicle traffic in the macrophages generally. Macrophages were fed a mixture of PEG-PS and PCL, then were chased, pulsed with TRDx, washed, and chased again in a protocol like that of Fig. 6 *A*. The two kinds of particles could be distinguished inside macrophages by phase-contrast microscopy. In cells that contained both PEG-PS and PCL, PEG-PS phagolysosomes were inaccessible to subsequently added TRDx and did not exceed 4% colocalization, whereas PCL phagolysosomes remained accessible, reaching 61% colocalization at 90-min TRDx incubation time (data not shown).

Different Degradation Rates of Phagocytosed F-BSA

To determine whether proteins phagocytosed by mac-

Figure 4. The remodeling of phagolysosomes containing various particles. Texas red–negative phagosomes were scored for the presence of MPR (*A*), LAMP-1 (*B*), FDx that had been previously loaded into lysosomes (*C*), cathepsin D (*D*), or cathepsin L (*E*) at the indicated times after phagocytosis. Phagosomes of the same age were identified as described in Fig. 1, and the results are shown as the percentage of Texas red–negative phagosomes that contained FDx or fluorescein-labeled secondary antibody. Each point represents the average of two to four separate experiments ± SEM.

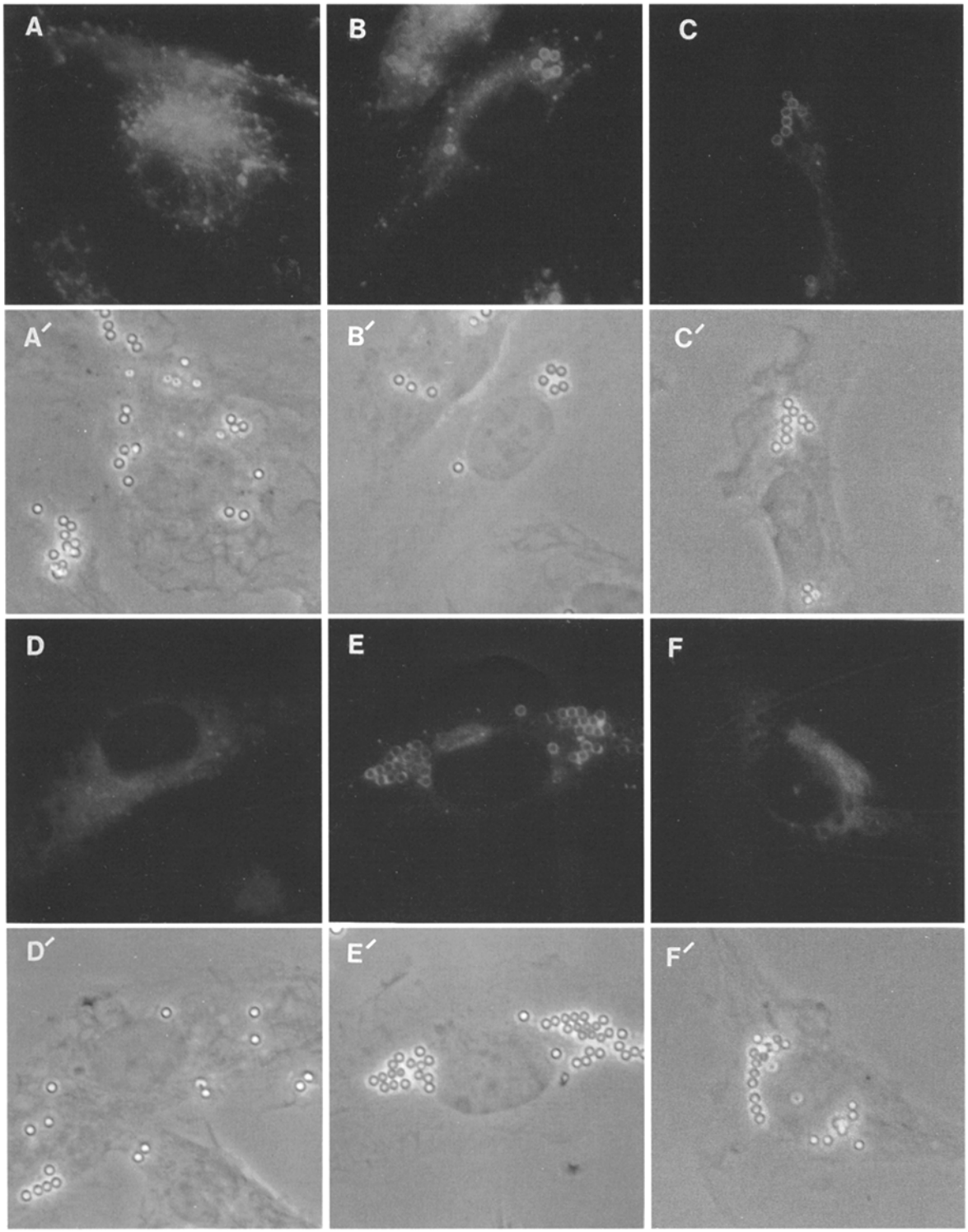


Figure 5. Distribution of cathepsin D and cathepsin L in phagosomes of different ages. Macrophages were fixed at 0 min (*A* and *D*), 15 min (*B* and *E*), and 4 h (*C* and *F*) after a pulse with PS, stained with primary antibodies against cathepsin D (*A–C*) or cathepsin L (*D–F*), and then stained with fluorescein-labeled secondary antibodies. Corresponding phase-contrast images (*A′–F′*) are shown in the row beneath each fluorescence image.

rophages are degraded differently than pinocytosed proteins, and if so, whether the nature of protein-carrying particles affects the protein degradation rate, we measured the degradation of F-BSA internalized by macrophages in various ways. F-BSA internalized by pinocytosis was degraded faster than F-BSA associated with particles, showing 65% degradation at 15 min and 85% at 30 min after pinocytosis (Fig. 7 *A*). Degradation rates of F-BSA associated with particles were slower and were different for different particles. The time required to degrade 50% of the

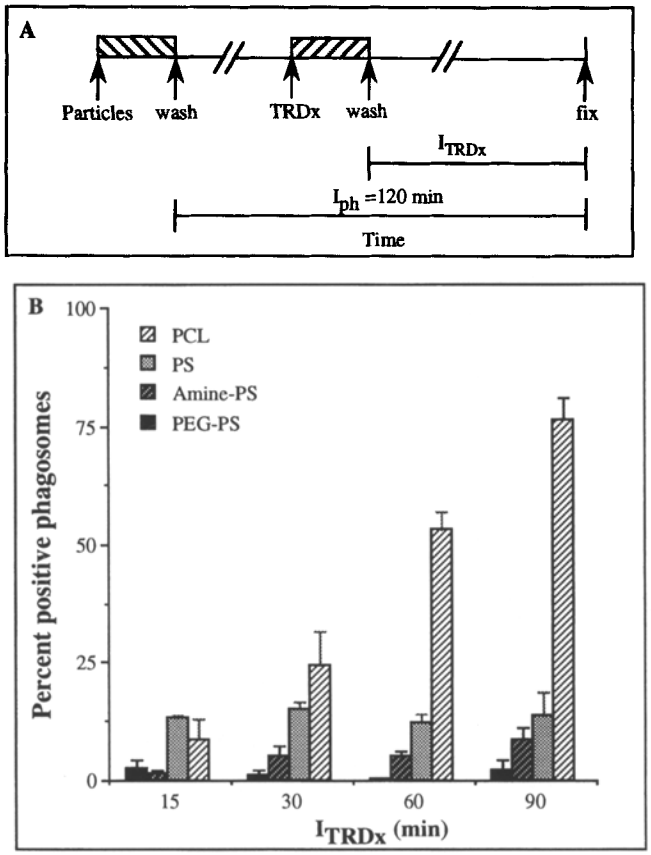


Figure 6. Different accessibility of various phagosomes to subsequently added endocytic tracers. (*A*) Macrophages were allowed to phagocytose PCL, PS, or PEG-PS, then were washed and chased, incubated with TRDx for 5 min, and washed and chased for a variable period (I_{TRDx}) before fixation. In all conditions, the interval between the removal of unphagocytosed particles and fixation (I_{ph}) was 120 min. (*B*) For each coverslip, at least 150 phagosomes were scored for the presence of TRDx. Each point represents the average of four separate experiments ± SEM.

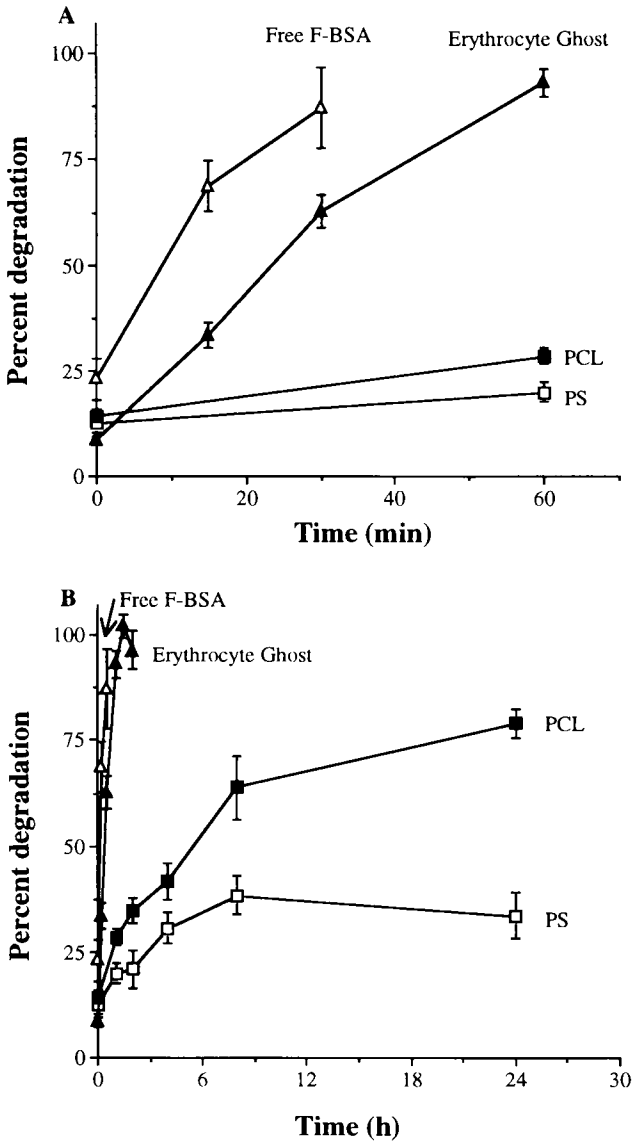


Figure 7. Degradation of F-BSA on various particles. Macrophages were incubated with free F-BSA or F-BSA–loaded particles. At various times after phagocytosis, cells were lysed with detergent and then total and TCA-soluble fluorescein fluorescence of these lysates were quantified. The percentage of F-BSA degradation was calculated as 100 × the fraction of TCA-soluble fluorescence divided by total cell fluorescence. (*A*) Short time points. (*B*) Long time points. Each point represents the average of four separate experiments ± SEM.

protein was 30 min for erythrocyte ghosts, 6 h for PCL, and longer than 24 h for PS (Fig. 7 *B*). The degradation kinetics of F-BSA on PS or PCL was not significantly affected by the extent of F-BSA coating on beads. Similar kinetics of degradation was observed for PCL with fivefold higher F-BSA per bead (data not shown). Thus, F-BSA degraded more slowly when associated with particles that became isolated from postlysosomal membrane traffic.

Discussion

Macrophages that ingest particles by phagocytosis deliver them quickly into the lysosomal compartment. This study found that although different kinds of particles all progressed to lysosomes with similar kinetics, the subsequent dynamics of phagolysosomes differed for different particles. Phagolysosomes varied in cathepsin L retention, accessibility to subsequently added endocytic tracers, and degradation of associated proteins. This indicates that continued interactions between phagolysosomes and lysosomes are necessary for the complete degradation of particle-associated molecules. These observations have implications for studies of phagocytosis and pathogenesis, and for the design of therapeutic protein delivery systems.

Early Events after Phagocytosis Are Similar for All Particles

Our results are consistent with earlier studies that found rapid progression of phagosomes to lysosomes. Desjardins et al. (1994*a*), using polystyrene latex beads, measured phagosomes that formed during a 20-min pulse and observed a sixfold increase in the concentration of LAMP-2 from 0 to 2 h after phagocytosis, their shortest chase time.

Pitt et al. (1992) found that phagosomes containing *S. aureus* rapidly lost markers of early endosomes and gained markers of late endosomes and lysosomes. In the present study, a method for identifying particles phagocytosed during a 2-min pulse made it possible to follow the progression of an age-matched cohort of phagosomes in intact cells. The refined kinetics revealed that the most active period for the progression of phagosomes to lysosomes occurred 5–10 min after phagocytosis. Phagosomes transiently acquired MPR, a marker commonly associated with late endosomes (Kornfeld and Mellman, 1989) and then merged completely with the lysosomal compartment. Although the study of Desjardins et al. (1994*a*) differs from ours in that the particles they followed were not opsonized with IgG, we have observed that the kinetics do not differ between phagosomes containing opsonized particles and unopsonized particles (data not shown). Furthermore, the size of particles in the range of 0.5–3 μm did not affect the kinetics of phagosome–lysosome fusion (data not shown).

The kinetics of phagosome maturation were similar to those of macropinosomes (Racoosin and Swanson, 1993). Like the phagosomes measured here, macropinosomes acquired lysosomal markers within 15 min of their formation. Inexplicably, the kinetics of MPR labeling of macropinosomes and phagosomes differed considerably. MPR labeling increased in macropinosomes coincident with other lysosomal markers, whereas MPR labeling of phagosomes increased shortly after phagocytosis and then decreased to low levels coincident with phagosome–lysosome fusion.

Later Events Vary

Although all phagosomes progressed to lysosomes at similar rates, their subsequent fates differed depending on the chemistry of the phagocytosed particles. In general, the more removed a particle's phagolysosome was from endocytic traffic, the more slowly its contents were degraded. The different accessibility of phagosomes containing both PCL and PEG-PS in the same macrophages indicated that PS or PEG-PS affected the dynamics of its phagosome locally rather than by inhibiting lysosome traffic throughout the cell. Although phagosomes containing more hydrophilic PEG-PS became somewhat more isolated than PS without PEG, phagolysosomes containing PEG-labeled PCL did not change cathepsin L staining after 4 h, relative to PCL (data not shown). Other attempts to manipulate the surface chemistry of these particles so as to modify their behavior have thus far not altered phagolysosome dynamics significantly. Therefore, we do not yet know what chemical features of particle surfaces determine their dynamics. In unpublished work, we have found that phagosomes containing zymosan are readily accessible to fluid-phase tracers, in agreement with previous reports that phagosomes containing zymosan can fuse with parasitophorous vacuoles, whereas phagosomes containing PS do not (Veras et al., 1992, 1994).

The selective loss of cathepsin L from PS phagolysosomes is most likely a consequence of their isolation within the macrophage cytoplasm. Although it is possible that cathepsin L was selectively removed by MPR-dependent transport (Prence et al., 1990), we consider this unlikely because cathepsin D, which is also subject to MPR-sorting mechanisms, remained in the PS phagolysosomes. Rather, we suggest that the loss of cathepsin L from PS phagolysosomes resulted from a combination of enzyme degradation without replacement. Cathepsin L has a short half-life (Portnoy et al., 1986). This would not normally result in loss of cathepsin L labeling of lysosomes because lysosomes usually continue to mix their contents with incoming endocytic vesicles and with other lysosomes (Ferris et al., 1987; Knapp and Swanson, 1990; Berthiaume et al., 1995). The isolation of PS phagolysosomes, as evidenced by their low accessibility to TRDx, indicates that the initial dose of cathepsin L that accompanies phagosome–lysosome fusion may be all that they get. Without continued interactions with lysosomes or Golgi-derived vesicles containing cathepsin L, the PS phagolysosomes would become depleted of the enzyme by proteolytic degradation.

The different levels of cathepsin L in phagolysosomes also correlate with the degradation rates of particle-associated F-BSA. Phagolysosomes with a higher content of cathepsin L showed gradual degradation of F-BSA, and phagolysosomes that lost cathepsin L showed little F-BSA degradation after 4 h. Furthermore, the removal of PS phagolysosomes from the normal endocytic pathway could limit the degradation of F-BSA delivered on PS. The similar progression kinetics of various phagosomes to lysosomes (Fig. 3) makes it unlikely that the lower degradation of F-BSA on PS is due to a limited initial fusion of PS phagosomes with lysosomes. Rather, it appears that efficient degradation of particle-associated proteins requires continuous and repeated engagement of phagolysosomes by lysosomes.

SE phagosomes could not be characterized with cathepsin L because their phagosomes fragmented rapidly and disintegrated to small fluorescent tubules and vesicles within 1 h (Knapp and Swanson, 1990; and Oh, Y.-K., unpublished observations). However, earlier studies indicated that SE phagolysosomes continue to interact with other endocytic compartments (Berthiaume et al., 1995). In this study, the rate of F-BSA degradation correlated well with SE disintegration in the cells: as more SE phagosomes disintegrated, the degradation percentage of F-BSA increased.

Implications for Pathogenesis

Some pathogenic microorganisms that survive inside macrophages do so because they can alter the normal course of phagosomes toward the lysosomes. In light of the present work, it may be worth considering whether some pathogens that go directly to lysosomes might survive by inhibiting further interactions with lysosomes. A microorganism might survive using properties like PS particles, reducing the continued engagement by lysosomes that usually follows phagosome–lysosome fusion.

The intracellular dynamics of synthetic particles have been used as controls in studying the dynamics of phagosomes containing bacteria (Garcia-del Portillo and Finlay, 1995; Rabinowitz et al., 1992; Sturgill-Koszycki et al., 1994). Our results indicating that different synthetic particles

behave differently make it difficult to generalize about the properties of phagosomes using only one kind of particle.

Implications for Antigen Presentation and Protein Delivery Systems

Synthetic particles serve not only as tools to study phagosome dynamics, but they have also shown promise as vaccine delivery systems (Alonso et al., 1993; Morris et al., 1994). As noted earlier, phagocytosis by macrophages can deliver antigens into either MHC class I or II presentation pathways. Presently it is not known how particle chemistry affects delivery into either system. From the observations reported here, that proteins associated with different particles degrade at different rates, we would suggest that some particles may deliver more efficiently than others.

Thus, the degradation of particles that begins with phagosome–lysosome fusion requires continued interaction with lysosomes for completion. Based on the kinetics of degradation, phagocytosed F-BSA resides in cells longer than F-BSA taken in by pinocytosis. Prolonged retention by association with particles could be exploited to increase the half-life of therapeutic molecules. This may also guide the rational design of synthetic particles as drug or antigen delivery systems.

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