Transport of Phagosomal Components to an Endosomal Compartment*

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The participation of phagosomes in interorganellar protein and membrane exchange is important to the maturation of phagosomes into phagolysosomes. To investigate this process, we have developed an assay to measure protein transport from phagosomes to other vesicle populations. J774-E clone macrophages phagocytosed 125I-anti-dinitrophenol IgG-coated Staphylococcus aureus for 3 min followed by chase for intervals of 0–30 min. Following cell fractionation, the intracellular distribution of radioiodinated protein was assayed. We observed a time-dependent increase radioiodinated protein in a non-phagosome vesicle fraction which displayed endosome characteristics. Concomitantly, radioiodinated protein within phagosomes decreased over the chase period. As assessed via Percoll density gradient fractionation, the phagocytosed radiiodinated protein migrated to both heavy (lysosome density) and light (endosome density) vesicle populations. Characterization of the fusogenic properties of the transport vesicles demonstrated that they are capable of in vitro fusion with early endosomes. Furthermore, this fusion event shares many of the biochemical requirements identified for phagosome-endosome and endosome-endosome fusion. Morphological analysis of phagosome maturation provides additional evidence for phagosome to endosome transport. These results suggest phagocytosed material is transferred from phagosomes to endosomes and then recycled out of the cell.

Phagocytosis, the receptor-mediated engulfment of large extracellular particles, is a specialized form of endocytosis (1). In vertebrates, mononuclear phagocytes and polymorphonuclear leukocytes are the primary phagocytic cells. Phagocytosis initiates when particles bind cell surface receptors and stimulate engulfment by the host cell plasma membrane. The distal regions of plasma membrane encompassing the phagocytic particle eventually meet and fuse, resulting in the formation of a phagosome. Following engulfment, extensive membrane exchange transforms the plasma membrane-derived phagosome into an acidic and protease-rich phagolysosome.

Membrane fusion among endosomes, lysosomes, and phagosomes is critical to phagolysosome formation. Mayorga et al. (2) recently demonstrated in vivo that extensive endosome-phagosome fusion precedes lysosome-phagosome fusion. Protein and membrane recycling from phagosomes also contributes to the formation of the phagolysosome. During periods of high phagocytic activity, up to 40% of the plasma membrane may be internalized in as short as 15 min (1). Because the cell surface area does not significantly change during this period, translocation of intracellular pools of membrane to the cell surface is postulated to occur (3). While endosomal and Golgi membranes may replenish some of the internalized plasma membrane, recycling phagosomal membrane may also contribute to this membrane flux. Muller et al. (4) showed that many phagosomal proteins rapidly recycle to the plasma membrane following phagosome formation. These authors postulated that small transport vesicles mediate this membrane recycling. In addition to membrane recycling, membrane synthesis may play a role in compensating for membrane loss from the cell surface. Werb and Cohn (5) reported that phagocytosis of indigestable latex beads leads to a stimulation of plasma membrane synthesis. They inferred that because the phagosome could not digest its contents, it could not undergo the size reduction necessary for membrane recycling. Consequently, new membrane synthesis was required. Thus, the influx and efflux of material from phagosomes is a vital element in their transformation to phagolysosomes and probably necessary to their function.

Employing an in vitro assay, our laboratory recently determined that the endosome-phagosome fusion event is biochemically very similar to endosome-endosome fusion (2). Furthermore, the introduction of early endosomal membrane and protein to early phagosomes may bestow early endosome functions, including protein recycling, to the early phagosome. Therefore, we have now developed an assay to investigate phagosome protein efflux. Herein we report on the time-dependent migration of a phagosome marker to small vesicles which display some features characteristic of endosomal vesicles. Furthermore, we provide morphological evidence that phagosome membrane efflux may involve the same recycling mechanism utilized by early endosomes.

**EXPERIMENTAL PROCEDURES**

*Biological Materials—J774-E clone, a mannose-receptor positive macrophage cell line, was grown in minimum essential medium containing Earle's salts and supplemented with 10% fetal calf serum. HDP-1, a mouse IgG1 monoclonal antibody specific for dinitrophenol (anti-DNP IgG)† was isolated as described previously (6). β-Glucu-

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1 The abbreviations used are: anti-DNP IgG, anti-dinitrophenol IgG; BSA, bovine serum albumin; DNP, dinitrophenol; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] aminoethanesulfonic acid; HBSA, HBSS with 0.1% BSA; EGTA, (ethylenebis(oxyethylenenitrilo)tetraacetic acid; GTPγS, guanosine 5'-O-(3-thiotriphosphate).
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Ronidase was isolated from rat preputial glands as described (7). Bovine serum albumin (BSA) and β-glucuronidase were derivatized with dinitrophenol (DNP) by using dinitrofluorobenzene (8). Anti-DNP IgG was radiolabeled with 125I using chloramine T (9). Cytosol was the high-speed supernatant of a cell homogenate obtained as described (10) and solution buffered with 10 mm HEPES, pH 7.4, and supplemented with 1% BSA. After incubating 200 μl of S. aureus (200 μl of a 10% suspension, approximately 4 x 10⁶ particles/μl, 2 mg of IgG/ml-binding capacity) with 200 μg of rabbit anti-mouse IgG polyclonal antibody (IgG fraction, Organon Teknika Corporation) for 1 h at 20 °C, the particles were washed three times in HBSA and incubated with 25 μg of anti-DNP IgG for 1 h at 20 °C. Coated S. aureus was washed three times and resuspended in HBSA. Phagosomal particles without 125I-anti-DNP IgG were inefficiently internalized, indicating that particle uptake is antibody- (i.e. Fc receptor)-mediated.

**Phagocytic Probe—**Formaldehyde-fixed Staphylococcus aureus (IgG-borne, The Enzyme Center) was washed with HBSA (Hanks' balanced salt solution buffered with 10 mm HEPES, pH 7.4, and supplemented with 1% BSA). After incubating 200 μl of S. aureus (200 μl of a 10% suspension, approximately 4 x 10⁶ particles/μl, 2 mg of IgG/ml-binding capacity) with 200 μg of rabbit anti-mouse IgG polyclonal antibody (IgG fraction, Organon Teknika Corporation) for 1 h at 20 °C, the particles were washed three times in HBSA and incubated with 25 μg of anti-DNP IgG for 1 h at 20 °C. The pellets from both high-speed centrifugations were resuspended in 50,000 g at 4 °C. The pellets were discarded. The supernatants were combined and centrifuged at 12,000 g for the indicated chase times. After cooling with prewarmed HBSA for the indicated times. After cooling with prewarmed HBSA for the indicated chase times. After cooling with prewarmed HBSA for the indicated chase times. After cooling with prewarmed HBSA for the indicated chase times. After cooling with prewarmed HBSA for the indicated chase times. After cooling with prewarmed HBSA for the indicated chase times.

**Fusion Reaction—**Vesicles fractions—J774-E clone macrophages (1 x 10⁶ cells) were incubated with antibody-coated S. aureus (100 μl) for 1 h at 4 °C. Uptake was initiated by the addition of prewarmed HBSA. After either 3 or 5 min at 37 °C, the incubation was stopped by the addition of ice-cold HBSA. Cells were subsequently washed three times (300 x 10⁵ cells at 4 °C and centrifuged at 4 °C and then chased by the addition of prewarmed HBSA for the indicated chase times. After cooling with HBSA at 4 °C, the cells were washed two times in 250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES-KOH, pH 7.2 (HBE), resuspended in HBE to 2 x 10⁶ cells/ml, and then homogenized in a ball-bearing homogenizer. Homogenates were centrifuged at 400 x 10⁵ for 3 min to eliminate nuclei and intact cells. The supernatants were quickly frozen in liquid nitrogen and stored at −80 °C. As described previously (2), phagosomes were prepared by diluting a quickly thawed postnuclear supernatant aliquot in 1 ml of HBE and centrifuging at 12,000 x 10⁵ g at 4 °C in a microfuge for 10 s at 4 °C. The pellet was resuspended in 0.5 ml of HBE at 4 °C and centrifuged at 12,000 x 10⁵ for 10 s, and then the centrifugation pellet was discarded. The supernatants were combined and centrifuged at 12,000 x 10⁵ g for 1.5 min at 4 °C. The pellet is referred to as the phagosome pellet, and the supernatant is referred to as the 12,000 g supernatant. Fractionation of the 12,000 g supernatant involved diluting the supernatant to 3 ml in HBE and centrifugation for 1 min at 55,000 x 10⁵ g at 4 °C in a Beckman TLA 100.3 rotor. The resultant supernatant was subsequently centrifuged for 5 min at 50,000 x 10⁵ g at 4 °C. The pellets from both high-speed centrifugations were resuspended in HBE. DNP-β-glucuronidase-containing early endosomes diluting the supernatant to 3 ml in HBE and centrifugation for 1 min at 55,000 x 10⁵ g at 4 °C in a Beckman TLA 100.3 rotor. The resultant supernatant was subsequently centrifuged for 5 min at 50,000 x 10⁵ g at 4 °C. The pellets from both high-speed centrifugations were resuspended in HBE. DNP-β-glucuronidase-containing early endosomes diluting the supernatant to 3 ml in HBE and centrifugation for 1 min at 55,000 x 10⁵ g at 4 °C in a Beckman TLA 100.3 rotor.
min at 37 °C. Thus, because all cell-associated radioactive protein was intracellular prior to the 37 °C chase, these results strongly suggest that an intracellular pathway exists which is capable of transporting protein from phagosomes to the cell surface.

To determine whether a non-phagosome vesicle population might contribute to the secretion of phagocytosed 125I-anti-DNP IgG and fragments thereof, we subfractionated cells which had phagocytosed S. aureus 125I-anti-DNP IgG for 3 min followed by incubation for 0–30 min at 37 °C. To separate phagosomes from other vesicles, post-nuclear supernatants were initially centrifuged at 12,000 × g for 1.5 min. This method efficiently pellets S. aureus-containing phagosomes while leaving smaller vesicles such as endosomes in the supernatant (2). As shown in Fig. 2A, the phagosome fraction displays a time-dependent decrease in radioiodinated protein. Concomitantly, the 12,000 × g supernatant progressively acquires radioiodinated protein. Also, as the radioiodinated protein remaining in the phagosomal fraction decreases with chase time, the percent of phagosomal-radioiodinated protein that remains specifically associated with the S. aureus particle decreased (Fig. 2A). That is, at 0 min of chase, approximately 90% of the radioactivity remained associated with the S. aureus following lysis of the phagosome with Triton X-100. However, after 30 min of chase, approximately 30% of the radioactivity remained associated with the S. aureus particle after lysis. Lastly, because formaldehyde-fixed S. aureus is not significantly degraded within the time course of this experiment (see electron micrographs below), the sedimentation properties of the phagosomes are probably unaffected over these chase periods. Therefore, the appearance of radioiodinated protein in the 12,000 × g supernatant represents 125I-anti-DNP IgG or fragments thereof that left the phagosome.

The 12,000 × g supernatant was further fractionated in order to localize the non-phagosomal radioiodinated protein. Centrifugation of the 12,000 × g supernatant at 35,000 × g for 1 min followed by centrifugation at 50,000 × g for 5 min served to isolate two membrane populations and the non-particulate, cytosol fraction. The 35,000 × g centrifugation served to partially remove large organelles and membrane sheets from the samples. The 35,000 × g pellet consistently contained 5–6% of the total cell-associated radioactivity regardless of chase time (data not shown). On the other hand, the 50,000 × g pellet displayed an increase in radioiodinated protein with increasing chase times (Fig. 2B). The radioactivity associated with this fraction is trypsin resistant and detergent soluble, indicating that the radioiodinated protein resides within sealed vesicles. Also, increasing amounts of radioiodinated protein were immunoprecipitable from detergent solubilized 50,000 × g pellets with immobilized rabbit anti-mouse antibody, indicating that a significant amount of the radioactivity represents intact protein and not free 125I-tyrosine. Finally, the supernatant after centrifugation at 50,000 × g also shows an increase in radioiodinated protein with increasing chase times (Fig. 2C). Because the radioiodinated protein in the 50,000 × g supernatant is cell-derived, this radiolabel most likely represents released fragments of 125I-anti-DNP IgG from broken phagosomes and other 125I-anti-DNP IgG-containing vesicles. Consistent with this interpretation is the inability of exogenously added immobilized rabbit anti-mouse antibody to immunoprecipitate radioactivity from this supernatant, suggesting that the radioiodinated protein was previously subjected to the proteolysis necessary for release from the S. aureus. Thus, the observed time-dependent accumulation of phagocytosed radioiodinated protein in a vesicle population distinct from phagosomes provides evidence that protein originally located in phagosomes can be transferred to a non-phagosome vesicle population.

Prior to leaving the phagosome, the radioiodinated protein must separate from the S. aureus particle. Evidence that the efflux of radioiodinated protein from phagosomes is dependent upon proteolytic activity in the phagosome is provided by the following experiments. Immune complexes associated with S. aureus are stable at the characteristic pH values of endosomes and lysosomes. Hence, pH-dependent dissociation is probably not significant. Two treatments which inhibit acid protease activity (i.e., incubating cells with monensin or a mixture of pepstatin A and leupeptin) inhibited transfer of radioiodinated protein from the phagosome pellet to the 50,000 × g pellet by 40–50% (data not shown). Evidence for early proteolytic activity in the phagosome is provided by the observed onset of radioiodinated protein release from phagosomal S. aureus (Fig. 2A). Also, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of radioiodinated material from the initial 12,000 × g pellet shows that significant degradation of the mouse-anti-DNP IgG heavy chain occurs during the first 15 min of phagocytosis (Fig. 3). These
results strongly suggest that phagocytosed radiiodinated protein is proteolytically released from the S. aureus particle prior to leaving the phagosome.

Characterization of Transport Vesicles—The buoyant density distribution of radiiodinated protein-containing vesicles from the 50,000 × g pellet (transport vesicles) was determined by Percoll gradient fractionation. Transport vesicles obtained from cells which phagocytosed S. aureus 125I-anti-DNP IgG for 5 min followed by chase for 0 or 15 min were fractionated by centrifugation in a 1.05 g/ml Percoll solution. Following fractionation of the gradient by downward displacement, each fraction was assayed for radioactivity. As seen in Fig. 4, the transport vesicles displayed a biphasic distribution with a majority of the radiiodinated protein at the top of the gradient (plasma membrane and endosome fraction) while the remaining radioactivity localized to the gradient bottom along with the β-hexosaminidase activity (i.e. lysosome fractions). This result indicates that phagocytosed 125I-anti-DNP IgG and fragments thereof migrate into a heterogeneous population of vesicles upon leaving the phagosome.

Because a large amount of radiiodinated protein appeared in a vesicle fraction of equivalent buoyant density to endosomes and the plasma membrane, and fusogenicity is an important characteristic of those membranes, we wished to determine if protein that leaves the phagosome enters a fusion competent compartment. The fusogenicity of the transport vesicles and phagosomes were assayed as previously described (2, 10). Briefly, isolated endosomes labeled by a 5 min uptake at 37 °C of DNP-derivatized β-glucuronidase were mixed with transport vesicles or phagosomes prepared as described above. Upon fusion and mixing of the vesicle contents, immune complexes can form between the DNP-β-glucuronidase and anti-DNP IgG (or fragments of anti-DNP IgG which retain antigen-binding domains). Determination of the amount of β-glucuronidase activity immunoprecipitated with immobilized rabbit anti-mouse antibody provides a measurement of complex formation. As shown in Fig. 5, the signal for fusion (i.e. the amount of β-glucuronidase activity immunoprecipitated) between equivalent amounts of phagosomes and early endosomes decreased with increasing chase time. To the contrary, the fusion signal obtained upon incubation of transport vesicles with early endosomes increased with increasing chase time. Characterization of the biochemical requirements for fusion between the 50,000 × g transport vesicles and endosomes demonstrated the cytosol, ATP, and K+ dependence of this reaction (Table I). Furthermore, GTPγS affects fusion similarly to its effect on endosome-endosome and endosome-phagosome fusion (2, 14). That is, at high cytosol concentrations, GTPγS inhibits fusion while at low cytosol concentrations, GTPγS stimulates fusion. Thus, these data indicate that with increasing chase time, phagocytosed protein can accumulate in vesicles capable of fusion with early endosomes.

Analysis of Phagosome Maturation by Electron Microscopy—Phagosome maturation was also studied by transmission electron microscopy. S. aureus coated with 20 nm of gold was internalized for various times by J774-E clone macrophages (Fig. 6). While most S. aureus-gold was internalized within 2 min at 37 °C, some particles were incompletely phagocytosed (Fig. 6A). At this stage, the S. aureus is completely and evenly surrounded by gold particles. Interestingly, coated pit regions budding from the forming phagosome were identified (arrow, Fig. 6A). Early phagosomes (<10 min at 37 °C) were frequently observed to contain tubular and vesicular membrane extensions (Fig. 6, B and C). After 10 min at
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Characterization of in vitro fusion between transport vesicles from the 50,000 x g pellet and early endosomes

Fusion was performed at 37 °C for 40 min under the following conditions: no treatment (1.5 mg protein/ml cytosol; ATP regenerating system, 125 mM KCl); absence of added cytosol; low cytosol (0.2 mg protein/ml); 20 μM GTPγS in the presence of either high (1.5 mg protein/ml) or low (0.2 mg protein/ml) concentrations of cytosol; pretreatment with N-ethylmaleimide followed by dithiothreitol; without KCl; with an ATP-depleting system; pretreatment of vesicles with trypsin.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative Fusion</th>
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<tbody>
<tr>
<td>None</td>
<td>1.0</td>
</tr>
<tr>
<td>(-) Cytosol</td>
<td>0.35</td>
</tr>
<tr>
<td>Low cytosol</td>
<td>0.43</td>
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<tr>
<td>GTPγS (low cytosol)</td>
<td>0.92</td>
</tr>
<tr>
<td>GTPγS (high cytosol)</td>
<td>0.37</td>
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<tr>
<td>NEM/DTT</td>
<td>0.25</td>
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<tr>
<td>(-) KCi</td>
<td>0.25</td>
</tr>
<tr>
<td>(-) ATP</td>
<td>0.27</td>
</tr>
<tr>
<td>Trypsin preincubation</td>
<td>0.09</td>
</tr>
</tbody>
</table>

37 °C, the phagosome membrane generally contained fewer tubules yet maintained a ruffled pattern (Fig. 6D). In addition, the phagosome volume increased slightly, gold within the phagosome no longer distributed evenly around the S. aureus, and the overall amount of gold observed within the phagosome decreased. After 18 min at 37 °C, phagosomes enlarged further and contained less gold (Fig. 6E). Finally, after 25 min at 37 °C, the phagosomes were greatly enlarged, contained little gold, and somewhat degraded regions of the S. aureus particle began to appear (arrowhead, Fig. 6F). Thus, the macrophage phagosome undergoes changes in its size and membrane topology during its transformation into a phagolysosome.

The tubular extensions acquired by phagosomes after 2 min at 37 °C occasionally contained free gold particles (arrow, Fig. 7A). Presumably, gold released from the S. aureus migrated to these extensions. Because the tubular nature of this membrane is reminiscent of previous work from our laboratory and others on tubular endosomes (15, 16), we determined if these extensions could be the result of endosome-phagosome fusion. Thus, early endosomes were labeled with mannose-BSA-coated colloidal gold as described under "Experimental Procedures." The same cells were then allowed to internalized prebound, antibody-coated S. aureus for 2 min at 37 °C. The cells were subsequently fixed and prepared for electron microscopy. Analysis of these preparations showed gold-containing tubules extending from phagosomes (Fig. 7B). Gold-containing vesicles were also seen attached to the phagosome (Fig. 7C). This result is consistent with our earlier descriptions of in vivo endosome and lysosome fusion with phagosomes (2). Thus, the tubular extensions observed on early phagosomes may originate from early endosomes.

**DISCUSSION**

Phagocytosis is a form of endocytosis employed by specialized cells to internalize and degrade bacteria and other relatively large particles. In macrophage phagocytosis, particle internalization is followed by a complex maturation process involving membrane and protein traffic between the maturing phagosome and organelles of the endosomal-lysosomal pathways. This process transforms the early phagosome into an acidic and protease-rich phagolysosome. While the occurrence of endosome-phagosome and lysosome-phagosome fusion during maturation is documented (2, 3), protein/membrane recycling from phagosomes is less well characterized (4, 17, 18).

We studied the efflux of phagocytosed protein from phagosomes using an assay that measures the in vivo migration of an exogenous phagosomal marker to other vesicle population.
tions. S. aureus coated with mouse 125I-anti-DNP IgG served as the phagocytic particle with J774-E clone macrophages as the phagocytic cell line. We observed that within 18 min of phagocytosis, a substantial fraction of phagocytosed radiiodinated protein migrated into a non-phagosome vesicle population that displays some endosomal characteristics.

A number of experiments suggest that the release of radiiodinated protein from the phagosome is proteolysis-dependent. The inhibition by protease inhibitors (either monensin or leupeptin and pepstatin A) of radiiodinated protein migration suggests that proteolysis of the phagocytic particle is necessary before radiiodinated protein export can occur. Also consistent with a role for proteolysis is the progressive increase in 125I-anti-DNP IgG degradation and its release from the S. aureus particle within the maturing phagosome (Figs. 2A and 3). Diment et al. (19) previously showed that within 2 min at 37°C, endocytosed ligand localizes to protease-rich endosomes. Thus, the early appearance of phagosomal proteolytic activity is likely provided by fusion of phagosomes with protease-containing early endosomes. However, the majority of the phagolysosome’s complement of hydrolases is probably not acquired until later in its maturation.

The fusogenic characteristics of the transport vesicles were analyzed in a vesicle fusion assay developed in our laboratory (8). We observe that as phagocytosed radiiodinated protein accumulates in a vesicle population which pellets in the 50,000 × g centrifugation step (transport vesicles), the signal for transport vesicle-early endosome fusion also increases. Characterization of the biochemical requirements for this fusion event indicates it is very similar to phagosome-endosome and endosome-endosome fusion (2, 8, 14, 20). Furthermore, the dual effect of GTPyS suggests that GTP-binding proteins participate in this fusion reaction (2). Recent work by Gruenberg et al. (21) indicates that rab 5, a member of the ras superfamily of low molecular weight GTP-binding proteins, participates in endosome fusion. As a consequence of the similar fusion requirements, transport vesicles may communicate extensively with endosomes. Accordingly, the contents of the transport vesicles may follow the endocytic recycling pathway to deliver luminal proteins to the extracellular media. Lastly, the common fusion requirements suggest that the transport vesicles may re-fuse to the phagosome.

Characterization of the transport vesicles by Percoll gradient fractionation showed that radiiodinated protein migrated into a heterogeneous vesicle population whose relative density distribution is independent of chase time. Although we cannot determine whether all of the isolated radiiodinated protein-containing vesicles derive directly from the phagosome or whether subpopulations of transport vesicles derive sequentially from one another, we predict a homogeneous population of vesicles derives from the phagosome and then quickly introduces its luminal contents to a heterogeneous vesicle population. For a heterogeneous population of transport vesicles to derive directly from the phagosome, individual phagosomes must maintain both light and heavy membrane domains, a situation that we believe is unlikely to occur. A density distribution profile of 125I-anti-DNP IgG similar to that shown in Fig. 4 was observed following fluid-phase uptake of non-aggregated 125I-anti-DNP IgG by J774-E clone (data not shown), suggesting that phagocytosed 125I-anti-DNP IgG and fragments thereof eventually enter compartments along the endocytic pathway in the fluid phase. The heterogeneous vesicle population observed in the Percoll gradients may thus represent endosomal and lysosomal vesicles. An interesting question which remains unanswered is whether the transfer of phagocytosed antibody to transport vesicles is receptor-mediated. As the FcII receptor has a high affinity for aggregated IgG, it is also possible that some aggregated 125I-anti-DNP IgG is sorted out of the phagosome by the FcII receptor. Other receptors (e.g. mannose receptor) may similarly be capable of removing material from the phagosome in the process of receptor recycling from the phagosome.

A morphological analysis of S. aureus-gold uptake by J774-E clone macrophages revealed many aspects of phagosome maturation. During particle engulfment, coated pits were frequently identified on forming phagosomes. Aggeler and Werb (22) have reported similar observations on forming phagosomes. This phenomenon may be due to receptor aggregation induced by the multivalent phagocytic particle. Once engulfed, however, coated regions were no longer observed, suggesting that the mechanism of phagosome membrane efflux is not mediated by clathrin-coated vesicles. Tubular regions extending from the phagosome may participate in phagosomal protein recycling. However, our fractionation studies demonstrate that phagosome efflux continues after 10 min of phagocytosis (Fig. 2B), when tubules are rarely identified. Thus, the tubular extensions observed on early phagosomes may participate in phagosome efflux but are probably not solely responsible for it. The lack of tubules in more mature phagosomes may be due to a decrease in the amount of endosome-phagosome fusion and diffusion of the tubular membrane into the expanding luminal membrane of the phagosome. With increasing chase times after phagocytosis, fewer gold particles were identified in the phagosome. At the same time, however, few gold-containing vesicles were seen

**Fig. 8. Proposed model for macrophage phagosome maturation.** We postulate that phagosomes mature in a manner similar to the mechanism previously proposed for endosome maturation (26, 27). Newly formed phagosomes undergo extensive membrane exchange with early endosomes. As a result, phagosomes acquire endosomal protein, membrane, and functions, including the capacity to recycle material. Transport vesicles may fuse either to the plasma membrane, early endosomes, or phagosomes. Early phagosomes gradually take on lysosome-like properties by increasing their fusion with both trans Golgi reticulum-derived vesicles and lysosomes. As a consequence of these fusion events, the phagosome volume increases over time. Degradation of the phagocytic particle is eventually followed by reduction in vacuole size. Recycling of material probably occurs at all stages of phagosome maturation, although the recycling rates and the destination of recycled membrane and contents may change (as indicated by the dotted line from the phagolysosome).
taining, non-phagosomal vesicles is probably due to diffusion of these vesicles out of the plane of the section. Also during this period, intraphagosomal gold particles clumped considerably. This may be the result of the hydrolysis of the gold's protein coating. Thus, our biochemical and morphologic studies describe phagosomes as dynamic organelles undergoing compositional and structural changes during their transformation into phagolysosomes.

The mechanisms responsible for transport of material endocytosed by clathrin-coated pits to lysosomes are currently a topic of great debate (23–26). In the endosome maturation model (26, 27), a newly formed endosome undergoes an initial period of extensive early endosome-early endosome exchange followed by progressive increases in communication of the endocytic vesicle with the trans Golgi network, late endosomes, and lysosomes. As a result of the sequential nature of these interactions, the endosome gradually acquires the composition and function of a lysosome. We postulate that macrophage phagosomes undergo a maturation process similar to that proposed for endosomes (Fig. 8). Accordingly, upon formation of the phagosome, extensive fusion occurs between early endosomes and the early phagosome. These fusion events have a number of effects on the phagosome. They introduce endosomal proteins including hydrolases, contribute membrane to the phagosome and thus increase its surface area, and bestow upon the phagosome functions of the early endosome, including the capacity to recycle protein and membrane. The phagosome subsequently decreases interactions with early endosomes and increases its communication with the trans Golgi network, late endosomes, and lysosomes. Eventually, the phagosome acquires the composition and functions of a phagolysosome. Thus, in this model, phagosomes communicate extensively with organelles of the endocytic pathway and, as a result, phagosomes sequentially acquire functions associated with early endosomes, late endosomes, and lysosomes. The phagosome-derived transport vesicles described in this report may consequently be similar to endosome-derived recycling vesicles.

Although phagosomes acquire many components of endosomes, maturing phagosomes may be compositionally different from their endosomal counterparts. For example, the respiratory burst in macrophages is due to an electron transport chain located in the phagosomal membrane. To date, this enzymatic activity has not been shown to exist in macrophage endosomes. Thus, some of the components of the electron transport chain necessary for functioning may be restricted to phagosomes.

Just as endosomes and lysosomes can recycle material to the cell surface (28–30), early phagosomes and phagolysosomes may recycle their contents. Such a mechanism may participate in antigen presentation. For example, during phagosome maturation or phagosome protein recycling, proteolytically released fragments of ingested particles may encounter proteins (e.g. class II major histocompatibility complex) involved in antigen presentation (31). Whether antigenic fragments recycle from either phagolysosomes or early phagosomes will depend on the susceptibility of each particular antigen to the phagosome's proteolytic environment. Alternatively, the proteins necessary for antigen presentation may be delivered to the phagosome via fusion with endosomes.

In conclusion, we have demonstrated that phagocytosed protein migrates from phagosomes into a heterogeneous population of vesicles that fuse to early endosomes. Further analysis of this pathway at the biochemical and morphological levels will certainly contribute to our understanding of other macrophage functions including antigen processing and presentation.

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