Phagosomes Are Fully Competent Antigen-Processing Organelles That Mediate the Formation of Peptide:Class II MHC Complexes

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During the processing of particulate Ags, it is unclear whether peptide:class II MHC (MHC-II) complexes are formed within phagosomes or within endocytic compartments that receive Ag fragments from phagosomes. Murine macrophages were pulsed with latex beads conjugated with OVA. Flow or Western blot analysis of isolated phagosomes showed extensive acquisition of MHC-II, H-2M, and invariant chain within 30 min, with concurrent degradation of OVA. T hybridoma responses to isolated phagosomes or within endocytic compartments that receive Ag fragments from phagosomes. Furthermore, when two physically separable sets of phagosomes were present within the same cell, OVA(323–339):I-A^d complexes were demonstrated in latex-OVA phagosomes but not in phagosomes containing latex beads conjugated with another protein. This implies that these complexes were formed specifically within phagosomes and were not formed elsewhere and subsequently transported to phagosomes. In addition, peptide:MHC-II complexes were shown to traffic from phagosomes to the cell surface. In conclusion, phagosomes are fully competent to process Ags and generate peptide:MHC-II complexes that are transported to the cell surface and presented to T cells. The Journal of Immunology, 1999, 162: 3263–3272.
MHC-II molecules for the presentation of newly generated peptides (25). It is clear that phagosomes acquire proteases by fusion with both endosomes (29) and lysosomes, and they certainly mediate the catabolism of particulate Ags. However, it is not clear whether phagosomes mediate the ultimate steps of Ag processing and the formation of peptide:MHC-II complexes, or whether Ag fragments are transported to other compartments in the endocytic pathway for ultimate processing and loading onto MHC-II molecules.

To define the role of phagosomes in Ag processing and presentation, we examined macrophage processing of OVA coupled to latex beads (latex-OVA). A recently developed technique for immunolabeling and flow analysis of isolated phagosomes (30) was applied to determine the levels of Ag, MHC-II, and other membrane proteins in phagosomes, and phagosomal proteins were analyzed by Western blot analysis for the presence of invariant chain and H-2M. Phagosomes were found to rapidly acquire high levels of MHC-II, H-2M, and invariant chain, while bead-associated OVA was degraded. In addition, subcellular fractionation was used to isolate phagosomes and other organelles to determine the presence of OVA(323–339):I-A^d complexes using a T hybridoma cell assay. OVA(323–339):I-A^d complexes were present in phagosomes and on the plasma membrane but not in the dense, late endocytic compartments that are active in the processing of soluble Ag. Other experiments indicated that the OVA(323–339):I-A^d complexes arose specifically within latex-OVA phagosomes and were not formed elsewhere with subsequent transport to the phagosomes. Additional evidence showed that phagosome-derived OVA(323–339):I-A^d complexes were transported to the cell surface for presentation to T cells. This is the first demonstration that phagosomes are fully competent Ag-processing organelles that mediate the formation of peptide:MHC-II complexes.

Materials and Methods

Cells and media

Peritoneal macrophages were harvested 10–14 days after i.p. inoculation of mice with Listeria monocytogenes (31). BD2F2/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). H-2^K/J mice were generously provided by L. Van Kaer (Vanderbilt University, Nashville, TN) and then bred at Case Western Reserve University (Cleveland, OH). The T cell hybridoma DOBW (32), which recognizes OVA(323–339) bound to I-A^d, derives from a transgenic mouse expressing the TCR from the OVA-specific DO11.10 T hybridoma (33) and was produced by Osami Omura (University of Iowa, Iowa City, IA). 34-5-3S and KL295 were obtained from the Developmental Hybridoma Bank (Johns Hopkins University, Baltimore, MD and University of Iowa, Iowa City, IA), 34-5-3S and KL295 were obtained from American Type Culture Collection (ATCC, Manassas, VA). In-1, a rat IgG2b specific for the cytoplasmic region of the invariant chain (34), was generously provided as a hybridoma supernatant by Andrea Sant (University of Chicago, Chicago, IL). Mouse antisera against OVA was generated in C57BL/6 mice. Rabbit antisera specific for the α-chain of H-2M was generously provided by John Monaco and Helena Russell (University of Cincinnati, Cincinnati, OH). Protein A-affinity purified MK-D6 (ATCC), an anti-I-A^d IgG2a, was iodinated by the chloramine T method.

Preparation of phagosomes for flow analysis

Macrophages were plated at 10^5 cells per well in 6-well plates. Three wells were used for each time point. Latex-OVA (10 μl) was added to each well in a final volume of 4 ml, and the beads were pelleted onto the cells by centrifugation at 900 × g for 5 min at 37°C. The cells were incubated at 37°C for an additional 5 min (providing a total pulse period of 10 min) and washed in ice-cold DMEM to remove extracellular beads. Prewarmed medium was added, and the cells were incubated for various periods up to 120 min. The cells were washed, detached by scraping, washed, resuspended in homogenization buffer (0.25 M sucrose and 10 mM HEPES (pH 7.2)) containing protease inhibitors (1.0 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, and 20 μg/ml leupeptin), and homogenized in a Dounce homogenizer (Kontes, Vineland, NJ) to obtain 80–85% lysis as described (30). Intact cells and nuclei were removed by centrifugation (400 × g, 10 min). The supernatant was then centrifuged at 1900 × g for 10 min to pellet the crude phagosome preparation, which was paraformaldehyde-fixed, washed, and resuspended in PBS as described (30). The resulting phagosome titer was ~10^7 phagosomes/ml. For flow analysis, phagosomes containing nonfluorescent beads were stained in 96-well round-bottom plates. The crude phagosome preparation (80–100 μl/well) was pelleted by centrifugation at 1900 × g for 10 min (providing a total pellet volume of 200 μl total volume) and incubated for 20–24 h. Supernatants (100 μl) were harvested and assessed for IL-2 content using the CTLL-2 proliferation assay (35). CTLL-2 proliferation was monitored by the addition of Alamar blue (Alamar Biosciences, Sacramento, CA) as an indicator dye and measured as the difference between absorbance at 550 nm and 595 nm after 24 h (36). Blanks for spectrophotometry were provided by wells containing medium alone (added at the same time as the Alamar blue and Alamar blue (added at the same time as for the other wells). All analyses were performed in triplicate.

Ag processing and presentation assays

Macrophages were plated for 2 h in 96-well plates at 2 × 10^5 cells/well, and nonadherent cells were removed by washing. Latex-OVA was added, and the plates were processed as above to achieve 10-min pulse and 0- to 120-min chase incubations. The macrophages were fixed with 0.5% paraformaldehyde and washed. Thy1.2+ peritoneal macrophages (1 × 10^6) were incubated with 2 ml of 62% sucrose, 10 mM HEPES (pH 7.4) containing protease inhibitors (1.0 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, and 20 μg/ml leupeptin), and homogenized in a Dounce homogenizer (Kontes, Vineland, NJ) to obtain 80–85% lysis as described (30). Intact cells and nuclei were removed by centrifugation (400 × g, 10 min). The supernatant was then centrifuged at 1900 × g for 10 min to pellet the crude phagosome preparation, which was paraformaldehyde-fixed, washed, and resuspended in PBS as described (30). The resulting phagosome titer was ~10^7 phagosomes/ml. For flow analysis, phagosomes containing nonfluorescent beads were stained in 96-well round-bottom plates. The crude phagosome preparation (80–100 μl/well) was pelleted by centrifugation at 1900 × g for 10 min (providing a total pellet volume of 200 μl total volume) and incubated for 20–24 h. Supernatants (100 μl) were harvested and assessed for IL-2 content using the CTLL-2 proliferation assay (35). CTLL-2 proliferation was monitored by the addition of Alamar blue (Alamar Biosciences, Sacramento, CA) as an indicator dye and measured as the difference between absorbance at 550 nm and 595 nm after 24 h (36). Blanks for spectrophotometry were provided by wells containing medium alone (added at the same time as for the other wells). All analyses were performed in triplicate.

Sucrose density gradient isolation of phagosomes and Western blot analysis

Phagosomes were isolated by sucrose density gradient fractionation as described by Desjardins et al. (37) with minor modifications. Two 6-well plates containing macrophages plated at 10^5 cells/well were used for each phagosome preparation. Fluorescent latex-OVA was added (10 μl/well), and the plates were processed as described above to achieve 10-min pulse and 30-min chase incubations. A crude phagosome preparation was prepared (above), resuspended in 2 ml of homogenization buffer, combined with 2 ml of 62% sucrose, 10 mM HEPES (pH 7.4) (resulting sucrose concentration = 40%), split into two equal samples, and loaded into two tubes containing a 1 ml cushion of 62% sucrose, 10 mM HEPES (pH 7.4). The following solutions (in 10 mM HEPES (pH 7.4)) were layered into the tubes: 2 ml of 32% sucrose, 2 ml of 26% sucrose, 2 ml of 21% sucrose, and 2.5 ml of 10% sucrose. The tubes were centrifuged in a swinging bucket rotor (SW40; Beckman Instruments, Palo Alto, CA) for 1 h at 100,000 × g at 4°C. The phagosomes were collected from the interface of the 10 and 21% sucrose solutions from both gradients, combined, diluted 3-fold in PBS, pelleted by centrifugation at 10,000 rpm for 5 min in an Eppendorf centrifuge at 4°C, immediately frozen in dry ice, and stored at −80°C.

Macrophages obtained from B6D2F1, CBA/J, or H-2M^q−/− mice were lysed at a concentration of 5 × 10^6/ml in lysis buffer (PBS containing 1% Nonidet P-40, 5 mM EDTA, 50 mM sodium citrate, 1 mM PMSF, 2 μg/ml pepstatin, and 20 μg/ml leupeptin; all from Sigma). Phagosomes isolated from sucrose density gradients were similarly lysed. Lysates were boiled in SDS-PAGE sample buffer under reducing conditions and electrophoresed on 12% SDS polyacrylamide gels (13). The proteins were blotted onto polyvinylidene difluoride membrane (Immobilon-P: Millipore, Bedford, MA) and probed with antibodies specific for the cytoplasmic region of the invariant chain (34), which was generously provided as a hybridoma supernatant by Andrea Sant (University of Chicago, Chicago, IL). Mouse antisera against OVA was generated in C57BL/6 mice. Rabbit antisera specific for the α-chain of H-2M was generously provided by John Monaco and Helena Russell (University of Cincinnati, Cincinnati, OH). Protein A-affinity purified MK-D6 (ATCC), an anti-I-A^d IgG2a, was iodinated by the chloramine T method.
Phagosomes containing magnetic latex-OVA beads were removed and the plates were processed as described above to achieve 10-min pulse and various chase incubations. The macrophages were washed, detached, and homogenized as described above in homogenization buffer without protease inhibitors. Intact cells and nuclei were removed by centrifugation at 200 × g for 10 min at 4°C, and the supernatant was collected. The phagosomes were pelleted by centrifugation at 850 × g for 10 min at 4°C, and the supernatant (containing “nonphagosomal membranes”) was transferred to a new tube.

Magnetic latex-OVA and nonmagnetic fluorescent latex-HEL. These experiments used six 6-well plates of macrophages (10⁶ cells/well). Magnetic latex-OVA (5 μl/well) and fluorescent latex-HEL (5 μl/well) were added, and the plates were processed as described above. The cells were resuspended in 1 ml of homogenization buffer using a Dounce homogenizer (20 strokes) to produce the nonphagosomal membrane sample.

Some experiments involved simultaneous exposure of macrophages to magnetic latex-OVA and nonmagnetic fluorescent latex-HEL. These experiments used six 6-well plates of macrophages (10⁶ cells/well). Magnetic latex-OVA (5 μl/well) and fluorescent latex-HEL (5 μl/well) were added, and the plates were processed as described above. The cells were resuspended in 1 ml of homogenization buffer and homogenized as described above. Phagosomes containing magnetic latex-OVA beads were removed with a magnetic particle concentrator (Dynal, Great Neck, NY), washed three times in 2 ml of homogenization buffer, and resuspended in 333 μl of 10% Percoll solution to achieve the Percoll concentration present in other phagosome fractions obtained from Percoll gradients (below). Microscopic examination showed that there were no intact cells contaminating the magnetic phagosome preparation. The remaining nonmagnetic material was used to generate latex-HEL phagosomes and nonphagosomal membranes, as described above.

Percoll density gradients were formed by layering 1 ml of sample on 9 ml of 20% or 23% Percoll in homogenization buffer, followed by centrifugation in a T50 fixed angle rotor (Beckman Instruments) for 60 min at 36,000 × g. The gradients were manually fractionated from the top into 30 fractions of 333 μl each. Each fraction was divided into replicate aliquots of 10, 50, or 100 μl and frozen at −80°C for subsequent biochemical or T cell analysis. For EM, phagosomes were isolated on the Percoll gradient, immediately diluted 3-fold in PBS, pelleted at 10,000 rpm for 10 min at 4°C in an Eppendorf centrifuge, and processed as described (30). Percoll density gradients were biochemically characterized as previously described (19) with minor modifications. To identify fractions containing plasma membrane, the plasma membrane was marked before homogenization by incubation of macrophages for 60 min at 4°C with 125I-labeled MK-D6. β-Hexosaminidase activity was measured as a representation of lysosomal enzyme distribution by combining 20 or 45 μl aliquots of each fraction.

T cell analysis of latex-OVA processing and presentation

Peritoneal macrophages from B6D2 mice were incubated with latex-OVA for 10 min, washed, and incubated for various periods in medium lacking latex-OVA. The cells were fixed, washed, and incubated with DOBW T hybridoma cells to assess the presentation of OVA(323–339):I-A^d complex. Processing was initiated rapidly, and presentation of OVA(323–339):I-A^d complexes at the cell surface began by 10 min (data not shown), with further delivery of additional complexes to the cell surface for at least an additional 1–4 h (see acidic stripping experiment, below). To confirm that uptake of latex-OVA and intracellular processing was required, the macrophages were incubated with or without cytochalasin D (to inhibit phagocytic uptake) beginning 15 min before the addition of beads. Cytochalasin D blocked latex-OVA processing (Fig. 1), although it had no effect on the processing of soluble OVA (data not shown), demonstrating that phagocytic uptake of latex-OVA was necessary for its processing. In addition, fixed macrophages were able to present OVA(323–339) peptide but not latex-OVA to DOBW cells (data not shown), confirming that intracellular processing was required and presentation was not due to peptide contamination of the latex-OVA.

Macrophages from B6D2 mice (H-2^d) were used in these studies, since preliminary studies established that these mice are an excellent source of high numbers of activated macrophages that efficiently process and present OVA (as required for subcellular fractionation). Although the OVA(323–339) epitope can also be presented by I-Ab under some circumstances (38), blocking studies with anti-I-A^d or anti-I-A^b mAbs showed that the signal detected in our systems for presentation of latex-OVA by B6D2 macrophages was entirely mediated by I-A^d.
Assessment of MHC-II and LAMP-1 expression in isolated phagosomes by flow analysis

Studies of the roles of phagosomes in Ag processing and presentation require analysis of phagosomes with exclusion of other organelles. This can be achieved either by physical isolation or by analytic isolation upon flow analysis, as recently described (30). With flow analysis, analytic isolation of latex bead phagosomes from other organelles and cell fragments is achieved by gating to select events based on the unique optical scatter properties of latex bead phagosomes. Phagosomal membrane proteins and components of the phagocytosed particle can then be quantitatively detected by immunolabeling.

To prepare phagosomes for flow analysis, macrophages were exposed to latex-OVA beads for a 10-min pulse incubation, washed, and subjected to an additional chase incubation of 0–120 min. The cells were then homogenized, and a crude phagosome preparation was prepared by differential centrifugation (see Materials and Methods). EM analysis of this preparation showed that the isolated phagosomes were intact and similar in structure to those within whole macrophages (data not shown), as seen in previous studies (30). Most phagosomes contained a single bead with a closely apposed limiting membrane, while some were more spacious, with significant luminal volume outside of the bead. In addition, a smaller number of phagosomes contained two beads. Gating by optical scatter analysis, as previously described, allowed the selection of exclusively single-bead phagosomes for immunolabeling analysis.

For flow analysis, the crude phagosome preparation was fixed with 4% paraformaldehyde, permeabilized with saponin, stained for LAMP-1, MHC-II, or OVA, and analyzed by flow cytometry. Gating by optical scatter parameters was used to select single-bead phagosomes (gate indicated in A) for immunolabeling analysis. MHC-II and LAMP-1 were detected with the indicated mAbs, and negative-control staining with isotype-matched control Abs was used to define the H1 gate (negative events). The H2 gate represents positive events. A-E. Data obtained with 10-min pulse and 30-min chase incubations. For detection of OVA, phagosomes were stained with OVA-specific mouse antiserum (or normal mouse serum as the negative control). J. Summary of OVA labeling in phagosomes of different ages.
fluorochrome value (MFV) of phagosomes in the LAMP-1-positive peak increased from 79 with no chase to 104 after a 120-min chase. The percentage of phagosomes that contained MHC-II increased from 59% with no chase to 84% following a 120-min chase (with subtraction of the 5% background in the positive gate). The mean level of MHC-II within the positively stained phagosomes, as determined by the MFV, remained relatively constant, although there was a wide range of MHC-II expression in different phagosomes. Thus, the major shift with time was an increase in the proportion of phagosomes that contained MHC-II, not an increase in the mean level of MHC-II in phagosomes. The level of MHC-II in positive phagosomes may reflect a balance of import and export of MHC-II by phagosomes.

**Flow analysis of phagosomal Ag degradation**

Immunolabeling and flow analysis can be used to detect particle-associated Ags, as well as phagosomal membrane proteins, in saponin-permeabilized phagosomes. We used this approach to assess Ag degradation after phagocytosis (Fig. 2). Latex-OVA beads and latex-OVA phagosomes of different ages were stained for OVA and analyzed by flow cytometry. The MFV for OVA staining decreased from 666 in latex-OVA beads (data not shown) to 581 in latex-OVA phagosomes following a 10-min pulse (Fig. 2G), 397 following a 10-min pulse and 30-min chase (Fig. 2H), and 54 following a 10-min pulse and 120-min chase (Fig. 2I). In the same time frame, 125I-OVA associated with latex beads was degraded to trichloroacetic acid-soluble catabolites (data not shown), confirming that the disappearance of OVA-labeling from phagosomes was accompanied by OVA degradation. Thus, OVA degradation was initiated within 10 min and was 92% complete by 120 min.

In subsequent subcellular fractionation experiments, phagosomes obtained after a 10-min pulse and 15- or 30-min chase were selected for detailed study, since these phagosomes were actively degrading Ag, contained high levels of MHC-II and LAMP-1, and could be obtained with high purity and minimal contamination with plasma membrane.

**Western blot analysis of H-2M and invariant chain in phagosomes**

Additional experiments determined phagosomal levels of other Ag-processing components, e.g., H-2M and invariant chain. Since the H-2M-specific antiserum was more effective for Western blotting than immunolabeling for flow analysis, phagosomes were first purified by sucrose gradient fractionation and then analyzed by Western blot analysis.Sucrose gradient fractionation produced highly purified phagosomes as judged by EM and the low degree of contamination by plasma membrane marker (<0.4% of radioactive plasma membrane marker, assessed as described below). Western blot analyses revealed that the purified phagosomes contained H-2M and invariant chain, as well as MHC-II and LAMP-1 (Fig. 3). The invariant chain-specific Ab, In-1, detected three distinct forms of invariant chain. Two of these appeared to correspond to the p41 and p33 forms of invariant chain (39). The third form was enriched in phagosomes relative to whole cells, and corresponded to p10, a degradation intermediate produced during the processing of invariant chain (18, 39). The presence of p10 suggests that the phagosomes were actively processing invariant chain, consistent with an active role of these compartments in Ag processing.

**Preparative isolation of phagosomes for T cell assays by Percoll density gradient centrifugation**

The previous studies showed that phagosomes mediated Ag degradation and contained high levels of MHC-II molecules and other proteins involved in Ag processing. However, it was still unclear whether peptide:MHC-II complexes were formed within the phagosome or within endocytic compartments that received phagosome-derived Ag fragments. To determine the intracellular site of formation of complexes during latex-OVA processing, we modified a technique previously developed for detection of peptide:MHC-II complexes in isolated endocytic organelles using a T cell assay (12, 19). This approach uses subcellular fractionation to isolate organelle membranes, which are disrupted by freezing and thawing steps to allow access to lumenal MHC-II Ag-presenting domains and then probed for the presence of specific peptide:MHC-II complexes using a T cell hybridoma assay.

Percoll density gradient fractionation was used to isolate latex bead phagosomes, since sucrose gradients were unsatisfactory due to deleterious effects of the sucrose on subsequent T cell assay steps. Macrophages were pulsed with fluorescent latex-OVA for 10 min, chased for 15 or 30 min, and homogenized. A crude phagosome preparation was separated from a nonphagosomal membrane preparation by differential centrifugation. The nonphagosomal membrane preparation (contaminated by ~10% of the total phagosomes) was fractionated on a 23% Percoll density gradient (Fig. 4A), while the crude phagosome preparation was fractionated on either a 23% (Fig. 4B) or 20% Percoll density gradient (Fig. 4C).

The relative distributions of plasma membrane and phagosomes were determined by fractionating macrophages that were allowed...
to phagocytose latex-OVA and were then surface-labeled with $^{125}$I-MK-D6 (anti-I-A<sup>d</sup>) at 4°C. The plasma membrane-associated radioactivity consistently localized to fractions 10–15 in the low density region of the 23% gradients (Fig. 4A) and fractions 11–16 on 20% gradients (data not shown). Phagosome localization was determined by microscopic examination of the fractions. When the crude phagosome preparation was fractionated, phagosomes were localized in fractions 20–22 in 20% Percoll gradients and in three adjacent fractions located with some variation between positions 15–19 in 23% Percoll gradients. The 23% Percoll gradient provided maximal separation of phagosomes from late endocytic compartments active in Ag processing, including MIIC, which were previously established to localize to fractions 21–28 in these 23% gradients by immuno-EM (12), as confirmed in these studies by MHC-II distribution in fractions from cells without phagocytic challenge (data not shown). The 20% Percoll gradient provided maximal separation of phagosomes from the plasma membrane. Of the total plasma membrane-associated radioactivity remaining in the phagosomal and nonphagosomal samples, 13% was present in the crude phagosome preparation, but <1.5% localized to the phagosomal fractions on a 20% Percoll gradient (20–22, Fig. 4C). Thus, this fractionation protocol produced phagosomes with minimal contamination by plasma membrane.

$\beta$-Hexosaminidase activity was assessed as a measure of lysosomal enzyme distribution. As expected, most $\beta$-hexosaminidase was associated with high density lysosomal fractions (fractions 25–30) in both 20% and 23% Percoll density gradients. Distinct peaks of $\beta$-hexosaminidase activity were also found in the phagosomal fractions in both 20% and 23% Percoll density gradients. Low levels of $\beta$-hexosaminidase activity were found in lighter fractions, presumably associated with endosomes that overlap with the plasma membrane.

Phagosomes isolated on the 20% Percoll gradient were also analyzed by EM (Fig. 4D). Consistent with the morphology observed in the crude phagosome preparation (data not shown), most of the phagosomes contained a single latex bead surrounded by a tightly apposed membrane containing occasional discontinuities. No significant contamination of the phagosomes with any other organelle was observed by EM. We conclude that the phagosomes isolated on Percoll density gradients were intact and highly purified.

**Analysis of subcellular fractions for OVA(323–339):I-A<sup>d</sup> complexes using a T cell assay**

To determine the intracellular site of formation of OVA(323–339):I-A<sup>d</sup> complexes, macrophages were pulsed with latex-OVA for 10 min, chased for 30 min, and fractionated as described above (similar results were observed with a 10-min pulse and 15-min chase, data not shown). An aliquot of each fraction was frozen, thawed, and incubated with DOBW T hybridoma cells. T cell detection of OVA(323–339):I-A<sup>d</sup> complexes was monitored by IL-2 secretion (Fig. 5).

Analysis of the nonphagosomal membrane preparation on 23% Percoll gradients showed T cell responses exclusively in the plasma membrane fractions (fractions 10–15; Fig. 5A), except for a slight response observed in fractions 18–20 (which contain the small proportion of phagosomes present in this preparation). Significantly, no response was detected in fractions 21–28, which contained the late endocytic compartments that mediate the formation of most peptide:MHC-II complexes during processing of soluble Ags in these cells (Fig. 5). Thus, analysis of 23% Percoll gradients showed that late endocytic compartments were devoid of OVA(323–339):I-A<sup>d</sup> complexes in the course of latex-OVA processing, indicating that Ag and peptides were not removed from phagosomes and transported to late endocytic compartments for ultimate processing and binding of peptides to MHC-II molecules.

Fractionation and analysis of the crude phagosome preparation on 20% Percoll gradients showed strong T cell responses to the

![FIGURE 4. Isolation of phagosomes on Percoll density gradients.](http://www.jimmunol.org/)

Crude phagosomes and nonphagosomal membranes were separated after exposure of macrophages to latex-OVA for 10-min pulse and 30-min chase incubations. To localize plasma membrane fractions, macrophage plasma membranes were labeled with $^{125}$I-MK-D6 (anti I-A<sup>d</sup>) at 4°C before fractionation. $\beta$-Hexosaminidase activity was used as a measure of lysosomal enzyme distribution. A. Fractionation of nonphagosomal membranes on a 23% Percoll density gradient. B. Fractionation of crude phagosomes on a 20% Percoll gradient. C. Fractionation of crude phagosomes on a 23% Percoll density gradient. D. EM of phagosomes isolated on a 20% Percoll density gradient. Bar = 0.5 μm.
OVA(323–339):I-A^d complexes were present in the magnetic latex-OVA phagosomes. However, we sought to further exclude the possibility that these complexes were formed elsewhere (e.g., a low density early endosomal compartment) and were subsequently transported to phagosomes. If this were to happen, the complexes would target to latex-HEL as well as latex-OVA phagosomes, if both were present in the same cell. To evaluate this possibility, macrophages were simultaneously pulsed with magnetic latex-OVA beads and fluorescent (nonmagnetic) latex-HEL beads for 10 min, and then chased for an additional 30 min. Examination of 30 cells by microscopy demonstrated that all contained both magnetic latex-OVA (average of 7.4 phagosomes/cell by microscopy) and fluorescent latex-HEL (average of 6.7 phagosomes/cell by microscopy). Macrophages containing magnetic latex-OVA were also separated magnetically, and flow analysis confirmed that virtually all (99.3%) of these cells contained fluorescent latex-HEL (average of 8.2 phagosomes/cell).

Following homogenization of these macrophages, the magnetic latex-OVA phagosomes were removed by the application of a magnetic field and thoroughly washed. Differential centrifugation was again used to separate the nonmagnetic membranes into a crude (nonmagnetic) phagosome preparation, which was fractionated on a 20% Percoll density gradient, and a nonphagosomal membrane preparation, which was fractionated on a 23% Percoll gradient (Fig. 6). The results of the DOBW assay indicated that OVA(323–339):I-A^d complexes were present in the magnetic latex-OVA phagosomes (Fig. 6C) and on the plasma membrane (Fig. 6A), but were not present at detectable levels in latex-HEL phagosomes (Fig. 6B). Thus, transport from other compartments was not sufficient to explain the levels of OVA(323–339):I-A^d complexes present in latex-OVA phagosomes. This confirms that specific peptide:MHC-II complexes were directly formed within the phagosomes containing the specific Ag and excludes the possibility that the phagosomal peptide:MHC-II complexes were formed in either early or late endocytic compartments.

In addition, these results show that positive T cell responses to phagosomal fractions from Percoll gradients (Fig. 5) were not caused by peptide:MHC-II complexes in nonphagosomal membranes that colocalized with the phagosomes, since this artifact would have produced positive DOBW responses to the latex-HEL phagosomes (Fig. 6B). This provides an important confirmation that the complexes detected by this approach are truly within phagosomes.

FIGURE 5. T cell analysis of subcellular fractions detects the presence of OVA(323–339):I-A^d complexes in macrophage phagosomes. Macrophages were pulsed with latex-OVA for 10 min, chased for 30 min (similar results were obtained with a 15-min chase), and homogenized. Crude phagosome and nonphagosomal membrane samples were prepared and fractionated on Percoll density gradients (see Fig. 4). Fifty microliters of each fraction was frozen, thawed, and analyzed for expression of OVA(323–339):I-A^d complexes using DOBW T hybridoma cells. A diagram summarizes the positions of different compartments in the 20% or 23% gradients. The position of late endocytic compartments containing MHC-II is labeled “MIIC”. This was determined by immuno-EM in previous experiments using the same macrophage preparations and 23% Percoll gradients (12) and confirmed in the present studies using the same macrophage preparations and 20% Percoll gradients (12) and confirmed in the present studies for both 20% and 23% Percoll gradients by determining the distribution of MHC-II molecules in the gradient. Fractionation of nonphagosomal sample on a 23% Percoll gradient. A. Fractionation of crude membranes on a 23% Percoll gradient. B. Fractionation of crude membranes on a 20% Percoll gradient. C. Fractionation of crude membranes on a 23% Percoll gradient. D. To provide a positive control for T cell responses, OVA(323–339) peptide was added to 10 µl fraction aliquots (1 µM final concentration) just before the addition of the DOBW cells.
samples were used in the T cell assay. OVA(323–339):I-Ad complexes.

Discussion
We have used macrophages pulsed with Ag-conjugated latex beads to evaluate the role of phagosomes in Ag processing and presentation. Latex-OVA processing required phagocytosis and intracellular processing to generate OVA(323–339):I-A^d complexes, which were detected by a T hybridoma assay. Latex-OVA phagosomes were analyzed by immunolabeling and flow analysis, which allowed analytic isolation of these phagosomes from all other organelles and cell fragments by gating based on their distinct optical properties. This gating selected only single-bead phagosomes for analysis, eliminating the possibility that immunolabeling would measure expression of MHC-II on phagosomes of widely divergent size. Since relatively few phagosomes were required for this type of analysis, and extensive physical purification of the phagosomes was not required, phagosomes of various ages could be rapidly evaluated for a variety of membrane proteins or bead-associated Ag. Within the time frame that active processing and presentation was known to occur in intact cells, up to 84% of phagosomes were found to contain significant levels of MHC-II molecules, and degradation of bead-associated OVA was observed, suggesting that peptides were generated and available to bind to MHC-II molecules. Moreover, other Ag-processing components, such as H-2M and invariant chain, were also detected in phagosomes, suggesting that phagosomes contained a complete set of Ag-processing components.

Activated peritoneal macrophages elicited by our protocol express \(1 \times 10^5\) cell surface I-A^d molecules per cell, as determined by binding of \(^{125}\text{I-MK-D6}\) (data not shown), similar to the level of expression of other MHC-II molecules on elicited murine peritoneal macrophages (40). By using flow analysis and immunolabeling, we compared the levels of staining for I-A^d or I-A^b on phagosomes and on the surface of intact cells. The average phagosome contained \(~1\%\) as many MHC-II molecules as were expressed on the cell surface, indicating expression of \(~1 \times 10^3\) molecules of I-A^d per phagosome. Under the conditions employed in this experiment, macrophages contained a mean of nine phagosomes/cell. Since \(~60\%\) of total cellular MHC-II is on the surface.

Phagosome-derived peptide:MHC-II complexes traffic to the cell surface
While the previous analyses show that OVA(323–339):I-A^d complexes formed within phagosomes and were not detected in endocytic compartments during processing of latex-OVA, additional experiments were performed to confirm that peptide:MHC-II complexes could indeed traffic to the cell surface from phagosomes at the time points used in these studies (which used 15- or 30-min chase incubations). To achieve this goal, macrophages were first pulsed with latex-OVA for 10 min and chased for an additional 30 min. At this time point, latex-OVA is confined to phagosomes, and phagosomes are the only detectable site of formation of peptide:MHC-II complexes, as shown above. Peptides bound to surface MHC-II were then stripped by acid treatment of the cells at 4°C. The cells were then washed and either fixed immediately (without recovery) to determine the efficiency of the stripping or subjected to a 1–4 h recovery incubation at 37°C and then washed and fixed to determine whether previously or subsequently formed intracellular OVA(323–339):I-A^d complexes could traffic from phagosomes to the cell surface. Cell surface OVA(323–339):I-A^d complexes were detected with DOBW T hybridoma cells. Without a recovery incubation, acid stripping resulted in a 80–98% loss in DOBW response, as compared with unstripped cells or cells stripped with control citrate buffer at pH 7.4 (Fig. 7). However, with recovery at 37°C, the previously stripped cells acquired OVA(323–339):I-A^d complexes on the cell surface, as seen by increased DOBW response. Thus, after a 30-min chase, OVA(323–339):I-A^d complexes must continue to traffic from an intracellular site, implicated to be phagosomes by the previous studies.

Discussion
We have used macrophages pulsed with Ag-conjugated latex beads to evaluate the role of phagosomes in Ag processing and presentation. Latex-OVA processing required phagocytosis and intracellular processing to generate OVA(323–339):I-A^d complexes, which were detected by a T hybridoma assay. Latex-OVA phagosomes were analyzed by immunolabeling and flow analysis, which allowed analytic isolation of these phagosomes from all other organelles and cell fragments by gating based on their distinct optical properties. This gating selected only single-bead phagosomes for analysis, eliminating the possibility that immunolabeling would measure expression of MHC-II on phagosomes of widely divergent size. Since relatively few phagosomes were required for this type of analysis, and extensive physical purification of the phagosomes was not required, phagosomes of various ages could be rapidly evaluated for a variety of membrane proteins or bead-associated Ag. Within the time frame that active processing and presentation was known to occur in intact cells, up to 84% of phagosomes were found to contain significant levels of MHC-II molecules, and degradation of bead-associated OVA was observed, suggesting that peptides were generated and available to bind to MHC-II molecules. Moreover, other Ag-processing components, such as H-2M and invariant chain, were also detected in phagosomes, suggesting that phagosomes contained a complete set of Ag-processing components.

Activated peritoneal macrophages elicited by our protocol express \(1 \times 10^5\) cell surface I-A^d molecules per cell, as determined by binding of \(^{125}\text{I-MK-D6}\) (data not shown), similar to the level of expression of other MHC-II molecules on elicited murine peritoneal macrophages (40). By using flow analysis and immunolabeling, we compared the levels of staining for I-A^d or I-A^b on phagosomes and on the surface of intact cells. The average phagosome contained \(~1\%\) as many MHC-II molecules as were expressed on the cell surface, indicating expression of \(~1 \times 10^3\) molecules of I-A^d per phagosome. Under the conditions employed in this experiment, macrophages contained a mean of nine phagosomes/cell. Since \(~60\%\) of total cellular MHC-II is on the surface.
of these cells (24, 40), ~5–6% of total cellular MHC-II molecules (13–15% of intracellular MHC-II molecules) were contained within phagosomes. Thus, phagosomes contain a significant percentage of intracellular MHC-II, consistent with the possibility that they play a major role in Ag processing.

To test for the actual formation of OVA(323–339):I-A<sup>d</sup> complexes in phagosomes, we modified a technique that was previously developed for detection of peptide:MHC-II complexes in organelle membranes using a T hybridoma assay (12, 19). Macrophages incubated with latex-OVA were homogenized, and both a crude phagosome preparation and a nonphagosomal membrane preparation were fractionated on isotonic Percoll density gradients. EM analysis showed that the phagosomal membrane remained associated with phagosomes after their purification on Percoll gradients, although occasional membrane discontinuities were noted. When the phagosomal membranes were disrupted by freeze-thaw and assayed with DOBW cells for OVA(323–339):I-A<sup>d</sup> complexes, positive responses were consistently produced by the plasma membrane and phagosomal fractions.

The potential contribution of dense late endocytic compartments was also directly investigated. Phagosomal and late endocytic fractions do not overlap on the 23% Percoll gradient, so this gradient was used to best distinguish these two compartments. I-A<sup>d</sup> molecules were present in the late endocytic fractions and were able to bind and present synthetic OVA(323–339) peptide that was added to the isolated organelles at the time of the T cell assay. Following processing of latex-OVA, however, complexes were not detected in late endocytic fractions of either the nonphagosomal membrane gradient or the phagosome gradient. Thus, OVA(323–339):I-A<sup>d</sup> complexes appear in phagosomes that contain latex-OVA beads but are not detectable in late endocytic compartments during latex-OVA processing. This indicates that late endocytic compartments do not contribute to the formation of complexes that appear in phagosomes, which appear to arise directly within the phagosome containing the particulate Ag.

Several precautions were taken in these studies to exclude contributions of other organelles that were potential contaminants of the phagosome preparations. First, preliminary studies indicated that plasma membrane contamination was minimized by analyzing phagosomes that were formed with chase incubations of adequate length (e.g., for 15–30 min) to allow virtually complete internalization of the beads. These were valid time points to choose, since Ag processing was actively proceeding. MHC-II and other Ag-processing components were present in phagosomes, Ag degradation was in progress, and the acid stripping experiments (Fig. 7) showed that peptide:MHC-II complexes continued to traffic from phagosomes to the cell surface after this time period. As a second precaution to minimize contamination, we first achieved partial purification by differential centrifugation to separate the nonphagosomal membrane preparation (containing 87% of the plasma membrane and 10% of the phagosomes) and the crude phagosome preparation (including 13% of the plasma membrane and 90% of the phagosomes), and the two preparations were then further purified on separate Percoll gradients. This allowed the preparation of phagosomes with <1.5% plasma membrane contamination. Third, EM analysis of Percoll gradient-purified phagosomes also failed to reveal any significant contamination with other membrane organelles. Fourth, OVA(323–339):I-A<sup>d</sup> complexes were also detected in magnetically isolated latex-OVA phagosomes, which had even greater purity (<0.003% plasma membrane contamination) and were formed by an approach that is capable of separating phagosomes from other membranes of similar physical density. Finally, the lack of an OVA-specific response in the latex-HEL phagosomes when macrophages were simultaneously pulsed with magnetic latex-OVA and latex-HEL beads clearly confirms that contamination with nonphagosomal membranes did not contribute to the T cell responses to Percoll gradient phagosome fractions (Fig. 6). These results all indicate that the complexes detected in the phagosomal fractions were truly in phagosomes and not in other contaminating membranes. Furthermore, the latter experiment (Fig. 6) excludes the possibility that the complexes were initially formed in endocytic compartments of either high or low density and subsequently transported to phagosomes.

In conclusion, our observations indicate that phagosomes are fully competent Ag-processing organelles that mediate the formation of peptide:MHC-II complexes. These complexes may be transported via recycling vesicles to the plasma membrane (24), but their route to the cell surface requires further investigation. Since our observations are limited to the examination of latex bead phagosomes, future studies are needed to assess the Ag-processing function of different types of phagosomes, since the maturation and function of phagosomes can be greatly influenced by their content. For example, certain intracellular pathogens are able to modulate phagosome composition and function, and the impact of this on phagosomal Ag processing requires further exploration.

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References


