

# Membrane Sorting during Phagocytosis: Selective Exclusion of Major Histocompatibility Complex Molecules but Not Complement Receptor CR3 during Conventional and Coiling Phagocytosis

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## Summary

We have used immunocytochemical techniques and enzyme cytochemistry to examine the distribution of plasma membrane proteins during coiling phagocytosis of *Legionella pneumophila* and conventional phagocytosis of *Escherichia coli*. Whereas class I and class II major histocompatibility complex (MHC) molecules are relatively excluded from nascent phagosomes during conventional and coiling phagocytosis, the CR3 complement receptor persists in nascent phagosomes. The staining pattern for alkaline phosphatase activity resembles that of MHC molecules, with a marked exclusion of phosphatase activity from *L. pneumophila* coils and nascent phagosomes. The staining pattern for 5'-nucleotidase activity, on the other hand, resembles that of CR3 with intense staining in the inner layers of *L. pneumophila* coils. These results demonstrate that the cell has the ability to exclude selectively certain membrane proteins from the nascent phagosome during phagocytosis, thereby producing a phagosomal membrane markedly different from the plasma membrane from which it is derived.

Phagocytosis is central to the pathogenesis of intracellular pathogens, such as *Legionella pneumophila* (1), and to host defense against extracellular pathogens, such as *Escherichia coli* (2). Two different morphological forms of phagocytosis have been described: conventional phagocytosis and coiling phagocytosis (3). Most microbes, such as *Staphylococcus aureus* and *E. coli*, enter the macrophage by conventional phagocytosis. A few microbial pathogens, such as *L. pneumophila* (3), *Chlamydia psittaci* (4), and *Leishmania donovani* (5), enter by coiling phagocytosis. Both forms of phagocytosis are mediated by interactions between specific molecules on the surfaces of the microbe and the phagocyte. Phagocytosis of both *L. pneumophila* and *E. coli* is mediated by interactions between fragments of complement component C3 fixed to the surface of the microbes and phagocyte complement receptors (2, 6).

Our understanding of the fate of plasma membrane proteins during phagocytosis is incomplete. The sorting of plasma membrane proteins during phagocytosis determines the membrane composition of the early phagosome. Knowledge of the composition of the phagosomal membrane is important to the understanding of host-pathogen interactions, including

the processing and presentation of parasite antigens, and the intracellular flow and recycling of plasma membrane receptor molecules. To enhance our understanding of membrane sorting during and immediately after phagocytosis, we have used cytochemical techniques at the ultrastructural level. In this report, we shall describe the distribution of MHC molecules, complement receptor CR3, and the membrane molecules alkaline phosphatase and 5'-nucleotidase during coiling phagocytosis of *L. pneumophila* and conventional phagocytosis of *E. coli*.

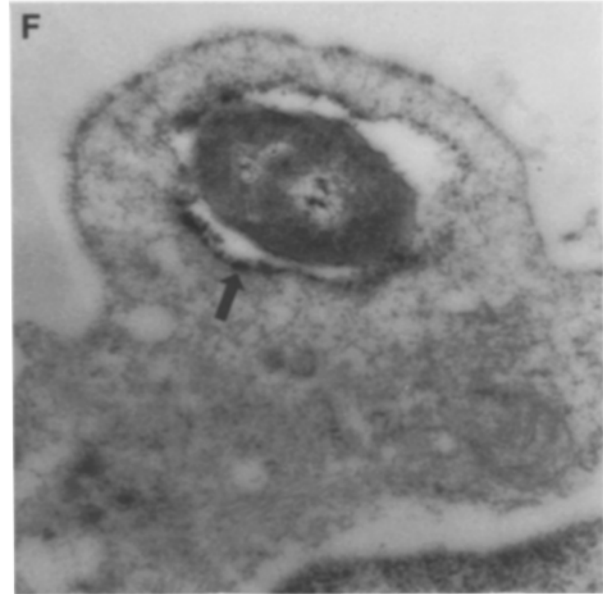
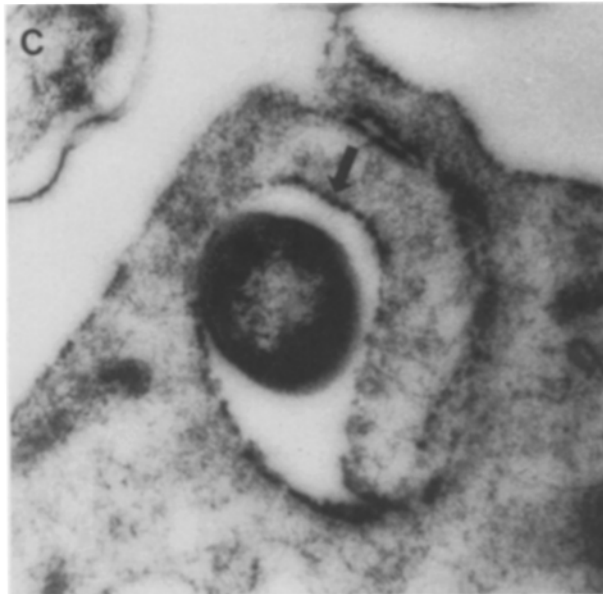
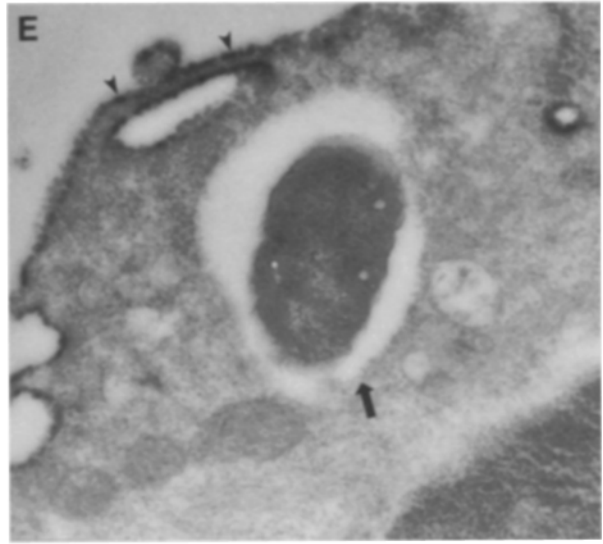
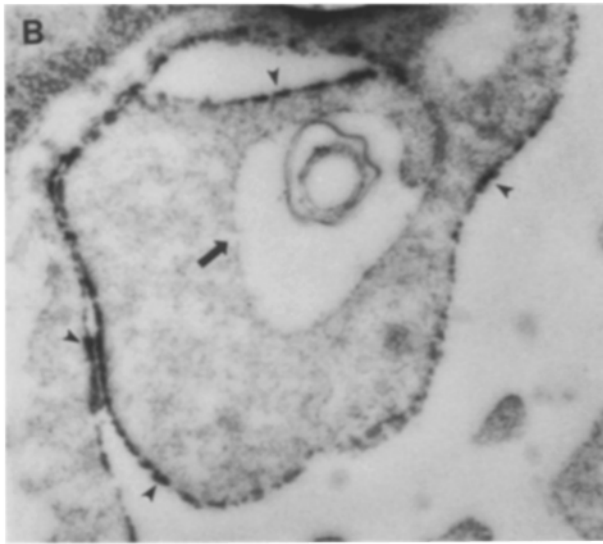
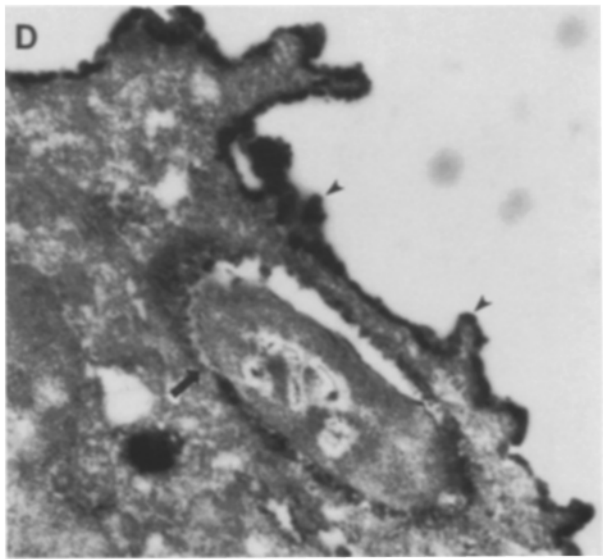
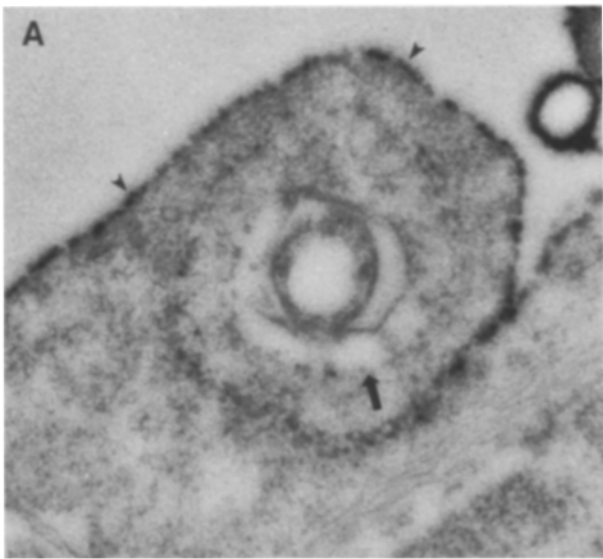
## Materials and Methods

**Reagents.** Mouse mAbs were purchased from the following sources: anti-HLA-DR (IgG2b, clone BL2) from AMAC, Inc. (Westbrook, ME), anti- $\beta_2$ -microglobulin (IgG1, clone BM-63) from Sigma Chemical Co. (St. Louis, MO), and anti-CR3 (IgG2a, clone D12) from Becton Dickinson & Co. (Mountain View, CA). Isotypic mouse myeloma control proteins were obtained from Cappel Organon-Technica (Westchester, PA). Horseradish peroxidase-conjugated goat anti-mouse IgG was purchased from Bio-Rad Laboratories (Richmond, VA). Goat anti-mouse colloidal gold (10 nm) was purchased from Sigma Chemical Co. Leucoprep was purchased from Gibco Laboratories (Grand Island, NY); glutaraldehyde, osmium tetroxide, and LR White embedding medium from Polysciences, Inc. (Warrington, PA); and Tween-20, lysine, sodium periodate, polyvinyl alcohol, polyvinylpyrrolidone, paraformaldehyde, hydrogen peroxide, and diaminobenzidine were from Sigma Chemical Co.

**Bacteria.** *L. pneumophila*, Philadelphia 1 strain, was grown in

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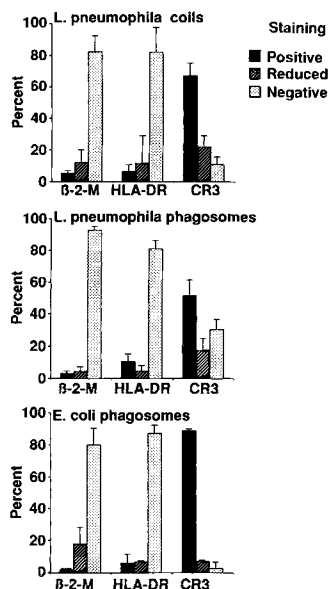
This work has been presented in part at the 1991 National Meeting of the Association of American Physicians, the American Society for Clinical Investigation, and the American Federation for Clinical Research, 3-6 May 1991, Seattle, WA, and published in abstract form.



embryonated hens' eggs, harvested, tested for viability and contaminants, and stored at  $-70^{\circ}\text{C}$ , as described (1). The egg yolk grown *L. pneumophila* were cultured one time only on charcoal yeast extract agar, harvested after 4 d of growth, and used immediately. *E. coli* (serotype O9:K9-:H12, strain x43) were grown in tryptic soy broth and harvested at log phase immediately before use (2).

**Immunoperoxidase Staining of Bacterial Phagosomes for MHC and Complement Receptor Molecules.** Optimal phagocytosis of *L. pneumophila* and *E. coli* was achieved using procedures described previously (3), and the cells were immunostained for electron microscopy by a preembedding immunoperoxidase technique (7). Human mononuclear cells were isolated from fresh heparinized blood using Leucoprep density gradients. Mononuclear cells ( $10^6/\text{ml}$  final concentration) and bacteria ( $10^{10}/\text{ml}$  *L. pneumophila* and  $10^9/\text{ml}$  *E. coli*) were mixed together in RPMI containing 10% autologous human serum and centrifuged first at 220 g for 10 min and then at 800 g for 10 min at  $4^{\circ}\text{C}$ , a temperature at which phagocytosis does not occur. The pellet was swirled gently for 3.5 min at  $37^{\circ}\text{C}$ , a temperature at which complement is deposited onto *L. pneumophila* and *E. coli*, and phagocytosis proceeds rapidly, and the supernate was removed by aspiration. The pellet was immediately fixed, first in 4% paraformaldehyde and 0.01% glutaraldehyde in 0.10 M cacodylate buffer, pH 7.30, containing 0.05% Tween-20 for 1 h, and subsequently in 10 mM periodate, 75 mM lysine, 2% paraformaldehyde in 37 mM sodium phosphate buffer, pH 7.30, containing 0.05% Tween-20 for 2 h at  $4^{\circ}\text{C}$  (7). Tween-20 (0.05%) was included to permeabilize the cells in these and all subsequent steps in which cells were incubated with antibody. Cell pellets were then washed in 0.15 M Tris-HCl, pH 7.50, incubated with 50% normal goat serum in Tris buffer for 1 h at room temperature to block nonspecific antibody binding sites, and incubated with primary mouse mAb or isotypic myeloma protein control (final mouse Ig concentration,  $5\ \mu\text{g}/\text{ml}$ ) in 50% normal goat serum, 0.15 M Tris buffer, pH 7.50, overnight at  $4^{\circ}\text{C}$ . The pellets were then washed three times in Tris buffer; incubated with peroxidase-conjugated goat anti-mouse IgG diluted 1:500 in 1% BSA, 10% autologous human serum, and 0.15 M Tris-HCl, pH 7.50, for 3 h at room temperature; washed four times in Tris buffer; incubated with 0.05% diaminobenzidine and 0.003% hydrogen peroxide in 0.15 M Tris-HCl, pH 7.60, for 30 min at room temperature to develop the peroxidase stain; washed three times; incubated with 1% osmium tetroxide for 1 h at room temperature; dehydrated with a graded series of ethanol concentrations; and embedded in hard grade LR White. Thin sections were collected on formvar-coated nickel grids, stained for 15 min with 2% uranyl acetate, and viewed with an electron microscope (100CX; JEOL USA, Cranford, NJ).

**Localization of MHC Molecules by Immunogold Staining of Cryosections.** The expression of MHC molecules on monocytes was increased by incubating them with IFN- $\gamma$  to facilitate study of the distribution of these molecules. Human mononuclear cells ( $5 \times 10^6/\text{ml}$ ) were incubated with 50 U/ml human rIFN- $\gamma$  in RPMI containing 20% autologous human serum overnight at  $37^{\circ}\text{C}$  in Teflon wells. Cells were released from the Teflon wells by placing the wells on ice for 30 min. Synchronized phagocytosis of *L. pneumophila* and *E. coli* by the monocytes was obtained as described above.

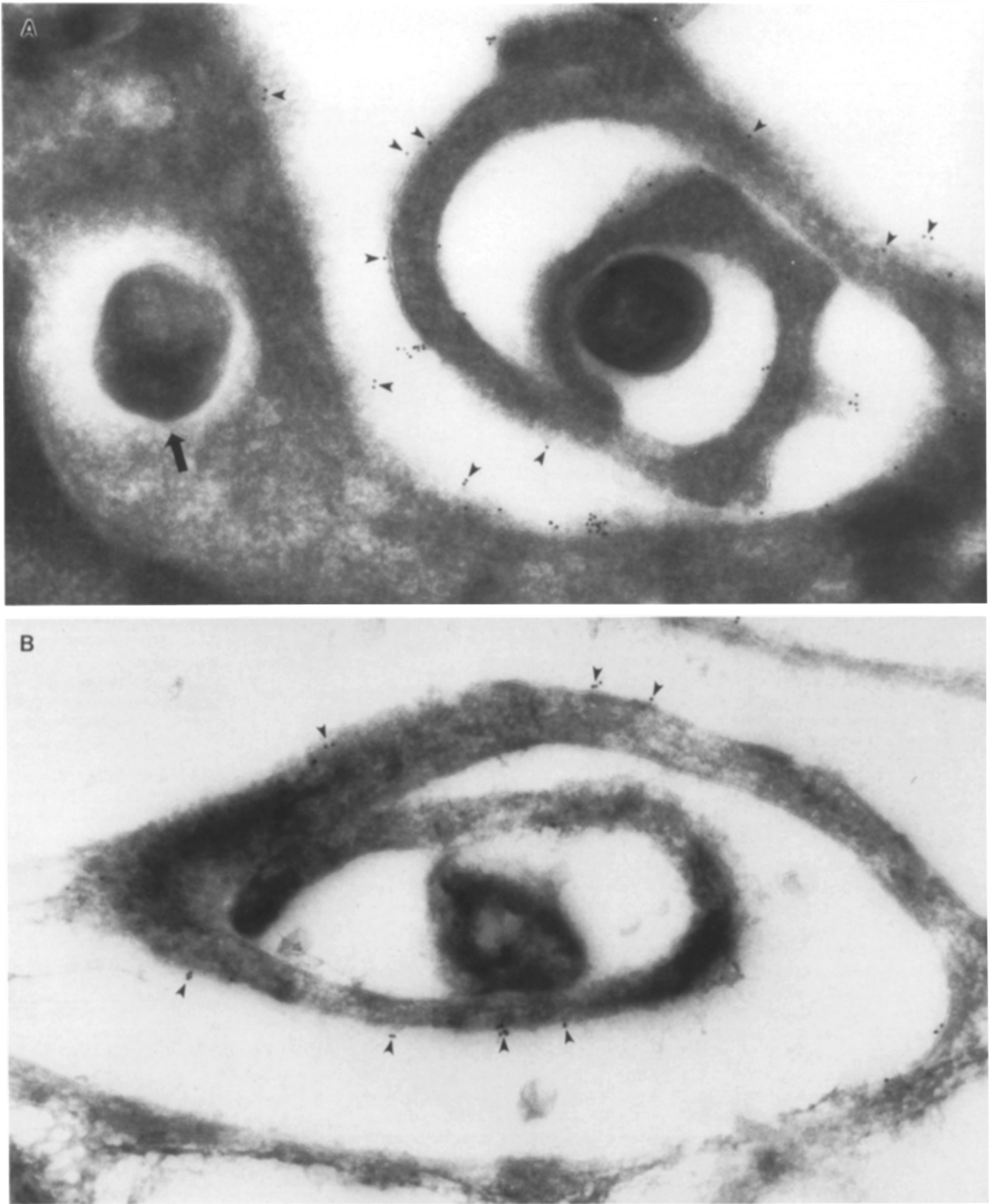


**Figure 2.** Exclusion of MHC molecules but not CR3 from nascent bacterial phagosomes. Immunoperoxidase staining for  $\beta_2$ -microglobulin, HLA-DR, and CR3 in *L. pneumophila* coils, *L. pneumophila* phagosomes, and *E. coli* phagosomes was rated as positive, reduced (compared with the plasma membrane), or negative. Data are the mean percentage  $\pm$  SD for at least 50 coils or phagosomes on each of at least two electron microscopy grids.

The monocytes were then fixed with 4% paraformaldehyde and 0.01% glutaraldehyde in 0.15 M sodium cacodylate, pH 7.30, for 2 h at  $4^{\circ}\text{C}$ . Cell pellets were washed with 0.15 M cacodylate buffer and incubated with 10 mM glycine-HCl in cacodylate buffer to quench free aldehyde groups on the cells. Cell pellets were resuspended in 0.15 M sodium cacodylate, pH 7.30, containing 6.25% acrylamide (prepared from 37.5:1 acrylamide/bis-acrylamide stock) for 1 h at  $4^{\circ}\text{C}$ , pelleted by centrifugation at 1,000 rpm for 10 min, and resuspended in freshly prepared 6.25% acrylamide containing 0.13% (vol/vol) TEMED and 0.83 mg/ml ammonium persulfate. The cells were pelleted again at 800 g for 10 min at  $4^{\circ}\text{C}$  and the acrylamide allowed to polymerize for 2 h at  $4^{\circ}\text{C}$ . The cell pellets were trimmed with a razor blade, infiltrated with 20% polyvinylpyrrolidone in 2.3 M sucrose overnight at  $4^{\circ}\text{C}$ , and cryosectioned at  $-90^{\circ}\text{C}$  with a CR-21 cryosectioning system (Research and Manufacturing Co., Inc., Tucson, AZ). Sections were transferred to nickel grids and incubated with 50% normal goat serum and 0.1% fish skin gelatin in 0.9% NaCl in 0.05 M Hepes, pH 7.40, for 1 h at  $4^{\circ}\text{C}$  to block nonspecific antibody binding. Sections were incubated with 10  $\mu\text{g}/\text{ml}$  mouse monoclonal primary Ig or control isotypic myeloma Ig diluted in Hepes buffer containing 50% normal goat serum and 0.1% fish skin gelatin overnight at  $4^{\circ}\text{C}$ , washed with Hepes buffer, and incubated with goat anti-mouse Ig conjugated to 10 nm colloidal gold for 90 min at room temperature. Sections were washed again with Hepes buffer, postfixed in 4% glutaraldehyde for 5 min, washed in distilled water, and embedded in 2% polyvinyl alcohol containing 0.3% uranyl acetate.

The distribution of MHC molecules at later time points after phagocytosis was studied by infecting monocytes in monolayer culture, washing away nonadherent bacteria, and then incubating the cells for 15 min to 1 h at  $37^{\circ}\text{C}$  before fixation. Human mononuclear cells were plated in 3.5-cm diameter polystyrene tissue culture wells

**Figure 1.** Preembedding immunoperoxidase staining of human monocytes within 3 min of engulfing *L. pneumophila* by coiling phagocytosis (A-C), or *E. coli* by conventional phagocytosis (D-F). Monocytes were fixed during phagocytosis and immunostained with mouse mAbs to  $\beta_2$ -microglobulin (A and D), HLA-DR (B and E), or CR3 (C and F). MHC molecules (A, B, D, and E) were abundant on the plasma membrane (arrowheads), but absent or greatly reduced on the inner layers of coils and on phagosomes (arrows). In contrast, CR3 (C and F) was abundant on the inner layer of coils (large arrows). A,  $\times 49,000$ ; B,  $\times 39,000$ ; C,  $\times 43,000$ ; D,  $\times 27,000$ ; E,  $\times 32,000$ ; F,  $\times 31,000$ .



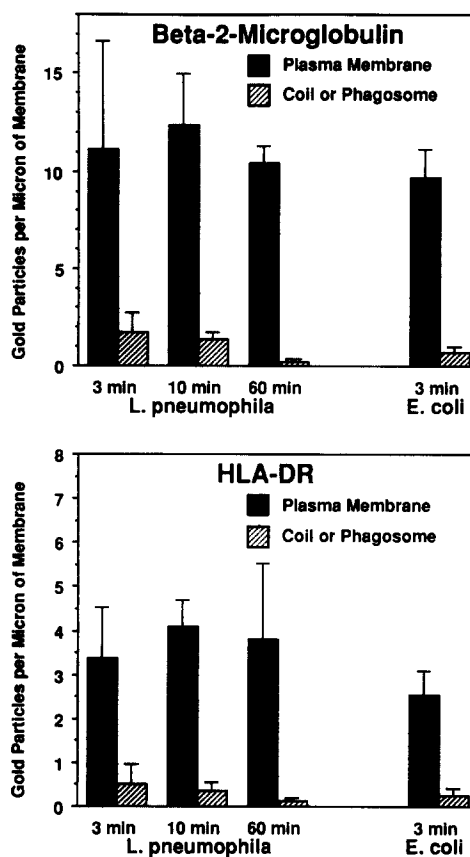
**Figure 3.** Cryosection immunogold staining for MHC molecules in *L. pneumophila* coils. IFN- $\gamma$ -treated human monocytes were fixed within 3 min of coiling phagocytosis of *L. pneumophila*. Cryosections were stained for  $\beta_2$ -microglobulin (A) or HLA-DR (B). Immunogold staining for MHC molecules was abundant on the plasma membrane (arrowheads) but absent or markedly diminished on the inner layers of coils (A and B) and nascent phagosomes (A, arrow). A,  $\times 78,000$ ; B,  $\times 70,000$ .

( $3 \times 10^6$  cells/ml, 2.5 ml/well) in RPMI containing 10% autologous serum. Monocytes were allowed to adhere to the plastic for 90 min at 37°C in 5% CO<sub>2</sub>, 95% air, washed three times with RPMI, and incubated with 50 U/ml of IFN- $\gamma$  in RPMI containing 10% autologous serum overnight. Monocyte monolayers were infected by incubating them with *L. pneumophila* ( $10^9$ /ml) in RPMI containing 10% fresh autologous human serum for 15 min at 37°C on a rotating platform (100 rpm). The monocytes were washed three times with RPMI to remove nonadherent bacteria and incubated for an additional 15 min to 1 h in RPMI containing 10% autologous serum. The infected monocytes were then fixed in 4% paraformaldehyde and 0.01% glutaraldehyde in 0.15 M cacodylate buffer, pH 7.30, for 2 h at 4°C, washed with cacodylate buffer, incubated with 10 mM glycine-HCl in cacodylate buffer for 30 min to quench aldehyde groups, and scraped from the tissue culture wells with a rubber policeman into cacodylate buffer containing 0.1% BSA. The scraped cells were pelleted by centrifugation at 1,000 rpm for 10 min, embedded in 6.25% acrylamide, cryosectioned, and stained by immunogold as described above.

**Cytochemical Staining of *L. pneumophila* cells and Phagosomes for Alkaline Phosphatase and 5'-Nucleotidase.** Human mononuclear cells were cultured in Teflon wells in RPMI containing 20% autologous serum for 7 d at 37°C to increase the expression of 5'-nucleotidase. Synchronized phagocytosis of *L. pneumophila* was obtained as described above, and the cells were fixed with 4% paraformaldehyde and 0.01% glutaraldehyde in 0.15 M cacodylate buffer, pH 7.3, for 1 h at 4°C. Alkaline phosphatase and 5'-nucleotidase cytochemistry was performed as described by Borgers and Thone (8). The fixed cells were pelleted by centrifugation at 800 g for 10 min, and washed twice with 0.22 M sucrose buffered with 60 mM Tris-maleate to either pH 9.0, for the detection of alkaline phosphatase, or pH 7.0 for the detection of 5'-nucleotidase activity. The cells were incubated for 30 min at 37°C in Tris-maleate-sucrose buffer containing 3 mM lead nitrate, 5 mM MgCl<sub>2</sub> and either 5 mM para-nitrophenyl phosphate for the detection of alkaline phosphatase or 5 mM 5'-adenosine monophosphate for the detection of 5'-nucleotidase. The cells were washed once with Tris-maleate-sucrose buffer, washed twice with 0.15 M cacodylate buffer, pH 7.3, and incubated with 1% osmium tetroxide for 1 h at room temperature. Cells were dehydrated, embedded in hard grade LR White, sectioned, and post-stained with uranyl acetate as described above.

## Results

**Exclusion of MHC Molecules but Not CR3 from Nascent Phagosomes.** By using a procedure to synchronize phagocytosis (copelleting bacteria and monocytes at 4°C and warming to 37°C for 3 min before fixation), we were able to observe numerous monocytes fixed in the process of phagocytosing bacteria. Most monocytes had one or more coils or nascent phagosomes in the sections viewed. Of the monocytes that had ingested bacteria in the plane of the section, most were positive for  $\beta_2$ -microglobulin and HLA-DR immunoperoxidase staining on the plasma membrane ( $96.1 \pm 1.0\%$  and  $90.9 \pm 0.01\%$ , respectively). However, in these monocytes,  $\beta_2$ -microglobulin and HLA-DR were excluded from the inner layer of the majority of *L. pneumophila* coils ( $82.5 \pm 10$  and  $81.1 \pm 5.3\%$ , respectively) and phagosomes ( $92.6 \pm 2.5$  and  $81.1 \pm 5.3\%$ , respectively; Figs. 1, A and B, and 2). Similarly,  $\beta_2$ -microglobulin and HLA-DR were excluded from nascent *E. coli* phagosomes; immunoperoxidase staining

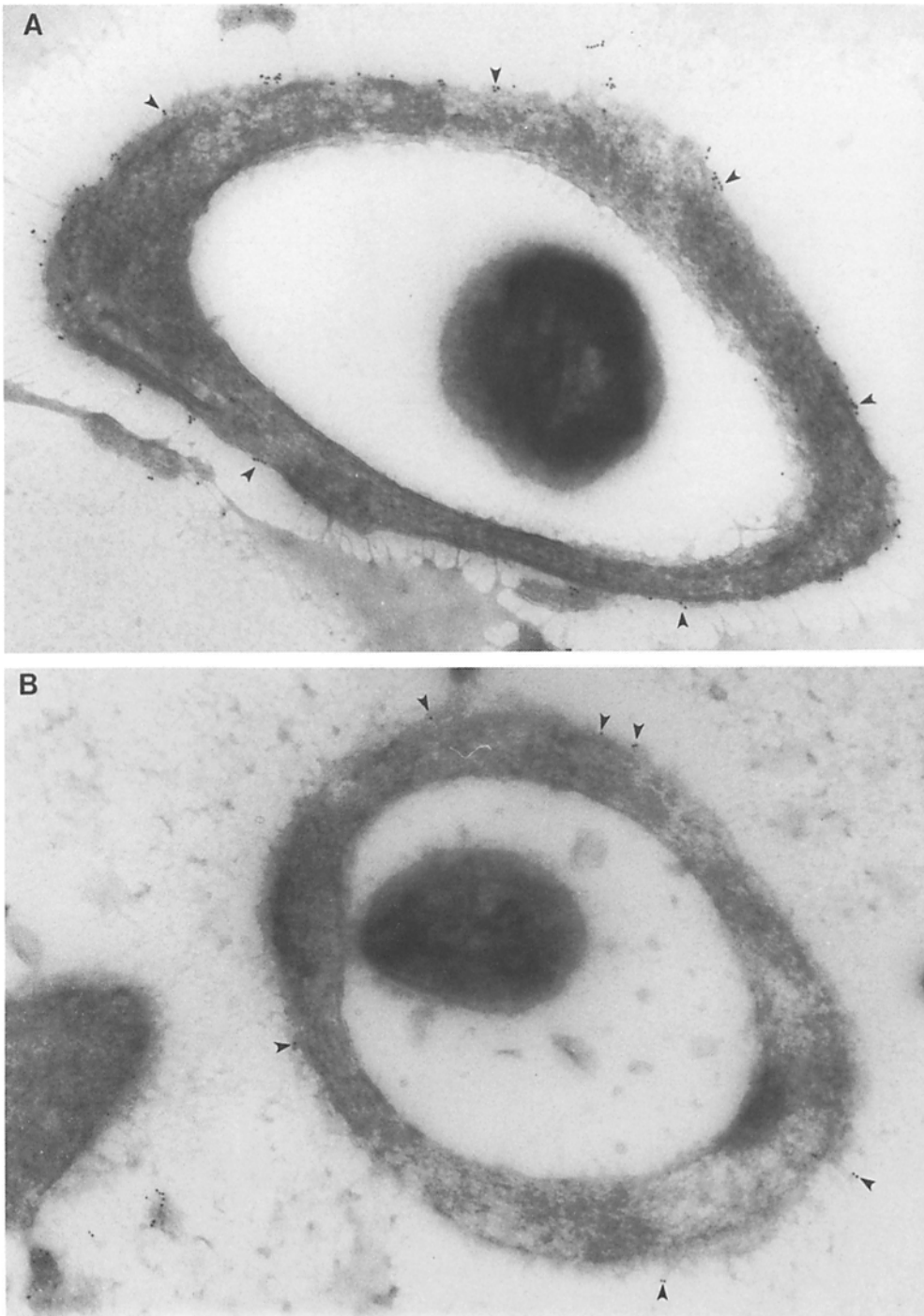


**Figure 4.** MHC molecules are excluded from *L. pneumophila* coils and phagosomes and *E. coli* phagosomes as assessed by cryosection immunogold staining. IFN- $\gamma$ -treated human monocytes were fixed at 3 min, 10 min, or 1 h after phagocytosis of *L. pneumophila*. Cryosections were stained by immunogold for  $\beta_2$ -microglobulin and HLA-DR. The number of gold particles per unit of membrane was counted on coils, phagosomes, and the plasma membrane. Data are the mean percentage  $\pm$  SD of gold particles per unit of membrane on coils or phagosomes relative to the plasma membrane for at least 20 cells on each of at least two electron microscopy grids.

for  $\beta_2$ -microglobulin and HLA-DR was absent from  $79.9 \pm 10.4$  and  $90.0 \pm 1.6\%$  of nascent *E. coli* phagosomes, respectively (Figs. 1, D and E, and 2).

In contrast to MHC molecules, CR3 immunoperoxidase staining persisted in *L. pneumophila* coils and nascent phagosomes. Of monocytes with ingested *L. pneumophila*, 91.7% were positive for CR3 by the immunoperoxidase technique. Of these monocytes, positive immunoperoxidase staining for CR3 was present in  $67.1 \pm 8.0$  and  $51.8 \pm 9.5\%$  of *L. pneumophila* coils and phagosomes, respectively (Fig. 2, A and B), and staining was frequently more intense in the inner layer of the coil than on the plasma membrane (Fig. 1 C). By the immunoperoxidase technique, CR3 staining also persisted in nascent *E. coli* phagosomes (Figs. 1 F and 2 C).

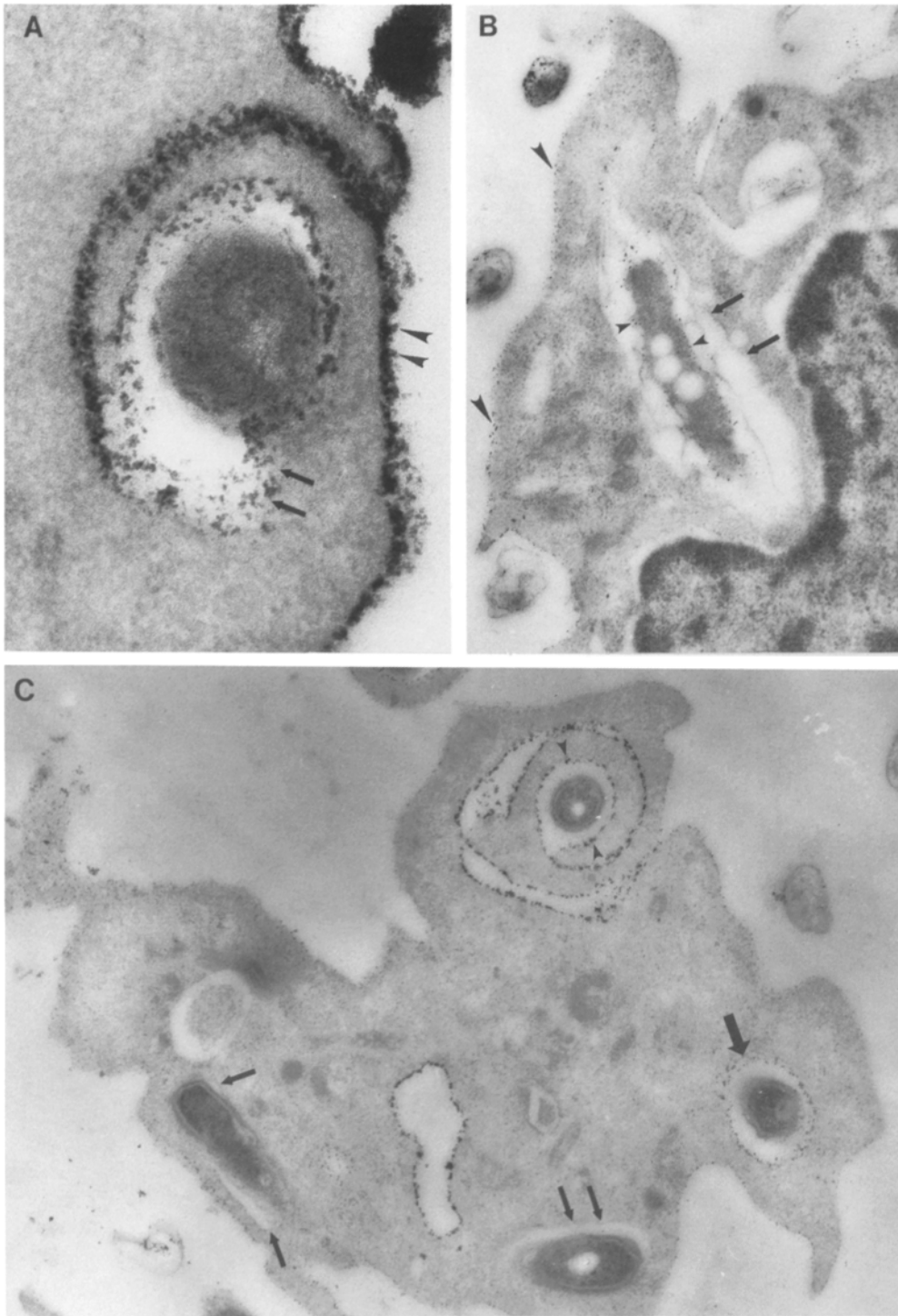
We used mouse myeloma Igs of IgG1, IgG2a, and IgG2b isotypes as controls for nonspecific staining in lieu of mAbs against MHC molecules and CR3. We observed no immunoperoxidase staining of monocytes with these myeloma proteins.



**Figure 5.** Cryosection immunogold staining for MHC molecules on nascent *E. coli* phagosomes. IFN- $\gamma$ -treated human monocytes were fixed within 3 min of engulfing *E. coli*. Cryosections were stained for  $\beta_2$ -microglobulin (A) or HLA-DR (B). Immunogold staining for MHC molecules was abundant on the plasma membrane (arrowheads) but absent or markedly diminished on phagosomal membranes. A,  $\times 47,000$ ; B,  $\times 25,000$ .

To verify the exclusion of MHC molecules from nascent *L. pneumophila* and *E. coli* phagosomes, and to confirm that the apparent exclusion was not due to incomplete permeabilization of the cells by the preembedding immunoperoxidase method, we determined the distribution of MHC molecules during phagocytosis by immunogold staining of ultrathin cryosections. To increase immunogold staining for MHC molecules, we incubated monocytes with 50 U/ml of human IFN- $\gamma$  for 1 d before infecting them with *L. pneumophila* or *E. coli*. Immunogold staining per unit of mem-

brane was quantitated for HLA-DR and  $\beta_2$ -microglobulin on the inner layer of *L. pneumophila* coils, on the membrane of *L. pneumophila* or *E. coli* phagosomes, and on the plasma membrane of the same monocytes. Immunogold staining of cryosections verified the relative exclusion of MHC molecules from nascent bacterial phagosomes observed by the preembedding immunoperoxidase method. At 3 min after phagocytosis,  $\beta_2$ -microglobulin and HLA-DR immunogold staining per unit of membrane were reduced to  $15.9 \pm 7.8$  and  $18.0 \pm 2.8\%$ , respectively, of the staining of the plasma



**Figure 6.** Phosphatase staining of human monocytes engulfing *L. pneumophila* by coiling phagocytosis. Human monocytes were fixed during coiling phagocytosis of *L. pneumophila* and stained for alkaline phosphatase (A and B) or 5'-nucleotidase (C). Alkaline phosphatase activity is present on the plasma membrane of monocytes (A and B, large arrowheads), but is greatly reduced (A) or absent (B) from the inner layers of coils and phagosomes (arrows). *L. pneumophila* alkaline phosphatase was often present between inner and outer bacterial membranes (B, small arrowheads), indicating that the staining reagents can permeate the inside of the phagosome. 5'-Nucleotidase activity stained intensely in the inner layers of coils (C, arrowheads), often staining more intensely than the plasma membrane. 5'-Nucleotidase activity was reduced or absent in most *L. pneumophila* phagosomes (C, small arrows) but not all (C, large arrow). A,  $\times 88,000$ ; B,  $\times 18,000$ ; C,  $\times 19,000$ .

membrane (Figs. 3, A and B, and 4). Immunogold staining for  $\beta_2$ -microglobulin and HLA-DR was decreased further at 10 min and 1 h after phagocytosis (Fig. 4).  $\beta_2$ -microglobulin and HLA-DR immunogold staining were similarly reduced in *E. coli* nascent phagosomes compared with that of the plasma membrane (Figs. 4 and 5).

Immunogold staining of cryosections for complement receptor CR3 was consistent with preembedding im-

munoperoxidase staining, but the overall level of immunogold staining was too low to be reliable.

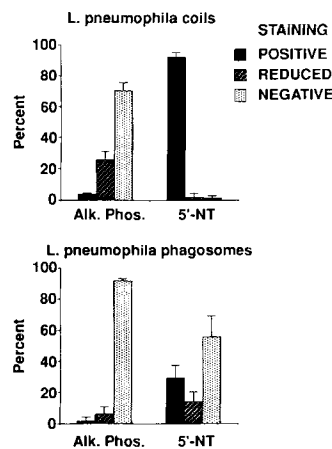
We again used isotypic mouse myeloma Igs as controls for nonspecific staining in lieu of mAbs against MHC molecules. Nonspecific staining of the plasma membrane of monocytes with control Igs was negligible (IgG2b,  $0.007 \pm 0.009$  gold particles per micron of plasma membrane; IgG1,  $0.04 \pm 0.02$  gold particles per micron of plasma membrane).

**Exclusion of Alkaline Phosphatase but Not 5'-Nucleotidase from *L. pneumophila* Coils.** To determine whether the rapid sorting of membrane proteins during phagocytosis is unique to MHC molecules and complement receptors or a more general phenomenon, we examined the distribution of the plasma membrane markers alkaline phosphatase and 5'-nucleotidase during phagocytosis of *L. pneumophila*. Whereas alkaline phosphatase staining was present on the plasma membrane of virtually all (90.6%) monocytes with ingested *L. pneumophila*, alkaline phosphatase staining was absent from  $70.3 \pm 5.2\%$  of the *L. pneumophila* coils, and from  $91.6 \pm 1.5\%$  of nascent *L. pneumophila* phagosomes (Figs. 6 and 7). *L. pneumophila* contains its own alkaline phosphatase enzyme, and alkaline phosphatase reaction product was present between the bacterial inner and outer membranes (Fig. 6 B). The percentage of *L. pneumophila* that were positive for alkaline phosphatase was similar for intracellular and extracellular *L. pneumophila* (56.9 and 54.9%, respectively). That *L. pneumophila* alkaline phosphatase phosphatase reaction product was present in coils and phagosomes from which monocyte alkaline phosphatase reaction product was absent indicates that the exclusion of monocyte alkaline phosphatase from *L. pneumophila* coils and phagosomes was not due to a failure of the enzyme substrate and lead nitrate capture reagents to penetrate into coils or phagosomes.

Whereas alkaline phosphatase activity was excluded from *L. pneumophila* coils, 5'-nucleotidase activity was present in coils. Staining for 5'-nucleotidase activity was present in  $91.9 \pm 3.3\%$  of *L. pneumophila* coils (Fig. 7). As with CR3, staining for 5'-nucleotidase activity was typically more intense on the inner layer of *L. pneumophila* coils than on the adjacent plasma membrane (Fig. 6 C). However, the persistence of 5'-nucleotidase activity appeared very transient, as  $56 \pm 13\%$  of phagosomes did not stain for 5'-nucleotidase (Figs. 6 C and 7), suggesting that 5'-nucleotidase activity is rapidly removed from nascent phagosomal membranes.

## Discussion

By synchronizing phagocytosis, we were able to study the distribution of membrane proteins during and immediately after formation of the phagosome. Our study demonstrates that monocytes selectively exclude some membrane proteins while other persist during formation of a phagosome (Fig. 8). Class I and class II MHC molecules, whose distribution during phagocytosis has not previously been reported, are rapidly excluded from the phagosomal membrane during coiling and conventional phagocytosis of *L. pneumophila* and *E. coli*, respectively. In marked contrast, CR3 persists in the nascent phagosomal membrane. That nascent *L. pneumophila* and *E. coli* phagosomes stained readily for CR3 by the preembedding immunoperoxidase technique indicates that the paucity of MHC staining by this technique was not due to inadequate permeabilization of the cells by reagents used in the preembedding immunoperoxidase staining method. Cryosection immunogold labeling, a method in which all cell compartments are equally accessible to antibody staining,



**Figure 7.** Exclusion of alkaline phosphatase but not 5'-nucleotidase from *L. pneumophila* coils. Alkaline phosphatase and 5'-nucleotidase staining of coils (top) and phagosomes (bottom) were rated as positive, reduced (compared with the plasma membrane), or negative. Data are the mean percentage  $\pm$  SD of at least 50 cells on at least two electron microscopy grids.

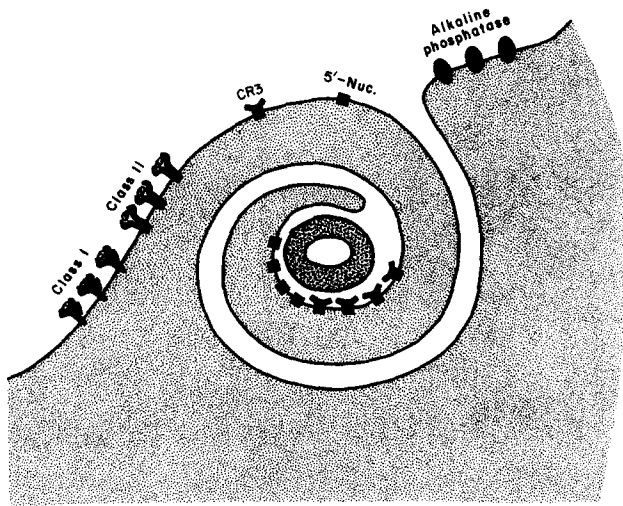
confirmed the relative exclusion of MHC molecules from nascent *L. pneumophila* and *E. coli* phagosomes.

As with MHC and CR3 molecules, the plasma membrane markers alkaline phosphatase and 5'-nucleotidase show different staining patterns during coiling phagocytosis. Alkaline phosphatase activity, like MHC molecules, is excluded from *L. pneumophila* coils and phagosomes; in contrast, 5'-nucleotidase activity, like CR3, persists in coils. However, the 5'-nucleotidase activity is rapidly lost as the coil matures into a phagosome. Although the staining for both CR3 and 5'-nucleotidase activity was typically stronger in the coil than on the plasma membrane, this does not necessarily indicate concentration of CR3 or 5'-nucleotidase activity in the coil, since immunoperoxidase or nucleotidase reaction product may be more free to diffuse away from the plasma membrane than the interior of the coils.

Both alkaline phosphatase and 5'-nucleotidase have been reported to have phosphatidylinositol-glycan anchors (9, 10). However, alkaline phosphatase is readily and completely solubilized from the plasma membrane with low concentrations of phosphatidylinositol-specific phospholipase, but 5'-nucleotidase is only 30–70% solubilized with higher concentrations and longer incubations with phosphatidylinositol-specific phospholipase (9, 10). The differences between alkaline phosphatase and 5'-nucleotidase both in susceptibility to phospholipase and in membrane sorting during phagocytosis may reflect the existence of additional forms of 5'-nucleotidase with a transmembrane protein anchor. Alternatively, the differences may reflect dissimilarities in the environment of the proteins in the membrane; e.g., 5'-nucleotidase may interact with other membrane proteins in a fashion that sterically limits access of phospholipase to its phosphatidylinositol anchor (10) and also alters its sorting during phagocytosis.

Other investigators have examined the distribution of various membrane proteins at later time points, subsequent to but within 30 min of phagocytosis, and also found evidence for exclusion or persistence of membrane molecules (11–14). Tsan and Berlin (11) found no change in the rate of transport of adenosine, adenine, or lysine after phagocytosis of latex beads by peritoneal exudate cells or alveolar macrophages,





**Figure 8.** Pictorial summary of the sorting of membrane proteins during phagocytosis. Alkaline phosphatase and class I and class II MHC molecules are relatively excluded from the phagosome during coiling phagocytosis. In contrast, 5'-nucleotidase and complement receptor CR3 persist on the inner layer of the coil during coiling phagocytosis.

despite internalization of 35–50% of the plasma membrane, indicating that the membrane transport systems are either excluded during phagocytosis or are rapidly and efficiently recycled to the surface. Similarly, North (12) found that  $\text{Ca}^{2+}/\text{Mg}^{2+}$  ATPase cytochemical staining was excluded from the phagosome after phagocytosis of erythrocytes by guinea pig peritoneal exudate macrophages. Pimenta and De Souza (13) reported that  $\text{Mg}^{2+}$ -ATPase and 5'-nucleotidase cytochemical staining was absent from *Leishmania amazonensis* parasitophorous vacuoles after coincubation of parasites and resident peritoneal macrophages for 30 min. They found, however, that NAD(P)H oxidase activity appeared to cointernalize with *L. amazonensis* promastigotes. These authors did not examine earlier time points, so it is not known if 5'-nucleotidase activity is present in the *L. amazonensis* phagosome during phagocytosis and then lost in the immediate postphagocytic period, as in our study.

Mellman et al. (14) have shown that Fc receptors are removed selectively from the plasma membrane during Fc receptor-mediated phagocytosis of IgG-coated particles. This observation is analogous to our finding that CR3 persists in nascent *L. pneumophila* and *E. coli* phagosomes. CR3 mediates phagocytosis of *L. pneumophila* and presumably *E. coli* (2, 6). These findings, taken together with our other findings, suggest that specific receptors involved in mediating phagocytosis are included in the nascent phagosome, while plasma membrane proteins not involved in phagocytosis may be excluded from the phagosome during its formation.

The distribution of MHC molecules either during phagocytosis or during the immediate postphagocytic period has not been studied previously. However, at 2 h after phagocytosis, Antoine et al. (15) observed that only 34% of *L. amazonensis* phagosomes stained positively for class II MHC. This relatively low percentage is consistent with our observation that MHC molecules are relatively excluded from the early

phagosome. Antoine et al. (15) also found that class II MHC staining of *L. amazonensis* phagosomes steadily increased to 87% between 2 and 48 h after infection. We have not observed a marked increase in MHC class I or class II staining of *L. pneumophila* phagosomes at 4 or 22 h after infection (our unpublished data), suggesting that the relationship of MHC molecules to the *L. pneumophila* phagosome is qualitatively different from that of MHC molecules to the *L. amazonensis* phagosome. This may reflect major differences between these two phagosomal compartments. For example, whereas the *L. pneumophila* phagosome does not fuse with lysosomes and is only mildly acidified (16, 17) the *L. amazonensis* phagosome does fuse with lysosomes and is highly acidified (18, 19). Thus, the relative exclusion of MHC molecules from the *L. pneumophila* phagosome may be a feature of the distinctive phagosomal compartment in which it resides.

Membrane sorting consequent to phagocytosis has not been consistently observed. Hubbard and Cohn (20), and Muller et al. (21) studied phagocytosis of lactoperoxidase-coated polystyrene beads by L cells and found that the phagosomal membrane is similar in lipid and protein content to the plasma membrane. They also observed that membrane proteins are rapidly and efficiently recycled back to the plasma membrane. Thus, the degree to which membrane proteins are sorted during phagocytosis may be dependent upon the particular phagocytic system studied.

During the postphagocytic period, the phagosomal membrane continues to maintain a dynamic state. The relative amounts of class I and class II MHC molecules continue to decline. The number of class I MHC molecules drops from  $15.9 \pm 7.8\%$  of the plasma membrane level at 3 min to  $2.1 \pm 0.2\%$  at 1 h, and the number of class II MHC molecules drops from  $18.0 \pm 2.8\%$  of the plasma membrane level at 3 min to  $3.2 \pm 1.8\%$  at 1 h. 5'-Nucleotidase activity is present in the inner layer of *L. pneumophila* coils, but rapidly disappears as the coils mature into phagosomes.

The decline in MHC immunogold staining and 5'-nucleotidase activity in the phagosome is unlikely to be due to inactivation by hydrolases, as the *L. pneumophila* phagosome does not fuse with lysosomes (16) and is only mildly acidified (17). Instead, the decline in activity likely represents active sorting and removal of membrane proteins from the nascent phagosome. However, since the plasma membrane markers 5'-nucleotidase and alkaline phosphatase activity were detected by their enzymatic activity rather than by immunochemical methods, we cannot rule out the possibility that the alterations in their staining pattern reflect modulation of enzyme activity rather than alterations in the concentrations of enzyme in the membrane compartments.

In conclusion, our results indicate that the cell is able to sort membrane proteins rapidly during phagocytosis and define a phagosomal membrane whose composition is markedly different from that of the plasma membrane from which it is derived. The mechanism by which the cell accomplishes this remains to be determined. The composition of the phagosomal membrane is important in host-parasite interactions, including the processing and presentation of parasite-

derived antigens. The relative exclusion of MHC molecules from bacterial phagosomes during phagocytosis suggests the possibility that bacterial antigens in the phagosome may not be presented by MHC molecules derived directly from the

plasma membrane, although it is possible that low levels of MHC molecules remaining in the phagosome are able to play an important role in antigen presentation.

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