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Douglas G. Perry, Gena L. Daugherty and William J. Martin, II

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Clathrin-Coated Pit-Associated Proteins Are Required for Alveolar Macrophage Phagocytosis¹

Douglas G. Perry,² Gena L. Daugherty, and William J. Martin II

During phagocytosis, phagocytic receptors and membrane material must be inserted in the pseudopod membrane as it extends over the phagocytic target. This may require a clathrin-mediated recycling mechanism similar to that postulated for leading edge formation during cell migration. To investigate this possibility, liposomes were used to deliver to intact rat alveolar macrophages (AMs): 1) Abs to clathrin, clathrin adaptor AP-2, and hsc70, and 2) amantadine. Phagocytosis was assayed by fluorometric and colorimetric techniques. Liposome-delivered Abs to clathrin and AP-2 inhibited AM phagocytosis of zymosan-coated, fluorescent liposomes from 16.3 ± 0.3 to 5.8 ± 0.3 , and 10.1 ± 0.9 to 4.8 ± 0.2 liposomes/cell ($p < 0.01$). Similarly, liposome-delivered Ab to clathrin also inhibited AM phagocytosis of IgG-opsonized RBCs from 11.7 ± 1.7 to 3.8 ± 0.7 RBCs/cell ($p < 0.01$). Amantadine, which blocks the budding of clathrin-coated vesicles, inhibited phagocytosis from 13.8 ± 0.8 to 5.7 ± 0.6 ($p < 0.01$). Ab blockade of hsc70, which catalyzes clathrin turnover, also inhibited phagocytosis from 9.1 ± 0.5 to 4.3 ± 0.2 ($p < 0.01$). These findings suggest that clathrin-mediated receptor/membrane recycling is required for phagocytosis. *The Journal of Immunology*, 1999, 162: 380–386.

Phagocytosis is the primary defense mechanism of all animal phyla (1). Even in vertebrates with advanced immune systems, phagocytes provide first-line protection against, and removal of, unwanted organisms and particles. Paradoxically, some pathogenic organisms take advantage of phagocytosis to circumvent detection by the host (2, 3). Much has been achieved in understanding this fundamental cell function, yet much remains to be explored (4). For example, in spite of the advance in knowledge of receptor interaction (5, 6), signal transduction (7), and cytoskeletal rearrangement (2, 8) during phagocytosis, questions remain about pseudopod extension: by itself, actin polymerization would simply stretch the cell membrane along the axis of the lipid bilayer; such stretching is severely limited by biophysical constraints (9). Clearly, new membrane material must be inserted in the pseudopod membrane as it extends over the phagocytic target. A similar challenge is faced by a migrating cell, which must rapidly provide surface receptors (and accompanying membrane) to its leading edge. In this context, Bretscher developed a theory of cell locomotion that postulates that receptor/membrane recycling occurs through a endocytic/exocytic pathway (10). Receptors at the trailing edge of the cell are endocytosed via clathrin-coated pits, transported through the cytoplasm by vesicles, and inserted at the leading edge by exocytosis (11). In describing this model, Bretscher drew an analogy to the migrating cell as a moving tank, with adhesive receptors and their associated bits of membrane serving as the rolling treads, guided by cytoskeletal rearrangement (12). Hopkins et al. demonstrated that receptors are indeed recycled

during cell migration (13). Other observers have noted the striking similarity between the formation of leading edges in migrating cells with the extension of pseudopods in phagocytes (14).

Clathrin has been implicated as a necessary component of phagocytosis (15). Clathrin-coated pits are found in peritoneal macrophages (14, 16), and are located at surface adhesion sites (17) and phagosomes (18) in the macrophage. The clathrin-coated pit-associated adaptor AP-2 is concentrated at the phagosome membrane (19, 20). Coated pits are associated with macrophage surface adhesion sites (21). In addition, clathrin redistributes from the Golgi to plasma membrane in IgG-challenged macrophages (22). FcR localize to underlying coated pits (23), and coated pits internalize surface receptors of macrophages (24).

In light of these findings, we hypothesized that clathrin-coated pits play a role in phagocytosis, as similarly postulated for cell migration. To determine the involvement of clathrin and related coated pit proteins in alveolar macrophage (AM)³ phagocytosis, we used liposomes to deliver, to intact AMs, Abs to clathrin-coated pit-related proteins and a drug affecting coated pit function. AMs were subsequently challenged for both opsonic and nonopsonic phagocytosis, and the level of phagocytic activity was quantified by fluorometric and colorimetric assays.

Materials and Methods

Materials

Ammonium chloride, bovine clathrin, BSA, calcium chloride, cholesterol, 2,7-diaminofluorene (DAF), dibasic phosphate, dipalmitoylphosphatidylcholine, EDTA, FITC-conjugated goat anti-mouse IgG (gt anti-ms IgG-FITC), FITC-conjugated rabbit anti-goat IgG (rb anti-gt IgG-FITC), goat polyclonal Ab against human IgA (anti-IgA pAb), goat polyclonal Ab against rat clathrin HC (anti-clathrin pAb), heparin, HRP-conjugated rabbit anti-goat IgG (rb anti-gt IgG-HRP), hydrogen peroxide, HEPES, LPS, monobasic phosphate, mouse mAb against bovine adaptor AP-2 (anti-AP-2

Division of Pulmonary and Critical Care Medicine, Indiana University School of Medicine, Indianapolis, IN 46202

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² Address correspondence and reprint requests to Dr. Douglas G. Perry, Division of Pulmonary and Critical Care Medicine, Indiana University School of Medicine, 1001 West 10th St., OPW 425, Indianapolis, IN 46202. E-mail address: dperry@iupui.edu

³ Abbreviations used in this paper: AM, alveolar macrophage; BCA, bicinchoninic acid; DAF, 2,7-diaminofluorene; DIC, differential interference contrast microscopy; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; F-PS, fluorescent polystyrene microspheres; gt anti-ms, goat anti-mouse; HC, heavy chain; HRP, horseradish peroxidase; MLV, multilamellar vesicle; pAb, polyclonal antibody; rb anti-gt, rabbit anti-goat; rb anti-SRBC, rabbit anti-sheep red blood cells; RT, room temperature.

mAb), mouse mAb against hsc70 (anti-hsc70 mAb), paraformaldehyde, *o*-phenylenediamine dichloride, potassium bicarbonate, sodium chloride, SDS, sodium hydroxide, *t*-octylphenoxypolyethoxyethanol (Triton X-100), tris(hydroxymethyl)aminomethane (Tris), trypsin, and urea were obtained from Sigma (St. Louis, MO). Mouse mAb against SRBC was obtained from Accurate Chemical & Scientific (Westbury, NY). Rat polyclonal Ab against mouse IgG (anti-mouse IgG pAb) was obtained from Pierce (Rockford, IL). Rabbit polyclonal Ab against SRBCs (rb anti-SRBC pAb) was purchased from Cappel/Organon Teknica (Durham, NC). 1-Adamantamine (amantadine) was purchased from Aldrich Chemical (Milwaukee, WI). Radioiodination kits, containing lactoperoxidase, H₂O₂, K₂HPO₄²⁻, Na¹²⁵I, and Sephadex G-10, were purchased from ICN (Costa Mesa, CA). Protein assay kits, consisting of albumin, bicinchoninic acid, CuSO₄, Na₂CO₃, NaHCO₃, and sodium tartrate, were obtained from Pierce. 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was obtained from Molecular Probes (Eugene, OR). Pathogen-free female rats with an average weight of 225 g were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and housed in the Laboratory Animal Resource Center at Indiana University Medical Center in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care. Sheep erythrocytes (RBCs) were purchased from Colorado Serum (Denver, CO). Chloroform and diethyl ether was purchased from Mallinckrodt Specialty Chemicals (Paris, KY). Beuthanasia-D (3.9 g/L pentobarbital sodium) was supplied by Schering-Plough Animal Health (Kenilworth, NJ). Mac-SFM culture medium (Life Technologies, Grand Island, NY) was supplemented with 4 mM glutamine (Sigma) and penicillin/streptomycin (BioWhittaker, Walkersville, MD). PBS (19 mM H₂PO₄⁻, 81 mM HPO₄²⁻, 150 mM NaCl, 1 mM CaCl₂) and HBSS, both titrated to pH 7.4, were obtained from Life Technologies. Polystyrene microspheres (50 nm diameter) tagged with a proprietary yellow-green fluorophore (F-PS) were obtained from Polysciences (Warrington, PA). Polystyrene 96-well plates were obtained from Becton Dickinson (Lincoln Park, NJ).

Liposome production

Liposome production in this laboratory has been previously described in detail (25). Briefly, liposomes were prepared by aqueous reconstitution (26). Dipalmitoylphosphatidylcholine (63 μmol), dicetyl phosphate (18 μmol), and cholesterol (9 μmol) were dissolved in 2 ml chloroform. After evaporation, the vial was capped and heated to 60°C in a water bath. A total of 2 ml of swelling solution (150 mM NaCl, 20 mM HEPES, pH 7.4) was added, vortexed, and subjected to five cycles of freezing and thawing. Thermal cycling enhances liposome formation and solute entrapment (26, 27). The liposome suspension was centrifuged at 13,000 × *g* for 5 min. The pellet was resuspended in 5 ml swelling solution and recentrifuged five times. The pellet was resuspended in 2 ml mac-SFM. The resulting suspension contained liposomes in the form of multilamellar vesicles (MLVs, 26), based on differential interference contrast light microscopy. The typical liposome concentration was 3.1 × 10⁸ MLVs/ml, based on repeated measurements by grid cytometry. The typical liposome feret diameter was 6.5 μm, based on repeated measurements by digital image analysis calibrated with beads of known diameter. This size is too large to allow AM uptake of liposomes by endocytosis, but is optimal for phagocytosis (28).

Variations in the above method were used to make liposomes for Ab delivery, drug delivery, or fluorescent markers for the phagocytosis assay (see below). For Ab delivery, liposomes were made in the presence of 1:40 anti-clathrin pAb, 1:40 anti-AP-2 mAb, or 1:40 anti-hsc70 mAb. For controls using nonspecific IgG, liposomes were prepared with either 1:40 rb anti-SRBC pAb or 1:40 anti-IgA pAb. For drug delivery, liposomes were prepared with 100 mg/ml amantadine. For fluorescent liposomes to be used in the phagocytosis assay, the swelling solution contained 0.1% zymosan and a 1/4 dilution of F-PS, creating a fluorescent artificial yeast cell that could be phagocytosed by AMs via the β-glucan receptor, an example of nonopsonic phagocytosis (6, 8). Liposome suspensions were centrifugally rinsed to remove untrapped material. We have documented previously that liposome membrane integrity is maintained during this procedure (25).

To determine the amount of Ab entrapment, liposomes were made with radioiodinated anti-clathrin pAb (described below). After freeze-thaw, 3 ml swelling solution was added, the suspension was centrifuged, and the activities of supernatant and resuspended pellet (corrected to the original volume) were measured with a gamma counter. After correcting for successive dilutions, the encapsulation ratio was calculated by dividing final pellet activity by total activity of pellet and supernatant.

Alveolar macrophage isolation

Rat AMs were obtained from pathogen-free female Sprague Dawley rats by standard methods (25, 29). Anesthetized rats were sacrificed, and AMs

were recovered by whole lung lavage with HBSS containing 0.2 g EDTA and 10 ml penicillin/streptomycin per liter. Lavage fluid was centrifuged at 1200 × *g* for 5 min at 4°C, supernatant was discarded, and pellet was resuspended in lysing solution (11 mM KHCO₃ and 152 mM NH₄Cl) to lyse RBCs. After rinsing, the pellet was resuspended in 2 ml mac-SFM and cells were counted by grid cytometry. Typically, 4–5 × 10⁶ cells were obtained per rat, 95% of which were AMs (30).

To label cells for enumeration in the fluorometric phagocytosis assay described below, AMs were incubated with DiI in 300 mM glucose for 10 min at room temperature (RT). Cells were spun at 1200 × *g* for 10 min at 4°C. The pellet was resuspended in 300 mM glucose, then spun again at 1200 × *g* for 10 min at 4°C. The final pellet was resuspended in an appropriate volume of mac-SFM to give a final cell concentration of 2.5 × 10⁶ cells/ml.

Radiolabeling of Abs

To radiolabel Abs for liposome entrapment studies, 10 μg anti-clathrin pAb, anti-AP-2, or anti-hsc70 in 25 μl K₂PO₄²⁻ buffer (pH 7.5) was mixed with 2 mCi Na¹²⁵I and 5 μg lactoperoxidase, and transferred to a Sephadex G-10 column. The mixture was activated with 3% H₂O₂. After 10 min, the reaction was stopped by adding 500 μl of buffer. The ¹²⁵I-labeled Ab was eluted from the column and collected in 5-ml fractions. Radioactive fractions were pooled and protein content was determined by BCA assay (see below). Specific activity was expressed as cpm/μg protein.

Determination of protein concentration

To determine Ab concentrations, a BCA protein assay was used (31). Anti-clathrin pAb, anti-AP-2 mAb, or anti-hsc70 mAb, diluted 1/100, was added along with protein standards to 96-well plate containing BCA working reagent and incubated for 30 min at 37°C. Absorbance was measured at 540 nm using a plate reader.

To determine clathrin concentration in liposomes and cells by ELISA, samples and standards were added to 96-well plates, incubated overnight at 4°C, then rinsed four times with 0.05% Tween-20/PBS. 1:500 anti-clathrin pAb was added, incubated for 1 h at 37°C, then rinsed four times with 0.05% Tween-20/PBS. Rb anti-gt IgG-HRP (1:40,000) was added and incubated at 37°C for 2 h, then rinsed four times with 0.05% Tween-20/PBS. Developer (6 mM *o*-phenylenediamine dichloride and 30% H₂O₂) was added and incubated at RT for 15 min, and the reaction was stopped by adding 500 mM H₂SO₄. Absorbance was measured at 492 nm using a plate reader.

Liposome delivery of Abs and drugs to macrophages

To quantify Ab content in liposomes, liposomes were prepared with anti-clathrin pAb, then centrifugally rinsed five times. Liposome concentration was measured by cytometry. A 1-ml sample of this liposome suspension was dissolved in 3 ml 0.5% SDS. The solution was centrifuged for 15 min at 13,000 × *g*, and the amount of anti-clathrin pAb present in the supernatant was determined by ELISA. Anti-clathrin pAb content in fg/liposome was calculated by dividing the anti-clathrin pAb concentration by the liposome concentration.

To demonstrate that anti-clathrin pAb can be delivered by liposomes to AMs, liposomes were prepared with 1:40 anti-clathrin pAb. AMs were incubated with the liposomes for 1 h at 37°C; control cells were incubated with empty liposomes. After rinsing, cells were mounted on standard microscope slides, then fixed in 4% paraformaldehyde, rinsed in PBS twice, permeabilized with 1% Triton X-100, and rinsed four times in PBS. Cells were exposed to 1:100 rb anti-gt IgG-FITC. After rinsing three times in PBS, cells were viewed by microscopy.

To quantify the delivery by liposomes of anti-clathrin pAb to AMs, cells were transferred to a 12-well culture plate and spun at 1200 × *g* for 5 min at 4°C. Liposomes containing anti-clathrin pAb were made, and the amount of Ab per liposome was measured by ELISA. The liposomes were incubated with AMs (1 × 10⁶ cells/ml) for 1 h at 37°C, the plate was rinsed, and lysing solution was added for 25 min to lyse adherent but nonphagocytosed liposomes. After rinsing, cell concentration was measured by cytometry. A total of 2 ml of 1% SDS/PBS was added, and the solution was centrifuged at 13,000 × *g* for 15 min at 4°C. The amount of anti-clathrin pAb was determined by ELISA and expressed as pg/cell.

To determine the effect of liposome-delivered anti-clathrin pAb, anti-AP-2 mAb, anti-hsc70 mAb, and amantadine on phagocytosis, AMs labeled with DiI were plated on 96-well plates (250,000 cells/well) in mac-SFM containing 1 μg/ml LPS. Liposomes containing either 1:40 anti-clathrin pAb, anti-AP-2 mAb, anti-hsc70 mAb, or 100 mg/ml amantadine were added to the wells; liposomes containing either 1% BSA or rb anti-SRBC IgG (as a nonspecific Ab) were added to the control wells. The cells and liposomes were incubated for 1 h at 37°C. All wells were aspirated,

leaving only adherent cells. AMs were then challenged with either zymosan-coated, fluorescent liposomes or opsonized RBCs, for the fluorometric and colorimetric phagocytosis assays, respectively.

Immunolocalization

To identify clathrin HC and adaptor AP-2 in AMs, cells were fixed in 4% paraformaldehyde, rinsed twice in PBS, permeabilized with 1% Triton X-100, and rinsed four times in PBS. Cells were then incubated with either 1:40 anti-clathrin pAb or 1:100 anti-AP-2 mAb for 20 min at RT, rinsed three times in PBS, and incubated with either 1:100 rb anti-gt IgG-FITC or 1:100 gt anti-ms IgG-FITC, respectively, for 30 min. Control cells received second Ab only. After rinsing, slides were viewed with a microscope fitted for fluorescence ($\lambda_{EX} = 485$ nm; $\lambda_{EM} = 530$ nm) and DIC microscopy.

Fluorometric phagocytosis assay

In most of the phagocytosis experiments, the level of phagocytic activity was quantified by a fluorometric assay (25, 32). AMs were activated with 1 μ g/ml LPS (33–35), then labeled with DiI and exposed to excess ($>10^6/100$ μ l) fluorescent liposomes in 96-well plates. After incubation for 1 h at 37°C on a shaker, wells were rinsed to remove nonphagocytosed liposomes and nonadherent cells, then filled with 100 μ l lysing solution to lyse any remaining adherent, nonphagocytosed liposomes, then rinsed again. Fluorescence intensity was measured in a plate fluorometer, with $\lambda_{EX} = 485$ nm and $\lambda_{EM} = 530$ nm for F-PS, and $\lambda_{EX} = 530$ nm and $\lambda_{EM} = 590$ nm for DiI. Engulfed liposome and cell concentrations were determined by calibrated linear regressions of F-PS and DiI, respectively, and level of phagocytic activity was expressed as liposomes/cell.

Colorimetric phagocytosis assay

To determine the effect of anti-clathrin pAb on FcR-mediated phagocytosis, a standard colorimetric assay was used (36). AMs ($2.5 \times 10^5/100$ μ l) were exposed to 10-fold excess SRBCs opsonized with mouse mAb against SRBC for 1 h at 37°C in a 96-well plate on a shaker. The wells were aspirated to remove nonphagocytosed RBCs, then filled with 100 μ l lysing solution to lyse any remaining adherent, nonphagocytosed RBCs. After rinsing three times with PBS, wells were filled with 200 μ l DAF solution (2.5 mM DAF, 0.3% H_2O_2 , 0.2 M Tris, 6 M urea) and incubated for 5 min at RT. Absorbance was measured at 620 nm using a plate reader. The concentration of phagocytosed RBCs was determined by calibrated linear regression, and the level of phagocytic activity was expressed as RBCs/cell.

Statistical analysis

Quantitative results were expressed as mean and SE. Differences between group means were tested for significance using either Student's *t* test (when comparing two groups) or ANOVA (when comparing three groups), with significance accepted for $p \leq 0.01$. After significant difference between two or more groups was identified by ANOVA, Tukey's test was applied post hoc to identify which groups were different (37).

Results

Clathrin coat-associated proteins are present in pulmonary alveolar macrophages

To determine whether clathrin HC had an intracellular distribution in pulmonary AMs that was similar to that reported for other cells, rat AMs were fixed, permeabilized, and labeled with anti-clathrin pAb. Viewed under fluorescence microscopy, the fluorescent label is well distributed throughout the cytoplasm and extends to the cell perimeter (Fig. 1A). Distinct and abundant punctate labeling could be seen, which is consistent with the known distribution pattern of clathrin in other cells (38, 39). Fig. 1B shows the corresponding DIC image of the AM. Control cells were unlabeled (not shown).

To quantify the amount of endogenous clathrin HC present in rat AMs, cells were permeabilized and assayed for clathrin HC by ELISA. The amount of clathrin HC present in AMs averaged 4.22 ± 0.02 pg/cell (2.34×10^{-17} mol/cell).

To determine the intracellular distribution of the clathrin coat-associated adaptor AP-2, rat AMs were labeled with Ab to AP-2. Control cells did not receive anti-AP-2 mAb. The punctate fluorescent label is preferentially restricted to the cell periphery (arrow, Fig. 2A), consistent with the known distribution pattern of AP-2 in other cells, where it is associated with clathrin-coated pits

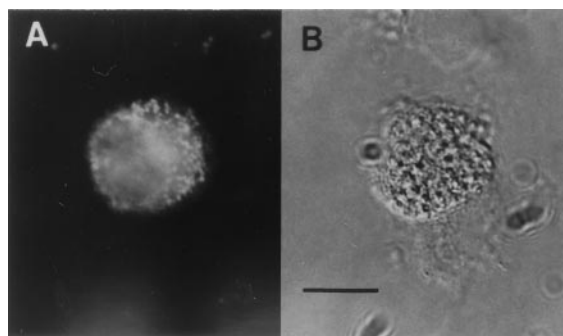


FIGURE 1. Immunolocalization of clathrin HC in AMs. Rat AMs were fixed in paraformaldehyde and permeabilized with Triton X-100. After rinsing, cells were exposed to 1:40 anti-clathrin pAb, incubated for 20 min, rinsed, then incubated with 1:100 rb anti-gt IgG-FITC. Control cells were exposed to second Ab only. A, The punctate fluorescent label is well distributed throughout the cytoplasm. B, Corresponding DIC image. Control cells were unlabeled (not shown). Bar = 10 μ m.

on the cytoplasmic surface of the cell membrane (40). Fig. 2B shows the corresponding DIC image of the AM. Control cells were unlabeled (not shown).

Liposomes can deliver Ab to the cytoplasm of macrophages

The liposome encapsulation ratio of radiolabeled anti-clathrin pAb was 86.9%. Based on ELISA, the actual amount of encapsulated anti-clathrin pAb was 0.14 ± 0.02 pg/liposome. Anti-clathrin pAb encapsulated in liposomes was delivered successfully to the cytoplasm of AMs, demonstrated by immunocytochemistry. A diffuse, intense fluorescent label, reflecting the presence of anti-clathrin pAb, was present throughout the cytoplasm (Fig. 3). Control cells, which were incubated without anti-clathrin pAb liposomes, were unlabeled (not shown). This indicates that the FITC-conjugated anti-goat Ab was specifically binding to the liposome-delivered anti-clathrin pAb (of which goat was the host animal), and not simply sticking to the cells by nonspecific interactions. The labeling pattern reflects the intracellular distribution of the liposome-delivered Ab, not the distribution of clathrin HC (compare to Fig. 1). The amount of anti-clathrin pAb delivered to AMs by liposomes was 0.94 ± 0.08 pg/cell, measured by ELISA. Based on the measured amount of anti-clathrin pAb encapsulated in liposomes (0.14 pg/liposome), this represents an average phagocytic uptake of 6.7 liposomes/cell.

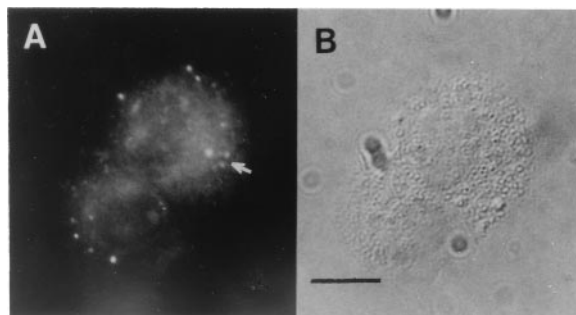


FIGURE 2. Immunolocalization of adaptor AP-2 in AMs. Rat AMs were fixed and permeabilized, then exposed to 1:100 anti-AP-2 mAb, incubated for 20 min, rinsed, then incubated with 1:100 gt anti-ms IgG-FITC. Control cells were exposed to second Ab only. A, The punctate fluorescent label is preferentially on the cell periphery (arrow). B, Corresponding DIC image. Control cells were unlabeled (not shown). Bar = 10 μ m.

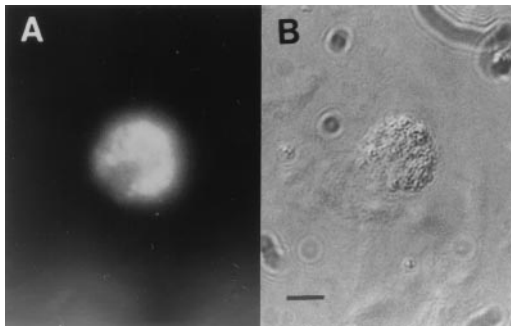


FIGURE 3. Liposome delivery of anti-clathrin pAb to macrophage cytoplasm. Rat AMs were incubated with liposomes containing anti-clathrin pAb for 1 h; control cells were exposed to empty liposomes. Cells were then exposed to 1:100 rb anti-gt IgG-FITC. *A*, A diffuse, intense fluorescent label is seen throughout the cytoplasm. *B*, Corresponding DIC image. Control cells were unlabeled (not shown). The labeling pattern reflects the intracellular distribution of the liposome-delivered Ab, not the distribution of clathrin HC (Fig. 1). Bar = 10 μ m.

Liposome-delivered anti-clathrin Ab inhibits phagocytosis

Liposome-delivered anti-clathrin pAb significantly attenuated phagocytosis compared with either liposome-delivered nonspecific Ab (rb anti-SRBC IgG) or 1% BSA liposomes (Fig. 4). The level of phagocytic activity for AMs receiving the 1% BSA (control) liposomes was 16.3 ± 0.3 liposomes/cell; similarly, phagocytic activity for cells receiving nonspecific Ab (anti-RBC) was 17.4 ± 2.6 liposomes/cell. In contrast, phagocytic activity for cells receiving anti-clathrin pAb (anti-clathrin) was only 5.8 ± 0.3 liposomes/cell ($n = 3$; $\star, p < 0.01$). These results show that anti-clathrin pAb had a significant effect on the level of phagocytic activity, implying that clathrin is directly involved in phagocytosis.

To establish the validity of the results obtained by the fluorometric phagocytosis assay, the effect of anti-clathrin Ab on the level of phagocytic activity was independently measured using a standard colorimetric assay for phagocytosis of opsonized RBCs, which is an accepted model of FcR-mediated phagocytosis (36). Similar to the results obtained by the fluorometric assay (Fig. 4), liposome-delivered anti-clathrin pAb markedly decreased phagocytosis compared with liposome-delivered nonspecific Ab (Fig. 5).

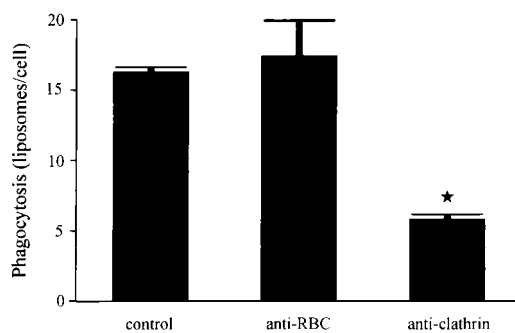


FIGURE 4. Effect of liposome-delivered anti-clathrin pAb on phagocytosis of zymosan-coated liposomes. Rat AMs were incubated with liposomes containing either 1% BSA (control), rb anti-SRBC IgG (anti-RBC), or 1:40 anti-clathrin pAb (anti-clathrin) for 1 h. After rinsing, cells were incubated with zymosan-coated, fluorescent liposomes for 1 h. After lysing adherent but nonphagocytosed liposomes and then rinsing, phagocytosis was measured by fluorometry and expressed as liposomes/cell. Liposome-delivered anti-clathrin pAb significantly attenuated phagocytosis compared with either liposome-delivered nonspecific Ab or empty liposomes ($n = 3$; $\star, p < 0.01$).

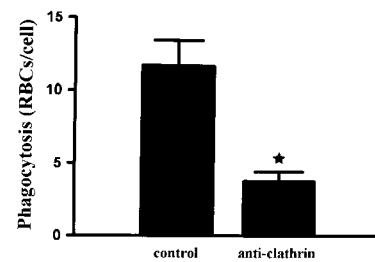


FIGURE 5. Effect of liposome-delivered anti-clathrin pAb on phagocytosis of opsonized RBCs. Rat AMs were incubated with liposomes containing either 1:40 anti-IgA pAb (control) or 1:40 anti-clathrin pAb (anti-clathrin) for 1 h. After rinsing, cells were incubated with zymosan-coated, fluorescent liposomes for 1 h. Lysing solution was added to lyse any adherent but nonphagocytosed liposomes. After rinsing, phagocytosis was measured by fluorometry and expressed as liposomes/cell. Liposome-delivered anti-clathrin pAb significantly attenuated phagocytosis compared with liposome-delivered nonspecific Ab ($n = 5$; $\star, p < 0.01$).

The level of phagocytic activity for AMs receiving control nonspecific Ab (control) was 11.7 ± 1.7 RBCs/cell; for AMs receiving anti-clathrin pAb (anti-clathrin), the level was only 3.8 ± 0.7 RBCs/cell ($n = 5$; $\star, p < 0.01$). The attenuation of phagocytosis by anti-clathrin Ab blockade was 67.5%. This compares almost exactly with the amount of attenuation observed using the fluorometric assay, 66.7%. This result shows that liposome delivery of anti-clathrin pAb inhibits phagocytic activities mediated by two different receptors following two different phagocytic pathways, β -glucan receptor/zymosan for nonopsonic phagocytosis (6) and the FcR/IgG-RBCs for opsonic phagocytosis (41).

The molar ratio of liposome-delivered anti-clathrin pAb to endogenous clathrin HC was determined by ELISA. Alveolar macrophages were allowed to phagocytose liposomes containing anti-clathrin pAb under the conditions specified above. The amount of anti-clathrin pAb delivered to these cells was 0.94 pg/cell. The amount of endogenous clathrin HC in macrophages was determined to be 4.22 pg/cell. Based on the molecular mass of anti-clathrin pAb ≈ 146 kDa and a molecular mass of clathrin HC = 180 kDa, this approximates a ratio of approximately 1 mol anti-clathrin pAb to 3.6 mol clathrin HC. The optimal molar ratio for Ab blockade in principle should favor a molar excess of Ab. The suboptimal ratio of approximately 1:3.6 (Ab:Ag) in these experiments could at least partially account for the observed attenuation, as opposed to complete blockade, of phagocytosis.

Liposome-delivered Ab to adaptor AP-2, a protein complex associated with clathrin-coated pits, inhibits phagocytosis

Similar to the above results for anti-clathrin pAb, liposome-delivered anti-AP-2 mAb significantly attenuated phagocytosis compared with control liposomes (Fig. 6). The control level of phagocytosis (control) was 10.1 ± 0.9 liposomes/cell; AMs that were incubated with anti-AP-2 mAb liposomes (anti-AP-2) had significantly lower phagocytosis, 4.8 ± 0.2 liposomes/cell ($n = 3$; $\star, p < 0.01$). This result shows that Ab blockade of a coated pit protein other than clathrin HC also disrupts phagocytosis, similar to Ab blockade of clathrin HC itself.

Amantadine, a drug that blocks clathrin-mediated endocytosis, inhibits phagocytosis

Liposome-delivered amantadine significantly attenuated phagocytosis compared with control liposomes (Fig. 7). AMs receiving control liposomes (control) had a level of phagocytic activity of 13.8 ± 0.8 liposomes/cell; in contrast, AMs receiving amantadine-laden liposomes (amantadine) had a phagocytic level of only $5.7 \pm$

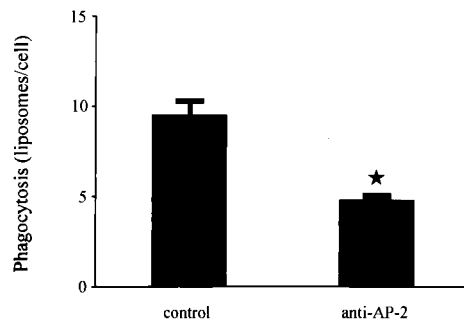


FIGURE 6. Effect of liposome-delivered Ab to clathrin-associated adaptor AP-2 on phagocytosis. Rat AMs were incubated with liposomes containing either 1% BSA (control) or 1:40 anti-AP-2 mAb (anti-AP-2) for 1 h. After rinsing, cells were incubated with fluorescent liposomes and assayed for phagocytosis. Liposome-delivered anti-AP-2 mAb significantly attenuated phagocytosis compared with empty liposomes ($n = 3$; $\star, p < 0.01$).

0.6 liposomes/cell ($n = 3$; $\star, p < 0.01$). Amantadine blocks coated pit invagination at the plasma membrane (42). This result shows that introduction of a drug that arrests clathrin-coated pit function at the plasma membrane also inhibits phagocytosis.

Liposome-delivered Ab to hsc70, the clathrin uncoating ATPase, inhibits phagocytosis

Experimental evidence suggests that hsc70 acts as a clathrin-dependent ATPase that removes individual clathrin triskelions from the coated vesicle (43, 44). Liposome-delivered anti-hsc70 mAb significantly attenuated phagocytosis compared with control liposomes (Fig. 8). AMs receiving control liposomes (control) had a level of phagocytic activity of 9.1 ± 0.5 liposomes/cell; AMs receiving liposomes containing anti-hsc70 mAb (anti-hsc70) had a phagocytic level of only 4.3 ± 0.2 liposomes/cell ($n = 3$; $\star, p < 0.01$). Since Ab blockade of hsc70 inhibits clathrin-mediated endocytosis, the result is consistent with the hypothesis that clathrin-coated pits participate in phagocytosis.

Discussion

The results of this study indicate that rat AM phagocytosis is inhibited by liposome delivery of Abs directed against clathrin HC, adaptor AP-2, and hsc70. In addition, amantadine, which inhibits clathrin-mediated coated pit function (42), also inhibits phagocytosis. The fact that all four of these agents significantly inhibited

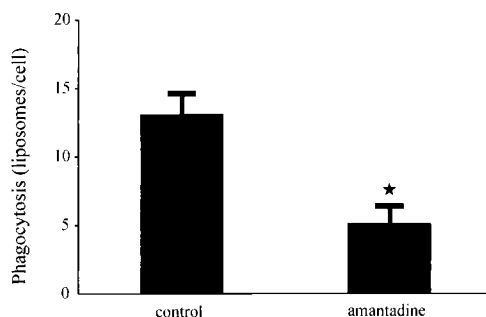


FIGURE 7. Effect of amantadine on phagocytosis. Rat AMs were incubated with liposomes containing either 1% BSA (control) or 100 mg/ml amantadine for 1 h, then rinsed, incubated with fluorescent liposomes for 1 h, and assayed for phagocytosis. Liposome-delivered amantadine significantly attenuated phagocytosis compared with 1% BSA liposomes ($n = 3$; $\star, p < 0.01$).

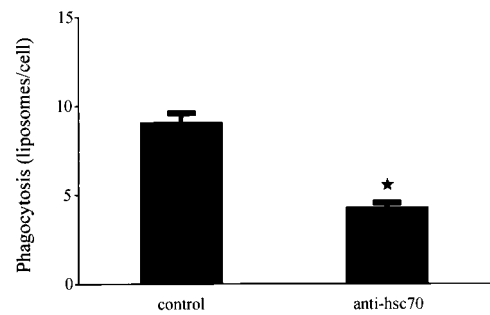


FIGURE 8. Effect of liposome-delivered Ab to uncoating ATPase hsc70 on phagocytosis. Rat AMs were incubated with liposomes containing either 1% BSA (control) or 1:40 anti-hsc70 mAb (anti-hsc70) for 1 h. After rinsing, cells were incubated with fluorescent liposomes for 1 h, then assayed for phagocytosis. Liposome-delivered anti-AP-2 mAb significantly attenuated phagocytosis compared with 1% BSA liposomes ($n = 3$; $\star, p < 0.01$).

phagocytosis, but did not completely abolish it, suggests that clathrin-mediated endocytosis is not the only endocytic pathway involved in receptor/membrane recycling during phagocytosis. Non-clathrin endocytosis in macrophages is known to occur (45), but its role in phagocytosis, if any, remains to be studied. Possibly, inhibition could represent partial abolishment of phagocytosis because of suboptimal (i.e., nonblocking) molar ratios of Ab to target protein, although this would not explain the partial inhibition by amantadine. Ideally, Ab blockade requires a 10-fold molar excess of Ab. In our measurements of liposome-delivered anti-clathrin pAb and endogenous clathrin HC, the molar ratio was 1 mol clathrin HC for every 3.6 mol anti-clathrin pAb. Although there was only partial inhibition by liposome-encapsulated Abs of phagocytosis, the inhibition was significant (up to 67.5%), which suggests that the liposome delivery system is potentially a useful approach to the study of AM function and that clathrin-associated proteins are most likely key factors in the phagocytic machinery of the cell.

Several facts rule out the possibility that liposome uptake is occurring by endocytosis or membrane fusion rather than by true phagocytosis: 1) The MLVs used in these experiments have an average feret diameter of $6.5 \mu\text{m}$, a scale far too large for endocytosis, but optimum for phagocytosis (28). 2) Previous work in our laboratory (25) established that AM uptake of liposomes occurs by bone fide phagocytosis, for the following reasons: We demonstrated that liposomes coated with zymosan and labeled with dichlorodihydrofluorescein (DHF) only fluoresce when ingested by AMs. Zymosan initiates a respiratory burst when phagocytosed by AMs (46), and DHF fluoresces only in the presence of free radicals generated in a respiratory burst (47). Neither free liposomes nor adherent, nonphagocytosed liposomes fluoresce under the same conditions. Liposomes also will not fluoresce during membrane fusion or endocytosis, because neither condition typically generates a respiratory burst. We determined that liposome uptake is prevented in the presence of cytochalasin B, which blocks phagocytosis but not endocytosis (48). We showed that AM uptake of liposomes is blocked at 4°C , which is permissive for membrane fusion, but not for phagocytosis. We directly visualized the phagocytosis of ferritin-containing liposomes by rat AMs at the electron-microscopic level. The electron-dense liposomes were seen in all stages of phagocytosis, from initial adherence on the AM surface, to engulfment by pseudopods, to incorporation in phagosomes.

Clathrin-coated pits and vesicles are known to participate in receptor-mediated endocytosis and intracellular sorting (19), but

heretofore no functional relationship between endocytosis and phagocytosis has been suggested. The reasons for assuming that there is no interaction is that the two cellular functions are clearly distinct: 1) endocytosis involves invagination of coated pits leading to internalization of a ligand within an endosome (49), whereas phagocytosis involves pseudopod extension over an external target leading to engulfment within a phagosome (50); 2) endocytosis can occur on a scale of tens of nanometers (51), whereas phagocytosis can occur on a scale of several micrometers (28); 3) endocytosis is not blocked by cytochalasin B (52), whereas phagocytosis is blocked (53); 4) receptors that can mediate both endocytosis and phagocytosis, such as Fc γ RI, involve different receptor subdomains for each of these functions (54), with phagocytosis requiring the interaction of Fc γ RI with an associated γ subunit that contains two YXXL sequences in a conserved motif (5); and 5) in the case of Fc γ RI, the signaling pathways also differ, with phagocytosis employing a protein tyrosine kinase pathway, and endocytosis employing a protein tyrosine kinase-independent pathway (55).

The marked difference between endocytosis and phagocytosis has engendered a presumption of mutual exclusivity for these two functions. Yet potassium depletion, long known to block endocytosis by interfering with clathrin function (56), also blocks phagocytosis (57). Moreover, inhibition of phagocytosis by potassium depletion has little effect on actin polymerization (15), suggesting that the mechanism of action is related to a process other than cytoskeletal rearrangement. In addition, potassium depletion abrogates fibroblast polarity, which is associated with directional cell migration (58). This last finding is important because endocytosis has been implicated as the mechanism of receptor/membrane recycling during cell migration (11, 13, 59), a process closely related to phagocytosis (60). As envisioned by Bretscher, the theory of receptor/membrane recycling during cell migration calls for an ongoing process of endocytosis and exocytosis (11). At the plasma membrane, where this recycling must occur, endocytosis is mediated by the coated pit-associated proteins clathrin and AP-2 (19), although not exclusively so (51). Blockade of clathrin function by microinjection of anti-clathrin Ab has been demonstrated to inhibit endocytosis (61). Ab blockade of clathrin and AP-2 would be expected to also decrease phagocytic activity if endocytic recycling is integral to the phagocytic process; by logical extension, amantadine, which arrests endocytosis by stabilizing coated pits at the plasma membrane (42), would also be expected to inhibit phagocytosis. Our findings reported in this study are consistent with this interpretation. A conflicting report on the involvement of clathrin in phagocytosis was published, in which antisense RNA was used to produce clathrin HC-deficient *Dictyostelium discoideum* (62). The authors reported that clathrin-deficient cells were impaired in the pinocytosis of fluid-phase markers, but competent in the phagocytosis of bacteria. However, this finding for phagocytosis was based only on photomicrographs of cells and fluorescently tagged bacteria, in which it is not possible to clearly distinguish between bacteria that were truly ingested versus merely adherent (63). The interpretation of the results is further called into question by the experimental condition under which phagocytosis putatively occurred: incubation for 10 min at 20°C, a condition that favors adherence, not phagocytosis. It is interesting to note that the authors also reported that clathrin-deficient cell colonies were incapable of migrating, which would be consistent with impaired phagocytosis (60).

The present study shows that Ab blockade of hsc70 diminishes phagocytic activity in rat AMs. A possible function of hsc70, also known as uncoating ATPase, is to amplify clathrin turnover (64). Endocytosis is an ongoing process of coated pit formation, invag-

ination, and budding to form a clathrin-coated vesicle (65). Once formed, clathrin of the outer (cytoplasmic) coat rapidly depolymerizes, liberating clathrin triskelions that can reenter the endocytic cycle by polymerizing at the plasma membrane at sites of AP-2 aggregation to form new coated pits (19). Clathrin depolymerization may be catalyzed by hsc70 (66), which could increase the available pool of free clathrin and subsequently increase amount of clathrin-coated pits.

The classic model of phagocytosis is the zipper model, in which phagocytosis occurs by progressive pseudopod extension over the phagocytic target, mediated by the “zippering” of receptor and ligand (e.g., FcR and opsonized Ig) (4). An alternative model, the trigger model, holds that, in certain circumstances, ligand-receptor interaction can trigger a complete, all-or-nothing phagocytic response (50). Both models recognize the central importance of receptor-ligand interaction in generating pseudopod extension; neither model addresses the problem of inserting new membrane material at the leading edge of the extending pseudopod. We regard the receptor/membrane recycling theory, adapted from Bretscher’s theory of cell migration (59), as complementary to existing theories of receptor signaling.

Clerc and Sansonetti demonstrated by immunolocalization in HEp2 cells undergoing phagocytosis of *Shigella flexneri* that clathrin accumulated in the regions of the cells in which internalization was occurring (15). Aggeler and Werb demonstrated by both scanning and transmission-electron microscopy of peritoneal macrophages undergoing phagocytosis of latex beads that the cytoplasmic surface nascent phagosomes had prominent basketworks of assembled clathrin (14). These phagosome patches of clathrin were larger than the coated pits typically seen on plasma membrane. Budding of these clathrin patches from the phagosome membrane was observed. Moreover, the surface area of membrane covered by the clathrin basketwork almost tripled in actively phagocytic cells compared with controls. The larger clathrin basketworks were also observed to be associated with the attached basal plasma membrane surface in spreading macrophages. The investigators surmised that, in terms of membrane dynamics, phagocytosis shares features in common with cell attachment and spreading.

Our own finding that anti-clathrin pAb inhibits the phagocytosis of both zymosan-coated liposomes and IgG-opsonized RBCs, mediated by β -glucan and Fc receptors, respectively, suggests that clathrin involvement is common to both types of phagocytosis, even though these receptors trigger different signal-transduction pathways (8). The common ground in all of these processes, including cell spreading and migration, is a demand for high turnover of plasma membrane and the involved surface receptor. Clearly, clathrin-mediated endocytosis should be considered a likely candidate in this turnover process.

In conclusion, our findings are consistent with the concept of endocytosis being required for phagocytosis, which in turn is consistent with the theory of receptor/membrane recycling during cell migration. It is possible that leading edges of migrating cells and pseudopods of phagocytes share a common mechanism for cellular protrusion, and therefore phagocytosis might be viewed as a specialized variation of cell locomotion.

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References

1. Van Oss, C. J. 1986. Phagocytosis: an overview. *Methods Enzymol.* 132:3.
2. Southwick, F. S., and D. L. Purich. 1994. Dynamic remodeling of the actin cytoskeleton: lessons learned from *Listeria* locomotion. *Bioessays* 16:885.

3. Downing, J. F., R. Pasula, J. R. Wright, H. L. Twigg, and W. J. Martin II. 1995. Surfactant protein A promotes attachment of *Mycobacterium tuberculosis* to alveolar macrophages during infection with human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* 92:4848.
4. Brown, E. J. 1995. Phagocytosis. *Bioessays* 17:109.
5. Indik, Z. K., J. G. Park, S. Hunter, and A. D. Schreiber. 1995. The molecular dissection of Fc γ receptor mediated phagocytosis. *Blood* 86:4389.
6. Ofek, I., J. Goldhar, Y. Keisari, and N. Sharon. 1995. Nonopsonic phagocytosis of microorganisms. *Annu. Rev. Microbiol.* 49:239.
7. Strzelecka, A., K. Kwiatkowska, and A. Sobota. 1997. Tyrosine phosphorylation and Fc γ receptor-mediated phagocytosis. *FEBS Lett.* 400:11.
8. Allen, L. A., and A. Aderem. 1996. Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. *J. Exp. Med.* 184:627.
9. Sassaroli, M., M. Vauhkonen, D. Perry, and J. Eisinger. 1990. Lateral diffusivity of lipid analogue excimeric probes in dimyristoylphosphatidylcholine bilayers. *Biophys. J.* 57:281.
10. Bretscher, M. S. 1984. Endocytosis: relation to capping and cell locomotion. *Science* 224:681.
11. Bretscher, M. S. 1989. Endocytosis and recycling of the fibronectin receptor in CHO cells. *EMBO J.* 8:1341.
12. Ruoslahti, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. *Science* 238:491.
13. Hopkins, C. R., A. Gibson, M. Shipman, D. K. Strickland, and I. S. Trowbridge. 1994. In migrating fibroblasts, recycling receptors are concentrated in narrow tubules in the pericentriolar area, and then routed to the plasma membrane of the leading lamella. *J. Cell Biol.* 125:1265.
14. Aggeler, J., and Z. Werb. 1982. Initial events during phagocytosis by macrophages viewed from outside and inside the cell: membrane-particle interactions and clathrin. *J. Cell Biol.* 94:613.
15. Clerc, P. L., and P. J. Sansonetti. 1989. Evidence for clathrin mobilization during directed phagocytosis of *Shigella flexneri* by HEp2 cells. *Microb. Pathog.* 7:329.
16. Kanaseki, T., and K. Kadota. 1969. The "vesicle in a basket." *J. Cell Biol.* 42:202.
17. Roth, T. F., and K. R. Porter. 1964. Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti* L. *J. Cell Biol.* 20:313.
18. Pearce, B. M. F. 1989. Characterization of coated-vesicle adaptors: their reassembly with clathrin and with recycling receptors. *Methods Cell Biol.* 31:229.
19. Pearce, B. M. F., and M. S. Robinson. 1990. Clathrin, adaptors, and sorting. *Annu. Rev. Cell Biol.* 6:151.
20. Pitt, A., L. S. Mayorga, P. D. Stahl, and A. L. Schwartz. 1992. Alterations in the protein composition of maturing phagosomes. *J. Clin. Invest.* 90:1978.
21. Robertson, T. A., and J. M. Papadimitriou. 1986. A morphometric study of coated pit formation on the subplasmalemmal surface of murine peritoneal macrophages after adhesion to glass. *J. Ultrastruct. Mol. Struct. Res.* 96:125.
22. Takemura, R., P. E. Stenberg, D. F. Bainton, and Z. Werb. 1986. Rapid redistribution of clathrin onto macrophage plasma membranes in response to Fc receptor-ligand interaction during frustrated phagocytosis. *J. Cell Biol.* 102:55.
23. Joiner, K. A., S. A. Fuhrman, H. M. Miettinen, L. H. Kasper, and I. Mellman. 1990. *Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. *Science* 249:641.
24. Ukkonen, P., V. Lewis, M. Marsh, A. Helenius, and I. Mellman. 1986. Transport of macrophage Fc receptors and Fc receptor-bound ligands to lysosomes. *J. Exp. Med.* 163:952.
25. Perry, D. G., and W. J. Martin II. 1995. Fluorescent liposomes as quantitative markers of phagocytosis by alveolar macrophages. *J. Immunol. Methods* 181:269.
26. Hope, M. J., M. B. Bally, L. D. Mayer, A. S. Janoff, and P. R. Cullis. 1986. Generation of multilamellar and unilamellar phospholipid vesicles. *Chem. Phys. Lipids* 40:89.
27. Dijkstra, J., J. L. Ryan, and F. C. Szoka. 1988. A procedure for the efficient incorporation of wild-type lipopolysaccharide into liposomes for use in immunological studies. *J. Immunol. Methods* 114:197.
28. Cannon, G. J., and J. A. Swanson. 1992. The macrophage capacity for phagocytosis. *J. Cell Sci.* 101:907.
29. Rehn, B., J. Bruch, T. Zou, and G. Hobsch. 1992. Recovery of rat alveolar macrophages by bronchoalveolar lavage under normal and activated conditions. *Environ. Health Perspect.* 97:11.
30. Merchant, R. K., D. A. Schwartz, R. A. Helmers, C. S. Dayton, and G. W. Hunninghake. 1992. Bronchoalveolar lavage cellularity. *Am. Rev. Respir. Dis.* 146:448.
31. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76.
32. Perry, D. G., P. Wisniewski, G. L. Daugherty, J. Downing, and W. J. Martin II. 1997. Nonimmune phagocytosis of liposomes by rat alveolar macrophages is enhanced by vitronectin and is vitronectin receptor-mediated. *Am. J. Respir. Cell Mol. Biol.* 17:462.
33. Tobias, P. S., J. Mathison, D. Mintz, J.-D. Lee, V. Kravchenko, K. Kato, J. Pugin, and R. J. Ulevitch. 1992. Participation of lipopolysaccharide-binding protein in lipopolysaccharide-dependent macrophage activation. *Am. J. Respir. Cell Mol. Biol.* 7:239.
34. Takahashi, H., M. Abe, S. Hashimoto, K. Takayama, and M. Miyazaki. 1993. In vivo effect of lipopolysaccharide on alveolar and peritoneal macrophages of rats: superoxide anion generation and 5-lipoxygenase metabolism of arachidonic acid. *Am. J. Respir. Cell Mol. Biol.* 8:291.
35. Cardozo, C., J. Edelman, and M. Lesser. 1992. Lipopolysaccharide-induced stimulation of alveolar macrophage opsonin-independent phagocytosis. *J. Surg. Res.* 53:170.
36. Gebran, S. J., E. L. Romano, H. A. Pons, L. Cariani, and A. N. Soyano. 1992. A modified colorimetric method for the measurement of phagocytosis and antibody-dependent cell cytotoxicity using 2,7-diaminofluorene. *J. Immunol. Methods* 151:255.
37. Zar, J. H. 1984. *Biostatistical Analysis*. Prentice-Hall, Englewood Cliffs, p. 718.
38. Lin, H. C., M. S. Moore, D. A. Sanan, and R. G. W. Anderson. 1991. Reconstitution of clathrin-coated pit budding from plasma membranes. *J. Cell Biol.* 114:881.
39. Larkin, J. M., M. S. Brown, J. L. Goldstein, and R. G. W. Anderson. 1983. Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. *Cell* 33:273.
40. Chang, M. P., W. G. Mallet, K. E. Mostov, and F. M. Brodsky. 1993. Adaptor self-aggregation, adaptor-receptor recognition and binding of α -adaptin subunits to the plasma membrane contribute to recruitment of adaptor (AP2) components of clathrin-coated pits. *EMBO J.* 12:2169.
41. Strzelecka, A., K. Kwiatkowska, and A. Sobota. 1997. Tyrosine phosphorylation and Fc γ receptor-mediated phagocytosis. *FEBS Lett.* 400:11.
42. Phonphok, Y., and K. S. Rosenthal. 1991. Stabilization of clathrin coated vesicles by amantadine, tromantadine and other hydrophobic amines. *FEBS Lett.* 281:188.
43. Pley, U., and P. Parham. 1993. Clathrin: its role in receptor-mediated vesicular transport and specialized functions in neurons. *Crit. Rev. Biochem. Mol. Biol.* 28:431.
44. Buxbaum, E., and P. G. Woodman. 1996. Binding of ATP and ATP analogues to the uncoating ATPase Hsc70 (70 kDa heat-shock cognate protein). *Biochem. J.* 318:923.
45. Kiss, A. L., and H. J. Geuze. 1997. Caveolae can be alternative endocytotic structures in elicited macrophages. *Eur. J. Cell Biol.* 73:19.
46. Kuroiwa, A., K. Igisu, T. Yano, N. Okada, and H. Okada. 1988. Fibronectin enhances respiratory burst of phagocytes stimulated by zymosan and immune complexes. *Immunology* 65:177.
47. Ryan, T. C., G. J. Weil, P. E. Newburger, R. Haugland, and E. R. Simons. 1990. Measurement of superoxide release in the phagocytosis of immune complex-stimulated human neutrophils. *J. Immunol. Methods* 130:223.
48. Painter, R. G., J. Whisenand, and A. T. McIntosh. 1981. Effects of cytochalasin B on actin and myosin association with particle binding sites in mouse macrophages: implications with regard to the mechanism of action of the cytochalasins. *J. Cell Biol.* 91:373.
49. Mukherjee, S., R. N. Ghosh, and F. R. Maxfield. 1997. Endocytosis. *Physiol. Rev.* 77:759.
50. Swanson, J. A., and S. C. Baer. 1995. Phagocytosis by zippers and triggers. *Trends Cell Biol.* 5:89.
51. Watts, C., and M. Marsh. 1992. Endocytosis: what goes in and how? *J. Cell Sci.* 103:1.
52. Lamaze, C., T.-H. Chuang, L. J. Terlecky, G. M. Bokoch, and S. L. Schmid. 1996. Regulation of receptor-mediated endocytosis by Rho and Rac. *Nature* 382:177.
53. Rikihisa, Y., Y. Zhang, and J. Park. 1994. Inhibition of infection of macrophages with *Ehrlichia risticii* by cytochalasins, monodansylcadaverine, and taxol. *Infect. Immun.* 62:5126.
54. Davis, W., P. T. Harrison, M. J. Hutchinson, and J. M. Allen. 1995. Two distinct regions of Fc γ RI initiate separate signalling pathways involved in endocytosis and phagocytosis. *EMBO J.* 14:432.
55. Allen, L. A., and A. Aderem. 1996. Mechanisms of phagocytosis. *Curr. Opin. Immunol.* 8:36.
56. Hansen, S. H., K. Sandvig, and B. van Deurs. 1993. Clathrin and HA2 adaptors: effects of potassium depletion, hypertonic medium, and cytosol acidification. *J. Cell Biol.* 121:61.
57. Matsui, H., L. G. Johnson, S. H. Randell, and R. C. Boucher. 1997. Loss of binding and entry of liposome-DNA complexes decreases transfection efficiency in differentiated airway epithelial cells. *J. Biol. Chem.* 272:1117.
58. Altankov, G., and F. Grinnell. 1993. Depletion of intracellular potassium disrupts coated pits and reversibly inhibits cell polarization during fibroblast spreading. *J. Cell Biol.* 120:1449.
59. Bretscher, M. S. 1996. Moving membrane up to the front of migrating cells. *Cell* 85:465.
60. Bearer, E. L. 1993. Role of actin polymerization in cell locomotion: molecules and models. *Am. J. Respir. Cell Mol. Biol.* 8:582.
61. Doxey, S. J., F. M. Brodsky, G. S. Blank, and A. Helenius. 1987. Inhibition of endocytosis by anti-clathrin antibodies. *Cell* 50:453.
62. O'Halloran, T. J., and R. G. Anderson. 1992. Clathrin heavy chain is required for pinocytosis, the presence of large vacuoles, and development in Dictyostelium. *J. Cell Biol.* 118:1371.
63. Sveum, R. J., T. M. Chused, M. M. Frank, and E. J. Brown. 1986. A quantitative fluorescent method for measurement of bacterial adherence and phagocytosis. *J. Immunol. Methods* 90:257.
64. Barouch, W., K. Prasad, L. E. Greene, and E. Eisenberg. 1994. ATPase activity associated with the uncoating of clathrin baskets by Hsp70. *J. Biol. Chem.* 269:28563.
65. Goldstein, J. L., R. G. W. Anderson, and M. S. Brown. 1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. *Nature* 279:679.
66. Höning, S., G. Kreimer, H. Robenek, and B. M. Jockusch. 1994. Receptor-mediated endocytosis is sensitive to antibodies against the uncoating ATPase (hsc70). *J. Cell Sci.* 107:1185.