Abstract. We have observed increases in assembled clathrin on the plasma membrane during "frustrated phagocytosis," the spreading of macrophages on immobilized immune complexes. Resident macrophages freshly harvested from the peritoneal cavity of mice and attached to bovine serum albumin (BSA)-anti-BSA-coated surfaces at 4°C had almost no clathrin basketworks on their adherent plasma membrane (<0.01 coated patch/μm²), as observed by immunofluorescence, immunoperoxidase, and platinum-carbon replica techniques, although abundant assembled clathrin was observed in the perinuclear Golgi region. When the cells were warmed to 37°C they started to spread by 4 min and reached their maximum extent by 20 min. Spreading preceded clathrin assembly at the plasma membrane. Clathrin-coated patches were first observed on the adherent plasma membrane at 6 min. Between 12 and 20 min assembled clathrin coats appeared on both adherent and nonadherent plasma membranes with a concomitant decrease in identifiable clathrin in the perinuclear region. A new steady state emerged by 2 h, as perinuclear clathrin began to reappear. At 20 min at 37°C the adherent plasma membranes of macrophages spreading on BSA alone had 0.9 coated patch/μm², whereas in cells spread on immune complex-coated surfaces, the clathrin patches increased, dependent on ligand concentration, to a maximum of 2.1 coated patches/μm². Because frustrated phagocytosis of immune complex-coated surfaces at 37°C increased the area of adherent plasma membrane, the total area coated by clathrin basketworks increased 5-fold (28 μm²/cell) as compared with cells plated on BSA alone (5.6 μm²/cell) and 200-fold as compared with cells adhering to immune complexes at 4°C.

We then determined that macrophages cultured on BSA-coated coverslips for 24 h already have abundant surface clathrin. When immune complexes were formed by the addition of anti-BSA IgG to already spread macrophages cultured on BSA-coated coverslips for 24 h, clathrin assembled at the sites of ligand-receptor interaction even at 4°C, before spreading, and a 2.6-fold increase in assembled clathrin was observed on the adherent plasma membrane of cells on immune complexes as compared with cells on BSA alone. Clathrin was reversibly redistributed to the Golgi region, returning to the steady state by 2 h. These data indicate that receptor-ligand interaction during frustrated phagocytosis induces clathrin assembly on the plasma membrane and reversibly diminishes the assembly of clathrin in the perinuclear Golgi region, and suggest that assembled clathrin may play a role in the dynamic changes in membrane distribution in these cells.

Clathrin-coated pits and vesicles are involved in receptor-mediated endocytosis (1, 5), but little is known about the factors that influence recruitment of clathrin at the cell surface. Although the total number of coated pits has been reported to be the same before and after ligands are given to cells (58), capping of surface IgG on lymphocytes induces an increase in surface-associated coated pits (49), and clathrin-coated pits appear at the cell surface in increased amounts during the development of mosquito oocytes to the vitellogenic stage (46, 47). A cycle of assembly and disassembly of clathrin basketworks probably is involved in endocytosis (23). A significant pool of soluble or unassembled clathrin triskelions (20, 25, 31, 46), which may serve as intermediates in assembly and disassembly, has been demonstrated in cells. In addition to the endocytic clathrin-coated pits and vesicles near the surface of the cells, clathrin-coated vesicles are observed within cells near Golgi complexes, where they are probably involved in the transport of membranes and contents vectorially through the Golgi complexes and secretion vacuoles (7, 16, 48).
High-resolution electron microscopy has been used to identify large clathrin-coated patches on phagosomes in macrophages that have ingested latex beads (1), and clathrin-coated patches have also been observed at sites of interactions of macrophages with opsonized erythrocytes (3, 36). These observations have suggested that clathrin assembly may be triggered by receptor-ligand interactions in macrophages. The spreading of macrophages on immune complex–coated surfaces is believed to represent an attempt to phagocytose these surfaces and has been used to analyze membrane receptor mobilization (21, 32, 60). This model system, called frustrated phagocytosis (24), is suitable for studying clathrin assembly because a large area of plasma membrane surface that interacts with the ligand is fixed on the ligand-coated surface and is thus available for examination. In the present study we have determined that clathrin assembly at the cell surface is induced by receptor-ligand interaction in this model system of Fc receptor–mediated phagocytosis and occurs with a concomitant decrease in clathrin-coated structures in the Golgi region.

Materials and Methods

Macrophages

Resident peritoneal macrophages were obtained from female CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA) by lavaging the peritoneum with phosphate-buffered saline (PBS) containing 50 U/ml heparin (54).

Antibodies

An IgG fraction of rabbit anti-bovine serum albumin (BSA) antibody, rhodamine-conjugated IgG fraction of sheep anti-rabbit IgG, and rhodamine-conjugated IgG fraction of affinity-purified goat anti-rabbit IgG were obtained from Cappel Laboratories (West Chester, PA). Mouse anti-BSA antibody was raised by injecting BSA in Freund’s complete adjuvant into CD-1 mice and then by boosting twice with BSA alone. The IgG fraction was purified from the serum by means of protein A-Sepharose (13). Affinity-purified rabbit anticalathrin antibody, a gift of Dr. Daniel Lionoud (Pasteur Institute, Paris) has been described previously (26). This polyclonal anticalathrin antibody, which was raised against empty clathrin cages prepared from pig brain coated vesicles by extraction with 2 M urea, recognizes both heavy and light chains of clathrin from various species, as tested by Western blotting (26).

Immune Complex–coated Coverslips

Coverslips coated with immune complexes were prepared by the procedure of Michl et al. (33) using 3 mg/ml BSA and rabbit or mouse anti-BSA IgG at various concentrations. Glass coverslips were treated with polyl-lysine (M, 150,000–350,000, Sigma Chemical Co., St. Louis, MO) and then with glutaraldehyde before binding with BSA and anti-BSA IgG. For all immunocytochemistry experiments, macrophages were spread on coverslips on which immune complexes were formed with BSA and 0.12 mg/ml IgG from a mouse anti-BSA antiserum, a concentration shown to induce maximum spreading.

The immobilized immune complexes used in these experiments were in an apparently homogeneous layer; they were uniform as observed by light microscopy with fluorescein-labeled anti-BSA IgG and by thin-section transmission electron microscopy using a peroxidase-conjugated rabbit anti-rabbit IgG reactive against rabbit anti-BSA IgG (52). Up to 2.5 h macrophages did not appear to ingest or degrade immune complexes. This was tested by using immune complex–coated surfaces formed by fluorescein-labeled anti-BSA IgG. If macrophages had taken up or degraded the immune complexes, darkened spots would have appeared in the bright carpet, as has been observed with cells plated on fibronectin-coated surfaces (6, 9). Occasional clearing of small areas was observed, but this was always mediated by polymorphonuclear leukocytes, which contaminate these preparations at a low concentration (<1%).

Determination of IgG Density on Immune Complex–coated Coverslips

Coverslips coated with BSA were incubated with fluorescein-conjugated rabbit anti-BSA (IgG fraction or Fab')_2 fragments. After the unbound IgG or Fab' (2) was washed away, the bound conjugate was eluted in 0.01 M NH_4OH and fluorescence was measured by excitation of 495 nm and emission of 519 nm. The amount of anti–BSA IgG was calculated by using a standard curve of known concentrations of anti–BSA IgG in the same pH buffer.

Spreading of Macrophages on Immune Complex–coated Coverslips

Freshly harvested macrophages were attached to and spread onto preformed immobilized BSA-anti-BSA immune complexes by a procedure modified from Rabinovitch and DeStefano (45). For precise temperature control, an aluminum cooling block (32) that allowed a rapid switch from 4 to 37°C was used. After the switch from 4°C to warm water, 34°C was attained by 1 min. The temperature of the block was monitored by a surface temperature probe connected to a telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH), and the temperatures were maintained at ± 0.1°C. BSA-anti-BSA-coated coverslips were placed on the cooling block, which had been precooled to 4°C. Macrophages suspended in ice-cold Eagle’s minimal essential medium supplemented with 25 mM Hepes buffer, pH 7.4, were overlaid onto coverslips (1–10 × 10^5 cells/cm^2) and allowed to attach for 60 min at 4°C. The coverslip cultures were washed free of nonadherent peritoneal cells and covered with a drop of ice-cold Eagle’s minimal essential medium–Hepes. Then the coverslips were warmed to 37°C by changing from the circulating water bath that perfused the block to the warm circulating water bath. For incubations longer than 20 min, the coverslips were transferred into medium in a multiwell plate and cultured at 37°C in a humid 5% CO_2 incubator. In some experiments cells were attached to the BSA-anti-BSA-coated coverslips at room temperature for 5–10 min.

A second method for formation of immune complexes, called the underlay method, was used for macrophages first cultured on BSA alone for 24 h. Anti-BSA IgG was then added to form immune complexes, and cells were allowed to spread according to the procedure of Michl et al. (32, 33). Macrophages suspended in Dulbecco’s modified Eagle’s medium supplemented with penci- lin, streptomycin, and 0.2% lactalbumin hydrolysate were plated onto BSA-coated coverslips in a multiwell plate (1–2.5 × 10^5 cells/cm^2) and incubated for 2 h at 37°C in a humid 5% CO_2 incubator before being washed free of nonadherent peritoneal cells and cultured overnight in Dulbecco’s modified Eagle’s medium–lactalbumin hydrolysate. The cells cultured overnight on BSA were washed with warm Dulbecco’s modified Eagle’s medium–lactalbumin hydrolysate and placed onto the cooling block at 4 ± 0.1°C. The anti-BSA IgG was added and the coverslips were incubated for 60 min at 4°C on the cooling block. Anti-BSA IgG penetrated under most parts of the cells and formed a uniform immune complex, as shown by the use of fluorescein-labeled anti-BSA IgG. After the excess soluble anti–BSA IgG was removed, the coverslips were returned to the cooling block and covered with Eagle’s minimum essential medium–Hepes, and the cooling block was warmed to 37°C. For incubations longer than 20 min, the coverslips were transferred to a CO_2 incubator at 37°C.

Indirect Immunofluorescence

Macrophages were fixed in 2% paraformaldehyde in PBS for 10–20 min at ambient temperature. Cells were then rinsed with PBS twice, incubated for 10 min in PBS containing 0.1 M glycine or 0.05 M NH_4Cl to quench any remaining aldehyde reactivity, and rinsed again in PBS. The fixed cells were then rendered permeable to protein reagents by 10-min exposure to 0.1% Triton X-100 in PBS. The cells were washed in PBS containing 3% normal sheep serum, and coverslips were overlaid with rabbit anticalathrin antibody diluted 1:200 in PBS containing 3% normal sheep serum. After a 60–90-min incubation at ambient temperature, the coverslips were washed thoroughly with PBS or with PBS that contained 3% normal sheep serum. Samples were then incubated for 30–60 min at ambient temperature with rhodamine-conjugated IgG fraction of sheep anti-rabbit IgG or rhodamine-conjugated affinity-purified goat anti–rabbit IgG diluted 1:100. The coverslips were then washed vigorously in PBS and then observed with a Zeiss Photomicroscope III equipped for epi-illumination.

Immunoperoxidase Procedures

The procedures used were essentially the same as those described previously (51). Macrophages were fixed in 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at ambient temperature. After incubation at ambient temperature for 90 min with rabbit anticalathrin antibody or control substance, the cells were washed six times and then incubated for 90 min at ambient temperature in Fab fragments of sheep anti-rabbit IgG conjugated to peroxidase (Pasteur Institute) at a dilution of 1:50. The cells were washed and then fixed at ambient temperature for 1 h with 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, containing 5% sucrose. The cells were then incubated overnight at 4°C in 0.1 M sodium cacodylate buffer, pH 7.4.
Preparation of Platinum-Carbon Replicas

Platinum-carbon replicas of broken-open macrophages were prepared by either quick-freeze deep-etch or critical-point-dry methods, as described previously (1, 2). In brief, cells cultured on coverslips were broken open in inside buffer (100 mM KCl, 3.5 mM MgCl₂, 3 mM EGTA, and 20 mM Hepes, pH 6.8) by placing a poly-L-lysine-coated coverslip over them and pulling it away. For quick-freeze deep-etch, broken-open cells were fixed, rapidly frozen by contact with a copper block that had been cooled to liquid helium temperature (4K) (25). Freeze-dried in a Balzers freeze-fracture apparatus (Balzers, Hudson, NH), and then rotary replicated with platinum-carbon at an angle of 25°. For critical-point drying, broken-open cells were fixed with lysine, glutaraldehyde, OsO₄, and tannic acid, dehydrated through graded alcohols, critical-point dried out of bone-dry liquid CO₂, and rotary replicated (2). Replicas were examined in a JEOL 100CX or Philips 300 electron microscope at 80-100 kV.

Morphometric Analysis

For detection of clathrin basketwork assembly, photographs were taken randomly from large open areas that were not heavily obstructed by cytoskeleton and where plasma membrane was easily visible. Obstructed areas were excluded from the reference area. About 25 photographs of ~10 μm² each were taken from each sample, and the amount of clathrin-coated area and reference area was quantified with a digitizer (Hewlett-Packard Co., Palo Alto, CA). Preliminary studies showed that the area in ~20 photographs was summed, the coefficient of variation of the percentage of coated areas became smaller. Because some of the coated patches were rounded, these coated areas were measured separately. Although it was not possible to measure the surface area of round coats directly, the area of round surfaces was estimated by multiplying the apparent area by two. The number of coated patches was determined from the same micrographs as those used for morphometric analysis. In addition, for some samples the number of coated patches only was determined from lower magnification micrographs of ~30 μm². Approximately 25 micrographs were used in each sample.

Results

Observations on Macrophages Freshly Explanted into Culture

Clathrin Is Redistributed During Adherence and Spreading of Freshly Harvested Macrophages on Immune Complex-coated Surfaces. For the study of clathrin redistribution during the spreading of macrophages on immune complex-coated surfaces, freshly harvested macrophages were attached to the coverslips at 4°C for 60 min, warmed to 37°C for various periods, and then fixed and stained with anti-clathrin antibody and observed by indirect immunofluorescence. After 60 min of adherence at 4°C, cells remained round with an area of contact of 200–300 μm² (Fig. 1). By 24 h, when strong Golgi staining was observed, most of the cells had regained clathrin staining in the perinuclear area, although peripheral punctate staining persisted. By 24 h, when strong Golgi staining at one side of the nucleus had developed, abundant peripheral clathrin staining was still seen (Fig. 2). Although the spread areas of cells were similar at 2 and 24 h, there was more clathrin in cells cultured for 24 h than in macrophages during their first 2 h in culture, which suggests that clathrin synthesis had taken place.

Ultrastructural Immunoperoxidase Observations Reveal the Redistribution of Clathrin from the Golgi Region During Ligand-induced Spreading. The distribution of clathrin in macrophages before and after spreading was investigated by antigen localization using a polyclonal antibody against clathrin and an immunoperoxidase procedure. In the nonspread cells, few coated vesicles were seen along the plasma membrane, and little immunoperoxidase staining was present (Fig. 3). This paucity of surface clathrin was observed both in cells fixed in vivo in the peritoneal cavity and in cells attached to immune complexes for 60 min at 4°C. Most of the staining for clathrin was seen on coated vesicles along and near the Golgi elements (Fig. 3). Abundant clathrin basketworks were also observed in the Golgi region by replica methods (data not shown).

When the macrophages were allowed to spread on immune complexes for 20-40 min at 37°C, there was a major redistribution of clathrin (Fig. 4). The clathrin staining in the Golgi region diminished (Fig. 4, a and b), and clathrin staining was viewed by interference-reflection microscopy, macrophages had extensive black areas at their spreading edges, which indicated close contact in these peripheral regions (data not shown). From 12 to 40 min, when the cells were fully spread, much of the punctate immunofluorescence was seen in the peripheral area, and peripheral staining was depleted (Fig. 2, d-i). There was little change in the concentrated intracellular clathrin in cells plated on BSA alone, although some clathrin was seen in the small veil of spreading at 20 min. Cells just beginning to spread had many microvilli and folds on their surface, whereas fully spread cells were completely flattened and had few surface microvilli. After 40 min the spread area began to decrease, and by 2 h the cells became more heterogeneous in shape and reached a plateau of ~600 μm². By 80-120 min, most of the cells had regained clathrin staining in the perinuclear area, although peripheral punctate staining persisted (Fig. 2, g-h). By 24 h, when strong Golgi staining at one side of the nucleus had developed, abundant peripheral clathrin staining was still seen (Fig. 2). Although the spread areas of cells were similar at 2 and 24 h, there was more clathrin in cells cultured for 24 h than in macrophages during their first 2 h in culture, which suggests that clathrin synthesis had taken place.

Figure 1. Time course of spreading of newly harvested macrophages on immune complexes. Coverslips were coated with 3 mg/ml BSA and then with 0.12 mg/ml mouse anti-BSA IgG. Macrophages were attached to the coverslips for 60 min at 4°C, then warmed at 37°C for 0-120 min. The spread area was determined by tracing the profiles of >100 cells for each sample on a digitizer. The data were collected from four different experiments, indicated by different lines. The standard error of each point was <5%.

7.4, containing 7.5% sucrose, and then washed three times during a 15-min period in 50 mM Tris-HCl buffer, pH 7.4, with 7.5% sucrose. They were preincubated for 5 min in Tris-HCl buffer containing 1 mg/ml 3,3′-diaminobenzidine (Grade I, Sigma Chemical Co.) and then incubated for 10 min with diaminobenzidine containing 0.01% (vol/vol) H₂O₂. After being washed they were exposed to 1% unbuffered OsO₄ for 2 min and then postfixed in reduced OsO₄ (which was produced by adding 3 mg of K₂Fe(CN)₆ to 2 ml of 1% unbuffered OsO₄) for 20 min at 4°C. After the cells were rinsed for 2 min in 1% unbuffered OsO₄, they were dehydrated in alcohols, washed off the plates with propylene oxide, and prepared for transmission electron microscopy. Control procedures included substitution of nonimmune rabbit IgG or buffer for the primary antiserum.
Figure 2. Redistribution of clathrin during initial spreading of freshly harvested macrophages on immune complexes. Macrophages were attached to the coverslips coated with BSA and 0.12 mg/ml mouse anti-BSA IgG for 60 min at 4°C, then warmed to 37°C for (a) 0, (b) 4, (c) 8, (d) 12, (e) 20, (f) 40, (g) 80, and (h) 120 min, and (i) 24 h, and stained with rabbit anticlathrin antibody. Matched pairs of immunofluorescent images and Nomarski-optic images are shown. The edges of the advancing plasma membrane are shown by small double arrows, and the clathrin staining in the perinuclear Golgi area by large arrows.
Figure 3. Electron micrographs of nonspread, resident peritoneal macrophages. Labeling of clathrin using an immunoperoxidase procedure on cells made permeable by saponin reveals dark reaction product on the cytoplasmic surface of vesicles, which are presumably coated (arrows). Most reactive vesicles are located in the Golgi region (Gc, inset), or cytoplasm, and few are on the plasma membrane. Note that the reaction product diffuses and spreads beyond the antigenic sites, a limitation of the immunoperoxidase procedure noted by Courtoy et al. (11). N, nucleus; m, mitochondria.

seen on both the nonadherent (Fig. 4a) and adherent (Fig. 4c) plasma membrane surfaces. The weaker staining of the adherent surface was probably due to the greater difficulty in penetration of anticlathrin antibodies to this region of the cell; however, abundant clathrin basketworks on the adherent surface were observed by replica procedures (Fig. 5).

Clathrin Assembly on Adherent Plasma Membrane Begins by 6 Min at 37°C in Freshly Harvested Macrophages. Clathrin basketworks on the inside adherent surface of macrophages spreading on immune complexes were studied in platinum-carbon replicas of broken-open cells. When suspended macrophages were attached to the immune complex-coated surfaces at 4°C for 60 min, no clathrin-coated patches were observed on the adherent membranes (Table I and Fig. 5a), although distinct cytoskeletal foci had developed (Figs. 5a and 6a). As macrophages were warmed to 37°C on immune complexes, clathrin-coated patches were not observed until 6 min (Fig. 6c), and abundant surface clathrin was seen later (Table I and Figs. 5b and 6, d-f). This contrasts with spreading, which was already under way by 4 min at 37°C (Figs. 1 and 2).

IgG Density That Induces Clathrin Assembly on Plasma Membrane Is Similar to the Density That Induces Spreading. To study whether the interaction of Fc receptors and immune complexes induces the clathrin-coated patches on plasma membrane, we performed quantitative analysis on cells spread on immune complexes. First, the relationship between the concentration of immune complexes and the extent of spreading was studied by coating BSA bound to coverslips with increasing amounts of anti-BSA IgG and examining the spreading of macrophages on them (Fig. 7). A few cells spread on BSA alone, but their contact area was always small (<500 μm²). A significant increase in spreading was first observed when 3 × 10⁻³ mg/ml of IgG was used to form complexes. At this concentration the contact area of the spread cells was 800–900 μm² each, but only 20% of cells were spread (Fig. 7a). Interestingly, at ≥3 × 10⁻² mg/ml of IgG, the percentage of spread cells reached a maximum (>80%), but the spread
Electron micrographs of newly harvested macrophages spread on immune complexes for 20 min at 37°C. Cells were labeled with the anticlathrin antibody using an immunoperoxidase procedure. Note the dramatic redistribution of clathrin (arrows) from the Golgi region (Gc) of the cell to the adherent and nonadherent membranes. s, adherent surface.

Figure 4. Electron micrographs of newly harvested macrophages spread on immune complexes for 20 min at 37°C. Cells were labeled with the anticlathrin antibody using an immunoperoxidase procedure. Note the dramatic redistribution of clathrin (arrows) from the Golgi region (Gc) of the cell to the adherent and nonadherent membranes. s, adherent surface.

area of the cells stayed the same (800–900 μm²). When the amount of IgG present in immune complexes was quantified by preparing the coverslips using fluorescein-labeled anti-BSA IgG, it was evident that spreading of macrophages was induced at 300 IgG molecules/μm² and reached a maximum at 1,000 IgG molecules/μm² (Fig. 7b). The densities of IgG that induced spreading on BSA-anti-BSA immune complexes were similar to those that induced rosette formation and phagocytosis of IgG-coated erythrocytes (52; data not shown). Anti-BSA F(ab′)2 did not induce spreading, which indicated that spreading was dependent on the Fc portion of IgG. It was necessary to present IgG to macrophages as immune complexes, because anti-BSA IgG that was coupled directly to coverslips using poly-L-lysine and glutaraldehyde did not induce spreading, even at 10 times the necessary density of IgG.

When clathrin coats were quantified in spreading macrophages, a dose-dependent increase in the density of coated patches on the adherent membrane surface and in the total number of coated patches per cell was observed over the range of IgG concentrations that induced spreading (Table II). There was also a slight increase in the density of coated patches on the nonadherent top surfaces of the macrophages. At maximum spreading on immune complexes, there was a more than fourfold increase in the number of clathrin patches on the adherent surface as compared with cells spread on BSA alone (Table II), and a remarkable increase of >200-fold as compared with the macrophages freshly lavaged from the peritoneal cavity (Table I). These data support the concept that Fc receptor-mediated spreading induces clathrin assembly at the site of ligand-receptor interaction in macrophages, but other physiologic changes induced by placing cells in culture, presumably endocytic rate, may also have profound effects on clathrin distribution in macrophages.

Coated patches on the adherent plasma membrane varied in size from a few assembled hexagons of basketworks to large patches encompassing 8–10 coated vesicle equivalents. To take the patch size into account, the area of plasma membrane coated with clathrin basketworks was determined (Table III). Macrophages spread on BSA alone had 1.4% of the adherent surface coated with clathrin basketworks, whereas those spread on immune complexes had 3.5% of the adherent membrane coated (Table III). The density of clathrin basketworks on the nonadherent surface did not change when the macrophages were spread on the immune complexes, indicating that the increase in clathrin coats was primarily on the adherent plasma membrane surface (Tables II and III). However, because cells spread on BSA had only ~400 μm² of adherent plasma membrane, whereas cells spread on immune complex-coated surfaces had ~800 μm², the actual increase in clathrin basketworks was fivefold (5.6 to 28 μm²). These data suggest that the site of ligand-receptor interaction is important for clathrin assembly. Clathrin coats were not distributed uniformly over the adherent surface of the macrophages, although large areas were coated (Fig. 5).
Figure 5. Adherent surface of newly harvested macrophages viewed from the cytoplasmic side in platinum-carbon replicas of critical point-dried broken-open cells. (a) Macrophages were attached to the immune complex-coated surface formed from 3 mg/ml BSA followed by 3 mg/ml of rabbit anti-BSA IgG for 60 min at 4°C. Foci-like cytoskeletal structures were present near the rim of the exposed membrane (arrows), but no clathrin was observed. (b) Macrophages were spread on the immune complex-coated surface for 20 min at 37°C. Numerous coated patches (arrowheads) can be seen. Many of the coated patches are made up of areas comprising several fused coated vesicle equivalents (asterisk).
Observations on Cultured Macrophages

Up to this point our study on redistribution of assembled clathrin by ligand-receptor interaction was made with macrophages freshly explanted into culture, which initially are unspread and have little surface clathrin. We were interested in comparing the clathrin kinetics in those cells with that in spread, cultured macrophages, which already have abundant surface clathrin (1) and quantitatively increased total clathrin, as determined by immunocytochemistry (Fig. 2 i). In addition, because most previous work on endocytosis and clathrin dynamics in macrophages (1–3, 30, 33, 36) or fibroblasts (5, 19, 28) has used cells in culture, an analysis of clathrin distribution in response to frustrated phagocytosis of immune complexes would be most directly comparable to these studies.

Clathrin Is Reversibly Redistributed in Cultured Macrophages Spreading on Immune Complex–coated Surfaces. We first studied the distribution of clathrin in macrophages that already had substantial amounts of surface clathrin basket-works by being placed in culture for 24 h. Such macrophages that have attached to and spread on coverslips coated with BSA during 24 h in culture can be induced to spread further: immune complexes are formed under the cells at 4°C by their being incubated with anti-BSA IgG by the underlay method and then warmed to 37°C. Macrophages cultured on BSA alone for 24 h appeared flattened, with a spread area of 600–900 μm² (Fig. 8), and had strong clathrin staining concentrated in the Golgi area (Fig. 9a). When these cells were incubated with anti-BSA IgG at 4°C, the edges of the membranes ruffled, which indicates that even at 4°C the membranes were dynamic; although distribution of clathrin in the Golgi region changed little, there appeared to be some additional peripheral staining in these cells (Fig. 9b). As soon as the cells were warmed to 37°C, they became flattened, increasing their area of contact with the coverslip to 1,100–1,200 μm² by 4–8 min (Fig. 9, c and d). Clathrin staining in the Golgi area was rapidly depleted (as early as 4 min) (Fig. 9, c–g). After 80–120 min the strong staining of clathrin began to reappear in the Golgi area in some cells (Fig. 9, h and i). At this time cells altered their morphology and reassumed shapes similar to those of cells cultured on BSA for 24 h. That redistribution was not blocked by cycloheximide suggests that synthesis of new clathrin was not required, at least in the first 1–2 h.

Clathrin Assembly Occurs at 4°C in Cultured Macrophages. When coated patches were observed on the adherent membrane of macrophages cultured on BSA for 24 h, their density was found to be similar to that of patches on newly adherent macrophages spread on BSA for 20 min (Fig. 10a...
Figure 7. Dose response for spreading of macrophages on immune complexes. (a) Dependence of macrophage spreading on the concentration of anti-BSA IgG used to form immune complexes. Coverslips were coated with 3 mg/ml BSA and then with 0–3 mg/ml rabbit anti-BSA IgG. Freshly harvested macrophages were spread for 40 min at 37°C. The adherent area of spread cells (○) was determined by a digitizer. Profiles of 20 cells were traced for each sample. The bar indicates the standard error of the mean. The area of contact of unspread cells (△) is also indicated. The percentage of spread cells (●) was determined by differential counting of spread and unspread cells (>100 total). The data point for 3 mg/ml anti-BSA IgG is from a separate experiment. (b) Dependence of macrophage spreading on IgG or F(ab')2 density. The number of IgG or F(ab')2 molecules bound to coverslips was determined as described in Materials and Methods. The percentage of spread cells was determined as described in a.

Table II. Dose Response of Clathrin-coated Patches Observed in Freshly Explanted Macrophages Spreading on Immune Complexes

| Anti-BSA IgG | Nonadherent surface* | Adherent surface | Nonadherent surface* | Adherent surface |
|--------------|---------------------|------------------|---------------------|------------------|
| mg/ml        | n                   | n                | n                   | n                |
| 0            | 0.3 ± 0.1 (25)      | 0.9 ± 0.2 (24)   | 120                 | 360              |
| 0.003        | 0.6 ± 0.1 (25)      | 1.3 ± 0.3 (27)   | 300                 | 650              |
| 0.3          | ND                  | 2.4 ± 0.2 (23)   | ND                  | 1,920            |
| 3.0          | 0.6 ± 0.1 (22)      | 2.1 ± 0.6 (26)   | 480                 | 1,680            |

Macrophages were attached to coverslips coated with 3 mg/ml BSA and various concentrations of rabbit anti-BSA IgG at 4°C for 60 min, then warmed to 37°C for 20 min. Both tops and bottoms of broken-open macrophages were fixed, critical point-dried, and replicated. For details of quantification, see Table I. The numbers of clathrin-coated patches per square micrometer on the adherent surface of cells spread on BSA alone were significantly different (P < 0.05) from the values for cells spread on immune complexes.

* When estimating the nonadherent surface area by light microscopy, we did not take curvature, surface ruffles, and microvilli into account; therefore, the true area is underestimated by a factor of from ~1.5 (for spread cells) to 3 (for unspread cells).

Discussion

In this report we have described dynamic changes in the distribution of morphologically recognizable assembled clathrin during Fc receptor-mediated frustrated phagocytosis. The kinetics of clathrin redistribution are summarized diagrammatically in Fig. 11. We found that clathrin-coated patches assemble on the plasma membrane adherent to immune complexes. In cultured macrophages, clathrin assembled rapidly on the membrane, even at 4°C, whereas in macrophages freshly lavaged from the peritoneum, the two processes were separated kinetically. Spreading preceded clathrin assembly in newly harvested macrophages but followed clathrin assembly in cultured macrophages, which suggests that they are not causally related. Concomitantly, clathrin from the Golgi region of the macrophages diminished, and then was restored by 2 h.

One unexpected observation was the low number of clathrin-coated patches on the plasma membrane of resident macrophages both in vivo and freshly harvested from the peritoneal cavity and attached to immune complexes at 4°C. Al-
Table III. Comparison of Membrane Area Coated with Clathrin Observed in Freshly Explanted and Cultured Macrophages Spreading on Immune Complexes

| Macrophage          | Anti-BSA IgG mg/ml | Clathrin-coated surface area (% of total) | Estimated surface area per cell μm² | Total clathrin-coated surface area per cell μm² |
|---------------------|-------------------|------------------------------------------|-----------------------------------|-----------------------------------------------|
|                     |                   | Nonadherent surface                        | Adherent surface                  |                                               |
| Freshly explanted   | 0                 | 0.8 ± 0.2 (25)                             | 1.4 ± 0.4 (24)                   | 400                                           |
|                     | 3.0               | 0.6 ± 0.2 (23)                             | 3.5 ± 0.8 (26)                   | 800                                           |
| Cultured for 24 h   | 0                 | ND*                                       | 1.5 ± 0.4 (23)                   | 900                                           |
|                     | 0.3               | ND                                        | 3.1 ± 0.5 (26)                   | 1,200                                         |

For freshly explanted macrophages, coverslips were coated with 3 mg/ml BSA and then with 3 mg/ml of rabbit anti-BSA IgG. Cells were attached for 10 min at room temperature and then spread for 20 min at 37°C. Both tops and bottoms of broken-open macrophages were fixed, critical point-dried, and replicated. For cultured macrophages, cells were cultured for 24 h on coverslips coated with 3 mg/ml BSA, and then the coverslips were incubated with 0.3 mg/ml of rabbit anti-BSA IgG for 60 min at 4°C. After unbound IgG was washed off, macrophages were incubated for 20 min at 37°C. The bottom membranes of macrophages were fixed, critical point-dried, and replicated. For quantification, micrographs with 5-10 μm² of membrane that was unobstructed by cytoskeleton at a final relative intensity (26), and recent estimates indicate that ~30-50% of cellular clathrin is in its unassembled form in a wide variety of cell types (20). Thus, clathrin is probably redistributed to macrophage plasma membranes from intracellular pools.

Our data on clathrin distribution in macrophages in vivo have important implications for the extrapolation of in vivo rates of membrane turnover, receptor turnover, fluid uptake, and membrane trafficking from rates derived from cells in culture (29, 30, 50). Previous studies have demonstrated relatively few plasma membrane-coated vesicles in macrophages in vivo (3) except in specialized sites such as regions in which macrophages interact with Leydig cells (35). That mutant cells defective in endocytosis require only ferric ions to proliferate (27) suggests that transferrin may be the only normal metabolite that must be taken into cells by endocytosis. Resident macrophages do not proliferate (56), have very few transferrin receptors (22), and thus may be endocytically inactive in the absence of specific ligands. However, clathrin coats are abundant in cells actively engaged in endocytosis in vivo (37) and in culture (1, 3, 36). In previous studies, concentrations of clathrin-coated pits and vesicles have been observed on the basal surfaces (28) or trailing edges (43) of cells, which suggests that endocytic activity may occur preferentially in these areas.

The redistribution of clathrin during Fc receptor-mediated spreading was not confined to the freshly harvested macro-

patches after 20 min at 37°C was at least 30-fold, even on the nonadherent surface of the attaching macrophages, and was as much as 200-fold on plasma membrane surfaces adherent to immune complexes.

Clathrin appears to be in a dynamic state and can be induced to assemble in increased amounts at the cell surface in many types of cells (1, 10, 15, 36, 38, 47, 48). Clathrin is usually seen within cells in a membrane-associated assembled basketwork structure (1, 4, 17, 59); however, unassembled clathrin triskelions stored in the cytoplasm are probably the source for the induced assembly at the plasma membrane (20, 46). Large aggregates of unassembled clathrin (46) have not been observed in most cells; nonetheless, diffusely distributed unassembled forms have been detected by immunocytochemistry (26), and recent estimates indicate that ~30-50% of cellular clathrin is in its unassembled form in a wide variety of cell types (20). Thus, clathrin is probably redistributed to macrophage plasma membranes from intracellular pools.
Figure 9. Redistribution of clathrin during spreading of cultured macrophages on immune complexes. Macrophages were cultured on BSA-coated coverslips for 24 h (a); then 0.12 mg/ml mouse anti-BSA IgG was added at 4°C for 60 min. Cells were then warmed to 37°C for (b) 0, (c) 4, (d) 8, (e) 12, (f) 20, (g) 40, (h) 80, and (i) 120 min, and stained with rabbit anticalathrin antibody. Matched pairs of immunofluorescent images and Nomarski-optic images are shown. The clathrin staining in the Golgi area is shown by arrows.
Assembly of coated patches on adherent membranes of cultured macrophages spread on immune complexes observed by platinum-carbon replica techniques. Macrophages were cultured for 24 h on BSA-coated coverslips, then incubated with anti-BSA IgG, the assembly of clathrin coats at 4°C was observed before spreading (Fig. 11). One previous study indicated that an arrest of endocytosis by cold treatment did not alter the number of coated vesicles seen at the cell surface (19); however, in another study twice as many coated vesicles were seen immediately after warming of cells that bound low-density lipoproteins in the cold than under steady state conditions (4). Our data clearly demonstrate that the signal for clathrin assembly can be transduced in the cold via the Fc receptor. Clathrin also assembles within 2 min on latex phagosomes in cultured macrophages (1). The striking contrast between the lack of assembly of clathrin coats at 4°C followed by a 6-min delay in clathrin assembly at 37°C in newly harvested macrophages, on one hand, and rapid assembly of clathrin coats at 4°C in cultured macrophages, on the other hand, suggests that a fundamental change in the plasma membrane takes place during culture of macrophages. The possibilities for this change include (a) an alteration of membrane fluidity, allowing migration of proteins in the plane of the membrane, such as that observed for the complement receptor (21); (b) an alteration in the state of the Fc receptor so that the signal for clathrin coat assembly is more rapid; or (c) a rate-limiting requirement for assembling or modifying clathrin-associated proteins (40, 42) at sites that bridge the plasma membrane and the clathrin basketwork.

Clathrin Redistributes During Frustrated Phagocytosis. What is the reason for the clathrin redistribution during frustrated phagocytosis? Several observations by others on the nature of the clathrin-coated compartments related to the Golgi complex are instructive. Brown and his colleagues (7, 8) have shown that mannose-6-phosphate receptors accumulate in coated vesicles along Golgi cisternae in ligand-deficient cells, at the presumptive sorting site. Similarly, a clathrin-coated Golgi-related compartment accumulates in proinsulin-secreting cells when secretion is blocked by monensin, presumably at a sorting site (39). In both cases, the steady state distribution of clathrin has been perturbed by disruption of the normal intracellular traffic through the Golgi complex. In frustrated phagocytosis the rapid addition of membrane to the cell surface may also disturb intracellular traffic, thus increasing the turnover of membrane-associated clathrin from the Golgi region (Takemura, R., P. E. Stenberg, D. F. Bainton, and Z. Werb, manuscript submitted for publication), with a resulting decrease in assembled clathrin in that region. A redistribution of other membrane compartments such as secretory granules and endosomes communicating with the plasma membrane takes place concomitantly with clathrin redistribution during the time before establishment of a new steady state, when intracellular traffic of membrane is balanced. Secretion products are also directed to the adherent surface engaged in frustrated phagocytosis (24; Werb, Z., and D. F. Bainton, manuscript in preparation).
in the intracellular transport of membranes and proteins (41), especially deep in the cytoplasm and around the Golgi complex. There have been only a few cases in which such movement of clathrin redistribution occur more rapidly than in freshly harvested cells shown in a. 

Anti-BSA Time at Clathrin-coated patches Clathrin-coated patches Estimated adherent surface Total coated patches on
IgG 37°C Total area of samples counted per μm² area per cell surface per cell

| Anti-BSA IgG | mg/ml | min | μm² | n | μm² | n |
|-------------|-------|-----|-----|---|-----|---|
| None        | 0     | 0   | 868 (40) | 1,925 | 1.0 | 800 | 820 |
| None        | 0.3   | 0   | 868 (40) | 1,925 | 1.0 | 800 | 820 |
| None        | 0.3   | 20  | 625 (27) | 1,073 | 1.7 | 1,200 | 2,060 |

Macrophages were cultured on coverslips coated with 3 mg/ml BSA for 24 h, and then incubated with 0.3 mg/ml rabbit anti-BSA IgG at 4°C for 60 min. After the unbound IgG was washed off, the macrophages were warmed to 37°C for 0 or 20 min, and the bottom surfaces of the cells were fixed, critical-point-dried, and replicated. Coated patches were counted in representative micrographs of 20-30 μm² of unobstructed membrane. The number of micrographs is shown in parentheses. The adherent membrane surface area, estimated from Fig. 8, was used to calculate the total number of coated patches on the adherent surface. Data are derived from two independent experiments. Some of the coated patches were large, consisting of up to 10 fused, individually discernible coated pits. If these are taken into account, the total number of coated patches increases by 10-35%.

**Table IV. Quantification of Clathrin-coated Patches Observed on Adherent Surfaces of Cultured Macrophages Spreading on Immune Complexes**

![Diagram](https://example.com/diagram.png)

*Figure 11. A diagrammatic summary of spreading and clathrin distribution in macrophages adhering and spreading on immobile immune complexes at 37°C. The data are qualitative and are deduced from the results. (a) In freshly explanted macrophages, spreading precedes clathrin assembly at the cell surface, and Golgi region clathrin redistribution is reversible. (b) In cultured macrophages the point at ~60 min represents cells cultured on BSA for 24 h; then anti-BSA IgG was added at 4°C. The interval between ~60 and 0 min represents the 60 min at 4°C. Note that clathrin associates with the cell surface at 4°C and precedes spreading, and that spreading and clathrin redistribution occur more rapidly than in freshly harvested cells shown in a.*

Because a large amount of cellular clathrin is found as coated vesicles located away from the plasma membrane, especially deep in the cytoplasm and around the Golgi complex, clathrin may also be supplied to the cell surface by the redistribution of coated vesicles deep in the cytoplasm. Despite the fact that coated vesicles are believed to be involved in the intracellular transport of membranes and proteins (41), there have been only a few cases in which such movement of coated vesicles has been demonstrated directly (17, 18). Because of the delay in redistribution of clathrin from the Golgi region, as compared with the assembly of clathrin on the adherent surface and the resultant increase in surface area, there is probably not a direct movement of assembled clathrin-coated vesicles. It is more likely that a soluble pool of clathrin triskelions is the intermediate in redistribution, and this mechanism probably accounts for the marked assembly of clathrin on the adherent surface of cultured macrophages spreading on immune complexes at 4°C. It is apparent that tissues that are more endocytically active have smaller pools of unassembled clathrin (20). Studies are in progress to determine the distribution of clathrin between assembled and soluble pools in macrophages during frustrated phagocytosis.

**Fc Receptor Interactions Signal Clathrin Basket Assembly in Macrophages.** We observed a two- to threefold increase in density of clathrin coats in cells spread on immune complexes as compared with cells spread on BSA alone. When the increase in the total plasma membrane in spread macrophages is taken into account, the increase in coated structures was fivefold. It has been estimated that 0.2-0.8% of cellular protein of macrophages is clathrin (3, 20). Assuming that macrophage protein is ~100 μg/10⁶ cells, each cell would have enough clathrin to cover between 80 and 200 μm² of membrane surface (12). The maximum area covered by clathrin basketworks on the plasma membrane of spreading macrophages used in this study was ~30 μm² per cell; therefore, nearly half the clathrin in these cells was assembled at the plasma membrane. Macrophages have ~1.3 × 10⁷ Fc receptors (57), most of which are captured at the bottom surface during spreading onto immune complexes (14, 32-34). At 3 mg/ml of anti-BSA IgG, the density of the immune complex on the dishes was ~10,000 molecules/μm²; therefore, the average spacing of IgG molecules was ~10 nm, which is comparable to the distance between the vertices of the clathrin basketwork. Furthermore, although the average percentage of coated area of the cells spread on immune complexes was only 3.5%, some regions of the cells were coated up to 17% (for examples see Fig. 5b). Such dense concentrations of clathrin coats were never observed in control cells. Because the half-life of coated vesicles involved in receptor-mediated endocytosis is only 2–3 min at 37°C (5, 58), one interpretation is that regions with the highest density of clathrin coats are temporal foci for basket assembly. Although membrane spreading preceded the appearance of assembled clathrin at the plasma membrane by several minutes, we cannot rule out the possibility that the act of cell spreading on a substratum...
may itself trigger clathrin assembly, even without defined ligands or receptors. Extensive assembled clathrin has also been observed at attachment sites of fibroblasts (28).

The frustrated phagocytosis system modeled Fc receptor-mediated phagocytosis well. IgG concentrations inducing phagocytosis were the same as those inducing spreading. The time course of macrophage spreading corresponded to that for the disappearance of Fc receptors from the upper surface observed previously in cultured macrophages (32). The fact that discrete coated vesicles are seen at the sites of close interaction of IgG-coated erythrocytes with macrophages (3, 36), rather than the large planar areas at sites of attachment of latex particles (1), suggests that Fc receptors may be clustered. Macrophage Fc receptors are internalized and degraded after attachment to mobile immune complexes (29, 30). The precise fate of Fc receptors in macrophages spreading on immobilized immune complexes remains to be determined. Spreading of cultured macrophages is also induced by agents, such as phorbol diesters (44), that do not act selectively at the adherent surface. It will be interesting to determine if such stimuli also induce clathrin assembly.

Why does clathrin assemble at the sites of membrane-receptor interaction? Because membrane spreading and clathrin assembly at the adherent surface of the macrophage are separated in time (Fig. 11), the signals transduced by ligand-receptor interaction, the exact mechanism and the role of this increased clathrin assembly have yet to be determined. Frustrated phagocytosis provides a useful model for perturbing and analyzing clathrin dynamics.

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References

1. 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. 94:613–623.
2. Aggeler, J., R. Takemura, and Z. Werb. 1983. High-resolution three-dimensional views of membrane-associated clathrin and cytoskeleton in critical-point-dried macrophages. J. Cell Biol. 97:1452–1458.
3. Aggeler, J., R. Takemura, B. A. Nichols, and Z. Werb. 1985. Macrophage clathrin and clathrin. In Mononuclear Phagocytes and Inflammation. R. van Furth, editor. Martinus Nijhoff, Medical Division, The Hague.
4. Anderson, R. G. W., J. L. Goldstein, and M. S. Brown. 1976. Localization of a density lipoprotein receptor on the surface of normal human fibroblasts and their absence in cells from a familial hypercholesterolemia homozygote. Proc. Natl. Acad. Sci. USA. 73:2434–2438.
5. Avnir, Z., and B. Geiger. 1981. The removal of extracellular fibronec tin from areas of cell-substrate contact. Cell 25:121–132.
6. Brown, W. J., and M. G. Farquhar. 1984. The mannose-6-phosphate receptor for lysosomal enzymes is concentrated in cis Golgi cisternae. Cell. 36:295–307.
7. Brown, W. J., E. Constantinescu, and M. G. Farquhar. 1984. Redistribution of mannose-6-phosphate receptors induced by tunicamycin and chloroquine. J. Cell Biol. 99:320–326.
8. Chen, W. T., K. Olden, B. A. Bernard, and F.-F. Chu. 1984. Expression of transformation-associated protease(s) that degrade fibronec tin at cell contact sites. J. Cell Biol. 98:1546–1555.
9. Connolly, J. L., S. A. Green, and L. A. Greene. 1984. Comparison of rapid changes in surface morphology and coated pit formation of PC12 cells in response to nerve growth factor, epidermal growth factor, and dibutyryl cyclic AMP. J. Cell Biol. 98:457–465.
10. Courtoy, P. J., D. H. Picton, and M. G. Farquhar. 1983. Resolution and limitations of the immunoperoxidase procedure in the localization of extracellular matrix antigens. J. Histochem. Cytochem. 31:945–951.
11. Crowther, R. A., and B. M. F. Pearse. 1981. Assembly and packing of clathrin into coats. J. Cell Biol. 91:780–797.
12. Ey, P. L., S. J. Prowse, and C. R. Jenkin. 1978. Isolation of pure IgG, IgG2a, and IgG1 immunoglobulin receptors from mouse serum using A-Septaphase. J. Immunotherapy. 15:429–436.
13. Ezekowitz, R. A. B., M. Bampton, and S. Gordon. 1983. Macrophage activation selectively enhances expression of Fc receptors for IgG2a. J. Exp. Med. 157:807–812.
14. Fisher, G. W., and L. I. Rehbin. 1983. Sea urchin egg cortical granule exocytosis is followed by a burst of membrane retrieval via uptake into coated vesicles. Dev. Biol. 99:456–472.
15. Franke, W. W., M. R. Luder, J. Kartenbeck, H. Zerban, and T. W. Keenan. 1976. Involvement of vesicle coat material in casein secretion and surface regeneration, J. Cell Biol. 69:173–195.
16. Friend, D. S., and M. G. Farquhar. 1967. Functions of coated vesicles during protein absorption in the rat vas deferens. J. Cell Biol. 35:357–376.
17. Garner, J. A., and J. R. Lasek. 1981. Clathrin is axonally transported as part of slow component b: the microfilament complex. J. Cell Biol. 88:172–178.
18. Goldenthal, K. L., I. Pastan, and M. C. Willingham. 1984. Initial steps in receptor-mediated endocytosis. The influence of temperature on the shape and distribution of plasma membrane clathrin-coated pits in cultured hamster cells. Exp. Cell. Res. 152:558–564.
19. Goldenthal, K. L., I. Pastan, and M. C. Willingham. 1984. Initial steps in receptor-mediated endocytosis. A quantitative study using an enzyme immunoassay. J. Cell Biol. 100:521–527.
20. Griffin, F. M., Jr., and P. J. Mullinan. 1981. Augmentation of macrophage complement receptor function in vitro. III. C3b receptors that promote phagocytosis migrate within the plane of the macrophage plasma membrane. J. Exp. Med. 154:291–305.
21. Hamilton, T. A., J. E. Weel, and D. O. Adams. 1984. Expression of the transferrin receptor in murine peritoneal macrophages is modulated in the different stages of activation. J. Immunol. 122:2285–2290.
22. Harrison, S. C., and T. Kirchhausen. 1983. Clathrin, cages, and coated vesicles. Cell. 37:650–652.
23. Henson, P. M. 1971. Interaction of cells with immune complexes: adherence, release of constituents, and tissue injury. J. Exp. Med. 134(3, Pt. 2):1148–1155.
24. Heuser, J. E., T. S. Reese, M. J. Dennis, Y. Jan, L. Jan, and L. Evans. 1979. Synaptosome exocytosis captured by quick freezing and correlated with quanta1 transmitter release. J. Cell Biol. 81:275–300.
25. Lownard, D., C. Morris, G. Warren, K. Stanley, F. Winkler, and H. Reggio. 1983. A monoclonal antibody to the heavy chain of clathrin. EMBO (Eur. Mol. Biol. Organ.) J. 2:1655–1664.
26. Marnell, M. H., L. S. Mathis, M. Stooksky, S.-P. Shia, D. K. Stone, and R. K. Draper. 1984. A Chinese hamster ovary cell mutant with a heat-sensitive, conditional-lethal defect in vacuolar function. J. Cell Biol. 99:1907–1916.
27. Maupin, P., and T. D. Pollard. 1983. Improved preservation and staining of HeLa cell actin filaments, clathrin-coated membranes, and other cytoplasmic structures by nannic acid-glutaraldehyde-saponin fixation. J. Cell Biol. 96:51–62.
28. Mollman, I., and H. Plutner. 1984. Internalization and degradation of macrophage Fc receptors bound to polyvalent immune complexes. J. Cell Biol. 98:1170–1177.
29. Mollman, I. S., H. Plutner, R. M. Steinman, J. C. Unkeless, and Z. A.
Cohn. 1983. Internalization and degradation of macrophage Fc receptors during receptor-mediated endocytosis. J. Cell Biol. 96:887–895.

31. Merisko, E. M., M. G. Farquhar, and G. E. Palade. 1983. Changes in clathrin distribution in pancreatic exocrine cells under anoxic conditions. J. Cell Biol. 97(5), Pt: 21:74a (Abstr.)

32. Michi, J., M. M. Pieczonka, J. C. Unkeless, G. I. Bell, and S. C. Silverstein. 1983. Fc receptor modulation in mononuclear phagocytes maintained on immobilized immune complexes occurs by diffusion of the receptor molecule. J. Exp. Med. 157:2121–2139.

33. Michi, J., M. M. Pieczonka, J. C. Unkeless, and S. C. Silverstein. 1979. Effects of immobilized immune complexes on Fc- and complement-receptor function in resident and thioglycollate-elicited mouse peritoneal macrophages. J. Exp. Med. 150:607–621.

34. Michi, J., J. C. Unkeless, M. M. Pieczonka, and S. C. Silverstein. 1983. Modulation of Fc receptors of mononuclear phagocytes by immobilized anti-amyloid antibodies. Quantitative analysis of the relationship between ligand number and Fc receptor response. J. Exp. Med. 157:1746–1757.

35. Miller, S. C., B. M. Bowman, and H. G. Rowland. 1983. Structure, cytochemistry, endocytic activity, and immunoglobulin (Fc) receptors of rat testicular interstitial-tissue macrophages. Am. J. Anat. 168:1–13.

36. Montesano, R., A. Mosaz, P. Vassalli, and L. Orci. 1983. Specialization of the macrophage plasma membrane at sites of interaction with opsonized erythrocytes. J. Cell Biol. 96:1227–1233.

37. Nichols, B. A. 1982. Uptake and digestion of horseradish peroxidase in rabbit alveolar macrophages. Formation of a pathway connecting lysosomes to the cell surface. Lab. Invest. 47:235–246.

38. O’Neill, R. M., and J. W. La Claire II. 1984. Mechanical wounding induces the formation of extensive coated membranes in giant algal cells. Science (Wash. DC). 225:331–333.

39. Orci, L., P. Hallan, M. Amherdt, M. Ravazzola, J.-D. Vassalli, and A. Perrelet. 1984. A clathrin-coated, Golgi-related compartment of the insulin secreting cell accumulates proinsulin in the presence of monensin. Cell. 39:39–47.

40. Pearse, B. M. F. 1978. On the structural and functional components of coated vesicles. J. Mol. Biol. 126:803–812.

41. Pearse, B. M. F., and M. S. Bretscher. 1981. Membrane recycling by coated vesicles. Annu. Rev. Biochem. 50:85–101.

42. Pfeiffer, J. R., J. M. Oliver, and R. D. Berlin. 1983. Identification of three coated vesicle components as α- and β-tubulin linked to a phosphorylated 50,000-dalton polypeptide. J. Cell Biol. 97:40–47.

43. Pfeiffer, J. R., J. M. Oliver, and R. D. Berlin. 1983. Topographical distribution of coated pits. Nature (Lond.). 286:727–729.

44. Phaire-Washington, L., E. Wang, and S. C. Silverstein. 1980. Phorbol myristate acetate stimulates pinocytosis and membrane spreading in mouse peritoneal macrophages. J. Cell Biol. 86:634–640.

45. Rabionovitch, M., and M. J. DeStefano. 1973. Macrophage spreading in vitro. I. Inducers of spreading. Exp. Cell Res. 77:323–334.

46. Raikhel, A. S. 1984. Accumulations of membrane-free clathrin-like lattices in the mosquito oocyte. Eur. J. Cell Biol. 35:279–283.

47. Roth, T. F., and K. R. Porter. 1964. Yolk protein uptake in the oocyte of the mosquito Aedes aegypti. J. Cell Biol. 20:313–312.

48. Rothman, J. E., H. Bursztyn-Pettegrew, and R. E. Fine. 1980. Transport of the membrane glycoprotein of vesicular stomatitis virus to the cell surface in two stages by clathrin-coated vesicles. J. Cell Biol. 86:162–171.

49. Salisbury, J. L., S. J. Condeelis, and P. Satir. 1980. Role of coated vesicles, microfilaments, and calmodulin in receptor-mediated endocytosis by cultured B lymphoblastoid cells. J. Cell Biol. 87:122–141.

50. Steinman, R. M., S. E. Brodie, and Z. A. Cohn. 1976. Membrane flow during pinocytosis. A stereologic analysis. J. Cell Biol. 68:665–687.

51. Stenberg, P. E., M. A. Shuman, S. P. Levine, and D. F. Bainton. 1980. Redistribution of alpha-granules and their contents in thrombin-stimulated platelets. J. Cell Biol. 98:748–760.

52. Takemura, R. 1984. Dynamics of clathrin distribution during macrophage spreading induced by receptor-ligand interaction. Ph.D. thesis, University of California, San Francisco. 189 pp.

53. Takemura, R., and D. F. Bainton. 1983. Redistribution of clathrin during spreading of mouse macrophages on immobilized immune complexes. J. Cell Biol. 97(5, Pt. 2):174a (Abstr.)

54. Takemura, R., and Z. Werb. 1984. Regulation of elastase and plasminogen activator secretion in resident and inflammatory macrophages by receptors for the Fc domain of immunoglobulin G. J. Exp. Med. 159:152–166.

55. Takemura, R., J. Aggeler, and Z. Werb. 1982. Initial events during frustrated phagocytosis by mouse macrophages. J. Cell Biol. 95(5, Pt. 2):423a. (Abstr.)

56. Tushinski, R. J., J. T. Oliver, L. J. Guibert, P. W. Tynan, J. R. Warner, and E. R. Stanley. 1982. Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cells selectively destroy. Cell. 28:71–81.

57. Unkeless, J. C., H. Fleit, and I. S. Mellman. 1981. Structural aspects and heterogeneity of immunoglobulin Fc receptors. Adv. Immunol. 31:247–270.

58. Wall, D. A., G. Wilson, and A. L. Hubbard. 1980. The galactose-specific recognition system of mammalian liver: the route of ligand internalization in rat hepatocytes. Cell. 21:79–93.

59. Willingham, M. C., J. H. Keen, and I. H. Pastan. 1981. Ultrastructural immunoocytochemical localization of clathrin in cultured fibroblasts. Exp. Cell Res. 132:329–338.

60. Wright, S. D., and S. C. Silverstein. 1984. Phagocytosing macrophages exclude proteins from the zones of contact with opsonized targets. Nature (Lond.). 309:359–361.