Disappearance of Macrophage Surface Folds after Antibody-dependent Phagocytosis

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ABSTRACT
We have employed the method of Burwen and Satir (J. Cell Biol., 1977, 74:690) to measure the disappearance of surface folds from resident guinea pig peritoneal macrophages after antibody-dependent phagocytosis. Unilamellar phospholipid vesicles containing dimyristoylphosphatidylcholine and 1 mol% dinitrophenyl-\(\varepsilon\)-aminocaproyl-phosphatidylethanolamine, a lipid that possesses a hapten headgroup, were prepared by an ether injection technique. These vesicles were taken up by macrophages in a time- and temperature-dependent fashion. Vesicles that contained ferritin trapped in the internal aqueous volume were identified within macrophages by transmission electron microscopy. Scanning electron microscopy has shown that macrophage surface folds decrease dramatically after phagocytosis. The surface fold length (\(\mu m\)) per unit smooth sphere surface area (\(\mu m^2\)) decreases from 1.3 ± 0.3 \(\mu m^{-1}\) to 0.53 ± 0.25 \(\mu m^{-1}\) when cells are incubated in the presence of specific anti-DNP antibody and vesicles at 37°C. No significant effect was observed in the presence of antibody only or vesicles only. Our studies show that phagocytosis is associated with a loss of cell surface folds and a loss of cell surface area, which is consonant with current views of the endocytic process. On the basis of our uptake data, we estimate that \(\sim 400 \mu m^2\) of vesicle surface membrane is internalized. The guinea pig macrophage plasma membrane has a total area of \(\sim 400 \mu m^2\) in control studies, whereas the cells have roughly 300 \(\mu m^2\) after phagocytosis. These estimates of surface areas include membrane ruffles and changes directly related to changes in cell volume. We suggest that during antibody-dependent phagocytosis a membrane reservoir is made available to the cell surface.

The macrophage plasma membrane possesses receptors for the Fc domain of the immunoglobulin molecule that can mediate immunologic recognition leading to phagocytosis or cytolysis of a target (for reviews see references 21–23). A variety of targets has been employed as models of antibody-dependent and antibody-independent phagocytosis (e.g., sheep erythrocytes and latex beads, respectively). Recent research in this laboratory (12, 16) and others (11) has examined the antibody-dependent phagocytosis of lipid-hapten-containing phospholipid vesicles. This system has several distinct advantages over the more conventional methods because: (a) it is chemically well characterized, (b) the physical characteristics ("fluidity") of the membrane can be controlled, (c) membrane hapten density is known and can be varied, (d) surface charge density can be altered, (e) the cholesterol-phospholipid ratio can be changed, and (f) various materials can be delivered to the cells by entrapment in the internal aqueous volume of the vesicle. It is clear that such vesicles have many potential advantages for studies of molecular events involved in immunologic recognition, triggering, and subsequent effector functions.

To understand the role of cell surface components during antibody-dependent phagocytosis it is helpful to have an understanding of variations in plasma membrane surface area as well as turnover. This description requires knowledge of four interrelated parameters: (a) cell surface area before phagocytosis, (b) cell surface area after phagocytosis, (c) area of cell surface membrane internalized, and (d) the area of membrane shuttled to the plasma membrane during this process. In the present study we have measured parameters \(a, b,\) and \(c\) to infer the magnitude of \(d\). We have used resident guinea pig peritoneal macrophages in a study of the antibody-dependent phagocytosis of single-shell lipid vesicles containing lipid hapten, and radiolabeled lipid, or ferritin (FT) trapped in the internal aqueous volume.
MATERIALS AND METHODS

**Materials**

Cell buffer was composed of 2.0 mM CaCl₂, 1.5 mM MgCl₂, 5.4 mM KCl, 1 mM Na₂HPO₄, 5.6 mM glucose, 120 mM NaCl, 0.2% bovine serum albumin (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.; fatty acid poor), and 25 mM HEPES at pH 7.4. Horse spleen FT was obtained from Sigma Chemical Co. (St. Louis, Mo.). Chitraldehyde, colidine buffer, and O₃O₄ were obtained from Polysciences, Inc. (Warrington, Pa.). Scanning electron microscopy (SEM) specimen stubs were obtained from Pelco, Inc. (Tustin, Calif.).

**Phospholipid Vesicles**

FT-containing, ~1-μm unilamellar vesicles were prepared by a modification of the ether injection method of Deamer and Bangham (5) as described previously (12). Vesicle lipid composition was 99 mol% dimyristoylphosphatidylcholine (DMPC) and 1 mol% dimyristoylphosphatidylglycerol (DMG) capped with 1 mol% radiolabeled phospholipid L-[1-¹⁴C]α-dipalmitoylphosphatidylethanolamine (55 μCi/mmol, Applied Science Labs, Inc., State College, Pa.) and 1 mol% of the fluorescent lipid N-4-nitrobenz-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE), prepared from egg lecithin (Avanti Biochemicals, Inc., Birmingham, Ala.) were included in the lipid mixture with a concomitant decrease in the mole fraction of 20,000. The cell radius (neglecting surface projections) was determined from the-vesicle uptake, control experiments were performed in the presence of radiolabeled phospholipid vesicles and the absence of anti-DNP antibody. After incubation with vesicles, an aliquot of 0.5 ml was then layered with a polypropylene pipette tip onto 0.5 ml of a 32% mixture of 10% Ficoll (Pharmacia, Uppsala, Sweden) in cell buffer and Ficoll-Hypaque (density = 1.077) in a 1.0-ml polystyrene centrifuge tube (Fisher Scientific Co., Pittsburgh, Pa.). The tube was capped and spun at 500 g for 20 min, frozen, and the lower tip was removed with a hack saw. The contents of the tip were digested overnight with 0.5 ml of NCS solubilizer (Amersham Corp., Arlington Heights, Ill.) and the radioactivity was determined by adding 10 ml of a toluene-based scintillation fluid (Prebland 2x70; RPI Corp., Elk Grove Village, Ill.) and counting in a liquid scintillation counter. The channel discriminating were set to exclude background light caused by chemiluminescence-phosphorescence, and the relative counting efficiency was determined later by adding an internal ¹⁴C standard.

**Phagocytes**

Macrophages were critical-point dried in C0₂. Cellswere dispersed on a stub (pore size 1.0 μm) affixed to one end (15). The cell pellets were resuspended in 0.1 ml of PBS and centrifuged at 800 g for 10 min. The lower portion of the centrifuge tube containing the cells was then removed. The macrophages were critical-point dried in C0₂. Cells were fixed in suspension with 1% glutaraldehyde in PBS for 1 h at room temperature. The macrophages were postfixed with 1% O₃O₄ in 0.1 M collidine buffer (pH 7.0) at room temperature for 1 h. The cells were dehydrated in acetone and embedded in Spurr’s resin (18). The embedded cells were thin-sectioned on an AMR ultramicrotome (AMR Corp., Bedford, Mass.) equipped with a diamond knife. The thin sections were examined in a Hitachi HU-11E electron microscope.

**Measurement of Surface Fold Length**

Macrophages were fixed with 1% glutaraldehyde in PBS for 1 h at room temperature. The macrophages were postfixed with 1% O₃O₄ in 0.1 M collidine buffer (pH 7.0) at room temperature for 1 h. The cells were dehydrated in acetone and embedded in Spurr’s resin (18). The embedded cells were sectioned on an AMR ultramicrotome (AMR Corp., Bedford, Mass.) equipped with a diamond knife. The thin sections were examined in the ISI-40 scanning electron microscope. Micrographs were taken with Polaron type 55 P/N film.

**Phagocytosis of Vesicles**

To measure vesicle uptake (binding followed by phagocytosis) by macrophages, we employed ¹⁴C-labeled lipids (see above for preparation). In all determinations of vesicle uptake, control experiments were performed in the presence of radiolabeled vesicles and the absence of anti-DNP antibody. After incubation with vesicles, an aliquot of 0.5 ml was then layered with a polypropylene pipette tip onto 0.5 ml of a 32% mixture of 10% Ficoll (Pharmacia, Uppsala, Sweden) in cell buffer and Ficoll-Hypaque (density = 1.077) in a 1.0-ml polystyrene centrifuge tube (Fisher Scientific Co., Pittsburgh, Pa.). The tube was capped and spun at 500 g for 20 min, frozen, and the lower tip was removed with a hack saw. The contents of the tip were digested overnight with 0.5 ml of NCS solubilizer (Amersham Corp., Arlington Heights, Ill.) and the radioactivity was determined by adding 10 ml of a toluene-based scintillation fluid (Prebland 2x70; RPI Corp., Elk Grove Village, Ill.) and counting in a liquid scintillation counter. The channel discriminating were set to exclude background light caused by chemiluminescence-phosphorescence, and the relative counting efficiency was determined later by adding an internal ¹⁴C standard.

**Cell Volume**

The intracellular volume accessible to water was determined using methods similar to those previously described (4). [¹³C]Polyethylene glycol 4000 (21.7 mCi/g) and H₂O (5 Ci/ml) were obtained from Amersham Corp. Incubation of macrophages with or without vesicles was carried out as described under Phagocytosis of Vesicles, except that all volumes and reagents were increased by a factor of 2.5. After a 30-min incubation with vesicles, 5 x 10⁶ macrophages were pelleted by centrifuging at 800 g for 10 min and resuspended to 140 μl in HBSS. 15 μl of a mixture of [¹³C]polyethylene glycol (50 μCi/ml) and H₂O (50 μCi/ml) was added and the suspension was incubated at 25°C for 30 min. The suspended cells were then layered over a 250-μl cushion of dibutyl phthalate and paraffin oil in a 400-μl polypropylene centrifuge tube and centrifuged at 1,000 g for 2 min. The lower portion of the centrifuge tubes containing the cells was cut off. The cell pellet was resuspended in 0.1 ml of H₂O and 0.5 ml of NCS solubilizer and digested overnight. ¹⁴C and ³H were determined as described under Phagocytosis of Vesicles in a dual-channel Beckman LS-230 counter.

**Transmission Electron Microscopy**

Macrophages were fixed in suspension with 1% glutaraldehyde in PBS for 1 h at room temperature. The macrophages were postfixed for 1 h in 1% O₃O₄ in collidine buffer at room temperature. The cells were dehydrated in an increasing series of acetone in water with three changes in 100% acetone. The cells were then placed in modified BEEM capsules (Better Equipment for Electron Microscopy, Inc., Bronx, N.Y.) with a Millipore filter (Millipore Corp., Bedford, Mass.; pore size 1.0 μm) affixed to one end (15). The macrophages were critical-point dried in CO₂. Cells were dispersed on a stub coat with double-sticky tape before sputter coating with gold. Specimens were examined in the ISI-40 scanning electron microscope. Micrographs were taken with Polaroid type 55 P/N film.

**Scanning Electron Microscopy**

To measure vesicle uptake (binding followed by phagocytosis) by macrophages, we employed ¹³C-labeled lipids (see above for preparation). In all determinations of vesicle uptake, control experiments were performed in the presence of radiolabeled vesicles and the absence of anti-DNP antibody. After incubation with vesicles, an aliquot of 0.5 ml was then layered with a polypropylene pipette tip onto 0.5 ml of a 32% mixture of 10% Ficoll (Pharmacia, Uppsala, Sweden) in cell buffer and Ficoll-Hypaque (density = 1.077) in a 1.0-ml polystyrene centrifuge tube (Fisher Scientific Co., Pittsburgh, Pa.). The tube was capped and spun at 500 g for 20 min, frozen, and the lower tip was removed with a hack saw. The contents of the tip were digested overnight with 0.5 ml of NCS solubilizer (Amersham Corp., Arlington Heights, Ill.) and the radioactivity was determined by adding 10 ml of a toluene-based scintillation fluid (Prebland 2x70; RPI Corp., Elk Grove Village, Ill.) and counting in a liquid scintillation counter. The channel discriminating were set to exclude background light caused by chemiluminescence-phosphorescence, and the relative counting efficiency was determined later by adding an internal ¹⁴C standard.

**Measurement of Surface Fold Length**

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**Results**

**Uptake of Vesicles**

Macrophages bearing Fc receptors bind specific antibody liganded phospholipid vesicles in a time-dependent fashion. The rate of binding is dependent on the surface density of bound antibody molecules and on the physical properties of the vesicle lipid bilayer (12). The time-course of binding a large excess of DMPC vesicles containing 1 mol% DNP-cap-PE at 37°C to guinea pig macrophages is shown in Fig. 1. After 30
cells observed are spherical in shape. The folds were similar in cell that was incubated at 37°C for 30 min is shown in Fig. 3 macrophages possess many folds and ridges. A representative using epifluorescence microscopy. the fluorescent vesicles was confirmed by direct observation antibody-dependent binding and subsequent phagocytosis of DNP antibody vs. 4% in its absence. Incubation on ice for 15 min resulted in insignificant cell-associated radioactivity. The resultswere obtained on four separate occasions, each consisting min, 16% of the vesicle lipid was bound in the presence of anti-DNP antibody vs. 4% in its absence. Incubation on ice for 15 min resulted in insignificant cell-associated radioactivity. The antibody-dependent binding and subsequent phagocytosis of the fluorescent vesicles was confirmed by direct observation using epifluorescence microscopy.

TEM of FT Vesicles

The uptake of FT vesicles was examined by thin-section TEM. A representative cell is shown in Fig. 2. FT-containing vesicular structures may be seen throughout the cytoplasm. Such structures have not been observed in untreated macrophages (data not shown). It is also known that FT is not present in peritoneal macrophages under normal conditions (9). We suggest that these structures are the internalized FT vesicles. The phagocytic vesicles may have fused with lysosomes.

Scanning Electron Microscopy

The surfaces of untreated resident guinea pig peritoneal macrophages possess many folds and ridges. A representative cell that was incubated at 37°C for 30 min is shown in Fig. 3 a. A few microvilli can be seen on occasional cells. Nearly all cells observed are spherical in shape. The folds were similar in width, although fold height appears to vary more in macrophages than in mast cells (3). Macrophages are heterogeneous with regard to both cell size (Table I) and fold length. To provide a significant amount of quantitative data in convenient form, we have employed the method of Burwen and Satir (3) to assess the ratio of surface fold length to surface area. In Fig. 4A we show a histogram of this ratio for untreated cells. These data represent a composite of three separate experiments. The mean fold length:area ratio of 1.1 μm⁻¹ is similar to that of 1.3 μm⁻¹ reported for rat peritoneal mast cells (3). In additional control experiments, we have shown that macrophages treated with antibody alone (Fig. 3 b) or FT vesicles alone (Fig. 3 c) are very similar to the untreated cells. Macrophages were exposed to either reagent for 30 min at 37°C.

A substantial reduction in the number of surface folds occurs when macrophages are incubated with FT vesicles in the presence of specific antihapten antibodies. We show a typical cell in Fig. 3 d. These cells were treated with antibodies and FT vesicles for 30 min at 37°C as described above. Identical results were obtained on four separate occasions, each consisting of pooled cells from several animals in each. In Fig. 4 B we show fold length:area ratio for this cell population, which is significantly less than that of control cells (Fig. 4 A). A break in the histogram of Fig. 4 B can be observed. However, the reason for this discontinuity is uncertain. It may, for example, represent subpopulations of macrophages that differ in surface properties after phagocytosis. Additional experiments (data not shown) have indicated that the change in surface folds is time-dependent.

In addition to data on surface folds, this technique also provides information with regard to cell size. The mean cell radii, neglecting surface projections, in control (absence of both antibody and vesicles) and phagocytosis experiments are 3.3 ± 0.06 and 3.8 ± 0.06 μm, respectively (see also Table I). The cell radius, as measured by SEM, increased ~15%.

Cell Volume

Because cell radius is an indirect measure of cell volume, we considered it advisable to determine cell volume by a more direct means. We have employed ³H₂O as a probe for total pellet volume and ¹¹Cpolyethylene glycol as a probe of extracellular volume to measure total cell volume. Macrophage volume was found to be 160 ± 50 μm³/cell and 250 ± 70 μm³/cell before and after phagocytosis, respectively (see Table I). Extracellular volume was somewhat greater after phagocytosis, suggesting a different packing behavior of the cells. These values are similar to those calculated from cell radii with the assumption that the cells are smooth spheres (see Table I).

Calculation of Macrophage and Vesicle Surface Areas

From the data obtained, one may directly calculate the influence of membrane folds upon macrophage surface area. The fold length:surface area ratio is first adjusted by a small, but significant, geometrical factor (1.16) that corrects for image foreshortening (up to 0.8 x) in the SEM micrographs. The only assumption is that the cells are randomly placed on the SEM stub. The area of the fold is then calculated as described (3) with fold height estimated as 0.6 μm, fold width is 0.1 μm, and the area of a smooth sphere of the cell radius is 140 μm². Fold width was estimated with TEM and SEM. Fold height was estimated from thin-section TEM micrographs by measuring the distance from cell body to end of the fold. This underestimates fold height and decreases slightly the magnitude of the differences we have observed. The surface area of a cell, including surface fold length and our estimates of height and width, is calculated to be ~400 μm² (values are summarized in Table I). Surface area after phagocytosis (fold height ~0.2 μm), including the increase in volume, is ~300 μm².

The surface area internalized may be calculated from the data given in Fig. 1. With the assumption of random vesicle uptake, we calculate an internalized surface area of ~400 μm²/cell. We have employed the value of 50 A²/lipid molecule and assumed 0.3 mol of exposed lipid/mole lipid in this calculation, as previously determined for similar vesicles (17). In experiments utilizing the fluorescent phospholipid NBD-PE and fluorescence microscopy as described (12), we estimate that at least one-half of the vesicles are internalized under the conditions employed for guinea pig macrophages. Previous workers have established that the surface area of internalized particles is very nearly equal to the surface area of plasmalemma

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**Figure 1** Rate of vesicle uptake by macrophages. DMPC vesicles contained 0.25 mol % radio labeled phospholipid, 1 mol % fluores- scent NBD-PE, and 1 mol % DNP-cap-PE hapten (○, with anti-DNP rabbit IgG; ●, without). Points show the mean of duplicate determinations; standard deviations are ±10% of the measured values.
FIGURE 2  TEM of an FT vesicle and specific antibody-treated resident guinea pig peritoneal macrophage. Macrophages were exposed to FT vesicles and antihapten antibody for 30 min at 37°C with agitation as described in Materials and Methods. A number of vesicular cytoplasmic structures that contain the FT label may be discerned. The vesicle membranes are not visualized because the saturated fatty acids employed in this study are not osmiophilic. This thin section has not been counterstained. x 17,000.

internalized. This has also been demonstrated (by M. McCloskey in this laboratory) for the antibody-dependent uptake of lipid vesicles with freeze-fracture techniques.

The above calculations show that the amount of surface area internalized is approximately equal to that originally present at the cell surface. Although the cell surface area decreases after phagocytosis (while the volume increases), this does not account for the amount of plasmalemma internalized.

DISCUSSION

Our studies have shown that there is a dramatic reduction of macrophage surface folds after antibody-dependent phagocytosis of lipid-hapten and ferritin-containing phospholipid vesicles. The disappearance of surface folds requires the presence of both specific antibody and FT vesicles because antibody or FT vesicles alone have no effect. The reduction in fold length
Figure 3  (a) SEM of a resident guinea pig peritoneal macrophage incubated at 37°C for 30 min. These cells possess many surface folds. (b) A typical SEM of a macrophage treated with anti-DNP antibody at 37°C for 30 min. (c) SEM of a macrophage exposed to FT vesicles at 37°C for 30 min. This cell population and that of b are indistinguishable from the cells of a. (d) In this SEM, macrophages were treated with both specific antibody and FT vesicles under identical conditions at 37°C for 30 min. A substantial reduction in the number of surface folds has taken place. All micrographs are x 7,200.
was concomitant with the appearance of FT vesicles in the cytoplasm and the binding of $^{14}$C-labeled phospholipid vesicles to macrophages. The data indicate that the internalization of plasma membrane during phagocytosis results in a decrease in surface folds.

In previous studies (14, 15) one of us has shown that the adsorptive pinocytosis of concanavalin A results in a global decrease in macrophage surface folds. Various workers (for a review see reference 22) have suggested that localized decreases in surface folds occur near the location of phagocytosis. However, the latter qualitative experiments are complicated by the facts that it is difficult to know a posteriori where the phagocytic event took place and that the responses are generally not high (for example, because of antibody-independent conditions). We have, therefore, employed the method of Burwen and Satir to measure the decrease of surface folds in a highly responsive antibody-dependent system. Our data (Table 1) show that a significant area of the cell surface membrane is removed.

TEM studies of the uptake of FT vesicles have shown clearly the presence of FT-containing vesicular structures within macrophages. These structures probably represent the internalized FT vesicles. The presence of these structures indicates that vesicle morphology is not altered dramatically by phagocytosis. The vesicle membrane was not visualized in the thin sections because the saturated fatty acid employed in these preparations is not osmiophilic. This method of visualizing vesicle phagocytosis might be useful in examining vesicle clearance in drug delivery systems.

From our binding and SEM experiments, we suggest that a membrane reservoir is made available to the plasmalemma during phagocytosis. This is based upon the following data: (a) cell surface area before phagocytosis is $400 \mu m^2$; (b) this value after phagocytosis is $300 \mu m^2$; and (c) the binding data indicate that $300 \mu m^2$ of vesicle surface area is internalized. (This value is analogous to results in experiments utilizing RAW264 macrophages where these cells took up an area equivalent to 100% of the cell surface area during phagocytosis [12].) Roughly $300 \mu m^2$ of surface area in addition to the $100 \mu m^2$ contributed by the decrease in membrane folds would need to be made available to the cell surface. We have not yet examined the possible sources of this area; however, the Golgi apparatus and the plasmalemma-like membrane which is recycled during pinocytosis (20) are likely candidates.

Previous studies of macrophage membrane flow have focused upon (a) surface area internalized during bulk phase pinocytosis in resident (20) and activated (7) macrophages; (b) the recycling of trypsin-α macroglobulin receptors after adsorptive pinocytosis (10, 19); and (c) the recycling of membrane components after antibody-independent phagocytosis of latex beads (13).

Antibody-dependent lipid vesicle phagocytosis has been examined in our laboratory (12, 16) and in others (11). In the presence of specific antibody, these vesicles activate the CN$^-$-insensitive respiratory burst of RAW264 macrophages (12) and guinea pig macrophages (unpublished observations). The dependence of this respiratory burst on vesicle lipid composition ("fluid" or "solid") is similar for both types of macrophages. The binding of vesicles to cells has been examined by fluorescence microscopy (12) and a fluorescence-activated cell sorter (11). A quantitative theory of Fc receptor motion and depletion after antibody-dependent phagocytosis of vesicles has been presented (12) and this theory has been supported by rosetting experiments (16). The experiments described herein provide additional important ultrastructural data relevant to the mechanism of Fc receptor depletion and membrane turnover.

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### Table 1

Properties of Macrophages

| Quantities                             | Control   | Phagocytosis |
|----------------------------------------|-----------|--------------|
| Measured                               |           |              |
| Cell radius ($\mu m^*$)                 | 3.3 ± 0.06| 3.8 ± 0.06   |
| Surface fold length/surface area ($\mu m^2$) | 1.1 ± 0.03| 0.53 ± 0.04  |
| Cell volume ($\mu m^3$/cell)           | 160 ± 50  | 250 ± 70     |
| Calculated                             |           |              |
| Cell volume ($\mu m^3$/cell)           | 150       | 230          |
| Surface area of smooth sphere ($\mu m^2$) | 140       | 180          |
| Surface area including folds ($\mu m^2$) | 410       | 300          |
| Approximate surface area of internal vesicles ($\mu m^2$)§ | —        | ~400         |

* Based on SEM data (mean ± SEM); n = 41 for each entry.
§ Based on $^3$H$_2$O tracer volume data (mean ± SD of duplicate determinations).
§ Based on binding data. See text for details.
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