Identification of Phagocytosis-associated Surface Proteins of Macrophages by Two-dimensional Gel Electrophoresis

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ABSTRACT Two-dimensional PAGE (P. Z. O'Farrell, H. M. Goodman, and P. H. O'Farrell. 1977. *Cell.* 12:1133–1142) has been employed to assess the effects of antibody-dependent phagocytosis on the cell surface protein composition of RAW264 macrophages. Unilamellar phospholipid vesicles containing 1% dinitrophenyl-aminocaproyl-phosphatidylethanolamine (DNP-cap-PE) were used as the target particle. Macrophages were exposed to anti-DNP antibody alone, vesicles alone, or vesicles in the presence of antibody for 1 h at 37°C. Cell surface proteins were then labeled by lactoperoxidase-catalyzed radioiodination at 4°C. After detergent solubilization, membrane proteins were analyzed by two-dimensional gel electrophoresis. The resulting pattern of spots was compared to that of standard proteins. We have identified several surface proteins, not apparently associated with the phagocytic process, which are present either in a multichain structure or in several discretely charged forms. After phagocytosis, we have observed the appearance of two proteins of 45 and 50 kdaltons in nonreducing gels. In addition, we have noted the disappearance of a 140-kdalton protein in gels run under reducing conditions. These alterations would not be detected in the conventional one-dimensional gel electrophoresis. This evidence shows that phagocytosis leads to a modification of cell surface protein composition. Our results support the concept of specific enrichment and depletion of membrane components during antibody-dependent phagocytosis.

Macrophages are involved in a wide variety of immunological functions such as antigen presentation, host defense against tumors, and resistance to certain pathogens (18). Many macrophage-mediated responses require the participation of certain cell surface components (35). The recognition and effector steps of antibody- or complement-dependent phagocytosis, clearance of antigen or lysosomal enzyme-α_2 macroglobulin complexes, cytolysis, and cell-cell collaboration are examples of specific responses involving macrophage surface molecules. Since cell surface proteins and glycoproteins have been shown to play an important role in many cellular phenomena (for examples see reference 6, 14), one might expect that cell membrane proteins are involved in macrophage function.

Many workers have analyzed the membrane proteins of macrophages (3, 10, 16, 17, 25, 34), neutrophils (1, 15, 27, 30, 32, 33) and eosinophils (2, 28) with one-dimensional SDS PAGE. Yin et al. (34) have demonstrated differences in the plasma membrane protein composition between resident and activated macrophages, Remold-O'Donnell (25) has examined trypsin-sensitive surface proteins, and Muller et al. (16, 17) have studied the effects of the antibody-independent phagocytosis of latex beads on the membrane proteins of murine macrophages. In addition, Watt and Burgess (31) have examined neutrophil proteins with two-dimensional electrophoresis.

In this study we have used high-resolution, two-dimensional gel electrophoresis (19) to identify ~40 proteins or glycoproteins in the plasma membranes of the macrophage cell line RAW264. Three of these proteins are associated with the specific antibody-dependent phagocytosis of lipid-hapten-containing lipid vesicles. Two proteins of 45,000 and 50,000 mol wt were shown to appear after antibody-dependent phagocytosis, while one protein of 140,000 mol wt was shown to disappear. We chose the RAW264 cell line because it is highly responsive in specific antibody phagocytosis of lipid-hapten-containing vesicles. This system is particularly well defined; previously we have studied the kinetics of this vesicle-macrophage binding and phagocytosis (13), the macrophage respiratory burst (7), Fc receptor depletion (22), macrophage surface internalization associated with specific antibody-dependent phagocytosis (23), and phagosome-lysosome fusion (H. R. Petty and H. M. McConnell, submitted for publication). For this study, the lipid vesicles containing lipid hapten in the presence of specific antibodies offer additional special advantages: unlike nonspecific phagocytosis of substances such as latex beads,

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the specific antibody-dependent uptake of vesicles results in the loss of some receptors (Fc) but not others (C3b) (22). Thus, changes in plasma membrane protein composition are likely to be simpler and more closely related to a single specific function.

MATERIALS AND METHODS

Cells

The RAW264 murine macrophage line was obtained from Dr. P. Ralph (Sloan-Kettering Institute for Cancer Research) (24). The cells were grown in RPMI 1640 medium with 10 mM HEPES buffer (GIBCO Laboratories, Grand Island Biological Co., Santa Clara, Calif.), 10% fetal calf serum, and 40 μg/ml gentamicin sulfate. The cells were adherent to polystyrene culture flasks (No. 25120, Corning Glass Works, Science Products Div., Corning, N. Y.) and were removed by incubation for 30–45 min with Earle's balanced salt solution without Mg^{2+} or Ca^{2+} (GIBCO Laboratories) containing 2 mM EDTA, followed by vigorous shaking. Cells were centrifuged at 200 *g* and resuspended in cell buffer (2.0 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5.4 mM KCl, 1 mM Na_2HPO_4, 5.6 mM glucose, 120 mM NaCl, 0.2% bovine serum albumin [Calbiochem-Behring Corp., American Hoescht Corp., La Jolla, Calif.; fatty acid poor], and 25 mM HEPES at pH 7.4).

Phospholipid Vesicles

Large unilamellar vesicles were prepared by a modification of the ether injection method of Deamer and Bangham (4) as described previously (22). The principal component of these vesicles is egg phosphatidylcholine or dimyristoylphosphatidylcholine (DMPC) (94–98 mol%). In addition, they contained 1 mol% dinitrophenyl-ϵ-aminocaproyl-phosphatidylethanolamine (DNP-cap-PE). In some experiments, vesicles contained 4 mol% phosphatidylserine and/or 1 mol% *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE) prepared from egg lecithin (Avanti Biochemicals, Inc., Birmingham, Ala.) to enable fluorescent visualization.

Antihapten Antibodies

Rabbit anti-DNP antiserum was a gift from Dr. A. Esser, (Scripps Clinic and Research Foundation). The anti-DNP IgG was fractionated from serum, using protein A coupled to cyanogen bromide-activated Sepharose (5). In all experiments, the antibodies were centrifuged at 110,000 *g* for 30 min in a Beckman airfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) to remove aggregates.

Phagocytosis of Vesicles

To 1.2 × 5.0-cm polypropylene vials (E & K Scientific Products, Inc., Saratoga, Calif.), vesicles (0.025 μmol total lipid), suspended in 0.01 M sodium phosphate and 0.15 M NaCl, pH 7.4 [PBS]), and antibody diluted into 500 μl of cell buffer were added and thoroughly mixed. 4×10^6 macrophages were added in cell buffer to a final volume of 1 ml. The mixtures were incubated at 37°C in a gyratory water-bath shaker (Model G76, New Brunswick Scientific Co., New Brunswick, N. J.). After 1 h, the vials were chilled in ice. These experimental conditions are very similar to those employed in previous work (7, 13, 22, 23; H. R. Petty and H. M. McConnell, submitted for publication).

Radioiodination and Solubilization of Intact Cells

Macrophages were washed with PBS five to six times by centrifugation at 4°C. Iodination, solubilization, and two-dimensional electrophoresis were carried out essentially as described by Ledbetter et al. (12) and Jones (8). Briefly, to 5–10 × 10^6 macrophages suspended in 1.0 ml of PBS were added 50 μg of lactoperoxidase (B grade; Calbiochem-Behring Corp., San Diego, Calif.), 1 mCi of ^{125}I (Amersham Corp., Arlington Heights, Ill.), and successive 10-μl pulses of H_2O_2 (0.3 mM, 1 mM, 3 mM, and 9 mM) at 10-min intervals. The iodination procedure was carried out at 4°C. Cells were washed three times with PBS and extracted in 200 μl of lysis buffer (0.5% Nonidet P-40 [NP-40; Particle Data, Inc., Elmhurst, Ill.]; 50 mM Tris, 50 mM NaCl; 0.02% NaN_3; 5 mM EDTA; 50 mM phenylmethyl sulfonyl fluoride; 0.2 TIU [trypsin-inhibitor unit]/ml Aprotinin, [Sigma Chemical Co., St. Louis, Mo.]; 1 μg/ml pepstatin A [Sigma Chemical Co.]; and 50 mM iodoacetamide, pH 8.0) for 30 min at 4°C Nuclei were removed by centrifugation at 7,000 *g* for 15 min. After solubilization in a urea-containing sample buffer, the extracts were analyzed by nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension followed by SDS polyacrylamide slab gel electrophoresis in the second dimension as described by Jones (8). The pH gradient in the first dimension established under these conditions (500 V for 6 h) has been shown to be linear (19). Radioautography was done with intensifying screens (Cronex lighting plus screens; E. I. DuPont de Nemours & Co., Wilmington, Del.) using Kodak X-Omat R film (Eastman Kodak Co., Rochester, N. Y.) at −70°C. Molecular weight markers (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) run on some gels were visualized by staining with Coomassie Brilliant Blue (Bio-Rad Laboratories, Richmond, Calif.). Apparent isoelectric points (pI) of membrane components were determined with reference to protein of known pI (20).

RESULTS

Two-dimensional gel electrophoresis of radioiodinated RAW264 surface proteins (before phagocytosis) results in the autoradiograph shown in Fig. 1 *A*. This pattern was quite reproducible from experiment to experiment, and in no case was actin (M_r = 43,500, pI = 6.1) detected. This strongly implies that the labeling was confined to the cell surface.

The proteins are shown schematically in Fig. 2, where the more heavily labeled proteins are shown as solid spots and the less labeled proteins as open spots. Since the degree of exposure of a given protein depends on both its surface density and labeling efficiency, no direct inference can be made about the relative amounts of various proteins on the surface.

Several large spots in the high molecular weight acidic region which appear to be one protein in Fig. 1 *A* are actually composites of several proteins which migrate close together. Thus, proteins *3* and *4*, *6–11*, and *16–18* in Fig. 2 each appear to be one large spot in Fig. 1 *A*. These large spots are resolved into their various components using lower acrylamide concentrations in the second-dimension gels (data not shown).

Some proteins appear to occur in several discretely charged forms (proteins *2*, *12*, *19*, and *24*). Thus, each of these proteins runs as a series of closely spaced spots spread out over roughly 1 pH unit in the first dimension, and all running at the same M_r in the second dimension. This distinct pattern is presumably due to varying amounts of terminal glycosylation with sialic acid or other post-translational processing involving charge modification. In addition, some proteins seem to exhibit a multichain structure as evidenced by their sensitivity to reducing conditions. Thus, the mobilities of proteins *12*, *15*, *19*, *23*, and *25* change dramatically when the proteins are run under reducing conditions (i.e., in the presence of 2-ME, data not shown). The properties of the multiply-charged and 2-ME-sensitive proteins are summarized Table I.

Representative autoradiographs of radioiodinated cell surface proteins before and after antibody-dependent phagocytosis are shown in Fig. 1 *A* and *B*, respectively. In addition, the following controls were performed: antibody plus cells, vesicles plus cells, and vesicles plus nonspecific antibody plus cells. In all cases, these were essentially indistinguishable from the blank (cells alone) (Fig. 1 *A*). These gels were NEPHGE in the first dimension followed by SDS PAGE (10% acrylamide) in the second dimension and were run under nonreducing conditions.

The antibody dilutions employed in this experiment was 1:5 and, based on previous work, corresponds to maximal Fc receptor depletion (80% inhibition of erythrocyte rosetting [22]). The broad area in the upper portion of Fig. 2 *B* is surface-bound antibody (denoted *AB*) which has not been internalized. The same broad area was found on gels of antibody alone (data not shown). The middle portion of the gel in Fig. 2 *B* shows two proteins which appeared after phagocytosis. The proteins are at M_r = 45 and apparent pI = 5.1, and M_r = 50

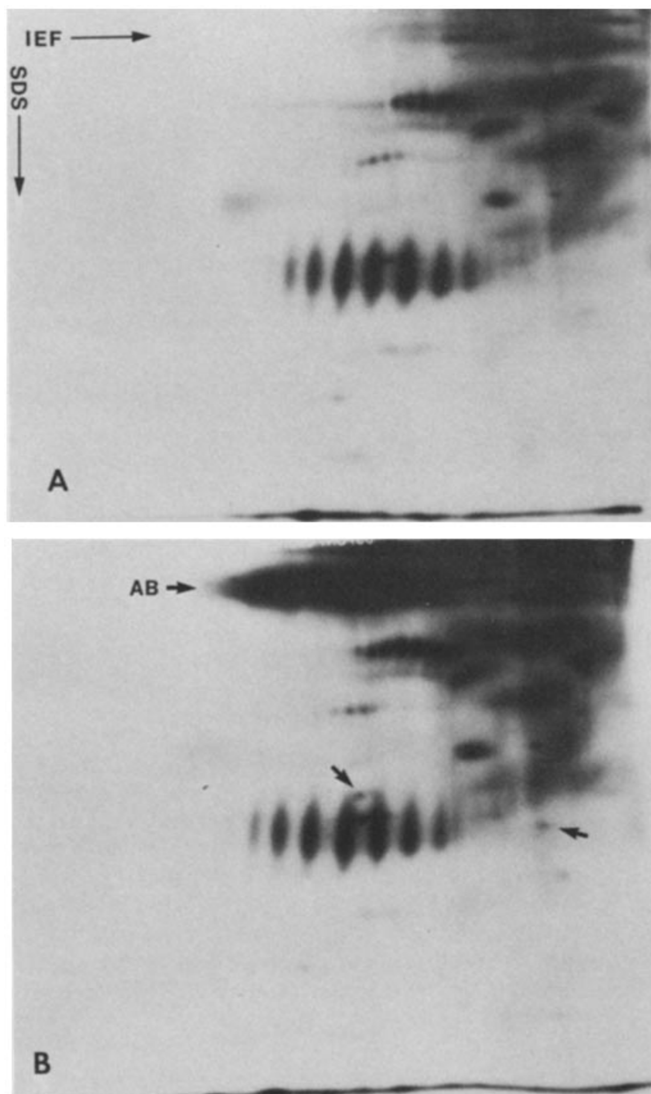

FIGURE 1 Two-dimensional gel electrophoresis of ^{125}I surface-labeled RAW264 macrophage proteins. 2×10^6 TCA-precipitable cpm were applied to each gel and the autoradiograms were exposed for 3 d. First-dimension gels were NEPHGE and second-dimension gels were 10% acrylamide, nonreducing conditions. (*A*) Macrophages before phagocytosis. (*B*) Macrophages after antibody-dependent phagocytosis. The unmarked areas in *B* denote the appearing spots. The arrow marked *AB* in *B* denotes the surface-bound antibody. Antibody dilution used was 1:5.

and apparent pI = 6.9. These proteins do not represent fragments of the antibody since: (*a*) the molecular weights and pIs do not correspond to those of the light chain of IgG; (*b*) the proteins run as very tight spots whereas the antibody is quite diffuse and covers a very large pH range, and (*c*) the more basic appearing spot can be detected, albeit somewhat more weakly, under limiting dose conditions (1:25 antibody dilution) where no surface antibody can be detected (data not shown). At the 1:25 antibody dilution, the depletion of Fc receptors (as measured by inhibition of rosetting) is very nearly the same as at the 1:5 antibody dilution (22). At higher antibody dilutions, the vesicle binding drops rapidly (22). Control experiments employing ^{125}I-labeled antibody and nonlabeled cells showed no detectable antibody fragments. Thus, gels run under conditions identical to those in Fig. 1 had only the major antibody band but no smaller units (data not shown). Fig. 3 is an enlarged view of the region around proteins *24* and *25* from an experiment similar to the one shown in Fig. 1 (NEPHGE, 10% polyacrylamide, nonreducing). Although the control gel, Fig. 3 *A*, is more heavily exposed, one can detect no protein which corresponds to the appearing spot denoted in Fig. 3 *B*.

In Fig. 4 we show the most acidic portions of two gels before and after phagocytosis (Fig. 4 *A* and *B*, respectively) which cover the entire molecular weight range in the second dimension. These gels were NEPHGE in the first dimension followed by 7.5% slab gels under reducing conditions in the second dimension. This acrylamide concentration allows for greater

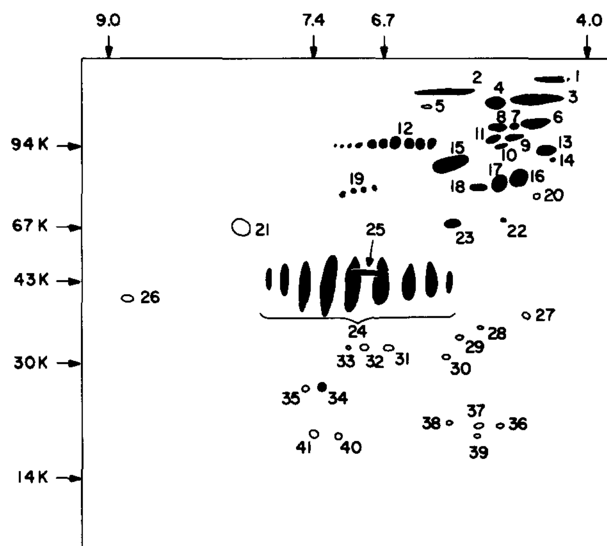

FIGURE 2 Schematic representation of the two-dimensional gel electrophoretic pattern of RAW264 macrophage membrane proteins before phagocytosis as shown in Fig. 1. Open spots represent lightly labeled protein; closed spots represent more heavily labeled protein. The numbering is from highest M_r, most acidic pI, to lowest M_r, most basic pI.

TABLE I

Properties of Selected Major ^{125}I-labeled RAW264 Macrophage Membrane Proteins *

Protein reference number‡	M_r§	pI(apparent)‖
	kdaltons	
Multicharged proteins¶		
2	131	5.5–6.1
12	97	5.9–6.6
19	76	6.5–6.8
24	35–50**	5.7–7.6
2-Mercaptoethanol sensitive proteins‡‡		
12	97	5.9–6.6
15	88	5.5
19	76	6.5–6.8
23	62	5.6
25	48	6.7

* Major proteins are defined as those which gave a dark spot on autoradiograms exposed under the conditions specified in Fig. 1.
‡ Protein reference number taken from Fig. 2.
§ Molecular weights were calculated with respect to the standards in Fig. 1.
‖ The nonequilibrium conditions of NEPHGE do not allow absolute measurement of pI. Apparent pI values were calculated by linear interpolation between known pI reference standards run in NEPHGE gels (18).
¶ These proteins run as several discretely charged components on the NEPHGE dimension.
** This protein runs as a broad component in the SDS PAGE dimension.
‡‡ The mobility of these proteins differs when the proteins run under reducing conditions as compared to nonreducing conditions. See text.

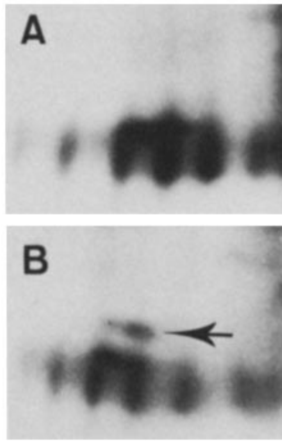

FIGURE 3 Two-dimensional gel electrophoresis pattern of I-125 surface-labeled RAW264 macrophages. The conditions used were 1 × 10^6 TCA-precipitable cpm/gel, exposed for 3 d. 10% gels, nonreducing. Only the pattern in the region of proteins 24 and 25 is shown. (*A*) Macrophages before phagocytosis. (*B*) After phagocytosis. The arrow in *B* denotes the appearing spot.

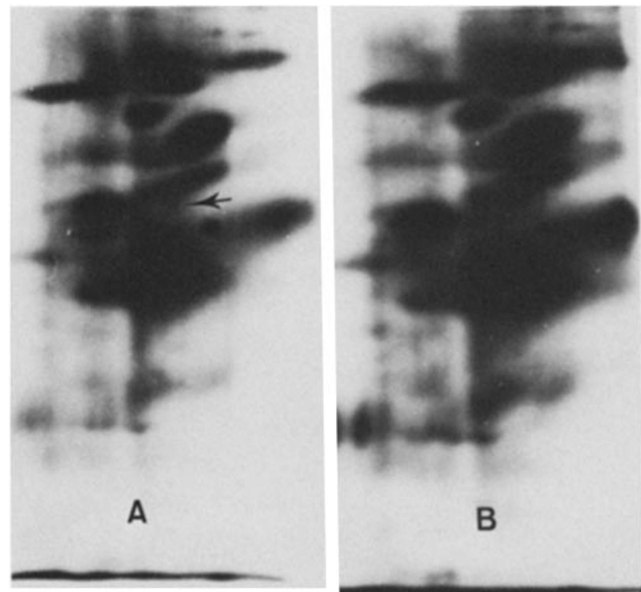

FIGURE 4 Two-dimensional gel electrophoresis of ^{125}I surface-labeled RAW 264 macrophages. Conditions were identical to those of Fig. 3, except that the second dimension was 7.5% acrylamide and run under reducing conditions. Only the acidic region (~pH 4–6) is shown. (*A*) Before phagocytosis. (*B*) After phagocytosis. The arrow in A denotes the disappearing protein. Antibody dilution was 1:25. Vesicle uptake is nearly the same as in Fig. 1 *B* (See reference 22).

resolution of the high molecular weight components. A spot of M_r = 140,000, apparent pI = 5.1 disappears after phagocytosis. It should be noted that at these dose conditions the antibody cannot be detected in this experiment (one would expect to observe the heavy and light chains, as we have in gels of antibody alone under reducing conditions). This protein may be associated with the membrane internalized during antibody-dependent phagocytosis.

DISCUSSION

The macrophage cell surface plays a major role in the recognition and destruction of foreign material. One well-characterized effector function of macrophages is antibody-dependent phagocytosis. Crucial to the understanding of the initial events involved in particle recognition and phagocytosis is a knowledge of the macrophage surface protein composition, since it is likely that several of the surface proteins participate in the processes. The murine macrophage tumor line RAW264 is a convenient and well-characterized model system for the study of antibody-dependent phagocytosis (22). Using two-dimensional PAGE, we have identified ~40 proteins on the RAW264 cell surface which can be labeled by lactoperoxidase-catalyzed radioiodination. Although this number is larger than that found by other workers employing one-dimensional gels, this is presumably due to the enhanced resolution of two-dimensional gels. The absence of any detectable actin (which comprises ~10% of total cell protein [26]) indicates that the labeling was confined to the cell surface.

Twenty of the labeled cell surface proteins were major iodinated membrane proteins. Of these proteins, at least four appear to occur in several discretely charged forms on the cell surface. This suggests that these proteins are glycoproteins which differ in the degree of terminal glycosylation, as is the case for several well-characterized cell surface proteins of the lymphoid cell lineage (11). In addition, the mobility of at least five proteins was sensitive to the reducing agent 2-mercaptoethanol. Although it was not possible in every case to identfy the new position of the protein after reduction, the differences in the pattern suggest a multichain disulfide bond linked structure for some of the membrane proteins.

Previous studies characterizing macrophage membrane proteins have identified from 10–20 surface proteins (16, 25, 34). Direct comparison of our results with previous work is not possible due to differences in cell type and gel systems. Nonetheless, it is possible to identify similarities in molecular weight between some RAW264 surface proteins and those of normal macrophages (i.e., proteins *1*, *2*, *6–10* with bands 3, 4, and 7 from reference 34).

A critical question with regard to the mechanism of phagocytosis is the enumeration of specific composition alterations of the cell surface that are related to phagocytosis. In addition, it is important to ascertain whether the region of the plasmalemma that takes part in phagocytosis is enriched or depleted in certain components relative to the remaining bulk plasma membrane. The evidence presented in this study bears on the first of these questions. The second question is a particularly difficult one, and several approaches have been employed by different laboratories (16, 29). A quantitative approach to this problem should include, in our opinion, a knowledge of: (*a*) membrane area internalized along with that of the surface before and after phagocytosis—which would include an estimate of cell surface area which originates from an intracellular reservoir(s) during phagocytosis (the "source" and "sink" terms) (23); (*b*) alterations of steady state membrane turnover via pinocytosis, during phagocytosis; (*c*) the functional alterations of the cell surface, i.e., rosetting behavior of surface receptors (22), transport sites (29), and enzymatic activities; (*d*) the lateral distribution and motion of surface receptors (21); and (*e*) the structural alterations of the cell surface proteins and lipids during phagocytosis.

Here we have shown that several cell surface proteins are closely associated with phagocytosis. We have shown that (*a*) at least one protein of ~140,000 daltons disappears after phagocytosis, and (*b*) two proteins of 45,000 and 50,000 daltons appear after phagocytosis. These differences probably represent

alterations in the protein or glycoprotein composition of the cell surface. We cannot exclude the possibility that phagocytosis induced conformational changes of surface proteins, making them more or less accessible to iodination with lactoperoxidase, although this would also apply to many similar experiments of other workers. It is possible that the appearing 45,000- and/or 50,000-dalton protein is a fragment of the 140,000 disappearing protein. The simplest interpretation of our results is that the 140,000 protein is associated with (or identical to) the Fc receptor which disappears under identical conditions (22). This hypothesis is in agreement with certain studies by others (9), although there is considerable disagreement regarding the molecular weight of the Fc receptor (9). In addition, the 45,000- and 50,000-dalton proteins could be associated with the previously suggested membrane reservoir which is made available to the cell surface during phagocytosis (23). In view of the striking morphological (23) and functional (22) alterations of the plasma membrane, it is remarkable that so few changes in protein composition were detected. Additional alterations of the plasma membrane may have taken place but have gone unnoticed in this study because of the absence of iodination sites on some membrane proteins. Studies using metabolic labeling with amino acids and simple sugars followed by plasma membrane isolation might assist in detecting other phagocytosis-associated proteins.

We have made no attempt here to obtain a quantitative relation between antibody concentration and surface alterations. This is because, at antibody concentrations where vesicle phagocytosis is extensive, the quantitative uptake of vesicles (as well as Fc receptor-mediated rosetting) is independent of antibody concentration over a large range of antibody concentrations (13, 22). At lower antibody concentrations, vesicle uptake as well as loss of Fc receptor-mediated rosetting decreases rapidly with decreasing antibody concentration over a relatively narrow concentration range (13, 22). The qualitative data presented in Figs. 1 *B* and 4 *B* were obtained at two antibody concentrations where vesicle uptake as well as apparent loss of Fc receptors do not depend strongly on antibody concentration: (*a*) at the 1:5 antibody dilution, antibody is seen in the autoradiograms, and (*b*) at the 1:25 antibody dilution, antibody is not seen in the autoradiograms but vesicle uptake and loss of Fc receptors are similar if not identical (13, 22). At higher antibody dilutions (e.g., 1:50–1:200), there is a strong dependence of vesicle binding (and loss of Fc receptors) on antibody concentration (22). In principle, a quantitative study of the modulation of plasma membrane composition associated with vesicle phagocytosis might be possible at the higher antibody dilutions, but a partial or even extensive purification of membrane components could be a prerequisite for such a study.

Muller et al. (16–17) have concluded that the membrane proteins of the phagosome and plasma membrane are very similar or identical, following the antibody- and complement-independent phagocytosis of latex beads. We find few qualitative changes in plasma membrane composition following phagocytosis. However, some important differences have been observed here. The reason for the apparent discrepancy between the two studies is not known. We feel that there are two likely sources: (*a*) our study was antibody-dependent, involving a specific ligand-receptor interaction that specifically depletes the Fc receptor, and (*b*) the greater resolution of two-dimensional electrophoresis allows the identification of more proteins (proteins might overlap in molecular weight are often separated in the pH gradient dimension). Our results are in general agreement with those of Willinger et al. (32, 33) who studied polymorphonuclear leukocyte phagocytosis, and Thorne et al. who examined eosinophil adherence to an antibody-coated surface (28) and polymorphonuclear leukocyte phagocytosis (27). Following eosinophil adherence to an antibody-coated surface, newly accessible proteins of 55,000 and 58,000 daltons appear in one-dimensional gels. The antibody-dependent-appearing proteins may be analogous to the 45,000- and 50,000-dalton proteins we have noted. Willinger et al. (33) have also noted cell surface changes following uptake of C3-opsonized particles. Their observations are in qualitative but not quantitative agreement with ours; the differences may be due to the different mechanisms of opsonization or the different cell types employed.

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