Membrane Proteins of the Vacuolar System. III. Further Studies on the Composition and Recycling of Endocytic Vacuole Membrane in Cultured Macrophages

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ABSTRACT In previous publications (Muller, W. A., R. M. Steinman, Z. A. Cohn, 1980, *J. Cell Biol.* 86:292–314), we found that the membrane of macrophage phagolysosomes could be selectively radioiodinated in living cells. The technique required phagocytosis of lactoperoxidase covalently coupled to latex spheres (LPO-latex), followed by iodination on ice with $Na^{125}I$ and hydrogen peroxide. In this paper, we use the LPO-latex system to further analyze the composition and recycling of phagocytic vacuole membrane.

Three approaches were employed to examine the polypeptide composition of the phagolysosome (PL) and plasma membranes (PM). (*a*) The efficiency of intracellular iodination was increased by increasing lysosomal pH with chloroquine. By one-dimensional SDS PAGE, the heavily labeled chloroquine-treated PL exhibited the same labeled polypeptides as PM iodinated extracellularly with LPO-latex. (*b*) Iodinated PL and PM were compared by two-dimensional gel electrophoresis. No differences in the isoelectric point and molecular weight of the major iodinated species were detected. (*c*) Quantitative immune precipitation was performed with five specific antibodies directed against cell surface antigens. Four antibodies precipitated similar relative amounts of labeled antigen on the cell surface and endocytic vacuole. One antibody, secreted by hybridoma 2.6, detected a 21-kdalton polypeptide that was enriched sevenfold in PL membrane. This enrichment was cell surface-derived, since the amount of labeled 2.6 was increased sevenfold when iodinated PM was driven into the cell during latex uptake. Therefore, intracellular iodination primarily detects PL proteins that are identical to their PM counterparts.

Additional studies employed electron microscope autoradiography to monitor the centrifugal flow of radiolabeled polypeptides from PL to PM. Cells were iodinated intralysosomally and returned to culture for only 5–10 min at 37°C. Most of the cell-associated label then redistributed to the cell surface or its adjacent area. Significant movement out of the lysosome compartment occurred even at 2°C and 22°C. Extensive and rapid membrane flow through the secondary lysosome presumably contributes to the great similarity between PM and PL membrane polypeptides.

We have previously reported on the selective labeling of the luminal surface of both pinocytic and phagocytic vacuole limiting membranes (1, 2). Employing internalized lactoperoxidase (LPO) in either a soluble (1) or particulate form (2), we carried out radioiodination at low temperature, to minimize membrane movement, and in the presence of a hydrogen peroxide-generating system. It was of interest that labeled polypeptides of the exterior surface of the plasma membrane (PM) and those of the organelles of the vacuolar apparatus were similar on one-dimensional SDS PAGE analysis. In addition, it was shown that all the labeled membrane polypeptides of the latex phagolysosome (PL) compartment were flowing centrifugally and could be captured at the plasma membrane (3). Therefore, an extensive bidirectional flow of membrane polypeptides was occurring between intracellular vacuoles and the cell surface, with pinocytic vesicles the quantal carriers.

Here we extend these studies by (*a*) enhancing the efficiency

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of intracellular iodination, (*b*) examining the polypeptide composition of PM and PL by additional techniques, and (*c*) arriving at a more accurate estimate of the rapidity of polypeptide flow by electron microscope (EM) autoradiography (ARG).

MATERIALS AND METHODS

Materials: Most materials were described previously (2, 3). New reagents were: chloroquine (Sigma Chemical Co., St. Louis, MO), protein A Sepharose ampholines and cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ), Nonidet P-40 detergent (Bethesda Research Laboratories, Bethesda, MD). The composition of the principal buffers was:

ISOELECTRIC FOCUSING SAMPLE BUFFER = 9.5 M urea, 8% (wt/vol) NP-40, 5% (vol/vol) β-mercaptoethanol, 2% (vol/vol) ampholines (pH 3.5–10 range).

LYSIS BUFFER = 0.05% (wt/vol) NP-40, 1% (vol/vol) Trasylol, 2 mM phenylmethylsulfonyl fluoride in phosphate-buffered normal saline (pH 7.2).

NEVILLE GEL BUFFER = 2% (wt/vol) SDS, 5% (vol/vol) β-mercaptoethanol, 12% (wt/vol) sucrose, 0.01% (wt/vol) bromphenol blue in 50 mM carbonate buffer, pH 8.6.

Previously described methods: The following procedures were described elsewhere (2, 3): culture of mouse peritoneal macrophage (Mϕ) monolayers; covalent coupling of lactoperoxidase (LPO) to 0.8-μm latex spheres; delivery of LPO-latex to Mϕ phagolysosomes (PL); iodination of PL within living Mϕ following addition of $Na^{125}I$ and glucose-glucose oxidase at 4°C; isolation of PL from cell homogenates on discontinuous sucrose density gradients; autoradiography of cell monolayers at light and EM levels; quantitation of TCA-precipitable radioactivity in cell lysates and one-dimensional SDS PAGE.

Iodination of Plasma Membrane (PM) Using LPO-Latex: To efficiently iodinate the PM, a 1:800 dilution of LPO-latex stock in PBS was centrifuged on to Mϕ monolayers. The cultures were then maintained on ice to inhibit phagocytosis. Most of the beads were cell-associated, because our monolayers were dense (2×10^7 cells/35 mm dish, or 3×10^6 cell/16 mm well). In this paper, we always used LPO-latex to label the PM. In control experiments we showed that cells iodinated by LPO-latex had a similar pattern of radiolabeled proteins as Mϕ iodinated with soluble LPO in suspension (2). No labeled proteins corresponding to serum components could be identified by one-dimensional SDS PAGE.

Two-dimensional Gel Electrophoresis: 2×10^7 peritoneal cells were plated onto 35-mm dishes and cultured for 2 d. 0.4 ml of LPO latex was centrifuged onto the Mϕ monolayers in PBS. One dish was returned to culture for 30 min to allow ingestion of the beads (PL sample) while the other was kept on ice to prevent phagocytosis (PM sample). The cultures were iodinated with 100 μCi/ml $Na^{125}I$ (PM) or 1,000 μCi/ml (PL), and 0.24 mM/ml glucose oxidase–20 mM glucose. Smaller doses of radiolabel were needed for the PM sample because it was iodinated so much more efficiently. After iodination, the cultures were lysed in 1 ml of 0.05% NP-40 and pelleted to remove latex and nuclei. The supernatant was mixed with isoelectric focusing (IEF) sample buffer in a 1:2 ratio by volume, and 15 μl was pipetted into the sample well of the IEF gel. Electrophoresis was done according to Ames and Nikaido (4) as modified by Piperno et al. (5). IEF was performed at 300 V for 15½ h followed by 400 V for 2 h. Samples were run from cathode to anode. A 6-mm strip was cut from the center of each IEF lane for SDS PAGE in the second dimension, using a 4–11% acrylamide gradient run at 37.5 mA for 6–7 h. The two-dimensional gel was processed for autoradiography as described previously (2).

Quantitative Immune Precipitation: A group of five antibodies (Ab), four of them rat anti-mouse monoclonals, were used to precipitate specific membrane proteins from cell lysates subject to PL or PM iodination. The five Ab have been described elsewhere and are summarized in Table I. All of the antigens: (*a*) are radiolabeled when Mϕ are cultured in the presence of [^{35}S]methionine, (*b*) are present in large amounts on the PM with more than 10^5 antibody binding sites per cell, and (*c*) are accessible to LPO-mediated iodination.

For immunoprecipitation, the iodinated monolayers were dissolved in 0.2–0.5 ml of lysis buffer, centrifuged at 15,000 *g* for 10 min at 4°C, and the supernatant was preadsorbed against 50 μl of protein A Sepharose at 4°C for 30 min in a 1.5-ml microfuge tube with constant shaking. The Sepharose was pelleted by centrifugation and the supernatant used to form immune complexes. For each 200 μl of supernatant, we used 1 μg of monoclonal antibody or 1 μg of rabbit anti-FcR antiserum on ice for 30 min. The immune complexes were retrieved with 25 μl of Sepharose coupled with Fab_2' rabbit anti-rat Ig (for the monoclonal Ab) or protein-A Sepharose (for the rabbit anti-FcR complexes) with constant shaking for 1 h at 4°C. Alternatively, monoclonal Ab were themselves coupled to Sepharose 4 B and the radiolabeled antigens were retrieved directly. Sepharose beads bearing labeled antigen were centrifuged and washed by the procedure of Kaplan et al. (10) and boiled in Neville gel buffer (2% SDS, 5% β-mercaptoethanol, pH 8.6) for 3 min before analysis by SDS PAGE. To determine the amount of radioactivity in a particular polypeptide, a well-exposed autoradiogram was aligned over the original dried gel. The band was traced from the film onto the gel via an intervening piece of carbon paper. The traced area was cut from the gel and counted in a gamma scintillation spectrometer. Similar-sized pieces of the gel were used to determine background radioactivity. This immunoprecipitation procedure was quantitative in that duplicate samples gave similar results, and no additional radioactivity could be precipitated in a second sequential immunoprecipitation.

Analysis of EM autoradiograms: The probability circle method, with appropriate corrections for "cross-fire", was used to localize the source of radiolabel corresponding to particular silver grains. This technique was described previously (3) and is outlined in Results (Table IV). Cell profiles were selected at random for analysis, provided they contained at least one exposed silver grain, part of the nucleus, and at least one latex bead.

Our autoradiograms contained substantial amounts (~10% of total grains) of nuclear labeling. Nuclear grains likely represented background due to unincorporated ^{125}I that was not removed by our washing procedure. There is no obvious way whereby LPO, covalently coupled to latex, could iodinate the nucleus; and we have previously shown that all proteins labeled by LPO-latex are indistinguishable from PM proteins (2). Background labeling on other cellular compartments, that corresponded to nuclear labeling, was calculated by the formula:

$$\text{Background grains} = \frac{\text{total area of compartment}}{\text{total area of nucleus}} \times \text{grains on nucleus.}$$

RESULTS

Modification of Intralysosomal Iodination by Chloroquine

Cultured mouse peritoneal macrophages (Mϕ) phagocytose latex spheres to which lactoperoxidase (LPO) has been covalently attached. If the latex beads are centrifuged onto the Mϕ monolayer in the cold, and if phagocytosis is allowed to take place for 20 min at 37°C, all intracellular latex is sequestered in acid phosphatase-positive vacuoles. Most cells internalize 10–20 spheres, which can then mediate the selective iodination of phagolysosome (PL) membrane, as previously described (2).

A limitation to the use of LPO-latex for lysosomal labeling is that iodinating activity decreases when the pH falls below 6. Those LPO-latex containing PL which had the lowest pH would therefore be iodinated less effectively. This might prevent the detection of polypeptide differences between PM and PL membranes. We therefore carried out experiments in which the lysosomal pH was raised with 0.1 mM chloroquine, administered during phagocytosis and iodination. Ohkuma and Poole (11) have shown that this concentration of drug raises the pH of Mϕ lysosomes from 4.7 to 6.3 within 2 min.

As anticipated, chloroquine-treated Mϕ containing intracellular LPO-latex incorporated more TCA-precipitable radioactivity than nonchloroquine-treated controls (Table II). Mϕ

TABLE I

Antibodies to Mouse Macrophage Plasma Membrane Antigens

Antibody			Antigen-Molecular Weight	References
Rat anti-mouse hybridoma,		1.21J	"Mac-1;" 180,000 and 95,000	1, 6
"	"	F4/80	Macrophage restricted; 165,000	1, 6, 7
"	"	I 3/2	Leukocyte common; 200,000	9
"	"	2.6	Macrophages and other cells; 21,000	1, 6
Rabbit α-mouse FcR			Fc receptor; 60,000	8

TABLE II

Effect of Chloroquine on Intracellular Iodination by LPO-Latex (TCA-precipitable cpm/culture)

No Chloroquine	Chloroquine during phagocytosis & iodination	Chloroquine during phagocytosis only	Chloroquine during iodination only
35,558	126,330	84,240	34,230
69,278	373,000	NT	NT
30,756	361,600	108,126	57,087
25,283	79,840	33,778	34,500

100 μM chloroquine was added to macrophage cultures during (20 min, 37°C) phagocytosis and/or during iodination (10 min, 4°C). Four experiments are shown in which different amounts of cells and ^{125}I were used. *NT*, not tested.

remained more than 99% viable (trypan blue negative), and by light microscope autoradiography the label was cell-associated. Quantitation of the autoradiograms was performed by counting the number of cells with heavily labeled PL ("hot spots," see Fig. 4, reference 2). Under our experimental conditions, 66% of the chloroquine-treated cells vs. 6% of control cells contained heavily labeled PL.

One-dimensional SDS PAGE (Fig. 1) was used to analyze the iodinated proteins of PL in the presence or absence of chloroquine. PL proteins were compared to PM polypeptides labeled with LPO-latex spun onto the Mϕ surface at 4°C. For all conditions, the labeled polypeptides were identical in molecular weight and labeling intensity (Fig. 1). Therefore one-dimensional SDS PAGE detects no difference between PM and PL proteins accessible to LPO-latex, even when PL are iodinated at elevated intravacuolar pH.

Two-dimensional Gel Electrophoresis of Labeled PM and PL Polypeptides

Two-dimensional gel electrophoresis was next used to compare PM and PL membranes following iodination with LPO-latex. Under our experimental conditions, all of the radiolabel entered the gel. At least 12 species were identified, each with a broad pI range characteristic of membrane proteins (Fig. 2). The molecular weight and pI were similar for all components in iodinated PL and PM (Fig. 2). Several different pH ranges (3.6–6.6; 3.8–5.0; 4.1–5.7; 4.2–8.3; 4.6–8.5) were employed for the first, isoelectric focusing dimension, with similar results (not shown).

Membrane Analysis by Immune Precipitation

The third approach compared PL and PM by immunoprecipitation with five specific antibodies. This approach allowed us to detect and quantitate iodinated polypeptides independent of their extent of radiolabeling. The antigens were "mac-1," F4/80, FcR or the trypsin-resistant Fc receptor, T200 or the leukocyte common antigen, and 2.6, each of which has been defined in previous publications (Table I). Autoradiograms of the proteins retrieved by immunoprecipitation showed that the relative amount of label in four of the five antigens was similar in PL and PM (Fig. 3). The exception was a 21-kdalton species identified by monoclonal 2.6, which was more heavily labeled in the PL membrane (Fig. 3).

With the autoradiograms as a template, the portions of the gel containing the specific antigen were excised and counted. Radioactivity was normalized to the most heavily labeled species, the 180 and 95dalton complex termed "mac-1" (Table III). In three experiments, the amount of radioactivity in F4/80, FcR and T200 proteins—relative to "mac-1"—was reduced in PL relative to PM (e.g., Table III *A*). In contrast, seven times more label was introduced into the 2.6 polypeptide following intralysosomal iodination (Table III*A*).

It was possible that the enrichment in labeled 2.6 occurred at the level of the PM, rather than the PL itself. To test this, we spun LPO-latex onto duplicate Mϕ cultures and used it to iodinate the PM. One of the cultures was lysed immediately to provide a PM sample, whereas the other was warmed to 37°C for 10 min to allow phagocytosis. The PL were purified on sucrose density columns, as described (2), and the PL membrane antigens were immunoprecipitated in parallel with the PM sample. Again, the radiolabel precipitated by 2.6 was seven times more abundant in the PL than in the PM sample (Table III *B*). Because the PM was radioiodinated initially in both cultures, enrichment of the 2.6 protein must occur at the level of the PM either during or shortly after phagocytosis (see Discussion).

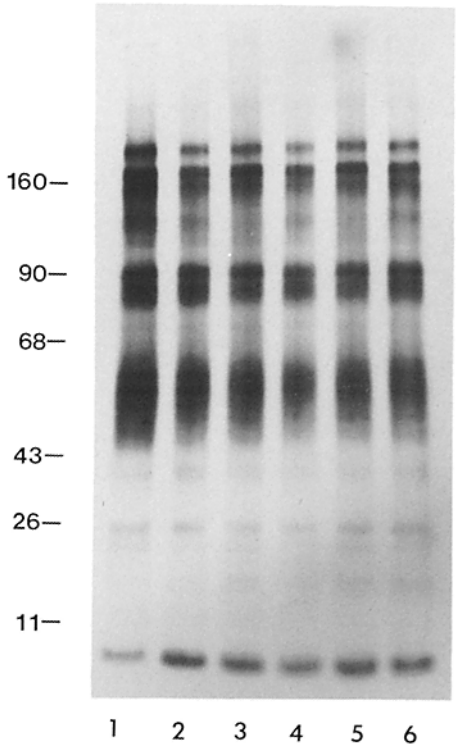

FIGURE 1 Polypeptides iodinated by LPO-latex in chloroquine-treated phagolysosomes. Macrophages were allowed to ingest LPO-latex in the presence or absence of 100 μM chloroquine, and then the monolayers were iodinated in the presence or absence of chloroquine. ~40,000 TCA-precipitable cpm from each sample were run on 4–11% SDS PAGE (same specimens as Experiment 4, Table II). 3-d exposure on Cronex film. Lane *1* is a plasma membrane sample iodinated extracellularly with LPO-latex, whereas lanes *2–6* were iodinated intracellularly with phagocytosed LPO-latex. (Lane *2*) Usual iodination procedure in the absence of chloroquine. (Lane *3*) Chloroquine present during phagocytosis and iodination steps. (Lane *4*) Chloroquine present during phagocytosis only. (Lane *5*) Chloroquine present during iodination only. (Lane *6*) A sucrose control to test the effect of lysosome swelling since chloroquine causes lysosomes to swell. Cells were incubated with 100 mM sucrose for 2 h before uptake with LPO-latex. This caused swelling of lysosomes including those containing LPO-latex; however, sucrose does not raise lysosomal pH (21) and did not change the efficiency of iodination. The numbers on the left are molecular weight standards in kilodaltons run on adjacent lanes.

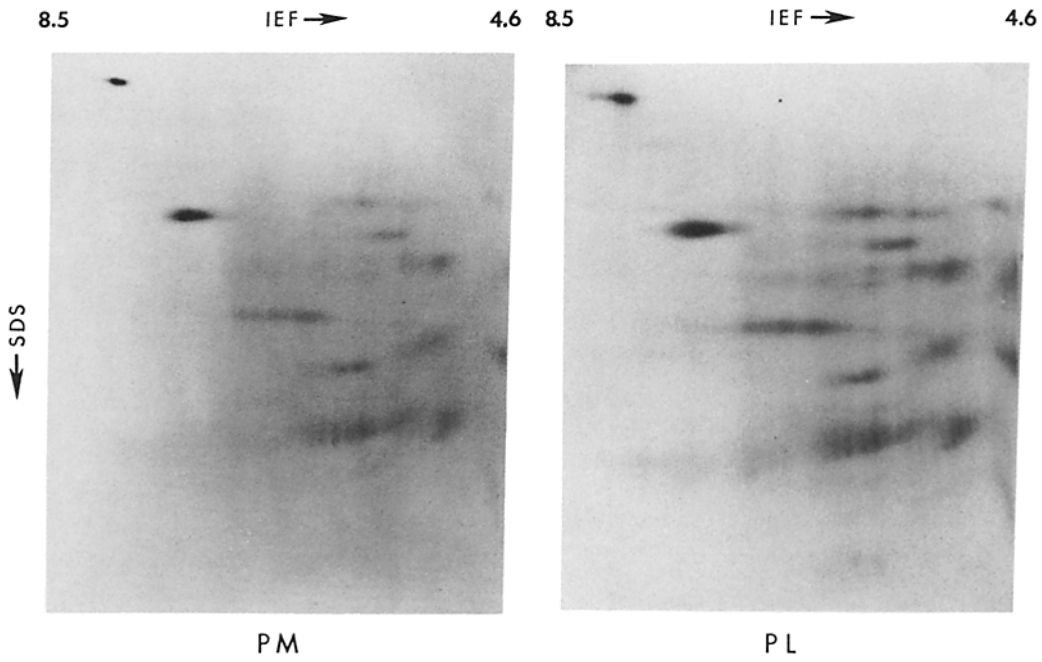

FIGURE 2 Comparison of iodinated plasma membrane (*PM*) and phagolysosome membrane (*PL*) by two-dimensional gel electrophoresis. In the first *IEF* dimension, the samples were run from cathode to anode over a pH gradient of 8.5 to 4.6. The second dimension was a 4–11% SDS polyacrylamide gel. The dried gels were exposed to Kodak XR-1 Film for 1 wk (*PM*) or 1 month (*PL*).

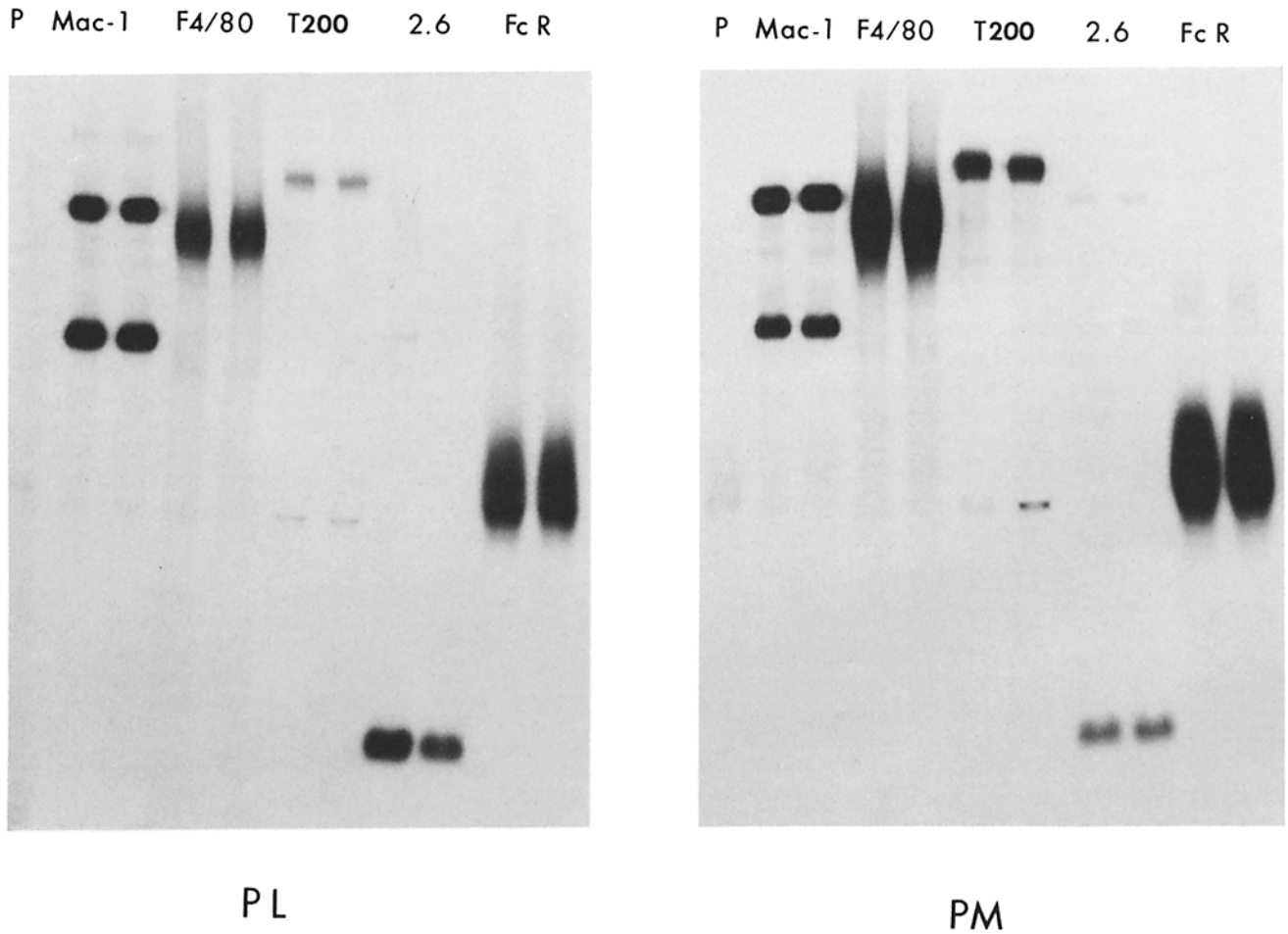

FIGURE 3 Immune precipitation of iodinated membrane proteins from phagolysosomes (*PL*) and plasma membrane (*PM*). Radioactive lysates were preadsorbed with preimmune rabbit serum and protein A-Sepharose (Lane *P*). The lysate was split and duplicate samples were serially precipitated with antibodies to *Mac-1*, *F4/80*, *T200*, 2.6, and Fc receptor (*FcR*) antigens. The precipitates were run on 4–11% SDS PAGE and exposed to Kodak XR-1. A nonspecific background band at 50-kdaltons appears inconsistently in some lanes. This figure shows that, under conditions in which most *PM* polypeptides are more heavily radiolabeled than their *PL* counterparts, the 2.6 protein at 21-kdaltons is more heavily radiolabeled in the *PL* sample.

TABLE III

Distribution of Specific Proteins in Phagolysosome and Plasma Membranes: Immunoprecipitation Analysis

A. *Separate iodination of phagolysosome and plasma membranes*

	Amount of ^{125}I-polypeptide retrieved by immunoprecipitation, relative to amount of labeled Mac-1				
	Mac-1	F4/80	FcR	T200	2.6
Plasma membrane	100	75	22	12	1
Phagolysosome	100	48	16	5	7.5

B. *Cell surface iodination, then phagocytosis and isolation of phagolysosomes*

	Amount of ^{125}I-polypeptide retrieved by immunoprecipitation, relative to amount of labeled Mac-1				
	Mac-1	F4/80	FcR	T200	2.6
Plasma membrane	100	40	27	ND	0.3
Phagolysosome	100	14	16	ND	2

Labeled phagolysosome membrane was obtained in two ways: by iodination intralysosomally (part A) or by PM iodination followed by phagocytosis and purification of the latex PL fraction (part B). In each case, the PL membrane was lysed and compared to iodinated PM using immunoprecipitation with five specific antibodies. The labeled antigens were isolated on SDS PAGE, and appropriate portions of the gel were excised and counted. Duplicate samples were always within 5% of one another. The amount of label is expressed relative to the amount of radioactivity recovered in the Mac-1 polypeptides. *ND*, not determined.

To show that the 2.6 antigen was located on an integral membrane protein, we immunoprecipitated cells that had been iodinated extracellularly and washed extensively with phosphate saline, water, 0.6 M NaI, 2 M NaCl, or 0.2 M carbonate, pH 10. These washes should remove peripheral or adsorbed membrane proteins. Regardless of the type of wash, the *amounts* of labeled 2.6 and T200 (an established transmembrane protein [9]) were the same (Fig. 4). Total TCA-precipitable counts were also similar in cultures exposed to the different salt washes, indicating that intrinsic PM proteins are the primary species iodinated by LPO-latex.

The Rapidity of Membrane Movement from Phagolysosome to Plasma Membrane

In our previous study (3), we found that iodinated membrane polypeptides recycled from PL to PM. The cells we studied were cultured for 30 min at 37°C. We now have examined earlier time points and lower temperatures.

Cells were iodinated for 10 min on ice and quickly washed (<30 s); the monolayers were either fixed immediately (time 0), kept on ice (2°C for 10 min), or returned to culture at 37°C for 5 min before fixation. The majority of the grains were over PL at time 0 (Table IV). Significant nuclear labeling was observed, which we consider to be background as discussed in Materials and Methods. All grain counts were corrected for this nuclear background. As a result, all the incorporated label at time 0 was associated with the PL. After 5 min at 37°C, 81% of the grains had redistributed to the PM. This result is similar to previous work in which the first time-point examined was at 30 min of reculture (3). Therefore, most of the radiolabel introduced into the PL recycles to the PM within 5 min at 37°C. A striking result was observed in iodinated cells that were left on ice for 10 min. Only 47% of the label remained associated with PL, and 40% of the grains were either on or close to the cell surface. Direct measurements verified that the culture medium was at 2°C during the 10 min before fixation.

In a second experiment (Table IV), we iodinated the cells for just 5 min on ice and then returned the cells to culture for 5 min at 2°C, 22°C and 37°C. Again, at time 0, all the radiolabel was associated with the PL (Table IV). After 5 min at 37°C, 63% of the label had redistributed to the PM; at 22°C, 40% of the grains were on or close to the PM; and at 2°C, 27% of the grains were on or close to the cell surface. Illustrative autoradiograms are shown in Fig. 5. There was some variation in the movement of radiolabel in the two experiments (Table IV). This could reflect differences in: the rate of which iodination occurred during intracellular labeling; the time spent at 2°C before iodination; or the state of macrophage activation which may in turn alter rates of membrane flow and fusion.

DISCUSSION

The Composition of Phagolysosome Membrane

Latex has been used extensively in studies of endocytosis, because latex phagocytic vacuoles can be purified by flotation in dense sucrose (12). Not unexpectedly, uptake of latex is accompanied by the internalization of several plasma membrane (PM) constituents, including lipids (13), ectoenzymes (14, 15), and a large spectrum of polypeptides radiolabeled with lactoperoxidase (LPO) (2, 14). Furthermore, the specific activities (amount/unit area of membrane) of several components are similar in internalized and non-internalized PM. The studies of Hubbard and Cohn (14) in L-cell fibroblasts were carried out under conditions in which cells internalized roughly 30% of their cell surface area. Latex phagolysosomes (PL) were

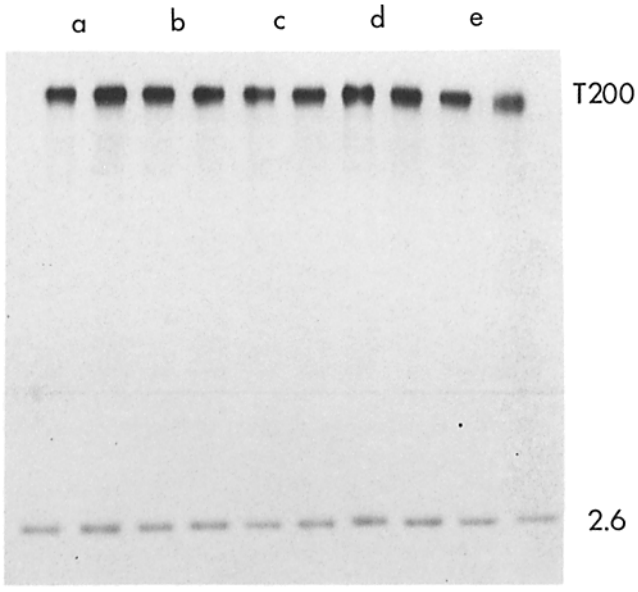

FIGURE 4 The 2.6 polypeptide is an intrinsic membrane protein. Following plasma membrane iodination of replicate cultures, macrophage monolayers were washed four times over 30 s with (*a*) PBS; (*b*) distilled water, (*c*) 0.6M NaI, (*d*) 2M NaCl, or (*e*) 0.2 M carbonate buffer at pH 10. The monolayers were then washed in PBS and lysed. Immune precipitation was carried out in duplicate using 2.6 and anti-T200 monoclonal antibodies combined. The immune precipitates were separated on 4–11% SDS PAGE, and the dried gel was exposed to Kodak XR-1 film for 20 h. Total TCA-precipitable cpm in *a*→*e* ranged from 8 to 12 × 10^6, which was within the range of replicate cultures treated identically.

purified in high yield and were noted to contain ~30% of the total content of the ectoenzyme 3′ phosphodiesterase and iodinated PM polypeptides. In recent work, Mills and Unkeless (personal communication) have compared the amount of F_c receptor, quantitated by radioimmunoassay, in enriched PM and *latex PL* fractions from J774 Mϕ. The Mϕ were iodinated before phagocytosis, and the specific activity of iodinated membrane (cpm/μg protein) was similar in PL and PM fractions. The concentration of F_c receptor (amount/cpm ^{125}I) in the PL fraction was one-half that in PM, indicating considerable internalization of this particular PM constituent.

The technique of intracellular iodination with LPO covalently coupled to latex might have detected novel constituents introduced by the lysosome component of the PL. Following phagocytosis the particles are all in vacuoles that contain acid phosphatase cytochemical reactivity (2). The PL membrane can then be radioiodinated within living cells. However, in this paper we show that no distinct lysosome-derived polypeptides can be detected. Rather, PL and PM membrane proteins are remarkably similar. First, by one-dimensional SDS PAGE, the patterns of polypeptides iodinated within cells resemble those labeled extracellularly. This is true even in chloroquine-treated cells, where lysosomal pH is greater than 6 and the efficiency of iodination is very high (Fig. 1). By two-dimensional gel electrophoresis, the molecular weight and pI of the major iodinated species of PL and PM are again similar (Fig. 2). Third, by immunoprecipitation, the relative amounts of four labeled, specific, polypeptides are analogous in PL and PM (Fig. 3; Table III). The F_c receptor is one of the proteins we studied and, as discussed above, the concentration of F_c receptor in PL membrane is roughly 50% of that found in PM. Therefore, similarity in the amount of radiolabeled polypeptides probably indicates similarity in absolute amounts of the four surface antigens we studied.

Clearly, the lysosomal membrane may still contain novel polypeptides that we do not detect because these proteins are not labeled with LPO-latex or are present in small amounts. Also, in our monoclonal Ab studies, we have used antibodies raised to whole cells, not purified PL. However, it is possible that the lysosome and endocytic vacuole membranes show considerable homology. This could occur if lysosomal hydrolases and PM were synthesized and packaged together in the Golgi complex. These primary lysosomes would then fuse with endocytic vacuoles, delivering acid hydrolases and plasma membrane constituents into the secondary lysosome. The newly synthesized PM would join a recycling pool of membrane (3). How acid hydrolases would be retained intracellularly during recycling is not clear, but the lysosomal membrane itself may escape digestion because it is continually and rapidly moving into and out of cells (3).

TABLE IV

Distribution of EM-ARG Grains in Macrophages After Iodination of Latex-Phagolysomes

Reculture time	Reculture temp.	Total grains		Corrected for crossfire				Corrected for crossfire and background		
				PL	PM	CYTO	NUC	PL	PM	CYTO
min	°C									
Cells Iodinated for 10 Min on Ice										
0	—	398	% Total grains	50	17	24	10	100	0	0
			% Total points	8	33	41	17	8	33	41
			Grain density	6.3	0.5	0.6	0.6	12.6	0	0
10	2	282	% Total grains	29	35	28	8	47	40	14
			% Total points	7	29	46	18	7	29	46
			Grain density	4.1	1.2	0.6	0.4	6.7	1.4	0.3
5	37	416	% Total grains	9	64	20	7	10	81	9
			% Total points	6	29	44	22	6	29	44
			Grain density	1.5	2.2	0.5	0.3	1.7	2.8	0.2
Cells Iodinated for 5 min on Ice										
0	—	669	% Total grains	61	19	12	8	95	5	0
			% Total points	4	42	33	21	4	42	33
			Grain density	15.3	0.5	0.4	0.4	24	0.1	0
5	2	522	% Total grains	41	30	20	9	67	27	6
			% Total points	3	34	40	22	3	34	40
			Grain density	13.7	0.9	0.5	0.4	22	0.8	0.2
5	22	451	% Total grains	32	39	20	9	53	40	6
			% Total points	3	37	38	21	3	37	38
			Grain density	10.7	1.1	0.5	0.4	17.7	1.1	0.2
5	37	412	% Total grains	26	59	11	4	31	63	6
			% Total points	4	40	34	23	4	40	34
			Grain density	6.4	1.5	0.3	0.3	7.7	1.6	0.2

The localization of silver grains ("% Total grains") with respect to four subcellular compartments was determined by a three-step method: (*a*) Around the center of each silver grain, a circle was applied that had a 50% probability of containing the source of the grain (the circle diameter was 1.73 times the half distance of 800 A.) Each grain was thereby assigned to any organelle contained partly or wholly within the circle. (*b*) Scattering of radioactivity from a heavily labeled compartment, i.e., the LPO-latex phagolysosome, may falsely elevate the labeling of adjacent areas. To correct for this error, termed "crossfire", we eliminated the few nuclear, cytoplasmic, and plasma membrane grains that were within 0.27 μm of LPO-latex spheres. (*c*) We then corrected for background labeling, determined by the extent of nuclear labeling, as described in Materials and Methods. To obtain a specific activity for the labeling of each compartment, we normalized the grain counts to the size of the compartment. The latter was determined by overlying the autoradiograms with a grid of random points, drawing 50% probability circles around each point, and counting any organelle within the circle. The "total points" falling on the compartment is an index of relative compartment size. The "relative grain density" is the % total grains in a compartment divided by the % total points. *CYTO*, cytoplasmic; *NUC*, nuclear.

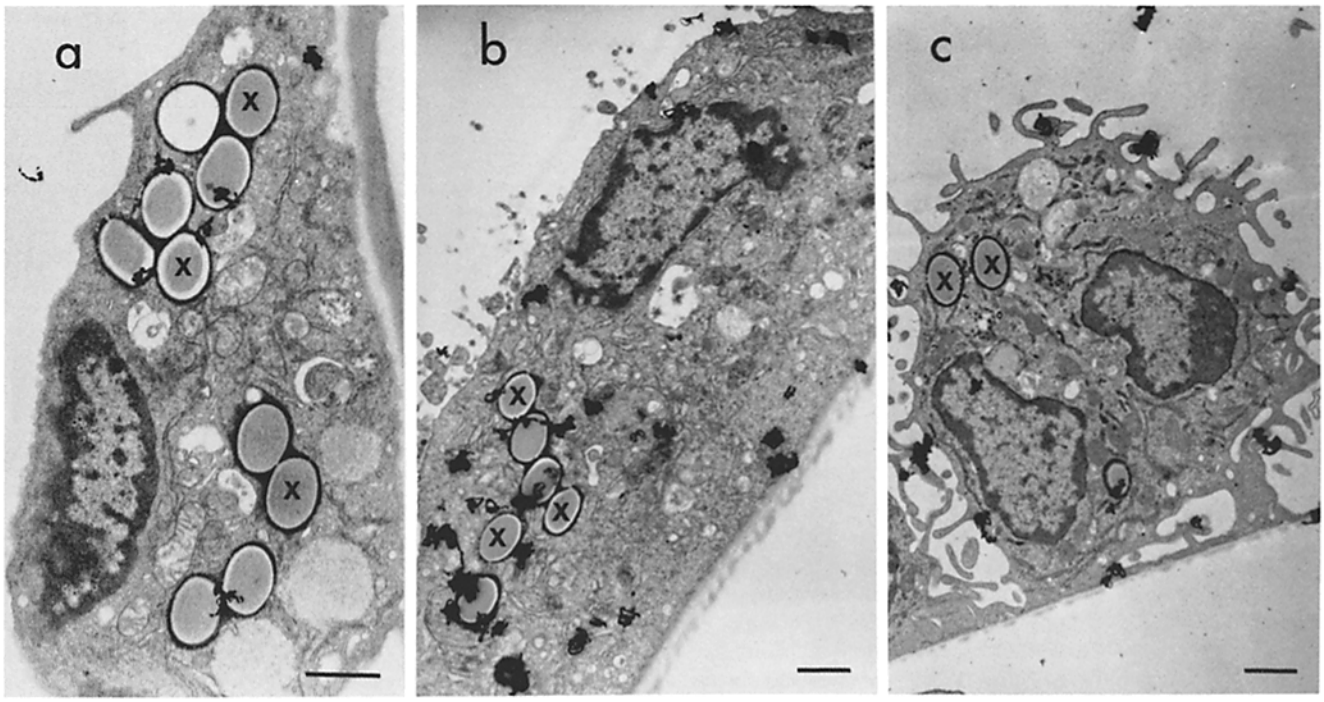

FIGURE 5 Rapid movement of radiolabeled polypeptides from phagolysosome to the plasma membrane. Macrophages were allowed to ingest LPO-latex particles (*x*), each of which is surrounded by a dense rim of peroxidatic cytochemical reaction product (diaminobenzidine - H_2O_2). After phagocytosis, the cells were chilled and were exposed to ^{125}I and H_2O_2 for 5 min on ice. The cells were washed and either fixed immediately (*a*), maintained for 5 min at 2°C before fixation (*b*), or cultured for 5 min at 37°C before fixation (*c*). At time 0 (*a*), radiolabel is bead associated; after 5 min on ice (*b*), some label moves out of the phagogysosome; after 5 min at 37°C (*c*), most of the radiolabel has redistributed to the plasma membrane. Bar, 1 μm.

The one protein that distinguishes the PL membrane from PM is a 21dalton polypeptide precipitated by the monoclonal antibody 2.6. The monoclonal was secured by Unkeless (1) who fused myeloma cells to rat spleen immunized to mouse Mϕ. The antigen is found on many cell types including Mϕ, granulocytes, dendritic cells, and platelets, but it is scarce on lymphocytes (6). The function of the 2.6 protein is unknown, but it likely is an intrinsic membrane component (Fig. 4). We found that the amount of labeled 2.6 was increased sevenfold in PL vs. PM. We think this reflects a sevenfold increase in protein concentration rather than enhanced iodination in the lysosome environment. Specifically, enrichment of labeled 2.6 occurs if PM is radioiodinated with LPO-latex extracellularly, and if then the labeled PM is internalized and isolated from PL fractions (Table III*B*). EM immunocytochemistry with this monoclonal antibody might clarify the mechanism of 2.6 enrichment. The protein may be sequestered within the forming latex phagosome; 2.6 may be enriched in nascent pinocytic vacuoles, e.g., in coated pits, which then fuse with intracellular latex PL; or the rate of 2.6 recycling may be very slow. Mellman et al. (1) have shown that radiolabeled 2.6 is also enriched in PV membrane iodinated intracellularly with pinocytosed soluble LPO.

Recycling of Membrane from Phagolysosome to the Cell Surface

When Mϕ are iodinated intracellularly at 4°C and returned to culture at 37°C, radiolabeled polypeptides move from PL to PM within 30 min as assessed by EM-ARG (3). Here we have examined earlier time points and the effects of temperature on the redistribution of radiolabel. The system is advantageous for studies of membrane flow. The iodinating enzyme, LPO, is covalently coupled to latex particles which in turn remain fully sequestered within intracellular vacuoles. The radioiodine is incorporated into membrane proteins, and labeling of lipids or vacuole content is not detectable. There is no reutilization of ^{125}I, either the free ^{125}I remaining after iodination or any ^{125}monoiodityrosine released by proteolysis (the amount of degradation would be small in any case, since the $t_{1/2}$ for the fast component of degradation is 60–90 min; 3).

By EM-ARG, the recycling of radiolabel from PL to PM is completed within 5–10 min at 37°C (Table IV; Fig. 5). The EM-ARG approach primarily documents movement of radiolabel out of the PL compartment; it is more difficult to be certain that radiolabel has been fully incorporated into PM. In previous experiments, when cells were cultured for 30 min (3), we isolated PM with a second phagocytic meal. We were then able to show that a representative sample of label PL had recycled to the cell surface. Because most membrane species recycle in tandem, the movement of label out of the PL compartment may be equated with the formation of vesicles composed of a typical lipid bilayer and iodinated membrane polypeptides. Other experiments with macrophages and other cell types provide additional evidence for a rapid vesicular flow of endocytosed membrane to the cell surface. For sucrose (16), horseradish peroxidase (17), asialoglycoprotein (18), mannosyl glycoprotein (19), and chemotactic peptide (20), a portion of internalized solute seems to be regurgitated shortly after uptake. Reflux probably occurs by exocytosis of recycling vesicles containing pinocytosed solute. The $t_{1/2}$ for reflux is <5 min in all systems studied to date.

We were surprised by the extent of movement at reduced temperatures, even 2°C. The LPO-latex system provides a

unique and possibly very sensitive method for monitoring membrane flow out of intracellular vacuoles, because all the radiolabel is placed into recycling membrane. Since we have not measured *rates* of movement, the flow of radiolabel at 2°C could be very slow relative to that of 37°C. One cannot assume that membrane flow ceases entirely at low temperatures. Fluid-phase pinocytosis has a Q10 of 3 and decreases progressively at reduced temperature rather than stopping altogether (22). A second possibility is that EM-ARG primarily monitors the formation of recycling vesicles. The Q10 for the latter process could be small i.e., $\ll 3$. It is possible that recycling vesicles form by the shrinkage and vesiculation of endocytic vacuoles, following movement of salt and water across the vacuole membrane (23, 24). Movement of salt and water out of the vacuole may not require significant metabolic energy, allowing substantial formation of vesicles at reduced temperature.

The compartment from which recycling occurs in our model is an intracellular vacuole accessible to an endocytic load (LPO-latex). Formally, the vacuole is a lysosome because it contains acid phosphatase cytochemical reaction product (2). In our experiments, phagocytosis always is allowed to occur for 20–30 min; acid phosphatase is not detectable in "phagosomes" when phagocytosis occurs for just 5 min (unpublished observation). We do not have further data on the kinetics or extent of lysosome fusion. Recent studies indicate that many events can occur in the endocytic pathway before obvious fusion with lysosomes, e.g., incoming pinocytic vesicles can fuse with one another, acidify, and release ligands attached to receptors (reviewed in reference 23). There is also evidence that lysosome fusion can begin within minutes of the internalization event, and these fusion rates may be influenced by the ligand and cell type being studied. In macrophages, one begins to detect digestion of ^{125}I-labeled ligand within 5–10 min at 37°C (^{125}I-acetylated low density lipoprotein (25); ^{125}I-DNP albumin anti-DNP complexes (I. S. Mellman, personal communication). Because both recycling and lysosome fusion can begin with minutes of endocytosis, recycling may occur from newly formed lysosomes, as in the LPO-latex vacuoles we have studied.

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