The Appearance of Lecithin-^{32}P, Synthesized from Lysolecithin-^{32}P, in Phagosomes of Polymorphonuclear Leukocytes

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ABSTRACT Rabbit polymorphonuclear leukocytes ingesting paraffin oil particles stabilized with albumin, converted more lysolecithin-^{32}P (added to the medium as an albumin complex) to cellular lecithin than did control cells.

Almost all of the increment in leukocyte lecithin-^{32}P is found in association with the isolated phagocytic vacuoles.

About half of lecithin-^{32}P of granulocytes incubated first with lysolecithin-^{32}P and then reincubated with paraffin particles in a nonradioactive medium is transferred from a sedimentable (presumably membrane) fraction to the phagosomes. Isolated phagosomes or granules by themselves are capable of acylating lysolecithin. The main source of lysolecithin-^{32}P for synthesis of cellular lecithin-^{32}P, however, appears to be extracellular rather than lysolecithin-^{32}P within the cytoplasm or the phagocytic vacuole. We interpret our findings therefore as indicating that lecithin-^{32}P in the phagosomes derives chiefly from the outer membrane.

INTRODUCTION

Previous studies in our laboratory have shown that during phagocytosis, the synthesis of lecithin from extracellular albumin-bound lysolecithin by phagocytes from various sources, is markedly stimulated (1, 2). It was proposed that this mechanism of substantial net addition of lecithin, a major membrane phospholipid of most mammalian cells, might represent at least one piece of the long sought biochemical evidence for increased membrane biosynthesis often assumed to take place during phagocytosis.

While considerable circumstantial evidence supports this view, we have not yet obtained direct proof that the increased conversion of monoacylphosphatides to their diacyl-derivatives is required for engulfment and its presumed need for new membrane formation, rather than a concomitant of the generally heightened metabolic activity of the engulfing phagocytes.

The recent development of a technique for isolation of intact phagosomes (3, 4), has enabled us to determine the extent to which the lecithin derived from medium lysolecithin becomes part of the phagosome membrane.

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The results indicate that: (*a*) during phagocytosis the bulk of the increment in newly formed lecithin becomes part of the phagosome fraction; (*b*) about half of prelabeled granulocyte lecithin is transferred from a sedimentable fraction, (presumably mainly membranes) to the phagosomes; (*c*) isolated phagosomes are capable of extensive conversion of lysolecithin to lecithin.

METHODS

The methods used in this study have all been described in detail before (1–4). In brief, rabbit granulocytes were obtained from sterile peritoneal exudates elicited by intraperitoneal injection of glycogen in saline.

Lysolecithin-^{32}P was prepared from biosynthetically labeled rat liver lecithin-^{32}P, by hydrolysis with snake venom phospholipase A_2.

Phagosomes were isolated after granulocytes had been incubated for 15 or 30 min with an emulsion of paraffin oil particles (3). These particles were prepared by sonication of heavy paraffin oil in a solution of bovine serum albumin (Fraction V, Pentex Biochemical, Kankakee, Ill., lot 147) in Hanks' medium (final albumin concentration 16 mg/ml).

Labeling of phagosome lecithin was examined in two ways: (*a*) granulocytes were incubated in a shaking waterbath at 37°C with lysolecithin-^{32}P, complexed to alubumin, and with or without paraffin particles; (*b*) granulocytes were first incubated with lysolecithin-^{32}P to label cellular lecithin. The cells were then resuspended in fresh (nonradioactive) medium and reincubated with or without paraffin oil. At the end of both types of incubation for 15 or 30 min, cells and medium were separated by centrifugation and the cells disrupted by homogenization (3). The amounts of lecithin-^{32}P in lipid extracts of whole homogenates and of various fractions (3) obtained by centrifugation, were determined by thin-layer chromatography and counting procedures that have been previously described (1).

For further details and calculation of results, see legends of figures. Recovery of all ^{32}P radioactivity in lipid extracts of the various fractions and in the water washes, expressed as per cent of the radioactivity in the whole homogenates of five samples was 113±4.2% (mean ±SEM). The recovery of lecithin-^{32}P in the fractions obtained from 10 homogenates was 102±7.5% (mean ±SEM).

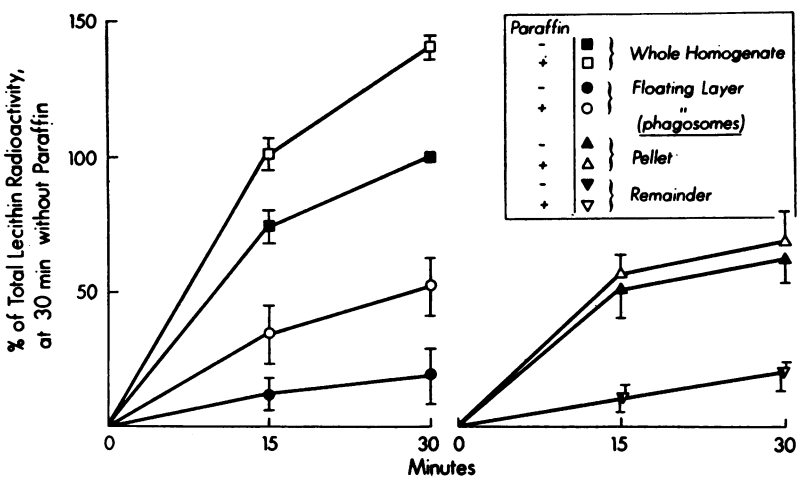

FIGURE 1 Incorporation of lysolecithin-^{32}P into lecithin of phagosomes during phagocytosis of paraffin particles by granulocytes. Each incubation mixture contained 2.5×10^8 granulocytes, 0.4 ml of paraffin particles in Hanks' solution, or Hanks' solution alone; 60 mg of albumin; 10 μg of linoleate; and, in different experiments, from 100,000 to 300,000 cpm of lysolecithin-^{32}P (representing from 20 to 60 nmoles), all in a total volume of 2.4 ml of Hanks' solution. At the end of incubation the suspensions were transferred to centrifuge tubes and diluted to a total volume of 40 ml with ice-cold Hanks' solution. To "control" suspensions, 0.4 ml of paraffin particles were added. All tubes were subjected to centrifugation at 20,000 *g* for 10 min. Floating fat layers were left in place and all liquid was carefully removed. The cell pellet and the floating fat layers were resuspended in 0.36 M sucrose that contained 1 mM Tris-HCl buffer (pH 7.5) 1 mM ethylenediaminetetraacetic acid (EDTA) and 500 U of heparin sodium (E. E. Squibb & Sons, Princeton, N. J.)/ml. This mixture was transferred to a chilled Dounce homogenizer. Homogenization and fractionation of the homogenate, using a discontinuous sucrose gradient were carried out as described previously (3). Total lipid radioactivity, radioactivity in the water washes of all lipid extracts, and the distribution of radioactivity between lecithin and lysolecithin, (separated by thin-layer chromatography) were determined on extracts of whole homogenate, the floating layer (paraffin alone (control) or phagosomes), the sediment, and the liquid layers (5, 6).

Counts per minute in lecithin of each fraction is given as per cent of total lecithin radioactivity in the whole homogenate of the control cells incubated for 30 min (without paraffin particles). The results are given as the mean and SEM of four experiments.

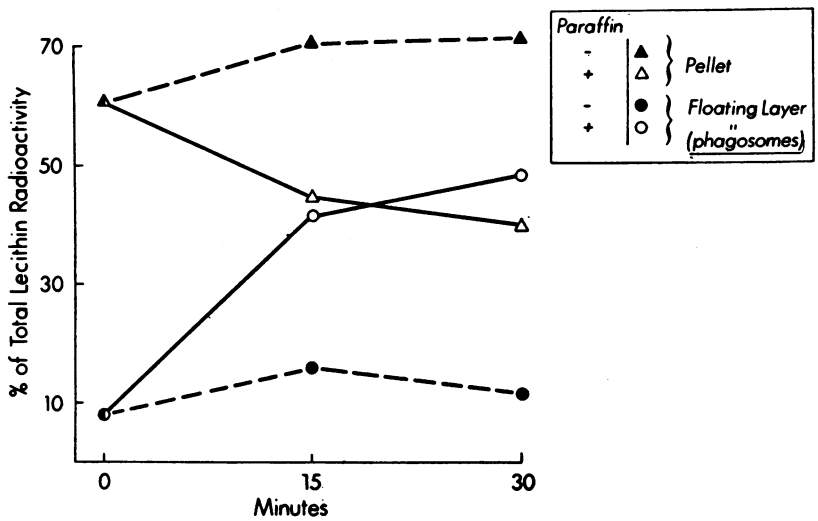

FIGURE 2 Appearance of lecithin-^{32}P in isolated phagosomes during phagocytosis of paraffin particles by prelabeled granulocytes. Granulocytes (1.25×10^9 cells) in 5 ml of Hanks'-albumin solution (40 mg/ml) were incubated for 45 min with lysolecithin-^{32}P (500,000 cpm, representing approximately 50 nmoles of lysolecithin). At the end of this labeling period, the cells were sedimented at 100 *g* for 10 min, resuspended in 5 ml of fresh, nonradioactive Hanks'-albumin solution, and reincubated for 15 min to permit acylation of remaining lysolecithin-^{32}P. The cells were then sedimented again, resuspended in 10 ml of Hanks' solution, and divided into five equal 2.0-ml portions. One of these served as a zero time, two received 0.4 ml of paraffin suspension, and two 0.4 ml of Hanks' solution. After 15 min at 37°C, one of each pair of cell suspensions was transferred to a chilled Dounce homogenizer and brought to a volume of 5 ml with a solution that provided a final concentration of 0.36 M sucrose, 1 mM Tris-HCl buffer (pH 7.5), 1 mM EDTA, and 500 U of heparin/ml. The other samples of cells were incubated for 30 min before homogenization.

The remaining of the procedures were carried out as described in the legend of Fig. 1. Counts per minute in lecithin in the indicated fraction are given as per cent of total lecithin radioactivity in each suspension. The results are presented as the means of two closely similar experiments.

RESULTS

Incubation of rabbit granulocytes with paraffin particles results in stimulation of the conversion of medium lysolecithin-^{32}P to lecithin-^{32}P (Fig. 1). This stimulation ($P < 0.01$) is of the same order of magnitude as previously observed using polystyrene spherules as ingestible material. The "control" floating layer carried lecithin-^{32}P, presumably reflecting artifactual transfer of membrane material during fractionation. The "phagosome" floating layer, however, contained much more lecithin-^{32}P ($P < 0.05$), and, in fact accounted for almost all of the increment observed in the whole homogenate. (Student's *t*-test was applied to the results of three paired observations at 15 min plus four paired observations at 30 min.

The effect of ingestion of paraffin particles on the redistribution of previously labeled granulocyte lecithin-^{32}P is shown in Fig. 2. Whereas from 60 to 70% of total granulocyte lecithin-^{32}P was present in the pellet that sedimented during centrifugation of the homogenized control cells and about 10% in the floating layer (control), after ingestion of paraffin particles the pellet contained approximately 45 and 40% of total lecithin-^{32}P at 15 and 30 min respectively, and the floating layer (phagosomes) 40 and 47%. Total lecithin-^{32}P radioactivity in the four portions of labeled cells that were incubated with or without paraffin, ranged from 82 to 109% of the zero time value in each of the two experiments that provided the data depicted in Fig. 2. The percentage recovery did not correlate with the length of incubation or the presence of paraffin oil. The variability in the lecithin-^{32}P content seems best explained by a small amount of clumping of the granulocytes causing differences in the number of cells actually distributed among the five portions. It appears therefore, that no substantial net gain or loss of lecithin-^{32}P took place during the course of these experiments.

These findings suggest that both the lecithin synthesized from medium lysolecithin during phagocytosis and cellular lecithin synthesized before engulfment is initiated become part of the phagosome membrane.

Lysolecithin-^{32}P radioactivity in the whole homogenates of prelabeled cells or in the fractions never exceeded 20%

of total lipid radioactivity. Although lysolecithin radioactivity exhibited no rise or fall indicative of its utilization for lecithin synthesis or its production because of lecithin-^{32}P breakdown during the 30 min of observation, it was of interest to determine whether isolated phagosomes are capable of converting lysolecithin to lecithin. Since the granule envelop fuses with the phagocytic vacuole (5), lecithin formation from lysolecithin by isolated granules was also determined. The results of six experiments in which the conversion of lysolecithin to lecithin by whole homogenates, isolated phagosomes, and the granule fraction (6) was compared are shown in Table I. As established previously (7, 8), acylation of lysolecithin by disrupted cells is stimulated by added ATP and CoA. This stimulation is about the same for whole homogenate, phagosomes, and granules. The acylating activity of the phagosome and granule fraction was roughly the same and, for each fraction, represents about 20% of the activity in whole homogenate. By contrast, less than 7% of total lecithin-^{32}P of intact granulocytes incubated for 30 min with lysolecithin-^{32}P is subsequently found in the granule fraction. We have not determined whether these values for the granule fraction represent, wholly or in part, contamination with outer membrane fragments.

DISCUSSION

Recent studies have demonstrated that the ingestion of a stable emulsion of paraffin particles provides a technique for the isolation of a subcellular granulocyte fraction, that, by a number of stringent criteria, can be considered to consist of intact phagosomes (3, 4).

The results of the present study show that these phagosomes contain most of the increase in granulocyte lecithin-^{32}P synthesized from medium lysolecithin during phagocytosis; thus, supporting the view that this source of net addition of membrane lipid might serve a need for increased membrane formation to accommodate ingested particles.

We have previously postulated that albumin-bound lysolecithin does not readily traverse the outer membrane, and that acylation of medium lysolecithin occurs within the plasma membrane (1). The finding that a large portion of lecithin-^{32}P of prelabeled cells was transferred during phagocytosis from the sedimentable (membrane) fractions in the homogenate to the floating phagosomes is consistent with the view that most of the lecithin, produced by acylation of lysolecithin in the resting cell, becomes part of the outer membrane, since it is this membrane that presumably is the origin of the phagocytic vacuole.

This interpretation could be challenged on the basis of the evidence that phagosomes are capable of converting lysolecithin to lecithin. It could be argued that exogenous lysolecithin may be available both within the cytoplasm and within the phagosome. The lysolecithin-^{32}P content of prelabeled cells that were reincubated with or without paraffin oil particles, underwent no change however, indicative of rapid utilization of cellular lysolecithin-^{32}P.

It also appears unlikely that enough albumin bound to the paraffin particles was incorporated by the phagocytizing granulocytes to account for the increased lecithin-^{32}P on the basis of increased delivery of substrate. The albumin used to stabilize the paraffin particles represents 6 mg/incubation mixture. Approximately one-fourth of this albumin is bound to the particles (3). No more than 5% of radiolabeled albumin become associated with roughly 5×10^8 granulocytes during incubation for 45 min with paraffin oil particles (3). Since the lysolecithin-^{32}P complexed to 60 mg of albumin was added last to the incubation mixture, only 0.5% of the total lysolecithin-albumin complex may have been delivered into the phagocytic vacuole (if it is assumed that the added albumin-lysolecithin complex completely equilibrated with the albumin coating the particles). In the four experiments depicted in Fig. 1, the increase in conversion of lysolecithin-^{32}P to lecithin-^{32}P represented on the average 10% of the total lysolecithin-^{32}P added. Thus, although the phagosomes as well as probably the granules that become part of them are capable of acylating lysolecithin, we do not believe this to be a major source of phagosome lecithin-^{32}P, but rather favor the view that the appearance of the lecithin-^{32}P in the phagosomes reflects the internalization of the outer membrane.

TABLE I

Conversion of Lysolecithin-^{32}P to Lecithin by Isolated Phagosomes and Granules

	− ATP, CoA, Mg^{++}		+ ATP, CoA, Mg^{++}	
Minutes of incubation....	15	30	15	30
	nmoles lecithin/10^9 granulocytes			
Whole homogenate	52±8.6	70±15.1	130±9.7	193±10.4
Phagosomes	12±3.5	16± 4.7	30±4.2	37± 4.2
Granules	8±0.9	11± 1.2	28±3.6	39± 5.4

Reaction mixtures of 0.5 ml contained 0.6 mM Tris-HCl buffer (pH 7.5), 0.6 mM EDTA; 150 U of heparin sodium; 0.1 ml of Hanks' balanced salt solution, or Hanks' solution containing ATP, CoA, and Mg^{++} (final concentration: 10, 0.2, 10 mM respectively); 0.3 ml of whole homogenate in 0.36 M sucrose (representing 2×10^7 cells), or 0.3 ml of phagosome fraction in 0.25 M sucrose (representing 1×10^8 cells); or 0.3 ml of granules in 0.36 M sucrose (representing 1×10^8 cells); and, in different experiments, from 20 to 70 nmoles of lysolecithin-^{32}P complexed to albumin. Incubation was carried out at 37°C in a shaking water bath. Lecithin formation was assayed as previously described (1, 7). Results (nmoles of lecithin formed per 10^9 granulocytes) are presented as the mean ±SE of six experiments.

ACKNOWLEDGMENTS

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