MEMBRANE FUSION OF PHAGOCYTIC VESICLES WITH LYSOSOMES IN POLYMORPHONUCLEAR LEUKOCYTES WITHOUT PHAGOCYTIC STIMULI

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1. Introduction

Many phagocytic cells incorporate foreign particles in phagocytic vesicles formed by invagination of the plasma membrane and these vesicles then fuse with lysosomes [1–3]. Although these events occur successively, they occur so rapidly [3] that the processes of incorporation and fusion have not been studied separately. Some authors have suggested that phagocytic stimuli may be needed for fusion of the vesicles with lysosomes [4], and others have suggested that the extent of their fusion with lysosomes is closely related to phagocytic activity [5]. Experiments in this laboratory suggested that phagocytic vesicles fused with lysosomes even after removal of phagocytizable particles [6], but precise investigations have not been made on this process. This paper reports studies showing that membrane fusion of phagocytic vesicles with lysosomes occurs in the absence of phagocytic stimuli, and also reports studies on the effects of various reagents on the process of fusion.

Abbreviations: PMNs, polymorphonuclear leukocytes; POE, paraffin oil droplets; BSA, bovine serum albumin; SH, sulfhydryl; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; PCMPSA, *p*-chloromercuriphenylsulfonic acid; DTT, dithiothreitol; β-ME, β-mercaptoethanol; 2-dGlc, 2-deoxyglucose; monoIA, monoiodoacetate; Con A, concanavalin A; RCA, *Ricinus communis* agglutinin; WGA, wheat germ agglutinin; α-MeMan, α-methylmannoside; Gal, galactose; GlcNAc, N-acetylglucosamine

2. Materials and methods

2.1. *Materials*

Paraffin oil was purchased from Fisher Scientific Co. (Fair Lawn, PA), oil red O from Chroma Gesellschaft (Stüttgart) and *p*-nitrophenylphosphate from Daiichi Pure Chem. (Tokyo). Lectins were generous gifts from Drs T. Osawa and M. Terao, Tokyo University. Cytochalasin B was obtained from Aldrich Chemical Co. (Milwaukee, WI) and dithiothreitol from Boehringer Mannheim GmBH. Other chemicals were of the purest commercial grade available.

2.2. *Isolation of the phagolysosome fraction*

Guinea pig PMNs were collected as in [4]. Then they were washed with saline and suspended at 4×10^7 cells/ml in Krebs-Ringer phosphate buffer (with 1/3rd the normal concentration of Ca^{2+}) (pH 7.4). Over 95% of the cells were PMNs and ⩾ 98% of them were viable. The paraffin oil was stained with oil red O before use as in [4] to assay the amount of the phagocytic vesicles. POE were emulsified with [4] and unbound BSA was removed by centrifugation. Phagocytosis of POE by PMNs and separation of the phagolysosome fraction were done as in [7].

2.3. *Reincubation of PMNs after phagocytosis*

For examination of whether phagocytic vesicles fuse with lysosomes without phagocytic stimuli, PMNs were allowed to phagocytize POE at 37°C for 5 min and then washed to remove POE on their cell surface. Then aliquots of PMNs were suspended

in Krebs-Ringer phosphate buffer (1/3rd Ca^{2+}) (pH 7.4) without POE at 4×10^7 cells/ml, and reincubated with shaking at 0°C or 37°C for 15 min, unless otherwise noted. After incubation, the cells were rapidly cooled and washed thoroughly with saline, and the phagolysosome fraction was separated as in [7]. Some reagents were added to the cell suspension just before reincubation of the cells at 37°C for 15 min.

2.4. *Assays*

The lysosomal enzyme acid phosphatase was assayed with *p*-nitrophenylphosphate at pH 5.0 [7]. Oil red O was extracted from POE with dioxane and the concentration was determined as in [4]. Protein concentration was determined as in [8] with BSA as a standard.

3. Results

3.1. *Phagocytosis of paraffin oil droplets*

PMNs phagocytized POE rapidly for the first 5 min and then gradually for another 15 min (fig.1). Microscopical observation showed that the POE were mostly inside the cells, with few droplets on the cell surface. After 5 min, 70–80% of the cells usually contained several POE.

3.2. *Fusion of phagocytic vesicles with lysosomes during phagocytosis*

The extent of fusion of phagocytic vesicles with lysosomes was expressed as the fusion index:

$$\text{Fusion index} = \frac{\text{(acid phosphatase in phagolysosome fraction)}}{\text{(oil red O in phagolysosome fraction)}}$$

The fusion index indicates the average extent of fusion of lysosomes per phagocytic vesicles, because in the fractionation procedure used here 80–90% of the phagocytic vesicles were recovered in the phagolysosome fraction, which contains no primary (unfused) lysosomes [4,7].

As shown in fig.2, the fusion index during phagocytosis rose more slowly than the phagocytic activity (fig.1) and after 20 min, the recovery of acid phosphatase in this fraction reached ~10% of the total enzyme activity.

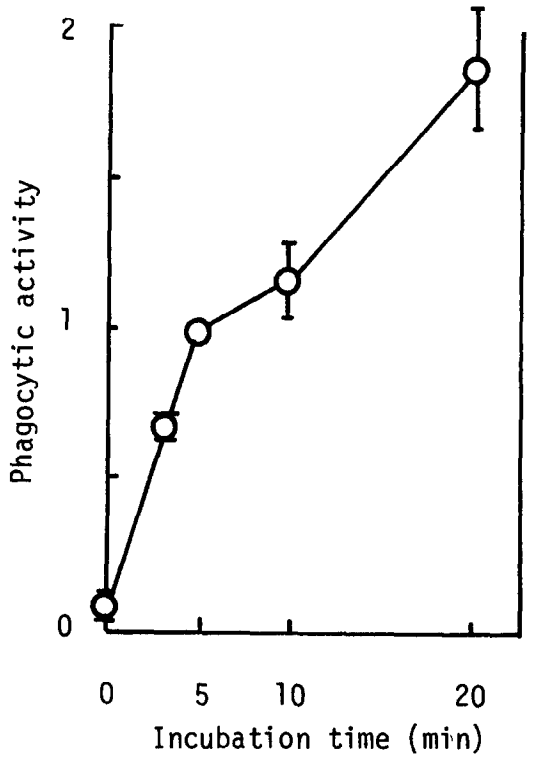

Fig.1. Time course of phagocytosis of paraffin oil droplets (POE). The number of POE was far in excess of the number of cells in this experiment. Phagocytic activity was calculated as nmol oil red O/mg cell protein in the cell homogenates. Values are shown relative to that after 5 min incubation.

3.3. *Fusion between phagocytic vesicles and lysosomes without phagocytic stimuli*

As shown in fig.3, the fusion index did not change at 0°C but increased at 37°C, reaching about twice the initial value after reincubation for 25 min. This indicates that fusion of phagocytic vesicles with lysosomes occurred without phagocytic stimuli at 37°C and that the reaction was temperature-dependent. The recovery of POE in the phagolysosome fraction was not affected by incubation at 0°C or 37°C. The increase of the fusion index was almost linear for the first 15 min and was higher than that in fig.2.

3.4. *Effects of various reagents on the fusion of vesicles without phagocytic stimuli*

At the concentrations tested, none of the reagents tested affected the assay system. Of the sulfhydryl (SH) inhibitors, NEM and PCMB strongly inhibited

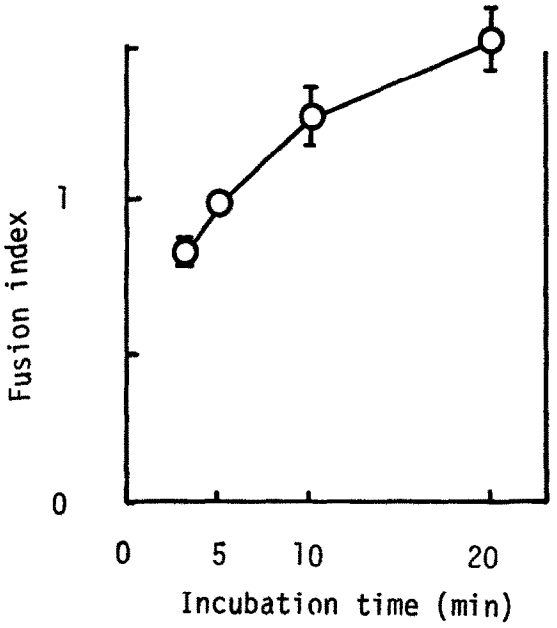

Fig.2. Time course of fusion during phagocytosis. Relative values for the fusion index are shown, as in fig.1. Values are means ± SE for ⩾5 experiments. The value at 0 time is omitted because the recovery of oil red O was too low to permit calculation of the fusion index. Acid phosphatase activity at 0 time was also very low (about 0.6% of the total activity).

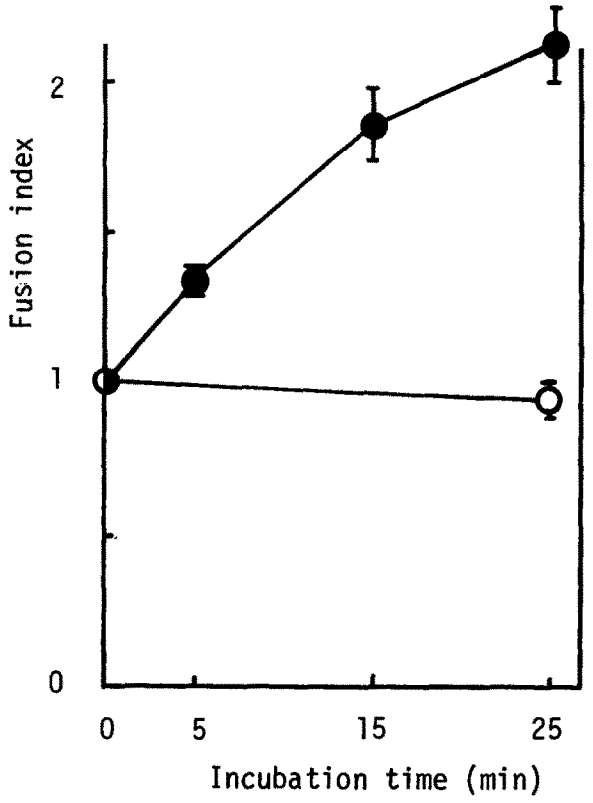

Fig.3. Time course of fusion without phagocytic stimulation. PMNs were reincubated in the same buffer as for phagocytosis, but without any phagocytizable particles either at 0°C (○) or at 37°C (●). The fusion indices were calculated and are expressed as relative values. Values are means ± SE for ⩾4 experiments.

the fusion, but PCMPSA had no effect, even at 500 μM. Unlike SH-inhibitors, some SH-reagents slightly enhanced the fusion when added at higher concentrations. Of the metabolic inhibitors tested, NaF and KCN had no effect, but NaN_3 was slightly inhibitory, 2-dGlc, more inhibitory and monoIA the most inhibitory. Of the lectins tested, Con A enhanced the fusion the most, and its effect was reversed by α-MeMan. Other lectins were less effective, but none was inhibitory. The fusion index was not changed by colchicine, but was enhanced by cytochlasin B at 10 μg/ml (table 1).

4. Discussion

Here we show that phagocytic vesicles fused with lysosomes in PMNs and that the fusion was essentially independent of phagocytic stimuli. Our system should thus be useful for further studies on fusion. It may be possible, for example, to study change in the nature of phagocytic vesicles during the process of their fusion with lysosomes [6], and the mechanisms regulating this fusion. With regard to the latter problem, we examined the effects of various reagents on this system. All the SH-inhibitors inhibited the fusion except PCMPSA which does not penetrate into the cells appreciably [9]. On the contrary, various SH-substances tended to enhance the fusion (table 1). Conceivably from these results some SH groups may play a role in regulating fusion inside the cells. Results in other laboratories have also suggested a role of SH-groups in cell fusion [10] and exocytosis [11], although the precise mechanism of their role in membrane fusion is unknown.

Table 1
Effects of various reagents on the fusion index

Reagent		(% of control) fusion index	n
1. SH inhibitors			
NEM	10 μM	103.9 ± 9.4	2
	100 μM	22.7 ± 1.8	2
	1 mM	15.8 ± 9.3	4
PCMB	10 μM	98.1 ± 11.7	2
	100 μM	58.3 ± 8.6	4
	500 μM	18.0 ± 1.6	2
PCMPSA	10 μM	108.7 ± 4.0	2
	100 μM	106.8 ± 12.3	2
	500 μM	84.7 ± 7.2	2
2. SH substances			
DTT	5 mM	127.1 ± 13.5	4
	50 mM	142.2 ± 0.3	2
β-ME	10 mM	128.1 ± 18.2	3
	100 mM	117.4 ± 0.1	2
Cysteine	10 mM	123.6 ± 11.6	4
Glutathione (reduced)	10 mM	100.7 ± 9.1	4
3. Metabolic inhibitors			
NaF	1 mM	89.5 ± 7.8	2
	10 mM	109.2 ± 20.4	4
monoIA	100 μM	110.1 ± 3.3	2
	1 mM	39.6 ± 13.2	2
	10 mM	−4.3 ± 9.4	2
2-dGlc	1 mM	79.5 ± 32.1	2
	5 mM	56.0 ± 5.4	2
KCN	1 mM	96.2 ± 10.7	2
NaN_3	5 mM	84.8 ± 3.3	2
	10 mM	145.4 ± 0.3	2
4. Lectins			
ConA + 1 mM α-MeMan	50 μg/ml	138.5 ± 8.4 99.3 ± 3.4	8 2
RCA + 1 mM Gal	50 μg/ml	108.7 ± 6.6 106.0 ± 11.4	3 2
WGA + 1 mM GlcNAc	50 μg/ml	116.9 ± 1.1 80.8 ± 16.5	4 2
5. Reagents affecting the cytoskeleton			
Colchicine	10 μM	102.0 ± 2.8	4
	100 μM	89.2 ± 13.5	2
Cytochlasin B	10 μg/ml	131.8 ± 6.0	10
	25 μg/ml	139.6 ± 24.3	4

Reagents were added to the PMN cell suspension just before reincubation at the final concentration indicated in the table. Fusion indices are expressed as percentages of the control (without reagents). Values are means ± SE for the numbers of experiments shown under '*n*'

This work also provided information on the relation between fusion of vesicles and the movement of cell surface receptors for lectins and of the cytoskeleton (table 1). All the lectins tested agglutinated PMNs, but only Con A caused enhanced fusion. Colchicine was not effective, but cytochlasin B enhanced the fusion. These findings suggest a relation between fusion of the vesicles and movement of Con A receptors or microfilaments [12,13], but not microtubules [5,14]. The effects of metabolic inhibitors also suggest that the fusion is energy-dependent, possibly owing to movement of microfilaments [15]. Phagocytized bacteria were reported killed in human monocytes after removal of noningested bacteria, but were not killed in these cells in the absence of serum in the medium [16]. Our system should be useful for studies on the mechanism of killing of bacteria and on factors regulating the killing of intracellular parasites [17].

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