Brief Definitive Report

MODULATION OF PHAGOSOME-LYSOSOME FUSION IN MOUSE MACROPHAGES*

BY MARGARET C. KIELIAN AND ZANVIL A. COHN

From The Rockefeller University, New York 10021

In a previous publication (1), we outlined a vital dye method for the quantitative study of phagosome-lysosome (P-L) fusion in intact cells, validated the technique by electron microscopic analysis, and described certain basic regulatory processes of P-L fusion in cultivated mouse macrophages. This report deals with factors that alter the normal pattern of P-L fusion. For this purpose, we have modified various structures and functions of the macrophage and examined the effects on the rate and extent of P-L fusion. Modification of the macrophage plasma membrane or the surface of the ingested particle did not alter P-L fusion. Fusion was also independent of drugs that affect the cytoskeleton. In vivo activation of macrophages dramatically increased both the rate and extent of P-L fusion.

Materials and Methods

Cell Cultures. Primary cultures of peritoneal macrophages were prepared from resident cells of female or male Nelson-Collins strain (NCS) mice and cultured as previously described (1).

Proteose-peptone-elicited macrophages were harvested from NCS mice 3 d after intraperitoneal injection of 1 ml sterile proteose-peptone solution (1% wt:vol in distilled H_2O).

In vivo activated macrophages (2, 3) were kindly supplied by Dr. N. Nogueira, The Rockefeller University, New York and Dr. H. Murray, Cornell Medical School, New York. NCS mice were primed with an i.p. injection of 5×10^6 live culture forms of *Trypanosoma cruzi* and boosted i.p. 4 wk later with 5×10^6 heat-killed *T. cruzi*. Cells were harvested 60 h after boosting. NCS mice were also chronically infected with *Toxoplasma gondii*, challenged i.p. with 5×10^6 heat-killed *T. gondii*, and peritoneal cells were harvested 3 d later.

Fluorescence Assay of P-L Fusion. P-L fusion in cover slip cultures of macrophages was assessed as previously described (1). Briefly, monolayers were labeled for 20 min at 37°C with 5 μg/ml acridine orange (AO), washed, and a dilute suspension of serum-opsonized, heat-killed yeast was then centrifuged onto the cell monolayer at 4°C. Unbound yeast was washed away, and the cultures were rapidly warmed to 37°C to synchronize particle ingestion. Cells were examined by fluorescence microscopy at various time points after an initial 10-min ingestion period. The presence of AO-stained intracellular yeast was considered positive for P-L fusion.

Electron Microscopic Evaluation of P-L Fusion. Horseradish peroxidase (HRP) was used as a marker for secondary lysosomes, as previously described (1).

Covalent Coupling of Concanavalin A to Latex Beads. Concanavalin A (Con A) (Sigma Chemical Co., St. Louis, Mo.) was directly coupled to carboxylate-modified 0.860-μm latex beads (Dow Chemical Co., Midland, Mich.) using a protocol similar to that described by Rutishauser and Edelman (4) for coupling to nylon fibers. 150 μl of latex (10% solids) was washed three times in 0.15 M NaCl, and resuspended in 1 ml of 0.15 M NaCl containing 5 mg Con A and 1.6×10^6 cpm of ^{3}H-labeled Con A (56 Ci/mmol) (Amersham Corp., Arlington Heights, Ill.) 25 mg of the water-soluble carbodiimide 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*P*-toluene sulfonate (CMC) (Pierce Chemical Co., Rockford, Ill.) was then added, and the bead suspension stirred at room temperature for 30 min. The beads were then washed four times

* Supported by grant AI-07012 from the National Institutes of Health.

TABLE I

Factors That Do Not Influence P-L Fusion

	Conditions	Time after particle ingestion	Positive staining with AO	
			Control	Treated
		min	%	
Enzyme* pretreatment of the cell surface	20 U/ml neuraminidase (37°C, 30 min)	40	48	53
	112 U/ml trypsin (37°C, 10 min)	40	65	71
	100 μg/ml pronase (37°C, 30 min)	120	64	76
	100 μg/ml chymotrypsin (37°C, 30 min)	120	64	61
Particle surface treatment	Whole rabbit anti-yeast antiserum versus normal mouse serum (37°C, 30 min)	140	25	29
			HRP stained by EM assay	
			Control	Treated
		min	%	
Particle surface treatment	Con A-coupled latex versus untreated latex	60	>95	>95
Cell surface cross-linking	Con A bound to cell surface 50 μg/ml (4°C, 30 min) versus untreated cells. Latex as test particle.	60	>95	>95

* *Vibrio cholera* neuraminidase was from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif., three-times-crystallized trypsin was from Worthington Biochemical Corp., Freehold, N. J., and pronase and chymotrypsin were from Sigma Chemical Co. EM, electron microscope.

with phosphate-buffered saline (PBS) and could be stored at −20°C in 50% glycerol-PBS for months without a noticeable change in binding activity.

Small samples of beads were assayed for labeled Con A by liquid scintillation counting in Aquasol. Under the above conditions, ~20% of the added Con A was adsorbed to the beads when CMC was not added. This adsorbed Con A could be removed by washing the beads once in 0.3 M NaCl, 10 mM Tris, pH 8.6, plus 0.1% sodium dodecyl sulphate and 0.05% Nonidet P-40. After washing, beads treated with CMC bound 17.6% of the added label or 880 μg Con A per 150 μl of original latex stock. When CMC was not added, 0.07% remained bound after washing.

Con A-coupled beads showed binding activity by their ability to agglutinate yeast into very large mixed aggregates.

Preparation of Anti-Yeast Antibodies. New Zealand white rabbits were injected in the marginal ear vein two to three times weekly for 5 wk with a suspension of whole *Saccharomyces cerivisiae* that had been autoclaved and processed as previously described (1). Each injection was 1 ml, and contained progressively increasing doses of yeast: from 2×10^6 to 5×10^7 cells/ml of 0.9% NaCl. 1 wk after the last injection, heat-inactivated sera gave a tube agglutination titer of 1:1,024, and this sera was used to opsonize yeast particles.

Results

Several intracellular parasites (5) and the plant lectin Con A (6) inhibit the fusion of endocytic vacuoles with lysosomes. These agents present on the luminal surface of the vacuole presumably influence the cytosolic face, inhibiting fusion by an as yet unknown mechanism. We have therefore evaluated several classes of cell surface perturbants for their ability to modify the intracellular fate of plasma membrane-derived phagosomes.

Enzymatic Modification of the Cell Surface. 3-d macrophage cultures were treated with a variety of proteolytic enzymes or with neuraminidase under conditions that are known to degrade protease-sensitive, exteriorly disposed polypeptides and receptors (7). The pretreated cells showed excellent cell viability and phagocytic indices. As shown in Table I, P-L fusion was in all cases similar to that seen in untreated cultures.

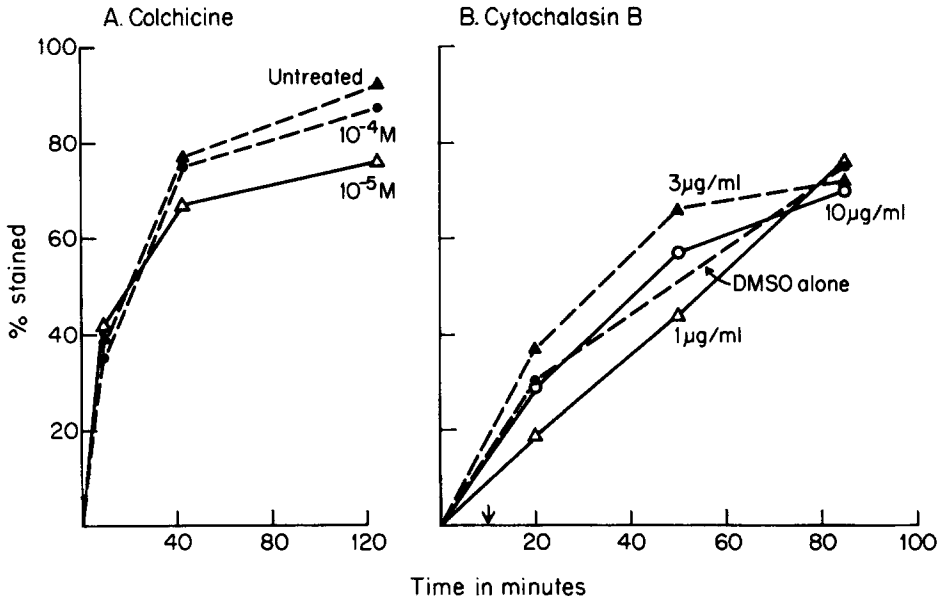

FIG. 1. Effect of cytoskeletal drugs on P-L fusion. (A) Rate of P-L fusion in 4-d cells pretreated for 2 h with 0, 10^{-5}, or 10^{-4} M colchicine. Assay was performed in the continuous presence of the drug. (B) Rate of P-L fusion in 3-d cells treated with 0–10 μg/ml cytochalasin B. Arrow on x axis marks the time of cytochalasin addition. 10 μg/ml is 2.1×10^{-5} M cytochalasin B. DMSO, dimethylsulfoxide.

Particle Opsonization. Fusion inhibition caused by several intracellular parasites can be reversed by coating the parasite with specific immune serum (5). We tested whether the rate of lysosomal fusion with yeast-containing vacuoles could be increased by opsonization with immune serum. Yeast was coated for 30 min at 37°C with either heat-inactivated, whole anti-yeast antiserum (Materials and Methods) or normal mouse serum, washed, and sonicated to break up clumps. As shown in Table I, 1-d cultures showed the usual slow fusion rate (1) after ingesting either particle preparation.

Con A—Soluble or Particle bound. Con A has been previously shown to inhibit pinosome-lysosome fusion in macrophages when added to the culture medium or prebound to the cell surface in the cold (6). The effect of Con A on P-L fusion was evaluated using latex beads covalently coupled to Con A or unmodified latex given to Con A-treated cells. Fusion was evaluated by electron microscopy, using HRP as a lysosomal marker and was in both cases similar to controls (Table I).

Drugs That Affect the Cytoskeleton. Various elements of the cytoskeleton are thought to be involved in the movement or anchoring of vesicles in the cytoplasm and thus could control the rate of fusion of these organelles. Drugs that affect the cytoskeleton were used to evaluate this involvement. Fig. 1A shows that pretreatment with colchicine has no effect on P-L fusion. AO-stained lysosomes in colchicine-treated cells were dispersed in the peripheral cytoplasm instead of being concentrated in the perinuclear region. This alteration of organelle polarization is a characteristic of microtubule depolymerization (8).

Because cytochalasin treatment blocks phagocytosis (9), AO-labeled cells were treated with cytochalasin B 10 min after the start of particle ingestion but before

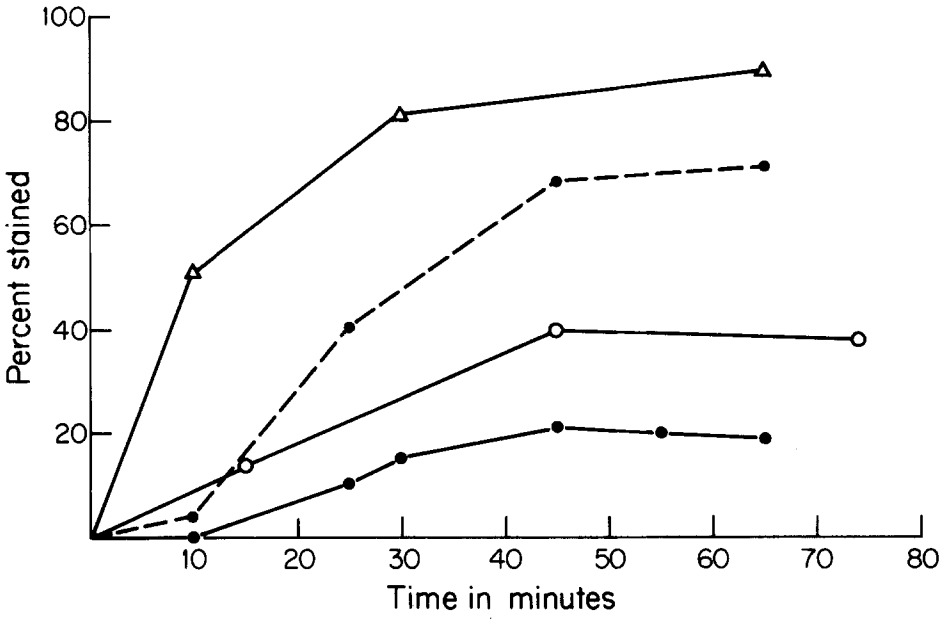

FIG. 2. Effect of in vivo activation on the rate and extent of P-L fusion. Shown are the results of three separate experiments in which *T. gondii* (△), *T. cruzi* (●--●), and proteose-peptone (○) cells were assayed 5–6 h after plating. Results were normalized to the average values for fusion in resident cells (●—●) plated 5–6 h. See Materials and Methods for immunization protocols.

extensive fusion. Fig. 1 B shows that this treatment did not affect fusion rate, although the cells showed the typical rounding and membrane blebbing associated with microfilament disruption (9). Similar results were obtained with cytochalasin D.

In Vivo Macrophage Activation. Activated macrophages have altered size, spreading, endocytic, microbicidal, and secretory properties (10). There is also evidence that the state of activation differs between macrophages involved in a nonspecific inflammation and those activated by sensitized lymphocytes or lymphokines. P-L fusion was assayed in several types of in vivo activated populations (Fig. 2) and was found to be considerably elevated compared with the low levels seen in resident cells soon after plating. The most striking increases were seen in microbicidal cells from *T. gondii*- or *T. cruzi*-infected and challenged mice. A smaller increase was observed in inflammatory cells elicited by proteose-peptone, a nonspecific irritant.

Discussion

Because the phagocytic vacuole is derived from the plasma membrane, it was postulated that some modification of this structure might result in organelles that would exhibit reduced fusion with lysosomes. Neither enzymatic digestion nor cell surface cross-linking by the lectin Con A had any influence on P-L fusion. Furthermore, opsonization with immune serum or Con A coating of the phagocytic particle itself did not modify P-L fusion. The presumed trans-plasma membrane inhibition of fusion caused by live parasites (5) or by Con A in pinocytic vesicles (6) is thus far without counterpart in this system.

The striking difference in the effect of Con A on the fusability of pinocytic and phagocytic vacuoles is another example of the distinction between these forms of endocytosis (11). Pinocytosis is a constitutive cell process in which extracellular fluid

and any plasma membrane-adsorbed ligands are interiorized. It is linear with temperature from 2 to 37°C, has an energy of activation of 18–25 kcal/mol (11, 12), and is little affected by cytoskeletal drugs (11). In contrast, phagocytosis is induced by the interaction of large particulates with the plasma membrane and results in their uptake while excluding most extracellular fluid. It is inhibited by cytochalasin and temperatures below 18–21°C and has an energy of activation of 54 kcal/mol (12). In terms of their fusion with lysosomes, both phagocytic vacuoles (1) and pinocytic vacuoles (13) appear not to fuse at temperatures below ~20°C, whereas only pinosome-lysosome fusion is inhibited by Con A.

Role of the Cytoskeleton in Vesicle Fusion. Macrophage microtubules have been postulated to control non-Brownian saltatory movements of organelles (8) and thus could influence vesicle contact and fusion. 2 h of pretreatment of macrophages with 10^{-6} M colchicine has been shown to abolish identifiable microtubules in electron microscopic samples of treated macrophages (14). Our detailed studies of P-L fusion show that its rate and extent are not affected by 10^{-5}–10^{-4} M colchicine. Thus, like Bhisey and Freed (8) and Pesanti and Axline (14), we conclude that microtubules do not seem necessary to direct P-L fusion.

During phagocytosis, a filamentous network enriched in actin, myosin, and actin-binding protein (11) assembles beneath the plasma membrane at the site of ingestion, excluding lysosomes and other organelles (15). Treatment of macrophages with cytochalasins B and D was used to evaluate the role of this network in the control of P-L fusion rate. Within 10 min after the addition of 1×10^{-5} M cytochalasin B, organized microfilaments in macrophages are disrupted and phagocytosis is inhibited (9). In vitro, this compound prevents the gelling of actin by actin-binding protein (16, 17). Within the time limits of our experimental system, these drugs did not affect P-L fusion, implying that the actomyosin system does not control fusion in any rate-limiting way.

P-L Fusion in Activated Macrophages. Parasites such as live *T. gondii* are known to inhibit P-L fusion in resident but not activated macrophages (5). This report demonstrates that the rate and extent of P-L fusion are greatly increased in activated cells when an inert particle such as killed yeast is considered. The increase in fusion correlates with the activation state of the macrophages, being highest in microbicidal cells and somewhat less in inflammatory cells. Inflammatory cells show increased size and spreading, stimulation of glucose oxidation and oxygen consumption, alterations of plasma membrane ectoenzyme levels, increased endocytic activity, and enhanced secretion of neutral proteases. Microbicidal cells express these properties and, in addition, display enhanced production of H_2O_2 and increased tumor cell and parasite killing (10). Enhanced P-L fusion is another trait of activated cells acquired during exposure to the humoral and cellular effectors of the activation process. It may be a consequence of the aforementioned metabolic and membrane alterations in these cells.

Summary

A previously described fluorescence assay has been used to characterize factors that modulate phagosome-lysosome (P-L) fusion in mouse macrophages. Fusion was not affected by enzymatic modification or by concanavalin A cross-linking of the plasma membrane or by coating the phagocytic particle with concanavalin A or immune serum. Pretreatment of cells with 10^{-5}–10^{-4} M colchicine, or treatment immediately after ingestion with 1–10 μg/ml cytochalasin did not alter P-L fusion; implying that

the cytoskeleton does not control fusion in a rate-limiting way. Fusion was strikingly elevated in 5-h cultures of activated macrophages from immune-boosted mice. A lower enhancement was seen in cells activated by proteose-peptone, a nonspecific inflammatory agent.

We thank Richard Huntley for technical assistance, Grace Silvestri for typing the manuscript, and Dr. Marcus Horwitz for help in raising the antisera.

Received for publication 30 December 1980.

References

1. Kielian, M. C., and Z. A. Cohn. 1980. Phagosome-lysosome fusion. Characterization of intracellular membrane fusion in mouse macrophages. *J. Cell Biol.* **85**:754.
2. Nogueira, N., S. Gordon, and Z. A. Cohn. 1977. *Trypanosoma cruzi*: modification of macrophage function during infection. *J. Exp. Med.* **146**:157.
3. Murray, H. W., C. W. Juangbhanich, C. F. Nathan, and Z. A. Cohn. 1979. Macrophage oxygen-dependent antimicrobial activity. II. The role of oxygen intermediates. *J. Exp. Med.* **150**:950.
4. Rutishauser, U., and G. Edelman. 1976. Fractionation and manipulation of cells with concanavalin A-coated fibers. *In* Concanavalin A as a Tool. H. Bittiger and H. P. Schnebli, editors. John Wiley & Sons Ltd., Chichester, England. 447.
5. Goren, M. B. 1977. Phagocyte lysosomes: interactions with infectious agents, phagosomes, and experimental perturbations in function. *Annu. Rev. Microbiol.* **31**:507.
6. Edelson, P. J., and Z. A. Cohn. 1974. Effects of concanavalin A on mouse peritoneal macrophages. I. Stimulation of endocytic activity and inhibition of phagolysosome formation. *J. Exp. Med.* **140**:1364.
7. Nogueira, N., and Z. A. Cohn. 1976. *Trypanosoma cruzi*: mechanism of entry and intracellular fate in mammalian cells. *J. Exp. Med.* **143**:1402.
8. Bhisey, A. N., and J. J. Freed. 1971. Ameboid movement induced in cultured macrophages by colchicine or vinblastine. *Exp. Cell Res.* **64**:419.
9. Axline, S. G., and E. P. Reaven. 1974. Inhibition of phagocytosis and plasma membrane mobility of the cultivated macrophage by cytochalasin B. *J. Cell Biol.* **62**:647.
10. Cohn, Z. A. 1978. The activation of mononuclear phagocytes: fact, fancy and future. *J. Immunol.* **121**:813.
11. Silverstein, S. C., R. M. Steinman, and Z. A. Cohn. 1977. Endocytosis. *Annu. Rev. Biochem.* **46**:669.
12. Mahoney, E. M., A. L. Hamill, W. A. Scott, and Z. A. Cohn. 1977. Response of endocytosis to altered fatty acyl composition of macrophage phospholipids. *Proc. Natl. Acad. Sci. U. S. A.* **74**:4895.
13. Dunn, W. A., A. L. Hubbard, and N. N. Aronson, Jr. 1980. Low temperature selectively inhibits fusion between pinocytic vesicles and lysosomes during heterophagy of ^{125}I-asialofetuin by the perfused rat liver. *J. Biol. Chem.* **255**:5971.
14. Pesanti, E. L., and S. Axline. 1975. Phagolysosome formation in normal and colchicine-treated macrophages. *J. Exp. Med.* **142**:903.
15. Korn, E. D., B. Bowers, S. Batzii, S. R. Simmons, and E. J. Victoria. 1974. Endocytosis and exocytosis: role of microfilaments and involvement of phospholipids in membrane fusion. *J. Supramol. Struct.* **2**:517.
16. Hartwig, J. H., and T. P. Stossel. 1976. Interactions of actin, myosin and an actin-binding protein of rabbit pulmonary macrophages. III. Effects of cytochalasin B. *J. Cell Biol.* **71**: 295.
17. Weihing, R. R. 1976. Cytochalasin B inhibits actin-related gelation of HeLa cell extracts. *J. Cell Biol.* **71**:303.