PHAGOSOME-LYSOSOME FUSION

Characterization of Intracellular Membrane Fusion in Mouse Macrophages

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ABSTRACT

Several approaches have been used to study the determinants of phagosome-lysosome fusion in intact mouse macrophages. Lysosomes were labeled with the fluorescent vital dye acridine orange and the rate and extent of their fusion with yeast-containing phagosomes was monitored by fluorescence microscopy. Fusion was also assayed by electron microscopy, using horseradish peroxidase or thorium dioxide as a marker for secondary lysosomes. Good agreement was found with results obtained from vitally stained cells, thin-section samples with an enzymatic marker, and thorium dioxide–labeled samples evaluated by stereology.

The rate of fusion as assayed by fluorescence was not affected by the number of particles ingested, serum concentration, or prior uptake of digestible or nondigestible substances. With this assay it was possible to observe the rate of fusion separate from and uninfluenced by the phagocytic rate. Both the rate and extent of fusion were dramatically increased after several days in culture and similar changes were found by use of the EM assays. Fusion was strongly affected by incubation temperature, having a Q_{10} of 2.5. No detectable fusion occurred below 15°C, and this inhibition was rapidly reversed when cells were returned to 37°C.

Intracellular membrane fusions serve to link discrete, discontinuous cellular compartments, leading to the mixing, export, or intake of exogenous and biosynthesized macromolecules. Major vectorial pathways include the centrifugal flow of secretory proteins from their point of synthesis in the rough endoplasmic reticulum (RER) to their exocytosis at the plasma membrane. The reverse flow of endocytic vesicles into the inner organelles of the vacuolar apparatus represents another route, one recently coupled to the recycling of interiorized plasma membrane (18, 27). Although membranes of apparently different origins fuse, the process is restricted within these limits and indiscriminate fusions do not occur. For this reason, some form of membrane-membrane recognition is likely. Other than a descriptive analysis of the ultrastructural and freeze-fracture patterns of fusions, little insight into the involved mechanism is available in eukaryotic systems.

One of the obstacles to gaining information about the fusion of phagosomes with lysosomes (P-L fusion) has been the lack of a rapid, quantitative assay for the rate and extent of fusion. For this purpose we have examined a sensitive vital dye method used by Hart and Young (12) and applied it to the study of the highly endocytic mouse peritoneal macrophage. In this report we

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establish conditions for the reproducible assessment of P-L fusion and compare conditions at both the light and EM level. In addition, we have determined the effects of particle number, composition, and size, ambient temperature, and prolonged in vitro cultivation on the fusion process.

MATERIALS AND METHODS

Cell Culture

Primary cultures of peritoneal macrophages were prepared from resident cells of female or male Nelson-Collins strain mice (22–30 g) (5). Cells were routinely cultured in 15–20% fetal calf serum (FCS; heat inactivated at 56°C for 30 min) in Dulbecco's modified Eagle's medium (MEM) containing 100 U/ml penicillin and 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B, and were given fresh medium at least every other day. Cells were plated at densities of 4×10^5 peritoneal exudate cells per 12-mm glass coverslip for fluorescence studies, or at $6–10 \times 10^6$ peritoneal cells per 35-mm dish for electron microscopy.

$P388D_1$, a macrophagelike cell line, was obtained from Dr. J. Unkeless and maintained in spinner culture in 10% FCS/MEM (30).

Culture dishes with 16-mm wells were obtained from Costar, Data Packaging, Cambridge, Mass.

Particle Preparation for Fusion Studies

Fresh baker's yeast was processed by the method of Lachman and Hobart (15). This procedure consists of autoclaving, reduction by mercaptoethanol, alkylation with iodoacetamide, and extensive washing in phosphate-buffered saline (PBS). The resulting preparation could be stored indefinitely in PBS without Ca^{2+} and Mg^{2+} plus 0.02% NaN_3.

To opsonize particles with complement components, 300 μl of a 5% suspension of yeast in veronal buffer was mixed with 300 μl of mouse serum (fresh or stored at −70°C), incubated 30 min at 37°C, and washed several times in cold veronal buffer. Preparations were used within 3 d of opsonization.

Latex particles of 0.50, 1.10, 2.02, 3.14, 4.27, 5.7, and 8.0 μm Diam were obtained from Dow Chemical Co., Indianapolis, Ind., and Duke Scientific Corp., Palo Alto, Calif., washed five times in PBS, and stored at 4°C until used.

Fluorescence Assay of P-L Fusion

Monolayer cultures on 12-mm glass coverslips were labeled for 20 min at 37°C in 15% FCS/MEM by adding acridine orange (AO) stock solution to a final concentration of 5 μg/ml. A stock solution of 100 μg AO/ml PBS could be stored in the dark at 4°C for 3–4 wk. Each coverslip was then placed in a 16-mm-Diam well of a plastic culture dish containing 1 ml of prewarmed medium, and incubated for 10 min at 37°C to reduce background fluorescence and decrease photodamage. The medium was then aspirated and 1 ml of a cold 0.004% (vol/vol) suspension of opsonized yeast was added per well. The yeast suspension was centrifuged onto the cell monolayer by placing the culture dish in a centrifuge carrier designed for microtiter plates (Cooke Laboratory Products, Alexandria, Va.) and centrifuging in an International centrifuge (International Equipment Co., Needham Heights, Mass.) at 1,200 rpm for 2 min at 4°C. The yeast particles bound to the macrophage complement receptor, and any unbound particles were removed by washing with cold MEM. Cold 5% FCS/MEM was added, the cultures were rapidly warmed to 37°C, and relatively synchronous ingestion of the bound yeast resulted. The usual number of yeast particles bound and ingested per cell was 2. Cells were examined by fluorescence microscopy at various time points after an initial 10-min ingestion period. The presence of orange-stained intracellular yeast was considered positive for P-L fusion.

AO Quantitation

Coverslip cultures were pulsed with 5 μg/ml AO in FCS/MEM for 20 min, washed, and extracted in 95% ethanol. Fluorescence of samples was compared to standards in the same solvent, using an MPF-44 fluorometer (Perkin-Elmer Corp., Norwalk, Conn.) with an excitation wavelength of 490 nm and emission wavelength of 520 nm. Values were related to the amount of cell protein on parallel coverslips, using the fluorescamine protein assay (3) with bovine serum albumin as a standard. Acridine standards in solvent with or without unlabeled cells were comparable.

Temperature Studies

After an initial phagocytosis step at 37°C, cultures were maintained at temperatures from 15° to 30°C in a water-jacketed chamber (17) gassed with 5% CO_2/95% air. Temperature was controlled by use of a constant-temperature circulating water bath and cultures were kept at 2°C in an ice bath.

Fluorescence Microscopy

AO-labeled cells were prepared by inverting coverslips over a drop of ice-cold PBS, blotting, and rimming with nail polish. These live specimens were then examined with a Zeiss photomicroscope III, using a mercury lamp adjusted for epi-illumination, BG-12 filter, and fluorescein dichroic mirror for excitation, and a 53 barrier filter. Black and white pictures were taken with Kodak Tri-X film.

Electron Microscopy

Monolayers were fixed for 30 min at room temperature in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After three saline washes, horseradish peroxidase (HRP) was visualized in labeled cultures by the Graham and Karnovsky diaminobenzidine method (11). Cultures were then postfixed in 1% osmium tetroxide in 0.1 M cacodylate (pH 7.4) for 60 min on ice, and stained with 0.25% uranyl acetate in 0.1 M sodium acetate buffer for 30 min at room temperature. Specimens were dehydrated in graded alcohols and embedded in Epon. Thin sections were examined in a Siemens Elmiskop II with or without uranyl acetate and lead citrate staining.

Scanning electron microscopy was kindly performed by Dr. Gilla Kaplan. Monolayer cultures on 12-mm coverslips were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Specimens were dehydrated in alcohol, transferred to amyl acetate and critical point dried (Sorvall critical point drying system; DuPont Co., Sorvall Biomedical Div., Norwalk, Conn.) in liquid CO_2. The cells were gold coated (Edwards 5150 sputter coater, Grand Island, N. Y.) and viewed with an ETEC autoscan.

EM Evaluation of P-L Fusion

Two markers for secondary lysosomes were used, HRP and colloidal thorium dioxide (Thorotrast). Cultures were pulsed with 2 mg/ml HRP in medium for 2–3 h, washed four times with

MEM, and cultured 20–60 min longer in medium without HRP. Cells were then given yeast or latex particles and fixed 1 h after ingestion. Specimens were processed for EM as described above, and thin sections examined without uranyl acetate and lead citrate staining. Phagocytic vacuoles were evaluated for the presence of a diffuse "rim" of electron-dense HRP reaction product.

Nondigestible Thorotrast is a useful marker because cell populations can be labeled at identical times in culture, when pinocytic uptake of the marker is similar. Cells were washed as usual 2 h after plating, exposed to a 1:100 dilution (vol/vol) of colloid in medium for 12 h, washed four times with MEM, and then cultured for a total of 1–4 d. Cells were fixed 1 h after particle ingestion. Stereology was used to evaluate the extent of fusion in specimens labeled with this particulate marker.

Stereological Analysis of Thorotrast-Labeled Preparations

Random samples were obtained from monolayer cultures fixed and processed as described above. At the propylene oxide step, the monolayer floated off the plastic dish and could then be further washed and embedded as a pellet in Epon. Random sections through this pellet were selected, and micrographs of most cells on a section were taken at the same final magnification. These negatives were projected through a 3 × enlarger onto a grid of 1-cm squares. Counts were made, for both horizontal and vertical lines, of the number of times a line crossed the membrane of the phagocytic vacuole vs. the number of times the line crossed Thorotrast in the vacuole. Thorotrast was present as discrete particles and distributed linearly around the rim of the vacuole. Counts were expressed as percent of total possible crossings that were Thorotrast positive.

For each determination, 25–60 vacuoles were evaluated, and total line crossings of 600–1,500 were obtained.

Reagents

FCS, newborn calf serum (NCS), and MEM were obtained from the Grand Island Biological Co., Grand Island, N. Y. Diaminobenzidine was obtained from Sigma Chemical Co., St. Louis, Mo., and AO, from the National Biological Stains Department of Allied Chemical Corp., New York, N. Y. Fluorescamine was obtained from Roche Diagnostics Div., Hoffmann-La Roche Inc., Nutley, N. J. and Thorotrast from Fellows Testagar Div., Fellows Mfg. Co., Inc., Detroit, Mich.

RESULTS

A Fluorescence Assay for P-L Fusion

The general method of Hart and Young (12) was employed to evaluate the extent of P-L fusion at the light microscope level. This method uses the fluorescent, vital dye acridine orange, a weak base that is selectively concentrated in the acid environment of lysosomes (1, 13, 25). Prestained, viable cells are exposed to phagocytosable particles and the transfer of lysosomal fluorescence into phagocytic vacuoles is evaluated by fluorescence microscopy. Most of the following experiments used nonviable yeast particles that were fully permeable to the dye and fluoresced uniformly when AO was transferred into the vacuole. To compare rates of P-L fusion in different cell populations, we analyzed the experimental variables involved.

The Effect of Phagocytic Rate

To study the rate of P-L fusion, it was necessary to dissociate this process from phagocytosis. This was examined by means of scanning electron microscopy. Opsonized yeast was centrifuged onto 1-d macrophage monolayers at 4°C and either fixed immediately (Fig. 1 *a*) or after a 10-min incubation at 37°C (Fig. 1 *b*). At 4°C, an average of 1.4 particles was bound to the cell surface, whereas after 10 min at 37°C, 94% of the particles had been ingested. Thus, phagocytosis was essentially complete at the first time point of the fusion assay, and additional particle uptake would not complicate long-term assays. Similar results were obtained by phase and fluorescence microscopy.

The Influence of Phagocytic Load on the Extent of P-L Fusion

By use of this assay system, the effects of phagocytic load could be examined on an individual cell basis. Fig. 2 shows the percent of yeast particles that were positively stained as a function of the number of particles ingested per cell. At 60 min after ingestion, the percent of stained particles was not affected by the amount of uptake, and similar results were observed at 30 min. Thus, the extent of P-L fusion is independent of the number of particles ingested, and there is enough AO concentrated by a cell to stain at least five particles. Therefore, variations in the number of particles ingested do not affect the subsequent results.

Choice of Particle

The particles used for this assay were heat treated, reduced, alkylated yeast opsonized with fresh mouse serum. These particles are very uniform in binding, uptake, and fluorescent staining characteristics. Because the particles are permeable to the dye but do not concentrate it, their color in the cytoplasm is an indication of the dye concentration in the phagocytic vacuole and therefore the extent of P-L fusion. The concentration of the dye is higher for orange-stained vacuoles than for green-stained vacuoles, as is known from the metachromatic properties of AO (16, 31). Thus, although it cannot be concluded that no fusion has occurred in green-stained vacuoles, the relative amount is less, and orange is the endpoint counted

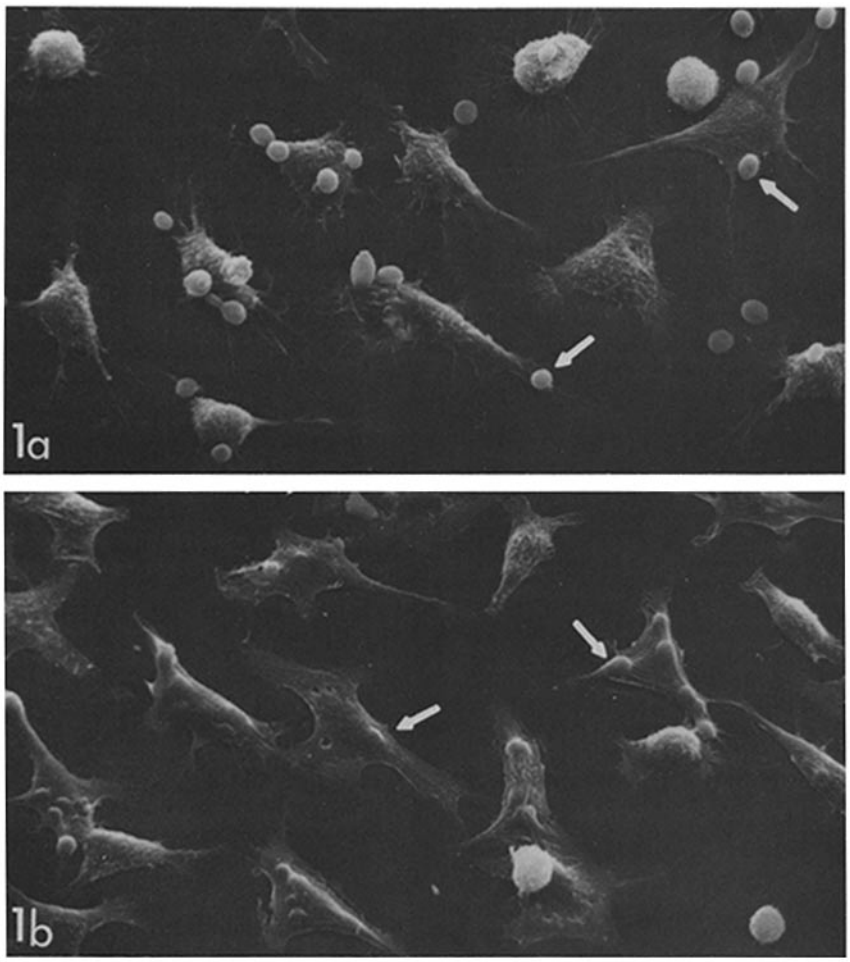


FIGURE 1 Scanning EM of cells before and after phagocytic pulse. Performed by Dr. Gilla Kaplan. (*a*) 24-h monolayer cultures were exposed to opsonized yeast and fixed immediately without a 37°C incubation. Almost all yeast particles (arrows) are extracellular and associated with either the cell body or a cytoplasmic process. The average number of particles per cell is 1.4. × 1,080. (*b*) As in *a*, except that cells were incubated for 10 min at 37°C. Cells have respread and 94% of the particles have been ingested at this time point. The outlines of phagocytosed yeast (arrows) can be seen. × 540.

as positive for fusion. This endpoint is easily distinguished and is stable for hours in viable cells, allowing long-term experiments.

Several other particles were tested in the fluorescence assay and proved less suitable for this type of analysis. Live yeast did not appear freely permeable to AO, but showed instead a rim of fluorescence that was more difficult to distinguish. Viable yeast cells are also capable of delaying the fusion process, as has been previously noted (12). Antibody-coated red blood cells were not appropriate particles, partly because of their rapid intracellular digestion. Finally, polystyrene latex spheres proved unsuitable because they bound AO nonspecifically in the extracellular milieu.

Fig. 3 *a* and *b* shows representative fluorescence pictures of 1- and 4-d-old cultures after ingestion of opsonized yeast. Pinpoint lysosomal staining can be seen, with the absence of appreciable nuclear or cytoplasmic background. Positive yeast appear very brightly fluorescent, whereas negative yeast are much more faintly stained and show a greenish fluorescence under the microscope. In 1-d cultures, numerous negative particles are seen 160 min after ingestion, whereas, in 4-d cultures, almost all particles are positive after 40 min.

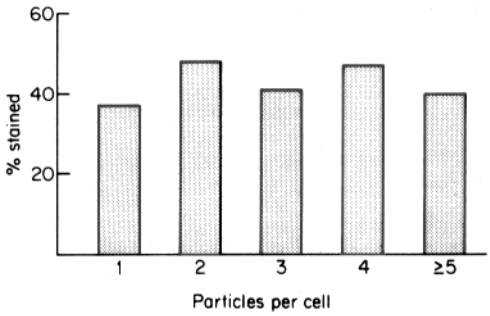


FIGURE 2 Effect of the number of interiorized particles on the rate of P-L fusion. A fusion assay of a 2-d culture, 60 min after the start of ingestion. The percent of intracellular particles stained is plotted as a function of the number of particles that each cell has ingested.

Effect of Culture Time on the Rate of P-L Fusion

This difference in fusion rate was examined in greater detail. These results are presented in Fig. 4, which shows the fusion rate in cells cultured in vitro for 5–96 h. At early times after culture, the majority of yeast particles failed to fuse even several hours after ingestion and a plateau was reached at ~60 min. When these cells were cultured for an additional 24 h, both the rate and extent of fusion increased; this trend continued up to 96 h of culture. In 4-d cells, the initial rate of fusion was about eightfold higher than at 5 h and resulted in the positive staining of 90% of the particles 60 min after ingestion. When 1-d cells with intracellular particles were cultured overnight, 90% of the particles then stained positively, showing that P-L fusion was continuing at this slow rate.

These striking differences in the rate of P-L fusion might be the result of the amount of AO concentrated by the cells. For this reason, quantitative studies on the uptake of AO by 1- and 4-d cells were conducted and are presented in Table I. No significant difference in dye uptake was noted between parallel coverslip cultures or in data normalized for cell protein, and this mechanism could not explain the progressive change in fusion rates.

The rate of P-L fusion was also determined in the phagocytic, macrophagelike cell line $P388D_1$ (14, 30). These cells replicate in culture, unlike the nondividing freshly explanted peritoneal macrophage. When $P388D_1$ cells growing in spinner culture were plated on coverslips and examined 1 and 24 h thereafter, the rate and extent of P-L fusion were rapid and similar to those of 4-d cultures of peritoneal macrophages.

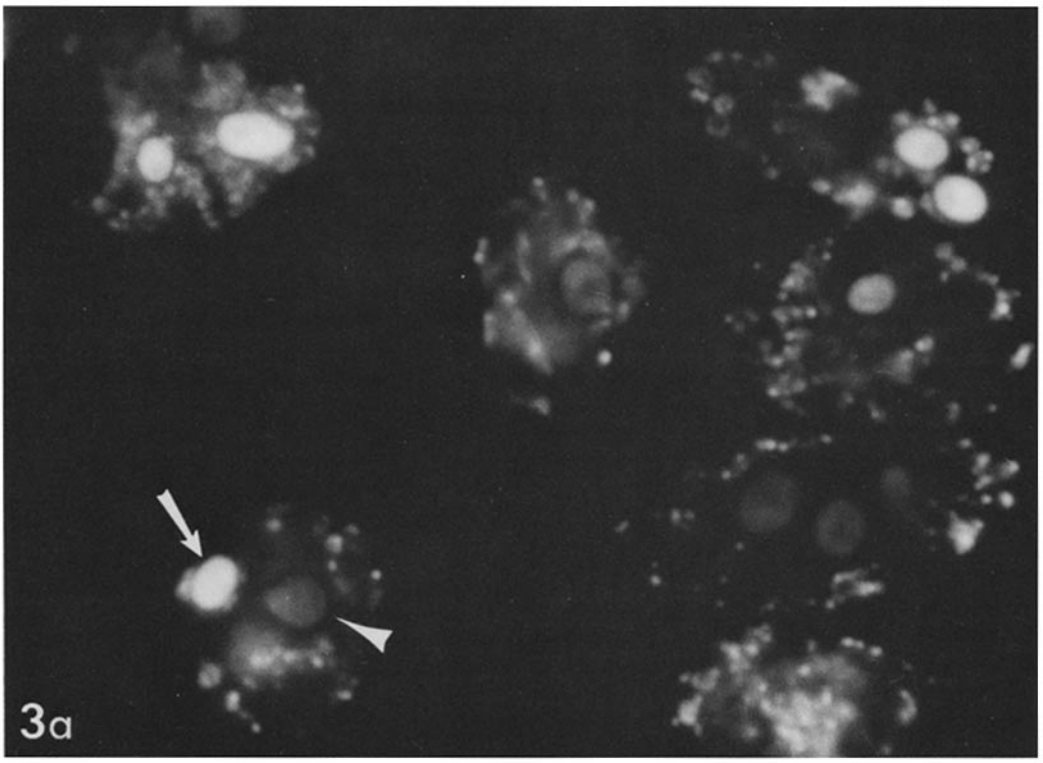
Correlation with EM Assays

We next thought it important to show a correlation of these results with other assay systems and therefore followed the fusion of phagosomes with lysosomes that had been prelabeled with electron-dense or enzymatic markers. Cultures were pulsed with 2 mg/ml HRP for 3 h, chased for 60 min with normal medium, given test particles to ingest, and fixed 1 h after the phagocytic pulse. Fig. 5 *a* shows an example of P-L fusion in these cells after ingestion of 3.14-μm latex spheres. Electron-dense reaction product from the diaminobenzidine staining is seen in secondary lysosomes and also in a rim around the particles. Fig. 5 *b* shows a similar assay in cells that were labeled for 12 h with Thorotrast and then cultured for 4 d. Labeling of secondary lysosomes and of a yeast-containing vacuole is seen. Stereology (see Materials and Methods) was used to compare the extent of fusion in specimens labeled with this particulate marker.

Results from all of these assays for P-L fusion are presented in Table II. In each assay system, fusion is greater after the macrophages had been cultured for 4 d. Differences between 1- and 4-d cultures are about twofold for all assays. Thus, these three assay systems are in relative agreement, in spite of the use of different markers, stereological analysis, and thin sections as opposed to whole cell preparations.

We noticed in the course of these studies that lysosome fusion, as evaluated by transfer of HRP, occurred to a much greater extent with latex phagosomes than with yeast phagosomes. When this was evaluated in 1-d cultures (Table II), latex phagosomes showed more fusion than yeast-containing vacuoles in either 1- or 4-d cultures. This

FIGURE 3 The appearance of acridine orange–stained cells in 1- and 4-d cultures. (*a*) 1-d cells 160 min after yeast ingestion. Both positively (arrow) and negatively (arrowhead) stained yeast are seen several hours after ingestion, as well as abundant lysosomal staining. × 2,000. (*b*) 4-d cells 40 min after ingestion. All ingested particles are brightly stained (arrow) and lysosomal staining is seen in cells containing several positively stained yeast. × 2,130.



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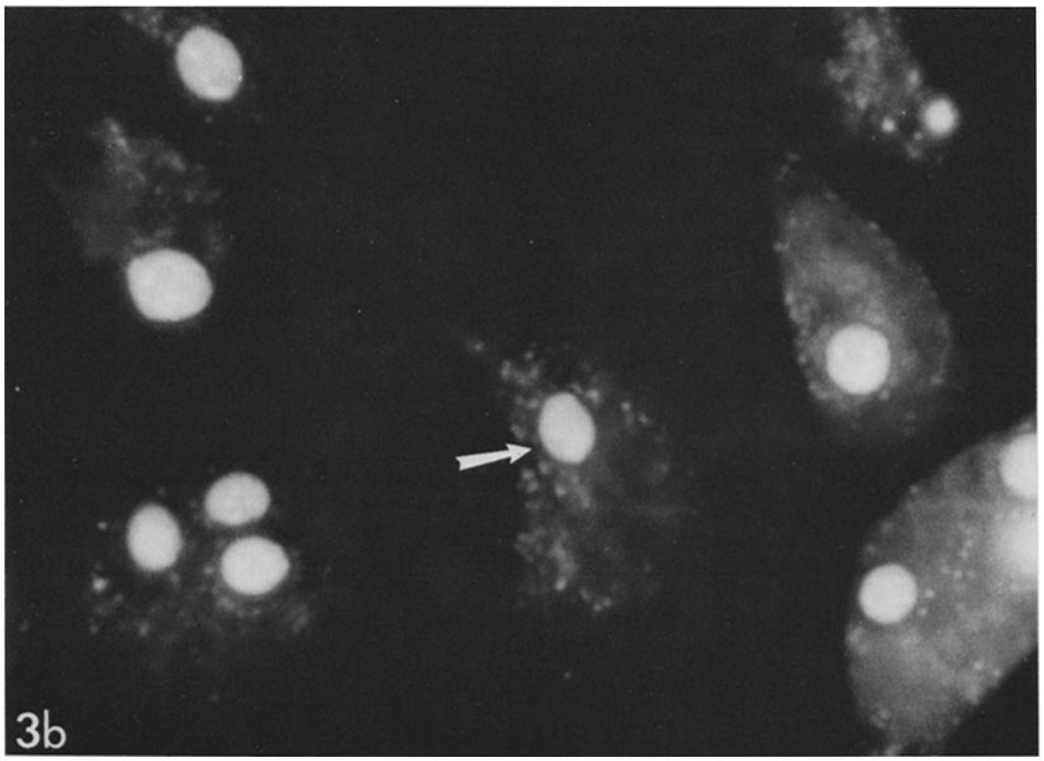

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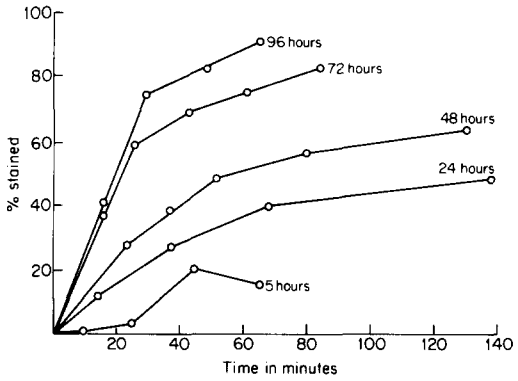


FIGURE 4 Effect of in vitro cultivation on the rate and extent of P-L fusion. The percent of intracellular particles stained is plotted vs. time after particle ingestion. Time in culture is shown in hours. Average results from three separate experiments.

result does not appear to be attributable to the smaller size of 1.1-μm latex particles, because in another experiment, latex particles of 0.5, 1.1, 2.02, 3.14, 4.27, 5.7, and 8 μm Diam showed from 80 to 90% fusion within 1 h after ingestion.

Effect of Lysosome Size on the Rate of P-L Fusion

The increased rate of P-L fusion in cultured macrophages might also reflect modifications in the size of the secondary lysosomal compartment. This possibility was examined by use of two previously described methods for increasing the size of the lysosome and the relative intracytoplasmic volume of the lysosomal pool. For this purpose, cells were cultured either in 40% NCS (6) or in medium containing 0.3 M sucrose (7). At 48 h after cultivation in 40% NCS, AO-stained cells showed the increase in lysosome size (Fig. 6). When such cells were employed for studies of lysosomal fusion (Fig. 7*b*), there was no alteration in either the rate or extent of P-L fusion. Similar results were obtained with sucrose-laden cells (Fig. 7*a*). In addition, cells preloaded with either 1.1-μm latex or *Micrococcus lysodeikticus* showed no change in fusion rate. Thus, prior uptake of digestible or nondigestible agents of either a soluble or particulate nature did not influence fusion with lysosomes.

TABLE I

Comparison of Acridine Orange Uptake by 1- and 4-d Cultures

Sample	AO/protein*
	pmol/μg
1-d Cultures	49.3 ± 16 (6)
4-d Cultures	54.3 ± 15 (6)

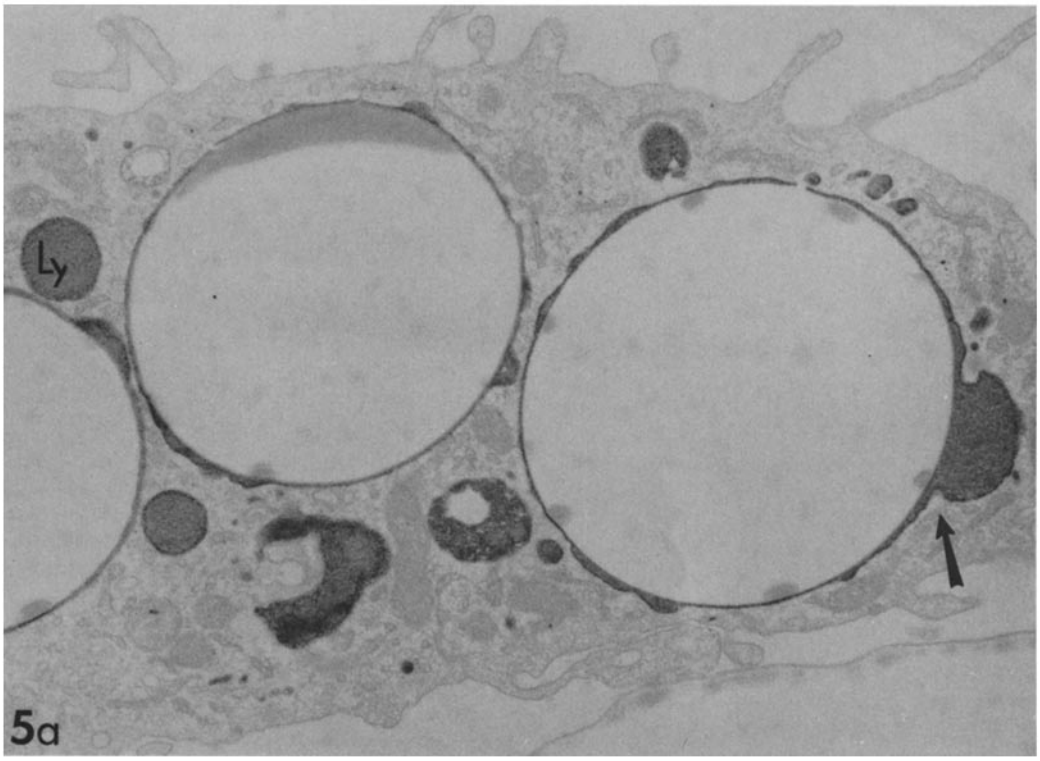
* Mean and standard deviation of six separate experiments.

Effect of Temperature

Phagocytosis and pinocytosis require membrane fusion to form the endocytic vacuole, and both processes show a strong temperature dependence. We examined the effect of temperature on P-L fusion, taking advantage of the observation that in early cultures fusion continues for several hours after ingestion is complete. AO-labeled 2-d cells were pulsed with opsonized yeast and, after 10 min of phagocytosis at 37°C, were shifted to various temperatures. Fig. 8 shows the effect of these incubation temperatures on subsequent P-L fusion. A strong temperature dependence was observed. Fusion continued but with decreasing rates for temperatures from 37° to 20°C. At 15°C no further fusion was observed and the inhibition appeared as great as that seen at 2°C. Fig. 9 shows that the inhibition at 2°C is readily reversible. Fusion resumes when these cells are returned to 37°C, and the rate is similar to that in control cells until a similar extent of fusion is reached.

The log of the initial rate of fusion for temperatures from 20° to 37°C was plotted against the reciprocal of the incubation temperature in degrees Kelvin, giving a linear plot with a correlation coefficient of 0.97 (see *inset*, Fig. 8). From this Arrhenius plot, an energy of activation E_{act} for P-L fusion was obtained of 16.4 kcal/mol, with a Q_{10} of 2.5. This is comparable to the E_{act} for pinocy-

FIGURE 5 EM evaluation of P-L fusion, using electron-dense lysosomal markers. (*a*) 2-d cells pulsed with HRP, and given 3.14 μm latex spheres. Note reaction product in secondary lysosomes (*Ly*), one of which has partially fused with a latex-containing phagosome (arrow). All latex vacuoles have a rim of reaction product. Much of the latex was dissolved by the propylene oxide. × 6,807. (*b*) 4-day cells prelabeled with Thorotrast and given opsonized yeast. Thorotrast is seen in secondary lysosomes (*Ly*), and distributed linearly around an ingested yeast (*Y*). × 15,000.



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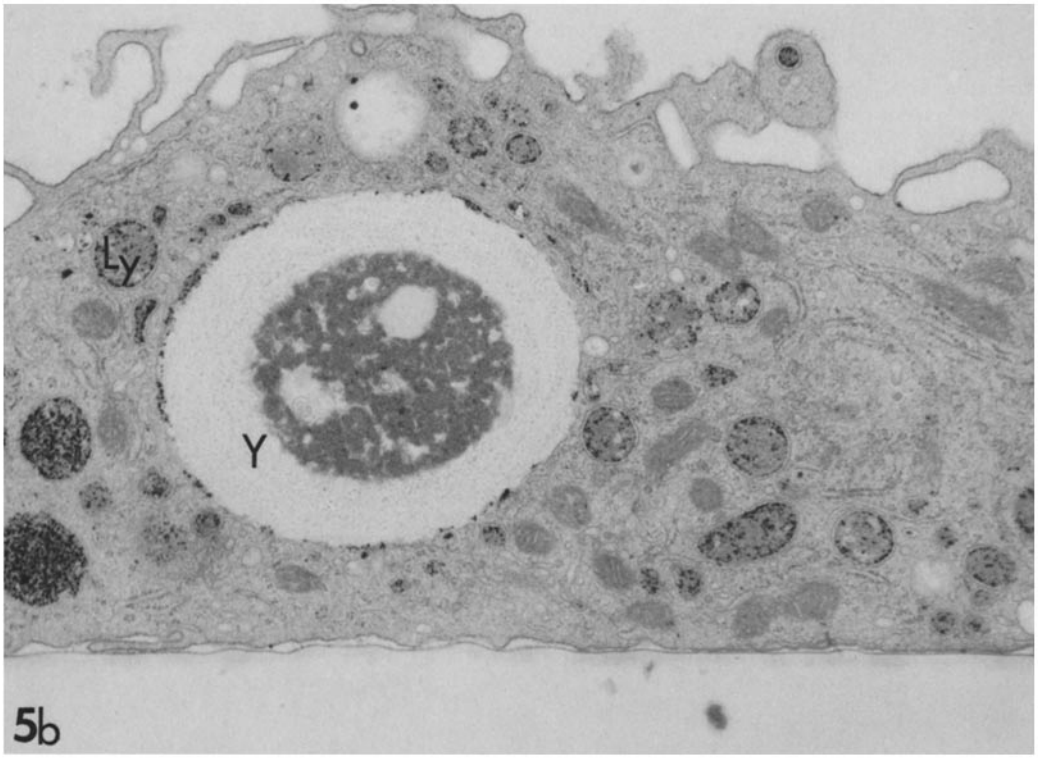

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TABLE II

Comparison of Various Assay Systems for P-L Fusion

Assay conditions	Fusion after 1 h*		
	Acridine assay	HRP-EM assay	Thorotrast-EM assay
Cultured 1 d	37 ± 5.8 (4)‡	25.9 ± 6.2 (3)	16.9 (2)
Cultured 1–2 d; 0.5–3.14 μm latex particles	—	85.5 ± 7.6 (5)	—
Cultured 4 d	85 ± 13.6 (5)	57.5 (2)	37.3 ± 4.9 (3)

* Percent and standard deviation.

‡ The number of experiments is given in parentheses.

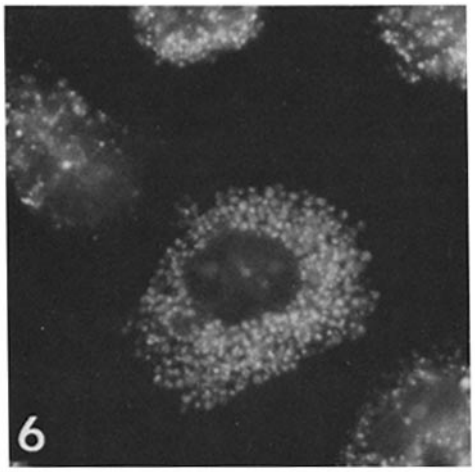

FIGURE 6 Cells cultured for 2 d in 40% newborn calf serum before acridine orange staining. The size of the AO-stained lysosomes is increased as compared to the cells cultured in normal medium (see Fig. 3). Cells grown in 0.3 M sucrose show a similar increase. × 1,200.

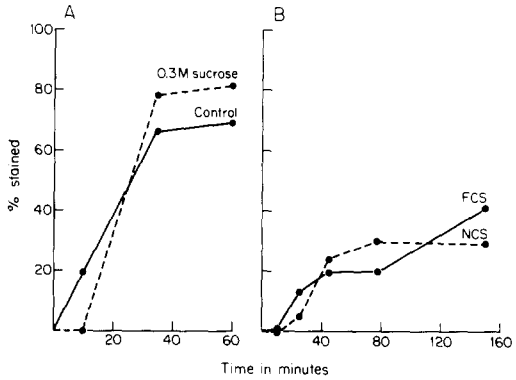

FIGURE 7 Effect of increased lysosome size on the rate of P-L fusion. (*A*) Rate of P-L fusion in 3-d cells cultured in medium with or without 0.3 M sucrose. (*B*) Rate of P-L fusion in 2-d cells cultured in 20% fetal calf serum (*FCS*) or 40% newborn calf serum (*NCS*).

tosis, 18–25 kcal/mol (17, 28) and considerably less than that seen for phagocytosis, 54 kcal/mol (17). P-L fusion as detected by this assay has a threshold temperature below which no fusion occurs. In this sense it is similar to phagocytosis, which has a cut-off temperature between 18° and 21°C (26), and contrasts with pinocytosis, which is linear from 2° to 27°C (28).

DISCUSSION

We describe studies on intracellular membrane fusion in mouse macrophages, using both an in vivo fluorescence assay and EM analysis. Several aspects of the techniques should first be considered. Our studies employed an opsonized yeast preparation as the test particle. This particle was well suited for fluorescence studies, and its relatively slow fusion rate allowed the investigation of variables, such as temperature, requiring the separation of ingestion and fusion. It is interesting that latex spheres showed a more rapid fusion rate in EM studies, making them impractical for several types of experiments. This more rapid fusion may be an intrinsic property of the polystyrene latex. Alternatively, the yeast cell surface may have some inhibitory activity (12) interacting with the membrane of the phagocytic vacuole and modifying its subsequent fusion with lysosomes.

Secondly, mouse peritoneal macrophages were useful for these studies because these cells have specific membrane receptors and phagocytose avidly. Also, many properties of macrophage plasma membrane and lysosomes have been characterized, as well as the fate in macrophages of several intracellular parasites that can inhibit P-L fusion (9). Stable macrophagelike cell lines such as $P388D_1$ are available and show similar P-L fusion in the fluorescence assay.

We have strived to establish conditions under

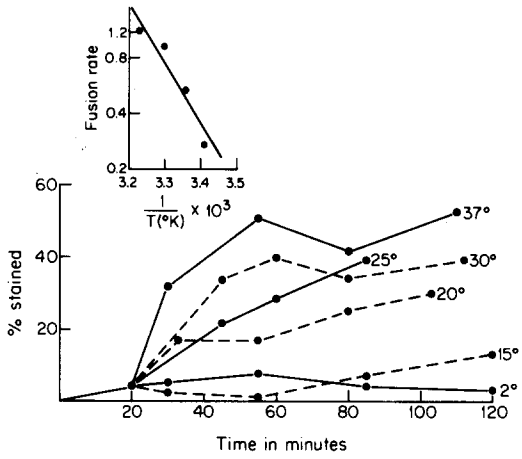

FIGURE 8 Effect of incubation temperature on fusion rate. After 10 min of phagocytosis at 37°C, 2-d cultures were shifted to the indicated temperatures and the effect on subsequent fusion was monitored. The abscissa gives the time since the start of the phagocytic pulse. *Inset* shows an Arrhenius plot of the initial rates for temperatures from 20° to 37°C. The slope defines E_{act}.

which we can examine the determinants of P-L fusion in the natural milieu of the cytoplasm. For this purpose we have adapted and characterized a vital dye method that can be employed at the level of the fluorescence microscope. It is now well known that many vital dyes are segregated within secondary lysosomes and serve as useful markers to trace the presence of lysosomes and their fusion with endocytic vacuoles (2, 8). The present technique, which is based upon the acridine orange method of Hart and Young (12), has been used effectively in the examination of the inhibitory influence of microorganisms and their products on the fusion process (10). Other approaches to this question have been based upon either the transfer of acid hydrolases into a paraffin oil (29) or latex phagolysosome fraction (23) or the fusion of isolated phagolysosomes (19, 20). Both, however, require the disruption of large numbers of phagocytic cells and are not as readily adapted to the study of rates of reaction. Other restraints are apparent in the high-resolution EM techniques in which thin sections make sampling a tedious process.

The fluorescence assay has many advantages but, similarly, can only be used under certain conditions. Thus, agents modifying the intralysosomal pH or causing quenching of AO fluorescence interfere with the assay. For example, ammonium chloride, chloroquine, or treatment with 2-deoxyglucose and sodium azide all cause an increase in intralysosomal pH (21) and also cause a visible decrease in lysosomal staining by AO (our unpublished observations). Therefore, it is important that the use of thin sections and markers other than vital dyes gives comparable information. The effect on fusion of lysosome uptake of fluorescence or EM markers is not known.

Effects of Particle Uptake

To approximate the degree of degranulation and the extent of P-L fusion, it is useful to consider the size of the vacuolar compartments involved in the fusion. From stereological analysis of the secondary lysosome compartment in mouse macrophages (27), the total volume of the secondary lysosome compartment was ~10 μm^3 per average cell volume of 395 μm^3. From light micrographs, the diameter of the yeast used in the experiments was estimated to be ~3.8 μm. Assuming that the yeast are spherical, their volume is then (by $v = \pi D^3/6$) ~30 μm^3. At least five particles of ~150 μm^3 total volume can be positively stained by the amount of AO sequestered by one cell. Because the volume of the total lysosome compartment is considerably less than that of the yeast phagocytic vacuole, AO is diluted upon P-L fusion, although still sufficiently concentrated to stain the yeast orange. Even in the case of high particle uptake, in which the particle volume is estimated to be at least 15 × that of the total secondary lysosome volume, fusion can occur with all particles and considerable lysosomal staining is still observed. This is similar to results obtained from quantitating the transfer of acid phosphatase to latex phagolysosomes (23), by which Pesanti and Axline demonstrated that, even at the maximum particle dose,

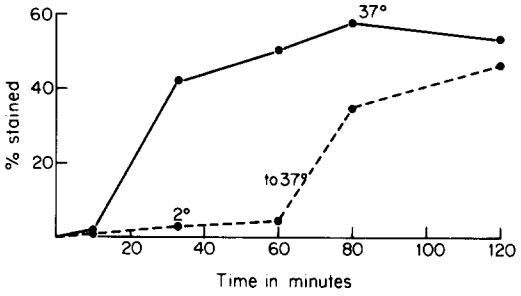

FIGURE 9 Reversibility of low-temperature inhibition of P-L fusion. Incubation temperature was downshifted as in Fig. 8. At 60 min after phagocytosis, 2°C cultures were shifted back to 37°C.

only 35% of the total acid phosphatase was found in the latex phagolysosome fraction. Thus, total degranulation does not occur, although the volume of the phagosomal compartment is much greater than that of the lysosomal compartment.

Effect of Lysosome Size

Increasing the surface area of vesicle membrane available for fusion might be expected to have several effects. First, it might greatly increase the random contacts of phagosomes with lysosomes, thus increasing the opportunities for fusion to occur. Conversely, theoretical considerations of charge repulsion between two membranes would argue that close contact can only be made by microvesicles with a low (<0.1 μm) radius of curvature (24). If charge repulsion affects P-L fusion, an increase in lysosome size might thus be expected to decrease the probability of fusion.

Several experiments examined the effect of loading lysosomes with sucrose, serum, bacteria, or latex on their subsequent fusion with phagocytic vacuoles. In these experiments, only a part of the lysosome population was influenced, although in the case of sucrose or NCS treatment this part is probably quite large (6, 7). None of these treatments altered the rate or extent of P-L fusion. Although obviously these results are not conclusive, they do suggest that the rate-limiting step in P-L fusion is not random vesicle contact but a more selective event.

Effects of In Vitro Cultivation

All assays of P-L fusion showed a progressive increase in fusion rate after several days of cell culture. The expression of this macrophage activity can thus vary, similar to that of other previously studied processes. These include the specific activity of plasma membrane ectoenzymes and lysosomal acid hydrolases, as well as pinocytic uptake and phagocytosis (4). These processes and P-L fusion are influenced by in vitro cultivation and/or cell activation. That particles such as latex, yeast, and parasites show different ısion rates may imply that a postendocytic modification of the phagocytic vacuole occurs that alters its recognition and fusion with lysosomes.

Effect of Temperature

The effect of temperature on membrane fusion has probably been best studied in liposome systems. Calcium-induced fusion of phosphatidylserine vesicles, for example (22), is apparently dependent on the phase change from fluid to solid state. Thus vesicle fusion is most likely to occur at a temperature where the acyl chains will be fluid before addition of divalent cation, and crystalline after addition. Both fusion and this cation-controlled phase change are inhibited at temperatures below the transition temperature.

Macrophage membrane phenomena such as phagocytosis and pinocytosis are strongly affected by ambient temperature. Similary, P-L fusion was dependent on temperature, with an E_{act} similar to that found for pinocytosis and a cut-off temperature below which no fusion was detectable.

The E_{act} was considerably lower than that observed for phagocytosis, which probably refelcts other requirements, as well as alterations in membrane fluidity. These include the circumferential attachment of ligand to receptor (26) that then induces the aggregation of contractile proteins and the subsequent engulfment of the particle. In contrast, it is not clear whether pinocytosis requires any involvement of the actomyosin system (26).

These studies suggest that the effects of membrane fluidity and particle interaction with the vacuole membrane may be fruitful areas for further investigation. Our subsequent reports will deal with both positive and negative effectors of P-L fusion, and evaluate the contributions of membrane composition, the contractile network, and the nature of the ingested particle.

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