INFECTION AND IMMUNITY, July 1987, p. 1663–1667
0019-9567/87/071663-05$02.00/0

Vol. 55, No. 7

Discrepancy in Assessment of Phagosome-Lysosome Fusion with Two Lysosomal Markers in Murine Macrophages Infected with *Candida albicans*

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Received 5 December 1986/Accepted 19 March 1987

Phagosome-lysosome fusion (P-LF) was studied in cultured mouse resident peritoneal macrophages after phagocytosis of *Candida albicans*. The macrophages were labeled with acridine orange (AO), the electron-opaque colloidal Thorotrast, or both markers. After phagocytosis of heat-killed *C. albicans*, both markers were delivered to more than 95% of phagosomes. After ingestion of viable *C. albicans* by labeled cells, delivery of AO to phagosomes was highly suppressed (90%), and yet Thorotrast delivery was almost universal. After phagocytosis and 60 min of incubation, about 10 to 20% of the yeasts were killed, and a similar fraction of phagosomes was stained by the fluorescent marker. The evidence from Thorotrast transfer and assessment of yeast viability indicates that *C. albicans* largely resists intracellular killing by resident macrophages in the face of entirely uninhibited P-LF. We infer that AO must transfer to nearly all of the phagosomes but that it is evidently recognizable only in those in which the yeasts have been killed or possibly severely injured. This conclusion constitutes yet another limitation in the usefulness of AO for studying P-LF.

In earlier studies partly reported (4), we compared the digestion of various endocytosed radiolabeled substrates by control resident mouse peritoneal macrophages (Mϕs) with that by cells previously exposed to certain polyanionic agents. In the course of these studies, digestive processing of ^{125}I-labeled keyhole limpet hemocyanin, ^{32}P-labeled *Saccharomyces cerevisiae*, and also similarly labeled *Candida albicans* was examined. The release of trichloroacetic acid-soluble labels from control and polyanion monolayers was essentially the same for a particular substrate. However, digestive processing of viable *S. cerevisiae* was much more rapid than that of viable *C. albicans* (M. Goren, L. S. Swendsen, and J. Henson, reported at the 14th Joint Conference on Tuberculosis, The U.S.-Japan Cooperative Medical Science Program, Denver, Colo., October 3 to 5, 1979).

Parallel examination of the control monolayers labeled with the lysosomotropic fluorochrome acridine orange (AO) suggested that phagosome-lysosome fusion (P-LF) was suppressed in the macrophages that ingested viable *C. albicans*, since the lysosomal marker was only poorly transferred to these phagosomes. In contrast, we regularly observed abundant AO delivery to phagosomes in cells that had ingested viable *S. cerevisiae*, as has often been described (3, 5, 8). Accordingly, the results of the P-LF studies with *C. albicans* reinforced those of the digestion studies and appeared to allow the conclusion that viable *C. albicans* inhibits P-LF from within the phagosomes, as several intracellular pathogens have shown (*Mycobacterium tuberculosis* [1], *Toxoplasma gondii* [11], *Legionella pneumophila* [10], and others).

Because more recent studies suggested that unequivocal evidence of P-LF with AO as a marker required that the trapped organism (specifically *S. cerevisiae*) be killed or at least severely damaged and digesting (6, 12, 17, 20, 21), we reexamined the *C. albicans* system more rigorously in Mϕs. We studied P-LF by using both the electron-opaque lysosomal marker Thorotrast and AO to compare results obtained with the two probes for both viable and heat-killed *C. albicans*.

MATERIALS AND METHODS

Two strains of *C. albicans* were used. Strain UC 820 was kindly provided by Robert Lehrer (2, 14, 15), in whose laboratory, as well as in others (16), this strain has been extensively investigated. Pamela Lindholm-Levy of our Clinical Microbiology Laboratory (National Jewish Center for Immunology and Respiratory Medicine) kindly gave us a recent isolate of *C. albicans* from a patient. We examined this isolate in parallel with strain UC 820. The behavior of the two strains was essentially identical. *C. albicans* was cultured in the yeast phase in Sabouraud dextrose broth (Difco Laboratories, Detroit, Mich.) without antibiotics at 32°C, usually for 2 to 3 days with constant shaking. *Candida parapsilosis*, a species that is susceptible to rapid killing by Mϕs (18), was also used for comparison with the more pathogenic analogs.

Candida suspensions were well vortexed and passed through a 27-gauge needle to break up clumps. Dilutions were counted in a hemacytometer to obtain total counts and also plated on Sabouraud dextrose agar (BBL Microbiology Systems, Cockeysville, Md.) for determining CFU after incubation at 32°C (48 h). Unelicited Mϕs were harvested from 8- to 10-week-old male Swiss Webster mice and cultured at 37°C in plastic Leighton tubes (Costar, Cambridge, Mass.) with removable cover slips in Dulbecco modified Eagle medium containing 20% heat-inactivated fetal bovine serum and supplemented with glutamine and antibiotics. The medium was ordinarily changed every 2 days. The tubes were loosely capped to allow equilibration with the 5% CO_2-air mixture in the incubator (37°C). Mϕs were used after 3 to 5 days in culture, when they are capable of a brisk P-LF response (13).

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Thorotrast was kindly given to us by A. E. Vatter (Webb-Waring Lung Institute). Secondary lysosomes were labeled by exposure to 2.5 mg of the marker per ml in complete medium for 18 to 24 h, after which the cells were washed and chased in fresh medium for at least 2 h before yeasts were offered for phagocytosis. For labeling with AO, Mϕs were exposed to the fluorochrome in Hanks balanced salts solution (7.5 μg of AO per ml) for 15 min, well washed, chased in balanced salts solution for 15 min, and then offered yeasts. For some experiments, cells were labeled with both Thorotrast and AO.

Candida spp. were washed by centrifugation in balanced salts solution, opsonized for 10 min in balanced salts solution-fresh mouse serum (1:1, vol/vol), and diluted to contain about 1.5×10^6 yeasts per ml. They were provided to the Mϕ monolayer at a multiplicity of about 5:1 for a 45-min uptake. The monolayers were washed to remove unattached yeasts, fresh complete medium was restored, and the cells were incubated for an additional 30 or 60 min, when samples were cut from the plastic cover slips and examined. When monolayers were labeled with both Thorotrast and AO, portions cut from the cover slips at the same time were processed for electron microscopy (EM) or fluorescence microscopy. For EM processing, the cut sample with attached cells was fixed by the method of Hirsch and Fedorko (9) with a combination of glutaraldehyde and osmium tetroxide. The cover slips were dehydrated and then infiltrated and embedded in Spurr medium. The monolayer adhered to the hardened polymer when the cover slip was detached. Interrupted thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 400 EM at 60 kV. For vital fluorescence microscopy, the freshly cut piece of plastic was placed cell side up on a microscope slide holding a few drops of medium, and the cells, flooded with additional medium, were covered with a glass cover slip (no. 1) for immediate examination. We used a Zeiss photomicroscope with dark-field illumination for fluorescence (blue-violet irradiation [heat filter, BG-38 and BG-12 exciter filters, and a 530 barrier filter]). Yeast phagosomes were scored as fused if they exhibited any of the appropriate patterns (fusion figures) previously described for AO-labeled cells, that is, either well-defined yellow-to-orange rims surrounding the yeast or uniform staining of bright green, yellow, or orange (5, 8). *C. albicans* heat killed by keeping a suspension of the yeast cells in a boiling water bath for 30 min were used as positive controls for fusion in both Thorotrast- and AO-labeled Mϕs (6).

Extracellular in vitro staining of viable and heat-killed *C. albicans* by AO was assessed as described previously for *S. cerevisiae* (6). Briefly, equal volumes of AO solution (200 μg/ml) and yeast suspension were mixed, and a drop was applied to a slide, covered, and immediately examined with blue-violet irradiation and filters as described above. Under these conditions, viable *S. cerevisiae* are stained a dim dull green, except for pinpoint orange nucleoli, and killed cells become a uniformly bright orange. Viable and heat-killed *C. albicans* were examined in this manner to compare the behavior with that of *S. cerevisiae*. Parallel examination for methylene blue exclusion was also done by mixing 1 drop of yeast suspension with 3 drops of 0.01% methylene blue. Dead yeasts are colored almost instantly with this reagent (14, 19).

RESULTS

Phagocytosis experiments with Mϕ monolayers labeled with AO and ingesting viable *C. albicans* gave us immediate evidence suggesting that P-LF was profoundly suppressed, as judged from the very limited transfer of this lysosomal label to only a few phagosomes. The apparent degree of suppression (about 90%) was essentially the same for both *C. albicans* isolates and was quantitatively the same (by scoring percentage of rims or uniformly stained yeasts) at both 30 and 60 min after the phagocytosis pulse. However, when either heat-killed *C. albicans* or viable *C. parapsilosis* or *S. cerevisiae* was the target for phagocytosis, an abundance of intensely colored phagosomes was seen, especially with the killed *C. albicans*, after 30 min. For the killed yeasts, almost every yeast cell was solidly brilliant with delivered AO. Figure 1 compares photomicrographs of representative fields 60 min after phagocytosis of viable *C. albicans* (panel A) and 30 min after phagocytosis of heat-killed *C. albicans* (panel B). Figure 1A was selected to show several illuminated phagosomes in a portion of a field which in toto contained about 40 to 50 *Candida* organisms. The various fusion figures are seen either as complete rims or as more uniformly stained yeasts. One of these phagosomes shows a developing pseudo germ tube. The majority of the phagosomes, however, are dark against a background of glowing lysosomes and therefore would be judged as not fused. By contrast, Fig. 1B shows a field of heat-killed cells in which every yeast is uniformly brilliant with color. No rims have formed because AO rapidly penetrated the dead target (6).

Examination by EM of counterpart monolayers labeled with Thorotrast revealed a different picture. Instead of the prevalent nonfusion pattern for the viable *C. albicans* shown with AO, the EM technique showed almost universal delivery of the marker. Figure 2 is a typical example, showing secondary lysosomes well labeled with the electron-opaque marker, a phagosome enclosing a single *Candida* organism with an emerging pseudo hypha, and abundant delivery of the Thorotrast marker along the entire periphery of the phagosomal membrane. The yeast cell and offspring appear to be intact and uninjured.

Table 1, which combines the results from three experiments, compares scores of marker transfer to phagosomes as obtained by EM and vital fluorescence microscopy for viable and heat-killed *C. albicans*. As described below, in this system, transfer of Thorotrast and probably of AO is likely valid evidence of P-LF, but suppression of AO transfer is probably not valid evidence of fusion inhibition. Therefore, in Table 1, scores for Thorotrast transfer are labeled percent fusion, whereas scores for AO transfer are labeled presumed percent fusion. Clearly there is a great difference between these assessments.

In some experiments, the cells were labeled with both Thorotrast and AO. In others, counterpart monolayers were labeled with only one marker. Single or double labeling did not affect the results. During these experiments, over 500 phagosomes were examined by EM, and more than 3,000 were scored by the much simpler AO technique. With heat-killed *C. albicans* as targets, a very high incidence (more than 95%) of marker transfer to phagosomes was documented by both EM and vital fluorescence microscopy. The incidence of Thorotrast transfer to phagosomes enclosing viable *C. albicans* was also very high (about 90%). But AO delivery, and thus presumed P-LF, in this system was discernible in fewer than 10% of phagosomes examined. The differences in fusion scores by the two techniques when Mϕs ingested viable yeasts is clearly highly significant ($P < 0.001$ by chi-square analysis).

During the 60-min (or longer) intracellular residence at

37°C, some of the yeasts began to form pseudo germ tubes. The incidence increased with time, consistent with the viability of many of the phagocytosed yeasts. These yeasts too were rarely illuminated when viewed by fluorescence microscopy. Only occasionally were they seen in AO-stained phagosomes. A single one is evident in Fig. 1A. By contrast, however, EM consistently showed these yeasts also to be held in phagolysosomes, as indicated by the delivery of the electron-opaque marker, again in contradiction of the evidence from AO observations.

When freshly cultured viable *C. albicans* were exposed to AO (100 μg/ml) and examined by fluorescence microscopy, only 6 of 730 yeasts (0.8%) stained a very bright uniform orange. The remainder were not colored to any recognizable extent. Parallel examination of another sample for methylene blue exclusion showed that 0.8% of the cells were dead and stained (493 scored) and that the remainder were free of dye and presumably viable. Thus, the behavior of viable and killed *C. albicans* toward AO and methylene blue is similar to that of *S. cerevisiae*. The *Candida* cells (either extracellular or intraphagosomal) that stain with AO can therefore be judged to be dead (or at least severely injured), in agreement with many earlier observations (4, 6, 12, 17, 20, 21). Accordingly, as judged from Table 1, only about 10% of the yeasts engulfed by the AO-labeled Mϕs were killed (and therefore stained). This finding was confirmed by plate counting of CFU recovered from the macrophages when the monolayers were lysed. In three experiments, 78 ± 8 CFU (mean ± standard deviation) were obtained per 100 yeasts, as enumerated by direct counting. Thus, the fraction of candida-containing phagosomes stained by the lysosomal AO (about 10%) approximates fairly closely the fraction in the recovered yeasts that is no longer viable (about 15 to 20%). Sasada and Johnston (18) reported that counts of viable *Candida* spp. obtained by dye exclusion were consistently about 15% higher than those obtained by counting colonies. This result may be accounted for in part by clumping of the yeasts.

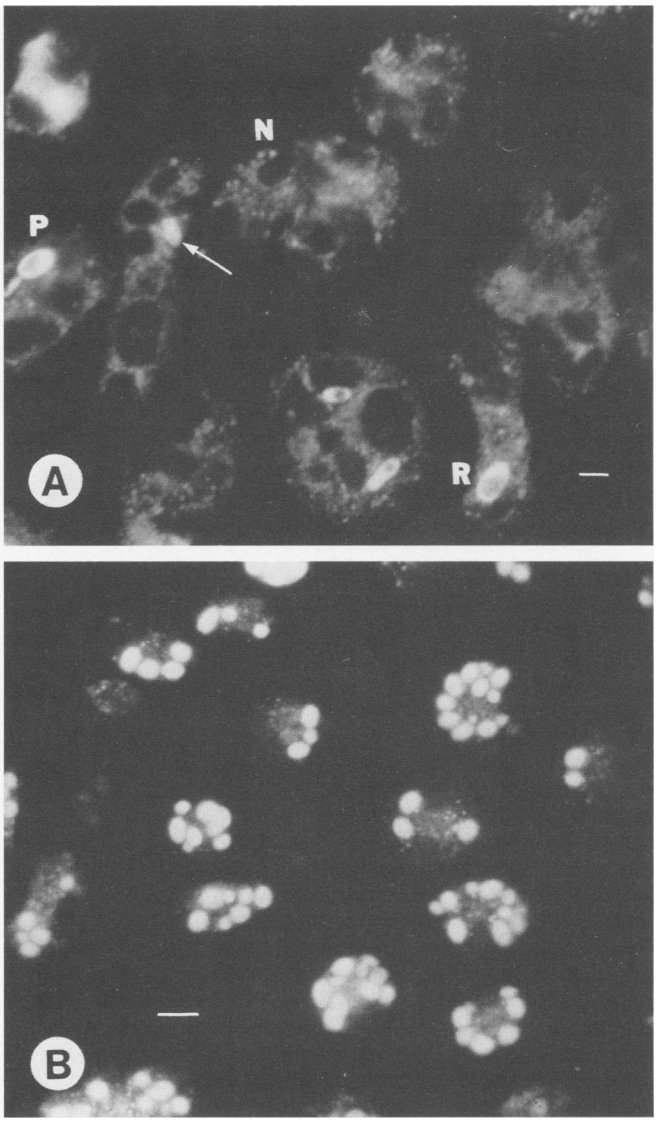

FIG. 1. Monitoring of marker delivery to phagosomes in Mϕs (lysosomally labeled with AO) after a 45-min phagocytotic pulse followed by a period of incubation. (A) Viable *C. albicans* as targets, 60-min incubation. Most of the phagosomes are devoid of AO marker, suggesting that they have not fused (N) with labeled lysosomes. A few yeasts exhibit various degrees of fusion and have complete, well-formed rims of AO (R). Several yeasts are more uniformly stained (arrow), including one that has formed a pseudo germ tube (P). Bar, 5 μm. (B) Heat-killed *C. albicans* as targets, 30 min after completion of the uptake pulse. Almost all of the yeasts are a uniform bright orange in the original color photograph. Bar, 10 μm.

DISCUSSION

Our earlier studies suggested that *C. albicans* prevents fusion of lysosomes with the phagosome in which the yeast is trapped. The judgment was based on negative evidence from vital fluorescence microscopy with AO-labeled cells, on the very limited digestive processing of radiolabeled yeasts after phagocytosis, and on destruction of the Mϕ monolayers as the yeasts continued to grow. The present results on the transfer of fluorescent marker duplicate the earlier ones. By the AO technique, lysosomal fusion with phagosomes enclosing viable *C. albicans* would be judged severely inhibited, since only about 10% of the phagosomes contained sufficient dye to be recognized. And yet the EM results deny this conclusion. Instead of inhibition, about 90% of the phagosomes had to be judged as having fused because they contained the electron-opaque colloidal marker (Fig. 1B). This result is compelling evidence, and indeed the transfer of the lysosomal Thorotrast (or Ferritin) to phagosomes is widely accepted as a valid indication of P-LF (1, 5, 8, 10, 11, 13). It may be asked how else the transfer could be convincingly accounted for. If the nearly universal fusion implicit in the EM data is accepted, then about 90% of the phagosomes must have fused with AO-labeled lysosomes, and yet the process remained cryptic, as is evident from Fig. 1A.

Our data indicate that about 10 to 20% of the yeasts were killed by the cultured resident mouse peritoneal Mϕs. Comparable findings have been reported by others. Lehrer et al. (15) reported 15% killing by resident rabbit peritoneal macrophages. Sasada and Johnston (18) reported about 10% *C. albicans* killed in 2 h by cultured resident mouse peritoneal Mϕs. In our studies, a quite similar fraction of intraphagosomal *C. albicans* was also found to be stained by AO. It is unlikely that this relationship is merely coincidence. We suggest, as we and others have before (6, 12, 17, 21), that the retention of AO by the yeasts is evidence of their death or severe injury. In our interpretation, it is the resistance of the yeasts to killing (or digestion) in the face of unrestricted

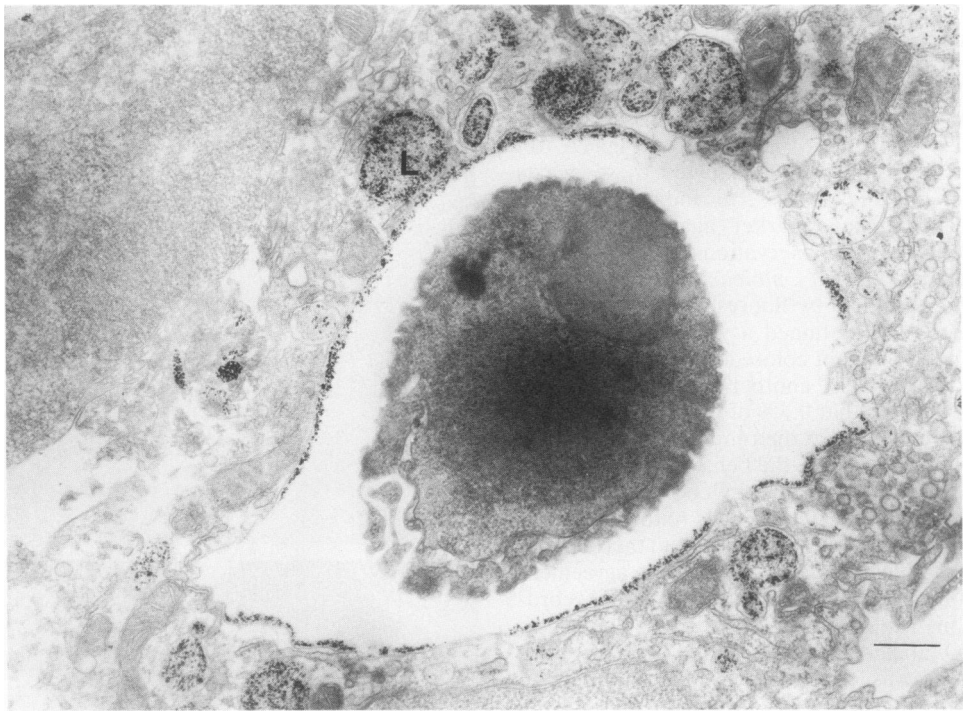

FIG. 2. P-LF documented by marker delivery after Mϕs labeled with Thorotrast ingested viable *C. albicans*. The cell has many lysosomes (L) well labeled with the electron-opaque marker. The yeast cell from which a pseudo germ tube is forming is enclosed in a phagolysosome into which marker has been delivered in abundance. This appearance was almost universal in these cells. Bar, 0.5 μm.

lysosomal fusion that accounts for the inability of the fluorochrome to penetrate the intact *C. albicans* and thus to reveal the fusion process. From Fig. 2 we estimate that the rim of Thorotrast (which clearly also cannot penetrate the intact yeast cell) is probably no thicker than about 0.1 μm. With the limits of resolution imposed by light microscopy, a halo of AO of these dimensions would not be recognized with certainty, as is evident from Fig. 1a, where more than 90% of the phagosomes must surely have fused with the secondary lysosomes (EM data) and yet remain dark. AO delivery seen in its earliest recognizable stages is present in only a small fraction. The dye has at least penetrated into presumably injured yeasts. The just-visible halos of AO in this figure, judged to be about 0.5 μm in width, are evidence of this limited penetration.

TABLE 1. Scoring of secondary lysosomal marker transfer to phagosomes in Mϕs ingesting viable or heat-killed *C. albicans*[a]

Condition of *C. albicans*	With Thorotrast			With AO		
	No. of phagosomes[b]		% Fusion	No. of phagosomes[c]		Presumed % fusion
	Marker transferred	Marker not transferred		Marker transferred	Marker not transferred	
Viable[d]	291	32	90	109	1,493	7
Dead[e]	235	11	95	1,433	7	99

[a] Results were pooled from three experiments, each of which was tested for significance by a chi-square test. The overall difference in apparent fusion scored by Thorotrast versus AO transfer is highly significant ($P < 0.001$).

[b] Sections through more than 100 Mϕs containing *C. albicans* were examined.

[c] More than 800 Mϕs were counted by light microscopy.

[d] Viability of the *Candida* culture was found to be about 90% by plate counting and 99% by methylene blue exclusion.

[e] Prepared by being boiled for 30 min.

In summary, the evidence for P-LF or its inhibition obtained with AO as lysosomal marker must be interpreted with caution. Lysosomally sequestered polyanionic agents can trap and immobilize the dye even while P-LF is unrestricted (7). In a closed system, AO can be drained from phagosomes to other domains that exert a greater affinity for the dye, including intra- or extracellular cation exchange particles and polyanion-containing lysosomes, even in other macrophages (4, 6). As a corollary, microorganisms with strongly acidic surfaces (*M. tuberculosis*) can trap the freely permeating dye without the intervention of P-LF (4, 6). Finally, evidence from the present study indicates that with viable *C. albicans* as the target, P-LF may not be recognized with AO labeling if the yeast cell is not at least severely injured or undergoing digestion. This interpretation seems valid for *C. albicans* and *S. cerevisiae* and probably will be found valid for a wide variety of other microorganisms as well.

ACKNOWLEDGMENTS

This investigation was supported by grant AI08401 from the U.S.-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases. M.B.G. is the Margaret Regan Investigator in Chemical Pathology at the National Jewish Center for Immunology and Respiratory Medicine. N.M. is a visiting scientist from Hebrew University Hadassah Medical School, Jerusalem, Israel.

We thank Shirley Downs for careful preparation of the manuscript and Nadia de Stackelburg for some of the figures.

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