Ammonium Chloride, an Inhibitor of Phagosome-Lysosome Fusion in Macrophages, Concurrently Induces Phagosome-Endosome Fusion, and Opens a Novel Pathway: Studies of a Pathogenic Mycobacterium and a Nonpathogenic Yeast

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Summary

The weak base ammonium chloride has been previously reported to inhibit lysosomal movements and phagosome-lysosome (Ph-L) fusion in cultured mouse macrophages (Mø), thus reducing delivery, to an intraphagosomal infection, of endocytosed solutes that have concentrated in secondary lysosomes. We have now addressed the question, whether NH_4Cl might affect any direct interaction (if it exists) between such infection phagosomes and earlier, nonlysosomal compartments of the endocytic pathway, i.e., solute-containing endosomes. The phagosomes studied were formed after ingestion of the mouse pathogen *Mycobacterium microti* and the nonpathogenic yeast *Saccharomyces cerevisiae*; and the endosomes were formed after nonreceptor-mediated endocytosis of electronopaque and fluorescent soluble markers. By electron microscopy, survey of the cell profiles of Mø that had been treated with 10 mM NH_4Cl so that Ph-L fusion was prevented, and that displayed many ferritin-labeled endosomes, revealed numerous examples of the fusion of electronlucent endosomes, revealed numerous examples of the fusion of electronlucent vesicles with phagosomes, whether containing *M. microti* bacilli or *S. cerevisiae* yeasts. Fusion was recognized by transfer of label and by morphological evidence of fusion in progress. The fusing vesicles were classed as endosomes, not NH_4Cl-lysosomes, by their appearance and provenance, and because lysosome participation was excluded by the concurrent, NH_4Cl-caused block of Ph-L fusion and associated lysosomal stasis. No evidence of such phagosome-endosome (Ph-E) fusion was observed in profiles from Mø treated with chloroquine, nor in those from normal, untreated Mø. NH_4Cl-treated living Mø that had ingested yeasts at 37°C, followed by endocytosis of lucifer yellow at 17°C (to accumulate labeled endosomes and postpone label passing to lysosomes), were then restored to 37°C. Fluorescence microscopy showed that as many as half of the yeast phagosomes (previously unlabeled) rapidly became colored. We inferred that this transfer was from endosomes (by Ph-E fusion) because Ph-L passage was blocked (by the NH_4Cl). We conclude that NH_4Cl induces Ph-E fusion at the same time as it suppresses Ph-L fusion. We discuss the mechanisms of these concurrent effects and suggest that they are independent; and we consider the implications of NH_4Cl opening a direct route for endocytosed molecules to reach an intraphagosomal infection without involving lysosomes.

After ingestion by cultured macrophages (Mø),[1] many microorganisms (e.g., the human pathogens *Leishmania donovani* and *Salmonella typhimurium*, the mouse pathogen *Mycobacterium lepraemurium*, and the nonpathogenic yeast *Saccharomyces cerevisiae*) come to reside in phagolysosomes, formed by fusion of host cell lysosomes with the microbe-containing phagosomes. A number of microorganisms, on the other hand, reside in unfused phagosomes (e.g., the human pathogens *Mycobacterium tuberculosis* and *Legionella pneumophila*, and the mouse pathogen *Mycobacterium microti* [vole tubercle bacillus]) (reviewed by Horwitz [1]). Certain chemical agents, notably ammonium chloride, can inhibit the normal fusion of lysosomes with phagosomes that is associated with the first group of microorganisms (judged by *S. cerevisiae* and *M. lapraemurium* [2–5]), and would be expected to add to the analogous

[1] *Abbreviations used in this paper:* AO, acridine orange; CF, cationized ferritin; FM, fluorescence microscopy; LY, lucifer yellow; Mø, macrophage; NF, native ferritin; Ph-E, phagosome-endosome; Ph-L, phagosome-lysosome.

natural inhibition of such fusion that is associated with the second group (exemplified by *M. microti* [6, 7]). In either case, lysosomal delivery to the intraphagosomal infection would be curtailed or cut off. These agents produce slowing or stasis of the saltatory lysosome movements throughout the Mø; we have suggested that their ability to inhibit phagosome-lysosome (Ph-L) fusion is the consequence (2–4); but the mechanism of the lysosomal stasis is not yet solved.

Preliminary experiments on Mø by fluorescence microscopy (FM) during the blocking of Ph-L fusion by NH_4Cl suggested unexpectedly that *S. cerevisiae* yeasts residing in (virtually lysosome-free) phagosomes were still receiving substantial amounts of lucifer yellow (LY) marker, endocytosed from the medium. We therefore sought the immediate vacuolar source of this delivery, extending our inquiries to EM of the cells after ingestion of these yeasts or of *M. microti*; and we obtained evidence, under the NH_4Cl block of Ph-L fusion, that endocytosed solutes could reach these intraphagosomal microorganisms directly, bypassing the lysosomes; the phagosomes were fusing with nonlysosomal vesicles. Our exploration of this phenomenon forms the subject of the present report, in which we give evidence to justify classifying the fusing vesicles as endosomes and ascribing their phagosome-endosome (Ph-E) fusion to an effect of the NH_4Cl other than its concurrent blocking of Ph-L fusion. We discuss possible mechanisms involved in the opening of this novel (nonlysosomal) pathway from the exterior to intraphagosomal infections, in which ammonium chloride has been essential to inducing Ph-E fusion, with formation of "phagoendosomes". Normal, untreated cells showed no evidence of this phenomenon, the two organisms displaying their natural patterns of Ph-L fusion (*S. cerevisiae*, fusiogenic [2, 8, 9]; *M. microti*, fusion inhibitory [6, 7]).

Materials and Methods

Cell Culture. Resident peritoneal macrophages from female mice of the albino P strain were established as monolayers on 10.5 × 35-mm glass coverslips in Leighton tubes closed with silicone-rubber bungs. The culture medium (1 ml/tube) was composed of 50% NCTC 199 (code 041-01150; Gibco Laboratories, Paisley, Scotland); 40% heat-inactivated horse serum (code 034-06050; Gibco Laboratories); 10% beef embryo extract (Gibco Laboratories or Flow Labs Rickmansworth, Herts, UK); and 60 U/ml penicillin (10). The cultures were used after 1–2 wk of incubation at 37°C and without a medium change.

Microorganisms. *M. microti* was maintained, and suspensions of live organisms prepared, as described previously (7, method *a*), and diluted 1:5 in HBSS containing 3.5% mouse serum for a 1-h ingestion at 37°C. The infection, giving a mean of 20–40 bacilli/cell, was well tolerated (7). Fresh commercial *S. cerevisiae* yeasts were suspended in HBSS. Macrophage monolayers in HBSS, or in 10 mM NH_4Cl/HBSS, were pulsed for 30 min at 37°C with the yeasts at 10^6 to 10^7 cells/ml, followed by a wash and chase in HBSS, plain or containing NH_4Cl.

Labeling of Mø for EM. The label was either native ferritin (NF) fluid-phase endocytic tracer, or cationized ferritin (CF) endocytosed after nonreceptor (ionic) binding. Ferritin was used in two ways. (*a*) To label secondary lysosomes exclusively, Mø were exposed to NF at 10 mg/ml in culture medium for 3 h at 37°C, followed by a 20-h chase in ferritin-free medium (11). (*b*) To label endosomes preferentially, Mø were exposed to NF at 10 mg/ml in culture medium for 3 h or to CF at 0.05–0.20 mg/ml in PBS for 30 min to 3 h, without the long chase; alternatively, the exposure to CF (0.175 mg/ml) was extended to 18 h continuously in culture medium at 37°C (some passing to lysosomes; see later).

Processing for EM. The Mø monolayers were fixed in situ on the coverslips with 1% (wt/vol) paraformaldehyde and 1.25% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at room temperature for 1 h. The cells were then gently pushed off the coverslips (still elongated) and pelleted, and left in fixative at 4°C overnight. Then, the cells were rinsed in 0.1 M cacodylate buffer and postfixed in 2% (wt/vol) osmium tetroxide for 2 h. Cells were prestained with 1% (wt/vol) uranyl acetate, dehydrated in a graded ethanol series, and embedded in araldite. Thin sections were stained with saturated ethanolic uranyl acetate and Reynolds lead citrate.

Assessment of Ph-L and Ph-E fusion by EM. The criterion was the transfer of lysosomal or endosomal label to the phagosomes and/or the appearance of fusion in progress (11, 12). When suitable for quantitative assessment, ferritin-labeled phagosomes were quantified by a system previously used and subject to statistical analysis (7, 9, 11).

Labeling of Mø for FM. The fluid-phase endocytic marker, Ly (13), was used (at 1.0–7.5 mg/ml) to label lysosomes exclusively or endosomes preferentially, depending on sequence (see Results). Acridine orange (AO) was used (at 5 μg/ml) terminally to label lysosomes; after 5 min at 37°C, the monolayers were quickly washed and examined (2, 7, 9, 10).

Assessment of Ph-L and Ph-E Fusion by FM (Yeast Target). The criterion of fusion was: (*a*) the presence of intraphagosomal "rims" of LY or AO transferred from the lysosomes, or of LY transferred from endosomes; or (*b*) coloration of the yeasts by LY or AO. The labeled phagosomes were scored using an overall semiquantitative evaluation (2, 3, 9). A critique of the use of fluorescent lysosomotropic dyes to assess Ph-L fusion has been previously given, including the possibility that AO in the presence of NH_4Cl might give an erroneous assessment (2, 7); however, experiments then performed indicated that under the conditions used then and now by ourselves, AO assays of Ph-L fusion in the presence of NH_4Cl are reliable. The reliability of AO assessment of Ph-L fusion (yeast target) is supported by agreement with results obtained with FITC-dextran, LY, and cascade blue, as well as with ferritin (2, 7, 12). Lysosomes could be distinguished from endosomes (using FM and phase microscopy) by size, shape, phase density, saltatory movements, central location, and brilliant coloration by AO (2, 4).

Chemicals. NF (2× crystallized, cadmium-free) was obtained from Pentex (Kanakee, IL), and CF (code F-7879) from Sigma Chemical Co. (St. Louis, MO); FITC-cationized ferritin was from Molecular Probes (Eugene, OR); NH_4Cl (AR grade) was from British Drug Houses (Poole, Dorset, UK); LY, carbohydrazide dilithium salt, was from Sigma Chemical Co.

Results

Electron Microscopy. The phagosomes studied were membrane-bound vacuoles containing *M. microti* or *S. cerevisiae* ingested by the Mø. Endosomes were confined to those formed after nonreceptor-mediated endocytosis of the electronopaque markers NF and CF. Lysosomes were host cell secondary lysosomes.

Confirmation of Inhibition of Ph-L Fusion by NH_4Cl. The yeast *S. cerevisiae*, a known promoter of Ph-L fusion in Mø

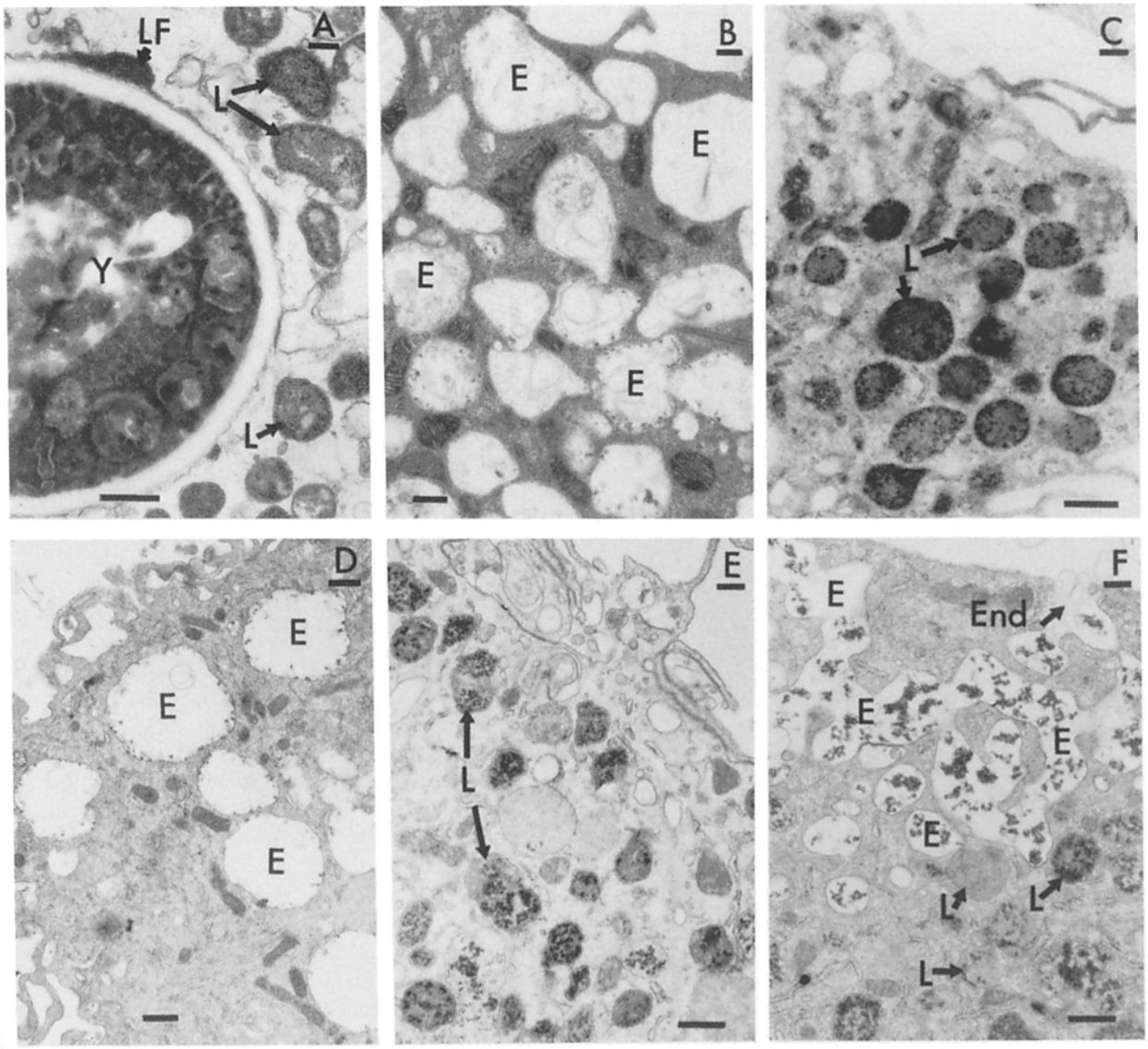

Figure 1. EM cell profiles of ferritin-labeled Mø; NH_4Cl (10 mM)-treated or normal untreated. (*A*) Ph-L fusion in untreated cells. Mø had been pulsed at 37°C with NF, followed by a 20-h chase to label lysosomes; live *S. cerevisiae* had been ingested. Shown are labeled dense secondary lysosomes (L), some of which are fusing (LF) with a yeast phagosome (Y) and transferring their label. This fusion was prevented in NH_4Cl-treated cells (micrographs not shown). (*B–F*) Further illustrations of contrasting appearances of endosomes and lysosomes in the ferritin-labeled Mø studied, their production depending on timing and on presence or absence of NH_4Cl (see Text). (*B*) Cells treated with NH_4Cl. Shown are endosomes (E), electronlucent, many multivesicular, lightly labeled with NF mainly peripherally. (*C*) NH_4Cl-treated cells. Shown are secondary lysosomes (L), dense, heavily labeled with NF. (*D*) Cells treated with NH_4Cl. Early (peripherally situated) endosomes (E), induced at 17°C and previously identified in the living monolayers by the fluorescence of the FITC-CF (see Text), are recognized by lucency and by the ferritin of the same conjugate in sequential EM profile of cell from same monolayers; other micrographs (not shown) included centrally situated dense lysosomes, most of them not labeled. (*E*) Cells treated with NH_4Cl. Lysosomes (L), previously identified by FITC-CF label, are recognized in EM by density and heavy ferritin label. (*F*) From same monolayers as *E*. Shown here are centrally located labeled dense vesicles (L) and peripherally situated labeled lucent vesicles (E), corresponding to endosomes and lysosomes, respectively, as seen by FM. Endocytosis (End) of label in progress is also apparent (Bars = 0.5 μm).

and commonly used in fusion assays (2, 8, 9), was used as intraphagosomal target. Mø were pulsed for 3 h with NF (10 mg/ml), followed by a 20-h chase; this sequence had been observed previously to concentrate label exclusively in secondary lysosomes (11) (see Materials and Methods). Then, some of the monolayers were exposed to 10 mM NH_4Cl in HBSS at 37°C for 1 h before, during, and 1.5 h after a 30-min yeast pulse, and fixed for EM. Comparable monolayers were treated similarly but in HBSS without NH_4Cl. Survey of profiles of the normal, untreated Mø showed the expected numerous well-labeled dense secondary lysosomes and frequent typical Ph-L fusion, judged both by transfer of ferritin label and the appearance of fusion in progress (Fig. 1 *A*). Low-density vesicles were rarely seen. The NH_4Cl-treated Mø also showed numerous lysosomes, though these were slightly swollen. In contrast to the untreated cells, Ph-L fu-

sion was very rare. Typical fusion scores by a quantitative screening survey method (7, 11) were 46% for untreated and 4% for treated ($p < 0.01$).

Production of Endosomes in Mø Exposed to Ferritin. After exposure of macrophage monolayers to CF for short periods (30–60 min) at 37°C, electronlucent vesicular structures, variable in size, often quite large, irregular in shape, and often multivesicular, were seen in cell profiles; like those seen after longer exposures to CF or to NF, they had the characteristic appearances described for endosomes by many authors (e.g., 14, 15) (Fig. 1, *B* and *F*). They were distinguishable from normal and also from NH_4Cl-containing, secondary lysosomes; the latter, although slightly swollen, retained their fairly uniform spherical shape and much of their density (Fig. 1, *C*, *E*, and *F*). We considered them prima facie to be endosomes. Further evidence of identity is given in the Discussion. In our Mø, such short periods of exposure to CF were inadequate for ensuring that sufficient well-labeled endosomes were made available to the ingested microorganisms; they were too few and peripherally located. Longer periods were indicated. Similar well-labeled, and more widely distributed, electronlucent vesicles were formed in profusion if the Mø had been exposed to 0.175 mg/ml CF in culture medium throughout 18 h of incubation at 37°C. The longer exposure to CF inevitably led to passage into lysosomes of a proportion of label, though unexpectedly small if under NH_4Cl treatment; however, we considered any error thus introduced in the assessment of phagosome-vesicle fusion to be insignificant for reasons given below.

Ph-E Fusion in M. microti–ingested Mø. Monolayers that had been exposed to 0.175 mg/ml CF in culture medium for 18 h at 37°C were pulsed with *M. microti*, under 10 mM NH_4Cl/HBSS treatment, or in HBSS alone, for 1 h before, during, and 1.5 h after, the 60-min pulse, before fixation for EM. Survey of EM cell profiles of the normal untreated Mø showed many labeled endosomes present, but failed to detect CF label in the *M. microti* phagosomes; also, no actual fusion in progress was observed (Fig. 2 *C*). These observations indicated absence of fusion of the phagosomes with any visible host cell vesicle. Absence of conventional Ph-L fusion conformed to the known inhibition of fusion of lysosomes with phagosomes containing this mycobacterium (6, 7). In the treated cells, this inhibition of Ph-L fusion by the intraphagosomal mycobacterium itself had been added to by the blocking effect of the NH_4Cl present, and, in confirmation, again no fusion of phagosomes with lysosomes was observed; but, remarkably, numerous instances were seen of fusion in progress of phagosomes with labeled vesicles having the characteristic appearance of endosomes, and much CF label had been transferred to the phagosomes (Fig. 2, *A* and *B*). We considered the possibility that, arising from passage of label from endosomes to lysosomes during the 18-h exposure, these vesicles fusing with phagosomes might be secondary lysosomes, not endosomes; but we concluded that this error was insignificant, not only because of the distinctive appearances of endosomes, but also because the inherent mycobacterial inhibition of lysosomes with phagosomes, plus the blocking effect of the NH_4Cl, must have prevented lysosomal participation, leaving the field clear for nonlysosomal fusion to be recognized.

Unlike Ph-L fusion, Ph-E fusion in thin sections could not be satisfactorily quantified, owing to the marked variability of the number of intraphagosomal *M. microti*; hence, our assessments were qualitative. To check for toxicity and that the NH_4Cl block of Ph-L fusion was effective throughout these experiments, additional and comparable living monolayers undergoing the same *M. microti* infection and other procedures, or without the infection, were tested terminally by a yeast-AO technique (2, 9) (see Materials and Methods); typical Ph-L fusion scores were 70% for untreated and 5% for treated; there was no apparent toxicity.

Ph-E Fusion in Mø after Ingestion of S. cerevisiae. Monolayers were treated with 10 mM NH_4Cl in HBSS for 40 min at 37°C before pulsing with the yeasts for 15 or 30 min, followed by a 20-min chase. They were then pulsed with NF (10 mg/ml) in culture medium, or CF (0.20 mg/ml) in HBSS, for 2.5 or 3 h, and fixed for EM (NH_4Cl [10 mM] present throughout). Other monolayers were treated similarly with HBSS without the NH_4Cl. Survey of EM cell profiles of the normal, untreated Mø showed, since *S. cerevisiae* is a promoter of Ph-L fusion (2, 8, 9) (consequently residing in phagolysosomes), much evidence of this fusion with yeast phagosomes in progress (as in Fig. 1 *A*) the dense lysosomes being easily recognizable; but (as was the case with *M. microti*) no lucent structures resembling endosomes were seen fusing. In contrast, in the treated cells, the profiles showed no morphological evidence of Ph-L fusion, which in any case had been suppressed by the NH_4Cl); however (again as with the *M. microti* infection), instances were frequently seen of Ph-E fusion, judged mainly by appearances of fusion in progress, with the formation of "phagoendosomes" (Fig. 2 *D*). Thus, again, the presence of NH_4Cl in these cells appeared instrumental in revealing Ph-E fusion: (*a*) by inducing this fusion; and (*b*) by excluding Ph-L fusion from participation and thus facilitating the recognition of the induced (Ph-E) fusion.

Fluorescence Microscopy. Support was obtained in living Mø infected with *S. cerevisiae*, using the endocytic fluid-phase tracer LY (13), and with NH_4Cl (10 mM) present throughout. Monolayers were pulsed with the yeasts for 30 min at 37°C, followed by LY (7.5 mg/ml) in NH_4Cl/HBSS for 2 h at 17°C to accumulate labeled endosomes (of characteristic appearance and mobility) without lysosomes being labeled (endolysosome formation having been reported to be impeded at this temperature [16]). Finally, the monolayers were restored to 37°C for 45 min, to permit onward passage to occur along the conventional endocytic pathway. Many of the intraphagosomal yeasts (which, like the lysosomes, were unlabeled at 17°C) were observed now to have become stained by the LY. An overall semiquantitative assay by FM of these labeled yeast phagosomes (2, 9) gave, typically, a 50% score, which we inferred to indicate substantial Ph-E fusion. As with EM, this assumption depended on the efficacy of the concurrent NH_4Cl suppression of Ph-L fusion, which we checked by labeling lysosomes (recognizable by features listed in Materials and Methods) with a pulse of LY (1.0 mg/ml) for

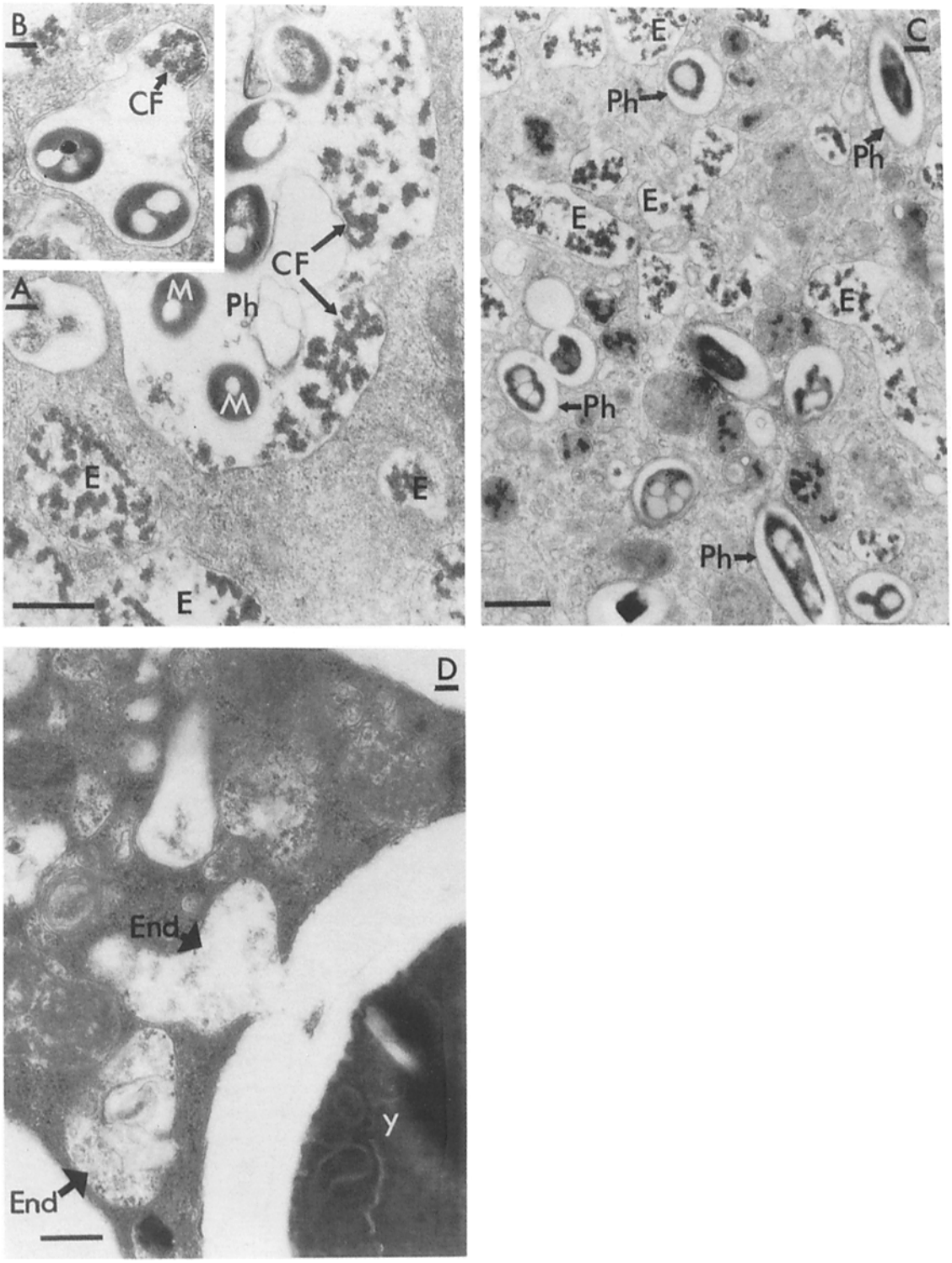

Figure 2. EM cell profiles of ferritin-labeled Mø; NH_4Cl-treated or normal untreated. Ph-E fusion in the NH_4Cl-treated cells. (*A*) NH_4Cl-treated cell after ingestion of *M. microti*. Mø had been exposed in culture to CF for 18 h; then treated for 1 h with 10 mM NH_4Cl at 37°C before, during and for 1.5 h after an *M. microti* pulse. Shown are endosomes (E) labeled with CF; and phagosomes (Ph) of *M. microti* (M) containing much transferred label. (*B*) From similar micrograph. Shown are fusion and transfer of ferritin (CF) in progress. (*C*) To be compared with *A*. Normal untreated *M. microti*–infected cell, exposed in culture to CF for 18 h. Shown is that endosomes (E) contain label, but *M. microti* phagosomes (Ph) do not. (*D*) NH_4Cl-treated cell after ingestion of *S. cerevisiae*. Mø were pulsed with the yeasts at 37°C, followed by NF for 2.5 h (NH_4Cl [10 mM] being present for 40 min before the start and thereafter). Shown are fusion of one of several labeled endosomes (End) with a yeast phagosome (Y), and transfer of label in progress (Bars = 0.5 μm).

20 h at 37°C, followed by NH_4Cl (10 mM/HBSS) for 60 min, and a 30-min yeast pulse in NH_4Cl/HBSS. The LY-positive yeast phagosomes (resulting from transfer of LY label from the lysosomes) were scored; the low figure, typically 10%, contrasted with 60% in controls not treated with NH_4Cl. Additional monolayers undergoing the same procedure, but without LY, were tested for Ph-L fusion after labeling the lysosomes with AO (2, 9). A typical score for AO-containing phagosomes (again indicating Ph-L fusion) was 5% in NH_4Cl-treated and 70% in untreated, thus confirming the blocking effect of NH_4Cl. No evidence of toxicity attributable to NH_4Cl was observed in these experiments.

Discussion

Evidence for NH_4Cl-induced Ph-E Fusion (with "Phagoendosome" Formation). As a necessary preliminary, using *S. cerevisiae* (Ph-L fusion promoter) as intraphagosomal target, and lysosomotropic endocytic markers, we have confirmed the inhibition (crucial to this study) of Ph-L fusion by NH_4Cl (2–4); with the procedure followed here, this fusion (with phagolysosome formation) was virtually blocked by this agent. We have now shown by EM that, concurrently, NH_4Cl can under some conditions induce the fusion of ferritin-labeled cytoplasmic vesicles with phagosomes containing two widely differing intracellular microbial species, the pathogenic *M. microti* and nonpathogenic *S. cerevisiae*. The criteria of fusion were transfer of label and the visible presence of fusion in progress. We inferred that these fusing vesicles were not lysosomes because Ph-L fusion had been excluded by its NH_4Cl block; a further compelling reason is the associated lysosomal stasis produced by this amine (2, 4). The main nonlysosomal candidate was therefore the endosomes formed after internalizations of the ferritin from which the vesicles seen in the act of fusion with phagosomes appeared indistinguishable. Besides their distinctive appearance (differentiating them, for example, from NH_4Cl-lysosomes; see Results and Fig. 1), there was other positive evidence for identifying these vesicles as endosomes. Thus, CF-labeled vesicles having the appearance of endosomes were similar after 18 h at 37°C to the fewer seen after 30 min at this temperature, suggesting the same origin; moreover, such vesicles could be seen being formed at the periphery of cell profiles (Fig. 1 *F*).

As a further test of identity, we used double labeling with a fluorescent cationized ferritin, so that organelles could be identified in the living cells by FM and then visualized in fixed thin sections of the same monolayers by EM. (*a*) Some Mø monolayers were treated with 10 mM NH_4Cl/HBSS for 60 min at 37°C, and then exposed to FITC-CF (0.5 mg/ml) in NH_4Cl/culture medium for 3 h at 17°C, when FM recognized fluorescein-labeled endosomes, situated mainly in the cell periphery, while the central region did not fluorescence (the low temperature slowing endocytosis and impeding formation of endolysosomes; see above). (*b*) In other monolayers, lysosomes were selectively labeled by FITC-CF (0.18 mg/ml) in the culture medium for 18 h at 37°CL, followed by a 3-h chase in 10 mM NH_4Cl/HBSS. The lysosomes, characteristically located centrally, fluoresced but, in addition, some cells showed labeled endosomes remaining at the periphery. The two sets of monolayers were now fixed for EM, which in *a* showed ferritin labeling mainly in peripherally situated electronlucent vesicles similar to those classified as endosomes, except that the label was inside the circumference of the organelles (presumably early endosomes) (Fig. 1 *D*); most of the lysosomes (centrally situated and electron dense) were unlabeled. In *b*, ferritin labeling mainly in centrally situated dense vesicles similar to those that we have classified as secondary lysosomes (Fig. 1 *E*); but in some cells, well-labeled electronlucent vesicles were still at the periphery (corresponding to those seen peripherally by FM) (Fig. 1 *F*). In both cases, labeled vesicles seen by EM should have been the same labeled vesicles seen by FM, and therefore, be predominantly endosomes and lysosomes, respectively (Fig. 1, *D* and *E*), and, in some cells, a mixture (Fig. 1 *F*).

We have set out, because it is crucial to our conclusions, our reasons for classifying as endosomes (and not confusing them with, in particular, lysosomes) the marker-induced electronlucent vesicles that had fused with phagosomes in NH_4Cl-treated Mø. In summary, the appearances were characteristic, and the NH_4Cl-induced block of Ph-L fusion, and (previously reported) lysosomal stasis, would have ensured that the fusing vesicles were nonlysosomal.

As a contrast to the Ph-E fusion (and consequent phagoendosome formation) appearing during NH_4Cl treatment, EM preparations from untreated monolayers infected either with *M. microti* or with *S. cerevisiae* showed no evidence of such an event. With *M. microti*, this organism's inherent inhibition of Ph-L fusion (6, 7) allowed nonfusion to be inferred by absence of label in the phagosomes and by failure to detect the appearance of Ph-E fusion in progress; in the case of *S. cerevisiae*, since Ph-L fusion was, as expected (2, 7, 9), frequently observed, only the second criterion (fusion in progress) could be used, but, on the basis of the evidence presented here, we consider this safe. We conclude that Ph-E fusion is not a natural event.

We have also tested chloroquine, another amine weak base, the procedures being closely similar to those for NH_4Cl, but substituting 20 μM chloroquine diphosphate. No evidence of Ph-E fusion was obtained from the profiles, these results differing from those from untreated cells only in that Ph-L fusion (easily recognized; see above) was characteristically enhanced (2, 7, 17) (micrographs not shown).

The experiments on NH_4Cl-treated monolayers of living Mø, using LY as endocytic tracer and phase-contrast and fluorescence microscopy, had some advantages over EM. After a yeast pulse at 37°C, addition of LY, and immediate transfer to 17°C, the characteristic slow progression of LY-labeled endosomes of recognizable appearance could be followed from the periphery towards the central area of the cells, where, enlarged by interfusion, they accumulated, as expected at this low temperature (14, 16). When the monolayers were restored to 37°C, the striking rapid staining of the intraphagosomal yeasts (previously unlabeled at 17°C), now seen in many cells,

suggested a considerable incidence of phagoendosome formation since the normal lysosome-phagosome pathway had been blocked by the NH_4Cl. At the same time, formation of endolysosomes proceeded at a slower pace.

Mechanism. The conditions for NH_4Cl treatment of these Mø to induce Ph-E fusion appeared to include adequate accumulation of labeled endosomes; but this was not enough, since many endosomes were evident not only in the experiments where long-term CF was used before *M. microti* ingestion under NH_4Cl treatment, but also when under chloroquine treatment or even with no treatment. Yet, only the first procedure yielded positive results. It may be relevant that in living Mø monolayers that had received LY the passage of this fluorescent endocytic probe into lysosomes when 10 mM NH_4Cl was being administered at 37°C (the incubation temperature of our EM experiments) was delayed, compared with untreated monolayers (unpublished observations). This delay could have favored diversion of the endosomes from endosome-lysosome fusion towards contact with the phagosomes and consequent Ph-E fusion. Chloroquine administration (which did not induce Ph-E fusion) did not produce this delay when a similar protocol was followed.

We considered the possibility that Ph-E fusion was a compensatory response to the block of the Ph-L pathway by NH_4Cl. However, failure to detect such fusion in untreated *M. microti*, in spite of its own, natural, inhibition of Ph-L fusion, made this connection unlikely; the NH_4Cl was required. The two, concurrent, effects of NH_4Cl, i.e., to block Ph-L fusion and to induce Ph-E fusion, were apparently independent. Put another way, the NH_4Cl induced Ph-E fusion, and its concurrent blocking of Ph-L fusion opportunely (but crucially) excluded lysosomes from their participation in fusion with phagosomes and so permitted the induced fusion to be revealed, and identified as endosomal.

Various changes in activity of lysosomes and endosomes in Mø after application of weak bases have been attributed to a rise in intravacuolar pH from the normal acidity (18). In most cases, NH_4Cl and chloroquine (both of which raise the pH [19]) have rather similar effects on many activities, but in some cases (e.g., Ph-L fusion, lysosome saltatory movements, and stability of tubular lysosomes) NH_4Cl is active but chloroquine is either inactive or has an opposite effect (e.g., enhancing Ph-L fusion [see reference 10]). Since chloroquine did not induce Ph-E fusion, it seems unlikely that vacuolar pH is relevant. However, these animals are known to be biphasic in activity (10), and a relationship is still a possibility. A more relevant factor might be the pH of the cytosol, which can be changed by NH_4Cl added to the medium (20); and a possible connection with Ph-E fusion is being investigated (though an acidification experiment suggested by Dr. J. Heuser did not show an effect on Ph-L fusion).

Some progress towards elucidating the mechanism of inhibition of Ph-L fusion has been made recently, in two directions. One is the observation that microbial inhibitors of this fusion themselves prevent intraphagosomal acidification (see reference 1). This too seems of doubtful relevance to Ph-E fusion induced by NH_4Cl, since the endosomes fused with self-acidified phagolysosomes (*S. cerevisiae*), as well as with putative self-alkalized phagosomes (*M. microti*). The other advance is the observation (by fluorescence and phase microscopy) that lysosomal saltatory movements were slowed or static throughout the Mø monolayers during NH_4Cl treatment (2, 4) and also in the area around live *M. microti* within their phagosomes (7); these inhibitions could plausibly explain the nonfusions by impeding Ph-L contact. In the present study, however, NH_4Cl treatment and Ph-E fusion were not accompanied by any appreciable change in speed of endosome movements; such a simple explanation seems therefore unlikely.

Biological Significance. The existence in infected Mø of a direct post-endocytic route opened by Ph-E fusion, such as we have described here, was first proposed by a Pasteur Institute (Paris) group (21–23), who used (21, 22), using, in particular, the nonpathogen *Bacillus subtilis* without treatment by any added agent. We, on the other hand, were unable to detect evidence of Ph-E fusion with the nonpathogenic *S. cerevisiae* unless treated with NH_4Cl. It is possible that the horseradish peroxidase (used by these authors as electronopaque fluid-phase tracer) that was present in the *B. subtilis* phagosomes could have come from peroxidase-labeled secondary lysosomes by the conventional route (i.e., by Ph-L fusion) rather than directly from the labeled endosomes; we believe that we have avoided this contingency by the NH_4Cl-induced block of Ph-L fusion. Moreover, there is no mention of any EM morphological evidence of Ph-E fusion in actual progress in their macrophages. We have placed much reliance on such evidence.

We conclude that NH_4Cl, which closes one pathway for endocytosed molecules to reach an infection in Mø (i.e., by the commonly travelled endosome-lysosome-phagosome route) can, concurrently, open another pathway, direct from endosomes to phagosomes (bypassing the lysosomes). If this is so, a complex situation could arise. Considering the two species here studied as representative types, the intraphagosomal microorganisms would presumably benefit by their freedom from contact with potentially inimical lysosomal contents, possibly including a drug (such contact being already reduced by the natural inhibition of Ph-L fusion by *M. microti*). However, the new potential nonlysosomal approach route for endocytosed molecules could also confer disadvantages as well as advantages to the infecting microbe.

On the other hand, a direct pathway for endocytosed molecules to reach an intracellular infection should offer new facilitates for transport manipulation in Mø studies, avoiding the lysosomal environment. To the organisms that themselves prevent passage from lysosomes to infected phagosomes by inhibiting Ph-L fusion (*M. tuberculosis* and *microti*, *Toxoplasma gondii*, *Legionella pneumophila*, etc. [1]) it could provide an alternative delivery route. Unless (or until) an acceptable substitute for NH_4Cl is discovered, the likelihood of any practical usefulness would seem remote.

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Note added in proof: Mayorga, L. S., F. Bertini, and P. D. Stahl (1991. *J. Biol. Chem.* 266:6511) also present evidence of phagosome-endosome fusion. Their observations and ours seem complementary. In the macrophage-like cell line J1774-E, receptor-mediated endocytosis of the marker dinitrophenol-derivatized β-glucuronidase was followed by an immunological-biochemical assay, which revealed the transfer of marker to phagosomes containing *Staphylococcus aureus*. Other salient differences (in intact cells) include: (*a*) internalization of marker was far more rapid than with our nonreceptor-mediated endocytosis; (*b*) no inducing agent (such as NH_4Cl) appeared necessary; (*c*) the localization of marker in (early) endosomes depended on the duration of endocytosis and chase, and no agent was used to block fusion of the phagosomes with probe-acquired secondary lysosomes; in our systems such a block (by NH_4Cl) was a crucial aid in validation of the direct Ph-E fusion; (*d*) the *S. aureus* had been killed before its phagocytosis and thus induced phagosome-*lysosome* fusion very rapidly; (*e*) in all experiments the phagocytosis followed the (timed) endocytosis of marker; in many of ours the order was the opposite; (*f*) experiments are also presented to confirm Ph-E fusion in a cell-free system, which included mannose-BSA-coated gold particles as marker in organelle preparations from cells broken after receptor-mediated endocytosis; this in vitro fusion was stimulated by various additions; (*g*) in EM profiles our endosomes varied in size but averaged at least six times those described (their Fig. 5 a).

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