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Evidence that Vesicles Containing Living, Virulent *Mycobacterium tuberculosis* or *Mycobacterium avium* in Cultured Human Macrophages Are Not Acidic

ALFRED J. CROWLE,* ROLF DAHL, ELISE ROSS, AND MARY H. MAY

Webb-Waring Lung Institute, Department of Microbiology and Immunology, University of Colorado Health Sciences Center, B-122, 4200 East 9th Avenue, Denver, Colorado 80262

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***Mycobacterium tuberculosis* and *Mycobacterium avium* multiply in cultured human macrophages (MP) within membrane-enclosed vesicles. These vesicles are generally assumed to be acidic. The evidence most frequently cited for this assumption is that pyrazinamide, which requires an acid pH to be effective, is effective and streptomycin, which loses most of its activity at a low pH, is poorly effective against tubercle bacilli. This assumption was tested by using the two weak bases chloroquine and NH_4Cl to raise the pH of acidic vesicles in MP experimentally infected with *M. tuberculosis* or *M. avium*. An immunocytochemical locator of acidic regions in the MP was used to monitor the association of intracellular bacilli with acidity. MP were infected with *M. tuberculosis* or *M. avium* and incubated with various combinations of the drugs and the weak bases. Replication of the bacteria in the MP was measured by culture counts. Intracellular associations of the mycobacteria with acidity were assessed by electron micrographs and by using the weak base 3-(2,4-dinitroanilino)-3′-amino-*N*-methyl dipropylamine, which was detected with colloidal gold-labeled antibodies. It was confirmed by immunocytochemistry that both chloroquine and NH_4Cl raise the pH of acidic vesicles in the infected MP. However, neither caused any pH-related change in the antimycobacterial activities of pyrazinamide or streptomycin or of the pH-independent drug isoniazid. Immunochemical analyses showed acidity to be associated with killed but not living mycobacteria in the MP. These findings suggest that living *M. tuberculosis* and *M. avium* are located in human MP in vesicles which are not acidic.**

Mycobacterium tuberculosis and *Mycobacterium avium* in infected subjects live and replicate within monocytic phagocytes (4, 5, 26, 38, 39). They enter these cells by phagocytosis (31) and lodge and multiply in vesicles (37, 38). Early experiments with the two drugs pyrazinamide (PZA) and streptomycin (SM) suggested that the vesicles are acidic (38–40). Thus, PZA inhibits *M. tuberculosis* at acid but not neutral pH (40), and it was found to be effective intracellularly in macrophages (MP) but not extracellularly (39). On the other hand, SM inhibits *M. tuberculosis* at neutral but not acid pH (19), and it was found to be nearly ineffective against *M. tuberculosis* in MP (38). At the time these experiments were being reported, Sprick used color indicator-stained *M. tuberculosis* and *Mycobacterium smegmatis* to show directly that the pH of these nonliving bacteria in mouse MP is between 4.5 and 5.0 (50). MP were known from much earlier work (47) to actively maintain the low pH of acidic vesicles. Subsequently, DeDuve et al. (18) identified such organelles as lysosomes and phagolysosomes which normally have a pH of about 5. Such converging information seemed to be strong evidence that *M. tuberculosis* and *M. avium* must exist in MP in acidic vesicles, probably phagolysosomes. This came to be an accepted idea and is currently widely used to interpret many aspects of mycobacterium-MP relationships (10, 24, 51).

However, this idea seems incompatible with the rate of multiplication of these bacteria in MP, which is as rapid and copious as that in the best bacteriologic media (10, 12, 17), because the low pH of these vesicles should stop their growth or even kill them (9). Furthermore, it is not supported by the recent finding that chloroquine (CQ), which raises the pH of MP phagolysosomes, enhances rather than inhibits the anti-*M. tuberculosis* activity of PZA (11). This concept that mycobacteria exist in acidic vesicles in infected MP therefore was tested with *M. tuberculosis* and *M. avium* in cultured MP by using the pH-sensitive drugs PZA and SM and the pH-raising weak bases CQ and NH_4Cl and also directly by using electron micrographic detection of acidic compartments of the infected MP. The results, reported here, indicate that dead *M. tuberculosis* and *M. avium* are associated with acidic vesicles in the MP but that living ones are not.

MATERIALS AND METHODS

Bacteria and infections. The bacteria and their preparation and their use for infecting cultured human MP were as previously described in detail (10–17) and, briefly, as follows. *M. tuberculosis* Erdman and a virulent phenotype of *M. avium* 7497, serovar 4, were used. Previously standardized stock suspensions of these bacteria, stored at −70°C, were used to infect the cultured MP so that from a 30-min period of infection about 10% of the MP became infected with 1 or 2 bacteria each. An infection resulting in 20 times more bacteria per MP was used for the immunocytochemical examination of the distribution of living or heat-killed bacteria and acidic vesicles in the MP immediately following a 30-min period of infection, as noted below. For that kind of experiment, suspensions of killed bacilli were prepared by heating the bacteria for 30 min at 62°C.

Human subjects MP isolation and culture. These experiments used MP from six white males, five white females, two black males, and one black female (informed consent was

* Corresponding author.

obtained). The subjects were healthy adults ranging in age from their early 20s to late 50s. One white male donor was tuberculin positive; the other donors were tuberculin negative. Tuberculin reactivity had no obvious effect on the results.

MP were isolated from donor peripheral blood and cultured as previously described (10, 12, 14). Seven days after the MP isolation, the culture medium (RPMI 1640 supplemented with 1% unheated AB serum, no antibiotics added) was replaced with culture medium containing infecting bacilli, and they were incubated for 30 min. Then, the infecting medium was removed, the cells were washed twice with RPMI 1640, and incubation was continued for 7 days more in culture medium alone (negative control) or in medium with the drugs or reagents being tested as described below. Usually, the MP were infected with either *M. tuberculosis* or *M. avium* alone, but in some experiments they were infected with both simultaneously, by using a mixture of the two species at concentrations of each that were the same as when they were used alone (12).

The maximum concentrations of CQ and of NH_4Cl which could be tolerated by the cultured MP for the required 7 days of infection without harm (as detected by morphological degeneration or loss of cells from culture) were determined by preliminary experiments. The numbers and morphologically apparent health of the MP cultures were monitored continuously during experiments by inverted phase microscopy and were equivalent in all the groups of the experiments reported here. Appropriate concentrations of antimycobacterial drugs were selected from data of earlier experiments (10, 11, 13–15, 16, 48).

Drugs. Reagent-grade NH_4Cl was used. Tissue culture medium and components and the drugs PZA, SM, isoniazid (INH), and CQ were purchased from Sigma Chemical Company (St. Louis, Mo.). Bacteriologic media (7H9 and 7H10) were the products of BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, Md. 3-(2,4-dinitroanilino)-3′-amino-*N*-methyl dipropylamine (DAMP) was purchased from Oxford Biomedical Research Inc., Oxford, Miss.

CFU counts. The antimycobacterial effects of PZA, SM, and INH in MP were determined as previously described (14–16) by mycobacterial CFU counts made of dilutions of samples of lysed, infected MP taken at 0, 4, and 7 days after infection. The results will be presented here as bar graphs for 7-day CFU counts (± standard error of the mean), where the baseline for the bars in each graph is the mean CFU count for the infected MP immediately after infection.

Immunocytochemical localization of acidity with DAMP. The technique of Anderson et al. (2; see also references 1 and 34) was used to reveal MP compartments and contents associated with acid pH. In this technique, the weak base DAMP accumulates in acidic compartments of the MP. It can be detected by electron microscopy with colloidal gold-labeled antidinitrophenol antibodies, which bind to it. The technique was applied as follows.

Infected MP were removed from their original culture medium and washed with fresh RPMI 1640. Then, they were incubated for 30 min with DAMP as described by Anderson and Pthak (2). The DAMP was removed, and the cells were washed three times with RPMI 1640. Then, the cells were fixed for 60 min at room temperature with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.35, containing 2.5% sucrose. The fixed cells were dehydrated with ethanol, removed from the culture plates with propylene oxide, and centrifuged at 1,000 rpm for 5 min. The resulting pellet of cells was embedded in LR White (Polysciences, Warrington, Pa.). Ultrathin sections were cut and mounted on Formvar-coated nickel grids.

The mounted sections were processed for immunocytochemistry (2) by the following sequence of incubations and washings at room temperature: (i) incubation with 0.1 M HCl for 10 min, (ii) washing with water, (iii) incubation with 5% normal goat serum in 0.5 M NaCl–0.01 M sodium phosphate (pH 7.0)–0.1% Tween 20 (NBST) for 20 min, (iv) draining, (v) incubation for 90 min with mouse anti-2,4-dinitrophenol antiserum (kindly provided by John Moorhead, University of Colorado Health Sciences Center) diluted 1:1,000 in NBST, (vi) washing with NBST containing 0.5% Tween 20, (vii) incubation for 30 min with goat anti-mouse immunoglobulin G antiserum conjugated to 5 nm of colloidal gold (Janssen; Ted Pella Inc., Redding, Calif.) diluted 1:20 in NBST, and (viii) washing with NBST containing 0.5% Tween 20 and then with water. For improved observation, the colloidal gold was intensified with silver (Janssen; Ted Pella, Inc.) for 4 min. The grid was stained with 2% aqueous uranyl acetate.

In the above sequence, appropriate controls showed that the colloidal gold staining depended upon both the exposure of cells to DAMP and the presence of the mouse anti-2,4-dinitrophenol antiserum.

The association between intracellular bacilli, easily identified by morphologic characteristics, and DAMP, located by electron-dense gold particles, was determined by counting of gold particles, of vesicles, and of bacteria. This was done without letting the counter know which group of MP was being evaluated. As noted in description of one of the experiments, this procedure is only semiquantitative and is largely subjective. For added meaning, electron micrographs were examined in the same way by other readers, and then the evaluations of all the readers were combined for final expression of the results.

RESULTS

Influence of CQ on the effectiveness of SM against *M. tuberculosis* in MP. If the inefficiency of SM against *M. tuberculosis* in MP were due to the low pH of the phagosomes or phagolysosomes containing *M. tuberculosis* (35, 46), then CQ should improve the intracellular inhibition of *M. tuberculosis* by SM by raising phagolysosomal pH. Fifteen experiments to examine this hypothesis were done with MP from 10 different donors (4 white females, 3 white males, 3 black males). In all of the experiments CQ did significantly increase the effectiveness of SM against the *M. tuberculosis*. Figure 1 shows results from one of the experiments in which concentrations of SM were varied, and Fig. 2 shows results from one in which the concentrations of CQ were varied. However, as both figures show, the CQ alone inhibited *M. tuberculosis*, confirming earlier results (11). Therefore, its enhancement of the effectiveness of SM could be due more to direct inhibition of *M. tuberculosis* than to its ability to change vesicular pH.

Effects of CQ used with INH. This interpretation of a pH-independent cooperation between CQ and other antituberculosis drugs was tested with the pH-independent antituberculosis drug INH. INH was used in various combinations with CQ in seven experiments using MP from four different donors. CQ improved the effectiveness of INH against *M. tuberculosis* in every experiment. Typically, as shown in Fig. 3, the combination of CQ and INH was bactericidal, while INH alone was bacteriostatic. In the experiment in

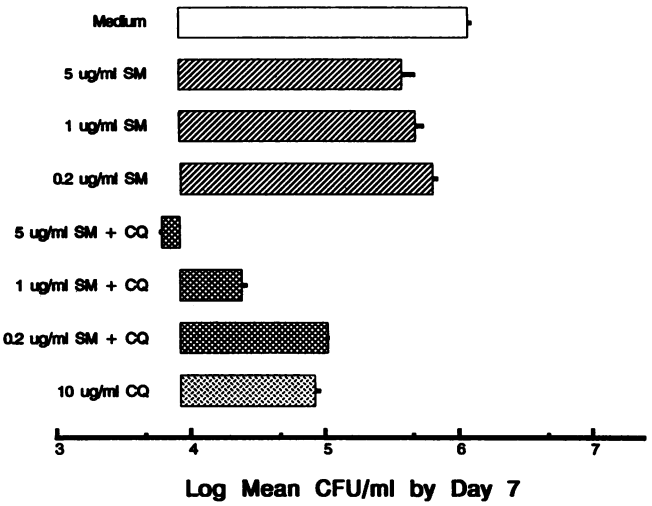

FIG. 1. Cooperation between CQ at 10 μg/ml and SM at the indicated concentrations against tubercle bacilli in infected MP. In this and succeeding figures, the baseline is mean CFU of tubercle bacilli per milliliter of MP lysate (1 ml contained an average of 10^5 MP) at zero time (immediately after infection), and the bars show mean CFU per milliliter of macrophage lysate (± standard error of the mean) at 7 days after infection. For this and the experiments shown in other figures, the drugs were added to the medium once, at the concentrations shown, immediately after infection.

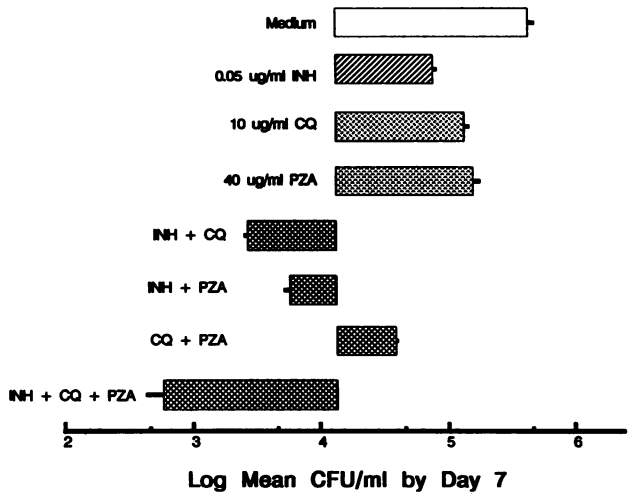

FIG. 3. Cooperation of CQ with the pH-independent drug INH against *M. tuberculosis* in MP and with the acid-dependent drug PZA. The same concentrations of drugs were used in the various combinations as were used in their use individually. The drugs were added immediately after infection.

Fig. 3, CQ also enhanced the inhibition of *M. tuberculosis* by PZA, a finding which is confirmed and extended below.

Effects of CQ used with SM against *M. avium*. SM is able to inhibit the strain of *M. avium* used in these experiments, but CQ is completely ineffective against it (no inhibition at up to 8,000 μg/ml [data not shown]). The ability of CQ to enhance the effectiveness of SM against *M. avium* in MP was tested in two separate experiments by using MP infected with both *M. avium* and *M. tuberculosis*. Results from the two experiments agreed and data from one of them are represented by Fig. 4. Thus, CQ enhanced SM activity in MP against *M. tuberculosis* but not that against *M. avium*.

Effects of CQ used with PZA against *M. tuberculosis*. Since PZA requires a low pH to inhibit *M. tuberculosis* (40) and CQ raises the pH of low-pH vesicles in MP (43, 44), CQ should inhibit PZA anti-*M. tuberculosis* activity in MP if the *M. tuberculosis* are in acidic vesicles. This idea was tested by using CQ in various combinations with PZA against *M. tuberculosis* in 15 experiments with MP from nine different donors. CQ did not inhibit the anti-*M. tuberculosis* activity of PZA in any of the experiments. In 14 of 15 experiments it improved inhibition of *M. tuberculosis* by PZA. Figure 5 shows representative results from an experiment in which one concentration of CQ was used with three different concentrations of PZA (see also Fig. 3, above).

PZA was not tested with *M. avium*, in combination with CQ or with NH_4Cl, because *M. avium* is not sensitive to inhibition by PZA (27).

Effects of NH_4Cl on SM inhibition of *M. tuberculosis* in MP. NH_4Cl is a weak base which can raise the pH of acidic vesicles in MP (8, 44) but has no ability of its own to inhibit *M. tuberculosis* (data not shown). It was used at the maximum concentration which could be tolerated by MP (5 mM) as a substitute for CQ to test whether the anti-*M. tuberculosis* activity of SM in MP can be increased solely by increasing vesicular pH. In 9 of 12 experiments using MP from eight different donors, the presence of the NH_4Cl did not affect the effectiveness of SM against *M. tuberculosis*, while in three it did increase the effectiveness slightly. However, in these three experiments, the presence of NH_4Cl alone tended to inhibit the growth of the *M. tuberculosis* in the MP. Results most representative of this series of experiments are shown from two experiments in Fig. 6. In Fig. 6A, the NH_4Cl did not affect the effectiveness of SM against *M. tuberculosis* at any of three concentrations of SM. In Fig. 6B, at either of two concentrations it failed to enhance the effectiveness of SM, while CQ at each of three concentrations synergized with the SM against the *M. tuberculosis*.

Effects of NH_4Cl and PZA against *M. tuberculosis* in MP. NH_4Cl was used together with PZA in *M. tuberculosis*-infected MP cultures in eight experiments using five different donors. In six experiments it did not affect the effectiveness of PZA against *M. tuberculosis*; in two it decreased the

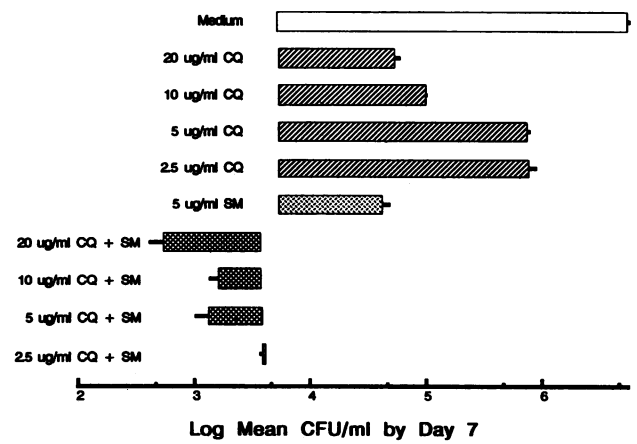

FIG. 2. Titration of the ability of CQ to inhibit intracellular *M. tuberculosis* and to synergize with 5 μg of SM per ml to kill intracellular *M. tuberculosis*.

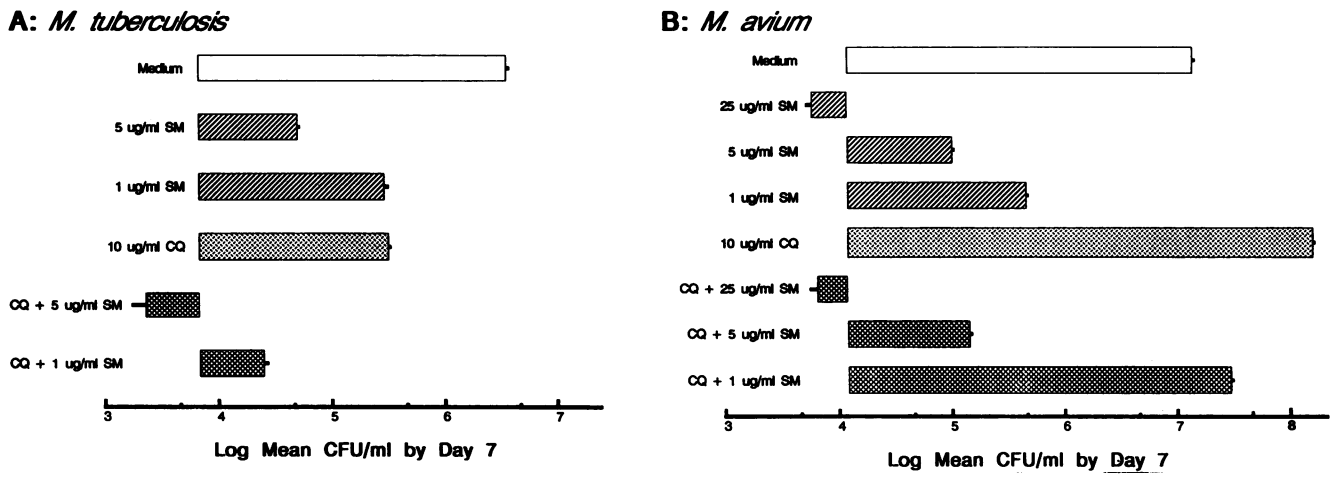

FIG. 4. Results from an experiment showing that while CQ synergizes with SM to inhibit or kill *M. tuberculosis* and while it is itself inhibitory for *M. tuberculosis* (A), it is unable to improve inhibition by SM of *M. avium*, for which it alone is not inhibitory (B).

effectiveness moderately. Figure 7 illustrates the findings of these experiments in an experiment in which both PZA and the NH_4Cl were used at different concentrations.

Electron microscopic examination of intracellular association between acid vesicles and *M. tuberculosis* and *M. avium*. The association between acidity and *M. tuberculosis* or *M. avium* in MP was examined directly by immunochemical electron microscopy, using the weak base DAMP. The selective binding of the DAMP to acidic regions of the infected MP was detected with colloidal gold-labeled antibodies to DAMP. MP were examined for three different conditions of infection: (i) at 7 days after infection with the same concentration of bacteria as that used in the drug experiments, (ii) after a 30-min period of infection with suspensions of living bacteria at concentrations 100 times greater than were used in the drug experiments, and (iii) after a 30-min infection with the 100-times-greater concentration of bacteria which had been killed before the infection by heating at 62°C for 30 min. Representative electron micrographs of these cells are shown in Fig. 8.

Figure 8A and B show that at the end of a 7-day period of normal infection there was practically no association between DAMP (i.e., low pH) and the intracellular bacteria. This was confirmed by actual blind counts for 12 different MP for association between *M. tuberculosis* or *M. avium* bacteria and DAMP. Thus, 138 *M. avium* were negative and 3 were positive, and 207 *M. tuberculosis* were negative and 33 were positive.

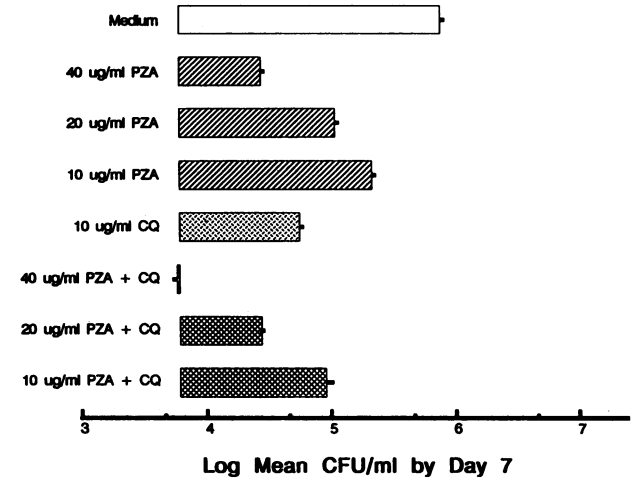

FIG. 5. Experiment demonstrating that CQ does not inhibit suppression of *M. tuberculosis* growth in MP by PZA.

The results of counts for MP infected for 30 min with the dense suspensions of living or heat-killed bacteria are shown in Fig. 9. They show that DAMP was associated in the MP more with dead than with live bacteria.

CQ and NH_4Cl effects on vesicular pH in MP. To learn whether the NH_4Cl and CQ used in the chemotherapy experiments described above raised vesicular pH of the infected MP during the 7-day period of infection, infected cultures were incubated in medium alone, in NH_4Cl, or in CQ as done in those experiments. Samples were taken for incubation with DAMP, fixation, and electron micrographic examination as already described. It was not possible to use any simple objective measure of labeling for the electron micrographs made of the samples, mainly because of the heterogeneity of the intracellular acidic compartments and their uneven intracellular distribution. Therefore, several sets of representative electron micrographs of the infected cells were examined by four different readers without letting them know which groups they were viewing. They looked for frequency of association of gold particles with intracellular bacteria and for the number and distribution of gold-labeled cellular organelles. Their observations were translated into a scale of 0 to 4, where 4 indicates that the number and distribution of labeled organelles were the same as in MP incubated in culture medium alone and 0 indicates no labeling.

The consensus ratings are graphed in Fig. 10 and indicate the following. While the results confirmed that living *M. tuberculosis* were not associated with acid vesicles at 7 days, they indicated that some were at 4 days. Both NH_4Cl and CQ suppressed the moderate association of the bacteria with acid vesicles that was evident even at 4 days. Both drugs diminished the DAMP labeling of vesicles in the MP on both day 4 and day 7. However, despite additions of NH_4Cl to the cultures at 2, 4, and 6 days after infection, there was a rise in the number of DAMP-labeled vesicles after 7 days compared

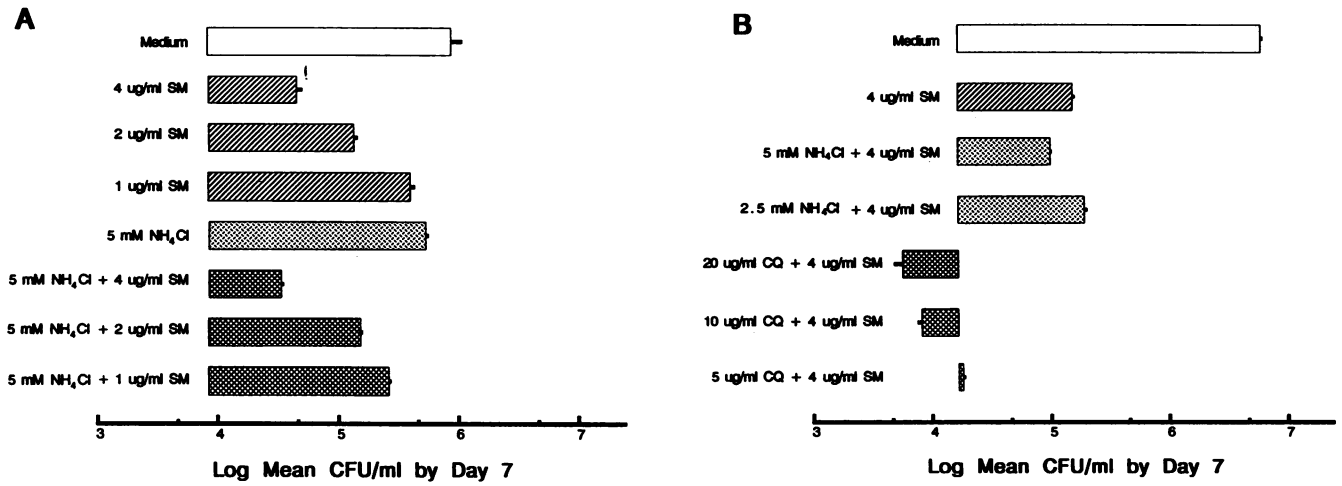

FIG. 6. Results from paired experiments showing that NH_4Cl will not enhance inhibition of *M. tuberculosis* in MP by SM (A) under experimental conditions in which CQ will (B).

with the number after 4 days. There also seemed to be a rise in numbers of acidic vesicles at day 7 compared with day 4 for CQ. CQ was added to the medium only on day 0 of the infection. This experiment thus shows directly that both chemicals, when used as described for these chemotherapy experiments, were able to raise the pH of acid vesicles in the MP. It also confirms directly that the mycobacteria multiplying in the MP are in vesicles that are not acidic, regardless of whether NH_4Cl or CQ is added to the culture medium.

DISCUSSION

The results reported here confirm earlier observations (10, 17) that *M. tuberculosis* and *M. avium* are found in vesicles but not in the cytoplasm of cultured human MP. The bacteria are similarly located in cultured mouse MP (3, 22, 25, 26, 35, 36, 38, 39, 45, 50) and probably also in the MP of infected animals and people (4, 35–37).

The vesicles which contain these mycobacteria are commonly assumed to be phagolysosomes with a pH of approximately 5, and this assumption has become an essential part of most interpretations of *M. tuberculosis* and *M. avium* existence in MP (14, 15, 24, 30, 35, 51). According to results presented here, however, this belief probably is correct only for dead or impaired *M. tuberculosis* and *M. avium*. Both indirect chemotherapeutic evidence and direct localization of intracellular acidity indicated that the vesicles in cultured human MP which contain living and healthy *M. tuberculosis* or *M. avium* are probably phagosomes which are not acidic.

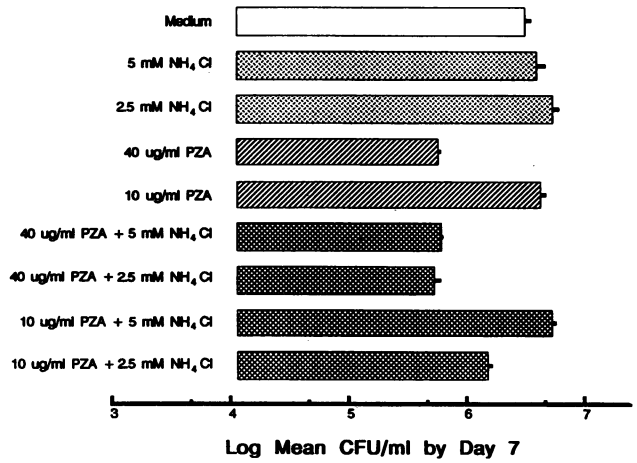

FIG. 7. NH_4Cl does not inhibit suppression by PZA of *M. tuberculosis* growth in MP.

If the mycobacteria had been in acidic vesicles, as is conventionally assumed, then the actions of PZA and SM against them should have been changed by the addition of the lysosomotropic weak bases CQ and NH_4Cl to the infected MP, which raised the pH of the vesicles (5, 8, 33, 42–44). PZA should have lost and SM should have gained effectiveness. However, neither base had these predicted effects. Inhibition of *M. tuberculosis* by PZA was consistently enhanced by CQ rather than inhibited, and it was not inhibited by NH_4Cl as it should have been. The effectiveness of SM against *M. tuberculosis* was also enhanced by CQ. However, this was not related to pH change but rather to the direct anti-*M. tuberculosis* effectiveness of CQ, as previously described (11). CQ is ineffective against *M. avium* (11), and it did not improve inhibition of *M. avium* by SM.

Both CQ and NH_4Cl, as used in these experiments, were determined by direct immunolabeling of acidic compartments of the MP to raise the pH of these compartments (1, 2). The immunolabeling further showed with the high resolution of electron microscopy that the vesicles containing living *M. tuberculosis* and *M. avium* in MP were not acidic. They did show, however, that killed mycobacteria are associated with acidic vesicles. This latter observation agrees with the 1956 findings of Sprick (50), made with mouse MP and killed *M. smegmatis* and *M. tuberculosis* which had been labeled with pH indicator dyes.

Newly ingested *M. tuberculosis* and *M. avium* are found in phagosomes, which should have the same pH as the medium in which the ingesting MP are being cultured (29, 31, 42). Originally, these phagosomes were thought to have to fuse with lysosomes to become acidic, and phagosome-lysosome fusion was therefore believed to be important in the control of mycobacterial infections. Since both *M. tuberculosis* (3, 23, 25, 26) and *M. avium* (22, 45) were found to suppress

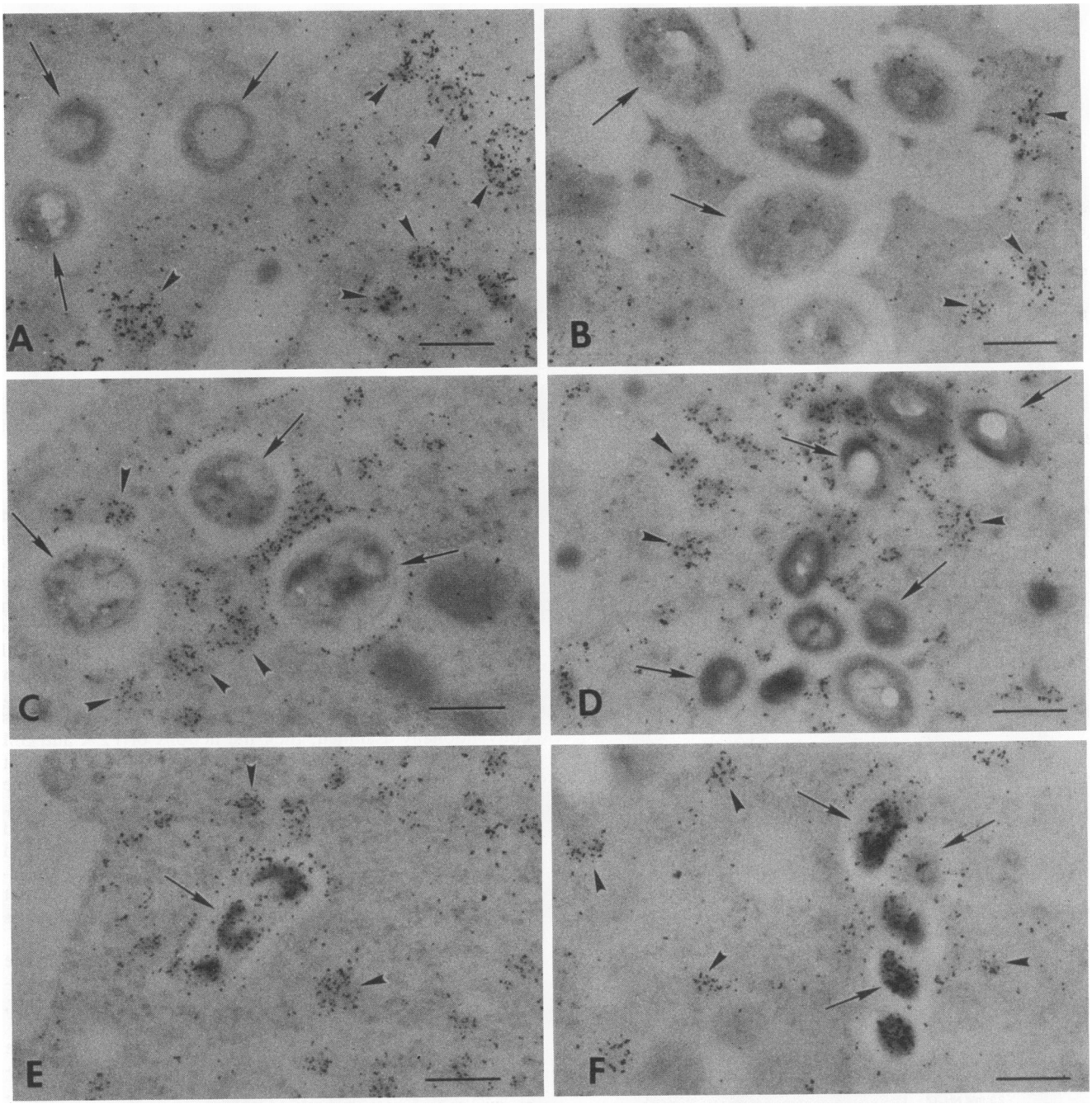

FIG. 8. Electron micrographs of MP infected with *M. avium* or *M. tuberculosis* and exposed to DAMP. The acidified components of the cells have been revealed by the immunocytochemical localization of DAMP with colloidal gold-labeled antibodies to DAMP. Panels A and B are of macrophages after 7 days of infection with live *M. avium* and *M. tuberculosis*, respectively. Panels C and D were prepared after 30 min of infection with live *M. avium* and *M. tuberculosis*, respectively. Panels E and F were prepared after 30 min of infection with heat-killed *M. avium* and *M. tuberculosis*. The arrowheads show aggregates of silver-intensified colloidal gold indicating acidified vesicles, including lysosomes. The arrows show bacteria within phagosomes. As these micrographs illustrate, the DAMP was not present in phagosomes containing (presumed) live bacteria but was found in those containing heat-killed bacteria. The bacteria were contained within phagosomes 7 days as well as 30 min after infection, and the phagosomes generally contained single *M. avium* but multiple *M. tuberculosis* bacilli. The cells in A and B were not washed after exposure to DAMP and prior to fixation and therefore exhibit a greater amount of nonvesicular DAMP than do the cells in the other panels. Bars, 0.5 nm.

phagosome-lysosome fusion, it was supposed that they might use this ability to avoid acidification and exposure to the antibacterial acid-dependent contents of lysosomes. However, the pathobiological significance of this avoidance of fusion by these bacteria became questionable when Armstrong and Hart (3) demonstrated that *M. tuberculosis* coated with antibody before phagocytosis became unable to block fusion but nevertheless multiplied in mouse MP and

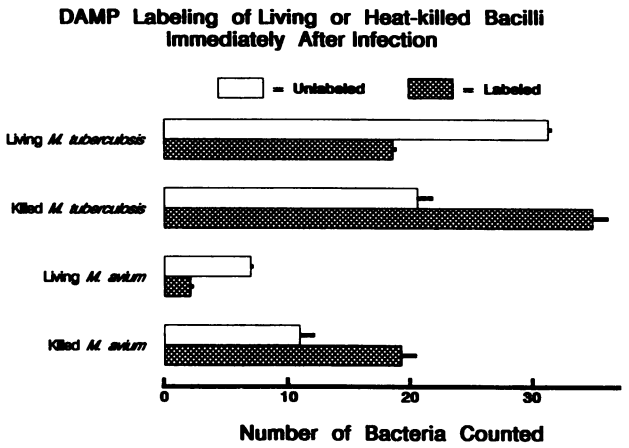

FIG. 9. Counts of *M. tuberculosis* and of *M. avium* in MP that are associated with DAMP, detected by colloidal gold labeling, in experiments in which the macrophages were infected for 30 min with live or heat-killed bacteria at a ratio of bacteria to macrophages of 20:1. The bars show the numbers of bacteria counted which were or were not associated with gold label. □, unlabeled; ▩, labeled.

that *M. tuberculosis* which had not been coated with antibody was able to block fusion.

The importance of phagosome-lysosome fusion in vesicle acidification has been diminished still further by recent findings that mammalian cells have several different kinds of vesicles, including phagosomes, which are all actively acidified by an ATPase-dependent proton pump. Phagosomes do not have to fuse with lysosomes to become acidic (42, 49). In fact, phagosome acidification may be a condition which promotes phagosome-lysosome fusion (6, 34, 49). The findings presented here suggest that living, virulent *M. tuberculosis* and *M. avium* are able to neutralize the acidifying effects of this proton pump. This would result in their being able to maintain a microenvironmental pH suitable to their survival and multiplication, to retard phagosome-lysosome fusion, and to counteract the antimicrobial effectiveness of acid-dependent lysosomal constituents if phagosome-lysosome fusion were to occur. The results also showed, however, that killed *M. tuberculosis* and *M. avium* are associated with acidic vesicles. Thus, once they are injured or killed, these mycobacteria probably are subject to conventional acidification and consequent phagolysosomal digestion and disposal.

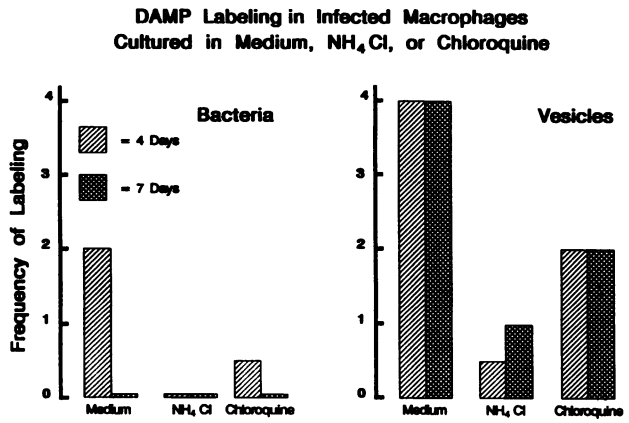

FIG. 10. Estimates on a scale of 0 to 4 of intracellular bacteria or of MP vesicles which were acidic (i.e., DAMP-labeled) at 4 or 7 days after infection in cultures with medium only, with 5 mM NH_4Cl added at 0, 3, and 6 days, or with 10 μg of CQ per ml added at zero time after infection.

These two species of mycobacteria may counteract phagosome acidification by production of ammonia (23). Cultures of *M. tuberculosis* are able to make higher concentrations of ammonia (20 mM) than were used in the present experiments to artificially raise vesicular pH. Ammonia is produced copiously by other species of mycobacteria including *M. avium* (20, 21, 41, 46). *M. avium* is reported to be especially effective in a reversible reaction for ATP and NH_3 production (21). Interestingly, *M. avium* was found to increase production of NH_3 considerably if starved of carbohydrates (38) or oxygen (20), both of which might occur within MP.

Since these bacteria, especially *M. tuberculosis* (9), are inhibited by acidic pH, the present observations that *M. tuberculosis* and *M. avium* are in nonacidic vesicles in MP helps explain how they avoid inhibition and instead multiply abundantly within MP (10, 12, 17). These observations also suggest some new explanations for antimycobacterial activities of MP associated with the expression of immunity and with the effectiveness of certain antituberculosis drugs. Thus, any set of circumstances favoring vesicle acidification (e.g., increased activity of the MP proton pump or decreased ability of the mycobacteria to neutralize products of the pump) should protect MP from the mycobacteria and vice versa. Other intracellular acid-sensitive parasites have also been shown to block acidification of the MP vacuoles containing them. Horwitz and Maxfield (28) were the first to show this, observing that living *Legionella pneumophila* but not the killed bacteria nor living *Escherichia coli* blocked acidification of vesicles containing them equally well in both quiescent and activated human monocytes. Sibley et al. (49) observed the same for *Toxoplasma gondii* in cultured mouse MP. In both reports the investigators concluded that the ability of these parasites to block acidification of their intracellular microenvironment is critical to their survival in the infected phagocytes. Our results support this interpretation for the intracellular survival of *M. tuberculosis* and *M. avium*.

These findings suggest some changes in thoughts of how PZA and SM inhibit intracellular mycobacteria. An inherently low pH of phagolysosomes no longer alone suitably explains the intracellular effectiveness of PZA against *M. tuberculosis* (15, 51), nor does it explain the poor effectiveness of SM against *M. tuberculosis* and *M. avium* (14, 38). Although low pH probably is associated with the effectiveness of PZA, its effectiveness most likely is more a consequence of bacterial than of host cell activity. Pyrazinoic acid is made by bacterial pyrazinamidase from PZA (32, 48, 51). Unable to cross mammalian cell membranes, this acid becomes trapped with the bacteria in their vesicles (15, 48, 51). Its accumulation, perhaps helped by acidification by the proton pump of the phagocyte, probably overcomes the ability of *M. tuberculosis* to maintain the nonacidic microenvironment it needs to multiply in MP. This interpretation fits well with the *M. tuberculosis*-killing synergy that is seen between PZA and MP-activating molecules like the metabolites of vitamin D which protect human MP against *M. tuberculosis* (13), as well as the seemingly paradoxical synergy between PZA and CQ (11). In this latter synergy, the pyrazinoic acid produced by the *M. tuberculosis* from PZA could become a localized acidic sink for accumulation

of the basic and *M. tuberculosis*-toxic CQ and the concentration of CQ in direct contact with tubercle bacilli could rise enough to kill them.

The probably incorrect explanation of SM losing antimycobacterial activity in MP because of low pH around the bacteria could be replaced by one stating that the SM may be ineffective simply because it is not reaching the intracellular bacteria. While SM does enter and accumulate in MP (7), because it is a weak base it could be homing to acidic compartments of the MP rather than to the nonacidic vesicles which contain the target mycobacteria.

These results suggest that studying how to block or counteract the antiacidifying properties of *M. tuberculosis* and *M. avium* in the vesicles of infected MP may lead to better immunologic and chemotherapeutic control of infections caused by these mycobacteria.

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