

Inhibition of Tubercle Bacilli in Cultured Human Macrophages by Chloroquine Used Alone and in Combination with Streptomycin, Isoniazid, Pyrazinamide, and Two Metabolites of Vitamin D₃

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Intracellular tubercle bacilli (TB) reside in vacuoles in infected human macrophages (MPs). The relative impotency of streptomycin against TB in MPs and the contrary greatly increased potency of pyrazinamide (PZA) have been attributed to the fact that these vacuoles are phagolysosomes and, therefore, acidic. Chloroquine (CQ) is a lysosomotropic base which can be used to raise phagolysosomal pH. Consequently, it was tested for its ability to increase the anti-TB effectiveness of streptomycin and decrease that of PZA in cultured human MPs. MPs infected with virulent Erdman strain TB were incubated in medium with various combinations of the drugs. Samples were taken at 0, 4, and 7 days and lysed for CFU counts of viable TB on nutrient agar. As expected, CQ increased the effectiveness of SM, but unexpectedly, it did not decrease that of PZA. CQ alone was found to be able to inhibit intracellular TB. Because of this, it was also tested with isoniazid, 1,25(OH)₂-vitamin D₃, and 25-OH-vitamin D₃. It significantly enhanced the anti-TB protectiveness of both isoniazid and 25-OH-vitamin D₃. Some combinations of CQ and the various drugs tested were able to kill intracellular TB. These results suggest that CQ may be useful in the treatment of tuberculosis.

Tubercle bacilli (TB) in infected human macrophages (MPs) reside in vacuoles (4). These vacuoles might be phagolysosomes, which would be acidic and have several acid-dependent enzymes and activities (18). The presumed vacuolar acidity is thought to affect the activities of certain anti-TB drugs. It is supposed to decrease that of streptomycin (SM) (7) and increase that of pyrazinamide (PZA) (21, 22, 27).

Chloroquine (CQ) is a drug which is widely used against malaria and certain kinds of chronic inflammatory diseases (3, 28). It is also used experimentally to study the acidic vacuoles of animal cells (16, 24), because it is a lysosomotropic base (13, 28) which can increase the pH of these vacuoles and inhibit their acid-dependent activities (16, 24). Thus, it inhibits malaria parasites by concentrating in the parasite's food vacuoles (lysosomes), raising their pH, and interfering with their digestive functions. This deprives the parasites of critical nutrients (13). Similarly, CQ can raise the pH of phagolysosomes in mammalian cells and affect their digestive functions (16, 18, 24). If TB do live in acid phagolysosomes in MPs and CQ can raise the pH of these vacuoles, then this drug should increase the anti-TB effectiveness of SM and lower that of PZA. This was tested in the experiments described here. While CQ enhanced the effectiveness of SM, as expected, it did not suppress that of PZA. Also, unexpectedly, it was itself able to inhibit TB in MPs, and it enhanced the anti-TB activity of another drug, isoniazid (INH), whose activity is not pH sensitive.

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MATERIALS AND METHODS

Human subjects. Peripheral blood monocytes from five normal healthy donors (four males; one female; ages, 22 to 59 years) were used (informed consent was obtained). A 59-year-old male donor was tuberculin positive. Tuberculin reactivity, however, had no apparent effect on the results reported here.

MP cultures. MP cultures were prepared from adherent peripheral blood monocytes as described previously (4, 8). The cells to be infected were therefore 7-day-old macrophages distributed in three separate spots of approximately 5×10^4 MPs per spot or 1.5×10^5 MPs per 35-mm-diameter petri dish. The cells were cultured in 1.5 ml of RPMI 1640 medium per dish. The medium was supplemented with 1% unheated human type AB serum (obtained from a single healthy donor whose blood has been used for several years in such experiments) and 2 mM L-glutamine. No antibacterial drugs other than those used experimentally were added. The number and physical condition of the MPs were monitored during the experiments by phase microscopy. Throughout these experiments, there were no differences in number, appearance, or viability of test and control MPs that related to the activities of the various drugs.

Bacilli and macrophage infection. *Mycobacterium tuberculosis* Erdman TB were used, as described previously (4, 8), for a 30-min period of infection of the 7-day-old MP cultures. This infection was adjusted so that about 10% of the MPs ingested one or two TB each (4). The numbers of MPs that were infected and the numbers of bacteria in the infected MPs were determined by comparing the counts of acid-fast bacilli in fixed, stained samples of the MP cultures, the numbers of MP per culture, and the numbers of culturable bacteria per MP for lysates of the cultures, in which the numbers of MPs were known by direct counting (4). After infection, the infecting suspension was washed off with unsupplemented RPMI 1640 medium, and fresh supplemented medium was added to each dish. There was no

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extracellular growth of TB in these experiments (11). The experimental drugs were added to this medium once, immediately following the infection, at the concentrations indicated in descriptions of the experiments. The infected MPs were incubated with or without the drugs for 7 days following the infection.

Counts of bacilli. Counts of bacilli were made from samples of the infected MPs taken at 30 min after infection (time zero) and 4 and 7 days after infection. The procedure for lysing the samples of infected MPs with sodium dodecyl sulfate, neutralizing the lysate with bovine serum albumin, diluting it, and plating samples of it onto 7H10 agar has been described in detail previously (4, 11). The results obtained were CFU counts of TB growing on the 7H10 agar. These indicate the numbers of living TB in the MP lysates at the various times of sampling. The CFU is given as the number per ml of MP lysate. One milliliter of lysate was the product of an average of 10^7 lysed MPs. Samples of MPs were observed by phase microscopy and by conventional microscopy, after fixing and staining, for counts of the MPs and verification of cell culture health. The numbers and characteristics of the MPs in the various experimental groups reported in these experiments were comparable.

Effect of CQ against TB in bacteriologic medium. The effect of CQ against TB in bacteriologic medium was tested as described previously (9). Briefly, 0.15-ml volumes of TB-inoculated 7H9 medium were used to make twofold dilutions of CQ from a maximum concentration of 8 mg/ml downward. There was also a medium-only negative control. The titration was done in triplicate in 96-well microtiter dishes. The dishes were incubated for 7 days at 37°C. At the end of incubation the amount of bacterial growth was estimated on a 0 to 4+ scale in comparison with the growth in medium alone. Samples were also taken from each well, diluted to 2 ml with 7H9 medium, sonicated to disperse the bacteria, and diluted further in 10-fold steps for plating on 7H10 agar and counting of CFU.

Drugs. CQ (diphosphate salt; no. C-6628), PZA (no. P-7136), and INH (isonicotinic acid hydrazide, free base; no. I 3377) were purchased from Sigma Chemical Co., St. Louis, Mo. Streptomycin sulfate, USP, was purchased from Pfizer Inc., New York, N.Y. 1,25(OH)₂-vitamin D₃ (1,25-D₃) and 25-OH-vitamin D₃ (25-D₃) were gifts of Milan R. Uskokovic, Hoffmann-La Roche Inc., Nutley, N.J. CQ, SM, PZA, and INH were dissolved and diluted in RPMI 1640 medium to the final concentrations needed and were sterilized by passing them through a membrane filter. The vitamin D metabolites were made up in stock solutions of 1 mg in 1.5 ml of 95% ethanol and stored at -20°C. For use, they were diluted directly into the culture medium that was already added to the infected MPs (5). Previous experiments (5) have shown that the small amount of ethanol added to these cultures does not affect the results.

Method of reporting results. For all experiments, 0-, 4-, and 7-day counts of CFU were made. However, to simplify presentation of results, Fig. 1 to 5 show only the 0- (baseline) and 7-day counts, that is, the 7-day change in CFU. Figures 2 to 5 show results from a single experiment. However, every experiment was repeated for confirmation at least once by using blood from a different donor. Standard errors of the means given in Fig. 1 to 5 were determined by Student's *t* distribution from the five CFU counts recorded for each experimental condition.

RESULTS

Titration of CQ against TB in MPs. The range of the lowest CQ concentrations effective against TB in human MPs was determined from several experiments. The MPs were infected, and the drug was added to the MP culture medium once immediately after infection. Results from three experiments using blood from two different donors are given in Fig. 1A. Results are mean CFU of TB per ml of MP lysate at 7 days after infection. Each experiment included a control without drug. Also, for comparison, there was a group treated similarly with 40 µg of PZA per ml. This is the lowest concentration of PZA which is consistently effective in human MPs (8). CQ was effective at a lower concentration in subject 1 MPs than in subject 2 MPs. The lowest concentration that was regularly effective in all subjects for this regimen of use was 10 µg/ml.

Titration of CQ against TB in 7H9 broth. Figure 1B gives the results by CFU counts by titrating CQ directly against TB in bacteriologic culture medium. The concentrations are expressed in milligrams per milliliter, because high concentrations of CQ were needed to inhibit TB outside of MPs. Very high concentrations were able to kill TB. At 0.5 mg/ml, CQ inhibited TB in broth approximately as much as 10 µg/ml could in MP (cf. Fig. 1A).

CQ was also tested in 7H9 broth against the virulent 7497 strain of serovar 4 *Mycobacterium avium* (10). It did not inhibit *M. avium* even at the highest concentration used (8 mg/ml).

Cooperation between CQ and SM against TB in MPs. MPs from subject 3 were infected and then incubated for 7 days in three different concentrations of SM without or with 10 µg of CQ per ml. The drugs were added to the culture medium once, immediately after infection. The results are given in Fig. 2. When used alone, only the 5-µg/ml concentration of SM was significantly effective. When CQ was present, 1 µg of SM per ml became effective, and the effectiveness of the 5-µg/ml concentration was increased considerably.

Interactions between CQ, PZA, and INH against TB in MPs. CQ was tested in combination with PZA, INH, or both, which were used at their MICs against TB in human MPs (8, 9). MPs from subject 4 were used. The results are given in Fig. 3. CQ was somewhat more effective in this subject than it was in the others. It was significantly more inhibitory than PZA. INH alone at 0.05 µg/ml was bacteriostatic or slowly bactericidal, as reported previously (9). CQ and PZA used together were more effective than was either one used alone. CQ plus INH caused exponential killing of intracellular TB; this combination was more effective than the combination of PZA plus INH. The combination of CQ, PZA, and INH was more rapidly bactericidal than the combination of only CQ and INH was.

Effects of CQ and 1,25-D₃ against TB in MPs. Human MPs are reproducibly protected against TB by 4 µg of 1,25-D₃ per ml (5). The effect of CQ on this kind of resistance was tested. MPs from subject 5 were incubated with 1,25-D₃, CQ, or PZA and combinations of these drugs (Fig. 4). In this experiment, the protectiveness of all three drugs was strong and approximately the same. CQ and 1,25-D₃ did not interfere with each other; their combined effect was bacteriostatic. As reported previously (6), 1,25-D₃ and PZA cooperated to become mildly bactericidal. The combination of CQ and PZA was bacteriostatic. The combination of all three drugs killed TB somewhat faster than the combination of 1,25-D₃ and PZA did.

Cooperation between CQ and 25-D₃. The cooperation

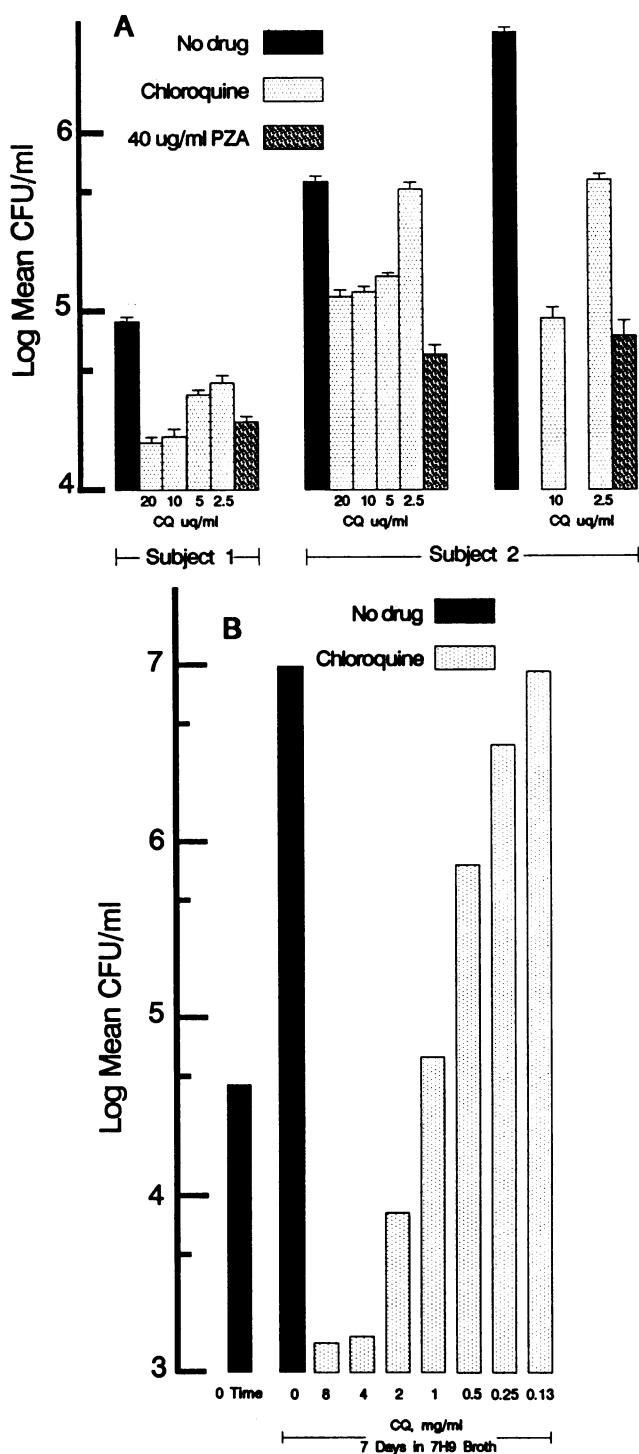


FIG. 1. Titration of CQ inhibition of TB in MPs (A) or 7H9 bacteriologic culture medium (B). For titrations in MPs, cells from subjects 1 (first experiment) and 2 (second and third experiments) were used. CQ was used in the MP culture medium at the indicated concentrations. The data shown are mean \pm standard error (for five values each) CFU per milliliter of MP lysate at 7 days after infection. Similar data are shown for CFU counts of samples of 7H9 medium taken after 7 days of incubation at 37°C. Note that the CQ concentration in MPs is given in micrograms per milliliter and the concentration in broth is given in milligrams per milliliter.

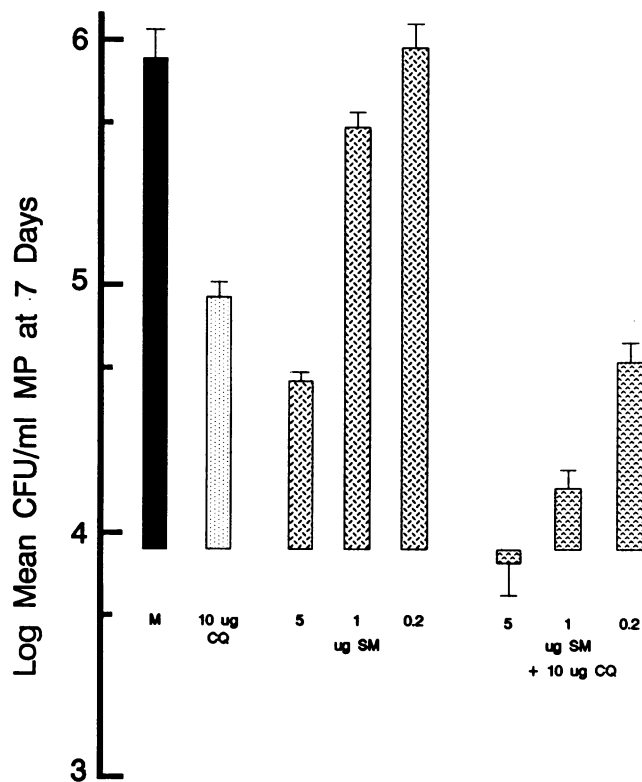


FIG. 2. Effects of SM at 5, 1, or 0.2 μ g/ml against TB in MPs used alone or with 10 μ g of CQ per ml. The base line for all bars is the count at time zero; each bar is the mean \pm standard error of the mean of five 7-day counts of CFU. Drugs were in the culture medium that was added to the MPs immediately following infection of the MPs.

between CQ and 25-D₃ was an unexpected discovery from experiments with the two drugs to test whether CQ could inhibit protection of MPs by this vitamin D metabolite precursor of 1,25-D₃. The experiments were suggested by clinical use of CQ for controlling hypercalcemia in patients with sarcoidosis by lowering the excessive levels of circulating 1,25-D₃ (23) that are thought to be produced from 25-D₃ by MPs (25). CQ was expected to block hydroxylative production from the nearly unprotective 25-D₃ (4a, 26) of the protective 1,25-D₃. CQ was used at 10 μ g/ml with 4 μ g of the two metabolites per ml. The results (Fig. 5) show that CQ and 25-D₃ used together provided stronger anti-TB protection than did either one alone. These results were confirmed in a separate experiment with blood from a different donor.

DISCUSSION

CQ originally was developed and selected in the United States as an antimalarial drug substitute for quinine, when quinine became unavailable at the beginning of World War II (17). It has been used as such in millions of people (17, 20, 28). It is also effective against rheumatoid arthritis and is used more for that now than it is against malaria (12, 20, 28).

CQ probably inhibits malaria plasmodia by concentrating in their lysosomal food vacuoles. Lysosomes use acid-dependent hydrolases to digest substances delivered to them by phagosomes and endosomes (18, 19). CQ is actively pumped into lysosomes (17, 20), where, as a weak base, it neutralizes phagolysosomal acidity (16, 20, 24). This sup-

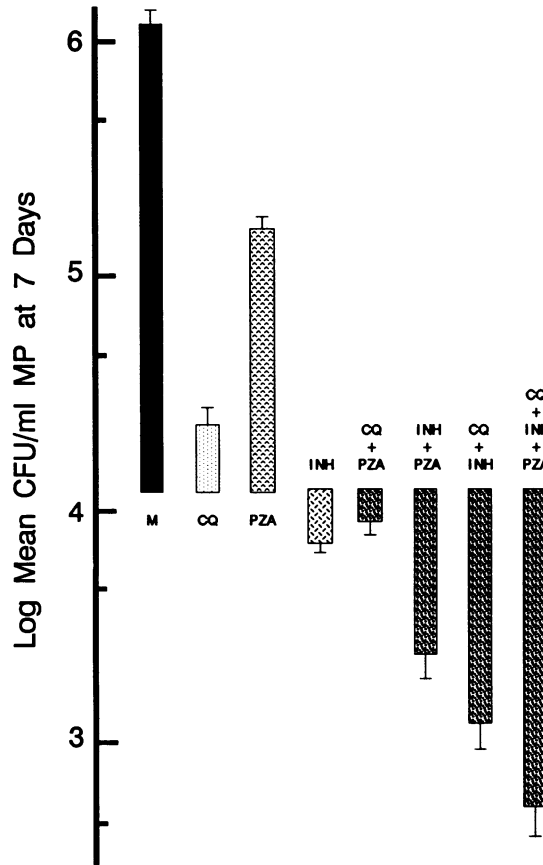


FIG. 3. Effects of INH (0.05 $\mu\text{g/ml}$), PZA (40 $\mu\text{g/ml}$), and CQ (10 $\mu\text{g/ml}$) used alone or in combinations against TB in MPs. Experimental conditions and data are as described in the legend to Fig. 2.

presses lysosome digestive activities, and in patients with malaria, treatment inhibits the parasites by depriving them of critical nutrients (13). These properties of CQ also have made it useful experimentally for changing the pH of lysosomes in mammalian cells to study various acid- and lysosome-dependent cell functions (16, 24).

In this study we tested CQ for its ability to enhance the anti-TB effectiveness of SM in human MPs by raising phagolysosomal pH, because virulent TB are thought to multiply intracellularly within phagolysosomes (4) and the low pH of these vesicles is assumed to diminish the antibacterial effectiveness of SM (7). CQ performed as expected, decreasing by fivefold the minimal concentration of SM that could inhibit TB in MPs. It enabled SM to kill some intracellular bacilli, when SM alone was only able to slow their growth. However, the reason for cooperation between CQ and SM is unclear, because CQ itself was found to be able to inhibit intracellular TB. CQ slowed or stopped the growth of the TB in MPs when CQ was added to the MP cultures at concentrations down to 2.5 $\mu\text{g/ml}$ (0.005 mM) (Fig. 1A). This concentration is in the range of 0.5 to 5 $\mu\text{g/ml}$ of plasma which is commonly attained in CQ-treated patients with rheumatoid arthritis (12). At 1 mM or higher, CQ can inhibit some species of bacteria by directly impairing DNA and RNA synthesis (2). TB apparently are among these susceptible species of bacteria, because they were inhibited by CQ in 7H9 broth at 500 $\mu\text{g/ml}$ (1 mM) and were killed by concentrations of 2,000 $\mu\text{g/ml}$ or more (Fig. 1B). These

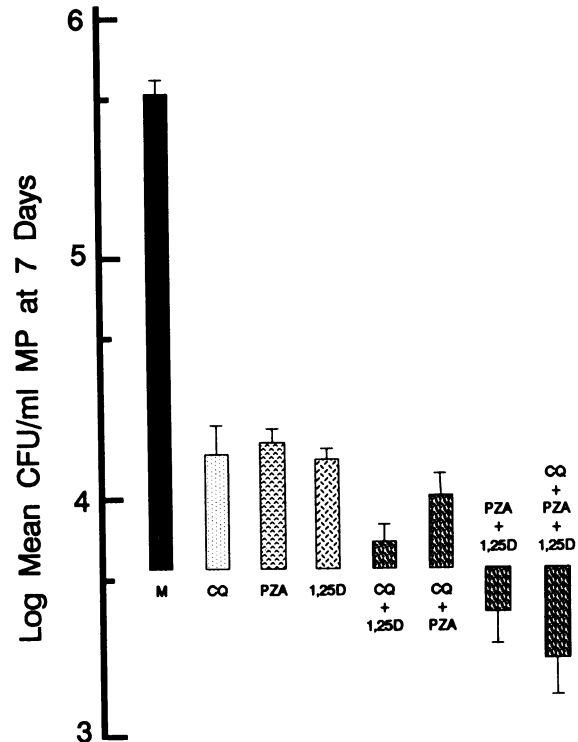


FIG. 4. Effects of 1,25- D_3 (4 $\mu\text{g/ml}$), PZA (40 $\mu\text{g/ml}$), and CQ (10 $\mu\text{g/ml}$) used alone or in combinations against TB in MPs. Experimental conditions and data are as described in the legend to Fig. 2.

concentrations were much higher than were needed for inhibition of TB in MPs. However, human monocytic phagocytes concentrate CQ rapidly as much as 100-fold, and at the concentrations achieved in plasma during therapy of rheumatoid arthritis, they accumulate CQ at concentrations of 150 $\mu\text{g/ml}$ and higher (1, 12). Furthermore, TB have acidic surfaces which could further increase CQ concentrations within infected phagolysosomes (14). When CQ was in contact with the TB in the infected phagolysosomes, CQ may have reached concentrations that could inhibit the bacteria directly. The ability of CQ to inhibit TB is selective, because it was not able to inhibit *M. avium*, even at 8,000 $\mu\text{g/ml}$ (16 mM).

If CQ enhanced the anti-TB effectiveness of SM solely by raising phagolysosomal pH (16, 19, 24) as was originally postulated for these experiments, then it should have proportionally decreased the effectiveness of PZA, because PZA requires a low pH to inhibit TB (21, 22, 27). CQ and PZA used together, however, were somewhat more effective against TB in MPs than was either one used alone. CQ also enhanced the anti-TB effectiveness of INH, a drug which, unlike SM or PZA, is not pH sensitive. These findings suggest that CQ cooperates with SM, PZA, and INH with anti-TB properties of its own and acts independently of the pH changes it might induce in MP phagolysosomes. High concentrations of CQ can cooperate with INH against TB outside of MPs in 7H9 broth (data not shown). So, it is possible to account for the cooperation between CQ and INH in MPs by two CQ properties: its direct anti-TB effect and its active concentration to inhibitory concentrations in TB-infected phagolysosomes. The rise in phagolysosomal pH which should have been induced by CQ under the conditions of these experiments would not seem to have any

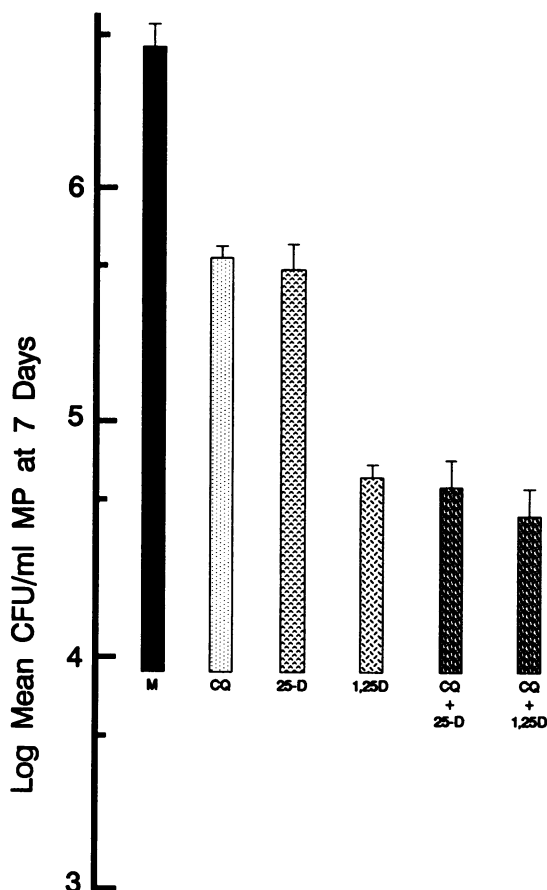


FIG. 5. Cooperation between 25-D₃ (4 μg/ml) and CQ (10 μg/ml) against TB in MPs in comparison with cooperation with 1,25-D₃ (4 μg/ml). Drugs were added to the MP cultures at the indicated concentrations immediately following infection of the MP. Experimental conditions and data are as described in the legend to Fig. 2.

direct role in the cooperation between CQ and the various drugs tested against the intracellular TB. The natively low pH of lysosomes could, however, be important indirectly in the anti-TB activity of CQ in MPs by promoting the intravacuolar accumulation of CQ (12, 19, 28).

The strong inhibition of TB in MPs by the combination of CQ and 25-D₃ was unexpected. CQ enabled this primary metabolite of vitamin D, which ordinarily increases MP resistance to TB only weakly (4a, 26), to make MPs as resistant as they became when they were incubated with the strongly protective secondary metabolite 1,25-D₃ (Fig. 5). CQ may be able to change the metabolism of 25-D₃ in the MPs (23; 25) to increase their production of a strongly protective metabolite. Since CQ suppresses synthesis by MP of 1,25-D₃ (23), the protective metabolite could be one of those recently reported to be more protective than 1,25-D₃ (4a) or some still unidentified metabolite. This finding could be valuable if CQ is used clinically against tuberculosis, because circulating concentrations of 25-D₃ are easily increased by oral supplementation or exposure to UV light (29), and CQ could enhance synthesis of protective metabolites from a relatively abundant 25-D₃ in the actual microenvironment of infection.

Apparently, CQ never has been used to treat tuberculosis. Nor are there any published data on whether people being treated with CQ for other reasons are more resistant to

tuberculosis than people not receiving it. Hart et al. (15) mentioned briefly some experiments on lysosome-phagosome fusion in which they found that 0.02 mM (10 μg/ml) CQ was able to inhibit TB in cultured mouse MPs. The differences between CFU counts for treated and untreated MPs were not large (e.g., 0.5 log units; Fig. 1A). However, they were comparable to such differences as were produced in this experimental model by clinically relevant concentrations of PZA (6, 8) which, because of its notable cooperation with other antituberculosis drugs, has become one of the two drugs most widely used against tuberculosis. Our results suggest, therefore, that CQ and related drugs may be worth further investigation for possible use in antituberculosis chemotherapy.

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