Killing of *Mycobacterium avium* and *Mycobacterium tuberculosis* by a Mycobacteriophage Delivered by a Nonvirulent Mycobacterium: A Model for Phage Therapy of Intracellular Bacterial Pathogens

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Mycobacterium avium **causes disseminated infection in patients with acquired immune deficieny syndrome.** ***Mycobacterium tuberculosis*** **is a pathogen associated with the deaths of millions of people worldwide annually. Effective therapeutic regimens exist that are limited by the emergence of drug resistance and the inability of antibiotics to kill dormant organisms. The present study describes a system using** ***Mycobacterium smegmatis,*** **an avirulent mycobacterium, to deliver the lytic phage TM4 where both** ***M. avium*** **and** ***M. tuberculosis*** **reside within macrophages. These results showed that treatment of** ***M. avium*****–infected, as well as** ***M. tuberculosis*****–infected, RAW 264.7 macrophages, with** ***M. smegmatis*** **transiently infected with TM4, resulted in a significant time- and titer-dependent reduction in the number of viable intracellular bacilli. In addition, the** ***M. smegmatis*** **vacuole harboring TM4 fuses with the** ***M. avium*** **vacuole in macrophages. These results suggest a potentially novel concept to kill intracellular pathogenic bacteria and warrant future development.**

Tuberculosis is a serious public health problem that results in millions of deaths around the world each year [1]. Although tuberculosis is a treatable disease, the incorrect as well as erratic use of therapy often leads to the development of resistance to available regimens. In addition, the advent of the AIDS epidemic has accentuated the problem of drug intolerance in treating tuberculosis, and epidemics of multidrug resistant *Mycobacterium tuberculosis* infection have been reported [2].

Disseminated infection caused by organisms of the *Mycobacterium avium* complex are common in patients with AIDS with <50 $CD4^+$ T cells/mm^3 [3]. The introduction of protease inhibitors in the therapeutic armamentarium against human immunodeficiency virus type 1 (HIV-1) has had a significant impact on the incidence of *M. avium* bacteremia [4]; however, the infection rebounds as soon as the anti-HIV drugs are stopped or fail [5]. In addition, *M. avium* infection has been described with increasing frequency in non-AIDS populations [6, 7].

One of the hallmark characteristics of *M. avium* is its resistance to most of the antituberculosis drugs [8, 9], and only recently have a small number of compounds, such as the new macrolides (azithromycin, clarithromycin, and roxithromycin), been shown to have activity against the bacterium in vitro and in humans [10–12]. Despite the activity of macrolides, the challenge for medical practitioners persists, since those drugs are used for prolonged periods as prophylaxis for the infection, and, once resistance develops, it is generalized toward all macrolides, and effective alternative drug treatment is suboptimal [13].

It is, therefore, paramount that new drugs as well as new forms of therapy be investigated. We hypothesized, on the basis of a number of studies used in the past to treat extracellular bacterial infections such as *Escherichia coli* [14], that the use of a mycobacteriophage might be useful as either part of an antimycobacterial regimen or by itself. In the present report, we show that TM4, a lytic bacteriophage that does not form stable lysogens, can be delivered by a transiently-infected nonvirulent mycobacterium to kill both *M. tuberculosis* and *M. avium* inside macrophages [15, 16].

Materials and Methods

Mycobacteria and mycobacteriophage. *M. avium* strain 109 was isolated from the blood sample of a patient with AIDS and demonstrated to be virulent in the mouse model of *M. avium* infection (data not shown). *M. avium* 109 was chosen for these studies be-

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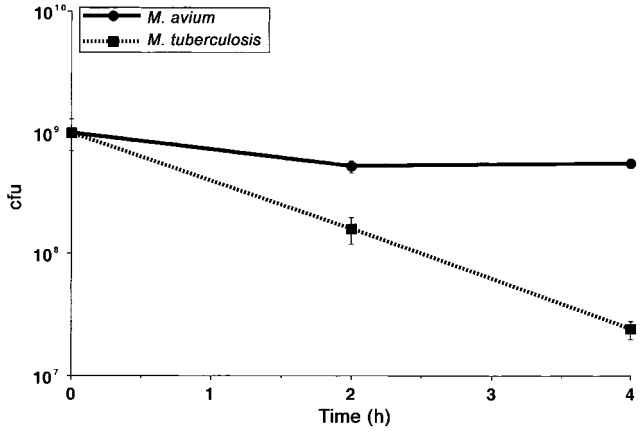

Figure 1. Effect of TM4 phage on *Mycobacterium avium* and *Mycobacterium tuberculosis* viability. *M. avium* and *M. tuberculosis* were incubated with TM4 for 1 h, and then the no. of viable bacteria was determined over time. There was a rapid killing of *M. avium* following exposure to the phage ($P < .05$), compared with the unexposed control. An even more significant bactericidal effect ($P < .01$) was obtained when TM4 was incubated with *M. tuberculosis.* The no. of *M. avium* and *M. tuberculosis* untreated with TM4 remained $1 \pm 0.1 \times 10^9$ cfu for the 4 h of the experiment.

cause it can be infected with the bacteriophage TM4, as reported elsewhere [16]. A spontaneous kanamycin-resistant (R) *M. avium* 109 was isolated by selection on plates containing 400 mg/mL kanamycin. The use of kanamycin-R *M. avium* helped in distinguishing between *M. avium* and *M. smegmatis* after plating. The phenotype was verified to be stable, and virulent assay in macrophage showed that the kanamycin-R *M. avium* was as virulent as the wild-type strain (data not shown). *M. tuberculosis* H37Rv was purchased from American Type Culture Collection. A spontaneous kanamycin-R *M. tuberculosis* was also isolated by selection on plates containing 400 mg/mL kanamycin. The phenotype was determined to be stable. *M. smegmatis* mc^2155 was a gift from William Jacobs, Jr. (Albert Einstein College of Medicine, Bronx, New York). Bacteria were grown on Middlebrook 7H11 agar plates supplemented with oleic acid, albumin, dextrose, and catalase, and individual colonies were subcultured in 7H9 broth for 5 days *(M. avium* and *M. tuberculosis)* or 1 day *(M. smegmatis)*. Bacteria were washed with Hank's balanced salt solution (HBSS) and processed to avoid clump formation, as reported elsewhere [17]. The dispersion of the inoculum and the viability of the organisms were verified by microscopy using the LIVE-DEAD assay (Molecular Probes), as described elsewhere [17].

Phage TM4 was propagated in *M. smegmatis,* as described by Jacobs, Jr. et al. [18] and Foley-Thomas et al. [16]. In brief, phage lysates (10^5 pfu/mL) were incubated with fresh cultures of *M. smegmatis* (10^7 cfu) at room temperature for 30 min. 7H9 soft agar was then added, and the cells were plated on 7H11 agar by use of the soft agar layer method and incubated at 37°C for 2 days or as described elsewhere [16]. Phage titers were determined at dilutions that gave single isolated plaques, to exclude the possibility of lysis from other sources.

Macrophages. Mouse peritoneal macrophage cell line, RAW 264.7, was obtained from the American Tissue Culture Collection. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Difco Laboratories) supplemented with 10% fetal bovine serum and 2 m*M* L-glutamine. For the assays described in this article, RAW 264.7 macrophages (10^5) were treated with trypsin, washed, and seeded on a 24-well tissue culture plate (Costar) and allowed to grow overnight at 37°C with an atmosphere of 5% CO_2.

Infection of macrophages. *M. avium* 109 and *M. tuberculosis* H37Rv were used to infect RAW 264.7 macrophages. Monolayers ($\sim 5 \times 10^5$ cells) were incubated with mycobacteria at a ratio of either 1 bacteria to 1 cell or 10 bacteria to 1 cell. Infection was allowed to occur for 2 h, and then the monolayers were washed with HBSS twice to remove the extracellular bacteria. Infected RAW 264.7 monolayers were incubated for either 24 or 48 h before being exposed to *M. smegmatis* carrying the TM4 phage. *M. avium*–infected and *M. tuberculosis*–infected monolayers were incubated for 2 h with either 0.1 mL of *M. smegmatis* (5×10^7) containing $\sim 7.8 \times 10^7$ pfu/mL of phage *(M. avium)* and 7.5×10^7 pfu/mL of phage *(M. tuberculosis)* or *M. smegmatis* without phage. The titer of the phage was confirmed by plating *M. smegmatis* infected with TM4 for plaques. The extracellular bacteria were removed by extensive washing (HBSS), and the cell cultures were harvested after 2 and 4 days to quantify the viable intracellular bacteria.

The numbers of macrophages on the monolayers were monitored throughout the experiment, as described elsewhere [17]. Approximately 10% of the cells detached after 4 days of infection. The percentage of macrophages infected with *M. avium* after 2 h of incubation varied from 46% ± 6% (with the bacteria-to-cell ratio of 1:1) to 68% ± 9% (with the bacteria-to-cell ratio of 10:1), whereas the percentage of macrophages infected with *M. tuberculosis* after 2 h ranged from 40% ± 4% (1:1 ratio of bactera to cells) to 73% ± 6% (10:1 ratio of bacteria to cells).

Quantitation of intracellular bacteria. The number of intracellular bacteria was determined 2 and 4 days following coinfection with *M. smegmatis*–infected transiently with TM4, *M. smegmatis* only, or phage TM4 only, as described elsewhere [13]. The lysate

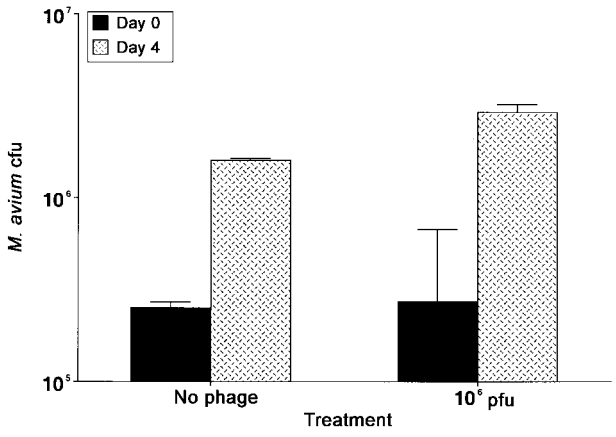

Figure 2. Effect of extracellular TM4 phage on the survival of intracellular bacteria. TM4 phage was added to macrophage monolayer infected with *Mycobacterium avium*. At 4 days after infection, the no. of viable bacteria was quantified, as described in Materials and Methods. No effect of TM4 on viability was evidenced.

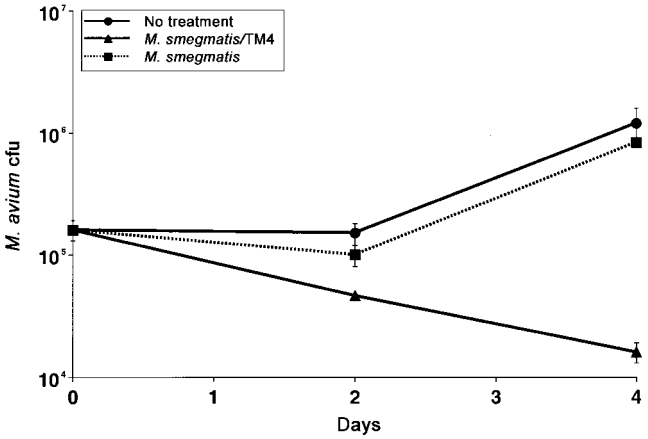

Figure 3. Effect of treatment of RAW 264.7 macrophages infected with *Mycobacterium avium* for 24 h with *Mycobacterium smegmatis* carrying the TM4 lytic phage. RAW 264.7 macrophage monolayers were infected with *M. avium* for 24 h and later were treated with *M. smegmatis* or *M. smegmatis* carrying TM4. $P < .05$, macrophages treated with *M. smegmatis*/TM4 vs. untreated macrophages at 2 and 4 days. $P < .05$, *M. smegmatis*/TM4 vs. *M. smegmatis* treatments.

was serially diluted and plated onto 7H11 agar containing 50 mg/mL kanamycin (a concentration that prevents *M. smegmatis* growth). The plates were incubated for 10 days, and the colony-forming unit count was determined. When plated on agar without kanamycin, it was observed that ~30% of *M. smegmatis* and ~20% of *M. smegmatis* were alive by day 2 and 4, respectively.

Observation of intracellular bacteria. To investigate a possible mechanism by which phages are delivered to *M. avium* vacuoles within macrophages, RAW 264.7 macrophage monolayers were infected with *M. avium* 109 and, after 24 h, coinfected with *M. smegmatis*. Cultures were maintained for 4 days and either observed daily using video microscopy or fixed with 2% paraformaldehyde, as described elsewhere [17]. We used 2 approaches. First, because *M. avium* is a short bacillus when cultured to logarithmic phase of growth and *M. smegmatis* is a long rod, both bacteria can be easily distinguished within cells. Therefore, the first approach was to determine, by real-time video microscopy (Optronics; DEI-700) and by phase contrast microscopy (Nikon OPTIPHOT) if both bacteria could coinhabit the same vacuole. As a variant of this approach, in some experiments, *M. smegmatis* was labeled with fluorescein-tagged succinimidyl ester (Molecular Probes) for 1 h and then used in video assays. The dye only stains viable cells. Second, by relying on the fact that *M. avium* vacuole does not acidify and *M. smegmatis* vacuole is acidic, we used acridine orange (Sigma Chemical) to determine whether fusion of both vacuoles would occur. In brief, macrophage monolayers were infected with *M. avium* and 24 h later coinfected with *M. smegmatis* for 4 days. The monolayers were incubated with acridine orange for 10 min using a protocol described elsewhere [19]. After washing, the slides were mounted and observed by fluorescent microscopy. *M. avium* is usually found in a non-acidic environment, and, therefore, the vacuole would not incorporate acridine orange. In contrast, *M. smegmatis* environment is acidic and therefore stains green-yellow. In case the *M. smegmatis* vacuole would fuse with the *M. avium* vacuole, *M. avium* and *M. smegmatis* would share the resulting compartment. It potentially would be acidic, and both bacteria would stain with acridine orange.

To determine whether intracellular *M. tuberculosis* was killed after *M. smegmatis*/TM4 treatment, infected monolayers in Lab-Tek Slides (Nunc) were fixed after the assay with 2% paraformaldehyde and stained by the Kinyoun method, as described elsewhere [17].

Statistical analysis. The assays were repeated at least 3 times, and the results were expressed as mean ± SD. Comparisons between groups at the same time point were analyzed using the Mann-Whitney nonparametric test.

Results

Mycobacteriophage TM4 kills extracellular M. avium *and* M. tuberculosis *in vitro.* We have shown that *M. avium* strain MAC 109 is infected by the TM4-derived luciferase reporter phages phAE39 and phAE40 [16]. To verify that the phage infection results in lytic killing in vitro, strain MAC 109 was infected with mycobacteriophage TM4, and the bactericidal effect of TM4 was measured over time. MAC 109 (1×10^5) was infected with 1×10^5 or 1×10^7 pfu of mycobacteriophage TM4, and, at various time points, phage absorption was stopped by centrifugation at 4°C for 10 min, and the pellet was resuspended in HBSS and plated onto 7H11 agar. The kill curve demonstrates that mycobacteriophage TM4 effectively kills MAC 109 in vitro with a 50% reduction in the number of viable bacilli observed after 120 min of phage infection (figure 1). The same method was used with *M. tuberculosis,* with the killing observed being even greater, with ~30-fold reduction in the number of viable bacteria after 4 h following phage infection (figure 1).

Mycobacteriophage TM4 alone cannot kill intracellular mycobacteria. Phage therapy of an intracellular infection would

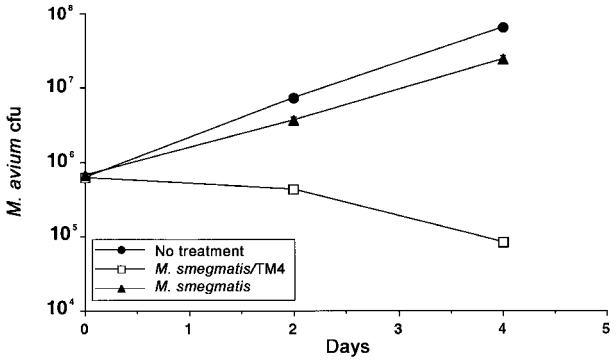

Figure 4. Effect of treatment of *Mycobacterium avium*–infected RAW 264.7 macrophages with *Mycobacterium smegmatis* carrying the TM4 lytic phage. Macrophage monolayers were infected with *M. avium* 109 for 48 h and then coinfected with *M. smegmatis* carrying TM4 or *M. smegmatis* alone. The no. of viable intracellular bacteria was determined after 2 and 4 days. $P < .05$, untreated monolayers vs. monolayers treated with *M. smegmatis*/TM4 for 2 and 4 days. $P > .05$, monolayers treated with *M. smegmatis* vs. *M. smegmatis*/TM4.

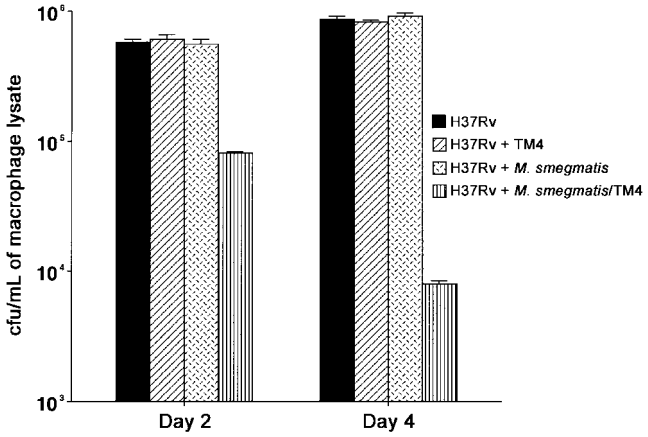

Figure 5. Effect of treatment of RAW 264.7 macrophages infected with *Mycobacterium tuberculosis* for 24 h with *Mycobacterium smegmatis* carrying the TM4 lytic phage. Macrophage monolayers were infected with *M.* tuberculosis H37Rv for 24 h and then coinfected with *M. smegmatis* carrying TM4 or *M.* smegmatis alone. The no. of viable intracellular bacteria was determined after 2 and 4 days following coinfection. $P < .05$, all the comparisons vs. treatment with *M. smegmatis*/TM4.

require the internalization of the mycobacteriophage to the site where the pathogen is located within the cell. It is possible, but not likely, that macrophages may phagocytose extracellular mycobacteriophage TM4 and directly deliver the phage to the *M. avium* or *M. tuberculosis* phagosome by intracellular trafficking prior to partial- or complete-phage degradation. To test this hypothesis, we treated *M. avium*–infected RAW 264.7 macrophage monolayers with TM4 phage (10^5, 10^6, and 10^7 pfu). *M. avium* continue to grow intracellularly, similarly to the growth observed in macrophage monolayers that received no treatment suggesting no effect of the phage (figure 2).

Effect of phage TM4 delivered intracellularly against M. avium. To deliver TM4 to the intracellular environment of macrophages, we used as a vehicle *M. smegmatis,* a nonpathogenic mycobacterium, infected with TM4. It was postulated that *M. smegmatis*/TM4 would be ingested by *M. avium*–infected macrophages and potentially deliver the phage to the site of *M. avium* in macrophages. To address this hypothesis, we used *M. smegmatis* infected for 30 min with 7.8×10^5 or 7.8×10^7 pfu to treat macrophage monolayers that had been infected with *M. avium* for 24 or 48 h. As shown in figure 3, the use of *M. smegmatis* infected with TM4 (7.8×10^7 pfu) for 30 min to treat macrophage-monolayers previously infected with *M. avium* for 24 h resulted in significant inhibitory activity at 2 days and killing of the intracellular *M. avium* at 4 days. Treatment with TM4 at a titer of 7.8×10^5 pfu was associated with an average 30%–40% less (but still significant) anti–*M. avium* activity than 7.8×10^7 pfu (data not shown).

Figure 4 shows the effect of *M. smegmatis*–TM4 in macrophages infected with *M. avium* for 48 h prior to treatment. Although a 10-fold decrease was observed when macrophages were treated with *M. smegmatis*–TM4 after 24 h of infection, a 100-fold decrease in the number of intracellular *M. avium* was seen as the result of treatment of infected monolayers with *M. smegmatis*–TM4 after 48 h of infection with *M. avium* (figure 4). In contrast, treatment with *M. smegmatis* without TM4 infection did not result in a significant inhibition or killing of intracellular *M. avium*. Control samples were tested to determine the number of viable cells. Infection of RAW 246.7 cells was not associated with decreased viability of the monolayer (data not shown).

M. smegmatis *TM4 kills intracellular* M. tuberculosis. To determine the effect of TM4 on intracellular *M. tuberculosis,* RAW 264.7 cells were infected with H37Rv, and 24 h after infection, treated with *M. smegmatis*, TM4 or *M. smegmatis*–infected with TM4 (*M. smegmatis* TM4, 6.7×10^7 pfu) for 2 and 4 days. As shown in figure 5, while neither *M. smegmatis* nor TM4 treatment was associated with decrease in viable intracellular *M. tuberculosis, M. smegmatis* TM4 resulted in ~10-fold reduction after 2 days and ~100-fold reduction in the number of intracellular bacteria after 4 days. The decrease in the number of intracellular *M. tuberculosis* was confirmed by light microscopy (data not shown).

Coinfection of macrophages with M. avium *and* M. smegmatis–*TM4 results in fusion of the infected vacuoles.* To elucidate the mechanisms by which *M. smegmatis* delivers the TM4 lytic phage to intracellular *M. avium,* we monitored the RAW 264.7 macrophage coinfection using time-lapse video microscopy. The use of this technique allowed us to observe where *M. smegmatis* taken up by macrophage went into the cell. Macrophage monolayers were infected with *M. avium* and coinfected with *M. smegmatis* 24 h later. Because *M. avium* grown to logarithmic phase is a short bacilli and *M. smegmatis* is always a long rod, it was feasible to follow the dynamics of the infection daily by real-time video microscopy. As shown in figure 6*A,* using this method, we could detect the fusion of *M. avium* and *M. smegmatis* vacuoles. In addition, we used acridine orange, a dye that concentrates in acidic compartments in the macrophages, to detect the fusion of *M. avium* and *M. smegmatis* vacuoles. Figures 6*B,* 6*C,* and 6*D* show that, in macrophages infected with *M. smegmatis,* the vacuole is acidic and therefore the bacterium stains green. In contrast, in macrophages infected with *M. avium* alone, we do not see the bacterium, which does not stain. Macrophages coinfected with *M. avium* and *M. smegmatis* (figure 6*D*) have *M. avium* in an acidic environment, which, in the figure, is shown by the appearance of green small rods characteristic of *M. avium.*

Discussion

Disseminated *M. avium* infection is a common complication in patients with AIDS with <50 $CD4^+$ T cells/mm^3. Although in the last 5 years treatment with new macrolides and rifabutin

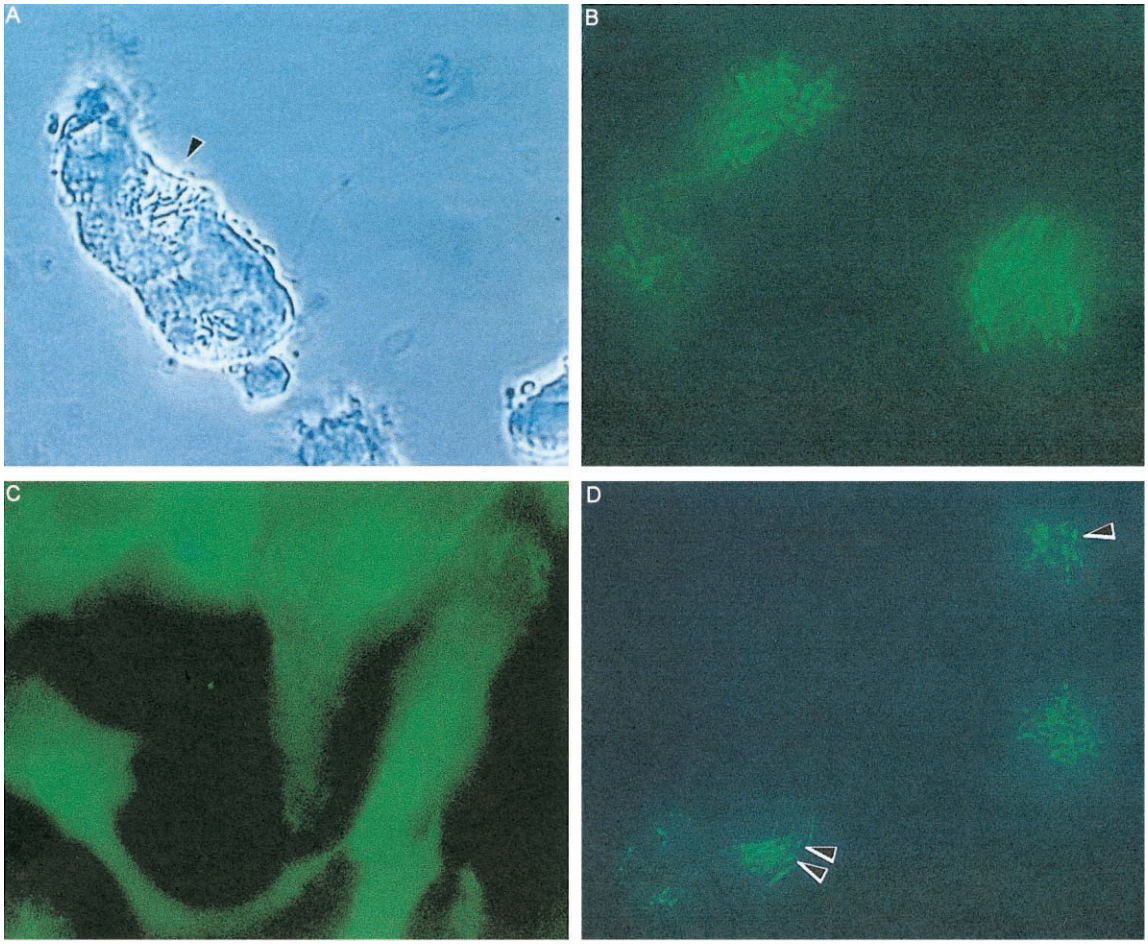

Figure 6. Fusion of *Mycobacterium smegmatis* and *Mycobacterium avium* phagosomes. *A,* Time lapse video microscopy showing fusion of *M. avium* and *M. smegmatis* vacuoles after 2 days of coinfection. *B,* Acridine orange staining of *M. smegmatis* in RAW 264.7 macrophages after 2 days of infection. Bacteria are observed incorporating the dye. *C, M. avium* (48 h)–infected macrophages stained with acridine orange. No bacterium incorporating the dye is seen, indicating that *M. avium* is in a nonacidic environment. *D,* RAW 264.7 macrophages infected with *M. avium* for 24 h and subsequently with *M. smegmatis* for 48 h. The figure shows *M. smegmatis* (long rods) and *M. avium* (short rods; *arrowheads*) stained with acridine orange, indicating that both are in acidic environment.

became available, killing of the bacteria in deep tissues (i.e., bone marrow) usually is not achieved [20]. In addition to the problem of *M. avium* and *M. tuberculosis* resistance to antibiotics, both bacteria are able to infect and grow within macrophages and monocytes, and in vivo undergo a latent or dormant phase of infection in the host [1]. A limitation of most antimicrobial agents is that their modes of action require having the microbial target in active replication.

Phage therapy to treat an infectious disease was conceived many years ago with limited success. Phages are specific to microorganisms and, even within a species, can fail to infect some strains [15, 16]. In addition, they need to be in the same environment as the pathogen to be able to infect it. Since phages, in contrast to antimicrobials, are not diffusable across membranes, strategies need to be devised to deliver the phage to the intracellular pathogen. Nevertheless, Sula et al. reported that the phage DS-6A was effective in killing *M. tuberculosis* in guinea pigs after parenteral administration [21]. A possible explanation for this result might have been that some *M. tuberculosis* cells were infected with the phage while extracellularly within the animals (e.g., in the extracellular milieu of the pulmonary cavity). In this scenario, transiently phage-infected *M. tuberculosis* cells may have delivered the phage to *M. tuberculosis* bacilli within macrophages. Nonetheless, this observation needs to be further substantiated.

Our results show that, when delivered to the site where the pathogenic bacterium resides inside the macrophages, TM4 was effective in lysing *M. avium* and, even more significantly, *M. tuberculosis*. In fact, the decrease in bacterial numbers after 4 days was similar to or better than the anti–*M. avium* effect obtained with macrolides clinically used as antituberculosis drugs in the same system [22, 23]. We had similar but more

compelling results with *M. tuberculosis*. Because not all *M. avium* vacuoles fused with *M. smegmatis* vacuoles after 4 days (figure 6*A*), it is possible that longer period of observation would lead to increased killing. Although both *M. avium* and *M. tuberculosis* inhibit phagosome-lysosome fusion in macrophages [24, 25], it is known that the mycobacterial vacuole still retains its ability to fuse with endosomes [26]. A recent study demonstrated that, in macrophages coinfected with *M. avium* or *M. tuberculosis* and *Coxiella burnetti,* fusion of the 2 vacuoles was observed after a period of ~24 h [27]. Interestingly, in this case, the new vacuole created after fusion, containing *Coxiella* and mycobacteria, was also acidic [27]. This seems to be the case in our experiments, in which the vacuoles containing *M. avium* and *M. smegmatis* incorporated acridine orange. Another possible explanation would be that the TM4 phage reached the *M. avium* vacuole by another mechanism and that, after lysing, the bacterium the vacuole then became acidic. Although possible, this explanation does not agree with our observation by video microscopy (fused vacuoles), indicating the presence of fused vacuoles.

In the present study, we described a novel strategy to deliver lytic phage to *M. avium,* as well as *M. tuberculosis,* vacuoles within macrophages. Our results show that this method of delivery can be useful and, above all, that the potential of this form of therapy needs to be explored. Although *M. smegmatis* certainly may not be the ideal delivery system, the results shown in this study can be seen as "proof of concept," and future studies should address this question. We are currently exploring the use of other mycobacteriophages and attenuated mycobacterial strains of *M. avium* and *M. tuberculosis*, as well as bacille Calmette-Guerin as potential phage delivery systems. Other delivery systems need to be developed if the administration of treatment into the airways proves to be efficacious.

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