



Bacteriophages: antibacterials with a future?

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Summary The hypothesis as to whether a benign species of bacteria could kill a virulent kind has to this point been untested.

Recently it was shown that in the macrophage, bacteriophages, when properly introduced through a nonvirulent microbe, had a killing rate for virulent AIDS *Mycobacterium tuberculosis* and *Mycobacterium avium* far in excess of modern day antibiotics.

The study in effect brought a natural phenomena, lysogeny, whereby one bacterial colony kills another thru phage weaponry, to bear in the conquest of hard-to-kill, antibiotic resistant pathogens.

This killing occurred intracellularly, within the white blood cell using *Mycobacterium smegmatis*, a benign bacterial species found generally in smegma secretions from human genitalia as well as soil, dust and water, and first identified in 1884.

The subsequent 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 unexpectedly large time- and titer-dependent reduction in the number of viable intracellular bacilli. In addition, the *M. smegmatis* vacuole harboring TM4 fused with the *M. avium* vacuole in macrophages.

These results suggested a potentially novel concept to kill intracellular pathogenic bacteria and warrant future development.

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Introduction

Ever since Sinclair Lewis popularized d'Herelle's epic laboratory saga of continual bacterial warring in his 1923 cutting edge *Arrowsmith* [1], the concept of injecting bacterial phage viruses into the body to kill serious bacterial disease has fascinated man. But the mechanics of such an attack systematically just didn't pan out.

Therapy with bacteriophages indeed has had a checkered history. Long before penicillin was

known, doctors carried these bacterial killing viruses as injections and potions and in the early 1930s drug giants Eli Lilly, ER Squibb and Abbott all manufactured "phage" preparations. But a pivotal, yet poorly designed 1934 US JAMA study by Eaton and Bayne-Jones [2] found systemic phages as having mixed results, concluding that body fluids strongly inhibited and destroyed most bacteriophages before they could reach target tissue.

So, by the late 1930s, phages, poorly understood, fell by the wayside as antibiotic use, seemingly infallible, soared. Canadian Felix d'Herelle, stumbled across bacteriophages in 1917 at the Pasteur Institute as accidentally as Fleming came across penicillin. Like many bacteriologists,

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d'Herelle long noticed clear spots on culture broths otherwise teeming with bacteria. But what he went on to find in further studies was a virtual killing field as these viral phages decimated their bacterial prey. Thru d'Herelle, mankind would in fact be drawn into an invisible microscopic war in which bacteria killed one another in their natural state using bacteriophages, viral weapons. Surreal in appearance, extraterrestrial tadpole-shaped phages, a fraction of their bacterial victims, were hurled towards and settled, tail first on the bacterial surface, like a staging platform, secreting enzymes to soften-up the targeted germ's cell wall before injecting their deadly DNA. Thus compromised, the bacteria, virtually under house arrest, were forced to churn out 100–150 new phages in the space of 10–20 min, often ending in the explosion of its cell wall and the death of the microbe. Less violent possibilities occurred when phage DNA either simply binded to bacterial DNA or the virus became merely a tenant in the outer cytoplasm of the germ. In either case, sooner or later phage DNA or actual phages left the bacteria with the ability to kill other bacteria.

Today, antibiotics are no longer infallible. Epidemics of drug-resistant (MDR) tuberculosis have been reported [3] and *Mycobacterium avium* infections, for which there never was a satisfactory treatment, are described with increasing frequency in non-AIDS populations [4].

A limitation of most antimicrobial agents is that their modes of action require having the microbial target in active replication. And pathogens such as *M. avium* and *Mycobacterium tuberculosis* have a latent or dormant phase of infection in humans [5].

The Russians took phages far more seriously than the West, soon producing some 80% effective against serious enterococcal infections. But even in the States, a combined University of Texas–Austin and Emory study by Levin and Bull showed that mice infected with fatal doses of *Escherichia coli* had a 92% survival rate with phage therapy as opposed to a 33% survival rate with the antibiotic streptomycin [6].

Perhaps the greatest test of bacteriophage therapy would lay in its ability to kill tuberculosis and the mycobacteria in their intracellular macrophage habitat. Nevertheless by 1981, Sula [7], in a Czech study, used parenterally injected mycobacteriophage DS-6A to destroy *M. tuberculosis* in guinea pigs with the approximate effectiveness of Isoniazid (INH), a first line TB drug.

Tuberculosis and the mycobacteria had caused 1 billion deaths between the years 1850 and 1950 alone [8], but seemed to be diminishing. Then, in the mid-1980s, tuberculosis, like a comeback pla-

gue, returned, with a vengeance, both in the US and abroad. Menacing new strains resistant to known antibiotics emerged. Yet in the last 30 years, no new classes of anti-TB drugs were developed [9].

Even in the case of *avium* (MAI or fowl tuberculosis), a leading infection in AIDS, though presently used Highly Active Retroviral Therapy (HAART) seem superficially to stay that infection to various degrees, when HAART was stopped or failed, *M. avium* returned, punishingly [10].

Waksman's "antibiotics"

Historically, antibiotics have been the mainstay of TB treatment ever since soil microbiologist Selman Waksman, a non-doctor, organized research in that direction. There was something in the soil that killed tuberculosis. By 1935, while Waksman knew that tuberculous germs survived in sterile soil, he also knew that they died out slowly in soil contaminated by other organisms. But unlike d'Herelle, Waksman saw bacterial struggle couched in terms of their chemical antibiotic weapons and not bacteria shooting viral phages at one another.

Rene Dubos had laid the groundwork with his discovery of Gramicidin and Fleming and Domagk followed with penicillin, and the sulfonamide Prontosil, respectively. This was the same Rene Dubos who in 1943 published one of the best early animal studies of phage treatment, supported by the US military, and answering virtually all of the scientific concerns raised in the flawed yet fatal Eaton and Bayne-Jones 1930s JAMA report. Dubos's [29] study used concentrated intraperitoneal phage injections to fight bacteria injected into the brain at lethal levels, and showed that there was no problem in the phages reaching and destroying them, even in this privileged site in the brain. The phages multiplied rapidly there and remained at substantial levels in the blood as well, as long as there were sensitive bacteria in which they could reproduce [29]. But despite this impressive study, antibiotics, soon thereafter, became seen as a much more general "magic bullet", easily produced and applied, while phage workers in the West became diverted to molecular biology.

Neither Dubos's, Fleming's nor Domagk's early studies killed Gram negative microbes effectively. Nor did they kill tuberculosis. It was Waksman's original interest in Gram negatives which led him to the Actinomyces, a family under the order Actinomycetales to which mycobacteria such as tuberculosis also belonged. Waksman had always been

fascinated by the use of the *Actinomyces* to produce antibiotics. Before him, Dubos had discovered that soil microbes were the creators of certain chemicals which Waksman first labeled "antibiotics" and Chester Rhines experiment of which Waksman [11] was a part, had proven that tuberculous germs added to certain soil, died. But the first two antibiotics Waksman isolated from soil *Actinomyces* were much too toxic, killing laboratory animals rather than curing them.

However, when Waksman's fellow, Schatz, finally isolated tuberculosis-killing streptomycin from an *actinomyces* in well-manured soil, both he and Waksman ignored the possibility that mycobacteriophages, also from soil, could have caused the death that TB faced from certain soils.

The general gene sequence and chromosomal organization of soil-born *Mycobacteria smegmatis* is very similar to that of the closely related *actinomyces* (the streptomycetes) with which Waksman and Schatz worked to isolate streptomycin [12]. In his publication on phage therapy Sula emphasized the subsequent need for a study on the phage-therapy of antibiotic resistant *M. avium*–*intracell-intracellulare* (MAI), or fowl tuberculosis, an ever spiraling disease affecting both man and animals. Since Sula [13], though not only have new phages been uncovered that attack MAI and TB, but so too has the development of 'shuttle phasmid' delivery systems.

Phagotherapy takes a turn

By 1982, Brenner [14], used the term "shuttle phasmid" to denote phages that replicated in non-pathogenic, non-disease causing forms of microorganisms such as *E. Coli* and *M. Smegmatis* as plasmids (extra chromosomal, circular, genetic materials) while at the same time have the capacity to multiply in pathogenic bacteria as potentially lethal agents. Such a scenario opened-up the theoretical possibility that bacteria, non-pathogenic to man, but with the capacity to generate phages known to kill virulent pathogens already in the body, could be parenterally injected to cure disease – all the while both protecting, nurturing and delivering these beneficial viral phages.

This phenomena, in nature, is referred to as "lysogeny". Lysogeny is how one colony of bacteria kills another by means of its phage weaponry without itself being harmed.

Recently our group appeared in *The Journal of Infectious Diseases* [15] in a study that showed that

in the macrophage, bacteriophages, when properly introduced by a nonvirulent microbe, had a killing rate for virulent AIDS MTB and MAI far in excess of modern day antibiotics.

We used *M. smegmatis*, a benign bacterial species [16]. First identified in 1884, *smegmatis* is abundantly found in the secretions that collect under the prepuce of the foreskin of the penis or of the clitoris, called smegma.

Rich [17], describing it as the smegma bacillus, citing it as an example of a non-pathogenic acid-fast bacillus repeatedly found not only in man but other animals. He mentions that injected into the body in appropriate numbers, these non-pathogenic acid-fast bacilli incite a mononuclear-like reaction with the formation of epitheloid cells, giant cells and tubercles, "but they do not survive long in tissues of warm-blooded animals and the lesions gradually disappear" [17].

Mycobacterium smegmatis was used to generate TM4 mycobacteriophage, a phage which were it compared to antibiotics would be called "broad-spectrum" in that it infects both fast (*M. smegmatis*) and slow (*M. tuberculosis*) growing *mycobacteria*. TM4, already known to have lytic action against the drug-resistant MAI and TB it was sent to kill, has been used extensively to deliver reporter genes to other *mycobacteria* [18]. Injecting TM4 laden *M. smegmatis* into a diseased organism in many ways serves as a veritable Trojan horse to conquer heretofore incurable disease.

Smegmatis was signaled out as to attack *M. tuberculosis* and MAI, both by virtue of its relatively benign nature, and the fact that lysogeny in nature usually occurs between like species. Inside *smegmatis* lay a host of TM4, the Greek soldiers within that horse.

The systemic use of live attenuated 'atypical mycobacteria' is nothing new, evidenced by the widespread use of the even more virulent *Mycobacteria bovis* as BCG vaccination, extensively used abroad and in which four of six studies showed a relatively high degree of immunization against future TB infections [16].

By 1996, the Russians, inspired by a WHO program for tuberculosis vaccine development, used *M. smegmatis* in mice without TM4 as a possible TB vaccination [19]. Although pure *smegmatis* did not possess protective activity against tuberculosis in susceptible mice, it did not have morbidity towards these subjects either.

Regardless, once systemic, certainly *M. smegmatis* shares antigenic determinants (epitopes) with TB and the other *mycobacteria*, aiding in its search and destroy mission. In addition, there is evidence of the advantageous biosynthesis of a lipase by

smegmatis once its infected with mycobacteriophages (D29) which share a common heritage with phage TM4 [20–22].

Such lipase is a fat-splitting, lipolytic, enzyme absent in uninfected *smegmatis*, yet biosynthesized shortly after phage infection. And although it possibly plays a role in the release of mature phage from *smegmatis* [21] such lipase could also facilitate transfection of TM4 into the target pathogens *M. tuberculosis* and *M. avium*, leading to their subsequent destruction.

Of great interest was the finding that neither *smegmatis* nor TM4 alone was able, in and of itself, to kill virulent mycobacteria. But combined, they did [15].

Although *smegmatis* is not considered a human pathogen, it can very occasionally cause skin or soft tissue disease sensitive to a variety of antibiotics [23]. But is an uncommon pathogen in humans [24], and disease from it is a rarity [25]. Furthermore, although MTB is far more virulent than *smegmatis*, it cannot confer its virulence to *smegmatis* [26].

Conclusion

There is much evidence in the literature that antibiotic resistant bacteria evolved from interspecies transfer of phages and that the germs Mankiewicz, for example, found in lung cancer were as a result of gene swapping thru phages among the *Actinomycetales* [27].

Likewise in AIDS, multi-drug-resistant MTB and *M. avium* can be traced to probable interspecies phage transfer.

In fact, many of man's deadly bacterial epidemics were and are a by-product of bacteria infecting other bacteria with their viral phages. It is when such an attack does not kill, cripple or maim that antibiotic-resistant pathogens are born for mankind to cope with [28]. And the proper way to deal with this is to learn from nature, how bacteria kill one another and to recruiting relatively benign bacteria to deliver lytic phage, destroying other-wise incurable infection thru lysogeny.

The implications of the novel technique to kill intracellular pathogenic bacteria that was presented are broad and include all classes of intracellular bacteria for both man and animals. It is intended to be used parenterally.

Since the study was done in the macrophage it can be seen as "proof of concept" only, and further animal and toxicity studies should be done before approaching human trials.

Mycobacteria were chosen because they have created a vast reservoir of disease on earth, the last chapter of which has yet to be written. And unfortunately we are a major host.

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