REVIEW ARTICLE

Origin and Development of RUTI, a New Therapeutic Vaccine Against *Mycobacterium tuberculosis* Infection

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This article reviews the pathophysiology of the latent form of *Mycobacterium tuberculosis* along with its natural history and progression in infected tissues. The proposed hypotheses regarding the relationship between *M tuberculosis* and the associated immune response, the cause of granuloma necrosis, the tolerance of a certain concentration of the bacillus in host tissues, the constant turnover of cells in the lung, and the effect of chemotherapy form the basis for the design of the therapeutic vaccine RUTI against latent *M tuberculosis* infection. This vaccine is generated from detoxified *M tuberculosis* cell fragments that facilitate a balanced T helper (Th) 1/Th2/Th3 response to a wide range of antigens along with intense antibody production. Treatment with RUTI following chemotherapy has been demonstrated to be effective in experimental models in mice and guinea pigs and does not exhibit toxicity.

Key words: *Latent bacilli. Immunotherapy.* Mycobacterium tuberculosis. *Koch phenomenon. Foamy macrophages. Experimental models. Tolerance. Chemotherapy. Th1/Th2.*

Origen y desarrollo de RUTI, una nueva vacuna terapéutica contra la infección por *Mycobacterium tuberculosis*

En este artículo se revisan la fisiopatología de la forma latente de *Mycobacterium tuberculosis*, su naturaleza y su evolución en los tejidos infectados. Las hipótesis planteadas entre la relación de este bacilo con la respuesta inmunitaria generada, el origen de la necrosis intragranulomatosa, la tolerancia hacia cierta concentración bacilar en los tejidos del hospedador, el constante recambio celular en los pulmonares y el efecto inducido por el tratamiento quimioterápico permiten conocer las bases para el diseño de la vacuna terapéutica RUTI contra la infección latente por *M. tuberculosis*. Se trata de una vacuna generada a partir de fragmentos celulares de *M. tuberculosis* biotransformados que permiten generar una respuesta equilibrada de tipo Th1/Th2/Th3 ante un amplio abanico de antígenos, además de una intensa producción de anticuerpos. El tratamiento con RUTI, posterior a la quimioterapia, ya ha demostrado su eficacia en modelos experimentales en ratones y cobayas, sin generar ninguna respuesta tóxica.

Palabras clave: *Bacilos latentes. Inmunoterapia.* Mycobacterium tuberculosis. *Fenómeno de Koch. Macrófagos* foamy. *Modelos experimentales. Tolerancia. Quimioterapia. Th1/Th2.*

Introduction

It is well known that *Mycobacterium tuberculosis* infection currently affects around 2000 million individuals, a third of the world's population. With such a reservoir, control of tuberculosis would seem to be a difficult task to approach. The rates of morbidity and mortality are extremely high: it is estimated that 8 million new cases of tuberculosis appear each year and 2 million individuals will die as a consequence of the disease.[1] The main strategy in countries with a high incidence of tuberculosis is to detect new cases—especially patients with a positive sputum smear who are thus capable of infecting others—and guarantee that they receive full treatment through the use of strategies such as DOTS (directly observed treatment, short course).[1] In addition, since the 1970s treatment of infected individuals has been promoted in countries with sufficient economic resources in an attempt to reduce this enormous reservoir and lower the risk of reactivation and cases of the disease in areas where the incidence is very low.[2] Unfortunately, that strategy has encountered a notable difficulty: the long period of isoniazid administration that is required (9 months) has made treatment compliance exceedingly poor, when in

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the majority of affected individuals the infection does not generate the slightest symptoms. Consequently, it is not surprising that in 1998 the Centers for Disease Control and Prevention in the USA recommended the development of a therapeutic vaccine against *M tuberculosis* infection.[3]

Without doubt, a key aspect of *M tuberculosis* infection, namely the appearance of the latent bacillus, has come to represent one of its most notorious characteristics. The appearance of this form of the bacillus has generated and continues to generate most interest on the part of tuberculosis researchers, to the extent that *M tuberculosis* infection has been termed "latent tuberculosis infection."[2] That this aspect of *M tuberculosis* infection is universally accepted does not alter the fact that it is an exaggeration of a biological characteristic that is extremely common in microorganisms. In fact, there are many microorganisms with an even greater capacity to persist for extended periods in host tissues. This applies principally to a large number of viruses, such as those of the herpes family.[4] In the case of bacteria, in all of the strains that it has been possible to study, researchers have observed the appearance of resistant forms and forms that display reduced metabolism in response to stress or simply a lack of nutrients when growing populations enter the stationary phase.[5] In addition, it has been demonstrated in all of these bacteria that the cells have a greater resistance towards noxious environmental stimuli in that state.[6] Genomic and proteomic analysis of *M tuberculosis* has confirmed that it is not all that different genetically from, say, *Escherichia coli*.[7] These approaches have also made it possible to confirm that *M tuberculosis* is incapable of generating an environmentally resistant form similar to a spore. At most, it has a highly complex hydrophobic cell wall[8] and a very slow growth capacity. However, in essence, those are not characteristics that we need to fear in a bacterium. What, then, is the origin and significance of this latent bacillus?

Without doubt, interest in this bacillus began with antibiotic treatment using streptomycin in the 1950s, when the introduction of multiple treatments led researchers to recognize disease recurrence that could not be accounted for by resistance to the antibiotics used.[9] Subsequently, towards the end of the 1970s, the group led by Mitchison,[10] having designed a "short-course" treatment regimen for *M tuberculosis* infection following the discovery of rifampicin, attempted to explain why treatment of tuberculosis nevertheless needed to be continued for 6 months. The action of rifampicin, capable of destroying those bacilli that activate their metabolism for short periods of time, led that author to predict that a population of bacilli existed that was either not metabolically active or had a drastically reduced metabolism. Those findings offered an explanation for earlier experimental observations regarding the capacity of *M tuberculosis* to persist in old cultures[11] and confirmed clinical observations regarding endogenous reactivation of tuberculosis.[12,13]

"Visualization" of the Latent Bacillus: the Importance of Foamy Macrophages

In the 1950s, the group of McCune[14] established an experimental model in mice—the Cornell model—that indirectly demonstrated the presence of the latent bacillus. Following a short period of infection (around 2 weeks) the animals were treated for 14 weeks with isoniazid and pyrazinamide, and it was confirmed that no culturable bacteria could be isolated from the tissues. Three months later, or after 1 month of hydrocortisone treatment, it was again possible to culture bacilli from the same tissues. It could be proposed, then, that during the period of time during which the tissues remain "sterile," the bacteria were present in a latent state. Those studies led various authors to address the different states of bacilli that were viable but not culturable, their recovery (technically, "resuscitation"), and the requirements for resuscitation to occur.[15,16] Again, many other researchers working with environmental bacteria have made numerous, decisive contributions in this area that have been of critical importance.[5,17]

Numerous authors have also undertaken experimental work to investigate the hypothesis that the latent bacillus must be a cell that is able to adapt its metabolism in order to persist under conditions of very low oxygen tension such as those which must occur in the necrotic tissue of tuberculous granulomas, one of the most important foci for the latent bacilli.[10,18] This led to the publication of a number of articles focusing on the metabolic changes occurring in *M tuberculosis* subjected to hypoxic and even anaerobic conditions. At least 1 significant metabolic change was identified, the glyoxylate shunt, with at least 2 outcomes: the generation of reduced NAD (nicotinamide adenine dinucleotide) to facilitate anaerobic respiration[19] or simply to allow energy to be obtained from lipids.[20]

Ian Orme, probably the living scientist who has worked most extensively with experimental models of tuberculosis, challenged assumptions in the scientific community 3 years ago in a discussion article entitled "The latent tuberculosis bacillus (I'll let you know if I ever meet one)."[21] Although the title of Professor Orme's paper was provocative, he put forth some very interesting theories. Firstly, he questioned the relationship between a positive tuberculin test and latent infection. In doing so, he attacked one of the pillars of immunology, namely the presence of immunologic memory, which in this case states that infection is not necessary in order to present a reaction to the tuberculin test. He also criticized the Cornell model and suggested that the bacillus probably becomes tolerant of isoniazid in that model.[22] Although defenders of the model have demonstrated that the recovered bacteria continue to be sensitive to isoniazid, Orme suggested that their recovery in a rich culture medium may lead to reversal of isoniazid resistance in order to be able to grow in

better conditions. This is linked to classical observations of the ability of mycobacteria to persist without cell wall synthesis, making them resistant to isoniazid.[23] This phenomenon of antibiotic resistance by virtue of no longer synthesizing the cell wall has been described in numerous other species of bacteria, the resistant cells being referred to as "L forms."[24]

In response to these challenges, our group normally states that "we have seen the latent bacillus." When studying histopathologic changes in granulomas using an experimental mouse model of tuberculosis, we realized that infected macrophages were surrounded by lymphocytes that were in turn surrounded by foamy macrophages that occupied the alveolar spaces surrounding the granulomas.[25,26] We were able to demonstrate this because there was a temporal sequence in a model in which a low dose of bacteria was inoculated as an aerosol. The most interesting finding was that some of the foamy macrophages contained a single bacillus and, later, the presence of multiple bacilli was observed in quite deteriorated foamy macrophages. It is likely to be at this point that the slow growth of *M tuberculosis* is important, since it allows the bacteria to pass unnoticed even when the aggressive stimulus generated against it has abated, and the time taken for it to multiply provides an extra period during which it may not be recognized. Finally, the bacillus can reproduce unhindered, far away from the granuloma foci, in the alveolar spaces, in which the concentrations of protective cytokines, mainly interferon gamma (IFN-γ), are vanishingly low.

In our opinion, what we saw was the latent bacillus. In other words, that was the bacillus that is able to survive in the stressful environment created by macrophages activated through the immune response, which destroys the majority of the bacilli. Those bacilli pass unnoticed by the macrophage or, if that macrophage is destroyed, by the immature macrophage attracted towards the granuloma that "cleans" the necrotic tissue and exits into the alveolar space to be drained, usually, towards the upper bronchi and trachea[27] to be swallowed or spat out. Subsequently, we showed that the presumed control of the infection occurring in the infected mouse that led to the belief that the mouse was able to display a degree of resistance against *M tuberculosis* similar to humans[28] was not quite that. In reality, although the concentration of bacilli was stabilized in the lung and remained fairly constant over an extended period of time, the same was not true for occupation of the lung parenchyma, in which an arithmetic increase was observed over time.[29] This observation has widespread implications, since in the end the mouse dies as a consequence of the total occupation of the lung parenchyma by the granulomatous structures caused by the bacillus, not as a consequence of an extraordinary inflammatory reaction.[30,31]

These findings, recently confirmed in a guinea pig model and even in human autopsy samples (unpublished data), provided us with much useful information for the design of the therapeutic vaccine RUTI. The hypothesis that we proposed was consistent with the theory that the immune response to *M tuberculosis* centered on the identification of peptides synthesized by actively multiplying bacilli.[32] Thus, we thought that to fight the latent bacillus, the carrier should be immunized with structural antigens of the bacillus that are present both in actively multiplying bacilli and in those that are in a stationary or latent phase.

The Origin of Granuloma Necrosis in the Tuberculous Granuloma

One of the most remarkable characteristics of *M tuberculosis* infection in humans is the occurrence of granuloma necrosis.[33] Unfortunately, this is not generated in the mouse.[28] This characteristic, considered by some authors to be an example of an effective, nontoxic response to the bacillus,[34] represented an enormous disadvantage, from our point of view, for the assessment of new antibiotics or vaccines, since it implied the absence of an extracellular population of bacilli. Consequently, we attempted to design a "humanized" model of tuberculosis in the mouse. Analysis of the literature was of great help. Firstly, the classical experiences of Lurie[35] in rabbits described the presence of granuloma necrosis at the onset of infection. In addition, other authors linked the Koch phenomenon, the classical term for the process that generates granuloma necrosis, with the Shwartzman reaction.[36] The Shwartzman reaction was initially described as a phenomenon of local cutaneous reactivity triggered by intradermal inoculation of an endotoxin (preparatory injection) followed 24 hours later by an intravenous injection of the same material (provoking injection). After approximately 4 hours, a hemorrhagic necrosis develops in the skin at the point where it had previously been inoculated with the endotoxin.[37] This phenomenon was subsequently correlated with local production of tumor necrosis factor (TNF).[36] Given that tuberculous granulomas contain high concentrations of TNF, and even higher concentrations when the bacillus concentration is highest (after 3 weeks of infection), we decided to employ intranasal inoculation of lipopolysaccharide to reproduce the phenomenon.[38] It worked.

The reproduction of the phenomenon allowed us to suggest a new hypothesis regarding the origin of the necrosis, separating it from delayed-type hypersensitivity (DTH). DTH was then considered to be a toxic cell-mediated immune response, in contrast to the effective cellular response, the response that generates IFN-γ in order to activate infected macrophages.[39] Furthermore, it indicated to us that if we wanted to inoculate infected individuals, we would have to ensure that no endotoxin was present if we were to prevent toxic reactions.

The Importance of Host Volume in the Inflammatory Response to *M tuberculosis*

The studies undertaken to develop a "humanized" model in the mouse also led us to think about an important aspect of *M tuberculosis* infection in experimental mouse models. Given that granuloma necrosis was induced by a nonspecific inflammatory process, the endotoxin had to be present in the structure of the bacillus itself, the concentration of the endotoxin increasing with the massive growth occurring at the end of the nonspecific response and provoking a phenomenon similar to the Shwartzman phenomenon. However, the marked increase in the concentration of endotoxins as a result of substantial growth of the bacterial population also occurs in the mouse. In that case, why is the massive inflammatory reaction that occurs in granuloma necrosis not provoked in that host?

The answer was found in the tolerance phenomenon that the mouse must display in response to various infections. This is supported by simply observing its volume. It is clear that the mouse would never be able to generate a tuberculous cavity like that generated in humans, among other reasons because the average volume of a cavity is greater than the total volume of a mouse. Therefore, it is not surprising that mice can "tolerate" a certain number of bacilli in their tissues; the mouse is only able to generate a "clean" response to the massive bacterial growth that could cause immediate death. In fact, studies undertaken in IFN-γ knockout mice indicate that death can occur after 4 weeks of infection.[40] In immunocompetent animals the control and reduction of the bacterial concentration does not begin until 3 weeks post infection.[25,31]

Another interesting finding is the dose of tuberculin necessary to trigger the DTH in the infected host. This is around 0.04 μg in humans,[41] but increases to 0.5 μg in guinea pigs and 5 μg in mice.[42] As well as reflecting the greater tolerance of the latter animals to proteins derived from *M tuberculosis*, this observation may also reflect the number of residual or latent bacilli in infected humans. Since that number is stabilized at around 4 $\log_{10}$ in mice and 3 $\log_{10}$ in guinea pigs infected with low—physiological—doses of *M tuberculosis* in aerosols (unpublished data), we can assume that the number in humans would be around 2 $\log_{10}$.

These findings suggest many things. Firstly, humans respond much more effectively to *M tuberculosis* infection. This is consistent with the most widely accepted epidemiologic data indicating that for every 100 humans infected, only 10 develop the disease and only half of these will die as a consequence.[43] In mice, for every 100 infected animals, 100 will die as a consequence of the infection.[31] These observations are illustrative of the type of effective immune response that can be developed against *M tuberculosis*. In humans, the response is a totally mixed T helper (Th) 1/Th2 response[33]: Th1 lymphocytes appear that are able to activate infected macrophages and generate granulomas around the bacilli (DTH response), alongside a significant Th2 response that stimulates the production of antibodies, which are able to control the extracellular bacilli and prevent their spread,[44,45] and the effective fibrosis of the granulomas through interleukin 4[46]; finally, a marked inflammatory response generates granuloma necrosis, initially preventing spread of the bacillus. Unfortunately, development of the disease appears to be linked to a purely anatomic characteristic of humans. The presence of tissues with high oxygen tensions, such as the apical lobes of the lung, favors local immunodepression and stimulates bacterial overgrowth.[47-50] Thus, the development of tuberculosis in immunocompetent patients would be the result of the adaptation of the bacillus to a highly specific ecological niche, the fruit to centuries of evolution and, therefore, very difficult to resolve.

We also have the tolerant response displayed by the mouse, based mainly on the synthesis of IFN-γ, which only allows control of multiplying bacilli, leading to a high concentration of bacilli in the tissues and constant spread of bacilli. Clearly, this type of response is not very effective.

These observations led us to conclude that the response generated to a therapeutic vaccine would have to be mixed, able to prevent intracellular multiplication of the bacillus, and also able to prevent extracellular spread. The low concentration of bacilli that can be expected in the tissues of carriers led us also to think about the requirement for such a vaccine to be multiantigenic, in order to increase the possibility that an extremely low concentration of antigen (tolerated) would be able to generate sufficient inflammatory response to mobilize immune cells to locate and destroy it.

The Dynamic Nature of the Latent Infection

Investigation of this process has always been hindered by an important aspect of host physiology, namely continuous turnover of cells. It is estimated that the average life of an alveolar macrophage is, at most, around 3 months.[51] Thus, it is quite clear that the bacillus must attempt to constantly replicate itself if it is to avoid drainage into the bronchial tree. It is relatively clear that necrotic tissue represents a primary source of these bacilli. Pathologists generally have difficulty observing acid alcohol-resistant bacilli inside lesions with granuloma necrosis indicative of being tuberculous in origin.[52] In our own group, we have difficulty in visualizing these bacilli in granulomas with spontaneous necrosis in guinea pigs or with induced necrosis in mice (unpublished data).

Another important aspect comes from the experimental mouse model. As mentioned, there is no necrotic tissue in this experimental model. However, as described in the Cornell model, whereas prolonged antibiotic treatment (eg, 14 weeks) with a combination of pyrazinamide and isoniazid results in the almost complete elimination of lesions from the lungs,

reactivation is still observed after a period of time.[14] In this case, there is no necrotic tissue to act as a reservoir; nor is there constant reactivation of the bacilli because this is prevented by the antibiotic treatment. There must be a third way through which the bacilli are maintained. This mechanism would have to involve the induction of apoptosis in the macrophages that phagocytize the latent bacilli.

Having arrived at this point, it is worth considering another interesting aspect of the latent bacillus that remains unresolved. It is well known that one of the characteristics of *M tuberculosis* is its capacity to prevent phagosome-lysosome fusion and thereby survive inside the macrophage.[53] However, this must require an active process on the part of the bacillus, since dead bacilli do not have that same capacity. What, then, would be the behavior of the latent bacillus? In our opinion, the latent bacillus, given its low metabolic capacity, would behave as if it were dead. That would mean that it could not prevent phagosome-lysosome fusion and it would then be immersed in a stressful environment, either due to the reduced pH, the presence of oxygen or nitrogen radicals, or exposure to lytic enzymes. This could lead to its destruction, but it probably does not do so by virtue of the fact that the bacillus is latent and, consequently, more resistant.[54] Equally, whereas the active bacillus attempts to prevent the macrophage that phagocytizes it from presenting its antigens at the cell surface, behaving as a dead cell prevents latent bacilli from doing the same thing.[55] Again, the bacillus requires an active metabolism in order to do this.

Once more, this is not something restricted to *M tuberculosis*. In general, intracellular bacteria prevent apoptosis of the phagocytic cells that contain them,[56] but this requires an active process on the part of the bacteria. This can be deduced, at least, from findings in *Brucella suis*, in which live cells prevent apoptosis, whereas dead cells cause apoptosis of the phagocytes.[57]

This third way in which the latent bacillus can participate is particularly worrying for its lack of predictability. On the one hand, it can be destroyed by a circulating alveolar macrophage, while on the other it can survive various stress processes by virtue of its resistance and induce apoptosis, with subsequent phagocytosis, etc. It is true that at any point one of the macrophages that phagocytize the bacillus can follow its usual dynamics and be drained out of the lung, meaning that the bacillus would disappear along with it. The problem comes if during the period between apoptosis and subsequent phagocytosis and processing, the bacillus momentarily stops being exposed to a hostile environment and is, therefore, provided with an opportunity to multiply. At this point, the macrophage is lost. The immune response of the host will not arrive in time and the bacillus will destroy it. Many of these bacilli will spread through the intraalveolar space, affect new macrophages, etc. New infectious foci will be generated, with the risk that one of them could develop in the apex of the lung, for instance.

Thus, this picture has further helped us to decide on the nature of the therapeutic vaccine. On the one hand, its administration must imply a restimulation of the immunity acquired with the infection and expand the range of recognizable epitopes in order to be able to recognize the macrophages carrying the latent bacillus. On the other hand, it should be able to increase the production of antibodies against different antigens in order to restrict the spread of bacilli towards new sites, in case one of those bacilli is able to replicate and destroy the macrophage that contains it. In these conditions, the most effective, immediate immune response is the humoral response.

Importance of Antibiotic Treatment of the Latent Infection

Antibiotic treatment of the latent infection is also a source of controversy. Many people do not understand why isoniazid is administered for 9 months. In fact, that is a very good question. Given that the latent bacilli are not susceptible to such chemotherapeutic drugs, why are they administered for such a long period? The answer is firstly to destroy the population of actively multiplying bacilli that can be found initially, and secondly, to prevent reactivation of any latent bacillus that attempts to multiply. Finally, an option that is not usually considered is that the macrophages themselves, following their normal dynamics, drain the bacilli towards the outside.

By reducing inflammation and preventing multiplication of the bacilli, antibiotic treatment is also highly recommendable to prevent the continuous formation of foamy macrophages and their accumulation or movement through the alveolar spaces. Another factor to take into account is that many of these macrophages are able to synthesize nitric oxide,[58,59] either through specific activation by IFN-γ or via nonspecific activation by components of the *M tuberculosis* cell wall.[60] In the first case, the problem appears with the macrophages that transport the latent bacilli; in the second case, the problem resides in the fact that nonspecific stimulation does not lead to sufficient activation of the macrophages and when they phagocytize an extracellular bacillus they will probably be unable to destroy it. Furthermore, the production of nitric oxide generates a transient immunodepression in the specific lymphocytes that try to activate these macrophages[61]; consequently, foamy macrophages cause a certain degree of local immunodepression that favors reactivation of the bacilli that are inside them.[58]

In conclusion, antibiotic treatment allows significant reduction of tissue inflammation and stabilization of the bacterial population towards a minimum that is completely inactive, it prevents local immunodepression phenomena, and it reduces the possibility of generating a toxic Koch-type reaction. Unfortunately, antibiotic treatment generates specific immune depression due to the fact that the concentration of bacilli is so low that it

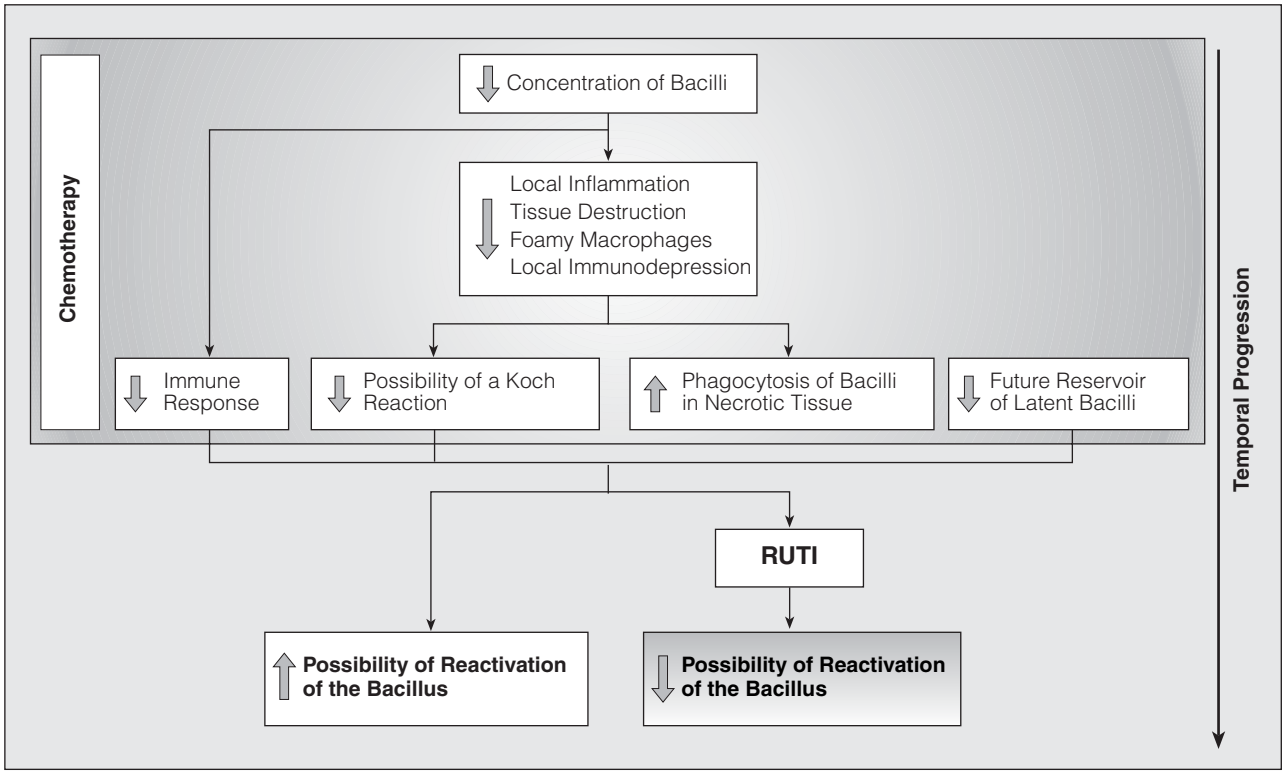

Figure. Temporal strategy for the use of RUTI, indicating the effects of short-course chemotherapy and the requirement for subsequent immunotherapy.

does not reach the stimulatory threshold for the generation of new effector cells.[62] This makes it of particular interest to undertake an immunotherapy treatment following chemotherapy in order to prevent reactivation of the bacillus as a consequence of the presence of latent bacilli in the tissues (after undertaking short-course chemotherapy) and the induction of local specific immunodepression. Also for this reason, it is very important to use an immunotherapy such as that provided by RUTI to be able to substantially reduce the period of chemotherapy by preventing this means of reactivation of latent bacilli (Figure).

What is the Future for RUTI?

The approach taken with RUTI came about in response to the questions that have been posed about the pathophysiology of *M tuberculosis* infection. With this approach, patients must complete a short course of chemotherapy, of no more than a month, and then be vaccinated in order to destroy the latent bacilli that they carry.

To date, we have been able to demonstrate that administration of RUTI, made up of detoxified fragments of *M tuberculosis* formed into liposomes, generates a substantial multiantigenic response with a well-balanced Th1/Th2/Th3 response and intense antibody production that is highly effective in various experimental mouse models[63] and even in the guinea pig (unpublished data). We are currently interested in assessing its efficacy in a large animal, such as the pig, that is capable of developing an inflammatory response very similar to that of humans.[64]

The RUTI vaccine represents a significant advance in immunotherapy for tuberculosis since it is the first prototype that has demonstrated efficacy without displaying toxicity for the host. Although initiatives to design new prototypes are currently underway, the RUTI vaccine is already being produced on a pilot scale for use in clinical trials. If the advantage is not lost and the necessary support is obtained, within a reasonable period of time (around 10 years) RUTI could represent a viable option to reduce the time required to treat *M tuberculosis* infection to a minimum that is both effective and practical, and of course, to subsequently reduce the time required for treatment of tuberculosis.

REFERENCES

1. World Health Organization. Global Tuberculosis Control: surveillance, planning, financing. Geneva: WHO Report; 2004.
2. American Thoracic Society. Targeted tuberculin testing and treatment of latent tuberculosis infection. Am J Respir Crit Care Med. 2000;161:S221-S47.
3. Centers for Disease Control and Prevention. Development of new vaccines for tuberculosis. Recommendations of the Advisory Council for the Elimination of Tuberculosis (ACET). MMWR Recomm Rep. 1998;47:1-6.
4. Bloom DC. HSV LAT and neuronal survival. Int Rev Immunol. 2004;23:187-98.

5. Mason CA, Egli T. Dynamics of microbial growth in the decelerating and stationary phase of batch culture. In: Kjelleberg S, editor. Starvation in bacteria. New York: Plenum Press; 1993. p. 81-102.
6. Hengge-Aronis R. The role of rpoS in early stationary-phase gene regulation in *Escherichia coli* K12. In: Kjelleberg S, editor. Starvation in bacteria. New York: Plenum Press; 1993. p. 171-200.
7. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature. 1998;393:537-44.
8. Brennan PJ. Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. Tuberculosis (Edinb). 2003;83:91-7.
9. Fox W, Ellard GA, Mitchison DA. Studies on the treatment of tuberculosis undertaken by the British Medical Research Council tuberculosis units, 1946-1986, with relevant subsequent publications. Int J Tuberc Lung Dis. 1999;3:S231-79.
10. Mitchison DA. The action of antituberculosis drugs in short-course chemotherapy. Tubercle. 1985;66:219-25.
11. Corper HJ, Cohn ML. The viability and virulence of old cultures of tubercle bacilli: studies on twelve-year broth cultures maintained at incubator temperature. Am Rev Tuberc. 1933;28: 856-74.
12. Canetti G. Exogenous reinfection: its relative impact with regard to development of pulmonary tuberculosis. A study of the pathology. Tubercle. 1950;31:224-33.
13. Opie EL, Aronson JD. Tubercle bacilli in latent tuberculous lesions and in lung tissue without tuberculous lesions. Arch Pathol. 1927;4:121.
14. McCune RM, Tompsett R, McDermott W. Fate of *Mycobacterium tuberculosis* in mouse tissues as determined by the microbial enumeration technique. I. The persistence of drug susceptible bacilli in the tissues despite prolonged antimicrobial therapy. J Exp Med. 1956;104:737-62.
15. Kaprelyants AS, Gottschal JC, Kell DB. Dormancy in non-sporulating bacteria. FEMS Microbiol Rev. 1993;10:271-85.
16. Shleeva MO, Bagramyan K, Telkov MV, Mukamolova GV, Young M, Kell DB, et al. Formation and resuscitation of "non-culturable" cells of *Rhodococcus rhodochrous* and *Mycobacterium tuberculosis* in prolonged stationary phase. Microbiology. 2002;148:1581-91.
17. Koch AL. Microbial physiology and ecology of slow growth. Microbiol Mol Biol Rev. 1997;61:305-18.
18. Wayne LG. Dormancy of *Mycobacterium tuberculosis* and latency of disease. Eur J Clin Microbiol Infect Dis. 1994;13:908-14.
19. Wayne LG, Lin KY. Glyoxylate metabolism and adaptation of *Mycobacterium tuberculosis* to survival under anaerobic conditions. Infect Immun. 1982;37:1042-9.
20. McKinney JD, Honer ZU, Bentrup K, Muñoz-Elias EJ, Miczak A, Chen B, et al. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature. 2000;406:735-8.
21. Orme IM. The latent tuberculosis bacillus (I'll let you know if I ever meet one). Int J Tuberc Lung Dis. 2001;5:589-93.
22. Wallis RS, Patil S, Cheon SH, Edmonds K, Phillips M, Perkins MD, et al. Drug tolerance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1999;43:2600-6.
23. Mattman KH, Tunstall KH, Mathews WW, Gordon DL. L variation in mycobacteria. Am Rev Respir Dis. 1960;82:202-11.
24. Mattman LH. Cell wall deficient forms. Stealth pathogens. Boca Ratón: CRC Press; 2001.
25. Cardona PJ, Llatjós R, Gordillo S, Díaz J, Ojanguren I, Ariza A, et al. Evolution of granulomas in mice infected aerogenically with *Mycobacterium tuberculosis*. Scan J Immunol. 2000;52:156-63.
26. Cardona PJ, Ausina V. Histopatología de la tuberculosis. Aproximación a la evolución de las lesiones pulmonares en modelos de experimentación animal inducidos mediante aerosol. Arch Bronconeumol. 2000;36:645-50.
27. Green GM. Alveolobronchiolar transport mechanisms. Arch Intern Med. 1973;131:109-14.
28. Lefford MJ. Diseases in mice and rats. In: Kubica GP, Wayne LG, editors. The mycobacteria: a source book. New York: Marcel Dekker Inc.; 1984; p. 947-77.
29. Cardona PJ, Gordillo S, Amat I, Díaz J, Lonca J, Vilaplana C, et al. Catalase-peroxidase activity has no influence on virulence in a murine model of tuberculosis. Tuberculosis (Edinb). 2003;83:351-9.
30. Cardona PJ, Cooper A, Luquin M, Ariza A, Filipo F, Orme IM, et al. The intravenous model of murine tuberculosis is less pathogenic than the aerogenic model owing to a more rapid induction of systemic immunity. Scand J Immunol. 1999;49:362-6.
31. Dunn PL, North RJ. Virulence ranking of some Mycobacterium tuberculosis and *Mycobacterium bovis* strains according to their ability to multiply in the lungs, induce lung pathology, and cause mortality in mice. Infect Immun. 1995;63:3428-37.
32. Andersen P. Host responses and antigens involved in protective immunity to *Mycobacterium tuberculosis*. Scand J Immunol. 1997;45:115-31.
33. Grange JM. Immunophysiology and immunopathology of tuberculosis. In: Davies PDO, editor. Clinical tuberculosis. London: Chapman & Hall; 1998. p. 113-27.
34. Dannenberg AM Jr. Rabbit model of tuberculosis. In: Bloom BR, editor. Tuberculosis: pathogenesis, protection, and control. Washington DC: American Society for Microbiology; 1994;10: 149-56.
35. Lurie MB. The correlation between the histological changes and the fate of living tubercle bacilli in the organs of tuberculous rabbits. J Exp Med. 1932;55:31-58.
36. Rook GA, Al Attiyah R. Cytokines and the Koch phenomenon. Tubercle. 1991;72:13-20.
37. Shwartzman G. Phenomenon of local tissue reactivity and its immunological, pathological and clinical significance. New York: Paul B. Hober; 1937.
38. Cardona PJ, Llatjós R, Gordillo S, Viñado B, Díaz J, Ariza A, et al. Towards a "human-like" model of tuberculosis: local inoculation of LPS in lungs of *Mycobacterium tuberculosis* aerogenically infected mice induces intragranulomatous necrosis. Scand J Immunol. 2001;53:65-71.
39. Dannenberg AM Jr. Delayed-type hypersensitivity and cell-mediated immunity in the pathogenesis of tuberculosis. Immunol Today. 1991;12:228-33.
40. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med. 1993;178:2243-7.
41. Comstock GW, Edwards LB, Philip RN, Winn WA. A comparison in the United States of America of tuberculins PPD and RT23. WHO Bull. 1964;31:2-45.
42. Baldwin SL, d'Souza C, Roberts AD, Kelly BP, Frank AA, Lui MA, et al. Evaluation of new vaccines in the mouse and guinea pig model of tuberculosis. Infect Immun. 1998;66:2951-9.
43. Costello AM, Kumar A, Narayan V, Akbar MS, Ahmed S, Abou-Zeid C, et al. Does antibody to mycobacterial antigens, including lipoarabinomannan, limit dissemination in childhood tuberculosis? Trans R Soc Trop Med Hyg. 1992;86:686-92.
44. Sánchez-Rodríguez C, Estrada-Chávez C, García-Vigil J, Laredo-Sánchez F, Halabe-Cherem J, Pereira-Suárez A, et al. An IgG antibody response to the antigen 85 complex is associated with good outcome in Mexican Totonaca Indians with pulmonary tuberculosis. Int J Tuberc Lung Dis. 2002;6:706-12.
45. Rook GA, Hernández-Pando R, Dheda K, Teng Seah G. IL-4 in tuberculosis: implications for vaccine design. Trends Immunol. 2004;25:483-8.
46. Meylan PRA, Richman DD, Konbluth RS. Reduced intracellular growth of mycobacteria in human macrophages cultivated at physiologic oxygen pressure. Am Rev Respir Dis. 1992;145:947-53.
47. Park MK, Myers RA, Marzella L. Oxygen tensions and infections: modulation of microbial growth, activity of antimicrobial agents, and immunologic responses. Clin Infect Dis. 1992;14:720-40.
48. Pérez-Padilla R, Franco-Marina F. The impact of altitude on mortality from tuberculosis and pneumonia. Int J Tuberc Lung Dis. 2004;8:1315-20.
49. Vargas MH, Furuya ME, Pérez-Guzmán C. Effect of altitude on the frequency of pulmonary tuberculosis. Int J Tuberc Lung Dis. 2004;8:1321-4.
50. Harmon KR, Marinelli WA, Henke CA, Bitterman PB. Regulation of cell replication, In: Crystal RG, West JB, Barnes PJ, Cherniack NS, Weibel ER, editors. The lung: scientific foundations. New York: Raven Press; 1991. p. 105-29.
51. Seiler P, Ulrichs T, Bandermann S, Pradl L, Jorg S, Krenn V, et al. Cell-wall alterations as an attribute of *Mycobacterium tuberculosis* in latent infection. J Infect Dis. 2003;188:1326-31.
52. Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK, et al. Lack of acidification in

Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science. 1994;263:678-81.
53. Wallace JG. The heat resistance of tubercle bacilli in the lungs of infected mice. Am Rev Respir Dis. 1961;83:866-71.
54. Ramachandra L, Noss E, Boom WH, Harding CV. Processing of *Mycobacterium tuberculosis* antigen 85B involves intraphagosomal formation of peptide-major histocompatibility complex II complexes and is inhibited by live bacilli that decrease phagosome maturation. J Exp Med. 2001;194:1421-32.
55. Moreno E, Pizarro-Cerdá J. Life and death of *Brucella* within cells. In: Govel JP, editor. Intracellular pathogens in membrane interactions and vacuole biogenesis. Georgetown: Eureka.com and Kluwer Academic/Plenum Publishers; 2004. p. 99-129.
56. Gross A, Terraza A, Ouahrani-Bettache S, Liautard JP, Dornand J. In vitro *Brucella* suis infection prevents the programmed cell death of human monocytic cells. Infect Immun. 2000;68:342-51.
57. Cardona PJ, Gordillo S, Díaz J, Tapia G, Amat I, Pallarés A, et al. Widespread bronchogenic dissemination makes DBA/2 mice more susceptible than C57BL/6 mice to experimental aerosol infection with *Mycobacterium tuberculosis*. Infect Immun. 2003;71:5845-54.
58. Scanga CA, Mohan VP, Yu K, Joseph H, Tanaka K, Chan J, Flynn JL. Depletion of CD4(+) T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon gamma and nitric oxide synthase 2. J Exp Med. 2000;192:347-58.
59. Rhoades ER, Orme IM. Susceptibility of a panel of virulent strains of *Mycobacterium tuberculosis* to reactive nitrogen intermediates. Infect Immun. 1997;65:1189-95.
60. Stumbles PA, McWilliam AS, Holt PG. Dendritic cells and mucosal macrophages. In: Ogra PL, Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, editors. Mucosal immunology. San Diego: Academic Press; 1999. p. 397-412.
61. Cardona PJ, Julián E, Vallés X, Gordillo S, Muñoz M, Luquin M, et al. Production of antibodies against glycolipids from the *Mycobacterium tuberculosis* cell wall in aerosol murine models of tuberculosis. Scand J Immunol. 2002;55:639-45.
62. Cardona PJ, Amat I, Gordillo S, Arcos V, Guirado E, Díaz J, et al. Immunotherapy with fragmented *Mycobacterium tuberculosis* cells increases the effectiveness of chemotherapy against a chronical infection in a murine model of tuberculosis. Vaccine. 2005;23:1393-8.
63. Bolin CA, Whipple DK, Khanna KV, Risdahl JM, Peterson PK, Molitor TW. Infection of swine with *Mycobacterium bovis* as a model of human tuberculosis. J Infect Dis. 1997;176:1559-66.