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On the nature of *Mycobacterium tuberculosis*-latent bacilli

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On the nature of Mycobacterium tuberculosis-*latent bacilli. P-J. Cardona, J. Ruiz-Manzano. ©ERS Journals Ltd 2004.*
ABSTRACT: *Mycobacterium tuberculosis*-latent bacilli are microorganisms that adapt to stressful conditions generated by the infected host against them. By slowing metabolism or becoming dormant, they may counterbalance these conditions and appear as silent to the immune system. Moreover, the dynamic turnover of the infected cells provokes a constant reactivation of the latent bacilli when the environmental conditions are favourable, or an activation after being dormant in necrotic and fibrotic lesions for a long period of time. Since there is no *in vivo* nor *in vitro* evidence for quick resuscitation of dormant bacilli, the current authors strongly favour the possibility that latent tuberculosis infection can be maintained for no longer than ~10 yrs, which is, nowadays, a time period very close to that considered for "primary" tuberculosis. This concept may also be helpful for newer epidemiological considerations regarding the real impact of reinfection in tuberculosis.
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One of the most remarkable features of *Mycobacterium tuberculosis* is its capacity to generate a latent infection. Far from the incidence reached in the 19th century, when tuberculosis (TB) was the first cause of death in the industrialised countries in Europe, the 21st century faces a fabulous reservoir of latent tuberculosis infection (LTBI). In fact, it is estimated that one-third of mankind (2,000,000,000 people) has LTBI [1]. Therefore, more efforts must be devoted to better control this disease, including more effective methods of diagnosis, prophylaxis and therapies. The methods currently used, such as the tuberculin test to diagnose the disease or the 6–12-month treatment with isoniazid, do not contribute towards improving the situation. The lack of new methodologies to reduce the reservoir of LTBI is one of the reasons for the ~8,000,000 newly diagnosed cases and 2,000,000–3,000,000 deaths by TB every year [1]. In this paper, the phenomenon of latency of *M. tuberculosis* is reviewed, focusing on the evidence for latent bacilli in clinical studies and experimental *in vitro* and *in vivo* models (table 1).

Estimations suggest that, once infected, only 10% of the hosts will develop TB. These data reveal how mankind has adapted to this infection. It is believed that 5% of the infected population will develop the disease after 5 yrs and the others will suffer from it at some time during their lives [30]. The first case is known as "primary" TB and usually affects children and immunosuppressed hosts, whereas the second case is known as "post-primary" TB. For years, post-primary TB has been related to the cases of TB in countries with a low risk of infection, and mostly to the elderly population (>65 yrs old). Nowadays, the use of molecular markers has shown that this idea needs to be reviewed. In fact, reinfection represents a large percentage of these "post-primary" cases [31]. Furthermore, in this form of TB, the concept of reactivation has been overemphasised, at least because of two facts: the lack of knowledge about the degree and duration of immunity conferred by *M. tuberculosis* infection; and the confusion between infected people (*i.e.* a positive tuberculin test, but with no lung radiograph images) and people that suffered TB but who resolved it without any antibiotic treatment. If only the concentration of bacilli is considered, the possibility of *M. tuberculosis* bacilli persisting seems to be higher in the second case.

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Clinical evidence of latent tuberculosis infection

The first evidence of LTBI was obtained with the treatment with antibiotics. After stating that TB should be treated simultaneously with at least two drugs in order to avoid spontaneous mutations, latency was the only explanation for the late reactivation of TB in patients who thoroughly followed the antibiotic treatments and the absence of drug resistance in isolated bacilli [2]. Soon after, chemoprophylaxis assays provided some idea about the nature of latency. In a trial conducted by the International Union Against Tuberculosis Committee on Prophylaxis, a protection of 93, 69 or 32% was demonstrated among "completer-compliers", following a period of treatment with isoniazid against LTBI for 12, 6 and

Table 1. – Evidence on the presence of latent bacilli

Clinical
Late reactivation after a large period of chemotherapy [2]
Chemoprophylaxis trials: the longer the period, the lower the chance of reactivation [3, 4]
Natural history: formation and healing of primary complex in basal zones of the lung. Development of post-primary TB a long time after the haematogenous dissemination to the apex [5–10]
Experiments *in vitro*
Higher bactericidal capacity of rifampicin in "intermittent" incubation [11]
Bacillary survival in sealed cultures at 37°C after 12 yrs [12]
Adaptation of bacilli to a low oxygen pressure [13–15]
Higher resistance to stressful conditions in bacilli from stationary-phase cultures [16]
Transcription of genes (*e.g. SigF*) related to the sporulation cascade in other bacteria, under stressful conditions [17–19]
Dormant "noncultureable" bacilli can be "resuscitated" with phospholipids and a specific factor (Rpf) synthesised by growing bacilli [20–24]
Experiments in animal models
The Cornell model in mice [25]
Bacilli from chronical lesions are better adapted to stressful conditions than the acute ones [26]
Specific *M. tuberculosis* gene knock-out strains have revealed the indispensable role of some genes expressed in stressful conditions for persistence in a chronic infection [27–29]

TB: tuberculosis; Rpf: resuscitation promoting factor; *M. tuberculosis*: *Mycobacterium tuberculosis*.

3 months, respectively [3]. Since susceptibility to antibiotics requires some level of metabolism and cell growth, this trial suggested that many bacilli involved in LTBI are constantly growing.

More evidence came from the natural history of pulmonary TB in humans [30]. Historically, "primary" TB appears when there is a formation of primary complexes by a parenchymal lesion, usually at the base of lungs, and by hilar lymphadenopathy, caused by a very low dose of infection (usually 1–5 tubercle bacilli) [5]. Anatomically, the basal and middle zones of the lungs are more prone to infection than the apical zones because of their volume, although sometimes ventilation seems to be favoured in the latter [6]. This is also supported by the finding of single calcified primary lesions observed in necropsies, in which 66% of infections were located in the lower half of the lung and only 12% were supraclavicular [7]. Haematogenous dissemination takes place after the initial infection [8], and the tuberculin test gives positive results ~3–8 weeks after infection. This primary complex resolves spontaneously with no symptoms in 95% of infected people, but 5% develop the disease, which may be local (*i.e.* causing pleurisy when there is rupture into the pleural cavity) or systemic (*i.e.* causing meningeal or even miliary TB).

A "post-primary" cavitary form of TB located at the apical zone of the lungs [9] has also been accepted and has traditionally been associated with the reactivation of an old lesion containing latent bacilli [10]. To support this natural history, it has been stated that bacilli lie dormant in a metastatic site, haematogenously seeded within a vulnerable region (*e.g.* in the upper pulmonary zone). Unlike infections in lower zones of the lung, the immune system would not be able to sterilise the infectious foci, thus maintaining the bacilli in a dormant state, even for life [5]. After the wane of immunity with time, which takes place mostly in the elderly at an estimated rate of 5% per year until complete disappearance of immunity [32, 33], the tubercle bacilli resume multiplication and increase their concentration in the apical focus. Once immunity is restored, the interaction with high quantities of antigen could lead to extensive caseation necrosis, liquefaction and cavity formation [5].

This post-primary pulmonary TB also accepts the presence of dormant bacilli constantly related with the immune system, waiting to reactivate due to immunosuppression. Nevertheless, other authors, such as CANETTI [34], were skeptical about this idea because, in most cases, the primary complex is sterile within 5 yrs. Since this author considered the metastatic foci as a part of the primary complex and, thus, suffered its same fate, and taking into account that the bacillary concentration would be even lower in the metastatic foci than in the original foci, it was believed that an exogenous reinfection would be the origin of post-primary pulmonary TB.

The first efforts to determine the nature of latent bacilli: the *in vitro* experiments

Soon after the clinical observations that led to the concept of LTBI, a new "miraculous" drug appeared: rifampicin. This new drug allowed a shorter period of treatment and is still the gold standard for the treatment of TB [35]. In order to explain why the use of rifampicin in the chemotherapeutic treatment of the disease could sterilise lesions in a remarkably short period of time compared with other drugs, MITCHISON and DICKINSON [11] observed that the culture of *M. tuberculosis* at 8°C in the presence of isoniazid or rifampicin did not affect bacillary concentration, whereas the culture at 37°C revealed a similar bactericidal capacity for both drugs. An "intermittent" incubation was then designed to demonstrate the higher bactericidal capacity of rifampicin. In fact, when a culture of *M. tuberculosis* at 8°C was incubated at 37°C for 6 $h \cdot day^{-1}$, rifampicin showed a higher bactericidal capacity than isoniazid. This experiment was the foundation of the theories on bacillary populations in TB lesions, based on the speed of growth of bacilli [36, 37]. Bacilli with a high metabolism were highly susceptible to chemotherapy. A medium speed of growth or "spurts of growth" were observed in those populations under acidic pH, or in bacilli sensitive to pyrazinamide or rifampicin but not to isoniazid. Finally, latent bacilli showed no metabolic activity and, thus, were not sensitive to chemotherapy. Therefore, post-primary infection or reactivation may be caused by the re-stimulation of the metabolism of these latent bacilli.

Tuberculous lesions are characterised by a consistent intragranulomatous necrosis, by compact macrophage and lymphocytic rings, and, finally, by a fibrotic layer. Taking into account the strict aerobic nature of *M. tuberculosis* when cultured in artificial media, WAYNE [13] hypothesised that latent bacilli might adapt to microaerobic and anaerobic environments. They were also encouraged by the studies carried out by CORPER and COHN [12], who kept several sealed cultures of human isolates at 37°C for 12 yrs and demonstrated a survival of 0.01%. WAYNE and LIN [14] conducted many experiments in artificial media to demonstrate the capacity of bacilli to adapt to oxygen-restricted

conditions. In those experiments, it was shown that after a progressive introduction to a low oxygen pressure, *M. tuberculosis* changed its metabolism by enhancing some enzymes, mainly isocitrate lyase and glycine dehydrogenase to generate a reduced nicotinamide adenine dinucleotide, so as to obtain energy through a fermentative metabolism [14]. However, this hypothesis did not solve the problem on how bacilli may survive the stress generated by surrounding activated macrophages, a low pH, and a high concentration of radical oxygen intermediates (ROI) and radical nitrogen intermediates (RNI) [38]. Latent bacilli must adapt to such conditions generated in activated infected macrophages. This hypothesis can also be criticised regarding the relative importance of low oxygen pressure in the growth of *M. tuberculosis* in host tissues. The microaerophilic conditions generated by WAYNE and coworkers [13, 14] in artificial media already exist in the macrophages physiologically [15]. Therefore, *M. tuberculosis* is usually well adapted to grow in these conditions *in vivo*. Conversely, data obtained in anaerobiosis showed that bacilli did not survive for more than a few months [14]. Moreover, *ex vivo* experiments conducted with macrophages demonstrated that a high oxygen pressure induced a higher bacillary growth than a low oxygen pressure [39], thus explaining why most cases of lung TB develop at the apex of the lungs, where oxygen concentrations are higher [6].

Different authors have also demonstrated higher resistance against different stress conditions of bacilli in the stationary phase of their growth compared with bacilli growing exponentially [26]. The stationary phase in a conventional culture is known to appear when bacteria are starving due to a lack of nutrients or the accumulation of toxins generated by bacterial metabolism [16]. This resistance capacity observed in all bacteria might reflect some kind of adaptation to hostile conditions, such as the ones generated in partially activated macrophages. The study of both the genomic and the proteomic expression of *M. tuberculosis* under stress conditions, such as acidity, low oxygen pressure, heat, cold, hydroxide peroxide, or even stationary growth phase, showed an increase in the expression of the RNA polymerase sigma (Sig)F unit, which was also related to the accumulation of an α-crystalline-like 16-kDa protein in the cell wall [17]. Interestingly, the presence of SigF during exponential growth was deleterious for *M. bovis* bacilli Calmette-Guérin (BCG) [18], perhaps because it directed the transcription of genes required for the stationary phase or a spore-like state when exponential growth was required. Whether the *M. tuberculosis* SigF protein primarily regulates a sporulation-like cascade (the same as *Bacillus subtilis* SigF and *Streptomyces coelicolor* SigF) is unknown [19].

Concerning an *in vitro* model of latency, KAPRELYANTS *et al.* [20] distinguished three major physiological states of bacilli states: 1) viable (cultureable) bacilli that may divide (*i.e.* forming a colony on an agar plate or proliferate in liquid medium); 2) dormant bacilli with a low metabolic activity that are unable to divide without a preceding resuscitation phase; and 3) nonviable (noncultureable) bacilli that cannot divide. KAPRELYANTS *et al.* [20] did not consider the bacilli obtained by WAYNE and SRAMEK [21] as dormant because they maintained a high viability and developed sensitivity to metronidazole when anaerobic, thus indicating an active metabolism. Nevertheless, prolonged cultures in stationary phase induced a true dormancy, generating "noncultureable" cells that had to be "resuscitated" before resuming active growing [22]. MUKAMOLOVA *et al.* [23] also described the resuscitation promoting factor (Rpf) in supernatants of growing *Micrococcus luteus* cultures. Rpf restored the ability to grow in *M. luteus* dormant cells [23]. This same test was repeated in dormant *M. tuberculosis* cells (from a 4-month-old culture), and it was found that recombinant Rpf from *M. luteus* and supernatant of growing *M. tuberculosis* cultures resuscitated dormant cells [22]. ZHANG *et al.* [24] repeated the test in a 1-yr-old culture, discovering that some phospholipids and an 8-kDa protein were responsible for resuscitation. However, this latter test can be criticised because dormant cells were generated after physiological entry into a stationary phase, giving enough time to the bacilli to perfectly adapt to this environment, as reported previously by CORPER and COHN [12]. It is difficult to extrapolate this to the real circumstances experienced by the bacilli inside macrophages, if they were to suddenly suffer stressful conditions like low pH, or ROI and RNI [38]. Therefore, if the test conducted by CORPER and COHN [12] showed 0.01% survival following 12 yrs of incubation, a minor survival rate of bacilli under stress may be extrapolated.

Nonacid-fast, cell wall-defective variants of tubercle bacilli were isolated from clinical specimens and mycobacterial cultures [40]. Far from the controversy generated about whether they really were *M. tuberculosis* or an environmental contaminant [41], or if they were really able to revert to the parent form or even to multiply [42], these forms seemed to be induced by the administration of antibiotics, as is the case with many other organisms [43]. Consequently, the lack of cell wall provides some advantage in the resistance against chemotherapeutic treatment by eliminating the targets to which the drugs are directed, although they would be more susceptible to changes in the environment and the chances to survive in an inflammatory area would be even lower. Hence, such bacilli may play a limited role in LTBI.

The search for latency in experimental models in animals

The Cornell model of latency was the first experimental evidence of the existence of latent bacilli in an *in vivo* model, and it is widely considered to be the experimental source of latent bacilli. MCCUNE and TOMPSETT [25] described "the persistence of drug-sensitive tubercle bacilli in tissues despite prolonged antimicrobial therapy". After infecting the animals, a 12-week course of drug administration with isoniazid and pyrazinamide was started, and a complete disappearance of cultureable *M. tuberculosis* was achieved in all the animals sacrificed at the end of treatment. The most interesting data were obtained after 12 weeks, when cultureable bacilli were recovered in two-thirds of infected mice. This percentage increased to 100% with 1 $mg \cdot day^{-1}$ of cortisone for 20 days [44]. SHLEEVA *et al.* [23] established a parallelism between this model and the one they generated *in vitro* after a long culture period. Unfortunately, the granuloma and the inflammatory response disappeared after chemotherapy, and, hence, the conditions of this model did not resemble those found in humans. Actually, it might have just been further evidence of the tolerance of mice to destroy *M. tuberculosis*, which was demonstrated by the triggering of a weak response only based on the control of a relevant concentration of growing bacilli [45].

Concerning the development of granulomas in a murine model of tuberculosis, the current authors' have observed how *M. tuberculosis* "escapes" from the granulomas (fig. 1). In fact, granulomas in mice are generated by an initial accumulation of macrophages in the infectious focus, which is surrounded by lymphocytes triggered by specific immunity. Subsequently, another cellular ring of foamy macrophages starts to surround these granulomas [46]. This is a consequence of the migration of macrophages, filled with tissue debris and bacilli, to the alveolar spaces [47]. Interestingly, most macrophages are activated either directly by specific lymphocytes or by ingesting cell wall components from

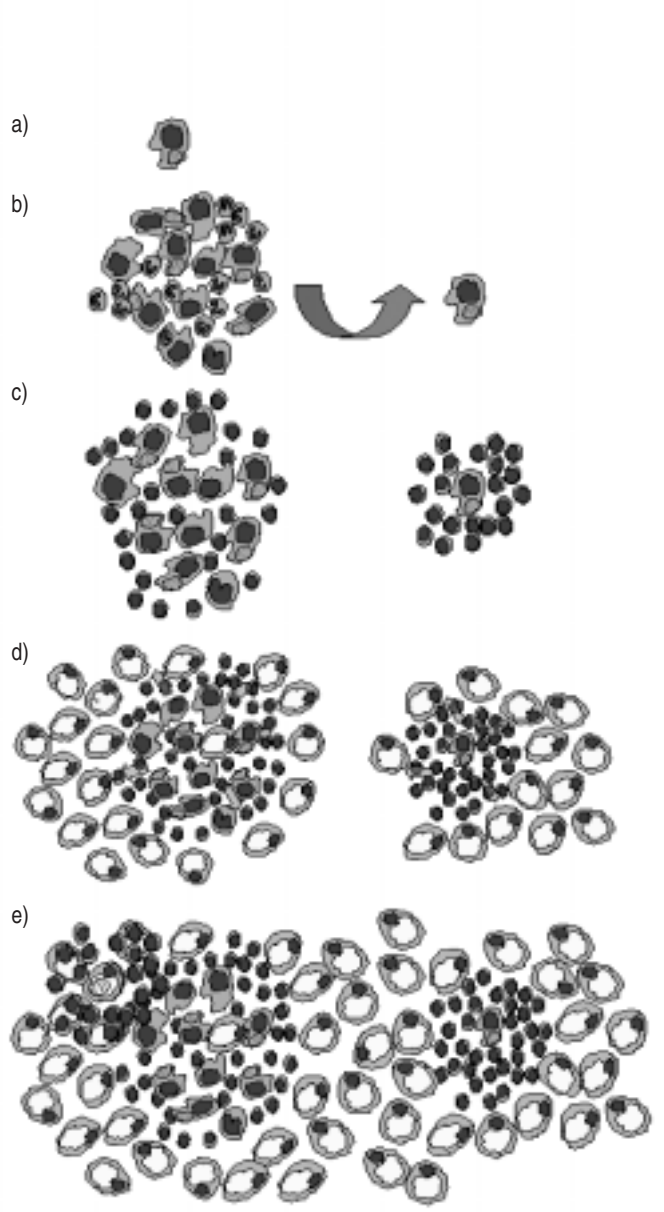

Fig. 1. – Evolution of pulmonary granulomas in the murine model of tuberculosis (based on [46, 48]). Immediately after the infection of alveolar macrophages (a) and the building of the first pre-granulomas, there is dissemination throughout the parenchyma generating the secondary granulomas (b). These are characterised by the scarcity of infected macrophages, heavily surrounded by a mantle of lymphocytes when the specific immunity is triggered (c). In the chronic phase of the infection, foamy macrophages leave the granuloma to the alveolar spaces, adding a second mantle to the lymphocytic one that connects different previous granulomas and generate the so-called "tertiary granulomas" (d). Interestingly, while acid-fast bacilli are hardly seen in the macrophages of the centre of the granulomas, a considerable number of foamy macrophages board single ones. Finally, some of them are able to grow inside these macrophages and generate the attraction of lymphocytes around them (e).

M. tuberculosis, as evidenced by the expression of nitric oxide synthase (NOS) [48]. It is difficult to explain why some of these cells have one or two bacilli inside. The answer may be that these bacilli withstand the bactericidal mechanisms by triggering a starvation response [16], thus stopping their growth. Taking into account that the immunity against *M. tuberculosis* seems to be directed against peptides synthesised by growing bacilli [49, 50], it may be hypothesised that these bacilli may not be recognised by the immune system. Another explanation may be related to the activated nature of these foamy macrophages, since, by synthesising NOS, these macrophages induce immunosuppression in any effector lymphocytes that lie in their vicinity [51]. Therefore, these foamy cells may be a kind of a "sanctuary" for the stressed bacilli. Finally, some of them start to grow inside the foamy macrophages, until they are destroyed [45, 46, 48]. This growth inside the alveolar space and outside the granulomas is especially harmful, as dissemination takes place very easily from this area. This might be the reason why all mice died due to an almost total occupation of the lung parenchyma [52, 53].

This dynamic nature of the infection in mice clearly contradicts the concept based on the classical data of REES and HART [54]. These authors hypothesised that a continuous bacterial turnover would lead to an accumulation of bacterial

bodies, since the remains of heat- or drug-killed *M. tuberculosis* were quite stable in the lungs of mice. As they observed a stable number of acid-fast bacilli in mouse lungs during persistent infection, they concluded that there was little turnover in *M. tuberculosis*. Both our observations of growing bacilli inside foamy macrophages and outside the initial granuloma (*i.e.* in the alveolar spaces), or even the constant expression of local interferon-γ [55], support the theory of a constant bacillary growth and destruction. Even the progressive occupation of the lung parenchyma during chronic infection [48, 52, 53] supports this idea. The findings in the murine model of TB must be considered with caution, since the human response against *M. tuberculosis* is not that tolerant, but the murine model might give us an idea about what happens at the beginning of infection when there is no well-established fibrosis in granulomas and the chances for trafficking material from these to the alveolar spaces is feasible.

In addition, the fact that phospholipids may resuscitate dormant bacilli is interesting because these molecules are found throughout host tissues, rendering possible the reactivation of latent bacilli. This phenomenon would be very difficult to explain if only Rfp was able to resuscitate these cells, as it would require the previous presence of growing bacilli to synthesise Rfp. It may be hypothesised that a nontoxic environment with phospholipids, such as the one found inside foamy macrophages, is a suitable scenario to start reactivation. Once growth has started, the synthesis of Rfp would enhance the process.

Special attention must be given to studies using strains of *M. tuberculosis* with one specific gene deleted. The infection of mice with those strains has provided a great amount of information on the role of each gene, regarding the capacity to survive inside a host. Out of these genes, the repression of isocitrate lyase (*icl1* mutant) or Hsp70 heat shock protein (*hspR* mutant) affects bacillary persistence in host tissues [27, 28], showing that both genes are important for the generation of latency. *ICL1* enhances the role of isocitrate lyase and the glyoxylate shunt to obtain carbon and energy for the metabolism of *M. tuberculosis* from the fatty acids of the host [29], instead of being a way to survive in an environment with a low oxygen pressure [14], whereas *HSPR* confirms the importance of counterbalancing the stress conditions generated against the bacillus.

How do latent bacilli deal with the dynamic nature of the pulmonary parenchyma?

Some authors have recently described the presence of *M. tuberculosis* DNA in human lung parenchyma inside endothelial and epithelial cells, or fibrocytes, and mainly outside granulomas [56], and they have stated that the bacilli would be responsible for the maintenance of LTBI. Without considering the limited significance of detecting DNA by *in situ* hibridisation in tissue [57–59], there is a paramount issue: the turnover of those pulmonary cells ranges 28–125 days [60]. If we accept that latent bacilli are reclused within this niche, latency is limited as some energy would be required to periodically invade younger parenchymal cells. Therefore, latent bacilli must deal with this dynamic nature of the pulmonary parenchyma. Careful analysis of published results led the current authors to consider two possibilities: bacteria constantly disseminate and reactivate, as supported by the murine model; or the bacteria are kept dormant inside the necrotic material of a fibrotic granuloma, where the movement of macrophages would be limited for a long time until being finally reabsorbed, that is, if they are not calcified or become a scar. Obviously, this resuscitation should be very fast, before the bacilli are drained out by the host. Experimental data from the *in vitro* model (*i.e.* from bacilli submitted to a stationary nonstressed culture) has shown that they require up to 4–5 months in the most ideal conditions [22] to reactivate them. At this point, bacilli would be drained out of the lungs. As a consequence, the idea of latent bacilli waiting for immunosuppression should be changed by a constant trend of bacilli to disseminate in order to reach an adequate environment for reactivation. If this reactivation takes place in an area where bacilli may grow quickly, such as the apex of the lungs, and where there is a lack of immunity, then a cavitary lesion (and, thus, pulmonary TB, which is the evidence of LTBI) may develop.

The histopathological characteristics of human TB seem to suggest that intragranulomatous necrosis is induced at the beginning of granuloma formation [45] and, thus, adds extra stress for bacilli. This would explain why humans develop a significant population of extracellular latent bacilli (which is hardly seen in the murine model of experimental TB) that would be phagocytosed by the new macrophages. In this case, the lack of growth may be beneficial for the survival of bacilli, since they would not activate the new macrophages, and then they would be easily removed from the granuloma once the infected cell had become a foamy macrophage.

Many questions arise when the pathologies observed in mice and humans are compared, but probably the most important is whether the initial lesions are "cleaned" by macrophages and then surrounded by foamy macrophages, such as in other chronic inflammatory responses in the lung [61]. The first results obtained from the current authors' studies carried out using material from autopsies of patients with TB seem to support this idea (data not published). Another concern is related to the time needed to become fibrotic and effectively close the granuloma in humans. This is not seen in mice, and that is why they die with extensive lung dissemination. The time taken to effectively turn a lesion fibrotic may help to establish the "risk period" to develop active TB.

Undoubtedly, the time for bronchogenic dissemination is limited, and it may be thought to be even more limited in the case of second-generation granulomas, since they would have developed under an immunological response, and the chances to grow would be lower than in the primary foci. Therefore, it is believed that chronicity also has a time limit. We must stress that, in order to be reactivated, latent bacilli must "escape" from the granuloma. Apparently, a low oxygen pressure and certain toxic materials from destroyed macrophages constitute a "nonresuscitating" medium. Moreover, once the granuloma is fibrotic or even calcified, the chances for these latent bacilli (probably dormant at this point) to escape are almost nonexistent. In this regard, nobody has demonstrated the ability of these bacilli to survive for years in an adverse environment. Conversely, classical studies showed that once intragranulomatous necrosis has been induced, the survival chances of bacilli decrease and are negligible after fibrosis and calcification. Up to 50% of necrotic lesions and 85% of calcified or fibrotic lesions are sterile [62]. Interestingly, OPIE and ARONSON [63] found that homogenates from fibrocaseous lesions in the upper areas of the lungs used to cause TB in guinea pigs, whereas homogenates from caseous encapsulated or calcified lesions rarely caused the disease. This study confirmed the difficulty in sterilising an infectious focus in the apical zone of the lung, and, consequently, both bacillary growth and inflammatory response were more significant, thus supporting the theory of a chronic active infection in this zone, rather than an induction of latency. Amazingly, in the same study, almost half of the samples from superficially

Table 2. – Facts supporting the limited length of the latent period

No experience on long survival period of bacilli under stressful macrophage-related bactericidal mechanisms (*i.e.* low pH, presence of ROI or RNI)
Dynamic turnover of pulmonary cells restricts latency to a period of months. Bacilli should behave in a dynamic way of constant reactivation
Regrowth of bacilli inside the necrotic tissue is nonprobable because of the presence of stressful environmental conditions. It depends on the chance to be phagocytosed again by macrophages in the reabsorbing process of the necrotic tissue
Experimental data about "resuscitation" of dormant bacilli, which would be the state adopted in the necrotic zones, do not demonstrate a quick reactivation, limiting the percentage of bacilli able to regrow before being removed by the macrophage turnover
Bacillary survival inside the encapsulated or calcified lesions is restricted
Estimated immunity (15–20 yrs) generated after *M. tuberculosis* infection ensures a prolonged period of protection against bacillary regrowth
Data from chemoprophylaxis trials monitoring reactivation after infection in control groups revealed a progression to a nonexistent probability after 8 yrs

ROI: radical oxygen intermediates; RNI: radical nitrogen intermediates; *M. tuberculosis*: *Mycobacterium tuberculosis*.

normal lung tissue were infectious in guinea pigs. These data support a constant dissemination through the alveolar spaces.

What are the chances of reactivation after a single infection?

The diagnosis of LTBI is based on the tuberculin test. The existence of live bacilli is not necessary to retain a strong immune memory, since these cells live for long periods of time [64] and many people with LTBI have already killed the bacilli; in this case, many LTBI bacilli will never reactivate. Infection with *M. tuberculosis* triggers protective immunity. It has been estimated that BCG vaccination can induce immunity for 15–20 yrs [3], therefore suggesting a similar period of protection after *M. tuberculosis* infection. Considering the current authors' hypothesis that the constant "escape" of bacilli from granulomas before fibrosis is the primary source of bacteria, reactivation would never occur after a specific time period, unless the host suffered an immunosuppressive episode. Another question is whether the immune system would be able to stop bacillary growth in the upper zones of the lungs due to high oxygen pressure [65]. The answer may be found in the classical literature. Since calcified primary lesions have also been detected in the upper zones of the lung [7], it seems clear that the immune system would be able to stop bacillary growth at this point.

The theory concerning the chances of developing the disease during life after a single infection needs to be reconsidered (table 2). The epidemiological data suggest that the risk of developing TB is higher immediately after infection with *M. tuberculosis*. Historical data from chemoprophylaxis trials using untreated TB-infected household contacts demonstrated that the disease occurred at a rate of $0.74\%\cdot yr^{-1}$ during years 1 and 2, $0.31\%\cdot yr^{-1}$ during years 3–5, and $0.16\%\cdot yr^{-1}$ during years 6 and 7 [4]. These data may be significantly adjusted to a decreasing linear regression, where the chances of developing TB would be nonexistent from 8 yrs after infection.

On the one hand, there are people who have suffered real active TB and were not cured with chemotherapy. These people with large lesions would have a greater chance of suffering dissemination and developing an important lesion, since final fibrosis and containment of bacilli would require more time. The analysis of the incidence of patients with fibrotic parenchymal lesions, where disease was naturally "arrested", showed that the risk of developing the disease might be up to 30-times higher compared with a healthy population [66]. In fact, patients with lesions <2 cm^2 had lower chances of developing tuberculosis than patients with lesions >2 cm^2 (0.116% *versus* 0.213%) [67].

On the other hand, data from molecular fingerprinting of TB cases seem to give a renewed role to reinfection [68] compared with the almost impossibility suggested in historical studies [69]. These positions need to be balanced definitively by looking at the epidemiological evidence. In populations with a high risk of infection, reinfection may be the major contributor to the rate of TB in adults. In populations with a low risk, the overall cases may probably be a result of reactivation [31].

In conclusion, latent *Mycobacterium tuberculosis* is a complex mixture of both slow metabolism and dormant bacilli (probably depending on the severity of the environmental stress suffered). In both cases, the fate of latent bacilli is determined by the dynamic physiology of the tissue where they remain. Thus, it seems feasible to suggest that there are only two possible mechanisms to establish latency and promote disease late in life due to reactivation: constant reactivation once the stressful conditions have disappeared (*e.g.* when bacilli leave the granuloma inside foamy macrophages); or keeping dormant inside the necrotic tissues, waiting for a late drainage, and then resuscitate in a short period of time before being definitively removed from the host. In both cases, it seems important that reactivation takes place without any specific immunity against the bacteria and after reaching a privileged zone, where they would be able to grow as much as possible and to generate a strong inflammatory response that, in turn, would induce liquefaction and form a cavitary lesion. Since there is no *in vivo* nor *in vitro* evidence for quick resuscitation of dormant bacilli, the current authors strongly favour the possibility that latent tuberculosis infection can be maintained for no longer than $\sim$10 yrs, which is, nowadays, a time period very close to that considered for "primary" tuberculosis.

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