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REVIEW

Survival perspectives from the world's most successful pathogen, *Mycobacterium tuberculosis*

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Studying defined mutants of *Mycobacterium tuberculosis* in the mouse model of infection has led to the discovery of attenuated mutants that fall into several phenotypic classes. These mutants are categorized by their growth characteristics compared with those of wild-type *M. tuberculosis*, and include severe growth *in vivo* mutants, growth *in vivo* mutants, persistence mutants, pathology mutants and dissemination mutants. Here, examples of each of these mutant phenotypes are described and classified accordingly. Defining the importance of mycobacterial gene products responsible for *in vivo* growth, persistence and the induction of immunopathology will lead to a greater understanding of the host-pathogen interaction and potentially to new antimycobacterial treatment options.

Tuberculosis remains the leading cause of mortality due to a bacterial pathogen, and infects approximately 32% of the world's human population. Tuberculosis was declared a global health emergency by the World Health Organization in 1993 and annually causes approximately 1.8 million deaths[1], which translates into 4,931 deaths per day (the equivalent of more than 10 Boeing 747 airplanes per day full of people succumbing to this global health crisis). The emergence of multi-drug-resistant strains of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, and the susceptibility of patients infected with human immunodeficiency virus to tuberculosis have fuelled the spread of the disease. *M. tuberculosis* was isolated by Koch, who conclusively proved the causal association between the bacillus and disease. The availability of the complete genome sequence of *M. tuberculosis*[2] and advances in techniques for gene transfer and disruption[3–5] have led to the discovery of several survival strategies used by this complex human pathogen.

Antituberculous immunity

The main route of infection for the tubercle bacillus is the respiratory tract, where the bacteria are inhaled in airborne droplets that proceed distally to the lung to establish an infection. After entering the lung, the first cell type encountered by the bacteria is the alveolar macrophage, which has the microbicidal armory to destroy most potential invaders. However, the tubercle bacillus has the extraordinary ability to persist and even to replicate in this extremely hostile environment, where most other pathogens perish.

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Tuberculous immunity relies mainly on cell-mediated immunity rather than humoral immunity. This immunity specificity is demonstrated by the considerably increased risk of tuberculosis in patients with reduced cell-mediated immunity, such as those infected with human immunodeficiency virus or individuals undergoing immunosuppressive therapy, compared with patients with defective humoral immunity, such as those with multiple myeloma, who show no increased predisposition to tuberculosis[6]. The macrophage has multiple functions in tuberculosis, including antigen processing and presentation, transport to deeper tissues and hence dissemination of infection[7], and effector cell functions. However, at least in the mouse model of infection, *M. tuberculosis* has the ability to evade the onslaught of innate immunity, as virulent bacilli replicate exponentially within mouse lung during the first few weeks after infection, yet after the onset of acquired immunity, the growth of the bacilli plateaus (**Fig. 1**). Protective acquired immunity to *M. tuberculosis* is dominated by $CD4^+$ and $CD8^+$ T cells with the T helper type 1 cytokine profile[8]. The idea of the importance of the key T helper type 1 cytokines interferon-γ (IFN-γ) and interleukin 12 is supported by the high susceptibility to tuberculosis of individuals with defects in interleukin 12, its receptor and the IFN-γ receptor[9–11].

M. tuberculosis can survive and replicate intracellularly, where most other invaders perish (**Fig. 2**). It is generally accepted that the tubercle bacilli's main niche is the host macrophage, and *M. tuberculosis* seems to have evolved effective mechanisms to survive most macrophage effector functions. These defensive mechanisms include the inhibition of phagosome-lysosome fusion[12], where *M. tuberculosis* was found to retard phagosomal maturation[13], and the use of complement receptors 1 and 3 for cell entry, which do not trigger the oxidative burst[14,15]. However, *M. tuberculosis* can enter the macrophage through multiple receptors without adversely effecting its survival[16]. Other effector mechanisms include the production of catalase and superoxide

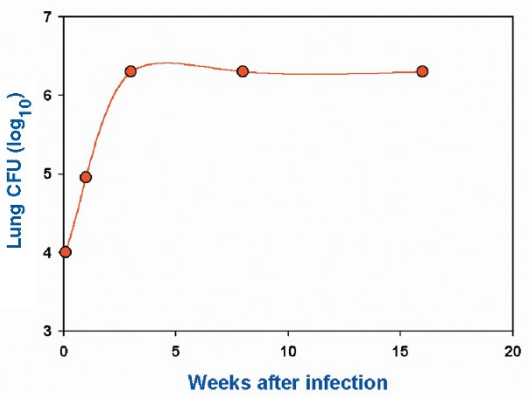

Figure 1 Growth of *M. tuberculosis* in the lungs of immunocompetent mice. C57BL/6 mice were infected intravenously with 1 10^6 colony-forming units (CFU) of *M. tuberculosis*, and growth kinetics were determined in the lungs.

dismutase, which are capable of degrading reactive oxygen intermediates[17,18]. The tubercle bacilli have even been reported to down-regulate some modulators of host immunity such as interleukin 12 (refs. 19,20), major histocompatibility complex class II (ref. 21), IFN-γ-mediated activation of the macrophage[22], the IFN-γ-induced gene *gamma.1* (ref. 23) and host cell apoptosis[24].

Classification of *in vivo* growth mutants

Studies of defined constructed mutants of *M. tuberculosis* in the mouse model of infection have led to the classification of attenuated mutants into several phenotypic classes (**Table 1**). These mutants are categorized by their growth characteristics, such as severe growth *in vivo* (*sgiv*) mutants, which show a very severe reduction in colony-forming units with time; growth *in vivo* (*giv*) mutants, which do not grow as robustly as wild-type *M. tuberculosis* in the lungs of immunocompetent mice, yet still grow better than *sgiv* mutants; persistence (*per*) mutants, which fail to grow or persist after the onset of acquired immunity; and mutants that have the same growth characteristics as *per* mutants, but show altered pathology (*pat*) compared with that of wild-type *M. tuberculosis* (**Fig. 3**).

Attenuated mutants of *M. tuberculosis* are of interest as potential vaccine candidates, with each of the different classes described above providing a different approach to vaccine development and potential disease prevention. A new vaccine for tuberculosis is urgently needed, as the present vaccine, *Mycobacterium bovis* bacille Calmette-Guerin (*M. bovis* BCG), shows vast differences in efficacy, with one study in India reporting an efficacy of 0% (ref. 25). In addition, despite being administered to over three billion people[26], the current vaccine has failed to curtail the spread of the disease. It would also be more advantageous to have a vaccine capable of blocking the initial infection, in contrast to BCG, which mainly protects against the uncontrolled replication and dissemination of the bacilli from the primary foci of infection to the rest of the lungs and body[27]. One approach for improving BCG as a vaccine vector would be to add antigens or functions that enhance its immunogenicity. For example, the ability of BCG to elicit CD8$^+$ T cell responses was improved by the addition of the ability to secrete listeriolysin from *Listeria monocytogenes*[28]. The immunogenicity of BCG has also been improved by the overexpression of key antigens[29,30], or the addition of the primary attenuating region of deletion of BCG, *RD1*, back to BCG[31].

A chief goal of new tuberculosis vaccine development is to have a vaccine derived directly from *M. tuberculosis*, the causative agent of tuberculosis in humans, rather than *M. bovis*, which causes tuberculosis in cattle. Recent advances in the molecular biology of mycobacteria have resulted in the construction of several attenuated mutants of *M. tuberculosis* as candidates for a vaccine[32–34]. The objective for generating a *M. tuberculosis*–derived vaccine is that it would be more efficacious and induce a qualitatively better protective immune response than a *M. bovis*–derived BCG vaccine. The decrease in protective quality of BCG may be due to the decrease in the immunogenicity of BCG[35]. Thus, *M. tuberculosis* mutants should offer better protection because of the expression of an antigenic profile that is nearly identical to that of virulent *M. tuberculosis*. Identifying and characterizing these classes of mutants could also provide insights into the interplay between the tubercle bacilli and the host. This knowledge could help to define the strategies this highly successful intracellular pathogen uses to subvert the hostile environment within the host.

Severe growth *in vivo* (*sgiv*) mutants

The *sgiv* mutants were initially considered to be ideal vaccine candidates. They are highly attenuated because of their inability to replicate and survive *in vivo* and would most likely be safer than BCG, in particular for immunocompromised individuals. However, the ability to replicate and persist may be a desirable trait in an antituberculous vaccine, as it would allow for a prolonged immune response, similar to that obtained with the live attenuated BCG vaccine. Most *sgiv* mutants cause considerably reduced pathology as a result of their severe growth defect. Many are defective in their ability to obtain the essential nutrients required for successful intracellular replication from the hostile phagosomal environment in which *M. tuberculosis* replicates. For example, the ability to acquire magnesium while residing in the phagosome seems to be essential for the virulence of both *M. tuberculosis* and another intracellular pathogen, *Salmonella enterica*. Thus, mutations in *mgt*, which is involved in magnesium transport, result in reduced replication *in vitro* in response to a combination of low magnesium and mildly acidic pH, representative of the *M. tuberculosis*–containing phagosome[36]. These *M. tuberculosis* mutants show substantially reduced growth in the lungs and spleens of mice, characteristic of an *sgiv* mutant.

Many auxotrophic mutants of *M. tuberculosis* also show the characteristic *sgiv* phenotype. Indeed, *M. tuberculosis* leucine (*leuD*)[32], lysine (*lysA*)[37], proline (*proC*)[38], tryptophan (*trpD*)[38] and purine (*purC*)[33] auxotrophs fail to grow in the mouse lung. As for the potential use of *sgiv* auxotrophic mutants as vaccine candidates, both *M. tuberculosis proC* and *trpD* gave the same protection as BCG after challenge with intravenously administered *M. tuberculosis* in mice[38], whereas the *purC* auxotroph[33] provided similar protection to that provided by BCG in an aerosolized guinea pig model of infection. These data indicate that *sgiv* mutants are potential candidates for an improved vaccine and warrant further investigation.

Growth *in vivo* (*giv*) mutants

Most constructed mutants fall into the *giv* class, showing reduced growth and pathology, resulting in an attenuated phenotype and associated increase in host survival time. These mutants may make ideal vaccine candidates because they maintain replication and are more likely to result in a long-lasting host immune response than would an *sgiv* mutant. However, a potentially dangerous situation could arise if the mutant regains its rate of growth, as may happen in immunocompromised individuals, resulting in disease. Thus, continued characterization of specific mutants of *M. tuberculosis* is required to develop strains that are able to elicit a strong protective immune response, but are unable to reactivate in individuals with severe immunodeficiency.

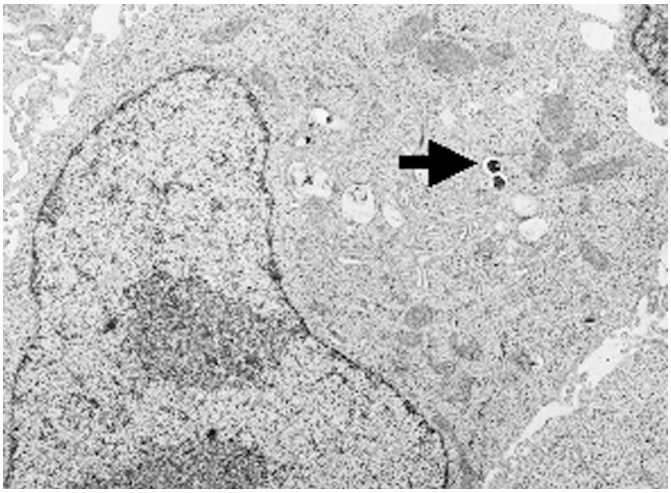

Figure 2 Electron micrograph of intracellular *M. tuberculosis* infection. Human A549 alveolar epithelial cell infected for 72 h with *M. tuberculosis* Erdman (arrow) at a multiplicity of infection of 10:1. Original magnification, 5000.

An example of a *giv* phenotype is caused by deletion of the gene encoding exported repetitive protein (*erp*) in both *M. bovis* BCG and *M. tuberculosis*[39]. The deletion of this gene, which has no ascribed function and is specific to mycobacterial species[40], impaired the growth of the bacilli both in the lungs and spleens of BALB/c mice, and in cultured macrophages. Therefore, Berthet *et al.* postulated that virulence is dependent on the ability of the bacilli to multiply[39]. Another example of a *giv* mutant of *M. tuberculosis* is the glutamine synthetase gene (*glnA1*) mutant. This gene product is important in nitrogen metabolism, and the *glnA1* mutant is attenuated both for growth in macrophages *in vitro* and in survival studies in the guinea pig model of infection[41]. The acquisition of iron by intracellular *M. tuberculosis* has been proposed to be mediated by mycobactins (2-hydroxyphenyloxazoline-containing siderophore molecules), and an *mbtB* deletion mutant, is restricted for growth in iron-limited media but not iron-replete media, and shows reduced growth in the human monocyte-derived cell line THP-1 (ref. 42). From these studies alone it seems that *M. tuberculosis* has evolved a variety of ways to synthesize or acquire essential nutrients for successful intracellular replication from the nutrient-limited phagosome in which the bacilli resides.

A means of identifying virulence-associated genes is the technique of signature-tagged mutagenesis[43], a powerful screening method for the identification of genes required for the growth of a pathogen in an animal model. This technique has been successfully used for many human pathogens, including *M. tuberculosis*[44,45]. In both studies[44,45], mutants were obtained that were in the *giv* category (showed a growth defect in the mouse lung) and that emphasized the importance of the distinctive cell wall–associated lipids of the tubercle bacillus. Camacho and colleagues[44] identified 16 attenuated mutants, most of which were involved in lipid metabolism or transport across the membrane. Several of the mutants had disruptions in a cluster of genes involved in the synthesis of the complex cell wall–associated lipid components pthiocerol and phenolpthiocerol derivatives, which are found only in pathogenic mycobacterial species. Similarly, in the study by Cox *et al.*[45], mutants were identified that had disruptions in the synthesis and transport of phthiocerol dimycocerosate. All the phthiocerol dimycocerosate mutants showed a change in colonial morphology, with overlapping tubular structures rather than the characteristic flat 'corded' structures of wild-type colonies, consistent with an alteration in the cell wall.

To sense and adapt to the changing environment encountered in the various stages of the host's immune response, systems such as two-component regulatory proteins of *M. tuberculosis* are believed to be important for intracellular survival. The *phoP* and *phoQ* two-component regulatory proteins control the transcription of genes important in the survival of many key human pathogens, including *Salmonella* sp., *Shigella* sp., *Yersinia* sp. and *Mycobacteria* sp. Indeed, a *M. tuberculosis phoP* deletion mutant is attenuated in the mouse model of infection. It has reduced growth in the liver, lung and spleen, which is characteristic of a *giv* mutant, and also shows reduced intracellular multiplication in bone marrow–derived macrophages *in vitro*[46]. The *phoP* mutant differed from wild-type in the relative abundance of monoacylated and triacylated mannosylated lipoarabinomannans (ManLAMs) and monoacylated ManLAMs. ManLAMs, which are found in the pathogenic slow-growing mycobacterium species, are considered to be key virulence factors and powerful anti-inflammatory mediators[47]. This may explain the *giv* phenotype of the *phoP* mutant, although the specific mechanisms remain to be elucidated.

As most bacterial virulence factors are exported, it can also be postulated that *M. tuberculosis* uses previously unknown secretion systems with which to secrete virulence factors. The deletion of the accessory secretion factor secA2, resulting in the attenuation of virulence of this mutant in mice, is again characteristic of a *giv* phenotype[48]. This observation indicates that *M. tuberculosis* can secrete immune mediators capable of counteracting the host innate immune response. As mentioned earlier, *M. tuberculosis* can partially overcome the potent toxicity of a key macrophage effector mechanism, the production of reactive oxygen intermediates, by secreting superoxide dismutase[18]. Superoxide dismutase lacks a classical signal sequence for protein export, yet the secretion of superoxide dismutase is secA2 dependent, and many other such immune mediators have been postulated to be secreted by this previously unknown pathway[48].

The importance of the *de novo* biosynthesis of pantothenate (vitamin B5) in the virulence of *M. tuberculosis* was demonstrated by the deletion of the pantothenate-synthesizing genes *panC* and *panD*[34]. The double *panCD* deletion mutant of *M. tuberculosis* was highly attenuated in survival studies, and much safer in immunocompromised SCID mice than the BCG vaccine. The Δ*panCD* mutant of *M. tuberculosis* retained the ability to undergo limited replication in immunocompetent mice and conferred substantial protection to mice after aerosol challenge with virulent *M. tuberculosis*.

Persistence (*per*) mutants

The tubercle bacillus is extremely successful in persisting within the human host, where it is generally considered to remain walled off within granulomas of the lung[7], although a recent study has suggested that bacilli may also persist in 'nonprofessional' phagocytes as well[49]. Indeed, most people infected with the bacillus show no signs of disease and only develop active disease when their immune system is perturbed—for example, after the onset of AIDS, with aging and with severe alcohol abuse. This asymptomatic infection is called latent disease and is notoriously difficult to treat, mainly because the bacilli are dormant and most antibiotics are directed against replicating bacteria. Indeed, the current protocol for treating active tuberculosis is a 6-month course of 'frontline' drugs to reach noncultivable stasis (in which no bacilli can be cultured *ex vivo*). However, dormant bacilli are likely to be noncultivable, and hence the disease potentially could still reactivate. Indeed, in the mouse model of latent infection (the Cornell model), general immunosuppressive treatment can reactivate *M. tuberculosis* infection after treatment to noncultivable stasis[50,51].

Table 1 Classification of the described *M. tuberculosis* mutants

Class	*M. tuberculosis* mutant	Gene function
sgiv	*leuD*[32]	Leucine synthesis
	lysA[37]	Lysine synthesis
	mgt[36]	Magnesium transport
	proC[38]	Proline synthesis
	trpD[38]	Tryptophan synthesis
	purC[33]	Purine synthesis
giv	*erp*[39]	Exported repetitive protein (function unknown)
	phoP[46]	Two-component regulatory protein
	secA2 (ref. 48)	Accessory secretion factor (secretes SOD)
	fadD28, pps-promoter, mmpL7 (ref. 45)	PDIM synthesis and transport
	glnA1 (ref. 41)	Glutamine synthetase (nitrogen metabolism)
	panCD[34]	Pantothenate synthesis
per	*pcaA*[53]	Proximal cyclopropanation of a-mycolates
	icl[54]	Isocitrate lyase (involved in cording phenotype)
	plcABCD[56]	Phospholipase C
	relMTB[57]	(p)ppGpp synthesis and hydrolysis
	dnaE2 (ref. 60)	DNA polymerase
	Hsp70 (ref. 61)	Hsp70
pat	*sigH*[63]	Sigma factor H
	rpoV(sigA)/whiB3 (ref. 65)	Sigma factor A and putative transcription factor
dis	*hbhA*[66]	HBHA

PDIM, phthiocerol dimycocerosate; SOD, superoxide dismutase.

To withstand host adaptive immune responses, *M. tuberculosis* may have adapted its persistance traits. Mutants would therefore be expected to be deficient in persisting in the face of this immunological onslaught (that is, *per* mutants). Characterization of this class of mutants could lead to the elucidation of the mechanisms *M. tuberculosis* uses to survive and persist while in this stage.

Koch first described the association of mycobacterial virulence with a colonial morphotype called 'cording'; that is, the formation of braided microscopic bundles by the bacteria[52]. A genetic screen for *M. bovis* BCG mutants that failed to cord led to the identification of a class of mutants that failed to persist[53]. These mutants, which replicated normally in the initial stages of infection, were disrupted in the gene encoding proximal cyclopropane of α mycolates (*pcaA*), which is required for cording and for the synthesis of the mycolic acid cyclopropane ring, thus defining a function for cyclopropanated lipids in mycobacterial pathogenesis. The same was also found for the *M. tuberculosis pcaA* mutant. The lungs of mice infected with *M. tuberculosis pcaA* mutant are characterized by large aggregations of lymphocytes, indicating that the cyclopropanation of mycobacterial lipids may be important in reducing the influx of lymphocytes, which are essential to the host's acquired immune response.

Other *per* mutants have mutations in the gene encoding isocitrate lyase (*icl*)[54], which is essential for the metabolism of fatty acids; mutations in the genes encoding phospholipase C (*plc*)[55], which are responsible for the cleaving of the phospholipid phosphatidylcholine and are associated with the most virulent mycobacterial species[56]; and the rel_{Mtb} mutant, which is compromised in the maintenance of long-term viability[57]. The *icl* mutant bacilli show normal growth in the acute phase of infection in mice, but very reduced growth after the onset of acquired immunity[54], indicating that persistence relies at least in part on fatty acid metabolism.

M. tuberculosis has four putative phospholipase C genes, *plcA*, *plcB*, *plcC* and *plcD*, and mutants with single and multiple gene deletions have been constructed[55]. All of the mutants showed a decrease in phospholipase C activity, with each gene product contributing equally to overall phospholipase activity[55]. The triple (*plcABC*-deficient) and quadruple (*plcABCD*-deficient) mutants showed the classical *per* phenotype; they were attenuated in the late phase of infection, thus indicating that phospholipase C activity has an as-yet-unidentified function in enabling *M. tuberculosis* to survive the onslaught of the T cell mediated immune response of the host.

After nutrient deprivation, all bacilli begin to grow more slowly and mount what is known as the 'stringent response'. This response is characterized by a reduction in the amounts of protein synthesis and also the synthesis of rRNA and tRNA. In *M. tuberculosis*, the stringent response is mediated by hyperphosphorylated guanine nucleotides, or (p)ppGpp, which are synthesized and hydrolyzed by the *M. tuberculosis* Rel_{Mtb} enzyme[58]. Mutants, which are deficient in the activity of this enzyme, are defective in long-term survival *in vitro*[59]. The phenotype of the mutant in the lungs and spleen of BALB/c mice is that of a *per* mutant, showing an impaired ability to grow during acquired immunity. This indicates that the stringent response is required for the persistence of *M. tuberculosis in vivo*.

Tubercle bacilli also modulate the stress-inducible 'SOS' response in adverse conditions. This response can alter the genetic mutation rate as a means of generating mutants more fit to survive than the parental strain, and Boshoff *et al.* hypothesized that the main replicative DNA polymerase (DnaE) in *M. tuberculosis* may be involved in error-prone DNA repair synthesis[60]. Indeed, *dnaE2* is upregulated *in vitro* in response to the DNA damaging agents, ultraviolet light and hydrogen peroxide. A *dnaE2* mutant showed reduced survival after ultraviolet irradiation and reduced colony counts in the mouse lung 9 months after infection and was attenuated for virulence in survival studies[60]. Drug resistance emerged more frequently in the wild-type and complemented strains than in the mutant, showing that the action of this polymerase contributes to the emergence of drug resistance *in vivo*. This extensive study establishes a function for DnaE2-mediated DNA repair during persistent infection.

Overexpression of the heat-shock protein 70 (Hsp70) proteins in *M. tuberculosis* also resulted in considerable impairment of persistence during the chronic stage of infection[61]. No obvious difference in growth was noted in bone marrow–derived macrophages *in vitro*, or in mice during the early phase of infection. However, the survival and growth of the mutant in the persistent stage of infection was substantially reduced and coincided with an increased number of $CD8^+$ IFN-γ-secreting T cells in the spleen. Overexpression of Hsp70 may favor the host rather than the pathogen during the chronic phase of infection, and thus the induction of Hsp expression may provide a means of fortifying host defenses during latent disease.

Pathology (*pat*) mutants

The *pat* mutants show no growth defect in the face of either innate or acquired immunity, but they do not elicit a typical antituberculous immune response and hence show a distinct difference in host

pathology. The identification of this group of mutants emphasizes the existence of genes within the *M. tuberculosis* genome that are required for increased immunopathology of the host. However, the fact that the tubercle bacillus has such pathology-escalating genes may be related to transmission of the bacilli, which is dependent on progressive lung damage and necrosis. Indeed, for *M. tuberculosis* to infect a new host, the bacteria must be coughed out into the surrounding atmosphere and hence must be extracellular to aid effective dissemination as droplets. The bacilli are thought to be extracellular only in the center of a liquefied necrotic lesion associated with extensive lung pathology and damage. The identification of *pat* mutants also emphasizes that colony-forming unit analyses alone are insufficient and that survival studies and pathology should also be examined when characterizing *in vivo* bacterial mutants in general[62].

A *M. tuberculosis* extracytoplasmic function sigma factor H gene (*sigH*) mutant was found to be attenuated for virulence in the mouse model of infection. However, this mutant showed no *in vivo* growth defect[63]. The attenuated phenotype correlated with fewer and smaller foci of infection, which do not progress as rapidly as in wild-type *M. tuberculosis*–infected mice. There was no difference in the virulence in immunocompromised SCID mice, indicating that T cell–mediated immunity is involved in this mutant phenotype. Indeed, flow cytometry showed that at 4 weeks after infection of immunocompetent mice, the mutant bacteria recruited only 10% the number of $CD4^+$ and $CD8^+$ T cells into the lung, with fewer cells producing IFN-γ and tumor necrosis factor-α. From these results, it seems that the modest T cell response elicited by the mutant bacteria is sufficient for control and that the overly exuberant response seen in wild-type infection is the cause of the increased tissue pathology and earlier death noted in wild-type *M. tuberculosis*–infected mice.

Another sigma factor mutant was discovered when genetic complementation analyses of attenuated and virulent strains of *M. bovis* identified a gene, *rpoV* (also called *sigA*), which restored virulence to the attenuated strain[64]. This gene showed a high degree of homology with principal transcription factors and sigma factors from other bacteria, and a more recent study identified the putative transcription factor with which it interacts, called *whiB3* (ref. 65). A *M. tuberculosis whiB3* deletion mutant showed no difference in bacterial burden compared with that of wild-type, yet showed an attenuated phenotype in immunocompetent mice, as observed from survival studies. The pathological changes noted in the wild-type infected mice were much more severe than those in the mutant infected mice, indicating that *RpoV-WhiB3*–mediated transcription influences the immune response of the host. Both the *SigH* and *whiB3 pat* mutants lack the control of a regulated set of genes, indicating that such regulons are required for the induction of typical tuberculous immunopathology.

Further elucidation of differences in the pathology induced by different *pat* mutants is required to fully define the function of the host immune response. For example, does the mutant-induced immunopathology involve different subtypes and percentages of T cells, as noted in the *sigH* and *Hsp70* mutants[61,63], or other cell types? Also, is there a difference in the production or secretion of immunomodulatory molecules induced by these mutants? Dissecting these interactions will lead to an increased understanding of advantageous and disadvantageous immunopathological responses and could result in the development of strategies that boost the hosts immune response during active disease. A *pat* mutant showing negligible pathology, but persisting at a high level of infection may also be a good vaccine candidate, stimulating the immune response but causing no harmful pathology or disease. In immunocompromised individuals, such a mutant might pose little threat, as it is unable to cause disease in the individual and also fails to undergo transmission to a new host.

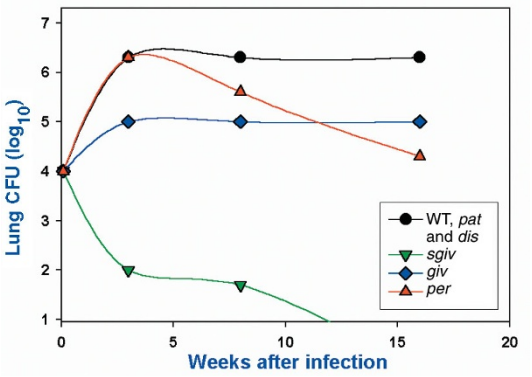

Figure 3 Classification of mutants based on their growth in the lungs of immunocompetent mice. C57BL/6 mice were infected intravenously with 1 10^6 colony-forming units (CFU) of wild-type (WT) or mutant bacilli.

Dissemination (*dis*) mutants

Although this classification system is a useful means of cataloging the various mutants encountered, there may be variations. One example is the heparin-binding hemagglutinin (*hbhA*) mutant, which shows no apparent growth defect when administered intravenously, but shows a defect in dissemination to extrapulmonary sites when administered intranasally[66]. This mutant constitutes a new class of mutants, the dissemination (*dis*) mutants. The development of a vaccine that cannot disseminate would be of particular utility in immunocompromized individuals. There have been many reports of disseminated BCG disease in children vaccinated with the current vaccine who also subsequently developed AIDS[67–69], and also in adults with AIDS who received the vaccine as an infant[70,71].

M. tuberculosis can persist within the host in cell types other than macrophages, including alveolar epithelial cells[49], in which the bacillus can survive and replicate *in vitro*[72]. Disruption of *M. tuberculosis hbhA* affected the adherence, invasion and survival in alveolar epithelial cells, but not macrophages, and showed reduced extrapulmonary colonization in BALB/c mice[66]. This indicates that passage through the alveolar epithelial cell layers is required for extrapulmonary dissemination of the bacillus. Incubating wild-type bacilli with antibodies to heparin-binding hemagglutinin adhesin (HBHA) can inhibit dissemination, which, as well as confirming this hypothesis, has implications for the potential use of such antibodies therapeutically. However, Mueller-Ortiz *et al.*[73], also working with a *hbha* mutant of *M. tuberculosis*, reported that there was an overall growth defect noted with this mutant in the lungs, liver and spleen of the more resistant mouse strain, C57BL/6. These differences in observation could be related to the different mouse strains used by the researchers. Nevertheless, the growth defect in C57BL/6 mice was still more pronounced in the livers and spleens than in the lungs, which may be the result of a defect in dissemination as well as growth. Further definition of the function of HBHA and other bacterial factors in dissemination is needed for a greater understanding of the host-pathogen interaction during this key phase of the disease.

Conclusion

Knowledge of the intracellular 'lifestyle' of the tubercle bacillus will lead to a greater understanding of the host-pathogen interaction and the mechanisms by which *M. tuberculosis* evades both the innate and

adaptive immune responses. Such knowledge should lead to the development of new, safe and efficacious vaccines and antimycobacterial treatment strategies.

An absolute requirement of a live *M. tuberculosis*–derived vaccine is that it be as safe or safer than BCG in humans. The *sgiv* mutants, which fail to grow *in vivo*, such as the leucine[32], proline[38], tryptophan[38] and purine[33] auxotrophs, show protection similar to that induced by BCG vaccination. The combination of two or three such deletions would provide a considerable safety advantage for use in humans. However, it remains to be determined whether limited replication of such a mutant would provide optimal immunogenicity and long-term protection. Another possibility is that such mutants would be too attenuated to elicit such protection.

The *giv* mutants are also defective for growth *in vivo*, yet not to the same extent as noted with the *sgiv* mutants. The *giv* mutants seem to be defective in their ability to interact with the innate immune responses, such as the phthiocerol dimycocerosate mutants[45], and *plcABCD*[56] and *secA2*[48] mutants. A combination of such mutations may reduce the virulence of a *M. tuberculosis* strain enough to provide safety in immunocompromised animals, as noted with the *panCD*[34] mutant, yet allow for limited replication and the elicitation of optimal immunogenicity. The addition of a *pat* mutation, with reduced immunopathology, or a *dis* mutation, with reduced extrapulmonary dissemination, would also increase the safety of such a vaccine by further reducing the risk of disease and dissemination.

More knowledge about *per* mutants, which are defective in persisting in the face of an adaptive immune response, will improve our understanding of the mechanisms used by *M. tuberculosis* to survive the adaptive mycobactericidal mechanisms. This knowledge should lead to new immunotherapies, as well as to strategies for enhancing the immunogenicity of an attenuated *M. tuberculosis* vaccine. It may even be possible to rationally design effective therapeutic vaccines that could eliminate a pre-existing *M. tuberculosis* infection. Thus, the ideal vaccine candidate would probably be a mix of these classes, providing a safer and more efficacious alternative to BCG. Because the development of new therapeutic strategies and vaccines are urgently required to curtail this global health crisis, efforts focusing on elucidating the strategies *M. tuberculosis* uses to be a successful pathogen should be actively pursued.

ACKNOWLEDGMENTS

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