TB is deeply rooted in populations where human rights and dignity are limited.
While anyone can contract TB, the disease thrives amongst the most vulnerable,
the marginalized, the discriminated against, and people living in poverty

The ritual of silence – Agustín Bejarano
Mixed/canvas
(175 x 250 cm)

'How many years must one man have
before he can hear people cry
How many deaths will it take till he knows
that too many people have died.'

Blowing in the Wind (Song) – Bob Dylan

Agustín Bejarano
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Agustín Bejarano graduated from the Higher Institute of Art in Havana, Cuba. Since the 1990s he has taken part in various art exhibitions held in Canada, Cuba, France, Mexico, Puerto Rico, Spain, Switzerland, and the United States. He received an award from the National Salon of Engraving (San Juan, Puerto Rico) during its IX Bienal de Grabado Latinoamericano y Caribeño, and the Grand Prize of the National Salon of Engraving, the Provincial Center of Fine Arts and Design, Havana.

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From Genomic Characterization to Improved BCG

Camille Locht

Introduction

Traditional BCG has been in use to protect humans against TB for more than 80 years now and it is one of the most widely administered vaccine, with an estimated three billion doses given worldwide. However, its effectiveness in reducing the incidence of TB is still under debate, and the molecular bases of its attenuation are still poorly understood. Recent comparative genomic studies have provided some clues as to the mechanism of attenuation of BCG and have also identified a number of genetic differences between BCG daughter strains. In particular, one region of difference—named RD1 and deleted in BCG—appears to be important for virulence. However, other, perhaps more subtle, genomic changes are likely to also contribute to attenuation. Perhaps some of this information will be helpful to improve the protective efficacy of BCG, one of the major challenges of current research. Many attempts have been undertaken in that direction, most of which have failed to show enhanced efficacy in animal models. Nevertheless, recently, some of the new recombinant BCG or MTB strains have shown promise in mouse and/or guinea pig models. However, since the protective efficacy of these new constructs in humans is still unknown, and most of them have not even entered clinical Phase I trials, BCG is probably here to stay for still a number of years or decades.

Genetic Mechanism of Attenuation

More than 85 years after the first use of BCG as an anti-TB vaccine, the precise mechanism of genetic attenuation is still unclear, although some of the important genes involved in MTB virulence that are lacking in BCG have been identified. Already, before the entire genome sequence of MTB and *M. bovis* became available, initial investigations on genetic differences between the virulent mycobacteria and BCG pointed to important genetic deletions in the BCG genome (1). By using subtractive genomic hybridization, three relatively large deletions were identified and called 'regions of difference' (RD) 1–3. RD1, a 9.5 kb DNA fragment, was absent in all BCG substrains tested, but present in all virulent *M. bovis* and MTB strains tested. In contrast, the 10.7 kb RD2 segment was conserved in all the virulent *M. bovis* and MTB, but absent only from BCG substrains derived from the BCG Pasteur strain after 1925, whereas the 9.3 kb RD3 segment was absent from all the BCG substrains, but only present in a minority of MTB clinical isolates. These observations suggested that the deletion of RD1 may have contributed to the attenuation of BCG. When reintroduced into the BCG genome, the RD1 sequence resulted in the repression of at least 10 proteins and the induction of at least 3 additional proteins, suggesting that it contains important regulatory elements that control the expression of multiple virulence-associated genes.

These studies were later confirmed and extended by using hybridization to whole-genome DNA microarrays (2). These microarrays were based on the then recently available complete genome sequence of MTB (3). The microarray analysis revealed a total of 16 regions absent in BCG compared to MTB (strain H37Rv), comprising the previous ones. These deletions were thus named RD1–RD16 and vary in length from 1,903 to 12,733 base pairs. Interestingly, many of them contain genes encoding putative regulatory functions. Of the 16 deletions, 9 are missing from BCG and all virulent *M. bovis* strains tested. Only RD1 is lacking in all the BCG strains. RD1 is also absent from *Mycobacterium microti* (4), a member of the MTB complex that has been used as anti-TB vaccine in humans. Other RD are lacking in some BCG strains, but present in others, suggesting that their deletion does not play a major role in the genetic attenuation of BCG. However, they were useful in studying the genealogy of the different BCG strains. The original strain, derived by Calmette and Guérin in 1921, has unfortunately been lost, and the current BCG strains have derived more or less directly from the progenitor. The analysis of the different RD regions in these daughter strains has helped in establishing the sequence of deletion events. RD1 was lost between 1908 and 1921, as BCG was derived from *M. bovis*. RD2 was then lost at the Institut Pasteur in Paris between 1927 and

1931. A further deletion (RD14) occurred in the BCG Pasteur strain between 1938 and 1961. RD8 and RD16 were lost between 1937 and 1948 in Montreal and, after 1925, in Uruguay or Brazil, respectively. Whether any of the deletions beyond RD1 have an impact on residual virulence or on protective efficacy of BCG is still a matter of debate.

When the RD1 region was deleted from the MTB chromosome, the mutant strain showed a growth defect in cultured macrophages (5). The growth defect of this strain was not as strong as that of BCG-Russia, a BCG strain that is closest to the original strain in that it only lacks RD1. In a mouse aerosol infection model, the mutated MTB strain behaved similarly to BCG-Russia. However, insertion of RD1 into the BCG chromosome did not result in virulence comparable to that of MTB (4, 6), although its virulence was enhanced compared to that of non-recombinant BCG. These observations suggest that other mutations in the BCG chromosome contribute to attenuation.

In addition to the relatively large deletions in the BCG chromosomes, more subtle genetic differences had also been detected between BCG and the virulent members of the MTB complex before the entire genome sequences became available. For example, an intergenic region between the two cistrons of a bi-cistronic operon encoding a two-component signal-transducing regulatory system, called *senX3-regX3*, was found to be different between BCG and virulent MTB complex strains (7). This intergenic region is composed of a mycobacteria-typical genetic element, named Mycobacterial Interspersed Repetitive Unit (MIRU) (8). The *senX3-regX3* intergenic region of BCG contains one, two, or three 77-bp MIRUs, whereas all clinical isolates of the MTB complex analysed so far contain, in addition to the 77-bp MIRUs, also a 53-bp truncated MIRU, as the last element of the repeats. The pathophysiological meaning of such subtle differences is not known. However, they can be used to rapidly and easily distinguish BCG from virulent MTB complex isolates in human or animal samples. Interestingly, the *senX3-regX3* intergenic region also displays some degree of polymorphism between BCG strains, and can be used to distinguish certain vaccine strains from each other.

The entire genomes of many mycobacteria, including MTB (3), *M. bovis* (9), and BCG (10) have now been sequenced, allowing for very precise comparison of the BCG genome with those of virulent mycobacteria. The genome of BCG Pasteur 1173P2 contains 4,374,522 base pairs and 3,954 predicted genes coding for proteins together with 34 pseudogenes. It is almost 30 kilobases larger than the *M. bovis* AF2122/97 genome because of 2 independent tandem duplications, named DU1 and DU2. The comparison of the genome sequences of MTB with those of *M. bovis* and BCG revealed a total of 42 RD. However, some of these RD were present in BCG but absent in the sequenced *M. bovis*

strain (AF2122/97), indicating that the parental BCG strain preceded the latter. Analyses of single nucleotide polymorphisms revealed 736 differences between BCG Pasteur and *M. bovis* AF2122/97, but more than 2,000 between BCG and MTB. The majority of the single nucleotide polymorphisms (83 per cent) between *M. bovis* and BCG occur within coding genes, and 68 per cent are non-synonymous.

Among the genes that were lost or inactivated in BCG strains, many code for regulatory proteins, such as extracytoplasmic function sigma factors, two-component systems, and cAMP-receptor protein. Consistent with this observation, comparative transcriptomic studies revealed additional important differences between BCG and virulent MTB complex strains. Several genes involved in important metabolic pathways were up- or down-regulated in BCG compared to *M. bovis*. It will be a long way before the role of each of these differences in the attenuation of BCG is sorted out. Some transcriptional modifications may seem logical to be involved in attenuation. For example, the expression of *mceG* is substantially lower in BCG than in *M. bovis*. This gene is essential for the function of the multiple *mce* locus in MTB. Mutations in the MTB *mceG* gene result in severe attenuation in vivo (11).

Many differences between the BCG strains were also detected by comparative genomics, and they may affect residual virulence and/or protective vaccine efficacy. However, establishing these links will be another tedious and time-consuming task. In addition, since TB is a very complex disease, and protection can be assessed in a number of different ways and models, it may be difficult to come to a definitive conclusion soon.

Improved BCG

There is much expectancy in the use of the new molecular tools and genetic data to improve BCG. With the technologies developed in the early 1990s that allowed scientists to introduce and express foreign genes in BCG (12), first attempts to improve the protective efficacy of BCG by recombinant means were undertaken. Based on the fact that protection against TB requires a strong T cell response, especially the production of Th1 cytokines such as IFN-γ, attempts were made to improve the inherent protective immunogenicity of BCG by engineering it such that it produces and secretes mammalian cytokines (13). A broad spectrum of murine cytokines could be produced and secreted by recombinant BCG. When mice were infected with some of these recombinant strains, the immune responses to PPD was found to be strongly modulated as compared to that of mice immunized with non-recombinant BCG. Sixteen

weeks after administration the mice that had received BCG producing IL-2, GM-CSF, or IFN-γ showed strongly enhanced T cell responses compared to mice vaccinated with non-recombinant BCG, as evidenced by immunoproliferative and cytokine responses of their splenocytes in response to PPD.

IL-18 is a cytokine that induces the production of IFN-γ. Since IL-18 is a monomeric protein, in contrast to the dimeric IFN-γ molecule, production of IL-18 in its active form may be easier to obtain in recombinant BCG than that of active IFN-γ. Recombinant BCG producing IL-18 has thus also been constructed (14). It was found to substantially increase the IFN-γ response in mice, compared to control BCG, especially at longer times after administration. However, the increased cytokine expression did not enhance the protective effect of BCG in the mouse models (unpublished observations). Nevertheless, the IL-18 producing BCG strain was found to suppress pulmonary Th2 responses, such as IL-5 production and airway eosinophilia, in a murine ovalbumin-challenge allergy model significantly more than did non-recombinant BCG (15). Therefore, such a strain may perhaps find a potential therapeutic application for the treatment of allergic reactions.

Following the idea that perhaps BCG does not produce sufficient amounts of protective antigens, Horwitz et al. (16) constructed recombinant BCG strains that overproduce the 30-kDa major secretory protein, also referred to as Antigen 85B. When guinea pigs were immunized with these recombinant BCG strains and subsequently challenged by aerosol with a highly virulent MTB strain, the bacterial load in spleen and liver 10 weeks after challenge were lower than in the animals vaccinated with conventional BCG. However, mortality and weight gain were similar after challenge in recombinant and conventional BCG vaccinated guinea pigs. Nevertheless, these studies suggest that it may be possible to engineer improved BCG vaccines.

A slightly different approach was taken by Pym et al. (4). They used a BCG strain containing RD1 inserted in the chromosome to test the hypothesis whether antigens present in MTB, but absent in conventional BCG, may help to improve the protective efficacy of BCG. The recombinant BCG was compared to the control strain in different animal models. In murine intravenous and aerosol challenge models, BCG::RD1 vaccination resulted in enhanced protection against MTB compared to vaccination with control BCG. However, this was only apparent when CFUs of MTB were counted in the spleen. Both BCG strains had comparable efficacy in the lungs. In a guinea pig aerosol challenge model, similar results were obtained in that 17 weeks after challenge CFUs in the spleen were significantly lower in the animals that had been vaccinated with BCG::RD1, compared to control BCG, whereas there was no significant difference in the lungs between the two vaccinated groups. This organ-specific

enhancement of protection is intriguing, and its mechanism has not been elucidated yet. Several explanations are possible, including a putative differential expression of RD1 genes in different organs, or a differential recruitment of RD1-specific effector T cells in different organs. It may also be possible that immune responses to RD1-encoded antigens restrict MTB growth in internal organs, but not in the lungs. RD1 contains a number of genes, including those coding for two potent T cell antigens, ESAT-6, and CFP-10, as well as those that are essential for ESAT-6 and CFP-10 secretion. Interestingly, not only production of the two T cell antigens by the recombinant BCG strain but also their secretion via the RD1-encoded secretion apparatus appeared to be crucial for maximal T cell immunogenicity. Obviously, any further development of this approach has to consider the narrow window between improved vaccine efficacy and increased virulence of the recombinant BCG.

A third approach to improve BCG was based on a recombinant strategy designed to increase the capacity of BCG to present mycobacterial antigens via the MHC class I pathway, which in turn should result in enhanced CD8+ T cell stimulation. This goal was achieved by constructing a BCG strain that produces and secretes recombinant listeriolysin from *Listeria monocytogenes* and is defective in urease production through the disruption of the *ureC* gene (17). The disruption of the *ureC* gene resulted in acidification of the recombinant BCG-containing phagosome, which otherwise remains neutral. An acidified environment is critical for optimal activity of listeriolysin. This toxin enables *Listeria* to escape from the phagosome into the cytosol. Since this is probably one of the mechanisms explaining the strong CD8+ T cell-inducing capacity of *L. monocytogenes*, the hypothesis behind this strategy was to cause phagosomal escape of the recombinant BCG due to the listeriolysin activity in an acidified phagosomal environment. Escape into the cytosol would then enhance MHC class I presentation of mycobacterial antigens.

Although phagosomal escape of the recombinant BCG could not be demonstrated, its vaccine efficacy in a murine aerosol challenge model was found to be superior to that of non-recombinant BCG. In the early or intermediate phase of infection, the protection levels did not significantly differ between the two vaccinated groups. However, at late stages (150 days post-challenge) the mice vaccinated with the listeriolysin-producing BCG had approximately one order of magnitude less MTB organisms in their lungs than the mice immunized with non-recombinant BCG. The differences were even more striking when a clinical MTB isolate from the Beijing/W family was used for challenge. In that case, non-recombinant BCG virtually failed to show any significant protection, whereas the listeriolysin-producing vaccine strain induced strong protection against the Beijing strain in the mice. The recombinant BCG strain was shown to be safe in mouse models,

including in Severe Combined Immunodeficiency (SCID) mice, even after intravenous administration of high doses, where it appeared in fact to be safer than non-recombinant BCG.

Although the recombinant BCG did not escape from the phagosome, perforation of the phagosomal membrane probably caused mycobacterial antigens to leak into the cytosol and to be loaded onto MHC class I molecules and allowed phagosomal proteases, such as cathepsin, to gain access to the cytosol, where they could activate apoptosis, which in turn may result in cross-priming of mycobacterial antigens. Both mechanisms were found to occur. Based on these observations, this vaccine candidate is now under clinical development.

Instead of building on existing BCG strains, an alternative strategy to develop improved vaccines against TB is to genetically modify virulent MTB in order to achieve safe attenuation yet improve protective immunogenicity over existing BCG strains. This approach initially followed strategies that had been successful in engineering other attenuated live vaccines, in particular *Salmonella*. It is based on the fact that auxotroph mutants of virulent bacteria are often attenuated. Thus, a variety of different auxotroph MTB mutants have been generated. Initial auxotroph mutants constructed by disruption of one of the purine biosynthetic genes were shown to provide some protection in guinea pigs, but the protection level was lower than that afforded by BCG (18).

Other auxotroph mutants showed more promise. A double-deletion mutant of MTB in the *panC* and *panD* genes, involved in the de novo synthesis of pantothenate (vitamin B5), was shown to be highly attenuated in mice, including SCID mice, yet able to confer protection in mice against aerosol challenge, which was comparable to that induced by vaccination with BCG (19). Pantothenate is an essential molecule required for the synthesis of coenzyme A and acyl carrier protein which are important in fatty-acid metabolism and for the tricarboxylic-acid cycle. SCID mice infected intravenously with this mutant strain survived substantially longer than SCID mice infected with BCG. Interestingly, however, after an initial drop in bacterial numbers in spleen, liver, and lungs, the number of the CFUs of the *panCD* mutant gradually increased in the SCID mice up to 10^8 CFU in the lungs, in contrast to other auxotrophs that were unable to grow in the SCID mice. In immunocompetent mice, the bacterial numbers of mutant strain initially increased roughly tenfold in the lungs and then declined gradually, causing much less pathology than the parent strain. It is thus possible that the enhanced persistence of this mutant strain over other auxotrophs may be one of the reasons for its improved immunoprotective potential, compared to the previously tested strains.

Even though these studies have shown that auxotroph mutants can be highly attenuated and still induce protective immunity in animal models,

none of them so far has shown protection superior than that afforded by BCG. Another strategy to genetically attenuate MTB was to alter its regulatory network by mutating genes encoding important transcription factors. One of them is *phoP*, a transcriptional activator that is part of a signal-transducing two-component system. The disruption of the *phoP* gene has led to strong attenuation in SCID mice infected by the aerosol route (20). When compared with BCG in the intravenous infection model of SCID mice, the *phoP* mutant was found to be much safer, even with a tenfold higher dose than BCG. When protection was assessed in mice by counting viable bacterial numbers of MTB upon intravenous challenge in the lungs and spleen four weeks after challenge, it was found to be similar to that afforded by BCG. Similar results were obtained in a low-dose aerosol challenge model in guinea pigs. However, in a high-dose aerosol challenge model of guinea pigs, animals that were vaccinated with the *phoP* mutant survived substantially longer than BCG-vaccinated mice. This improved protection was confirmed when pathology and bacterial counts in the lungs were determined. It will obviously be of interest to dissect the molecular consequences of the *phoP* mutation in order to better understand the mechanistic reason for attenuation. For safety reasons, the introduction of a second mutation, equally attenuating, possibly of a gene within the *phoP* regulon, may also perhaps have to be considered before this vaccine candidate can be tested in large clinical trials.

Finally, it may be possible to increase the protective effect of a live vaccine by interfering with important host functions that influence immunity, such as apoptosis. Recently, a mutant strain of MTB defective for the secretion of superoxide dismutase A, due to the inactivation of the *secA2* gene, was found to induce increased levels of apoptosis in infected macrophages, compared to the parent strain (21). This was associated with a significant enhancement of priming of antigen-specific CD8^{+} T cells, probably due to augmented cross-presentation by MHC class I molecules. Antigen-specific CD8^{+} T cells have been identified as major players in protective immunity against TB. When the *secA2* mutant was compared to BCG for its ability to induce protection in mice, it was indeed found to afford superior protection even against a Beijing strain of MTB in a low dose aerosol challenge model, as evidenced by longer survival and decreased bacterial load and lung pathology one month after challenge infection. Similar promising results were obtained in the guinea pig model, although survival in the guinea pigs was not monitored. It remains to be seen whether such a mutant can enter clinical development or if further mutations are required to obtain sufficient attenuation.

The Future of BCG

After more than 85 years of extensive use of BCG as an anti-TB vaccine, this vaccine is likely here to stay for at least a few more decades. Although many attempts have been made to improve the protective efficacy of BCG by recombinant means, most have failed to generate a vaccine that is superior to BCG. A few recent attempts appear promising, however, yet their clinical use is still far away. Most of them have not started Phase I safety trials in humans. It may perhaps be interesting to combine some of the most promising strategies into a single vaccine strain in order to achieve a quantum leap of enhanced protection. A major challenge will be to establish improved protective efficacy of these recombinant strains over BCG in humans—children or adults. This can probably only be tested in large efficacy trials conducted in high-incidence countries. However, in most high-incidence countries, BCG is routinely used in early childhood and whether, in a comparative Phase III efficacy trial, the new experimental vaccines can be assessed in parallel to the efficacy of BCG certainly raises important ethical issues.

As an alternative to the replacement of BCG, a heterologous prime-boost strategy may be easier to defend from the ethical point of view. Animal studies have shown promising results that indicate that boosting BCG-induced protective immunogenicity by purified antigens may substantially improve the vaccine efficacy.

Comparative genomics may perhaps help to unravel some of the conflicting findings on BCG efficacy. Genomic comparisons between different BCG strains have shown substantial differences between them. The initial reports on the protective efficacy of BCG by Calmette et al. (22) were much more promising than later reports, and it is possible that early BCG strains confer better protection than the more recent strains. There are indeed data that suggest that early BCG strains, such as BCG Japan, induce higher levels of Th1 cytokines and lower levels of Th2 cytokines in babies than do more recent strains, such as BCG Danish (23). Whether this reflects differences in efficacy remains to be seen.

One of the additional applications of BCG that has attracted much attention is its use as a vaccine vector to present heterologous antigens. Since the initial report on the production of recombinant BCG (12), many recombinant strains have been constructed that produce a variety of heterologous antigens [reviewed in (24)]. Some of them have shown promise in animal models, but none of them has yet reached clinical use or gone to extensive efficacy trials in humans.

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