Decrease in the Effectiveness of Bacille Calmette-Guérin Vaccine against Pulmonary Tuberculosis: A Consequence of Increased Immune Suppression by Microbial Antioxidants, Not Overattenuation

Douglas S. Kernodle
Departments of Medicine and of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee

Mutations that arose in bacille Calmette-Guérin (BCG) daughter strains during decades of in vitro cultivation have long been suspected of reducing the efficacy of the BCG vaccine against pulmonary tuberculosis. Although concern was raised 6 decades ago that BCG had become overattenuated, preferential use of relatively virulent BCG vaccines has not restored efficacy. The recent discovery that as BCG evolved its production of antioxidants increased as a consequence of genomic duplications and other mutations suggests the alternative hypothesis that BCG became better at suppressing oxidant-dependent immune responses. This new model of BCG evolution is supported by evidence indicating that reducing BCG antioxidants enhances immunogenicity. Furthermore, some previously unexplained aspects of the performance of the BCG vaccine in clinical trials now make sense in the context of the new model. Finally, the model suggests that the risk of developing pulmonary tuberculosis is influenced by the balance between host-generated oxidants and microbial antioxidants that activate and suppress, respectively, the antigen-presentation pathways that protect the lungs.

For almost 9 decades, the live vaccine Mycobacterium bovis bacille Calmette-Guérin (BCG) has been used against tuberculosis. In early studies, BCG vaccine was highly efficacious. In the 1920s, vaccination of nursing and medical students with BCG reduced pulmonary tuberculosis by 80% [1]. In the 1930s, a trial in North America found similarly high protection in the first 2 decades after vaccination, with continued benefit for 6 decades [2, 3]. Yet in subsequent studies BCG vaccine exhibited marked variability against pulmonary tuberculosis and occasionally even appeared to increase the risk of developing tuberculosis [4, 5]. This variability is highly significant ($P < .0001$) and is regarded as being indicative of true biological differences [4]. Yet despite its flawed record against pulmonary tuberculosis, BCG remains reliably efficacious in preventing tuberculosis meningitis and miliary tuberculosis in young children [6]. Because pulmonary tuberculosis is more common than disseminated tuberculosis, BCG has had a minimal effect on the global burden of tuberculosis, estimated at 9.4 million new active cases and 1.8 million deaths annually [7].

Several hypotheses attempt to explain the variable effectiveness of BCG against pulmonary tuberculosis. These hypotheses include differences between BCG daughter strains (substrains), an inadequate dosage of BCG in some trials, interference by environmental mycobacteria, genetic differences in human populations, and geographic differences in clinical isolates of M. tuberculosis. These hypotheses have been summarized elsewhere [4, 5, 8].

This Viewpoint offers a new perspective on the old idea that the variable efficacy of the BCG vaccine against pulmonary tuberculosis involves differences between BCG daughter strains. In the context of emerging data that reveal that mycobacterial antioxidants suppress host immunity [9–12] and that antioxidant production increased as BCG evolved [13, 14], the relevant literature is reexamined to reveal an association between BCG daughter strains that produce large amounts of antioxidants and poor efficacy against pulmonary tuberculosis. On the basis of partial but not fully conclusive evidence, a new model is proposed in which BCG, instead of becoming overattenuated, evolved to become better at suppressing the CD8+ T cell responses needed for protection against pulmonary tuberculosis.
EARLY CONCERN ABOUT OVERATTENUATION AND IDENTIFICATION OF PHENOTYPIC DIFFERENCES AMONG BCG SUBSTRAINS

An early theory regarding the apparent decline in efficacy of the BCG vaccine against pulmonary tuberculosis was that the vaccine had changed over time. In 1949, Irvine expressed concern that the "problem has now become one of over-attenuation. Separated from its natural habitat for 42 years, may not attenuation still be slowly progressing?" [15, p 25]. By the mid-1950s, it had been shown that BCG substrains differ in characteristics, including growth rate, their ability to persist in vivo, and their ability to protect mice against *M. tuberculosis* infection [16–19]. Daughter strains are descendents of BCG; before modern technologies for preserving bacteria became available, laboratories maintained BCG by serial passage (ie, by transferring part of an aging culture into fresh media). Some strains were passaged >1000 times before seed lots were prepared (eg, Pasteur 1173P2 and Danish 1331), whereas in Japan BCG was passaged only 172 times (Tokyo 172) [14, 20]. Serial passage at multiple sites caused divergent evolution. Thus, substrains differ from each other and from the original vaccine, which no longer exists.

SUPERIORITY OF RELATIVELY VIRULENT BCG VACCINES IN ANIMAL MODELS

In an attempt to identify BCG daughter strains still effective in humans, investigators turned to animal models and learned that protection correlated with the invasiveness and persistence of the vaccine strain. Dubos and Pierce summarized their findings in mice as follows: "Since the most invasive substrains of BCG are the most likely to elicit dependable and lasting immunity, it would appear at first sight that they are best suited to the practice of human vaccination.... On the other hand... greater invasiveness increases the incidence of adenopathies.... The choice of the optimal substrain of BCG involves a compromise between the requirements of the immunologist for dependable and lasting immunity and the concern of all for innocuousness" [19, p 713]. On the basis of studies in golden hamsters, bank voles, and guinea pigs, Bunch-Christensen et al also favored virulent BCG substrains, concluding that "the lower virulence [of some substrains] is a sign of genetic mutation [and] consistent with the general biological experience that virulence is often lost in vitro but that it practically never increases" [21, p 65]. Thus, virulent vaccines were assumed to be most like the original BCG, which was 80% efficacious in humans. This assumption influenced the selection of 2 virulent substrains, Pasteur 1173P2 and Danish 1331, for a vaccination trial in the Chingleput region of India [5, 22, 23]. However, despite their high rank (second and third, respectively) among the dozen vaccines evaluated in animals [22], neither vaccine protected humans against pulmonary tuberculosis [23].

SUPERIORITY OF BCG TOKYO 172 IN PREVENTING TUBERCULOSIS IN HUMANS

The failure to restore vaccination effectiveness in Chingleput with virulent substrains that worked best in animal models raised questions about whether overattenuation is the reason for the decline in BCG effectiveness and also about how well results in animal models correlate with results in humans. Concerned by the inconsistent ranking of vaccines by different laboratories [24], Comstock instead focused on the details of clinical trials to identify an effective BCG vaccine. He argued that case-control studies conducted over a time span in which one BCG daughter strain replaces another provide insight into their relative effectiveness [25–27]. Two studies, one in Indonesia involving disseminated and pulmonary tuberculosis (16 and 88 cases, respectively) and another in Colombia involving pul-

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**Figure 1.** Relative expression of genes encoding antioxidants and other microbial factors implicated in immune evasion. Figure panels were prepared by analysis of data in Table S4 of the article by Brosch et al [14]. First, the expression value for 2 reference isolates of *Mycobacterium bovis* was averaged, and the mean value was set as 1 (black line). Second, expression values for bacille Calmette-Guérin (BCG) Tokyo 172 and Pasteur 1173P2 were displayed relative to the mean values for the reference *M. bovis* isolates. A, Antioxidant and other immune evasion genes duplicated in Tokyo 172, Danish 1331, or Pasteur 1173P2 (full names are given in Table 1). B, Antioxidant genes outside the BCG duplication units. These include sodA (iron cofactorated superoxide dismutase), tpx (thiol peroxidase), ahpC and ahpD (alkylhydroperoxide reductases C and D), rubA (rubredoxin), the oxidoreductase Rv1774, and members of the whiB family of protein disulfide reductases.
Table 1. Antioxidant and Other Immune Evasion Genes in Bacille Calmette-Guérin (BCG) Duplication Units

<table>
<thead>
<tr>
<th>Protein function (gene name)</th>
<th>Duplication unit</th>
<th>Tokyo 172</th>
<th>Danish 1331</th>
<th>Pasteur 1173P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thioredoxin reductase (trxB2)</td>
<td>DU1</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thioredoxin (trxC)</td>
<td>DU1</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein disulfide reductase (whiB7)</td>
<td>DU2</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>Possible glutaredoxin (Rv3198A)</td>
<td>DU2</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein disulfide reductase (whiB1)</td>
<td>DU2</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Oxidative stress sigma factor (sigH)</td>
<td>DU2</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>NADPH quinone reductase (lpdA, Rv3303c)</td>
<td>DU2</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>PI3P phosphatase (sapM, Rv3110)</td>
<td>DU2</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. The genes within DU1 of BCG Pasteur 1173P2 were determined from BCGList (http://genolist.pasteur.fr/BCGList). To identify genes within DU2, H37Rv coordinates of the duplicated regions in each BCG daughter strain were obtained from Brosch et al [14] and included the following: Tokyo 172, 3,684,229–3,704,932; Danish 1331, 3,567,459–3,608,472 and 3,671,536–3,700,097; and Pasteur 1173P2, 3,590,902–3,608,472 and 3,671,536–3,690,127. Then TubercuList (http://genolist.pasteur.fr/TubercuList/) was used to identify the genes within the DU2 region of chromosome for each BCG daughter strain. NADPH, nicotinamide adenine dinucleotide phosphate; PI3P, phosphatidylinositol 3-phosphate.

In developing his argument, Comstock mentioned 2 other studies that compared BCG substrains [27]. The first was the Chingleput trial, in which neither virulent vaccine was effective. Comstock lamented the omission of a strain that ranked poorly in animal models while acknowledging that it was “understandable that making such an odious comparison was not politically possible for a World Health Organization–sponsored project” [27, p S251]. The second study involved newborns in Hong Kong [5]. Fourteen cases of tuberculosis involving lymph nodes, meninges, bone, joints, or multiple sites were observed in 150,000 persons vaccinated with BCG Pasteur, versus 31 cases in 150,000 recipients of BCG British/Glaxo. Of note, these vaccines ranked second and 11th, respectively, in animal models [22], and thus results in animals and humans correlated nicely. More recently, another study focused on protection against disseminated tuberculosis in early childhood, comparing intradermal Danish 1331 and percutaneous Tokyo 172 [31]. Both vaccines were highly effective, reducing disseminated tuberculosis by 87% overall compared with nonvaccinated individuals, yet Danish 1331 was 46% more effective than Tokyo 172. These results also correlate well with animal models, for which the vaccines ranked third and eighth, respectively [22].

In summary, in the 5 human studies in which BCG daughter strains have been compared, Tokyo 172 has demonstrated greater effectiveness against pulmonary tuberculosis, whereas BCG Pasteur and Danish 1331 have demonstrated greater protection against disseminated tuberculosis in early childhood. Furthermore, although rankings of BCG substrains in animal models [22, 29] correlate poorly with protection against pulmonary tuberculosis in humans [25–27], they correlate well with protection against disseminated tuberculosis. This dichotomy may reflect a requirement for qualitatively different immune responses for preventing pulmonary tuberculosis than disseminated tuberculosis [12]. Obviously, this type of analysis is limited because of the small number of studies that have compared BCG substrains in humans.

GENOMIC DELETIONS IN BCG SUBSTRAINS

Over the past few decades, the genetic evolution of BCG has been partly reconstructed. As BCG evolved from M. bovis, it lost the region of difference 1 (RD1). RD1 is absent from the chromosome of all BCG substrains and encodes a secretion system involved in virulence [32–34]. Additional genomic deletions are found in some BCG substrains, and despite the failure of virulent BCG vaccines in Chingleput, the hypothesis that the decline in the effectiveness of the BCG vaccine involved overattenuation has resurfaced [35–37]. In the reformulated hypothesis, deletions after RD1 are believed to cause overattenuation, yet no deletion has been clearly linked to attenuation [37].

INCREASED EXPRESSION OF ANTIOXIDANTS IN BCG SUBSTRAINS

The evolution of BCG vaccine also included the duplication of regions of its genome and greater expression of antioxidants (Figure 1 and Table 1) [13, 14]. Two duplication units, DU1 and DU2, exist. Although DU1 may be unique to BCG Pasteur, DU2 is widely distributed, assumes different forms, and is triplicated in some BCG daughter strains. Notable among the duplicated and highly expressed genes is the sigma factor SigH, which augments the expression of multiple antioxidants during oxidative stress, including thioredoxin, thioredoxin reductase, and iron-cofactored superoxide dismutase (SodA) [38]. SigH also induces enzymes that synthesize precursors of mycothiol [39].

The biological reason underlying the
Figure 2. Influence of oxidant-dependent immune signaling on antigen-presentation pathways and clinical outcome. After becoming infected with Mycobacterium tuberculosis, the host faces 2 challenges. The first is to halt the lymphohematogenous spread of bacilli and prevent the development of miliary tuberculosis; this is accomplished by macrophages and CD4+ T cells that produce interferon-$\gamma$ (IFN-$\gamma$). The second is to prevent foci of granulomatous inflammation from expanding and damaging normal tissue; this probably requires cytotoxic T lymphocytes (CTLs) that kill bacteria within infected macrophages. The induction of CTLs appears to involve Mycobacterium-infected phagocytes that first undergo apoptosis. Then mycobacterial antigens within apoptotic cell fragments are taken up and presented by dendritic cells. Apoptosis-associated cross-priming pathways leading to CTLs (black box) may be the reason that 90% of $M. \text{tuberculosis}$-infected humans never develop pulmonary tuberculosis. In contrast, the small-animal models commonly used to evaluate tuberculosis vaccines are not natural hosts for $M. \text{tuberculosis}$. In most of these models, the host can restrict dissemination unless it is starved or has certain genetic defects; however, disease in the lungs progresses and lifelong containment similar to latent tuberculosis in humans does not occur, possibly because of inadequate CD8+ T cell responses. MØ, macrophages; MHC, major histocompatibility complex; PMNs, polymorphonuclear neutrophils; TB, tuberculosis.

In $M. \text{tuberculosis}$, reducing the activity or secretion of SodA enhances the activation and apoptosis of mononuclear cells and strengthens antigen-specific CD8+ T cell responses [9–11]. Furthermore, SigH promotes lung immunopathology by an unknown mechanism [41]. To determine whether antioxidants that increased as BCG evolved suppress immune responses, Sadagopal et al [12] eliminated SigH and reduced SodA activity and secretion in an extensively passaged BCG strain. BCG-specific CD8+ T cell responses suppressed by the parent vaccine were unmasked during vaccination with the modified BCG vaccine. Memory immunity was also enhanced, and the modified BCG grew slower in vitro and survived less well in mice.

**EFFECT OF REDUCING BCG ANTIOXIDANTS ON IMMUNOGENICITY**

The evolution of BCG represents a paradox. Unlike other live vaccines that evolved to become more attenuated during in vitro cultivation, BCG instead became more virulent. The literature clearly documents that the extensively cultivated BCG substrains Pasteur 1173P2 and Danish 1331 exhibit greater virulence in animal models than the less extensively cultivated Tokyo 172 strain [12, 22, 28, 29]. Furthermore, Danish 1331 is more virulent than Danish 1077 (British/Glaxo). The arguments made in this article suggest that the evolution of the BCG vaccine toward greater virulence might also explain...
the decline in its effectiveness against pulmonary tuberculosis.

Decisions made in accordance with the overattenuation model of BCG evolution have repeatedly failed to improve protection against pulmonary tuberculosis, most notably in the Chingleput trial [23] but also in subsequent studies in which Tokyo 172 was replaced with a more virulent vaccine [25–27]. These failures now make sense in the context of a new model of BCG evolution in which overattenuation never occurred. Instead, BCG evolved to make more antioxidants, possibly to fulfill a physiologic need involving the mycobacterial cell wall [12]. By chance, this coincided with a mycobacterial immune evasion strategy and made BCG better at suppressing host immunity, particularly CD8+ T cell responses. Although gene duplication and presumably other more subtle mutations underlie most of the increased expression of antioxidants in current BCG vaccines, it is noteworthy that a genomic deletion also contributed. The increased expression of Rv1774, an oxidoreductase, involves the loss of its repressor within RD14 [42].

The mechanistic and clinical implications of BCG evolving to become more virulent by producing more antioxidants are enormous. First, rather than not surviving long enough to induce immunity, the extensively cultivated BCG daughter strains actively suppress CD8+ T cell responses and immune memory. Because interferon-γ production by CD4+ T cells and macrophage responses are less affected than CD8+ T cell responses by microbial antioxidants [12], when subsequently infected with M. tuberculosis, a BCG-vaccinated host still compartmentalizes infection within granulomata and thus is protected against disseminated tuberculosis. However, weak CD8+ T cell responses limit the host’s ability to kill infected macrophages and resolve granulomatous foci of infection. An increasing body of evidence suggests that CD8+ T cells may help protect against pulmonary tuberculosis [43, 44]. In effect, as BCG evolved it became better at suppressing immune responses needed for protection against pulmonary tuberculosis while largely retaining responses that protect against disseminated tuberculosis in early childhood.

Second, because of divergent evolution the immune-suppressive capacity of each BCG strain differs yet should correlate roughly with the number of passages before the seed lot was preserved. This claim is supported by the greater immunogenicity, including CD8+ T cell responses, of BCG Tokyo 172 than Danish 1331 [45].

Third, the virulent and immune suppressive vaccines are more effective against disseminated tuberculosis in early childhood, possibly because they survive longer in vivo. Vaccine persistence also correlates with protection in mice [19, 29], yet if antibiotic treatment is used to clear BCG Danish 1331 from mice, immune responses and protection against dissemination decrease to low levels within months [46]. In effect, vaccines that induce the greatest protection against pulmonary tuberculosis may be less effective against disseminated disease in early childhood because they induce immunity that clears the vaccine strain from the host [12].

Fourth, involvement of oxidant-dependent immune responses in protection against pulmonary tuberculosis (Figures 2 and 3) makes sense in the context of risk factors for pulmonary tuberculosis in humans. For example, persons with chronic granulomatous disease, a genetic disease in which nicotinamide adenine dinucleotide phosphate oxidase fails to assemble to produce superoxide, frequently develop pulmonary tuberculosis and occasionally develop disseminated tuberculosis or BCG-osis [48, 49]. In mice, p47phox deficiency causes a survival defect in CD8+ T cells that is partially corrected by adding oxidants [50]. Less severe defects in oxidant-generating capacity, such as a weak oxidative burst [51] and/or low polymorphonuclear neutrophil counts [52], may underlie the high rate of pulmonary tuberculosis in some racial/ethnic groups. The role played by apoptosis-associated cross-priming in the induction of CD8+ T cell responses seems plausible in the context of the proapoptotic effects of oxidants and evidence indicating that oxidation of phosphatidylserine in membranes of apoptotic cells enhances the uptake of cell fragments by other phagocytes [53]. A role for antigen cross-presentation in humans is suggested by the observation that persons with latent tuberculosis (ie, positive for purified protein derivative) exhibit predominant macrophage apoptosis, whereas persons cured of active tuberculosis exhibit greater necrosis [54].

Fifth, a model in which the balance between host-generated oxidants and mycobacterial antioxidants affects CD8+ T cell responses also provides insight into the pathogenesis of tuberculosis. By allowing CD4+ T cell responses to develop and limit dissemination while suppressing CD8+ T cell responses, M. tuberculosis ensures disease transmission. Instead of dying quickly, most infected hosts with CD8+ responses inadequate to maintain latent tuberculosis will develop granulomatous lung cavities and infectious aerosols. A third of the world’s population is infected with M. tuberculosis, demonstrating the success of this pathogenesis strategy.

Sixth, the increased immune suppression model of BCG evolution predicts that the virulent BCG daughter strains are the least like the early BCG vaccines that exhibited 80% protection against pulmonary tuberculosis [1, 2]. In the new model, the virulent vaccines are not only expected to be ineffective against pulmonary tuberculosis, but their use increases adverse reactions unnecessarily. This contrasts with the overattenuation model, which predicts that “increased efficacy will be achieved only at the cost of increased adverse reactions” [36, p 134]. These predictions are timely in the context of reports that vaccination with BCG Danish 1331 causes disseminated BCG disease in almost 1% of human immunodeficiency virus–infected infants [55].

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Figure 3. Determination of the outcome of infection by the balance between host-generated oxidants and microbial antioxidants. In this model, 90% of humans generate enough oxidants during early infection to activate cross-priming pathways of antigen presentation (A). The cytotoxic T lymphocyte responses heal granulomatous foci of infection and provide ongoing immune surveillance to prevent the development of pulmonary tuberculosis (Figure 2). However, in 10% of persons the host-generated oxidants are insufficient to overcome suppression by antioxidants, resulting in weak CD8+ cell responses and eventually in the development of active tuberculosis. The goal of vaccination is to prevent active tuberculosis from developing in the 10% of persons so predisposed (B). The original bacille Calmette-Guérin (BCG) vaccine reduced pulmonary tuberculosis by 80%, which may reflect greater cross-priming compared with natural infection, perhaps from the loss of genes within region of difference 1 that suppress the production of oxidants by antigen-presenting cells [47]. Alternatively, by inoculating thousands of vaccine bacilli during vaccination, more polymorphonuclear cells with highly potent oxidant-generating capacity are activated than after inhalation of a few tubercle bacilli. Because the increase in antioxidants in BCG daughter strains is roughly proportional to the number of times they were passaged, vaccination effectiveness remained about +50% with Tokyo 172 yet decreased to as low as −38% with Pasteur 1173P2 [25–27]. The increased immune suppression model predicts that BCG can be modified to exhibit diminished activity of antioxidants, thereby inducing strong immunity that prevents pulmonary tuberculosis. In effect, by reducing BCG antioxidants the redox balance during early infection shifts, and cross-priming can now occur in some persons with low oxidant-generating capacity. This should enable them to develop immune responses more like those that develop in the 90% majority during natural infection. Then if infection occurs the vaccination-induced immune responses are recalled and help to prevent active tuberculosis. The development of memory immunity is probably the reason that protection was observed for 6 decades in recipients of early BCG vaccines [2]. TB, tuberculosis.
Seventh, if increased antioxidant production is the primary reason for the decline in the effectiveness of the BCG vaccine against pulmonary tuberculosis, then the simplest way to restore its effectiveness is to reduce BCG antioxidants (Figure 3). If the new model is correct, such modifications should enhance protection against pulmonary tuberculosis in humans. Table 1 and Figure 1 show some of the antioxidant genes that underwent duplication and increased expression as BCG evolved and thus represent high-priority targets. Reconstructing a BCG vaccine that exhibits protection comparable or superior to the early BCG vaccines should be easier to accomplish with Tokyo 172 than with virulent BCG substrains. It is noteworthy that prioritization of vaccines for human trials will need to be different from the algorithm used for the Chingleput study [22, 23]. Because the immune responses of mice and other small animals commonly used in vaccination-challenge experiments are inadequate to induce latent tuberculosis, more relevant models are needed. In the context of controlling tuberculosis globally, it is more important to target the pulmonary, contagious form of tuberculosis than disseminated tuberculosis. Thus, vaccines that induce strong immunity should be favored even if they are less effective against dissemination than virulent vaccines. Secondary immune responses in a memory-immune model [12] may be particularly useful in identifying vaccines likely to exhibit greater protection against pulmonary tuberculosis.

Finally, this new hypothesis does not mean that other hypotheses regarding the suboptimal effectiveness of the BCG vaccine against pulmonary tuberculosis do not also contribute. However, when it comes to understanding the effect of phenotypic differences among BCG substrains on protection against pulmonary tuberculosis, we failed to grasp a paradox and had it backward all along.

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