Antibodies against BCG and *M. tuberculosis* H37Ra do not Consistently Recognize Pathogenic *M. tuberculosis* whole Cells but Recognize their Cytoplasmic Constituents. Implications for the Variability and Protective Efficacy of the Vaccine

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Abstract

The production of antibodies by mycobacterial pathogens and BCG vaccine is still poorly understood. This study showed that antibodies raised against cells of TB strain H37Ra and against whole sonicates of BCG do not react consistently with whole cells of TB pathogenic strains and very poorly with whole BCG cells. These antibodies react with the internal components of the BCG cell, i.e. A60 and cytoplasmic constituents. It is concluded that the failure of antibodies raised against BCG to recognize some pathogenic strains permits the unrestricted entrance of these pathogenic bacteria in a vaccinated host and may explain the variable efficacy of this vaccine. The reactivity of these antibodies with internal components of the mycobacteria may impede the progress of the infection toward disease. These protective antibodies are not consistently produced in BCG vaccinees and may explain the excess TB and leprosy cases sometimes observed after vaccination.

Keywords: BCG; TB; Leprosy; Vaccine; Antibodies

Introduction

We develop a flow-through method for the detection of the tuberculosis pathogen to provide a sensitive and affordable detection method in regions where the prevalence of tuberculosis is high. It is based on the labeling of the bacilli with antibodies raised against the cells of a-virulent TB strain H37Ra. The method was tested in the field and gave equivocal results with sputum samples shown positive by AFB staining. The causative agent was found to be the specificity of the primary rabbit antibodies used to label the bacilli. This observation led to wider conclusions on the activity of BCG used as a vaccine.

The efficacy and innocuity of BCG have been disputed since 1927 [1] and its general failure is now accepted [2], with the reservation that the vaccine is not consistently inactive but varies in efficacy according to geographic location and disease characteristics [3]. The variable efficacy has remained without explanation until today [4]. The adverse side effects were credited to immuno-compromission of the host [5] unable to defend itself against the attenuated BCG vaccine. Our data, based on an analysis of humoral antibodies elicited by various mycobacterial antigens, shows that the phenotypic variation of the outer membrane of the pathogen is likely to be the culprit for the variable efficacy of the vaccine, but more than immune-compromising of the host is at play in the excess cases of mycobacterial infections sometimes encountered in vaccinees.

Materials and Methods

The steps of the flow-through method are: a liquid sample is filtered on a 0.45 µm nitrocellulose membrane (Advanced Microdevices, Ambala, Cannt, India). The bacteria present in the sample are retained at the surface of the membrane and are labeled with rabbit antibodies against whole mycobacterial cells. The presence of rabbit gammaglobulins is visualized on the membrane with gold-labeled protein A.

The main difficulty encountered in the development is the solubilisation of sputum.

**Bacilli filtered on the retaining membrane were:**

- Freeze-dried inactivated bacilli of *M. tuberculosis* strain H37Ra, from Difco (France).
- Pathogenic bacilli in the fresh sputum of TB positive patients from Tehran.
- Pathogenic bacilli isolated by centrifugation from the sputum of patients diagnosed in Paris.
- Live biomass of BCG bacilli, obtained from the Institut Pasteur du Brabant (PIB).
- Freeze-dried live BCG bacilli from Aventis-Pasteur Monovax lot W5586-2.
- Live cultured pathogenic bacilli from the sputum of patients diagnosed in Tehran.

**Other patient material originated from:**

- Fresh sputa of non-TB patients (courtesy Dr. Fraisse, CHU Hautepierre, France). One of these samples was determined by our method to be positive, after reception.
Frozen spuata of non-TB patients (courtesy of Dr. D. Banerjee, Saint George Hospital Medical School, London). Frozen spuata of non-TB and TB Brazilian patients, courtesy of Dr. M. Perkins (WHO, Geneva, Switzerland).

Fresh spuata, positive and negative, were analyzed on site at the Pasteur Institute of Iran, by the analysis of 9 culture-isolated strains working dilution of the primary rabbit antibodies to Neosystem were provided by the authors and consisted of 3.75 x 10^8 heat-inactivated pathogenic TB cells in 500 μl of physiological solution and of 450 μg M. tuberculosis H37Ra inactivated freeze-dried cells in 500 μl physiological solution. Both vaccines were homogenized with an equal amount of Freund's incomplete adjuvant and inoculated monthly.

The rabbits were precipitated with ammonium sulfate, dissolved in a tenth volume phosphate-buffered saline, dialyzed and clarified by low speed centrifugation. The working dilution of the primary rabbit antibodies was evaluated by the reactivity of different concentrations of these antibodies with whole cells of M. tuberculosis H37Ra spotted on the membrane. The rabbit antibodies suitably diluted in an isotonic medium containing 1% bovine albumin and 0.1% tween 20 at pH 7.2 were contacted (80 μl) with the sensitized membrane and the antibodies attached to the sensitizing antigens were revealed with 80 μl of a suitably diluted Protein A-Gold (40 nanometers) solution purchased from British Biocell (UK). The intensity of the red coloration, indicative of the presence of rabbit gamma-globulins on the membrane, was visually estimated with a gradation extending from - , i.e. absence of coloration, to +++++, indicative of intense coloration. The limit of sensitivity was signaled by "+/-".

We were evaluated the interaction of antibodies with bacilli of tuberculosis strain H37Ra, cultured pathogenic TB bacilli originating from the Pasteur Institute of Iran, live BCG from Aventis, cytoplasm and A60 of BCG. The results obtained with the other pathogenic bacilli were restricted to their interaction with antibodies against strain H37Ra and were used essentially to verify the phenotypic variation of pathogenic strains. These antibodies were not anymore available for additional studies with other antibodies.

**Results**

Rabbits produced abundantly antibodies against whole bacilli of the M. tuberculosis H37Ra strain and allowed the set-up of the flow-through test wherewith three antibodies and various antigens were tested. A number of spuata collected from TB patients living in different parts of the world were analyzed. The size of the samples was not in our power to define. The results of this analysis are given in Table 1.

No difference in response was seen, whether the spuata were used fresh or thawed. Antibodies raised against bacilli of strain H37Ra failed to recognize pathogenic strains in biologic samples in Bangalore, observed by Pr. Chandramucki who found no response when analyzing CSF samples from tuberculous meningitis patients. The absence of recognition of pathogenic strains and BCG by anti-whole cell H37Ra and their poor recognition by anti-sonicate BCG antibodies was confirmed by the analysis of 9 culture-isolated strains obtained from the Pasteur Institute of Iran, grown on Löwenstein medium and suspended in buffered physiological saline at one Mac Farland turbidity. No immunological reaction was observed with antibodies against H37Ra and only 3 samples were poorly recognized (score +++) with anti-BCG. The two BCG's were concentrated in saline to 50 bacteria per microscopic field for the evaluation of their immunological reactivity.

These results were at odds with previous observations of positivity of a smear-positive sputum sample obtained in Strasbourg, of 12
smear-positive sputa from Brazilian TB patients, and also in diverse samples analyzed in Lucknow. Interestingly, a fairly good response was obtained there in extra-pulmonary TB specimens but equivocal results with sputum, in that the positivity of sputa asserted by microscopic examination was sometimes but not always confirmed by the flow-through method.

The intensity of the coloration obtained with antiserum against A60 of BCG was significantly lower (score between + and +++) and significantly higher (score between ++ and ++++) than those obtained with the antibodies raised against TB strains, yet were not due to a difference in concentration of antibodies applied. The observed differences indicated a difference in specificity.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Antibodies against</th>
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<tr>
<td></td>
<td>H37Ra</td>
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<tr>
<td>H37Ra</td>
<td>++++</td>
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<tr>
<td>France 1 pat.</td>
<td>++++</td>
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<td>Brazil 12 pat.</td>
<td>+ to +++</td>
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<td>India extra pulm 30 pat.</td>
<td>+ to +++</td>
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<tr>
<td>India 50 pat.</td>
<td>- to +++</td>
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<tr>
<td>Iran 50 pat.</td>
<td>-</td>
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<tr>
<td>Iran# 1.5 x 10^6 bact. 9 pat.</td>
<td>-</td>
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<tr>
<td>France# 12 pat.</td>
<td>-</td>
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<tr>
<td>Pakistan 5 pat.</td>
<td>-</td>
</tr>
<tr>
<td>PIB 5.7 x 10^6 bact.</td>
<td>+/-</td>
</tr>
<tr>
<td>Aventis 5.7 x 10^6 bact.</td>
<td>+/-</td>
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<tr>
<td>A 60 5 micrograms</td>
<td>++++</td>
</tr>
<tr>
<td>Cytoplasm 5 micrograms</td>
<td>++++</td>
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*not done; #cultured samples, analyzed at a concentration of 1 McFarland in buffer; ##concentrated sediment suspended in buffer after decontamination.

Table 1: Results of flow-through tests on positive samples from various origins using 3 different detection antibodies.

We assessed the immunological reactivity of antibodies raised in rabbits against a heat-inactivated strain of pathogenic bacilli. The antibody recognized A60 (score: ++++) as well as whole cells of TB strain H37Ra (score: ++++), less efficiently the cells of its own immunizing strain (score: ++), and scarcely BCG (Aventis) (++/−). It recognized very efficiently (score: ++++) the cytoplasm of the lysed cells of the wild strain used for immunization. After centrifugation during 30 minutes at 16,000 rpm, the immunological activity of the lysed cells remained confined to the supernatant (+++++) with little immunological reactivity observable with the re-suspended sediment, containing the cells debris and cell membranes.

The immunological specificity of the cell membranes of the different mycobacterial entities here analyzed could also be a property of the A60 and the cytoplasm. This was suggested by the slightly reduced reactivity of antibodies against A60 extracted from BCG with H37Ra cells (Table 1). The large amount of A60 used as capture antigen (5 µg) had been applied to unequivocally establish the interaction of the antibodies with it but could have been too great to assess any difference in specificity of the different antibodies analyzed. To investigate this possibility, the cytoplasm of a wild Iranian strain of TB, used to raise antibodies, and the cytoplasm of BCG (PIB) were spotted on a membrane and the reactivity of antibodies against the wild strain, against H37Ra and against A60 from BCG (PIB) were evaluated in a flow-through system. The results are given in Table 2.

Table 2: Reactivity of 3 different antibodies with the cytoplasm of a wild pathogenic TB strain and the cytoplasm of BCG.

Discussion

The lack of sensitivity of the test is not due to an inactivation of one of its components, since we obtained good sensitivity with positive samples in some cases. The problem was traced to the primary rabbit antibodies used, that do not consistently recognize pathogenic strains. Important issues uncovered by this study are the antigenic variation of the cell surface of wild tuberculosis strains, the failure of antibodies to BCG to recognize whole BCG cells as efficaciously as their cytoplasmic constituents, the protective activity of the BCG vaccine and the study of the humoral response elicited by TB infection and BCG vaccination. These issues are developed hereunder.

Antibody production against mycobacterial antigens

The table reveals that the anti-M. tuberculosis H37Ra antibodies recognize very well bacilli of strain M. tuberculosis H37Ra, very poorly BCG bacilli, not at all the pathogenic M. tuberculosis strains originating from some cities, with equivocal results in Lucknow and good results elsewhere. Recognition of the inner components (cytoplasm and A60) of the BCG cell is noted.

BCG induced a higher level of anti-BCG antibodies in mice when the bacilli were grown in asparagine-containing medium, versus starch- and peptone-containing medium [7]. This effect of growth medium on immunogenicity cannot explain our own observations, since the failure of recognition was observed when TB-positive sputa were directly analyzed, when bacilli were isolated by centrifugation from sputum and re-suspended in saline and when bacilli cultured on Löwenstein medium were washed and suspended in isotonic saline, but was not observed when TB strain H37Ra was analyzed, either suspended in sputum or in saline. The condition of the cells, namely fresh, frozen or freeze-dried was not at cause either, because freeze-dried H37Ra cells were recognized by all 4 antibodies, freeze-dried BCG was not and neither were many positive fresh and frozen sputum samples.

As with antibodies directed against whole H37Ra cells, antibodies against A60 of BCG and against whole sonicates of BCG recognized poorly whole BCG cells (PIB and Aventis) and did not recognize cells of pathogenic strains (Iran and France) under the experimental conditions applied. By contrast, they recognized the cytoplasmic constituents of BCG (A60 and cytoplasm) and also H37Ra cells, which is puzzling. Antigen 60 was itself recognized by all four antibodies used in this study. One concludes that this antigen would be accessible late
during an infection [8], when host cells lyse. This observation confirms that A60 is mainly intracellularly located [9]. Yet, anti-A60 antibodies recognize whole cells of a-virulent tuberculosis strain H37Ra. The paradox is elucidated by assuming that the a-virulent tuberculosis strain H37Ra presents on its surface much more of the constituents of A60, which is a complex bearing at least 30 antigenic constituents presenting about 85 particular epitopes. This is contrary to all other strains tested here, where the presence of A60 constituents on the surface would be too low to be evidenced by our method of detection. The BCG cells bear more epitopes corresponding to A60 on its surface than do wild TB strains because A60 antibodies extracted from BCG recognize the cell surface of BCG cells and, in addition, their specificity is optimal. Noteworthy is that the stained cells of strain H37Ra appeared under the microscope as slightly bent rods, which set it completely apart from all other strains here, analyzed, be they pathogenic or attenuated, which appeared coccoidal. Noteworthy is the difference observed in the reactivity of the different antibodies with the cytoplasm. There remains no doubt that the antigens 60 originating from different mycobacterial species are immunologically distinct. This aspect of the humoral immunity of mycobacteria will be studied in more detail in the following communication.

It is possible to recognize a geographical pattern of sensitivity? Samples from Brazil are detected by anti-H37 RA antibodies while those from Karachi and Tehran are not detected by any of the applied antibodies. Both types seem to occur in India and France. The evidence is still scant but is an attractive hypothesis because it provides a simple explanation for the variable efficacy of the vaccine. We assume that the reasons for this variation are multiple and hard to pinpoint at this stage. One may advance the premature abandon of treatment by patients, which favors the growth of resistant strains that may present alterations of their cell surface. These alterations may vary according to the regimen applied [10]. Another source of variation that could be operative among the Hindu population of India is the consumption of raw milk from infected cows.

Heat-inactivated pathogenic tuberculosis cells elicit the formation of antibodies against their cell surface. This production is not massive (score ++), compared to the response observed simultaneously against their soluble cytoplasm (score ++++). The reactivity of these antibodies with A60 of BCG is low compared to that of antibodies raised against A60 of BCG. We suppose that the destruction of the cells by the host during the vaccination process was responsible for the antibodies produced against intracellular constituents, which explains why H37Ra cells were also detected. A weak humoral response against BCG cells was observed with this fourth antibody, whose intensity (+/-) was similar to the one observed with the three other antibodies.

We do not negate the impact of virulence factors on the infectiousness of various mycobacterial species and strains. We cannot, however, ignore the compelling evidence of the powerful humoral immune response M. tuberculosis strain H37Ra elicits against its own cell surface and cytoplasmic constituents and attribute a considerable part of the avirulence of this strain to this dual response. This basic observation allows an explanation for the variable efficacy of BCG.

The antibodies produced by BCG

The failure of rabbit antibodies to protect rabbits passively against an aerobic TB challenge [11], the direct relation between delayed hypersensitivity and the resistance to infection in BCG vaccinated mice [12], and the partial to total failure of recognition of BCG cells and TB pathogenic strains by rabbit antibodies against BCG sonicates, which our study confirms, has led to the generally accepted conclusion that antibodies play no role or only an insignificant role in the host's immune defense against mycobacterial infections [13-15]. Our results indicate that, on the contrary, it may play an essential role.

An experimental approach to the study of this role is limited by the life span and the susceptibility to infection of animal models and the inordinate use of massive amounts of non-viable antigens potentiated by adjuvants (in our case, Freund incomplete adjuvant) to raise antibodies. Under these experimental conditions all vaccinated rabbits in this study produced antibodies against the cytoplasmic constituents of BCG. The reproach one could make against our experimental design is the use of BCG sonicates instead of whole BCG cells, in that whole cells may produce more readily antibodies against whole BCG cells. However, three observations we made support the idea that BCG fails to produce antibodies against the cell surface of most pathogenic TB strains. The first observation is that H37Ra antibodies were obtained with whole cells and these cells were found wanting in their ability to elicit antibodies against whole pathogenic cells from Iran and against whole BCG cells (PIB) (Table 1). The second is that the antibody response elicited against H37Ra cells fits almost perfectly with the response obtained with BCG sonicates. The third is that antibodies against pathogenic cells (Iran) do not recognize BCG cells (Aventis: score +/- with the same efficacy than wild strains (score ++).

Further to that, studies in humans [16-19] show firstly that BCG does not induce IgG antibodies against A60 in some vaccinees, and secondly that some TB patients seeking a specific treatment fail to produce IgG antibodies against A60 [20]. Rota monitored the production of antibodies against A60 and PPD by BCG-vaccinated two months-old infants in Ankara during three years. Her results [16] are that the BCG given to human newborns was found effective in the formation of antibodies of the IgG class against A60, although only in less than 50% of the vaccinated population [2,8,17]. The conclusion is that BCG would likely not prevent the entry of TB in most vaccinees because the antibodies raised against BCG do not recognize whole cells of most pathogenic strains and, in newborns raised in Ankara, would prevent the progression of a TB infection toward disease in less than half of the vaccinees because the BCG failed to induce the synthesis of IgG antibodies against the internal components of the bacilli in the other half. Ours and other animal studies [6,18,19] show however that A60 is per se an excellent immunogen, which induces production of anti-A60 IgG's in all cases, recognizing the A60 of other mycobacterial species, albeit with less efficacy.

We establish a link between protection against progression toward disease and production of IgG antibodies against cytoplasmic constituents. This correlation is based on the absence or much reduced presence of IgG antibodies against A60 in some patients seeking specific treatment, with the subsequent rise of anti-A60 antibodies in successfully treated patients [20,21]. The production of antibodies by these patients correlates with the disappearance of positive cultures [20,22]. We have two possible explanations for this delayed rise of anti-A60 antibodies: firstly, the cytoplasm of bacteria killed by the treatment becomes exposed to the immune system, which would be enough to explain the rise. A second possibility, which we favor, is that live bacteria are immunosuppressive [23]; it is their inactivation that allows the synthesis of the A60-antibodies.

The immunosuppression by live mycobacteria of antibodies' production against mycobacterial cytoplasmic constituents is an...
attractive concept because it sheds light on the infection process by TB and BCG.

The potentiation of mycobacterial infections by the BCG

The failure of rabbit antibodies to protect rabbits passively against an aerobic TB challenge [11] is traced by us in part to the absence of antibodies against the cell surface antigens of the challenging bacilli. We think that an additional factor plays a considerable role: the enhancing effect of antiserum on infections was reported two years later in mice [24] and this paradox was verified again in 1991 [25]. Both *M. leprae* and BCG are known to induce the activity of suppressor cells [26,27]. These act by releasing soluble suppressors of IL-2 [27] in the blood [28]. IL-2 promotes the synthesis of IgG gamma-globulins. Partial to total immunosuppression by BCG leading to cancellation of the immunological recognition by the host of cytoplasmic constituents of the challenging pathogen is discernable in humans [16]. This would potentiate mycobacterial infections in vaccinated subjects. An analysis of the combined results of several studies in humans gives us some clues. The already mentioned enhanced production of IgG antibodies against A60 in successfully treated tuberculosis patients is indicative of an immunosuppression of humoral antibodies induced by live mycobacteria against cytoplasmic constituents. The failure of about 50% of infants, vaccinated at birth in Ankara, to produce IgG antibodies against A60 may be caused by BCG immunosuppression of the humoral system. Note that in Rota's study, about 80% of vaccinated infants had DTH positivity.

The results of vaccination campaigns and sensitins' reactivity studies in vaccinated humans support the claim that BCG, on some occasions favors the proliferation of TB and other mycobacterial species. Tuberculosis cases among vaccinees were reported during the vaccination campaign initiated in Djibouti and Madagascar in the 1950s [2]. As early as 1960, the promotion of mycobacterial infections by BCG, including tuberculosis and leprosy, in some populations of humans and animals, was published [29-32]. Vaccinated Swedish children monitored with three different sensitins showed a 16-fold increase of reactivity against tuberculin versus controls, from 3% to 49%. This considerable increase was attributed by the authors [33] to BCG but could just as well be non-apparent infections due to TB [8]. We defend this view firstly because it has been established that the reactivity to tuberculin in Italian vaccinated subjects is not due to BCG reaction (<5%) but to a *M. tuberculosis* infection [34], secondly because neonatal BCG vaccination had no marked impact on tuberculin skin test reactivity in Ugandan children exposed to infectious cases [35] and thirdly because there was a simultaneous 2 and 3 fold excess cases among the vaccinated Swedish children, of asymptomatic *M. scrofulaceum* (from 32% to 58%) and *M. avium* (from 25% to 67%). In healthy Swedish infants, the asymptomatic TB infections were confounded with BCG [33]. Unfortunately, the consequences of a vaccination are not always so benign and symptomatic cases may flare up. An excess of about 170% pulmonary tuberculosis symptomatic cases, restricted mainly to adults, was observed in South-India during the first five years that followed BCG vaccination [3,36]. A 9 fold excess of leprosy cases was observed in New Guinea, a TB-free zone, during the first five years following BCG vaccination and affected only vaccines less than 5 years old [37]. The vaccination of a South American Indian population, which was not TB naive since that disease was present on the continent before the arrival of the Europeans, but that was exposed to new pathogenic strains by contact with Europeans, had devastating consequences [38]. BCG-vaccinated Gambian patients with proven pulmonary tuberculosis were significantly younger than those not vaccinated and were less likely to have nutritional oedema. None of the BCG-vaccinated TB patients died whereas 17.5% of the non-vaccinated TB patients died. Survival was attributed to the vaccination [39]. This is not disputed: the evidence is that the BCG induced a TB infection at an earlier age in immunologically better protected subjects, which allowed their recovery, whereas starving non-vaccinated elderly subjects had lost adequate immune defenses and succumbed. In Tehran, 27% of 30 vaccinated children and 30% of 70 non-vaccinated children died from tuberculous meningitis. The observed difference in mortality is not significant but speaks in favor of the vaccine. The vaccine was of some benefit since the CNS complications were much lower in the vaccinated compared with the non –vaccinated surviving patients [40]. The evidence is that the protective activity of BCG is due to a generally benign primo-infection that potentiates infections with TB and other mycobacterial species leading in some distinct populations, exposed to a defined mycobacterial entity, to symptoms, during the first years following vaccination.

Conclusion

The immunization with a sonicate of BCG (Copenhagen) induces antibodies that recognize very well cytoplasmic components of BCG, one standard strain of *M. tuberculosis* (H37Ra) and a few wild strains but whole BCG cells strains Aventis and PIB are not so well recognized. In addition, the A60 of BCG elicits antibodies that are not totally specific for the homologous components of pathogenic bacilli.

Our data supports the hypothesis that BCG vaccination does not effectively protect against tuberculosis because it induces the production of antibodies preferably against antigens that are only exposed when cells are lysed and thus when the infection is well established. We therewidewith provide a simple explanation why BCG does not protect against infection but prevents dissemination from the primary foci to other parts of the body [41]. The absence of creation of antibodies against the outer cell membrane of wild strains also explains the long persistence of the pathogen in the invaded host. The data further indicates that geographical variations in exposed antigens among *M. tuberculosis* strains may be the cause of the discrepant results found when BCG efficacy is analyzed in different parts of the world.

To elucidate why BCG vaccination sometimes leads to disaster, one has to take into consideration two additional factors. First, the phenotypic variation of some BCG strains may impheach effective immunological recognition of some pathogenic TB strains. This will be analyzed in the following publication. The second factor is the partial to total induction of humoral immune anergy that suppresses the formation of protective IgG antibodies against the internal constituents of the bacilli and thereby promotes mycobacterial infections by the vaccine. Pathogenic TB bacilli very probably possess a similar factor of virulence.

Acknowledgments

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