Immune Responses Stimulated by Percutaneous and Intradermal Bacille Calmette-Guérin

E. B. Kemp, R. B. Belshe, and D. F. Hoft

Healthy volunteers were randomized to receive percutaneous or intradermal bacille Calmette-Guérin (BCG) vaccination. Delayed-type hypersensitivity (DTH) to tuberculin, as well as proliferative and interferon-γ responses in peripheral blood mononuclear cells stimulated by whole cell lysates and culture filtrates of Mycobacterium tuberculosis and Mycobacterium avium-intracellulare, were compared before and after vaccination. Positive DTH reactions were detected in 83% of intradermal and 40% of percutaneous BCG recipients 3 months after vaccination (P < .004). M. tuberculosis-specific proliferation was increased after intradermal BCG (P < .01) but not after percutaneous BCG compared with prevaccination responses. In addition, M. tuberculosis-specific interferon-γ production was increased after intradermal BCG compared with both prevaccination responses (P < .04) and those measured after percutaneous BCG (P < .05). Predominant immune responses stimulated by BCG were directed against antigens present in mycobacterial whole cell lysate. These results indicate that the BCG vaccination route can affect both in vivo and in vitro immune responses.

Bacille Calmette-Guérin (BCG), an attenuated strain of Mycobacterium bovis, is the only vaccine available for the prevention of disease related to mycobacterial infection. Although originally given orally when used against human tuberculosis (TB) in 1921, percutaneous and intradermal methods of BCG administration have generally replaced oral vaccination [1, 2]. Cutaneous vaccination methods have largely supplanted oral vaccination for various reasons, including a lower dose requirement for the induction of positive delayed-type hypersensitivity (DTII) to intradermal tuberculin purified protein derivative (PPD), suitability for vaccination of infants, cost, and reactogenicity profiles [1, 3, 4]. Percutaneous administration with a multiple-puncture device is generally associated with lower complication rates, but intradermal injection using a needle and syringe is the most precise method of controlling the delivered dose and therefore may more consistently induce mycobacteria-specific immunity [1, 2, 4]. In the United States, percutaneous administration is the only route currently licensed for use of BCG as a TB vaccine.

The relative efficacy of percutaneous and intradermal routes of BCG vaccination is not known. No conclusive trials have compared the ability of these different techniques of BCG administration to protect against mycobacterial disease [1]. A recent metaanalysis of previous BCG efficacy trials has indicated that BCG can prevent 50% of cases of pulmonary TB and 70% of TB-related deaths, but no conclusions could be made concerning the effect of vaccine route on protective immunity [5]. Historically, emphasis has been placed on DTH responses to tuberculin as a measure of immunity. Early clinical trials focused on determinations of the optimal doses of freshly cultured BCG that would induce high DTH conversion rates to tuberculin (>90%) without producing unacceptable adverse events [3]. Reports of similarly high conversion rates after vaccination with freeze-dried BCG preparations are documented, although some investigators have reported less successful rates, particularly with percutaneous BCG administration [3, 6, 7]. However, in both animal and human studies, tuberculin conversion has not been found to be a strong correlate of protective mycobacterial immunity [1, 8–12]. To better understand the nature of protective immunity induced by BCG vaccination, including the effects of different vaccine routes, more detailed investigations of immune responses other than DTII reactions must be done.

We conducted a comparative trial using commercially available freeze-dried BCG administered either percutaneously by multiple puncture technique or intradermally by needle and syringe. For both methods, we used standard doses of BCG currently recommended for use in the United States and worldwide. Our specific aim was to test the hypothesis that different
methods of BCG vaccination induce quantitative or qualitative differences in mycobacterial specific immunity.

Materials and Methods

**Human subjects and vaccination.** Healthy volunteers between the ages of 18 and 45 were recruited and considered for enrollment if they had a negative history for previous infection with *Mycobacterium tuberculosis*, no past exposure to an active TB case, and no previous positive PPD skin test. In addition, all enrolled subjects had negative human immunodeficiency virus serology, a negative reaction to a 5-TU PPD skin test, unremarkable complete blood cell count, and a normal serum alanine liver transaminase level. Sixty volunteers were enrolled and randomly assigned to receive either $1.1 \times 10^6$ cfu (0.3 mL) of Tice BCG (Organon Teknika, Rockville, MD) intradermally or $2 \times 10^8$ cfu (0.1 mL diluted) of Tice BCG intradermally in the deltoid area. The percutaneous and intradermal groups were well matched for age (mean ages, 37.5 and 34.1, respectively), sex (12 male/18 female vs. 11 male/19 female), and race (28 white/2 African-American vs. 27 white/3 African-American).

The percutaneous immunization was done by spreading 0.3 mL of the vaccine preparation on the surface of intact skin, followed by superficial perforation of the epidermis through the vaccine preparation with multiple tiny prongs to a depth of <2-3 mm. The majority of the vaccine suspension used for percutaneous vaccination was absorbed by sterile bandaging applied over the site after vaccination. The intradermal vaccination involved injection of the entire vaccine dose into the dermis within a sequestered interstitial location. Therefore, the inoculative dose of BCG was probably much smaller after percutaneous than after intradermal vaccination, despite the increased inoculum size used for percutaneous vaccination.

Volunteers were monitored for adverse reactions by examination and phone contact. In vivo responses, including reactions at the vaccination site and DTH response to a 10-TU PPD skin test 2 months after vaccination, were measured in the 59 persons who completed the study. Additionally, repeat PPD responses to a 10-TU skin test were measured 3 months after vaccination in 47 of the volunteers. Although the 5-TU PPD skin test has been standardized for use in evaluating persons for evidence of infection with *M. tuberculosis*, the 10-TU PPD skin test has been widely used to evaluate the immunogenicity of commercially available BCG vaccine strains. Furthermore, measurements of PPD responses to the 10-TU test are approved by the US Food and Drug Administration for potency testing of each manufactured lot of BCG vaccine.

**Antigens.** *M. tuberculosis* (Erdman strain) and *Mycobacterium avium-intracellulare* (MAI) were grown to mid-log phase in glycerol-alanine salts broth. For whole cell lysate preparation, mycobacterial pellets were heat-killed for 1 h at 80°C, disrupted by sonication or bead vortex, and passed through 0.2-μm filters before protein quantification (BCA protein assay kit; Pierce, Rockford, IL). Culture filtrates, representative of actively secreted mycobacterial proteins, were prepared from mid-log culture supernatants passed through 0.2-μm filters and then concentrated and dialyzed using an Amicon 10 diaphragm (WR Grace, Danvers, MA). *M. tuberculosis* whole cell lysate and culture filtrate were provided by J. Belisle (Colorado State University; under terms of NIH contract AI-25147). The MAI whole cell lysate and culture filtrate were prepared at Saint Louis University. Preservative-free PPD for in vitro studies was provided by Connaught Laboratories (Swiftwater, PA).

**Lymphoproliferative responses.** Heparinized blood was collected on the day of vaccination and 2 months after vaccination from 10 volunteers selected randomly from each group. Cell suspensions of human peripheral blood mononuclear cells (PBMC) were prepared by Histopaque separation (Sigma, St. Louis, MO) and diluted in RPMI 1640 supplemented with glutamine, penicillin, streptomycin, β-mercaptoethanol, and 10% human AB serum before adding $10^6$ PBMC/100 μL of media to individual wells of 96-well flat-bottomed plates. Mycobacterial antigens or media were added and cells incubated for 7 days at 37°C with 5% CO₂. During the last 12-16 h of incubation, cell cultures were pulsed with 0.5 μCi of [³H]thymidine before harvesting cells onto filter mats (Skatron, Sterling, VA) and counting the incorporated radioactivity. On the basis of our previous studies that optimized the dose responses (unpublished data), 2-μg/mL and 10-μg/mL quantities of mycobacterial protein were selected for stimulating cultures of PBMC.

**Cytokine induction assays.** The same volunteers examined for mycobacteria-specific T cell proliferative responses were studied for T cell cytokine responses. PBMC were incubated in 24-well tissue culture plates (4 × 10⁶ cells in 1 mL/well) in the presence of medium alone or mycobacterial lysates for 5 days at 37°C with 5% CO₂. The supernatants were collected and stored at -70°C. Interferon (IFN)-γ and interleukin (IL)-4 levels were measured by ELISA. Immulon II microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated overnight at 4°C with mouse monoclonal anti-human IFN-γ (MAb 400-45/6; Chemicon, Temecula, CA) or anti-human IL-4 (1842-01; Genzyme, Cambridge, MA) in 0.1 M carbonate buffer, washed with PBS-Tween 20 (0.05%), blocked with PBS with 10% fetal calf serum, and washed again before experimental culture supernatants were added. After 3 h of incubation at 37°C, wells were washed again and bound cytokine was detected with polyclonal rabbit anti-human IFN-γ (Endogen, Cambridge, MA) or polyclonal sheep anti-human IL-4 (Genzyme). Finally, donkey anti-rabbit IgG (Jackson Immunologicals, West Grove, PA) or donkey anti-sheep IgG (Genzyme) conjugated to horseradish peroxidase, followed by ABTS substrate (Kirkegaard & Perry, Gaithersburg, MD), were added to complete the assays. Recombinant human IFN-γ (Pharmlingen, San Diego) and human IL-4 (Genzyme) were used as standards to determine cytokine concentration. The specificities of these cytokine-specific antibodies have been evaluated by the manufacturers and confirmed by us. The sensitivities of the IFN-γ and IL-4 assays were 1 ng/mL and 50 pg/mL, respectively, in our studies.

**Statistical analyses.** Nonparametric two-tailed Fisher's exact tests were used to compare the proportions of volunteers developing positive PPD responses and ulcer formation. Mean values of PPD size, duration of ulcer drainage, duration of erythema, and duration of tenderness at the vaccination site were compared between groups after vaccination with Mann-Whitney U tests reporting the two-tailed P values. Proliferative and cytokine responses were subjected to Wilcoxon matched pairs tests (for comparisons before and after vaccination within a group) or Mann-Whitney U tests (for comparisons between the percutaneous and
Table 1. Mean duration (weeks) of clinical reactogenicity after percutaneous and intradermal BCG vaccination.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Percutaneous</th>
<th>Intradermal</th>
<th>P*</th>
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<tbody>
<tr>
<td>Erythema</td>
<td>1.8 ± 0.2 (14/30)</td>
<td>2.8 ± 0.2 (28/29)</td>
<td>.11</td>
</tr>
<tr>
<td>Soreness or tenderness</td>
<td>1.4 ± 0.3 (5/30)</td>
<td>4.0 ± 0.3 (26/29)</td>
<td>&lt;.003</td>
</tr>
<tr>
<td>Ulcer drainage</td>
<td>1.2 ± 0.2 (5/30)</td>
<td>4.1 ± 0.4 (24/29)</td>
<td>&lt;.002</td>
</tr>
</tbody>
</table>

NOTE. Data in parentheses are no. of subjects with reaction/no. vaccinated.
* Mann-Whitney U test; two-tailed P values are shown.

Results

Reactogenicity. Percutaneous BCG vaccination (by the multiple puncture technique) was associated with a lower reactogenicity profile compared with intradermal vaccination (table 1). Durations of erythema, soreness or tenderness, and drainage at the site of vaccination were significantly shorter in the percutaneous group. In addition, the incidence of ulcer formation was much lower in the percutaneous group and resolved more rapidly. Only 5 of 30 volunteers vaccinated percutaneously noted drainage, while 24 of 29 intradermally vaccinated volunteers developed draining ulcers at the vaccination site (P < .001 by two-tailed Fisher's exact test), with a typical onset 4–5 days after vaccination and a mean duration of 4.1 weeks. Although there were significant differences between groups in terms of clinical adverse events, none of the volunteers lost working days or required specific treatment related to vaccination.

PPD responses. Mean size of induration of the DTH reaction to PPD was significantly larger for the intradermal BCG group than for the percutaneous BCG group at both 2 and 3 months after vaccination (table 2), although the range of responses varied from 0 to ≥30 mm of induration in each group. In addition, the proportions of volunteers converting to a positive PPD status, defined as ≥5 mm of induration 48 h after intradermal placement of 10 TU of tuberculin PPD, were greater in the intradermal group (table 2). A positive PPD response occurred in 76% of intradermal vaccinees (22/29), while 50% of the 30 percutaneous vaccinees converted to a positive PPD status at 2 months after vaccination (P = .06). A repeat PPD test with 10 TU of tuberculin at 3 months was done in 47 of the volunteers. The positive conversion rate increased to 83% (19/23) for intradermal vaccinees but decreased to 40% (10/25) for percutaneous vaccinees (P < .004).

Lymphoproliferative responses. To assess the relative levels of antigen-specific cellular immune memory induced by percutaneous and intradermal BCG vaccination, we measured the in vitro lymphoproliferative responses in PBMC stimulated by different mycobacterial lysates before and after vaccination (figure 1). Postvaccination lymphoproliferative responses to mycobacterial antigens were not significantly increased in the percutaneous BCG group compared with prevaccination responses. In contrast, significantly increased lymphoproliferative responses after vaccination were detected in the intradermal BCG group after in vitro stimulation with both doses of M. tuberculosis whole cell lysate and 10 μg/mL M. tuberculosis culture filtrate were significantly greater in the intradermal group than in the percutaneous group on day 56 after vaccination (P < .05, Mann-Whitney U tests). Lymphoproliferative responses stimulated by MA1 antigens were not increased in either group after BCG vaccination (figure 1). Mycobacterial whole cell lysates stimulated higher absolute responses than mycobacterial culture filtrates both before and after vaccination. The highest absolute levels of lymphoproliferative responses, as well as the most significant up-regulation of M. tuberculosis-specific responses, were detected when PBMC from intradermally vaccinated volunteers were stimulated with whole cell lysates of M. tuberculosis (figure 1B).

T cell cytokine responses. To measure immune responses representative of different subsets of CD4 lymphocytes, we did cytokine-specific ELISAs with PBMC stimulated in vitro with mycobacterial lysates. We studied IFN-γ and IL-4 responses before and after vaccination as representative cytokines produced by CD4 Th1 and Th2 lymphocytes, respectively. IFN-γ responses were not significantly increased after vaccination in the percutaneous BCG group after in vitro stimulation with any of the mycobacterial lysates tested (figure 2A). In addition, the levels of IFN-γ induced in PBMC before and after vaccination in the intradermal BCG group were similar after in vitro stimulation in the in vitro lymphoproliferative responses in PBMC stimulated by different mycobacterial lysates before and after vaccination (figure 1).

Table 2. Delayed-type hypersensitivity responses to tuberculin after percutaneous and intradermal BCG vaccination.

<table>
<thead>
<tr>
<th>Time point, response</th>
<th>Route of vaccination</th>
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<tbody>
<tr>
<td></td>
<td>Percutaneous</td>
</tr>
<tr>
<td>2 months</td>
<td></td>
</tr>
<tr>
<td>Induration*</td>
<td>7.0 (1.5)</td>
</tr>
<tr>
<td>Positivity†</td>
<td>15/30 (50)</td>
</tr>
<tr>
<td>3 months</td>
<td></td>
</tr>
<tr>
<td>Induration*</td>
<td>7.3 (1.9)</td>
</tr>
<tr>
<td>Positivity†</td>
<td>10/25 (40)</td>
</tr>
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* Mean ± SE in mm, 48–72 h after 10 TU of tuberculin. Groups were compared with Mann-Whitney U tests; two-tailed P values are shown.
† No. positive/total (%); positive response was defined as ≥5 mm of induration 48–72 h after 10 TU of tuberculin. Rates were compared by Fisher's exact tests; two-tailed P values are shown.
Figure 1. Mycobacterium-specific human lymphoproliferative responses are induced by intradermal but not percutaneous BCG vaccination. A, B, Proliferative responses in peripheral blood mononuclear cells after in vitro stimulation with 2 and 10 μg/mL of Mycobacterium tuberculosis whole cell lysate (Mtb WL) and Mycobacterium avium-intracellulare whole cell lysate (MAI WL); C, D, Proliferative responses stimulated by 2 and 10 μg/mL of M. tuberculosis culture filtrate (Mtb CF) and MAI culture filtrate (MAI CF). Data are means (n = 10 for each group) ± SEs for pre- (day 0) and postvaccination (day 56) responses. Significant differences between pre- and postvaccination responses (Wilcoxon matched pairs tests) are indicated.

stimulation with M. tuberculosis culture filtrate or MAI whole cell lysate (figure 2B). In contrast, the levels of IFN-γ induced by M. tuberculosis whole cell lysate were significantly greater in PBMC from intradermal vaccinees 2 months after vaccination compared with baseline responses (P < .04, Wilcoxon matched pairs test). Furthermore, the levels of IFN-γ stimulated by M. tuberculosis whole cell lysate were significantly greater in PBMC from intradermal BCG recipients than in PBMC from percutaneous BCG recipients on day 56 after vaccination (P < .05, Mann-Whitney U test). IL-4 responses were not detected in the culture supernatants of PBMC harvested from either vaccine group after stimulation with any of the mycobacterial lysates tested (data not shown).

In vivo and in vitro correlations. When we used Pearson product-moment correlation analyses with the data obtained from all 20 volunteers studied for in vivo and in vitro responses, significant correlations between reactogenicity, in vivo DTH responses, and in vitro immune responses were detected. The size (millimeters of induration) of the DTH response to PPD measured 3 months after vaccination correlated with the duration of ulcer drainage (R = .502; P = .04). The duration of soreness or tenderness at the vaccination site was correlated with both the up-regulation of in vitro proliferative responses after vaccination (R = .510; P = .022) and the up-regulation of in vitro IFN-γ production after vaccination (R = .606; P = .005) stimulated by the optimal dose (2 μg/mL) of M. tuberculosis whole cell lysate. Furthermore, in vivo responses to PPD (millimeters of induration) at 2 months after vaccination were correlated with both in vitro proliferative responses (R = .600; P = .005) and in vitro IFN-γ responses (R = .569; P = .009) stimulated by the optimal dose (2 μg/mL) of M. tuberculosis whole cell lysate 2 months after vaccination.
Discussion

Cellular immune responses are believed to be the most important protective responses that develop naturally during primary infection with *M. tuberculosis*. It is also reasonable to believe that cellular immunity is important after BCG vaccination in protecting humans against active TB, and work in animal models confirms this hypothesis [13, 14]. The detailed mechanisms responsible for the induction of this protective immunity are not known [1, 8], and there are no known clinical laboratory markers that can be used to accurately predict the protective efficacy of BCG vaccine strains. Analyses of the detailed immune subsets stimulated by BCG vaccination will be important for the rational design of more effective TB vaccines and for the most appropriate use of BCG as a vector for the construction of vaccines directed against other infectious pathogens.

The importance of cellular immunity in control of *M. tuberculosis* infection has led to the practice of monitoring the levels of DTH responses to PPD stimulated by BCG vaccination as a presumed measurement of vaccine efficacy. However, DTH responses measure only a subset of host cellular immune responses, and DTH responses to PPD do not closely correlate with BCG-induced protective immunity [1, 8]. In general, BCG-vaccinated animals that become PPD-positive have been observed to be better protected against virulent *M. tuberculosis* challenges than similarly vaccinated animals that do not develop detectable DTH to PPD [8, 9]. However, strong DTH responses have been stimulated in some trials of BCG vaccination without the induction of protective immunity [10]. Conversely, PPD-negative animals after BCG vaccination have been found to be protected from mortality after virulent *M. tuberculosis* challenge despite their negative DTH status [8]. In humans, although it has been reported that there is a reciprocal relationship between the extent of TB disease and the DTH reaction, large field trials have failed to show that PPD reactivity after BCG vaccination correlates with protection against TB [1, 8, 12].

Recent evidence has shown that two populations of CD4 T helper lymphocytes that produce distinct profiles of cytokines after antigenic stimulation can be differentially stimulated and can have opposing effects on resistance and susceptibility [15–17]. Th1 cells, which produce IFN-γ and IL-2 after antigenic stimulation, are increased in animals resistant to infection with various intracellular parasites. Th2 cells secrete IL-4, IL-5, IL-6, and IL-10 after antigenic stimulation and are preferentially expanded in mice with increased susceptibility to intracellular parasite infections. Furthermore, IFN-γ can increase the killing activity of murine macrophages for mycobacteria, although it has been difficult to demonstrate such killing activity in human macrophages [14, 18, 19]. Acquired immunity to TB appears to be more complex than exclusively Th1 or Th2 cytokine response profiles [20, 21], but it is reasonable to hypothesize that the induction of IFN-γ responses in Th1 cells is one important component of the protective immune responses induced by BCG against mycobacteria. Our results demonstrating that intradermal BCG vaccination induced a significantly increased Th1-like cytokine response to *M. tuberculosis* whole cell lysate antigens compared with percutaneous BCG vaccination suggest that intradermal BCG vaccination may induce higher levels of protective immunity.

Cellular immune responses induced by whole cell lysates of mycobacteria were the most consistent marker of a vaccine effect. Both T cell proliferative and cytokine responses induced by whole cell lysates were significantly increased after intradermal BCG vaccination. In contrast, only T cell proliferative responses induced by a single dose of *M. tuberculosis* culture filtrate were significantly increased after intradermal BCG vaccination. Recent work on mycobacterial immunity has focused
on antigens secreted by viable mycobacteria, using the proteins present in the culture filtrate or supernatant of mycobacterial cultures during an early logarithmic phase of growth as a source for these antigens [18, 22–28]. Major components of these secreted mycobacterial proteins have been shown to induce immune responses that protect animals from virulent *M. tuberculosis* challenge and are being developed as candidate subunit vaccines [27, 28]. Our results demonstrating that BCG vaccination induced only limited immunity directed against mycobacterial culture filtrate antigens suggest that BCG does not induce optimal immune responses against these important antigens. This may explain why BCG is only partially protective against tuberculosis. On the other hand, a recent metaanalysis concluded that BCG significantly reduces the risk of pulmonary TB by 50% and decreases TB-related deaths by 71% [5]. *M. tuberculosis* whole cell lysates may contain nonsecreted antigens not found in *M. tuberculosis* culture filtrates that are involved in mycobacterium-specific protective immunity.

In addition to the need to identify the subsets of cellular immunity and specific mycobacterial antigens important in the protective immunity induced by BCG vaccination, it will be important to consider the effects of different routes of BCG administration. Conclusive efficacy trials focusing on the role of vaccine route in humans have not been published. The induction of increased PPD reactions by intradermal BCG vaccination compared with percutaneous BCG vaccination has been documented [6, 7]. To our knowledge, our study is the first to report that in addition to increased DTH responses, both human lymphoproliferative and IFN-γ responses are greater in magnitude after intradermal than after percutaneous BCG vaccination. This was in spite of the use of 40–50 times more colony-forming units of the same lot of Tice BCG per volunteer for the percutaneous vaccination protocol than was used for the intradermal vaccination protocol. These results suggest that intradermal BCG vaccination may represent a more efficient method of inducing mycobacteria-specific cell-mediated immune responses.

The percutaneous dose of Tice BCG used in our trial is currently licensed by the US Food and Drug Administration. The intradermal dose used in our trial corresponds to a standard dose recommended by the World Health Organization for intradermal BCG vaccination. These doses for percutaneous and intradermal BCG vaccination were optimized in previous clinical trials based upon the highest doses that induced maximal PPD conversion rates with minimal reactogenicity. Although the amount of BCG required for percutaneous vaccination is ~50 times greater than the amount required for intradermal vaccination, the “effective” or “inoculative” dose is probably much smaller for percutaneous than for intradermal BCG vaccination. The vast majority of the percutaneous vaccine preparation never penetrates the stratum corneum and therefore cannot infect suitable host cells, replicate, or be involved in immune induction. Therefore, it is unlikely that the higher dose used for percutaneous vaccination could have induced high-dose tolerance as a potential cause for the lower levels of cell-mediated immune responses measured in our experiments.

Until more is known about how the specific subsets of immunity are related to protection, it is difficult to recommend one route of BCG vaccination over another. Our study supports others in noting a relatively high incidence of ulceration with drainage that may persist for several weeks after intradermal vaccination [29], although our volunteers considered these adverse reactions to be mild to moderate in severity and easily tolerated. The percutaneous route of BCG vaccination was associated with a much lower incidence of ulceration and drainage but was also less effective in the induction of immune responses. In addition, we detected significant correlations between reactogenicity, in vivo DTH responses, and in vitro immune responses in our volunteers. These results suggest that a higher level of in vivo vaccine replication is necessary to induce optimal immune responses, and it may be difficult to separate clinical reactogenicity from the stimulation of protective immunity by live BCG vaccination.

**References**

11. Workshop report: summary, conclusions, and recommendations from the

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