Lipoarabinomannan-Reactive Human Secretory Immunoglobulin A Responses Induced by Mucosal Bacille Calmette-Guérin Vaccination

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The ability of 17 recombinant mycobacterial proteins, native antigen 85 complex, lipoarabinomannan (LAM), and Mycobacterium tuberculosis lystate to detect antibody responses induced by bacille Calmette-Guérin (BCG) vaccination and active tuberculosis infection were studied in enzyme-linked immunosorbent assays. Only LAM-reactive serum immunoglobulin G responses were significantly increased in both BCG-vaccinated patients and patients with active tuberculosis (P < .05), and oral BCG vaccination also induced significant increases in LAM-reactive secretory immunoglobulin A (P < .05). LAM-reactive antibody assays can serve as markers of humoral and mucosal immunity in future trials of BCG and never attenuated mycobacterial vaccines.

Intradermal vaccination with bacille Calmette-Guérin (BCG) is currently used in many countries to prevent severe disease associated with Mycobacterium tuberculosis infection. However, widespread BCG use has not reduced the overall prevalence of M. tuberculosis infection worldwide [1], and more-effective tuberculosis (TB) vaccination strategies are needed. We have initiated a series of human trials designed to identify the partially protective immune responses induced by intradermal BCG vaccination and to investigate the possibility that changes in the method of BCG vaccination (e.g., route, dose, use of booster doses, and schedule of repeated vaccination) could result in enhanced efficacy. To optimize mucosal vaccine dosing, we need a relevant marker of vaccine-induced mucosal immunity that can be measured in human volunteers. Studies of secretory IgA are minimally invasive, and IgA responses generally are a good marker for induction of mucosal immunity, even if IgA may not be directly involved in protection. However, to our knowledge, there are no previous reports of human secretory IgA responses induced by BCG vaccination, and specific antigenic targets relevant for these measurements previously were unknown. We present comparisons of the ability of 20 different preparations of recombinant and native mycobacterial antigens to detect BCG-induced mucosal and systemic antibody responses.

Subjects and methods. Healthy individuals aged 15–50 years who had negative results of skin testing with purified protein derivative (PPD; 0 mm of induration) and of serological testing for human immunodeficiency virus (HIV) were enrolled in vaccine trials of intradermal and oral BCG vaccination [2]. Connaught strain BCG was administered intradermally (∼3 × 10^6 cfu) or orally (2 × 10^6 cfu ingested within 15 min of intake of 2 g of oral bicarbonate).

Serum was collected from coagulated blood. Feces (10 mg) were mixed with PBS (20 mL) overnight before collection of supernatants. Nasal washes were obtained by instilling 5 mL of PBS in each naris and collecting the exhaled fluid. Parotid secretions were collected with vacuum-cup devices [3]. Tears were induced with orange-peel mist and collected with disposable bulb syringes. Samples were clarified by centrifugation at 500 g and stored at −70°C before ELISA.

Serum samples also were collected from 10 patients who had recently begun therapy for active TB. Nine patients had sputum cultures that were positive for M. tuberculosis, and 1 patient had positive results of culture of a pleural biopsy specimen.

Recombinant MPT63, MPT64, KatG, MPT51, early secretory antigen 6 (ESAT-6), MTC28, 14-kDa protein, and 38-kDa protein were expressed in Escherichia coli and purified by fast protein liquid chromatography on a series of nickel, desalting, and anion-exchange columns at the Public Health Research Insti-
M. tuberculosis from the H37Ra and H37Rv strains of nickel-column chromatography at the Center for Biologics Evaluation and Research (Bethesda, MD) [4]. Lipooarabinomannan (LAM) was purified by high-performance liquid chromatography from the H37Ra and H37Rv strains of *M. tuberculosis* at the University of Iowa (Iowa City), as described elsewhere [5]. The native antigen 85 (Ag85) complex was purified from log-phase culture filtrates of the *M. tuberculosis* Erdman strain at Colorado State University (Fort Collins; National Institutes of Allergy and Infectious Diseases, National Institutes of Health, contract NO1 AI-75320). Whole lysates of *M. tuberculosis* Erdman strain were prepared from log-phase cultures grown in glycerol-alanine salts broth at Saint Louis University, as described elsewhere [6].

Immuno 2 plates (Dynatech Laboratories) were coated with recombinant proteins (1 µg/mL), Ag85 (5 µg/mL), or whole *M. tuberculosis* lysate (5 µg/mL) diluted in 0.1 M carbonate buffer (pH 9.0) and incubated overnight at 4°C. After blocking with 10% fetal calf serum, plates were washed, and samples (serum pools, individual serum samples, and mucosal specimens) were added to duplicate wells and incubated overnight at 4°C. Plates were washed and developed with goat anti–human IgG or goat anti–human IgA conjugated to horseradish peroxidase (Southern Biotechnology Associates) and then with ABTS substrate (Kirkegaard & Perry), and absorbance was read at 405 nm. Serum pools were added beginning at a dilution of 1:50 in PBS and further diluted 2-fold across plates. Individual serum and mucosal samples were used at dilutions of 1:50 and 1:100, respectively.

H37Ra LAM diluted in 100% ethanol (4 µg/mL) was added to Immuno 2 plates (100 µL/well). These plates were dried in a fume hood, washed with PBS and 0.05% Tween 20, blocked overnight at 4°C with 1% bovine serum albumin in PBS with 0.05% Tween 20, and developed as described above for protein ELISA.

Statistica (StatSoft) was used for data analyses. Pre- and postvaccination responses were compared using Wilcoxon matched-pairs tests. Groups were compared using Mann-Whitney U tests. Proportional responses were compared using Fisher’s exact 2-tailed tests. Repeated-measures analysis of variance was used to analyze LAM-reactive mucosal IgA after oral BCG vaccination.

**Results.** ELISAs with 20 different mycobacterial antigen preparations were completed using 3 serum pools prepared with individual serum samples from (1) 10 PPD-negative control subjects who were later vaccinated intradermally with BCG, (2) the same 10 individuals 2 months after vaccination with BCG, and (3) 10 patients with active TB disease (data not shown). ELISAs were repeated at least 3 times and yielded similar results. The pooled serum from BCG-vaccinated subjects was found to have increased antibody reactivity only with H37Ra LAM. The pooled serum from patients with TB had increased antibody titers specific for recombinant 38-kDa protein, native Ag85 complex, and H37Ra LAM. Smaller increases were seen in the pooled serum from patients with TB of antibody specific for the 14-kDa, 45/47, ESAT-6, KatG, MPT64, and MPT70 recombinant proteins.

Figure 1 presents ELISA data from assays measuring the reactivity of individual serum samples with antigens in which the largest differences among the 3 serum pools were detected. Serum samples diluted 1:50 from PPD-negative control subjects, BCG-vaccinated subjects, and patients with TB were incubated in ELISAs with H37Ra LAM, native Ag85 complex, 38-kDa recombinant protein, and whole *M. tuberculosis* lysate. The median values and ranges of individual responses to each of these antigens for all 3 groups of subjects are shown in figure 1.

LAM-reactive IgG responses were significantly increased among both BCG-vaccinated subjects (*P* < .01, by Wilcoxon matched-pairs test) and patients with TB (*P* < .01, by Mann-Whitney *U* test), compared with the PPD-negative control group. Ag85-specific IgG responses were significantly increased (*P* < .05, by Mann-Whitney *U* test) only among the patients with TB. High background reactivity to Ag85 was detected in some PPD-negative control subjects, including values as high as the highest responses in the group of patients with TB. Patients with TB had increased reactivity to the 38-kDa antigen, compared with the other 2 groups, although this increase did not achieve statistical significance. Median and maximum antibody reactions to *M. tuberculosis* whole lysate increased progressively from the PPD-negative control group to the BCG-vaccinated group to the TB group, but these differences were not statistically significant. Assays were repeated at least 3 times, with similar results. Further ELISAs were performed in which plates were coated with LAM purified from the *M. tuberculosis* H37Rv strain, which confirmed that BCG induced significantly increased levels of antibodies that were reactive with LAM expressed by a virulent *M. tuberculosis* strain (median OD, 0.68 for prevaccination serum samples and 1.09 for postvaccination samples; *P* < .02, by Wilcoxon matched-pairs test).

We analyzed the proportions of subjects with positive responses in each serum group for different ELISAs (“positive response” was defined as an OD ≥ 2 SD higher than the mean for PPD-negative control subjects). The proportion of subjects with positive responses for LAM-reactive IgG was significantly greater in the active TB group than in the PPD-negative control group (6 of 9 vs. 0 of 10 subjects, respectively; *P* < .01, by Fisher’s exact 2-tailed test). In addition, the proportion of subjects with positive responses for LAM-reactive IgG was significantly increased after BCG vaccination (5 of 10 subjects before vaccination vs. 0 of 10 after vaccination; *P* < .05, by Fisher’s
Figure 1. Individual serum IgG antibody responses. Comparison of lipoarabinomannan (LAM), antigen 85 (Ag85), 38-kDa protein, and whole Mycobacterium tuberculosis lysate (Mt) ELISA reactivity in individual serum samples harvested from subjects before vaccination with bacille Calmette-Guérin (BCG; n = 10) and after BCG vaccination (n = 10) and from patients with tuberculosis (TB; n = 9). Median values (open symbols) with quartile ranges are shown for each group. *P < .05, postvaccination results vs. results for matching purified protein derivative (PPD)–negative control subjects (Wilcoxon matched-pairs test). **P < .05, results from patients with TB vs. results from PPD-negative control subjects (Mann-Whitney U test). ***P < .05, results from patients with TB vs. both postvaccination results and results from PPD-negative control subjects (Mann-Whitney U test). 

Representative results from 5 similar experiments are shown. Max, maximum; min, minimum.

The proportion of subjects with positive LAM responses in the active TB group was not significantly different from that in the BCG-vaccination group (6 of 9 vs. 5 of 10, respectively; P = .7, by Fisher’s exact 2-tailed test). Few samples were positive for antibodies reactive with Ag85, 38-kDa protein, or M. tuberculosis lysate, with no significant differences between groups.

Mucosal specimens (fecal extracts, nasal washes, parotid secretions, and tears) harvested from 8 volunteers before and after 2 oral vaccinations with 2 × 10⁸ cfu of BCG administered at an interval of 6 months were studied in H37Ra LAM, Ag85, and M. tuberculosis lysate ELISAs. All assays were repeated at least once, with similar results. Total IgA levels present in tears and parotid secretions were 100–1000-fold greater than total IgA levels detected in fecal extracts and nasal washes (data not shown). Figure 2 demonstrates that levels of LAM-reactive secretory IgA antibodies were significantly increased in tears harvested 6 months after primary oral BCG vaccination and on days 7 and 42 after the second oral BCG vaccination (P < .05, by Wilcoxon matched-pairs test). In addition, on a repeated-measures 1-way analysis of variance, LAM-reactive IgA responses in tears were found to be significantly increased, compared with prevaccination responses, on day 42 after the second vaccination (P < .05, by Tukey’s honestly significant difference post hoc comparison). Similarly, LAM-reactive secretory IgA responses were significantly increased (P < .05, by Wilcoxon matched-pairs tests) in parotid secretions after oral BCG vaccination (data not shown). Significant increases in LAM-reactive IgA were not detected in fecal extracts or nasal washes after oral BCG vaccination, and no significant increases in Ag85 or M. tuberculosis whole lysate–specific IgA responses were detected in any mucosal specimens after BCG vaccination (data not shown).

Discussion. In our studies, LAMs purified from M. tuberculosis H37Ra and H37Rv strains proved to be the most useful antigens for detection of BCG effects on mucosal and systemic B cell responses. LAM-reactive antibodies in serum were significantly increased after BCG vaccination when individual samples obtained before and after vaccination were compared (figure 1). Furthermore, LAM-specific secretory IgA responses were significantly increased after oral BCG vaccination (figure 2). These results demonstrate that measurements of LAM-reactive secretory IgA can be used to detect mycobacteria-specific mucosal immune responses in future vaccine trials. No other antigen tested in our experiments was able to detect mycobacteria-specific antibody responses after BCG vaccination.

We initially thought that fecal samples would most likely contain detectable levels of mycobacteria-specific secretory IgA...
Figure 2. Lipoarabinomannan (LAM)–specific secretory IgA induced by bacille Calmette-Guérin (BCG) vaccination. Tears from volunteers who had received 2 oral vaccinations ($2 \times 10^6$ cfu of BCG) at an interval of 6 months were studied in LAM ELISAs. The optical density values detected in prevaccination samples from each volunteer were subtracted from postvaccination values to obtain the change in optical density results, which represents the increases in antibody reactivity induced by BCG vaccination. At week 24, a second oral BCG vaccination was administered to all of these volunteers. Median values (open symbols) with quartile ranges for the responses detected in all 8 individual volunteers are shown. * $P < .05$, postvaccination vs. prevaccination responses (Wilcoxon matched-pairs test). Representative results from 3 similar experiments are shown. Max, maximum; min, minimum.

It has been demonstrated elsewhere [7] that increased LAM-reactive antibody responses are present during active TB and lepromatous leprosy disease. Sada et al. [8] found that detection of LAM-reactive serum IgG by ELISA had a specificity of 91% and sensitivity of 72% for the diagnosis of active TB among Mexicans in a group with a high TB prevalence. In another study, the presence of mycobacteria-specific IgA in tears indicated that oral BCG vaccination induced mucosal immune responses capable of circulating to other mucosal tissues via the common mucosal immune network.

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In summary, we have studied a panel of mycobacterial antigens for the ability to detect antibody responses induced by BCG vaccination and/or active TB. Only the LAM ELISA could detect significant increases in serum IgG induced by either BCG vaccination or active TB, as well as increases in secretory IgA induced
by mucosal BCG vaccination. Measurements of LAM-specific antibody responses will be useful in future vaccine trials for detection of vaccine-induced humoral and mucosal immunity.

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References