Intranasal Boosting with an Adenovirus-Vectored Vaccine Markedly Enhances Protection by Parenteral Mycobacterium bovis BCG Immunization against Pulmonary Tuberculosis

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Parenterally administered Mycobacterium bovis BCG vaccine confers only limited immune protection from pulmonary tuberculosis in humans. There is a need for developing effective boosting vaccination strategies. We examined a heterologous prime-boost regimen utilizing BCG as a prime vaccine and our recently described adenoviral vector expressing Ag85A (AdAg85A) as a boost vaccine. Since we recently demonstrated that a single intranasal but not intramuscular immunization with AdAg85A was able to induce potent protection from pulmonary Mycobacterium tuberculosis challenge in a mouse model, we compared the protective effects of parenteral and mucosal booster immunizations following subcutaneous BCG priming. Protection by BCG prime immunization was not effectively boosted by subcutaneous BCG or intramuscular AdAg85A. In contrast, protection by BCG priming was remarkably boosted by intranasal AdAg85A. Such enhanced protection by intranasal AdAg85A was correlated to the numbers of gamma interferon-positive CD4 and CD8 T cells residing in the airway lumen of the lung. Our study demonstrates that intranasal administration of AdAg85A represents an effective way to boost immune protection by parenteral BCG vaccination.

Tuberculosis (TB) is the second leading cause of death by infectious disease worldwide. Mycobacterium bovis bacillus Calmette-Guérin (BCG) has been the vaccine of choice given to humans via the skin for over 80 years and has shown variable efficacies of between 0 and 80% in clinical studies (25). In spite of this fact, over 80% of the world population are BCG vaccinees; this vaccine is still being utilized and will likely continue to be utilized in the majority of the world as a part of childhood vaccination programs because of its efficacy in preventing adolescent and disseminated forms of TB, despite the fact that BCG has been shown to have variable efficacies in preventing adult pulmonary tuberculosis (1, 6). Its limited success in control of adult TB has been attributed at least in part to the fact that immune protection by BCG wanes within 10 to 15 years. Much of the past effort has focused on modifying the current BCG vaccine or identifying new vaccine platforms (11, 17, 22). However, these strategies are unlikely to accomplish long-term TB protection in humans. Furthermore, replacement of the currently used BCG with a different vaccine platform is increasingly considered an unrealistic goal because of the wide clinical usage of BCG and because any phase III clinical vaccine trial would not ethically be allowed to withhold BCG vaccination (12, 26). Therefore, the development of strategies that aim to boost BCG-mediated protection represents a priority (15). In this regard, BCG has been shown to be an ineffective booster vaccine for itself and may even be deleterious to protection against TB, as previously shown in clinical and experimental studies (2, 5, 8). Increasing evidence suggests that effective booster vaccines for BCG immunization ought to be nonmycobacterial (boosters heterologous to BCG) and may include those based on recombinant protein, plasmid DNA, and viral vector systems (3, 5, 7, 17, 21). These strategies focus on improvement of the current vaccination strategy without compromising the benefits conferred by BCG and thus may be the most feasible in the near future.

Because of their potent immunogenicity and natural tropism to the epithelium, viral vectors are the most promising anti-TB vaccine candidates (10, 14, 21). Indeed, the most effective vaccination strategy to date has been that of BCG immunization boosted by intranasal (i.n.) vaccinia virus expressing Ag85A in a mouse model, although clinically vaccinia-Ag85A was administered intradermally (7, 9, 10). This study demonstrated that vaccinia-Ag85A was able to be an effective booster when administered between 14 and 22 weeks after primary immunization. Nonetheless, vaccinia-Ag85A alone is not an effective vaccine in the absence of BCG to protect against Mycobacterium tuberculosis challenge (7).

Recently, our laboratory described a vaccine candidate, an adenoviral vector expressing the mycobacterial protein Ag85A (AdAg85A) (21), which when administered via the intranasal route was effective in providing superior protection compared to BCG in a mouse model. Recent studies from our laboratory showed that i.n. AdAg85A was able to provide protection because of the accumulation of CD4 and CD8 T cells in the airway lumen. An adoptive transfer model also showed that CD4 and CD8 T cells purified from the spleen of unprotected mice given AdAg85A intramuscularly, when transferred into the airway lumen, also provided protection against M. tuberculosis challenge (16). Based on these observations, we believe that although intranasal AdAg85A is an effective stand-alone vaccine in experimental systems, it may be best utilized as a potent booster to the current BCG vaccine.
In the current study, we investigated the boosting effect of AdAg85A on immune protection by subcutaneous BCG immunization. We demonstrate that intranasal AdAg85A potently enhances immune protection by BCG, and this protection is as stable as and better than that induced by BCG or AdAg85A alone. These findings justify further examination of AdAg85A as a booster vaccine for BCG in larger animals as well as potentially in humans.

MATERIALS AND METHODS

Mice. Six- to 10-week-old female BALB/c mice were purchased from Harlan Laboratories. All mice were housed in a specific-pathogen-free level B facility. All animal experiments were done in accordance with the guidelines of the animal ethics research board of McMaster University.

Immunizations. As shown in Fig. 1, Mycobacterium bovis BCG (Connaught strain) was utilized for the prime vaccination and prepared as described in the following section. Briefly, a total of 50,000 CFU of BCG was administered to two intracutaneously, 50 μl each, to the right and left hindquarter of BALB/c mice as previously described (20). The AdAg85A booster immunization was administered at a dose of 5 × 10⁶ PFU as depicted in Fig. 1 via either an intranasal or an intramuscular (i.m.) route as previously described (16). Briefly, anesthetized mice were either injected once in each quadriceps with 25 μl of phosphate-buffered saline (PBS) for a total of 50 μl containing 5 × 10⁶ PFU AdAg85A or allowed to slowly breathe in 25 μl of PBS containing 5 × 10⁶ PFU AdAg85A as previously described (17).

Mycobacterial challenge and colony-forming assay. Mycobacterium bovis BCG (Connaught strain) and Mycobacterium tuberculosis (H37Rv strain) (ATCC 27294) were grown in Middlebrook 7H9 broth supplemented with Middlebrook oleic acid-albumin-dextrose-catalase enrichment (Invitrogen Life Technologies, Gaithersburg, MD), 0.002% glycerol, and 0.05% Tween 80 for 70°C until needed. Before each use, BCG or M. tuberculosis bacilli were washed with PBS containing 0.05% Tween 80 twice and passed through a 27-gauge needle 10 times to disperse clumps. For the intranasal injection, each mouse was anesthetized with isoflurane and allowed to slowly breathe in 25 μl of PBS containing 5 × 10⁶ PFU AdAg85A as previously described (17).

Intranasal but not intramuscular AdAg85A boosting of BCG immunization leads to markedly enhanced and sustained protection against pulmonary M. tuberculosis challenge. To examine the effectiveness of AdAg85A as a potential booster vaccine for the current vaccine, BCG, we primed BALB/c mice with BCG subcutaneously and subsequently boosted with selected booster vaccine candidates via the skin or respiratory mucosal route (Fig. 1). Mice were then challenged with M. tuberculosis via the airway at the time depicted in Fig. 1. At 4 weeks postchallenge, we observed a 2-log reduction in the number of cultivatable bacilli in the lungs of mice receiving BCG immunization boosted by AdAg85A i.n. compared to naive mice (P < 0.001). This was by far the largest reduction and greater than that for the AdAg85A i.m. booster vaccination (P < 0.001), BCG vaccine alone (P < 0.05), or even i.n. AdAg85A alone (P < 0.05) (Fig. 2A). Although not as effective as mucosal AdAg85A boosting, i.m. AdAg85A boosting was more effective in reducing the number of cultivatable M. tuberculosis cells than BCG alone (P < 0.001) or i.m. AdAg85A alone (P < 0.01) (Fig. 2A). Our results also suggest that BCG is not an effective booster vaccine for BCG prime immunization (Fig. 2A), as shown in human clinical trials and experimental studies (2, 8). These reductions were also mirrored in the numbers of cultivatable bacilli observed in the spleen, in that BCG boosted by i.n. AdAg85A was the most effective in reducing the number of viable M. tuberculosis colonies (P <
0.05), although there is no statistically significant difference compared to i.n. AdAg85A alone (Fig. 2B).

We also examined the level of protection against pulmonary TB at a longer interval (8 weeks) after *M. tuberculosis* challenge to understand which booster vaccine could provide sustainable protection. Again, BCG failed to boost protection by BCG prime immunization both in the lung and in the spleen (Fig. 3). A relatively small but significant level of boosting effect by i.m. AdAg85A observed at 4 weeks (Fig. 2) had completely waned by 8 weeks after challenge, and the protection level was identical to that provided by BCG vaccine alone or the BCG/BCG regimen (Fig. 3). In sharp contrast, AdAg85A i.n. boosting continued to lead to a remarkable level of immune protection, a 2-log or a more than 2-log reduction of TB infection in the lung and spleen compared to naïve mice (Fig. 3) (*P < 0.001*) very similar to that observed at 4 weeks postchallenge. Of interest is that i.n. AdAg85A alone seemed to be less protective at 8 weeks than at 4 weeks postchallenge,
although it was still better than no immunization or BCG immunization ($P < 0.05$) (Fig. 3A). As an additional control, an empty adenoviral vector (Addl70.3) delivered via the intranasal or intramuscular route had little boosting effect on BCG prime immunization at both week 4 and week 8 postchallenge (data not shown), indicating that the boosting effect by AdAg85A is Ag85A specific. Together, the above results demonstrate that AdAg85A is an effective booster vaccine for BCG prime immunization and that the respiratory tract is by far the more effective route of boosting.

**Intranasal but not intramuscular AdAg85A boosting leads to potent primary CD4 and CD8 T-cell responses in the airway lumen.** To understand the immunogenicity, including airway luminal T-cell responses, induced by BCG-boosted AdAg85A regimens and to see if this correlated to protection, we examined the levels of IFN-γ⁺ CD4 and CD8 T cells and Ag85A

![Graph A](image)

**A**

Log₁₀ cfu/lung

![Graph B](image)

**B**

Log₁₀ cfu/spleen

**FIG. 3.** Immune protection by AdAg85A-boosted BCG immunization 8 weeks after *M. tuberculosis* challenge. BALB/c mice were immunized with BCG subcutaneously alone (BCG/None) or boosted 8 weeks later by AdAg85A given intranasally (BCG/AdAg85A IN) or intramuscularly (BCG/AdAg85A IM). Some mice received no immunization at all (Naïve) or were immunized only with AdAg85A i.m. or AdAg85A i.n. for 4 weeks. Mice were subsequently challenged with *M. tuberculosis* 4 weeks after boost vaccination. Mice were sacrificed 8 weeks later, and the lung (A) and spleen (B) were examined for the level of *M. tuberculosis* infection by colony enumeration assay. Data represent the means ± SEM of results from eight mice/group. *, $P < 0.05$ (versus naïve); **, $P < 0.005$ (versus naïve); ***, $P < 0.001$ (versus all other groups).
tetramer-positive CD8 T cells by ICCS and tetramer staining techniques, respectively. To this end, BALB/c mice were primed with BCG; 8 weeks later they were boosted by AdAg85A i.m., AdAg85A i.n., or nothing at all; and 4 weeks following booster immunization (Fig. 1) cells from the airway lumen as well as two systemic organs, the lung and spleen, were obtained and analyzed as described above. T cells were stimulated with either CD4 or CD8 T-cell epitopes of the Ag85A protein, and these cells were also stimulated with whole M. tuberculosis protein antigens (Ag85 complex plus M. tuberculosis culture filtrate [CF] proteins) to determine the overall T-cell responses of multiple antigen specificities following boosting immunization. While we observed increased numbers of antigen-specific IFN-γ CD4 and CD8 T cells in the airway lumen of mice that were boosted by AdAg85A i.n., no or few such T cells were found in the airway lumen of groups given BCG alone or BCG boosted with AdAg85A i.m. (P < 0.001) (Fig. 4A and B). Since Ag85A tetramer immunostaining techniques allow the detection of Ag85A peptide-specific CD8 T cells regardless of whether they produce IFN-γ, we also examined Ag85A tetramer-positive CD8 T cells in the airway lumen. A remarkably increased number of Ag85A tetramer-positive CD8 T cells was detected in the lung of only mice given BCG boosted with AdAg85A i.n. (P < 0.001) (Fig. 4C). Our tet-

**FIG. 4.** Primary Ag85A-specific T-cell responses in the airway lumen. BALB/c mice were immunized with BCG subcutaneously alone (BCG) or boosted 8 weeks later by AdAg85A given intranasally (BCG/AdAg85A IN) or intramuscularly (BCG/AdAg85A IM). Mice were sacrificed 4 weeks after booster vaccination. The frequency of IFN-γ+ CD8 and CD4 T cells was determined by stimulating airway luminal cells (bronchoalveolar lavage [BAL]) for 5 h in the presence of Ag85A CD8 (A) or CD4 (B) T-cell-specific peptides (black bars) or M. tuberculosis CF-Ag85 complex protein (gray bars). Cells were stained as described in Materials and Methods. Ag85A tetramer-positive CD8 T cells (C) were analyzed in the airway luminal cells as described in Materials and Methods. Data represent the means ± SEM of results from three mice/group and are representative of two independent experiments. The inset fluorescence-activated cell sorter dot plot panels depict the proportion of cells positive for both Ag85A tetramer and CD8 among all airway luminal cells. *** P < 0.001 (versus the BCG or BCG/AdAg85A IM group).
ramer results establish that the intracellular IFN-γ assay underestimates the magnitude of antigen-specific CD8 T-cell responses, but the trend within the airway lumen was identical. Thus, clearly the high level of Ag85A-specific T-cell responses in the airway lumen correlates well with markedly enhanced levels of immune protection conferred by AdAg85A i.n.-boosted BCG prime immunization, lending further support to our previous conviction of the importance of airway luminal T cells in anti-TB immunity (14, 16).

In the lung interstitium, we observed similar numbers of IFN-γ+ CD4 T cells in both groups given BCG boosted with AdAg85A i.m. and groups given BCG boosted with AdAg85A i.n. (Fig. 5B). Interestingly, slightly more antigen-specific IFN-γ+ CD8 T cells were observed in the lung tissue of the group given BCG boosted with AdAg85A i.m. than in the group given BCG boosted with AdAg85A i.n. or BCG alone (P < 0.05) (Fig. 5A). This observation by ICCS, however, does not entirely agree with that by Ag85A tetramer CD8 T-cell staining, as we found more tetramer-positive CD8 T cells in the lung interstitium of mice given BCG boosted with AdAg85A i.n. than those given BCG boosted with AdAg85A i.m. (P < 0.005) (Fig. 5A and C, respectively).
The T-cell activation profile in the spleen is in agreement with our previous understanding (16) and indicates that parenteral AdAg85A boost immunization leads to far better antigen-specific CD4 and CD8 T-cell responses in systemic lymphoid organs (spleen) than the mucosal boost route (Fig. 6). Likewise, BCG alone was able to induce potent antigen-specific CD4 and CD8 T-cell responses in the spleen as well as the lung interstitium (Fig. 5 and 6). Together, the above results suggest that the level of airway luminal CD4 and CD8 T-cell responses, but not that in the systemic tissue compartments, correlates to the potent protection triggered by genetic vaccination against pulmonary M. tuberculosis challenge.

FIG. 6. Primary Ag85A-specific T-cell responses in the spleen. BALB/c mice were immunized with BCG subcutaneously alone (BCG) or boosted 8 weeks later by AdAg85A given intranasally (BCG/AdAg85A IN) or intramuscularly (BCG/AdAg85A IM). Mice were sacrificed 4 weeks after booster vaccination. The frequency of IFN-γ+ CD8 and CD4 T cells was determined by stimulating splenocytes for 5 h in the presence of Ag85A CD8 (A) or CD4 (B) T-cell-specific peptides (black bars) or M. tuberculosis CF-Ag85 complex protein (gray bars). Cells were stained as described in Materials and Methods. Ag85A tetramer-positive CD8 T cells (C) were analyzed in the splenocytes as described in Materials and Methods. Data represent the means ± SEM of results from three mice/group and are representative of two independent experiments. For panels A and B: *, P < 0.05 (versus BCG/AdAg85A IN). For panel C: *, P < 0.05 (versus BCG and BCG/AdAg85A IN). ***, P < 0.001 (versus BCG and BCG/AdAg85A IN).
Intranasal AdAg85A boosting induces potent secondary T-cell responses in the airway lumen after mycobacterial challenge. Having observed higher levels of primary T-cell responses in the airway lumen of mice following BCG prime and AdAg85A i.n. boost immunization, we also examined the secondary T-cell responses in the lung in these mice upon pulmonary mycobacterial exposure. To this end, we primed BALB/c mice with BCG; 8 weeks later we boosted these mice by AdAg85A i.m., AdAg85A i.n., or nothing; and 4 weeks later we subsequently challenged these mice intratracheally with a higher dose of BCG and examined the secondary T-cell responses in the lung. Again, compared to mice given BCG or BCG boosted with AdAg85A i.m., the greatest number of Ag85A tetramer-positive CD8 T cells was found in the airway lumen (Fig. 7A) and lung interstitium (Fig. 7B) of mice given BCG boosted with AdAg85A i.n. The numbers of IFN-γ+ CD4 and CD8+ T cells were also greater (data not shown). As such levels of T-cell responses to mycobacterial challenge in both airway lumen and lung interstitium were much greater (Fig. 7) than the primary T-cell responses in unchallenged immunized mice (Fig. 4C and 5C), these represent a true secondary T-cell recall response. The numbers of tetramer-positive CD8 T cells in the spleen were found to be similar between various groups (data not shown). The above data together indicate that AdAg5A intranasally boosted BCG immunization leads to the strongest primary and secondary T-cell responses in the airway lumen, which correlate well to robust protection from pulmonary M. tuberculosis challenge conferred by this BCG prime-AdAg85A boost regimen.

**DISCUSSION**

The replacement of BCG with a brand-new platform may not be a realistic goal; however, a vaccine that can work as an effective booster as well as a stand-alone vaccine may be ideal because it will be most effective regardless of current vaccination status (26). Previously, we demonstrated that AdAg85A when delivered as a single intranasal inoculum was a more potent vaccine than BCG in a mouse model of pulmonary M. tuberculosis infection (21). Furthermore, we demonstrated that its protection was dependent on CD4 and CD8 T cells present in the airway lumen (16). Here we show that intranasal administration of AdAg85A works as a potent booster for BCG prime immunization, resulting in enhanced sustainable protection against pulmonary M. tuberculosis challenge in a mouse model. And such markedly enhanced protection is correlated with increased numbers of antigen-specific CD4 and CD8 T cells present in the airway lumen prior to and after mycobacterial challenge.

Compared with respiratory mucosal (i.n.) AdAg85A boosting, protection was moderately enhanced by intramuscular boosting only at 4 weeks after M. tuberculosis challenge. This suggests that, similar to the situation where AdAg85A was used as a stand-alone vaccine (16, 21), respiratory mucosal AdAg85A boosting of BCG immunization is a far more effective strategy than parenteral boosting. This dichotomy of parenteral immunization not being protective and mucosal immunization being protective has been demonstrated by us and others. For example, recombinant virus-vectorized TB vaccines, including AdAg85A and MVAAg85A, when used alone or as a boost vaccine, were not effective unless they were given intranasally (7, 16, 21). In our current study, we demonstrate that intranasal or intramuscular administration of empty adenoviral vector (Addl70.3) is unable to boost the protection achieved by BCG, suggesting that the boosted protection by AdAg85A works in an Ag85A-specific manner. Furthermore, we also provide the data in our current study that contrary to...
a remarkable boosting effect by AdAg85A, BCG itself completely fails to boost protection by BCG prime immunization. This finding is in agreement with previous clinical and experimental findings (2, 5, 8) and supports the need for developing non-mycobacterium-based boosting strategies for BCG prime immunization (26).

Similar to our previous results (16), we found that only AdAg85A i.n. boosting could induce large numbers of IFN-γ+ CD4 and CD8 T cells in the airway lumen. These increases also correlated to Ag85A peptide-specific tetramer-positive CD8 T cells in the airway lumen of mice given BCG boosted with AdAg85A i.n. These results demonstrate that total numbers of peptide-specific CD8 T cells are underestimated by ICCS alone, and the combination of both techniques allows a more thorough assessment of T cells in the airway lumen and elsewhere. Indeed, our results also showed that AdAg85A immunization was able to increase the number of IFN-γ+ CD4 and CD8 T cells in the lung and spleen when these T cells were stimulated with either specific peptides or whole proteins. Similar to data from the airway lumen, tetramer data from the lung suggest more potent CD8 T-cell responses than those estimated by intracellular IFN-γ staining. Moreover, the detection of tetramer-positive CD8 T cells in the spleen also supports the possibility that intranasal AdAg85A can induce a more potent systemic immune response than initially estimated by intracellular IFN-γ staining. These data indicate that AdAg85A is able to enhance an antigen-specific population in which the potency correlates geographically to the route of administration. Recent studies in cattle have also shown the ability of MVAAg85A to boost the immunogenicity of BCG by increasing the number of IFN-γ-secreting antigen-specific CD4 and CD8 T cells (19). These data support our current study and suggest that AdAg85A will be an effective booster for BCG in a cattle model (18). In our current study, we also detected a much greater number of airway luminal CD4 and CD8 T cells in mice given BCG boosted with AdAg85A i.n. after secondary mycobacterial exposure than before mycobacterial exposure. This reflects a secondary recall T-cell response, probably due to de novo T-cell proliferation and/or the recruitment into the airway of antigen-specific T cells from elsewhere following mycobacterial challenge. Current literature information suggests both mechanisms may play a role in protection (4, 23, 24).

Interestingly, in our current and previous studies (16, 20, 21) we found that, different from parenteral genetic vaccination, parenteral BCG alone is effective in conferring protection from pulmonary M. tuberculosis challenge, and as we have demonstrated in the current study, such protection does not correlate with the level of antigen-specific T cells present in the airway lumen prior to M. tuberculosis exposure (Fig. 4). This suggests that the T cells systemically activated by parenteral BCG vaccination differ from those activated by parenteral genetic vaccination. We are currently investigating the mechanisms underlying such differences.

One of the top priorities in the area of TB vaccine development is to identify the effective heterologous boosting strategies for BCG prime immunization. This is because (i) the majority of the world has been vaccinated with BCG and any new vaccination strategy must be compatible with current practices, (ii) the utilization of an effective stand-alone vector may be the most effective booster strategy because it should work well regardless of vaccination status, and (iii) with so many human populations (those in the Northern versus Southern hemisphere, those in developing versus developed nations, those with different human immunodeficiency virus statuses, etc.) in need of improved protection against TB it may be naïve to assume that one “magic” vaccine may be able to control the current TB epidemic (12, 26). Our current evidence supports intranasal AdAg85A delivery as an effective way to boost parenteral BCG prime immunization through the induction of airway luminal CD4 and CD8 T cells. Our promising results as well as the ones observed in the cattle model justify the continued examination of intranasal AdAg85A in other animal systems and its evaluation in humans.

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