

The ID93 Tuberculosis Vaccine Candidate Does Not Induce Sensitivity to Purified Protein Derivative

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The tuberculin skin test (TST) is a simple and inexpensive test to determine whether individuals have been exposed to *Mycobacterium tuberculosis*. This test is not always reliable, however, in people previously immunized with BCG and/or who have been exposed to environmental mycobacterial species due to a reaction to purified protein derivative (PPD) used in the skin test. An issue with BCG, therefore, is that the resulting sensitization to PPD in some individuals compromises the diagnostic use of the skin test. The ability to induce protective immune responses without sensitizing to the tuberculin skin test will be important properties of next-generation tuberculosis (TB) vaccine candidates. We show here that guinea pigs immunized with the candidate TB vaccine ID93/GLA-SE, currently in clinical trials, do not react to intradermal PPD administration. In contrast, positive DTH responses to both ID93 and components thereof were induced in ID93/GLA-SE-immunized animals, indicating robust but specific cellular responses were present in the immunized animals. Noninterference with the TST is an important factor for consideration in the development of a vaccine against *M. tuberculosis*.

Tuberculosis (TB) remains a huge global health problem, with nearly 1.3 million deaths and more than 8.5 million people developing TB in 2012 due, in part, to the lack of an effective vaccine for the prevention of pulmonary tuberculosis in adults (1). The bacillus Calmette-Guérin (BCG) vaccine, in widespread use for more than 60 years, appears to have minimal impact on the worldwide incidence, despite demonstrating reasonable efficacy against severe complications of infant TB (2). The identification of individuals infected with the tuberculosis bacilli, particularly in epidemiological studies, has been traditionally done using a tuberculin skin test (TST). This test is based on the delayed-type hypersensitivity (DTH) reaction elicited by *Mycobacterium tuberculosis* antigens (tuberculin). Intradermal (i.d.) injection of purified protein derivative (PPD) of tuberculin, which is a cell-free purified protein fraction from *M. tuberculosis* diluted in PBS (or buffer), polysorbate Tween 80 (as a stabilizer) and phenol (as a preservative), results in a DTH response in most immunocompetent infected individuals peaking 48 to 72 h after injection. Although this is a relatively simple and inexpensive way to identify individuals that have been exposed to tuberculosis, there are drawbacks to the TST. One of the biggest drawbacks results from a large complement of proteins within PPD that are expressed by BCG (3), which can compromise the ability of this test to distinguish between BCG-vaccinated and *M. tuberculosis*-exposed people in countries where BCG is routinely used. A subset of people vaccinated with BCG revert to a PPD-negative status over time: there is little effect on the TST if BCG is given only once at birth and if the TST is performed 10 years or more after BCG vaccination (4). If, however, BCG is given as a booster vaccination as a part of the country's BCG policy (5), false-positive TST responses are more prevalent and can lead to larger TST reactions following injection with PPD (4). Because of this, careful interpretation of TST results from this population and use of more sensitive methods of detection, such as gamma interferon (IFN- γ) release assays (IGRAs) discussed below, should be considered. The current Centers for Disease Control and Prevention guidelines suggest interpreting a TST ≥ 15 mm in diameter as positive for latent *M. tuberculosis* infection in people

with no known risk factors for TB (<http://www.cdc.gov/tb/publications/tbi/diagnosis.htm>). BCG is not currently used as a routine vaccine in the United States or the Netherlands and in the United States is considered only in situations that warrant its use, including cases where PPD-negative children are continually exposed to an *M. tuberculosis*-infected adult or in certain health care settings (www.cdc.gov/tb/topic/vaccines).

Other diagnostic tests are available, including IGRAs, which measure antigen-specific IFN- γ from the patient's whole blood or peripheral blood mononuclear cells after stimulation with selected *M. tuberculosis* peptides (ESAT-6, CFP-10, and/or TB7.7). These tests have been a useful addition to the diagnosis of TB due to their specificity (<http://www.cdc.gov/tb/publications/factsheets/testing/IGRA.pdf>). In addition, Gene Xpert technology has also been beneficial for diagnosing active TB, which employs real-time PCR to detect *M. tuberculosis* from the sputum (6). Even so, these diagnostic tools are not ideal for the detection of TB in countries that are not able to afford this technology.

The guinea pig has been widely used as a model for studying the efficacy of drugs and vaccines against tuberculosis (7–13). In addition, DTH responses are easily observed after the injection of PPD in both BCG vaccinated and *M. tuberculosis*-infected guinea pigs. Since one of the pitfalls of the BCG vaccine is that it can compromise the TST, experimental TB vaccines, with potential use in non-BCG-vaccinated individuals, could be designed so that the utility of this simple screening tool is maintained.

In the present study, we show that the candidate ID93/GLA-SE vaccine, now in human clinical trials in healthy adult volunteers

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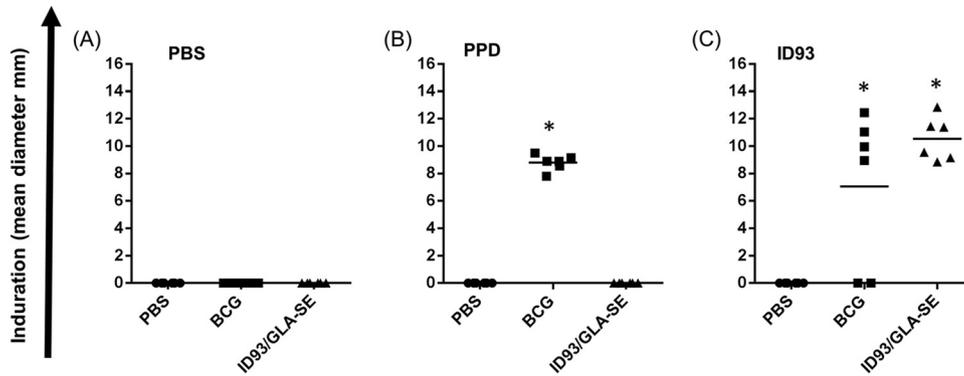


FIG 1 Immunization with ID93+GLA-SE does not compromise the tuberculin skin test. (A) PBS; (B) PPD; (C) ID93. Guinea pigs ($n = 6$ per group) were immunized i.m. three times, 3 weeks apart, with 10 μg of ID93 formulated in GLA-SE (5 μg) or were sensitized once, i.d., with BCG. DTH responses were performed 5 weeks after the third immunization. Dot blots of individual guinea pig DTH responses are indicated. The data represent the DTH responses 48 h after intradermal injection with either ID93 or PPD. The results are represented by the diameter of induration in mm (the line represents the mean response). The asterisk represents the statistical significance compared to PBS (*, $P < 0.05$).

(NCT01599897) and in BCG-vaccinated healthy adult volunteers (NCT01927159), was able to elicit a DTH response to the vaccine antigen, but did not compromise the PPD reaction. We also show by DTH responses that guinea pigs immunized with ID93 (a fusion protein containing four different tuberculosis proteins) were able to elicit T cell responses to all of the four proteins present in ID93 to different degrees. The use of ID93+GLA-SE has been shown to induce significant protection in the presence (9) or absence (7) of a BCG prime in the guinea pig model.

MATERIALS AND METHODS

Immunization. Five to six female Hartley guinea pigs (400 to 500 g) (Charles River Laboratories, Wilmington, MA) per group were housed in the Infectious Disease Research Institute animal care facility under specific-pathogen-free conditions. Animals were treated in accordance with the regulations and guidelines of the IDRI Animal Care and Use Committee. Guinea pigs were immunized with a fusion protein (ID93) comprised of four *M. tuberculosis* antigens: Rv3619, Rv1813, Rv3620, and Rv2608. ID93 was expressed and purified as previously described (9). Guinea pigs were immunized intramuscularly (i.m.) three times, 3 weeks apart, with ID93 (1 to 10 μg) formulated with 5 μg of GLA-SE (a synthetic TLR4 agonist adjuvant formulated in a 2% stable emulsion [SE]). Animals sensitized with BCG were immunized intradermally (i.d.) with a single dose (5×10^4) of live BCG Pasteur (Sanofi Pasteur) given during the same time as the first subunit vaccine immunization.

***M. tuberculosis* challenge.** A University of Wisconsin-Madison aerosol exposure chamber, calibrated to deliver 20 to 100 bacteria (*M. tuberculosis* H37Rv strain, ATCC 35718), was used to sensitize guinea pigs with *M. tuberculosis*.

DTH studies in guinea pigs. Guinea pigs ($n =$ either 5 or 6 per group) were immunized i.m. three times, 3 weeks apart, with 1 or 10 μg of ID93 formulated in GLA-SE (5 μg) as indicated in the figure legends or were sensitized once i.d. with BCG. The following reagents were included for the DTH skin tests: 5 tuberculin units (TU)/100 μl (Tubersol diagnostic antigen; Sanofi Pasteur), ID93 (1 or 5 μg /100 μl , as indicated), individual components of ID93 (1 μg /100 μl), and PBS (100 μl) were injected i.d. on the shaven backs of the guinea pigs with a standard tuberculin needle. For the first experiment (Fig. 1), the DTH response was performed 5 weeks after the third subunit immunization (or 11 weeks after the BCG immunization). For the second experiment (Fig. 2), the DTH response was performed 2 weeks after the third subunit immunization (or 8 weeks after the BCG immunization). DTH responses were measured 48 h after skin test injection and are represented by the diameter of induration in mm. Erythema measurements were not included.

Statistical analysis. For DTH responses, a one-way analysis of variance, followed by a Dunnett's multiple-comparison test, was performed, and the results were compared to the PBS control unless otherwise noted.

RESULTS

The PPD skin test is not compromised in guinea pigs given the ID93 vaccine. One problem associated with the attenuated live BCG vaccine is that the tuberculin PPD skin test is not always able to distinguish between individuals immunized with BCG and those exposed to *M. tuberculosis*. To determine whether or not our candidate TB vaccine compromised the PPD skin test, a DTH response to PPD was measured after immunization of guinea pigs with ID93 plus a synthetic TLR4 agonist mixed in a stable emulsion (ID93/GLA-SE). As expected, the BCG-immunized guinea pigs responded with a positive PPD skin test (Fig. 1B), whereas neither the ID93/GLA-SE vaccines nor the PBS control induced a DTH response to PPD (Fig. 1). No responses were observed in animals injected with the PBS control, as expected (Fig. 1A).

DTH responses elicited to the vaccine antigen. In order to determine whether guinea pigs immunized with ID93/GLA-SE would elicit a DTH response to the vaccine antigen, we included ID93 as a skin test antigen in these animals. We observed a statistically significant DTH response to ID93 in all of the ID93/GLA-SE immunized guinea pigs (Fig. 1C). A majority of the BCG-immunized animals (4 of 6) were also able to recognize ID93 (Fig. 1C).

DTH responses to individual component proteins of ID93. One of the interests of our group was to determine whether proteins included in the ID93 fusion protein were individually recognized by T cells in guinea pigs. For this reason, we evaluated DTH responses in guinea pigs 2 weeks after the third injection with ID93/GLA-SE. Because we have reproducibly shown that a lower dose of ID93 combined with GLA-SE is able to elicit better T cell responses in mice (unpublished data), we included one-tenth (1 μg) of the ID93 in the vaccine for the second DTH experiment rather than 10 μg . BCG-immunized guinea pigs were also included as a control for PPD in this experiment. All of the BCG-immunized guinea pigs responded to PPD, and there were no responses in the PBS controls as expected (Fig. 2A and B). In terms of responses to the individual antigens in ID93—there were 5/5 responders to the Rv1813 and Rv2608 proteins, 4/5 responders to

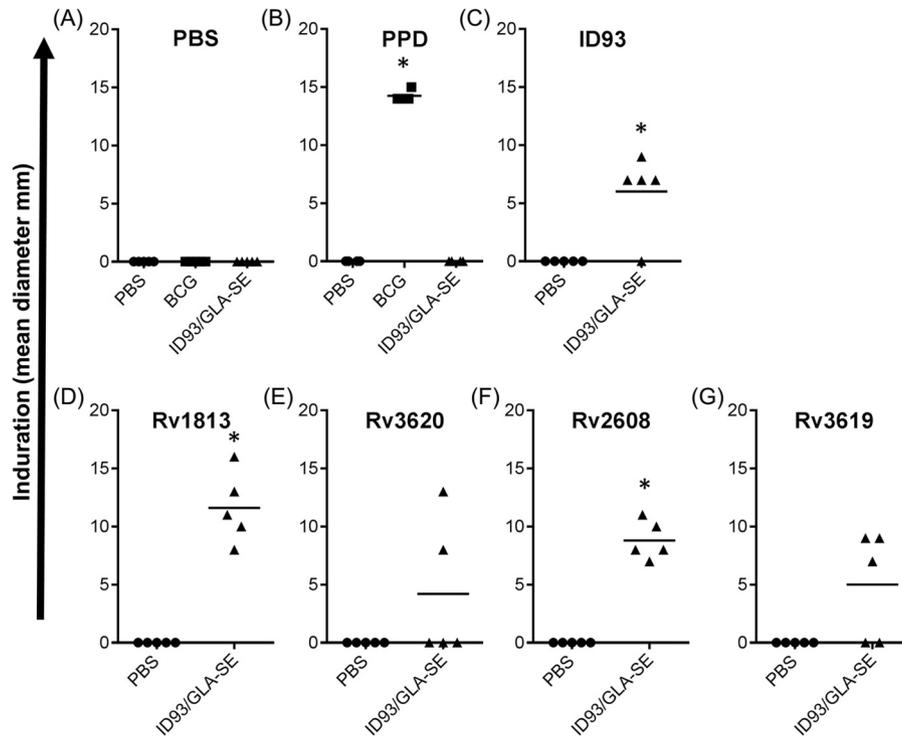


FIG 2 DTH responses to multiple components of the ID93 fusion protein. (A) PBS; (B) PPD; (C) ID93; (D) Rv1813; (E) Rv3620; (F) Rv2608; (G) Rv3619. Guinea pigs ($n = 5/\text{group}$) were immunized i.m. three times, 3 weeks apart, with 1 μg of ID93 and 5 μg of GLA-SE. DTH responses were determined 2 weeks after the third immunization with ID93/GLA-SE. PPD (Tubersol, 5 TU), ID93 (1 $\mu\text{g}/100 \mu\text{l}$), Rv1813 (1 $\mu\text{g}/100 \mu\text{l}$), Rv3620 (1 $\mu\text{g}/100 \mu\text{l}$), Rv2608 (1 $\mu\text{g}/100 \mu\text{l}$), or Rv3619 (1 $\mu\text{g}/100 \mu\text{l}$) were delivered i.d. on the back of the guinea pigs as skin test antigens. DTH responses were measured 48 h after injection. Dot plots of individual guinea pig DTH responses are indicated with either circles (PBS), squares (BCG), or triangles (ID93/GLA-SE). The results are represented by the diameter of induration in mm (the line represents the mean response). The asterisk represents statistical significance compared to PBS (*, $P < 0.05$).

ID93, 3/5 responders to Rv3619, and 2/5 responders to Rv3620 (Fig. 2). One reason why only 4 of 5 guinea pigs responded to ID93 and 5 of 5 guinea pigs responded to Rv1813 and Rv2608 may have been due to the set amount of protein (1 $\mu\text{g}/100 \mu\text{l}$) that was injected for each of the antigens rather than the use of molar concentrations for each protein. In addition, the reason there was one nonresponder to ID93 may have been due to the dose of antigen used for the immunization (1 μg of ID93) compared to the ID93 dose given for immunization in the first experiment (10 μg); 5 of 5 guinea pigs responded with a DTH response to the vaccine antigen in the first experiment (Fig. 1C).

DISCUSSION

Different strategies are being pursued to preserve the current skin test for diagnosis of individuals that have had prior exposure to *M. tuberculosis*. Some of the efforts that are under way to improve the TST include simplifying and/or optimizing the proteins that are in PPD for the development of second-generation PPD antigens. Ideally, these proteins would elicit responses only in *M. tuberculosis*-infected individuals. One study showed that the Rv3874 gene product CFP-10 is capable of inducing an *M. tuberculosis*-specific DTH response in *M. tuberculosis*-infected guinea pigs but not in guinea pigs sensitized with *M. avium* or *M. bovis* BCG strains (which lack the Rv3874 gene) (14). Some of the key proteins contained in the PPD that are responsible for inducing a DTH response have recently been identified. Two pools of defined proteins, including a combination of DnaK, GroEL2, and Rv0009 and another combination with DnaK, GroEL2, and Rv0685, were able

to induce the same level of DTH response that was observed with PPD in guinea pigs. However, the responses did not distinguish between animals sensitized to BCG or *M. tuberculosis* (15). Recently, several of the dominant proteins within PPD were defined (16). However, none of the 50 T cell antigens of PPD-S2 included the proteins in the ID93 fusion protein (Rv1813, Rv2620, Rv2608, or Rv3610). The *in vitro* IFN- γ release assay (IGRA) takes advantage of using *M. tuberculosis* specific antigens encoded in the region of difference 1 (RD1), such as ESAT-6, CFP-10, and TB7, that are not present in the BCG vaccine. These include the QuantiFERON-TB Gold-in-tube enzyme-linked immunosorbent assay (Cellestis, Australia) and the T-SPOT.TB enzyme-linked immunospot assay (Minus TB7.7; Oxford Immunotec, United Kingdom) (17). Both commercial assays employ two synthetic peptides from *M. tuberculosis*, ESAT-6 and CFP-10 (QFT-GIT and T-Spot), and one includes peptides from a third *M. tuberculosis* protein, TB7.7 (QFT-GIT). Although these assays offer an alternative to TST in BCG-vaccinated individuals, their widespread implementation in resource-limited settings has been limited by their cost and complexity.

Other efforts to preserve the TST involve improving TB immunization strategies so that the TST is not compromised. Hofst et al. showed that mucosal delivery (by the oral route) of BCG in humans spares the induction of a DTH response to PPD but still enables elicitation of a Th1 response (IFN- γ) to mycobacterial proteins included in either culture filtrates or whole lysates from *M. tuberculosis* (Erdman) (18). In the present study, we address

whether the candidate TB vaccine ID93/GLA-SE can be given without compromising the current TST.

We show that PPD (Tubersol) induced a significant DTH response in BCG- and TB-sensitized animals. To further characterize cellular DTH responses in ID93/GLA-SE immunized animals, we included individual proteins that make up the ID93 fusion protein as skin test antigens in the guinea pig model. All of the individual antigens that comprise ID93, including Rv1813, Rv2608, Rv3619, and Rv3620, were able to elicit DTH responses to differing degrees in ID93/GLA-SE-immunized guinea pigs, showing that each individual protein is capable of inducing a cellular response. The skin test antigens that resulted in generating the greatest number of responders included Rv1813 (a latency-associated protein), Rv2608 (a member of the PE/PPE family of TB proteins), and the ID93 fusion protein. We have previously shown that all of the individual proteins that make up the ID93 fusion protein are able to induce an IFN- γ response in PBMCs from PPD⁺ individuals (19).

We recently showed that ID93/GLA-SE, given in the absence of BCG, was able to protect against *M. tuberculosis* (7). In that study we describe the DTH responses in guinea pigs immunized with ID93/GLA-SE. We found that ID93/GLA-SE was able to elicit a DTH response to the ID93 immunogen when given i.d. in the skin test. Interestingly, guinea pigs immunized with ID93/SE (the SE adjuvant is a stable oil-in-water emulsion that lacks a TLR agonist) were also capable of inducing a DTH response to the ID93 antigen (data not shown) even though ID93/SE was not protective against *M. tuberculosis* (7). This suggests that DTH responses generated to a vaccine antigen may not be predictive of a protective immune response. Furthermore, we also previously showed that T cell proliferative responses in both the ID93/SE and ID93/GLA-SE-immunized guinea pigs were similar in response to ID93 protein or ID93 peptides (7). Nevertheless, only ID93 adjuvanted with GLA-SE induced protection. These results conflict with results shown by Hogarth et al. (20), where the efficacy of culture filtrate proteins (CFP) and CpG mixed in an emulsion was shown to elicit greater DTH responses to CFP and greater proliferative responses than CFP mixed in a MPL-containing adjuvant (which targets the TLR4) in guinea pigs. There are many differences in their study and ours, however, including the antigen(s) used, the route of administration, the immunization regimen, and the adjuvants included in the studies. Furthermore, a vehicle control alone (emulsion) was not included in the Hogarth study, nor was protection measured against challenge with *M. tuberculosis*.

More importantly, we show here that the TST was not compromised in the ID93/GLA-SE-immunized animals, whereas BCG-primed guinea pigs did elicit a DTH response to PPD. This is an important finding, since ID93/GLA-SE is currently in human clinical trials and if this vaccine were to be given to individuals unable to receive BCG, such as HIV⁺ individuals with adequate T cell numbers, the skin test could still be utilized to determine potential exposure to *M. tuberculosis*.

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S.G.R. is a founder of, and holds an equity interest in, Immune Design Corp., a licensee of certain rights associated with GLA.

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