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## Preclinical study and phase I clinical safety evaluation of recombinant *Mycobacterium tuberculosis* ESAT6 protein

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**Background:** To investigate the ability of rESAT6 to identify different mycobacteria-sensitized guinea pigs and its safety in preclinical and phase I clinical study.

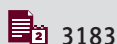
**Material/Methods:** Guinea pigs were sensitized with different *Mycobacteria*. After sensitization, all animals were intradermally injected with rESAT6 and either PPD or PPD-B. At 24 h after the injection, the erythema of the injection sites were measured using a double-blind method. For the preclinical safety study, different doses of rESAT6 and BSA were given 3 times intramuscularly to guinea pigs. On day 14 after the final immunization, the guinea pigs were intravenously injected with the same reagents in the hind legs and the allergic reactions were observed. A single-center, randomized, open phase I clinical trial was employed. The skin test was conducted in 32 healthy volunteers aged 19–65 years with 0.1 µg, 0.5 µg, and 1 µg rESAT6. Physical examination and laboratory tests were performed before and after the skin test and adverse reactions were monitored. The volunteers' local and systemic adverse reactions and adverse events were recorded for 7 days.

**Results:** Positive PPD or PPD-B skin tests were observed in all *Mycobacteria*-sensitized guinea pigs; the diameters of erythema were all >10 mm. The rESAT6 protein induced a positive skin test result in the guinea pigs sensitized with MTB, *M. bovis*, *M. africanum* and *M. kansasii*; the diameters of erythema were 14.7±2.0, 9.3±3.8, 18.7±2.4, and 14.8±4.2 mm, respectively. A negative skin test result was detected in BCG-vaccinated and other NTM-sensitized guinea pigs. The rESAT6 caused no allergic symptoms, but many allergic reactions, such as cough, dyspnea, and even death, were observed in the guinea pigs who were administered BSA. During the phase I clinical trial, no adverse reactions were found in the 0.1 µg rESAT6 group, but in the 0.5 µg rESAT6 group 2 volunteers reported pain and 1 reported itching, and in the 1 µg rESAT6 group there was 1 case of pain, 1 case of itching, and 1 case of blister. No other local or systemic adverse reactions or events were reported.

**Conclusions:** The rESAT6 can differentiate effectively among MTB infection, BCG vaccination, and NTM infection and is safe in healthy volunteers.

**Key words:** phase I clinical trial • recombinant protein • skin test • latent *M. tuberculosis* infection

**Full-text PDF:** <http://www.basic.medscimonit.com/download/index/idArt/883912>



## Background

The World Health Organization considers tuberculosis a global pandemic, with almost 20 million active pulmonary tuberculosis cases worldwide and approximately 8.8 million new cases each year [1,2]. In addition, an estimated one-third of the world population is infected with MTB [2], with 550 million infections in China alone. LTBI are the major source of new tuberculosis cases, and approximately 10% of LTBI will become active cases of pulmonary tuberculosis [3]. Therefore, the screening and prevention of LTBI is one of the main interventions used in attempts to control the disease.

The current method of screening LTBI predominantly depends on the classical tuberculin skin test. However, the complex PPD antigen is also expressed in both NTM and BCG, resulting in poor diagnostic specificity due to cross-reaction [4–6]. Tuberculin skin test results and their interpretation may be hampered by previous BCG vaccination. Given that BCG remains a planned immunization vaccine in most countries with high tuberculosis burdens, the PPD skin test is not able to differentiate between BCG vaccination and MTB infection. In some countries with high exposure to NTM, a false-positive PPD skin test can also be obtained [7,8]. The poor specificity of the PPD skin test limits its role in screening for LTBI.

IGRA is an *in vitro* diagnostic method for screening LTBI that was developed in recent years. There are 2 IGRA protocols: ELISA for quantitatively determining the IFN- $\gamma$  releasing level (such as the commercial QuantiFERON-TB Gold, Cellestis, Australia) and ELISPOT for enumerating the IFN- $\gamma$ -producing cells, the effective T cells, at the single cell level, such as the T-SPOT.TB (Oxford Immunotec, United Kingdom). Since these 2 methods are stimulated by MTB-specific antigen, their diagnostic results are more specific than that of the PPD skin test. Despite their high sensitivity and specificity [9–11], the main disadvantages of these tests are that they are difficult to perform and are expensive. In addition, the ELISPOT kit has a high requirement for experimental sites and apparatus. In addition, the fresh blood samples must be tested within short periods of time. All of these requirements limit the use of ELISA and ELISPOT in countries with high tuberculosis burdens and in undeveloped regions.

Compared with MTB, some regions of difference (RD) are lost in the BCG genome and the majority of NTM genome. The RD1 region genes are lost in all BCG genomes, which provide the possibility for selection of ideal antigens for differentiating between BCG vaccination and MTB infection [12,13,15]. *ESAT6* is an important RD1 region gene [13,14]. The present study expressed rESAT6 protein to replace the complex antigen PPD and developed a new method for screening LTBI based on a simple and easy skin test. The preclinical diagnostic ability and

safety evaluation of rESAT6 was then investigated, as was the safety of a phase I clinical trial of rESAT6.

## Material and Methods

### Guinea pigs

Guinea pigs aged 6–9 weeks and weighing 400–500 g each were purchased from the Resource Center for Experimental Animals of the National Institutes for Food and Drug Control, with a male: female ratio of approximately 1:1. The animals used for BCG vaccination and systemic allergic test were raised in clean rooms, and those used for the MTB and NTM sensitizations were raised in negative pressure rooms.

All animals used in this study were treated according to the animal welfare standards and reviewed by the Animal Care and Welfare Committee of the National Institutes for Food and Drug Control.

### Preparation of the rESAT6 protein

Genomic DNA of MTB was extracted and polymerase chain reaction was performed to amplify the target gene *ESAT6*. The purified target gene was linked to the plasmid vector, and positive clones were screened using digestion and sequencing techniques. The positive recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3), and the rESAT6 was induced and expressed using isopropyl  $\beta$ -D-1-thiogalactopyranoside [16]. Finally, the rESAT6 was purified using ion exchange chromatography and a molecular sieve, and an rESAT6 solution was obtained.

### Preparation of sensitized bacterial liquid

*M. tuberculosis* (H<sub>37</sub>Rv strain, ATCC27294), BCG (Danish strain), *M. bovis* (ATCC19210 strain), *M. africanum* (ATCC25420 strain), *M. kansasii* (ATCC12478 strain), *M. marinum* (ATCC927 strain), *M. smegmatis* (ATCC19420 strain), *M. phlei* (ATCC11758 strain), *M. vaccae* (ATCC15483 strain), *M. chelonae* (ATCC19977), and *M. fortuitum* (ATCC6841 strain) were all supplied by the National Center for the Management of Medical Bacteria Preservation of the National Institutes for Food and Drug Control. A 1-mg/mL aliquot of *M. tuberculosis* that had been stored at  $-70^{\circ}\text{C}$  with a live bacterial count of  $3 \times 10^7$  CFU/mL was diluted to a  $5 \times 10^3$  CFU/mL sensitized bacterial liquid using physiological saline. Frozen BCG (60 mg) was dissolved in 1 mL of physiological saline to yield the sensitized bacterial liquid. The freshly cultured *M. bovis*, *M. africanum*, *M. kansasii*, *M. marinum*, *M. smegmatis*, *M. phlei*, *M. vaccae*, *M. chelonae*, and *M. fortuitum* were washed with physiological saline, and the bacterial colonies were collected, ground, and formulated to a 1-mg/mL

bacterial liquid, which was then diluted to  $1 \times 10^5$  CFU/mL sensitized bacterial liquid.

### Reagents for skin test in guinea pigs

The rESAT6 solution was diluted to 2  $\mu\text{g/mL}$ , 5  $\mu\text{g/mL}$ , 20  $\mu\text{g/mL}$ , and 100  $\mu\text{g/mL}$  (2, 20, and 100  $\mu\text{g/mL}$  were used in the systemic allergic test) with 0.01 mol/L PBS (pH, 7.2–7.4) containing 0.0005% Tween-80 and 3 g/L phenol. As the control reagents, 50 IU/mL of PPD was prepared with *M. tuberculosis* (H<sub>37</sub>Rv) and 10  $\mu\text{g/mL}$  of PPD-B was prepared with *M. intracellulare* (ATCC13950). *M. intracellulare* was supplied by the National Center for the Management of Medical Bacteria Preservation of the National Institutes for Food and Drug Control.

### Clinical skin test reagents

The rESAT6 used in the current study was produced in compliance with good manufacturing process conditions and provided by Beijing Xiangrui Biological Products Co., Ltd. (Beijing, China) at different concentrations (1  $\mu\text{g/mL}$ , 5  $\mu\text{g/mL}$ , and 10  $\mu\text{g/mL}$ ).

### Guinea pig sensitization and skin tests

Fifty-eight guinea pigs were randomly assigned to 11 sensitized groups: *M. tuberculosis* (6 animals), BCG (6 animals), *M. bovis* (5 animals), *M. africanum* (5 animals), *M. kansasii* (6 animals), *M. marinum* (5 animals), *M. smegmatis* (5 animals), *M. phlei* (6 animals), *M. vaccae* (4 animals), *M. chelonae* (5 animals), and *M. fortuitum* (5 animals). Guinea pigs in each group were subcutaneously injected with the corresponding *Mycobacteria*-sensitized bacterial liquid (0.5 mL for each animal).

Six weeks after the sensitization, the hair was removed from both sides of the guinea pigs' spines in the *M. tuberculosis* and BCG groups and they were intradermally injected with 0.1 mL of rESAT6 protein (5  $\mu\text{g/mL}$ ) and 0.1 mL of PPD on both sides of the spine. The guinea pigs in the *M. bovis*, *M. africanum*, *M. kansasii*, *M. marinum*, *M. smegmatis*, *M. phlei*, *M. vaccae*, *M. chelonae*, and *M. fortuitum* groups were intradermally injected with 0.1 mL of rESAT6 protein (5  $\mu\text{g/mL}$ ) and 0.1 mL of PPD-B on both sides of the spine. The diameters of erythema were measured using the double-blind method and recorded 24 h after injection. Results are expressed as mean  $\pm$ SD.

### Preclinical safety assessment of guinea pigs

Guinea pigs were randomly assigned to 5 groups (8 animals in each): BSA positive control, low-dose, medium-dose, high-dose rESAT6 protein, and physiological saline negative controls. BSA (1 mg/mL) was used for the sensitization in the BSA-positive control group. Animals in the low-dose, medium-dose, and high-dose rESAT6 protein groups were sensitized using

2, 20, and 100  $\mu\text{g/mL}$  rESAT6, respectively. The prepared various sensitized liquids were given intramuscularly to guinea pigs at a dose of 0.5 mL/animal in the corresponding groups 3 times, once every other day. At day 14 after the final immunization, the guinea pigs were intravenously injected with the same reagents in the hind legs (in the positive control group the BSA injection dose was 4 mg/animal). At 1 h after challenge, allergic reactions including nose-catching, bristling, dyspnea, convulsion, shock, and death were observed and graded according to severity.

### Phase I clinical trial

The rESAT6 was approved by the State Food and Drug Administration and approved by the ethics review committee of Beijing Tuberculosis and Thoracic Tumor Research Institute. The clinical study was an open experiment that included a total of 32 healthy volunteers aged 19–65 years. Informed consent was obtained from all volunteers following a detailed description of the purpose and potential benefits of the study prior to the trial. The most important exclusion criteria were: current pulmonary tuberculosis, respiratory or other systemic symptoms, acute or chronic diseases, acute infectious disease, skin disease or skin allergy due to various causes, or close contact with individuals with TB. A comprehensive physical examination was performed in all volunteers prior to the trial, including vital signs (heart rate, blood pressure, and respiration), routine blood and urine tests, biochemical function tests, and electrocardiography (ECG). X-ray photography was performed to rule out patients with tuberculosis.

All volunteers were randomly assigned to 3 groups in which they underwent skin tests with intradermal injection of 0.1 mL of 1 (4 volunteers), 5 mL (18 volunteers), and 10  $\mu\text{g/mL}$  (10 volunteers) rESAT6 on the first 1/3 site of the ramus volaris antibrachii. The volunteers were injected with 0.1  $\mu\text{g}$  rESAT6, and 0.5  $\mu\text{g}$  was given after the volunteers were confirmed to be tolerant of 0.1  $\mu\text{g}$  rESAT6. Similarly, after volunteers were confirmed to be tolerant of 0.5  $\mu\text{g}$  rESAT6, 1  $\mu\text{g/mL}$  was given. All injections were given strictly according to the operation procedures of a clinical PPD trial.

### Safety evaluation

All volunteers were examined for systemic and local adverse reactions within 7 days after injection. Systemic adverse reactions included fever, allergy, headache, fatigue, weakness, nausea, vomiting, diarrhea, cramps, and cough. Local adverse reactions included pain, edema, itching, and blisters. The adverse reactions were stratified into light (Grade I), medium (Grade II), severe (Grade III), and potential death risk (Grade IV) levels [17]. At 24, 48, and 72 h, the vital signs were examined in all volunteers. On day 7 after the skin test, the vital signs

of all volunteers were examined and routine blood and urine tests, biochemical function tests, and ECG were performed.

### Data analysis

All statistical analyses were performed using the statistical software SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Fisher's exact test was performed to compare the occurrence of local and systemic adverse reactions and events in volunteers administered with different doses of rESAT6.

## Results

### Skin test to rESAT6 in sensitized guinea pigs

Positive PPD skin tests were observed in the *M. tuberculosis*- and BCG-sensitized guinea pigs, and positive PPD-B skin tests were observed in the guinea pigs sensitized to *M. bovis*, *M. africanum*, *M. kansasii*, *M. marinum*, *M. megmatitis*, *M. phlei*, *M. vaccae*, *M. chelonae*, and *M. fortuitum*. The rESAT6 induced a positive skin test in *M. tuberculosis*-sensitized guinea pigs, and positive skin tests were also observed in other *M. tuberculosis* complex-sensitized animals (*M. bovis*, *M. africanum*) and *M. kansasii*-sensitized guinea pigs. However, negative skin tests were detected in *M. marinum*-, *M. smegmatis*-, *M. phlei*-, *M. vaccae*-, *M. chelonae*-, and *M. fortuitum*-sensitized guinea pigs, and a similar negative reaction was observed in BCG-sensitized guinea pigs (Table 1).

### Preclinical safety of rESAT6

After challenge, there were no apparent abnormal reactions in the negative control group or the rESAT6 groups, whereas in the BSA-positive control group, 100% (6/6) of guinea pigs showed strong reaction (such as cough, dyspnea, and convulsion) and 50% (3/6) died after systemic anaphylaxis.

### Safety of rESAT6 in a phase I clinical trial

The laboratory test results showed that in the 0.5 µg group, only 1 volunteer had a high eosinophil count (3.9% before vs. 9.6% after injection) and another volunteer had a low lymphocyte count (23.2% before vs. 16.9% after injection). In the 1 µg group, only 1 volunteer had a high eosinophil count (3.9% before vs. 7.7% after the vaccination) and another 3 female volunteers had red blood cells in the urine (1+). All of the above adverse reactions were defined as Grade 1 (mildly abnormal) and disappeared within 7 days. General vital signs, routine blood and urine tests, biochemical tests, and ECG findings were normal in all volunteers.

All volunteers were found to be tolerant in the rESAT6 skin test. During the test period, several volunteers had local adverse

**Table 1.** Skin test of rESAT6 and PPD/PPD-B in guinea pigs sensitized with different *Mycobacteria*.

Groups	Number of guinea pigs	rESAT6 (mm, mean ±SD)	PPD or PPD-B (mm, mean ±SD)
<i>M. tuberculosis</i> sensitization	6	18.0±1.67	12.7±1.37*
BCG vaccination	6	–	12.7±1.03*
<i>M. bovis</i> sensitization	5	9.30±3.8	14.4±1.9
<i>M. africanum</i> sensitization	5	18.7±2.4	11.6±3.8
<i>M. kansasii</i> sensitization	6	14.8±4.2	16.5±3.0
<i>M. marinum</i> sensitization	5	–	14.7±1.8
<i>M. smegmatis</i> sensitization	5	–	11.1±3.4
<i>M. phlei</i> sensitization	6	–	14.3±2.9
<i>M. vaccae</i> sensitization	4	–	11.5±0.9
<i>M. chelonae</i> sensitization	5	–	16.8±2.1
<i>M. fortuitum</i> sensitization	5	–	18.2±2.2

\* The guinea pigs injected with PPD; others injected with PPD-B.

reactions like pain, itching, and blisters, but no other local or systemic adverse reactions or events were reported. No adverse reactions were found in the 0.1 µg group, but in the 0.5 µg group 2 volunteers had pain and 1 had itching, and in the 1 µg group 1 had pain, 1 had itching and 1 had blisters. The above adverse reactions were classified as Grade 1. Fisher's exact test was used to compare the incidence rates of various adverse reactions and events in volunteers administered with different doses of rESAT6, but no significant differences were detected (all *P* values >0.05) (Table 2).

## Discussion

Tuberculosis is a major infectious disease that seriously harms human health and has had a high prevalence and fatality rate over the past decade. Among infectious diseases, the mortality rate of TB ranks second only to that of HIV/AIDS. In addition to high prevalence and mortality, there are numerous LTBI worldwide, who are the main source of new cases of tuberculosis each year. Therefore, early diagnosis of tuberculosis and

**Table 2.** Adverse reactions following administration of different doses of rESAT6.

Adverse reaction type	Occurrence (Yes/No)	0.1 µg rESAT6 group		0.5 µg rESAT6 group		1 µg rESAT6 group		P
		Number	Percentage (%)	Number	Percentage (%)	Number	Percentage (%)	
Pain	Yes	0	0	2	11.1	1	10	1
	No	4	100	16	88.9	9	90	
Itching	Yes	0	0	1	5.6	1	10	1
	No	4	100	17	94.4	9	90	
Blisters	Yes	0	0	0	0	1	10	0.4375
	No	4	100	18	100	9	90	

screening of LTBI are of great importance in controlling the epidemic of tuberculosis.

Recent advances have been achieved in techniques for the early diagnosis of tuberculosis and screening of LTBI, especially ELISA- and ELISPOT-based detection of INF-γ release. Both methods select the RD1 encoded protein as a specific T cell antigen, which is present in the *M. tuberculosis* genome and is generally lost in the BCG and most of NTM genomes; therefore, these tests have high specificity [18–21]. However, the technique is complicated to perform and is not suitable for screening LTBI in countries like India and China that have high tuberculosis burdens.

The present study used *M. tuberculosis*-specific antigen (rESAT6) as a skin test reagent and developed a simple and rapid method for screening LTBI based on the skin test. Animal experiments showed that the PPD skin test was positive in BCG- and *M. tuberculosis*-sensitized guinea pigs, and the rESAT6 skin test was negative in BCG-sensitized guinea pigs and positive in *M. tuberculosis*-sensitized guinea pigs. The experiments indicated that the rESAT6 skin test was able to be used to make the differential diagnosis between BCG vaccination and *M. tuberculosis* infection, which was consistent with findings of other studies [22–25].

PPD-B is a patented skin test reagent developed in our laboratory for the detection of NTM sensitizations [26]. With the same purification technique of PPD, PPD-B is extracted from *M. intracellulare* and PPD is extracted from *M. tuberculosis*. Therefore, the PPD-B test is much more sensitive than the PPD test in guinea pigs sensitized with NTM. We used the PPD-B test in this study for the detection of NTM sensitizations.

The *ESAT6* gene is lost in BCG and most of NTM, and it is expressed in *M. tuberculosis* complex (including *M. bovis* and *M.*

*africanum*) and some NTM (such as *M. kansasii* and *M. marinum*). In animal experiments, we demonstrated that the *ESAT6* gene distribution, which exhibited similar results in that the rESAT6 skin test was positive in the *M. bovine*-, *M. africanum*- and *M. kansasii*-sensitized guinea pigs, whereas the skin test was negative in other NTM-sensitized guinea pigs, including *M. marinum*. However, Wu reported that the rESAT6 skin test was positive in *M. marinum*-sensitized guinea pigs [25]. We inferred that this difference in outcomes might have resulted from different strains used in the test. *M. marinum* (ATCC927 strain) was used in our study, whereas the TMC1218 strain was used in the Wu study. In our study, the negative skin test result of rESAT6 protein might be the reason that the *ESAT6* gene was incompletely expressed in the *M. marinum* strains [14,25]. Wu also demonstrated that the rESAT6 skin test was negative in the guinea pigs sensitized by *M. smegmatis*, *M. fortuitum* and *M. phlei*, as seen in our study. They also demonstrated that the rESAT6 skin test was negative in the guinea pigs sensitized to *M. avium*, *M. intracellulare*, *M. nonchromogenicum*, *M. gilvum* and *M. triviale*. In our research, we studied whether rESAT6 can induce cross-reactions in animals infected with *M. africanum*, *M. kansasii*, and *M. chelonae*, which has not been reported previously. These results suggest that the rESAT6 protein is capable of differentiating among *M. tuberculosis* infection, BCG vaccination, and NTM infection, indicating its specificity. Moreover, Weldingh studied the ability of a skin test response to ESAT6 to predict the later development of tuberculosis disease in a guinea pig model [22]. The results showed that ESAT6 protein allows for the identification of individuals with incipient disease who are at the highest risk of developing active tuberculosis in the near future. We presented similar results in another study and are prepared to further investigate this issue.

The rESAT6 belongs to an intradermal diagnostic reagent, the safety and effectiveness of which are equally important

in clinical application. It is, therefore, of great importance to assess the safety of ESAT6 prior to the clinical trial stage. Immunotoxicity is the most important issue for vaccines or intradermal diagnostic reagents that elicit immune system responses. The present study focused on the systemic allergic reaction of rESAT6, and the results showed that it caused no abnormal reaction in the systemic allergic test, indicating its safety.

Preclinical evaluation of rESAT6 also indicated safety; therefore, the phase I clinical trial of rESAT6 was approved. The present phase I clinical study was conducted according to the type, frequency, and degree of local and systemic adverse reactions following rESAT6 injection, and this method evaluated the reagent's safety. The results showed that pain and itching were the major adverse reactions. Blisters are considered a strong skin allergic reaction and were considered an adverse reaction in the present study. The incidence rates of all aforementioned adverse reactions caused by various doses of rESAT6 were all low. Fisher's exact test indicated no significant differences in safety among different doses of rESAT6. Bergstedt conducted a clinical trial of a combined rESAT-6 and rCFP-10 skin test reagent and reported no serious adverse events [27]. The results demonstrated that rESAT6 was safe and could be used for making clinical diagnoses.

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## Conclusions

As a novel intradermal diagnostic reagent, rESAT6 was used in the differential diagnosis for BCG vaccination and *M. tuberculosis* infection in the preclinical trial. rESAT6 has high specificity, as revealed by the preclinical results, and it is safe, as only minor adverse reactions were noted in the phase I clinical trial. Moreover, the skin test is simple, easy to perform, and requires no special equipment. Therefore, the rESAT6 skin test appears to be a promising method of screening LTBI. In the phase II clinical trial, we will study the diagnostic ability of rESAT6 for BCG vaccination in human.

## Abbreviations

**BSA** – bovine serum albumin; **BCG** –Bacille Calmette-Guérin; **ELISA** – enzyme-linked immunosorbent assay; **ELISPOT** – enzyme-linked immunosorbent spot test; **LTBI** –Latent *M. tuberculosis* infection; **MTB** – *Mycobacterium tuberculosis*; **NTM** – non-tuberculous *Mycobacteria*; **PPD** –tuberculin purified protein derivative; **PPD-B** –*Mycobacterium intracellulare* purified protein derivative; **rESAT6** –recombinant *Mycobacterium tuberculosis* ESAT6 protein; **IGRA** –  $\gamma$ -interferon release assay.

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