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Immunodiagnosis of latent tuberculosis : new answers to an old question?

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DOUBLE-BLIND RANDOMIZED PHASE I STUDY COMPARING RDESAT-6 TO TUBERCULIN AS SKIN TEST REAGENT IN THE DIAGNOSIS OF TUBERCULOSIS INFECTION

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ABSTRACT

Limited specificity of the tuberculin skin test incited the development of *in vitro* assays based on *Mycobacterium tuberculosis*-specific antigens such as ESAT-6 that are lacking in Bacillus Calmette Guérin (BCG). In animal studies, intradermal ESAT-6 was safe and induced specific skin test responses. The aim of the study was to assess the safety of intradermal recombinant dimer ESAT-6 (rdESAT-6) compared with tuberculin and to determine the human dose. The study design was a double-blind Phase I study with intrasubject randomization to the left and right forearm, comparing 2 Tuberculin Units (TU) intradermal tuberculin (RT23) with 0.01, 0.1, 1 or 10 µg rdESAT-6 in groups of five healthy controls or treated tuberculosis (TB) patients. The risk of sensitization after skin testing was assessed in healthy volunteers. All doses were tolerated well by healthy volunteers and responses to rdESAT-6 were limited to transient redness after 24 h only at the highest dose. No sensitization was observed. Because 1 µg rdESAT-6 induced large responses with local side effects in some TB patients, the 10 µg dose of rdESAT-6 was not tested. Mean responses to 0.01, 0.1 and 1 µg rdESAT-6 measured 14.0, 19.8 and 38.8 mm of redness, respectively, and 7.0, 13.4 and 14.6 mm of induration. The response to tuberculin was similar to the responses to 0.1 µg rdESAT-6. Mild local side effects due to tuberculin and rdESAT-6 were observed in 8/15, respectively, 6/15 patients, more pronounced at the highest rdESAT-6 dose. In conclusion, this pilot Phase I study of safety, feasibility and dose finding of intradermal rdESAT-6 provides proof of principle of a specific skin test for human use. No serious adverse events were observed but the study was not sufficiently powered to demonstrate complete safety. Intradermal rdESAT-6 did not seem to sensitize healthy volunteers. In treated TB patients, responses to rdESAT-6 were optimal at 0.1 µg. Further studies are needed to evaluate sensitization after repeated doses and to study the effect of additional CFP-10 on the sensitivity of a TB-specific skin test.

INTRODUCTION

Tuberculosis (TB) remains the single most important fatal infection of humans, with 8.8 million new cases every year and an estimated 3 million deaths. Tuberculin or purified protein derivative (PPD) has been used worldwide for almost a century as a skin test reagent to support the diagnosis of TB or for detection of latent TB infection (LTBI). The main disadvantage of the tuberculin skin test (TST) is the occurrence of false positive responses due to cross-reactivity to antigens in PPD that are shared by environmental mycobacterial species as well as by *Bacillus Calmette Guérin* (BCG) vaccine strains.^{1,2} The limited specificity of the TST is especially problematic in regions where BCG vaccination is standard practice and exposure to environmental mycobacteria is common, thus including most countries with high TB rates.

The discovery of antigens that are specific for *Mycobacterium tuberculosis*, being those lacking in BCG and most environmental mycobacteria, led to the development of *in vitro* assays based on antigen-stimulated interferon- γ (IFN- γ) production.^{3,4} Various formats of such IFN- γ release assays (IGRA) showed a high sensitivity and nearly complete specificity.⁵ Two IGRA have thus far been marketed. QuantiFERON[®]-TB Gold (QFT-G, Cellestis, Carnegie, Australia) is a whole-blood assay using ELISA for detection of IFN- γ responses. A novel in-tube version of QFT-G (QFT-GIT) contains an additional *M. tuberculosis*-specific peptide (TB.7.7) and has logistical advantages. T-SPOT[™].TB (Oxford Immunotec, Abingdon, UK) is based on the enzyme-linked immunospot technique (ELISPOT) and requires isolation of peripheral blood mononuclear cells. In various guidelines, IGRA have been recommended either as an alternative for or as confirmative test following TST testing guidelines.^{6,7}

During the past few years, IGRA are increasingly being used in various clinical-epidemiological settings for the detection of TB disease or LTBI, either in place of or as supplement to the TST. Compared to industrialized regions, the situation is different in less developed countries where the use of IGRA is limited by test costs and the need of laboratory facilities and trained personnel. In that setting, important issues are costs and practical feasibility even under critical circumstances such as interrupted power supply.

A skin test based on specific antigens would combine advantages of a high specificity of antigens with the logistics of a skin test without the need of laboratory facilities other than cold storage of the reagent. *In vivo* data of intradermal use of

specific antigens are limited. Use of intradermal ESAT6 reliably identified cattle infected with *Mycobacterium bovis*.⁸ Recombinant ESAT-6 elicited marked delayed type hypersensitivity skin reactions in guinea pigs infected with *M. tuberculosis*, but not in those infected with BCG or *Mycobacterium avium*.⁹ Recently, a recombinant dimer of ESAT-6 synthesized in *Lactococcus* (recombinant dimer ESAT6 (rdESAT-6)) has been produced following good laboratory practice and good manufacturing practices to be tested in humans. In pre-clinical studies in guinea pigs, mice, rats and dogs, no adverse events were seen following subcutaneous or intravenous administration of one or several excessive doses of rdESAT-6 to immunologically naïve animals.¹⁰

The aim of the present study was to evaluate the safety and diagnostic potential of intradermal rdESAT-6 in humans and to determine the human dose.

Subjects and methods

Aims of the study

Primary objective of the study was the assessment of the safety of four different doses of rdESAT-6 administered intradermally to healthy adult volunteers and to TB patients who had completed treatment. Secondary aims were to compare the safety and skin test responses to rdESAT-6 with 2 Tuberculin Units (TU) Tuberculin (RT23 SSI) as a reference. For healthy volunteers, the risk of sensitization to rdESAT-6 following a single intradermal administration was evaluated by comparing *in vitro* IFN- γ responses after ESAT-6 stimulation of whole-blood samples on days 2 and 28 after injection. In treated TB patients, *in vivo* responses to rdESAT-6 were compared with pre-study IFN- γ responses after *in vitro* ESAT-6 stimulation using the QuantiFERON[®]-TB Gold assay.

Study design

The study was designed as a single-center Phase I dose-escalating clinical trial. The study was double-blind, within subject controlled and randomized between the right and left arm allowing simultaneous testing of rdESAT-6 and 2 TU PPD (RT23, SSI, Copenhagen, Denmark). The randomization was performed by a statistician at the Biostatistics Unit at SSI, who did not participate in the data management or the statistical analysis of data from the trial. Each subject received only one test dose of rdESAT-6. rdESAT-6 was first given at the lowest dose level (i.e. 0.01

$\mu\text{g}/0.1\text{ mL}$) to five healthy adults. The trial was only allowed to proceed into a 10-fold higher dose level if the previous dose level had been considered safe by the principal investigator (S.A.) and the data safety monitoring board. After testing of the highest dose level in the healthy volunteer group (i.e. $10\ \mu\text{g}/0.1\text{ mL}$), the first treated TB patients were included at the lowest dose level (i.e. $0.01\ \mu\text{g}/0.1\text{ mL}$).

Study subjects

The study aimed to test 20 healthy male adults in groups of five and subsequently the same number of treated male or female TB patients. Before inclusion, pregnancy was excluded in women of childbearing age. General health was assessed by physical examination, routine blood and urine tests. Exclusion criteria were significantly abnormal blood or urine tests, presence of hepatitis B surface antigen, antibodies to HIV or hepatitis C virus, BCG vaccination within 5 years prior to the day of inclusion or BCG vaccination more than once, having received a TST within the past year, known immune deficiency or disease affecting the lymphoid organs (e.g., Hodgkin's disease, lymphoma, leukemia, sarcoidosis), use of immunosuppressive drugs within 3 months prior to the day of inclusion, vaccination with a live vaccine within 6 months prior to the day of inclusion or viral or bacterial infection at inclusion. To allow accurate evaluation of the size of the skin test reactions (induration and/or redness) subjects with black skin were not included in this first evaluation.

Exclusion criteria for healthy controls were a history of TB, known contact to a person with active TB or a positive response to ESAT-6 in QuantiFERON[®]-TB Gold. TB patients were eligible if they had completed treatment and were without symptoms of active TB disease at the time of inclusion. Responsiveness of the treated TB patients to ESAT6 was assessed before inclusion using the QuantiFERON[®]-TB Gold assay. In the first group, only those with a positive IFN- γ response (according to the manufacturer's instructions) after *in vitro* ESAT-6 stimulation were included. As bilateral skin test responses were observed already at the lowest dose level, the protocol was amended to allow for enrollment in subsequent groups of patients treated in the past for culture-confirmed TB but who did not respond to ESAT-6 *in vitro*.

Skin test reagents

Both skin test reagents, rdESAT-6 and 2 TU Tuberculin PPD (RT23 SSI), were manufactured at SSI in Denmark according to Good Manufacturing Practice standards. All strengths of rdESAT-6 were dissolved in phosphate buffered saline containing 0.01% Tween and with pH adjusted to between 6.5 and 7.5. The rdESAT-6 vials in the four strengths and the PPD RT 23 vials could not be distinguished from each other visually and labels only indicated the side of injection as per randomization.

The selected doses of 0.01–10 µg rdESAT-6 were based on results from pre-clinical studies with rdESAT-6 in guinea pigs, mice, rats and dogs.¹⁰ A standard dose of 2 TU Tuberculin PPD RT23 SSI corresponds to approximately 0.04 µg protein. Estimates from pre-clinical studies in guinea pigs suggested that the injection of 10 TU PPD RT23 SSI and 0.024 µg rdESAT-6 result in skin responses of similar sizes. However, the relationship between responses to PPD and rdESAT-6 in humans was not known and the optimal dose of rdESAT-6 had to be established experimentally.

Tuberculin and rdESAT-6 skin tests were administered intradermally following a standard protocol. All readings were independently performed by the principal investigator (S.A.) and research nurse (C.P.), who were trained and experienced in Mantoux testing. Discrepancies exceeding 2 mm were reassessed until consensus was reached. Measurements were done with calipers, and subsequently the distance between the calipers legs were measured with a ruler. On days 0 (skin test injection) through 4, digital images of both forearms including a size reference were taken vertically from a fixed distance from the arm using a tripod.

Safety and efficacy variables

Each trial subject was followed during 28 days after skin tests were administered (day 0, always on a Monday) with clinical observation during 2 h after injection and subsequently on days 1, 2, 3, 4 and 28. Both redness and induration were recorded because the characteristics of the response to the rdESAT-6 antigen in humans were not yet known, and redness could be a relevant parameter. Redness was defined as the visible red or pink discoloration of the skin around the injection sites.

The diameter of induration and/or redness at the injection sites was measured with calipers transversely to the long axis of the forearm at 24, 48, 72 and 96 h after injection and subsequently the distance between the calipers legs were

measured with a ruler. Local and systemic adverse reactions occurring within 28 days after application of the tests were recorded. Adverse events present on day 28 were followed up. Blood and urine parameters were retested on days 4 and 28. A physical examination was done on day 28. Definitions of adverse events were following the ICH E2A guideline (<http://www.fda.gov/cder/guidance/iche2a.pdf>). After each group, a safety report was presented to a predefined Trial Data Safety Monitoring Board of experts who advised on the safety of testing the next dose level. The routine laboratory assays were performed by the certified central laboratory at LUMC according to standard procedures. The QuantiFERON[®]-TB Gold 2nd generation assay using soluble ESAT-6 was performed at the laboratory of the Department of Infectious Diseases according to the manufacturer's instructions.

Statistical analysis

Descriptive statistical methods were used. The study was designed as an initial pilot study on safety and proof of principle of intradermal rdESAT-6 and the numbers tested were too small to determine a statistical significance. Oneway analysis of variance (ANOVA) was used to evaluate the effect of different parameters on the difference between the independent skin test measurements. Correlation between *in vivo* and *in vitro* responses was analyzed by Spearman correlation.

Role of the funding source

The study sponsor was involved in the study design and participated in the writing of the report. The study sponsor had no role in the collection, analysis, and interpretation of data or the decision to submit the paper for publication.

Results

Study participants

Twenty healthy uninfected volunteers and 15 treated TB patients in groups of five subjects were included on different dates, each new group being included after the previous dose level had been considered safe. Characteristics of participants are shown in Table 1. Between 0 and 2 subjects in each group had been BCG vaccinated, which was similar for patients and controls. The average age of treated TB patients was higher than the age of the controls, which was related to the recruitment procedure for healthy uninfected controls that mainly attracted

students. TB patients were recruited without selection criteria other than past TB diagnosis and completed treatment. They had been diagnosed with pulmonary ($N = 5$), pleural ($N = 3$), other extrapulmonary ($N = 5$; arthritis, spinal TB, peritonitis, brain abscess, renal) or disseminated TB ($N = 2$) between 3 and 60 years before the start of the present study. Nine of 15 cases were microbiologically proven. Four patients had a clinical diagnosis of TB (pulmonary TB in a technician after laboratory exposure, skin test conversion in association with pleuritis in a contact of a smear-positive colleague, Moroccan man with classical thoracic spine lesions, pulmonary infiltrate in partner of spouse with smear-positive TB) and all four responded favorably to TB treatment only. Two patients had not been treated with tuberculostatic drugs, because those were not available at the time of diagnosis, and reported spontaneous recovery of the disease. No clinical documentation was available for these two patients but the clinical presentation had been compelling, both were positive in QuantiFERON TB Gold and had significant skin test responses to rdESAT-6. All patients were without clinical signs or symptoms of TB disease at the time of the study.

Skin test responses

Skin test responses to tuberculin and rdESAT-6 at four dose levels are shown in Figures 1 and 2 and Table 1. In healthy controls no responses were observed to 0.01 μg rdESAT-6. After injection of 0.1 or 1 μg rdESAT-6, one of five subjects per group had transient local redness of 3 and 1 mm, respectively. The highest dose of 10 μg rdESAT-6 induced a transient redness of 19.4 ± 7.1 mm after 24 h in 5/5 healthy volunteers that was not associated with local complaints and had dissolved completely at 48 h (Figure 3A). Skin test responses to tuberculin were observed in several controls, including three BCG vaccinated (7, 9 and 10 mm induration) and one unvaccinated subject (9 mm induration).

In treated TB patients, the average of the maximal redness in response to 0.01, 0.1 and 1 μg rdESAT-6 was 14.0 ± 10.4 , 19.8 ± 12.6 and 38.8 ± 29.1 , respectively. The corresponding indurations were 7.0 ± 6.6 , 13.4 ± 8.5 and 14.6 ± 11.2 mm, respectively. The differences between skin test responses in the three dose groups did not reach a level of significance, but at the highest dose level two patients had edema and considerable local complaints (see below under Safety). Strong responses to rdESAT-6 were observed in both patients who had never received antibiotic treatment of TB (redness/induration of 29/21, respectively, 63/20 mm, compared to 29/14, respectively, 22/11 mm in response to 2 TU tuberculin). Notably, one patient in each of the three groups of treated TB patients was nonresponsive to

rdESAT-6 two of whom had culture-proven diagnosis and were Quantiferon TB Gold positive.

Responses to 2 TU tuberculin were similar in the three groups of TB patients that received different doses of rdESAT-6. Overall, skin test responses to 0.1 µg rdESAT-6 were almost equivalent to those to 2 TU of tuberculin (Figures 2 and 3B and Table 1), redness and induration being alike. At the time of the study, the investigators were blinded as to the allocation of rdESAT-6 and tuberculin to the left or right arm. During the study, it was noted that the characteristics of indurations on both arms were different with respect to the firmness and the border of induration, with one side feeling softer and the border being more gradually sloped compared to a firmer induration and welldefined border on the contralateral side. These characteristics were not part of the study design, however, and were therefore not consistently recorded.

Kinetics of skin test responses

Maximal redness in response to rdESAT-6 in TB patients was generally observed after 48 or 72 h, which roughly coincided with maximal redness in response to tuberculin. Indurations in response to rdESAT-6 peaked after 48–96 h in different patients. In patients with a maximal induration at 96 h, somewhat smaller but still comparable responses were also observed at 48–72 h. Most patients with maximal responses after 48 or 72 h had slightly smaller responses at 96 h, except for one patient with a 10 mm induration after 48 h which had decreased to 1 mm at 96 h. The optimal read-out time for both skin tests thus appeared to be 48–72 h after injection.

Inter-reader variability of skin test responses

The two independent readings of skin test responses were highly correlated; Spearman's correlation coefficient for the two independent measurements was 0.972 for redness and 0.965 for induration ($P < 0.001$). After exclusion of all readings that were zero for both readers, the correlation was 0.938 for redness and 0.934 for induration ($P < 0.001$). There was no digit preference of readings. The size of the difference between the independent readings of redness as well as of induration were only associated with being a control or TB patient ($P < 0.001$ both for redness and induration), but not with the injected substance being tuberculin or rdESAT-6, the side of injection, the rdESAT-6 dose level, the skin type (white or light brown), or the day of reading. After exclusion of all readings that were zero for both readers, the subject category was no longer significant.

Table 1 Characteristics of study population and skin test responses to tuberculin and rDESAT-6.

Group	No.	Dose rDESAT-6 (µg)	Avg (range) age (yr)	No. (%) BCG vaccinated	Years since diagnosis TB*	Avg (range) of maximal individual responses to rDESAT-6 (mm) [†]		Avg (range) of maximal individual responses to 2 TU PPD (mm) [†]	
						Redness	Induration	Redness	Induration
HC 1	5	0.01	30.1 (19–58)	0		0	0	0	0
HC 2	5	0.1	27.7 (19–45)	1 (20)		0.6 (0–3)	0.2 (0–1)	0.4 (0–2)	0
HC 3	5	1	23.1 (20–26)	1 (20)		0.2 (0–1)	0	3.2 (0–16)	1.8 (0–9)
HC 4	5	10	22.9 (20–25)	2 (40)		19.4 (8–24)	0	9.4 (0–15)	5.6 (0–10)
All HC	20		25.9 (19–58)	4 (20)		5.1 (0–24)	0.05 (0–1)	3.3 (0–16)	1.9 (0–10)
TB 1	5	0.01	63.8 (41–75)	1 (20)	3, 4, 5, 16, 20	14.0 (0–27)	7.0 (0–14)	27.8 (11–43)	13.4 (1–21)
TB 2	5	0.1	46.2 (32–76)	1 (20)	5, 8, 12, 15, 43 [‡]	19.8 (0–30)	13.4 (0–21)	23.6 (0–48)	13.0 (0–21)
TB 3	5	1	62.9 (45–78)	1 (20)	4, 8, 13, 30, 60 [‡]	38.8 (0–63)	14.6 (0–30)	18.8 (12–24)	12.6 (8–18)
TB 4 [§]	0	10							
All TB	15		57.6 (32–78)	3 (20)	Median 12 (3–60)	24.2 (0–63)	11.7 (0–30)	23.4 (0–8)	13.0 (0–21)

Abbreviations: BCG: *M. bovis* bacille Calmette Guérin, HC: healthy uninfected controls, TB: treated TB patients.

*Kruskal–Wallis test for comparison of the time since diagnosis TB between the three groups of TB patients not significant ($P = 0.53$).

[†]Data are expressed as the average (range) of the maximal individual responses observed during the 5 days after injection.

[‡]Denotes the interval since TB was diagnosed in the pre-antibiotic era in two patients.

[§]This group was not tested.

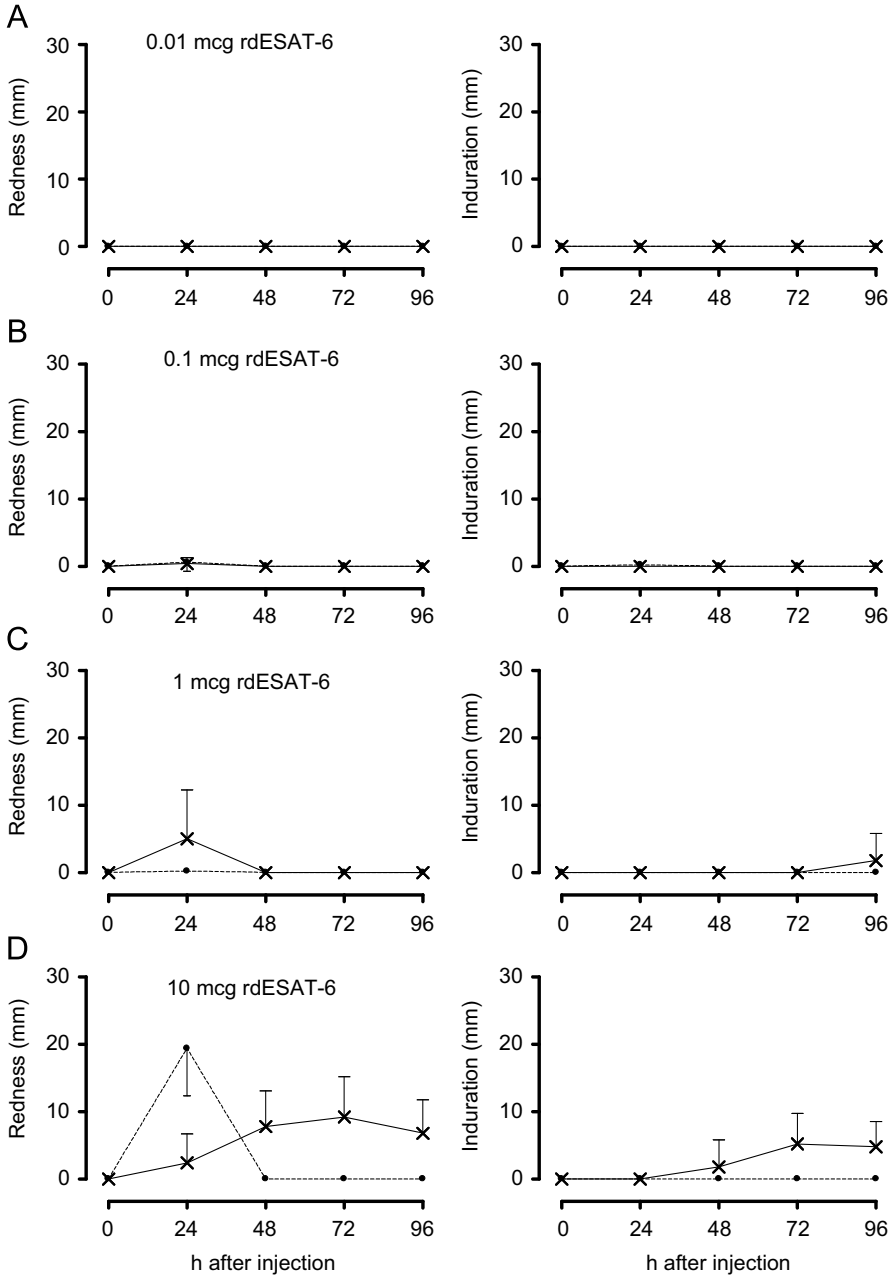


Figure 1 Skin test responses to rdESAT-6 and tuberculin in healthy controls. Average (SD) skin test responses as redness and induration during 4 days after injection of 2 TU tuberculin (solid line) and rdESAT-6 (dotted line) in groups of five healthy uninfected controls per dose level of rdESAT-6.

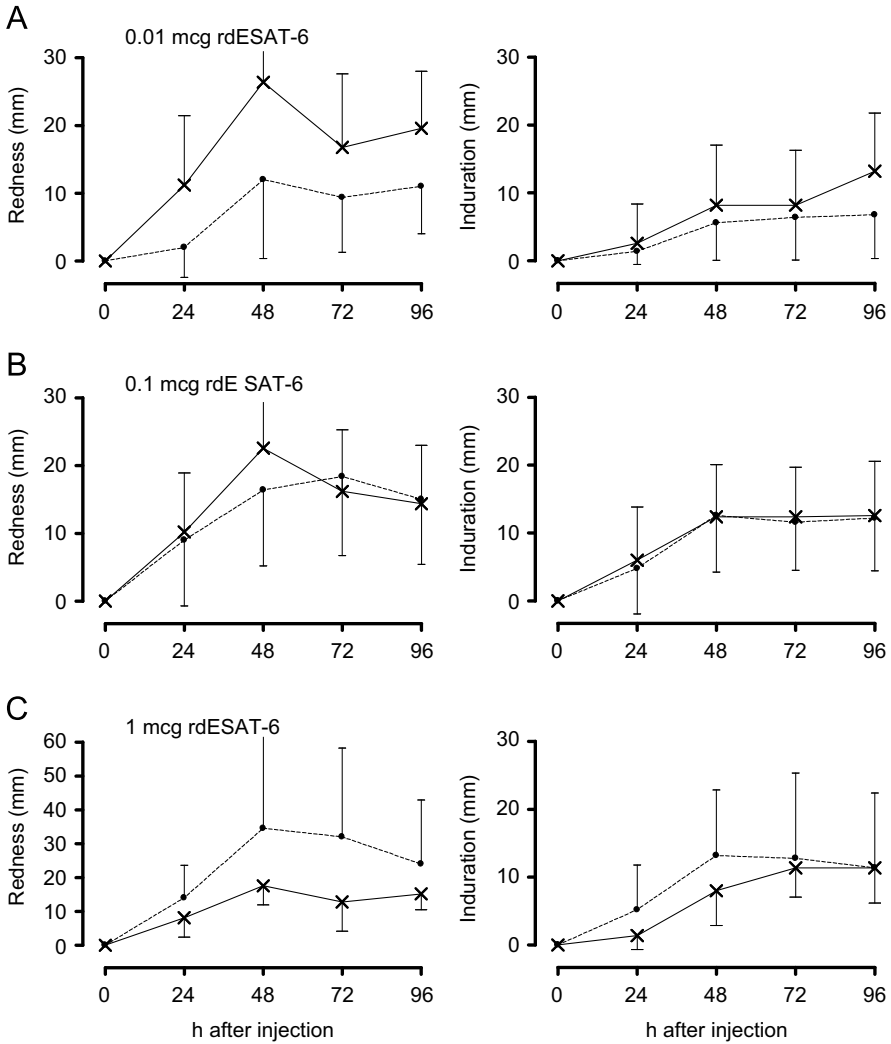
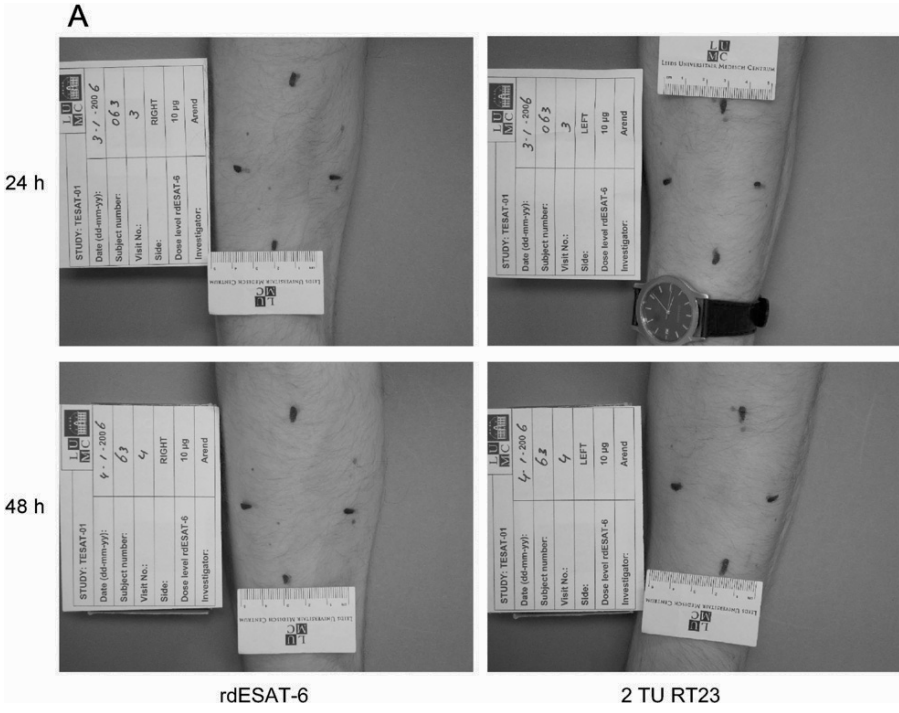
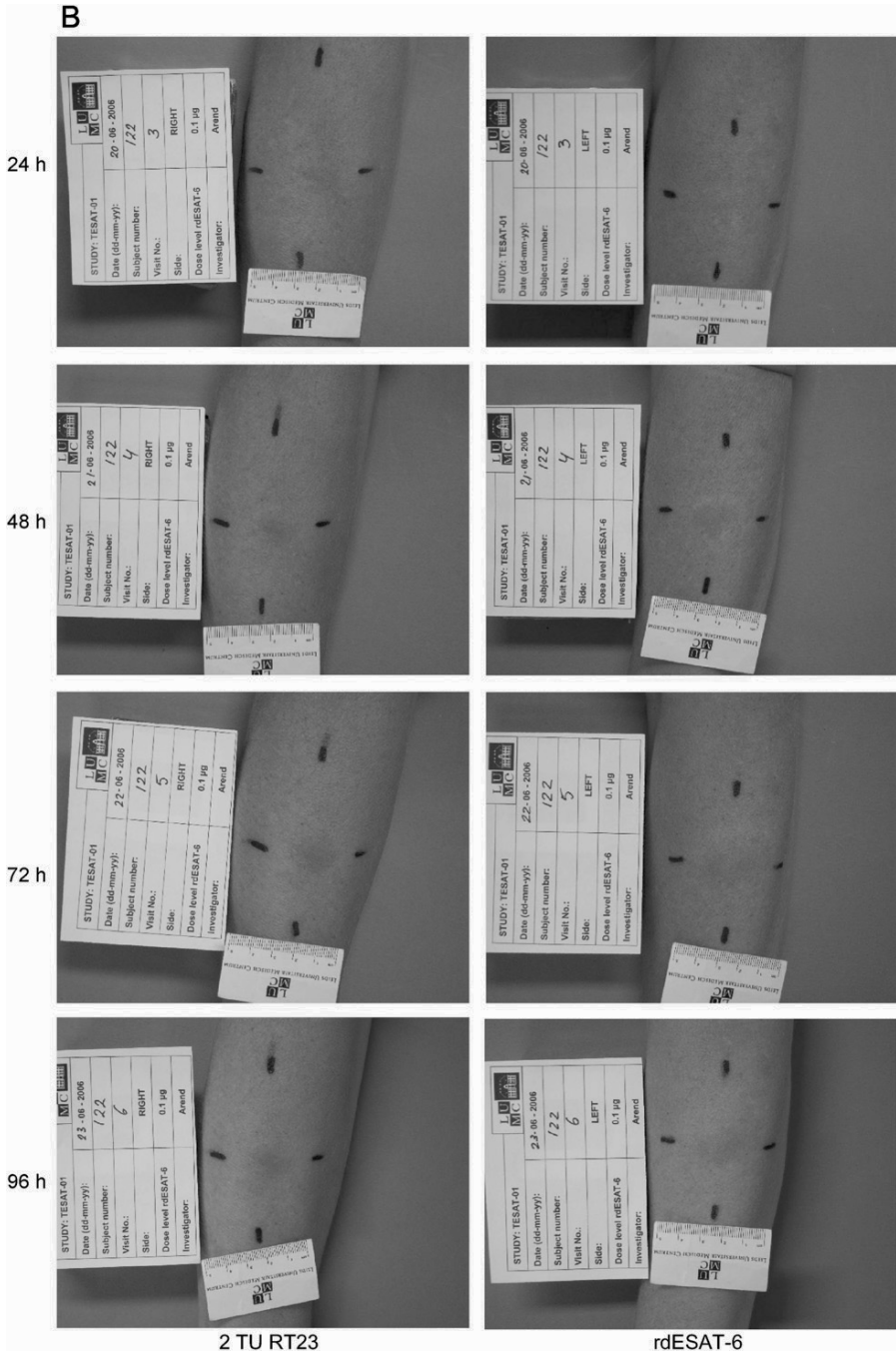


Figure 2 Skin test responses to rdESAT-6 and tuberculin in treated TB patients. Average (SD) skin test responses as redness and induration during 4 days after injection of 2 TU tuberculin (solid line) and rdESAT-6 (dotted line) in groups of five treated TB patients per dose level of rdESAT-6. Note that the Y-axis for redness in treated TB patients injected with 1 μ g rdESAT-6 is expanded to 60 mm.





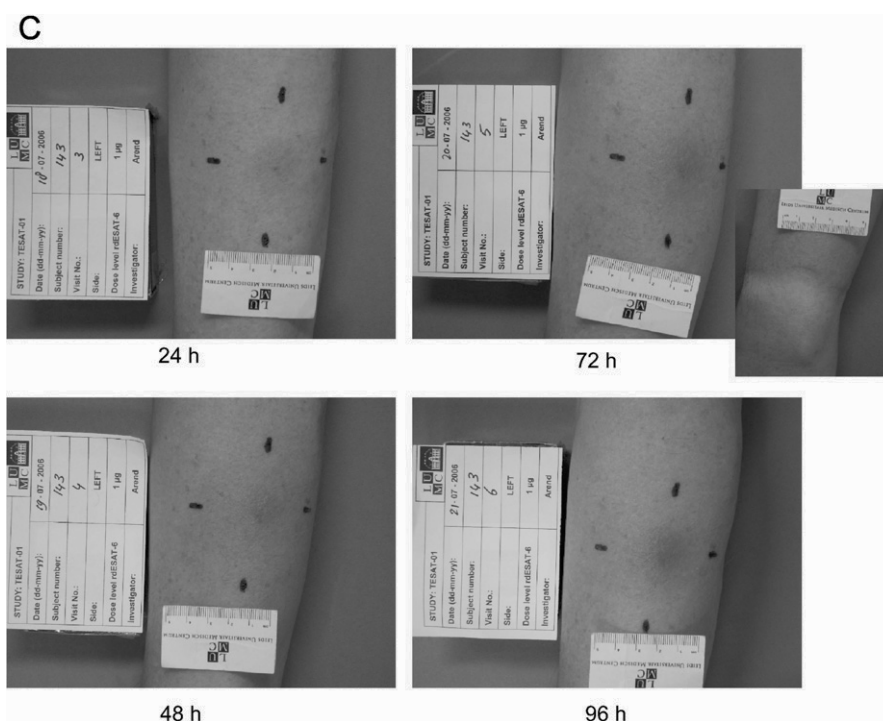


Figure 3 Examples of skin test responses. (A) Representative example of redness in response to 10 µg rESAT-6 at 24 h that has disappeared at 48 h in a BCG unvaccinated healthy control (right forearm). In comparison, a response to 2 TU RT23 is seen at 48 h (left forearm) that was maximal at 72 h (not shown). (B) Comparable responses to 0.1 µg rESAT-6 and 2 TU PPD RT23 in a TB patient. (C) Hyperresponsiveness to 1 µg rESAT-6 in a patient who had suffered from TB 60 years earlier. The maximal area of redness at 72 h measured 63 mm transversely and 123 mm longitudinally, induration was 20 mm (the inset shows wristwatch impression resulting from edema of the forearm).

Safety

Local side effects are listed in Table 2. Healthy uninfected controls all tolerated intradermal tuberculin as well as each of the four dose levels of rdESAT-6 without local complaints or changed laboratory parameters, except for the occurrence of mild local pain 24 h after injection of 10 mg rdESAT-6 in one subject that did not require the use of analgetics.

In TB patients, bilateral skin test responses were already observed at the lowest dose level of rdESAT-6 and mild itch in one person was the only adverse event. At the second dose level responses were comparable in size on both arms, with similar side effects of itch or mild pain. After injection of 1 µg rdESAT-6, local responses were clearly larger and caused considerable local complaints including itch, pain and edema of the forearm in two patients and axillary lymphadenitis and vesiculation in one patient each (responses of one patient are shown in Figure 3C). Based on these observations, it was decided not to proceed to the highest dose level of 10 µg rdESAT-6. After deblinding, the largest responses causing most side effects were confirmed to be related to the 1 µg dose of rdESAT-6.

Table 2 Side effects* caused by intradermal rDESAT-6 or tuberculin (scored as number of persons per group suffering from the side effect/sum of duration in days of the adverse event of all subjects per group).

Group	No.	Dose rDESAT-6 (µg)	Itch		Pain		Vesiculation		Axillary lymphadenitis		Other	
			rDESAT-6	PPD	rDESAT-6	PPD	rDESAT-6	PPD	rDESAT-6	PPD	rDESAT-6	PPD
HC 1	5	0.01	0	0	0	0	0	0	0	0	0	0
HC 2	5	0.1	0	0	0	0	0	0	0	0	0	0
HC 3	5	1	0	0	0	0	0	0	0	1/1*	0	1/1†
HC 4	5	10	0	0	1/1	0	0	0	0	0	0	0
TB 1	5	0.01	1/1	2/3	0	1/1	0	1/4	0	0	0	0
TB 2	5	0.1	3/5	3/7	2/5	3/6	0	0	0	0	0	1/1‡
TB 3	5	1	2/6	3/7	2/4	2/3	1/2	0	1/3	0	2/4§	0
TB 4¶	0	10										

Abbreviations: HC; healthy controls; PPD; purified protein derivative; rDESAT-6: recombinant dimer ESAT-6; TB: tuberculosis patients.

*The occurrence of side effects is expressed as number of individuals per group experiencing the side effect during the study/sum of the number of days that side effect was experienced per group (e.g. two subjects experienced side effect during 3, resp. 1 day is expressed as 2/4).

†Short-lasting stiffness of the muscle proximal to the injection site.

‡Tired feeling of the whole R arm, without loss of function.

§One patient experienced a tingling sensation at the injection site, edema of the forearm in association with itch, pain necessitating use of acetaminophen during 1 day and vesiculation lasting 5 days. The other subject experienced extensive edema of the forearm during 2 days (see inset in Figure 3C), in association with itch, mild pain that did not require treatment and axillary lymphadenitis.

¶This group was not tested.

***In vitro* responses to ESAT-6**

To assess the risk of sensitization by intradermal administration of rdESAT-6 to healthy uninfected controls, *in vitro* whole-blood responses to ESAT-6 were measured by Quanti-FERON TB Gold at 48 h and 4 weeks after injection of the skin tests. Test results of all 20 controls were negative at both follow-up time points indicating that one injection of rdESAT-6 up to 10 µg did not induce a positive *in vitro* T cell response to ESAT-6. Of note, only one injection of rdESAT-6 was given per individual thus *in vivo* sensitization induced by repeated testing was not evaluated.

The first group of TB patients all had a positive QuantiFERON TB Gold response, as indicated in the protocol. Coincidentally responses in this group were higher (10.9 ± 5.5 IU/mL IFN- γ), than responses in the subsequent groups (1.2 ± 1.0 , resp. 1.72 ± 1.86 IU/mL). Two patients in the two higher dose groups had negative pre-skin test QuantiFERON TB Gold responses to ESAT-6 (0.19 resp. 0.28 IU/mL IFN- γ , cut-off ≥ 0.35 IU/mL). The responses could not be determined in one patient in each of the dose groups 0.1 and 1 µg rdESAT-6 due to a failing NIL response and a high background, respectively. In TB patients, there was no statistically significant association between the pre-skin test *in vitro* response to ESAT-6 and skin test responses to rdESAT6 as redness or induration at any dose level or for combined groups (Figure 4). Three of four patients with a negative or indeterminate *in vitro* response at baseline had large-sized skin test responses (≥ 10 mm induration) to rdESAT-6. Of three patients with no skin test response to rdESAT-6 whatsoever, two had culture-proven diagnosis and had a positive QuantiFERON TB Gold response to ESAT-6 (11.83 and 0.52 IU/mL, respectively) and one was Quanti-FERON TB Gold negative but close to the cut-off level (0.28 IU/mL) in the dose groups of 0.01, 0.1 and 1 µg rdESAT6, respectively. The latter patient was the spouse of a person with smear-positive TB and had had a pulmonary infiltrate in association with TST conversion responding to TB treatment.

QuantiFERON TB Gold using ESAT-6 as well as QuantiFER-ON TB Gold in-tube measuring responses to the combination of ESAT-6, CFP-10 and TB7.7 were determined at day 28 after skin testing in all five patients with negative or indeterminate pre-skin test *in vitro* responses. Three now had a strong positive response, similar for both QuantiFERON formats (data not shown).

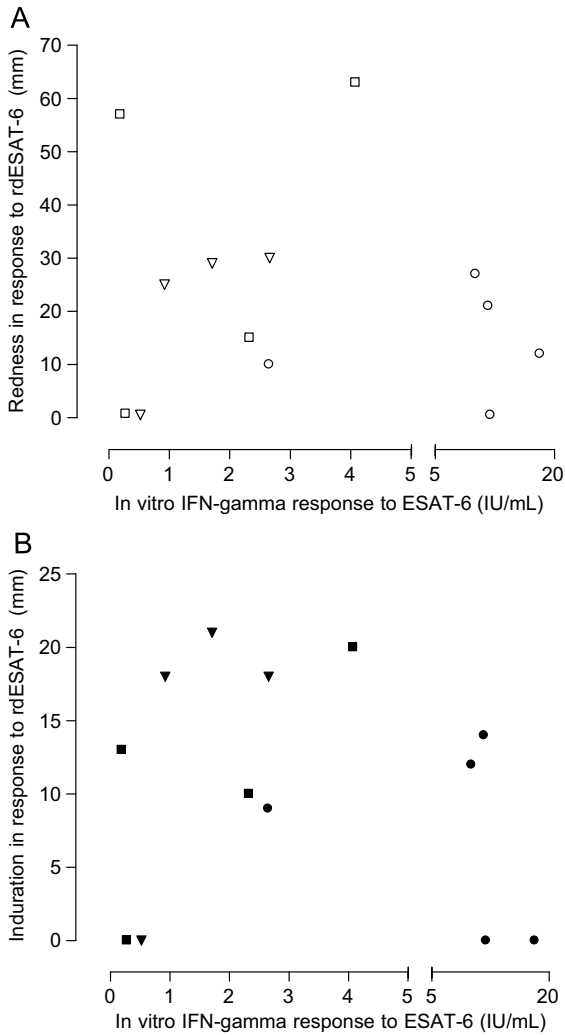


Figure 4 Relation between *in vitro* responses to ESAT-6 and *in vivo* skin test responses to rdESAT-6. Each data point depicts the *in vitro* response to ESAT-6 of one TB patient on the X-axis with the corresponding redness (A) or induration (B) in response to intradermal rdESAT-6 on the Y-axis. Five patients were tested at each dose level. Symbols represent redness (open symbols) or induration (closed symbols) in the different rdESAT-6 dose groups as follows: (○) redness (0.01 µg rdESAT-6); (●) induration (0.01 µg); (▽) redness (0.1 µg); (▼) induration (0.1 µg); (□) redness (1 µg); (■) induration (1 µg).

Discussion

This is the first study of a TB-specific skin test in humans. The results indicate that intradermal injection of rdESAT-6 was safe, that a single dose up to 10 μg did not sensitize uninfected subjects as measured by *in vitro* T cell responsiveness to ESAT-6 and that injection of 0.1 μg elicited a response in treated TB patients that was similar to 2 TU of tuberculin. No skin test responses to rdESAT-6 up to 1 μg were observed in healthy controls including those who were BCG vaccinated. Together these findings demonstrate the feasibility of a TB-specific skin test for human use.

For developing countries a skin test has obvious advantages over *in vitro* tests such as IGRA and most importantly no need for laboratory facilities. A major limitation of the TST is the occurrence of false-positive responses following BCG vaccination or infection with environmental mycobacteria and that limitation would be overcome with a skin test based on ESAT-6 and/or other *M. tuberculosis*-specific antigens. This is a major improvement in test characteristic, because worldwide most individuals still receive BCG vaccination after birth or at school age. The only expected source of cross-reactivity of human relevance could be the rare infections with *Mycobacterium kansasii* or *Mycobacterium marinum* that can induce responses to ESAT-6.^{10,11}

Based on the results of this study, it was concluded that 0.1 μg ESAT-6 was well tolerated, induced responses at 48–72 h of a size similar as those to tuberculin and therefore seems the optimal human dose for intradermal use, in agreement with the predicted human dose based on data obtained in animal studies.¹⁰ Our study provides no definite indication whether the specific skin test should be measured as redness or as induration, or which cut-off value should define a positive result. The only significant response that was observed in TB-uninfected subjects consisted of redness after 24 h at the 10 μg dose level that had disappeared completely after 48 h. This high dose was not tested in TB patients because the 1 μg dose already induced very large responses and local complaints. Thus, it is conceivable that any skin test response to 0.1 μg rdESAT-6, be it induration or redness, observed at 48–72 h can be regarded as relevant. However, whether induration or redness are eventually chosen as a read-out is still open for debate. The measurement of induration is notoriously unreliable and requires training and experience, whereas redness may be difficult to discern in subjects with dark skin color.

Three TB patients in our small study failed to respond to intradermal rdESAT-6 although being *in vitro* responsive to the antigen and *vice versa*. The biological background for this difference is unclear and our study was not designed to address such discrepant responses but it may relate to the different homing potential or activation kinetics of different lymphocyte subsets specific for ESAT-6. In this regard, discrepant positive TST with negative IGRA results have been associated with higher age, indicating that IGRA may be less sensitive to detect infection that was not recently acquired.¹² It is possible that the relatively short incubation time of 24 h that is used in IGRA allows detection of effector T cell responses but is not sufficiently long to detect memory T cell responses. This hypothesis was recently confirmed in two independent studies, showing that longer incubation times indeed increased sensitivity to more than 90% in subjects that were negative in a short incubation IGRA.^{13,14} In this context, a skin test may have the advantage that the antigen is available for recognition and immune stimulation for three as opposed to one day. This may explain the observation that, in contrast to IGRA, the TST has documented high sensitivity for recent as well as remote infection with *M. tuberculosis*.^{1,2} This is in agreement with the data from our study demonstrating that rdESAT-6 induced skin test responses in patients who suffered from TB between 3 and as long as 60 years earlier. There is an ongoing discussion in the IGRA field that attempts to better define recent TB infections as opposed to latent infection.¹⁵ Although more data on the performance of ESAT-6 based IGRA versus skin test in different clinical groups are needed, our data suggest that a novel *M. tuberculosis*-specific skin test could play a key role in a novel definition of LTBI. The idea of this study originated with the successful introduction of IGRA for immunodiagnosis of infection with *M. tuberculosis*. Measured by the number of recent publications, IGRA provide a much-wanted alternative to TST. However, IGRA have limitations and a skin test based on specific antigens might overcome at least some of those.¹⁵ In high-endemic and low-resource settings, the use of IGRA is often not feasible due to logistical and financial limitations, such as costs of the assay, lack of laboratory facilities and skilled personnel. The need of a blood sample has been named as a disadvantage especially in younger children and high rates of indeterminate results have been found in children and immunosuppressed adults.^{16,17} Most importantly, the detection and treatment of LTBI has thus far not been part of TB control policies in most high-endemic regions where the TST is almost uniformly positive and does not discriminate between BCG vaccination and LTBI. Even with a novel TB-specific skin test as described in the present

paper treatment of the immense numbers of latently infected individuals in these regions would be virtually impossible with existing resources and on a global level preventative therapy is recommended by World Health Organization only for HIV-infected persons with LTBI or other individuals at high risk of TB disease.¹⁸

A prognostic marker for TB disease that would allow targeted treatment of the small proportion of latently infected individuals in high-endemic regions who are at highest risk for developing TB disease is therefore an international research priority. A report by Doherty et al.¹⁹ has thus far remained the only indication based on clinical data that a positive IGRA result predicts progression to TB disease during a 2-year follow-up period, but data from a range of animal models have provided strong evidence for the correlation between high ESAT-6 responses and subsequent disease.²⁰⁻²² The results of several large ongoing follow-up studies, as summarized in Ref. 23 may provide more solid evidence for ESAT-6 based prediction of disease and pave the road for a skin test based on *M. tuberculosis*-specific antigens with prognostic potential, as was recently suggested.²³

It remains to be determined whether recombinant proteins are optimal for skin testing. In an *in vitro* culture system, overlapping peptides induced identical responses as recombinant ESAT-6.²⁴ In guinea pigs, individual peptides were compared with recombinant ESAT-6 for skin testing and skin test responses to the N-terminal peptide were observed.⁹ However, the use of overlapping peptides for a human skin test would have disadvantages compared with recombinant proteins with regard to the costs of large-scale production and complexity of quality control. In addition, it is not known whether small-sized peptides might diffuse from the injection site precluding local skin test responses. Our study used rDESAT-6 as a single antigen, but additional specific antigens such as CFP-10 in a skin test will most likely be necessary for maximal sensitivity, as has been demonstrated for IGRA. In guinea pigs, synergy was observed when ESAT-6 was combined with MTP-64 in a skin test.⁹

An important issue concerns the development of novel TB vaccines. Currently, ESAT-6 is tested as a component of a hybrid vaccine. The use of an antigen as vaccine component would preclude its use for diagnostic purposes. However, it is expected that ESAT-6 can be substituted for other immunogenic antigens, which will allow the highly specific and immunodominant ESAT-6 to be reserved for diagnostic purposes.

A limitation of our study was the small number of study subjects and the inclusion of only adult TB patients all but two with completed treatment. The power of the

study was therefore too low to exclude uncommon side effects.²⁵ Further studies should evaluate the safety and test performance in patients with untreated TB disease and those with recently acquired or past LTBI, including pregnant women and children. Repeated intradermal administration of a protein antigen can induce immune responses and a TB-specific skin test should be suitable for repeated use, e.g. in population surveys or yearly screening of risk groups, and should not sensitize uninfected subjects even after repeated use as was observed with high doses and short dosing intervals in a guinea pig model.¹⁰ The risk of sensitization of uninfected humans is currently being studied by repeated administration of rdESAT-6.

In conclusion, this pilot Phase I study of safety, feasibility and dose finding of intradermal rdESAT-6 provides proof of principle of a specific skin test for human use. No serious adverse events were observed among 20 uninfected subjects and 15 TB patients but the study was not sufficiently powered to demonstrate complete safety. In TB patients, a dose of 0.1 µg induced a skin test response of similar size as that to 2 TU of tuberculin. These findings justify more definitive studies of safety, the risk of sensitization after repeated use and the value of additional antigens.

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Ethical approval: The study protocol (EUDRACT No.: 2005001850-26, LUMC protocol No: 05.083), the Investigator's Brochure and the Investigational Medicinal Product Dossier and all remaining documents were approved by the Ethical Review Board of LUMC. All subjects gave informed consent for blood sampling and skin testing after written information was provided.

Data Safety Monitoring Board: The Data Safety Monitoring Board consisted of Dr. H.B. Thio, MD, PhD, (Head) Department of Dermatology, Erasmus Medical Center, Rotterdam, The Netherlands (h.thio@erasmusmc.nl); Dr. H.C. Rumke, PhD, Vaccine Center Rotterdam, Rotterdam, The Netherlands (h.rumke@erasmusmc.nl) and Prof.Dr. C.G.M. Kallenberg, MD, PhD, Department of Internal Medicine, University Medical Center Groningen, The Netherlands, (c.g.m.kallenberg@int.umcg.nl).

Competing Interests: S.A., W.F., C.P. and J.D.: no conflict of interest. H.A., B.T.C., P.N., K.W. and P.A. are all employed by the SSI that own patents on ESAT-6 and sponsored the study.

Contributions: S.A., K.W. and P.A. contributed conceptually; S.A., J.D. and B.T. designed the study; S.A., H.A., B.T. and K.W. took care of regulatory procedures; S.A. and C.P. recruited and screened the study subjects, S.A., W.F. and C.P. conducted the study; S.A. photographed all skin test responses; W.F. was responsible for the blood tests; S.A. documented all clinical data and study results; B.T. and P.N. were study monitors; S.A., W.F. and H.A. analyzed the data; S.A. wrote the first version of the manuscript; W.F., H.A., C.P., J.D., B.T., P.N., K.W. and P.A. commented on the manuscript. S.A. and P.A. finalized the manuscript. S.A. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tube.2007.11.004.

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