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Effectiveness and Safety of a Treatment Regimen Based on Isoniazid Plus Vaccination with *Mycobacterium tuberculosis* cells' Fragments: Field-Study with Naturally *Mycobacterium caprae*-Infected Goats

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Abstract

The identification of a herd of goats with tuberculosis let us test a new treatment regimen against latent tuberculosis infection (LTBI). Using large animal experimental models allows a better approach to understanding human tuberculosis according to immunopathological parameters. Based on an initial study showing a correlation between the ESAT-6-specific interferon (IFN)-γ secretion and the severity of pulmonary lesions, this parameter was used in combination with an X-ray examination to screen the animals to be included in the efficacy and safety studies. All the animals proved to be infected with *Mycobacterium caprae*. The efficacy study was run in animals distributed in three experimental groups according to treatment: untreated (CT), treated with isoniazid (INH), and treated with INH + RUTI (a vaccine based on *M. tuberculosis* cell fragments) inoculated twice. RUTI temporarily increased the IFN-γ production after stimulating the peripheral blood with ESAT-6, purified protein derivative and RUTI *in vitro*. The INH chemotherapy reduced both pulmonary and extra pulmonary affectation, but not disease in pulmonary lymph nodes. The addition of RUTI may have decreased extrapulmonar disease further but had no benefit to lung or lung lymph-nodes itself. Safety studies showed that inoculation of RUTI caused a temporary increase of rectal temperature (1–2 °C) and local swelling, both adverse effects being well tolerated. Neither systemic toxicity nor mortality was induced by the vaccination. The control of goats' infection by the therapeutic regimen consisting in INH chemotherapy + RUTI as well as its safety, represented a further step towards testing its effects in human LTBI in a future.

Introduction

It is estimated that around 30% of individuals exposed to the infective aerosol of patients with active tuberculosis (TB) develop a latent tuberculosis infection (LTBI) [1]. Once *Mycobacterium tuberculosis* enters into an alveolar macrophage and starts to proliferate, it attracts other macrophages to the dying infected macrophage in a process that causes further spread of the bacteria. When several macrophages have died and a specific immune response begins to be generated in the regional lymph nodes, specific lymphocytes that are able to recognize antigens produced by growing bacilli migrate to the site of infection to activate the infected macrophages by secreting interferon (IFN)-γ [2, 3]. This process eventually stops upon control of infection, although bacilli may persist inside the host for years. Reactivation of these persistent bacilli is thought to cause TB in a considerable percentage of population (5–25%) [1]. How these bacilli can persist for such a long time, in what is referred to as a LTBI, is not entirely known. It is traditionally considered that the bacillus becomes dormant and remains

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inside an old lesion until it reactivates its growth, in a context where resuscitation factors are crucial [4]. Alternatively, the dynamic hypothesis holds that bacilli are constantly drained from the lesion towards the alveolar spaces, where growth is reactivated, thus allowing a constant host reinfection [5].

Regardless of the mechanism of latent infection, one-third of human kind is estimated to have LTBI. The fact that this infection gives no symptoms helps persistence of the condition. The vast majority of LTBI cases are not objectively detected, as its diagnosis requires a specific test, the tuberculin skin test, followed by a chest X-ray examination to discard the presence of any lung lesion, and thus the possibility of suffering active TB [6]. Once diagnosed, the standard current therapeutic approach is based on a 9-month administration of isoniazid (INH) [7], although different attempts to reduce the treatment's length have been tried, to increase compliance: use of rifampicin for 4 months, or even for 3 months when combined with INH [8].

Some years ago, a new treatment regimen was proposed: the combination of chemotherapy and RUTI, a therapeutic vaccine based on cell fragments of *M. tuberculosis* [9]. The rational of this regime is based, first, on the ability of short-term chemotherapy to eliminate the growing bacilli, the inflammatory response and the local immunosuppressive environment generated inside the chronic lesions; and second, on a polyantigenic response following RUTI inoculation, which allows the detection of non-replicating bacilli (i.e. latent bacilli) by the immune system [10]. This regimen has already demonstrated its efficacy in different TB experimental models using small and middle size animals, including mice and guinea pigs [9–11] defying previous data indicating that the therapeutic administration of a vaccine would generate the toxic effect known as Koch's phenomenon [12–14]. A further concern with this combined regime was the size of the host used as a model of disease. Traditionally it has been stated that the murine model is the paradigm of resistance against *M. tuberculosis* infection, while susceptibility is reflected by the guinea pig model. These views are mainly based on the ability of mice or guinea pigs to survive or succumb to the infection, respectively [15]. However, when looking at the pathology, it is interesting to note the weak inflammatory response in mice, in contrast to the more 'human-like' immune response in guinea-pigs [15]. This difference between both animal models led to the concept that mice were in fact 'tolerant' to the presence of a high bacillary load and to systemic dissemination while guinea-pigs do try to induce this strong inflammatory response even if it finally kills them [10]. The concept that a strong immune response is a characteristic of larger animals (including humans) raised the need to test this therapeutic approach in appropriate animal models of human TB, to confirm both the therapeutic value and safety of treatment.

The detection of a TB case in a slaughter house led us to the identification of a herd of naturally infected goats. *Mycobacterium caprae*, member of *M. tuberculosis* complex, is the most frequent causative agent of tuberculosis outbreaks in Spanish goats, sometimes related to transmission to humans. Because of the major economical problem that an infected herd represents to the agriculture, several methods of genotyping are used in order to help the epidemiological analysis of these outbreaks [16, 17]. In both natural and experimental infections with *M. caprae*, goats usually generate lesions in the lungs and associated lymph nodes, even if tubercles may also be found in liver and spleen. Histologically, these lesions are similar to those observed in humans. Well-defined granulomas are observed, characterized by the presence of epithelioid cells and numerous giant cells. Acid fast bacilli are usually present, but in very low number [18, 19]. Previous experience with cows showed a relationship between the pathology scoring and the production of ESAT-6-specific secretion in peripheral blood [18]. We seized the opportunity to assay the combined therapy (chemotherapy + RUTI) in large animals, because previous data encouraged us to investigate animals presenting infection better resembling LTBI in humans, i.e. the ones with the lower pathology scoring. Data presented here confirm both RUTI's ability to increase the control of the infection when combined with INH, as well as the safety of this combined therapeutic regime.

Materials and methods

Animals. A herd of 500 Murciano-Granadina female goats was localized in Viladordis (Manresa, Barcelona) after detecting a TB case in the slaughterhouse. Consequently, the rest of the animals were tested for bovine TB infection, by stimulating the whole blood with bovine and avian purified protein derivative of tuberculin (PPD-B and PPD-A, respectively), obtained from Ingenasa (Madrid, Spain) and used in culture at 10 $\mu g/ml$. The specific IFN-γ production was determined using the BOVIGAM ELISA Kit (CSL, Melbourne, Australia) according to the manufacturer's recommendations. The results confirmed the presence of 150 positive infected animals.

Every infected goat was tested for signs of disease or injury before being selected for investigation and, as this was a field study, goats were kept in the farm where they were found. Goats were fed with commercial fodder and were observed daily by farm-workers and weekly by a veterinary to ensure their welfare.

All experimental proceedings were approved and supervised by the Animal Welfare Committee of the Universitat Autònoma de Barcelona in agreement with

the European Union Laws for protection of experimental animals.

Study design. All the infected animals were tested for specific-IFN-γ after whole blood (0.5 ml) stimulation with ESAT-6 (Statens Serum Institute, Copenhagen, Denmark; used at 5 μg/ml), using the BOVIGAM ELISA Kit. Results were recorded as optical density (OD) read at 450 nm.

After the ESAT-6 *ex vivo* assay, the infected animals were distributed in the following three studies, planned with different objectives: (1) a preliminary Correlation Study, to demonstrate the correlation between the ESAT-6-specific IFN-γ secretion and the Pathology Scoring (20 animals); subsequently (2) an Efficacy Study (42 animals); and (3) a Safety Study, to assess the safety of the RUTI vaccine (10 animals) (Fig. 1).

Correlation study. A total of 20 goats with different values of ESAT-6-specific secreted IFN-γ (according to the ESAT-6 *ex vivo* assay) were selected. The animals were euthanized and carefully examined *post-mortem*, in order to record precisely the pathology-score by counting and describing all the tuberculous lesions in lung lobes. Briefly, liver, spleen and extrapulmonary (mesenteric, hepatic and retropharyngeal) lymph nodes were removed for histopathological analysis; as well as seven lung lobes and three pulmonary (tracheobronchial, and cranial and caudal mediastinic) lymph nodes. After being removed, first all samples were carefully examined to detect peripheral lesions and sliced in order to evaluate any internal granulomatous lesion. The number and size of the lesions of each sample were recorded. Then, pathology scoring was made according to published standards [20] (Table 1).

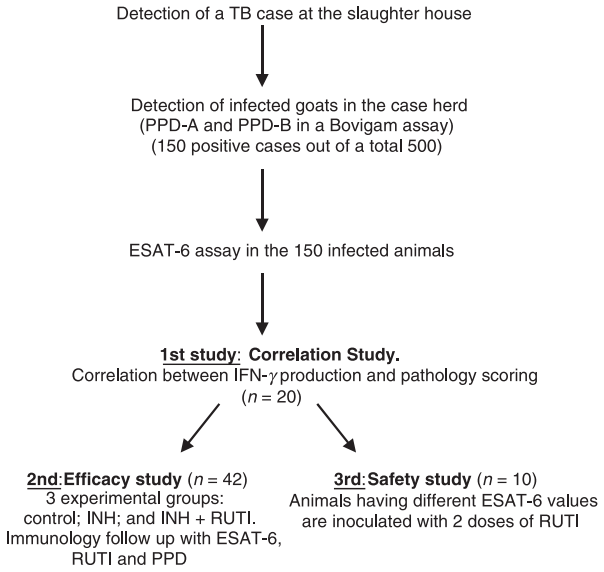

Figure 1 Schedule of all the procedures and study design.

Table 1 Semiquantitative pathology – scoring system (based on Ref [18]).

Score	Type of lesion
0	No visible lesions
1	Lesions <10 mm of diameter, only visible if slicing
2	≤5 lesions of <10 mm of diameter
3	>5 lesions of <10 mm of diameter or 1 lesion ≥10 mm of diameter
4	>1 lesion of ≥10 mm of diameter
5	Big coalescent lesions

The pathology score was compared with the ESAT-6-specific secreted IFN-γ (in means of OD values) using Spearman's test, in order to evaluate any correlation.

Efficacy study. After founding a correlation between the ESAT-6-specific response and the pathology scores, the animals of the herd were tested for a chest X-ray examination, using a portable X-ray unit PXP-20 HF PLUS (Poskom, Goyang, Korea) and the digital system Direct Digitizer Regius Model 110 (Konica Minolta, Tokyo, Japan), to be able to detect at least any lesion >5 mm. The 42 animals with the lowest results in the ESAT-6 *ex vivo* assay (IFN-γ OD <2.00) and a complete normal X-ray examination were selected for studying the therapeutic efficacy of RUTI in infected goats. After distributing the cohort randomly in three experimental groups, animals were followed-up and monitored for the specific cellular immunological response, by means of an assay of IFN-γ secretion *ex vivo*. Once killed, the bacterial load and the pathological scoring were evaluated. The three experimental groups (14 animals each) were: control non-treated; treated with INH (Cemidon, Alcala Farma, Madrid, Spain); and treated with INH + RUTI. INH was administered i.m. in the cervical region, as 300 mg twice a week for 4 weeks (from weeks 0 to 4). The vaccinated group also received two subcutaneous shots of RUTI (batch B04; 271 μg of FCMtb/0.4 ml), in the cervical region, at weeks 4 and 7. RUTI is the name of a therapeutic vaccine made from detoxified and liposomed *M. tuberculosis* cell fragments grown in stressful conditions. The vaccine was kindly provided by Archivel Farma SL (Badalona, Spain) where it is manufactured under strict good manufacturing practice standards, as published elsewhere [9].

For monitoring infection *ex vivo*, whole blood samples were collected at weeks 0, 4, 8 and 11, in order to determine the specific cellular immune response (by measuring secreted IFN-γ) at each time-point. A total of 0.5 ml of heparinized blood was cultured with each stimulus for 18 h at 37 °C in a humidified atmosphere of 5% CO_2. The stimuli used were: RUTI (at 20 μg/ml; Archivel Farma), PPD and ESAT-6 (at 10 μg/ml and 5 μg/ml, respectively, both from SSI, Copenhagen, Denmark). The harvested supernatants were tested for IFN-γ using the

ELISA BOVIGAM Kit (CSL) according to the manufacturer's recommendations, and the results recorded as OD read at 450 nm.

Animals were euthanized at week 11, by an i.v. sodium pentobarbital overdose, in order to evaluate the pathology scoring, as previously described in point 2.3. The removed lungs were submitted to an X-ray examination using the digital system Direct Digitizer Regius Mode 110 (Konica Minolta), with X-ray collimator Ralco, R302 (Beam Limiting Device, Lissone, Italy) located at the Veterinary Hospital in the Universitat Autònoma de Barcelona (Bellaterra, Catalonia, Spain), as well as to histopathological analysis by examining the samples after staining them with Haematoxylin and Eosin and Ziehl-Neelsen. The personnel performing the post-mortems were unaware of the vaccination status of the animals examined.

Homogenates from mediastinic lymph nodes (previously decontaminated according to the Krasnow method [21]) were plated on Middlebrook 7H11 agar (Biomedics, Madrid, Spain) and Lowenstein-Jensen (Biomedics) mediums, at 37 °C for 28 days, in order to evaluate the bacillary load. Colony Forming Units (CFUs) were counted. *Mycobacterium tuberculosis* complex was identified by polymerase chain reaction amplification of the fragments coding for rRNA 16S and MPB70 protein [22]. *Mycobacterium* species characterization was done using a Direct Variable Repeat spacer Oligonucleotide Typing (DVR-spoligotyping) [23]. Both techniques were carried out in the Health Surveillance Laboratory (VISAVET) in the Animal Health Department in the Veterinary School of the Universidad Complutense (Madrid, Spain).

Results of each treatment group were compared for statistically significant differences using a non-parametric test (Kruskal–Wallis) and a posterior Dunn's Multiple Comparison test.

RUTI Safety study. A total of 10 animals with different response to ESAT-6 in the *ex vivo* assay were chosen for inclusion in this study. Animals were all subcutaneously vaccinated twice (3 weeks apart) with RUTI (batch B04; 271 μg of FCMtb/0.4 ml) in the cervical region. Animals were followed for 30 days after the first inoculation. The safety evaluation was done by physical examinations and recording of rectal temperature (at 0, 4, 12 and 24 h and 2, 3 and 4 days after each inoculation), and by inspection of the injection site every 24 h during 4 days after each inoculation. Every abnormality was recorded.

Results

Correlation study: ESAT-6-specific cellular immune response (IFN-γ production) correlates positively with the pathology score

Figure 2 shows how values of IFN-γ production after challenging whole blood with ESAT-6 increase proportionally to the severity of the pathology score in lungs (Spearman r = 0.612 an P = 0.007). Only one animal presented a low IFN-γ production despite having one of the highest pathology scores (29), with a large lung cavity, a phenomenon attributable to an anergic process. The results from this one animal were discarded from the study. The correlation between cellular immune response and pathology allowed us to select animals for the efficacy study: we chose animals with the lowest pathology, avoiding inclusion of anergic animals by scanning all animals by chest X-ray for absence of lung cavities.

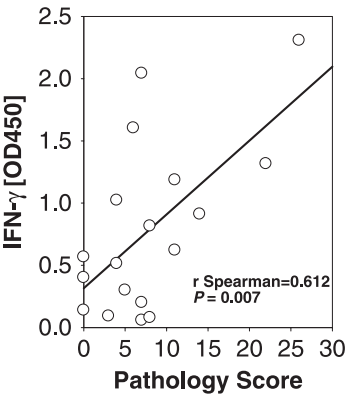

Figure 2 Correlation between the ESAT-6-specific interferon (IFN)-γ secretion and the pathology scores in the Correlation Study.

Efficacy study

The assay *ex vivo* with various stimuli shows a different profile of specific IFN-γ secretion in animals treated with RUTI compared to the other groups. Figure 3 shows the ESAT-6-specific IFN-γ production through the whole follow-up. While from control group did not show any characteristic pattern, INH treatment tended to slightly

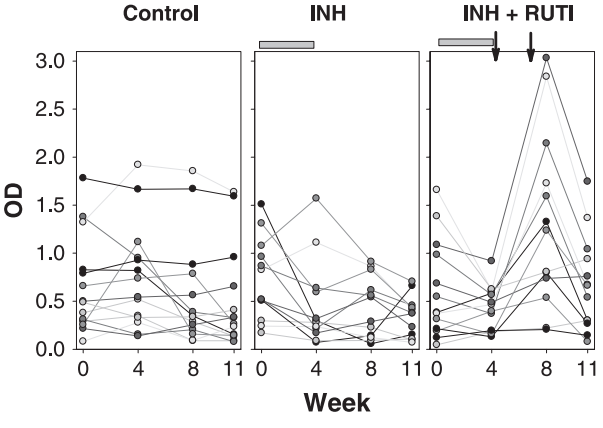

Figure 3 ESAT-6 specific IFN-γ production in the Efficacy Study: evolution through time. Each line reflects the evolution of the values for each animal.

reduce the IFN-γ production, and the RUTI-vaccinated goats experienced a clear increase after each inoculation. The same tendency was observed after stimulating whole blood with PPD or RUTI in the assay *ex vivo* (data not shown).

Treatment with INH changes the pathology scores both in lungs and extrapulmonary tissues. Including RUTI in the therapy seems to help to reduce extrapulmonary dissemination.

Cultures of samples from mediastinic lymph nodes showed an average of two $\log_{10}$ CFUs regardless of the experimental group. This confirmed that all animals were actually infected. The DVR-spoligotyping of all cultures showed a unique spoligotype (*spc-2*), belonging to *M. caprae.*

Mediastinic lymph nodes were usually affected in all animals, presenting a high degree of calcification (Fig. 4). Microscopic analysis of pulmonary lesions showed the presence of human-like granulomas, highly organized, with central necrosis surrounded by macrophages, some giant cells and a well-organized ring of fibroblasts (data not shown).

None of the treatments showed statistically significant differences compared with the untreated (CT) group, but a decreasing tendency for both of them could be observed. The administration of a short-term chemotherapy with INH induced a decrease of the pathology scores in the lungs and extrapulmonary organs. This was not observed in pulmonary lymph nodes. Adding RUTI to the INH did not improve the results in lungs, but seemed to help to prevent the dissemination, as the animals had less pathology scores at the extrapulmonary organs (Fig. 5).

No mortality related to RUTI vaccination was found. Even if one animal from the RUTI group had to be killed in order to ensure the compliance of the Ethical Committee requirements, the necropsy confirmed a hepatic esteatosis because of pregnancy. No other deaths were recorded during the study.

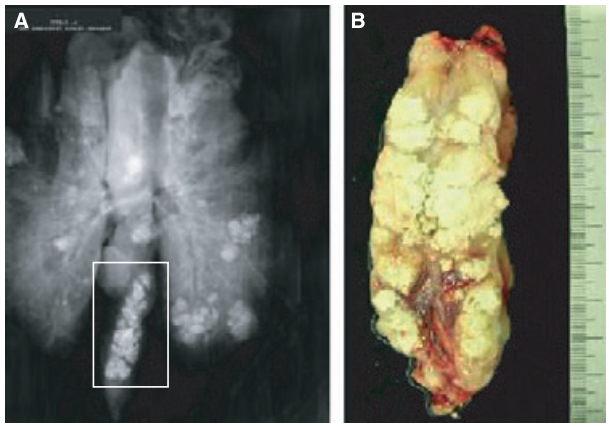

Figure 4 Pathology findings in the efficacy study. (A) A chest X-ray of an infected goat with a high pathology score (22) which correlated very well with the IFN-γ production after challenging peripheral blood with ESAT-6 (OD = 1.316). Note the profusion of calcified lesions, especially in the mediastinic lymph nodes (B).

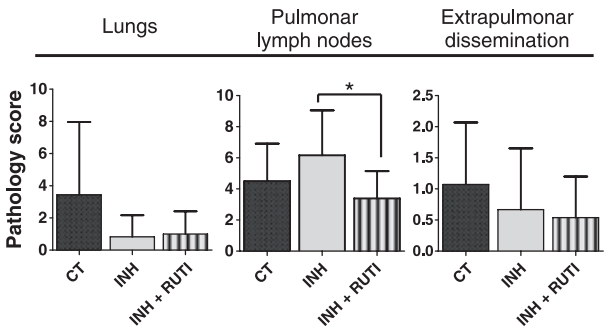

Figure 5 Results of the Efficacy Study, showing the average and standard deviation of the pathology scores in lungs, pulmonary lymph nodes and extrapulmonar organs for each different treatment group. *Statistically significant differences between groups.

Safety study

Neither mortality nor toxicity related to RUTI vaccination were observed when RUTI was administered to infected animals with various ESAT-6-specific responses (thus different pathology scores) without a previous INH treatment. A transient increase in the rectal temperature (1–2 °C) was observed after each inoculation. RUTI also induced an evident induration at the injection site that became stable 48 h after the inoculation (Fig. 6). Histopathology revealed the presence of a TB-like granuloma, very well organized with giant cells, with no acid-fast bacilli being detected (Fig. 7). No correlation between the severity of adverse effects and the initial pathology scores was found (data not shown). In general, RUTI was well tolerated and showed no toxicity in this safety study.

Discussion

Eradication of TB in goats is still a pending issue in Europe. The Catalan Government (Generalitat de Catalunya), through its Department of Agriculture, Livestock and Fishing works together with the CReSA in order to localize infected herds, thus limiting the spread of the disease. The identification of individual infected animals by using the BOVIGAM assay is an initiative to avoid the killing of healthy animals belonging to herds containing infected individuals. This scenario provided a good opportunity for designing an experimental model to evaluate the efficacy and safety of RUTI as a therapeutic vaccine.

Others have used a similar context to test new vaccines prior to testing in humans, especially using cattle

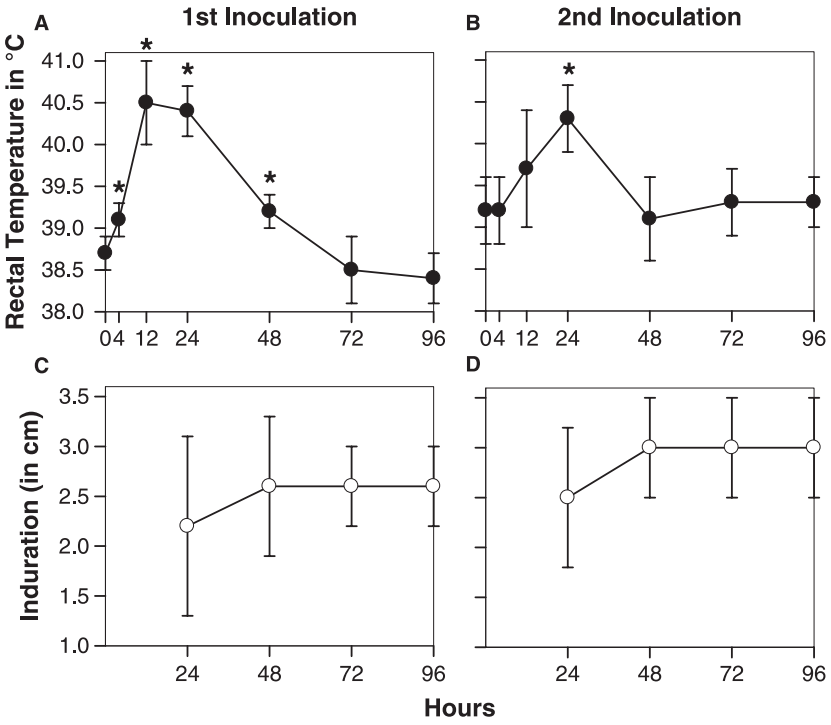

Figure 6 Safety Study. Evolution of rectal temperature (A and B) and local induration (C and D) after the 1st and 2nd inoculation of RUTI in goats not previously treated with chemotherapy.

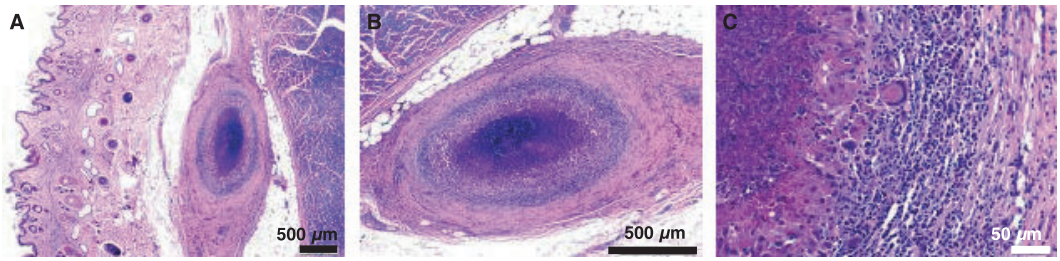

Figure 7 Microscopic aspect of the site of RUTI inoculation. Haematoxylin and eosin stain. Note the TB-like granuloma very well structured and with the presence of intragranulomatous necrosis and giant cells.

[24]. RUTI had previously been tested in murine and guinea pig experimental models, but even if the latter can provide a useful initial approach, and even if no problems of safety were found, a lot of potential uncertainties remained [9–11]. Using goats to test RUTI seemed to us as a good approach, because goats TB and human TB are very similar in the route of infection and the pathological characteristics [18, 19]. The size of the animals was another advantage of this model, being more similar to human's body but still permitting the easy manipulation for extracting samples, giving the treatments and doing the X-ray examinations. Goats also can induce strong inflammatory and immunological responses (as humans), thus giving a better idea about the potential safety profile prior to test any treatment in humans [10].

The major problems of the present study were related to the fact that the animals studied were naturally infected (i.e. the heterogeneity of the study population) and the difficulty of having to discern between LTBI and active TB. Using the previous knowledge provided from experience in cattle, where a high degree of correlation was found between ESAT-6-specific IFN-γ production and the severity of the pathology [20], we attempted to homogenize the study population. As the limit between infection and disease is not clearly defined in animal models, we chose the 42 animals with the lowest responses to ESAT-6 and a chest X-ray without visible lesions. Even if some of the ESAT-6 responses were substantial, thus a greater pathology expected, the X-ray helped to discard those infected animals with detectable active disease. That is the reason for using the both parameters (the ESAT-6 response and the X-ray examination) together in screening the animals, intending to be as much accurate as possible in the selection. With this measure, we also intended to mimic what is currently done in LTBI diagnosis, in order to obtain an animal

model that could resemble as much as possible the human LTBI.

Chemotherapeutic treatment was also a challenge, as no experience existed in treating goats with INH, so in the Efficacy Study we decided to administer INH i.m. twice a week, taking into account its prolonged post-antibiotic effect [25]. Interestingly, the effect of chemotherapy on the pathology was higher than we expected. INH treatment of goats seemed to decrease the protective effect of therapeutic vaccination with RUTI compared with previous studies. This decrease is surprising even when considering the influence of antigenic differences between *M. caprae* and *M. tuberculosis*, which is the basis of the vaccine. Unfortunately, logistic circumstances did not allow us to prolong further the length of the efficacy study, and thus it is not clear for how long the protective effect of the administration of INH alone would be maintained, and what would be the role of RUTI in this regard. The results of the efficacy study did not show statistically significant differences between the treatment regimens and the control group, but a tendency in decreasing the pathology score in lungs and extrapulmonar organs was observed for the INH. Adding RUTI to the INH treatment also seemed to tend to reduce the pathology score at the extrapulmonar organs, suggesting that it may help to prevent dissemination.

Regarding the ESAT-6-specific IFN-γ secretion, it was slightly decreased by the short-term chemotherapy with INH, in marked contrast to previous findings in humans [26] although in this case, the reduced cellular response observed in goats upon INH treatment can be a consequence of the reduction of the bacillary load [27]. Adding RUTI, there is a transient increase in the specific IFN-γ production against ESAT-6, RUTI and PPD, reflecting the boosting effect of RUTI on the immunological response.

Adverse effects related to RUTI administration were not serious and were, in fact, well tolerated even when no prior chemotherapy was administered, which might help decrease the chance of a Koch reaction effect. This finding can help to diminish the existing fear of using mycobacterial antigens for therapy (a fear arising from previous experiences using other formulations) [12–14]. In fact, no previous toxic effects had been related to RUTI in murine or guinea pigs models [9–11], so data presented here confirm its safety profile.

In conclusion, this study showed (in spite of the inconveniences of this being an in-field study) the utility of the goat model for vaccine strategies against tuberculosis. The results showed no increase of the INH effect on the pathology score in lungs when adding the vaccine, but seemed to reduce the extrapulmonar dissemination. RUTI also showed to increase the specific IFN-γ production and additional evidence of the vaccine's safety, the present study representing a further step towards testing its effects in human LTBI in a future.

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