T Cell Monitoring of Chemotherapy in Experimental Rat Tuberculosis

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Mycobacterium tuberculosis is the causative agent of a pulmonary epidemic that is estimated to infect one-third of the world’s population and that has an increased incidence of multidrug resistance. The evaluation of new chemical entities against M. tuberculosis is hampered by the lack of biological tools to help predict efficacy, from early drug development to clinical trials. As the rat is the animal species of choice in the pharmaceutical industry, we have developed a rat model of acute and chronic phases of M. tuberculosis infection for drug efficacy testing. In this model, we have evaluated the impact of tuberculosis drugs on T cell response using the enzyme-linked immunospot assay methodology. Infected rats treated with isoniazid (INH) or rifampin (RIF) responded to therapy, the potency of which was comparable to that seen in the mouse. Peripheral blood mononuclear cells from infected rats produced gamma interferon (IFN-γ) in response to RD-1 antigens, such as the 6-kDa early secretory antigen target (ESAT-6) and the 10-kDa culture filtrate protein (CFP-10). A decrease in IFN-γ spot-forming cells (SFCs) was consistently observed in response to drug treatment. In both the acute- and chronic-phase models, the T cell response was more sensitive to ESAT-6 than to CFP-10. The SFC count in response to ESAT-6 appears to be an indicator of bacterial killing in the rat. Collectively, our data suggest that the ESAT-6 response could be used as a potential surrogate of drug efficacy in the rat and that such a readout could help shorten drug testing during preclinical development.

Mycobacterium tuberculosis infection is among the world’s leading infectious diseases, causing about 2 million deaths annually. The emergence of multidrug-resistant M. tuberculosis strains along with the increase of HIV coinfected cases worsens the situation (42). In countries with a high incidence of tuberculosis (TB), TB control programs rely on a diagnostic methods and drugs that have been developed decades ago and that are inadequate to effectively control the epidemic. The urgent need to develop new diagnostic tools as well as new therapeutic interventions is hampered by long clinical trials, where markers of infection and drug response are lacking (38).

For decades, the tuberculin skin test (TST) has been used to diagnose TB (18). The TST measures cell-mediated immunity in the form of a delayed-type hypersensitivity response to the purified protein derivative (PPD), a crude mixture of antigens shared among M. tuberculosis, Mycobacterium bovis BCG, and several nontuberculous mycobacteria (NTM). As a result, the TST has lower specificity in populations with high BCG coverage and NTM exposure and shows poor sensitivity in immunocompromised individuals (30). The gamma interferon (IFN-γ) enzyme-linked immunospot (ELISpot) assay has emerged as an alternative to the TST. The assay consists of in vitro stimulation of peripheral blood mononuclear cells (PBMCs) using RD-1 antigens, the 6-kDa early secretory antigen target (ESAT-6) and the 10-kDa culture filtrate protein (CFP-10), which are early secretory antigens specific to M. tuberculosis (41). The presence of specific IFN-γ spot-forming cells (SFCs) is indicative of an M. tuberculosis infection and seems to correlate better than the TST with the level of exposure to M. tuberculosis (3). In this context, the IFN-γ ELISpot assay is considered to be a major advance in TB diagnostics.

The IFN-γ ELISpot assay has also been tested as a means to monitor the response to TB therapy, where efficacy is classically evaluated by measuring rates of relapse 6 to 12 months after treatment completion (11, 43). On the basis of the hypothesis that the IFN-γ ELISpot assay could function as a bacterial load sensor, a comparatively smaller amount of SFCs in response to ESAT-6 and/or CFP-10 over time would be indicative of a good response to treatment, leading to a lower probability of relapse. Though encouraging, the readings of recent exploratory studies were confounded by immune status, stage of infection, and previous drug treatment; and no clear conclusion was reached with regard to the predictive value of the ELISpot assay as a marker of therapy response (1, 5, 13, 19, 28). Relapse as a clinical trial endpoint is a key issue in drug development. Under optimal treatment conditions and compliance, relapse rates for nonmultidrug-resistant patients lie at about 2 to 4%. Therefore, efficacy trials aimed at significantly improving relapse rates require the recruitment of large patient cohorts and 4 to 5 years for completion. Hence, the identification of early markers of drug response would have a tremendous impact on therapeutic interventions and on the shortening of long clinical trials (40).
With a widely accepted predictive value in toxicology and pharmacokinetics, the rat has been a species of choice for early drug development. It has also helped to identify markers of disease and surrogate markers of drug response in chronic inflammation and age-related diseases (2, 17, 26). Recent studies, including some from our group, have also shown that M. tuberculosis-infected rats develop organized granulomas within the lungs while controlling the infection over time (32). This suggests that the rat may be a good alternative to the mouse model for in vivo drug efficacy studies (4, 34). The rat offers the additional advantage of providing larger volumes of body fluids and larger numbers of PBMCs than the mouse, which makes the model attractive to study the response of circulating immune cells as it is performed in tuberculosis patients.

In this context, we have established a model of acute and chronic phases of M. tuberculosis infection in the rat to test the pharmacokinetic and pharmacodynamic properties of antituberculosis compounds in early drug discovery stages. Here we report on the development of a rat version of the ELISpot assay used in clinical settings. We observe that this immunological assay appears to be suitable to understand some aspects of drug-mediated killing in the rat models of acute and chronic phases of M. tuberculosis infection. Our results show that while the current ELISpot assay has limitations in predicting drug efficacy, the rat model is a suitable tool to optimize immunological assays which could then be used in clinical studies for monitoring drug efficacy.

MATERIALS AND METHODS

Animals. Female Wistar rats 8 to 10 weeks of age were purchased from the Biological Resources Centre Singapore and housed in individually ventilated cages within the biosafety level 3 facility. Studies described in this report were approved and performed according to the guidelines and policies of the Institutional Animal Care and Use Committee (IACUC).

Peptides and bacterial strains. Synthetic peptides were synthesized by Mimotopes Pty. Ltd. (Clayton, Victoria, Australia) on the basis of the amino acid sequences. Each peptide contains 20 amino acid (aa) residues, with 10 residues overlapping adjacent peptides covering the full length of the corresponding proteins. The H37Rv M. tuberculosis strain (catalog no. 27294; ATCC) was cultured in 7H9 medium enriched with 10% ADS (albumin, dextrose, saline) and 0.05% Tween 80 (Sigma-Aldrich) for 2 to 5 days at 37°C. Mycobacteria were grown to an optical density at 600 nm (OD600) of 0.3 to 0.5 in 7H9-OADC medium and centrifuged at 2,200 × g for 10 min, and the pellet was washed once in warm 7H9-OADC medium. The washed pellet was resuspended at an OD600 of 1 (equivalent to 1.47 × 10^9 CFU) in 7H9-OADC medium supplemented with 15% glycerol and frozen at −80°C. On the day of infection, 1 ml of culture stock was thawed and added to 9 ml of 7H9 medium, before being subjected to sonication for 30 s. It was then serially diluted before being subjected to sonication for 30 s. It was then serially

Drug efficacy and ELISpot assay response in the rat model of acute phase of M. tuberculosis infection. In order to determine whether the IFN-γ ELISpot assay could be used to monitor chemotherapy and drug-mediated killing, we first treated rats with 10 mg/kg INH during the acute phase of infection and investigated the early specific ESAT-6 response at 48 h and 7 days posttreatment. The number of SFCs observed after 48 h of INH treatment (day 9 postinfection) is significantly higher than that in untreated infected animals (P = 0.024; Fig. 2A). After 1 week of treatment (day 14 postinfection), we did observe a significant decrease of bacterial load in the INH-
treated group (Fig. 2B), with concomitant drops in the IFN-γ/H9253 response and SFC counts which were below those observed in untreated infected animals. These results suggest that, in the TB rat model, INH-mediated killing appears to produce a burst of antigen release which in turn is reflected in transiently high SFC counts. Rats were then treated for a month with 10 mg/kg INH during the acute phase of infection, from day 7 postinoculation onwards. As expected, INH at 10 mg/kg was very effective at killing M. tuberculosis within 4 weeks (Fig. 3A), as previously described in the mouse model of the acute phase of M. tuberculosis infection (16). Using PBMCs isolated from M. tuberculosis-infected rats, we tested the impact of INH on the number of IFN-γ/H9253-producing cells specific to ESAT-6 and CFP-10 at 2 and 4 weeks post-treatment. A significant decrease in the number of IFN-γ/H9253-producing cells after ESAT-6 stimulation was observed in the treated group compared to the nontreated group after 2 weeks of treatment (*, P < 0.05; Student’s t test), comparison between unstimulated and stimulated cells. The experiment was carried out twice. CT, control (unstimulated) group.

We also observed that the average diameter of PBMC spots was smaller for INH-treated animals than for infected untreated rats. This suggests that the amount of IFN-γ produced by a single immune cell from a treated rat is smaller than the one produced by PBMCs of untreated infected rats (Fig. 4A). When we assessed the impact of INH treatment on the average spot diameter after 4 weeks of treatment, we observed a dramatic reduction in spot size diameter in response to both ESAT-6 and CFP-10 (Fig. 4B). We also evaluated the serum levels of IFN-γ during treatment, and we observed that serum IFN-γ decreased when infected rats were treated with INH (Fig. 4C). Taken together, our results indicated that INH treatment reduced the proportion of SFCs in response to ESAT-6 and CFP-10 and that these specific circulating cells were producing less cytokine, resulting in a decrease of secreted IFN-γ in serum.

Drug efficacy and ELISpot assay response in the rat model of chronic phase of M. tuberculosis infection. We then tested the ELISpot assay in a model of the chronic phase of infection where the rats were infected and treated for 4 weeks starting at 1 month postinfection. We observed a slow drop of lung CFU
counts following treatment with 25 mg/kg of INH once daily (Fig. 5A) that was significant only after 4 weeks of treatment ($P < 0.05$). This is in agreement with what is seen in the mouse model, where INH is less efficient during the chronic phase of infection. Interestingly, a significant decrease in ESAT-6 response was detected after 2 weeks of INH therapy ($P < 0.023$; Fig. 5B), a time point at which the CFU drop was not yet affected significantly. After 4 weeks of therapy, both lung CFU and ESAT-6 responses were significantly lower ($P < 0.0286$ and $P = 0.015$, respectively). The CFP-10 response was significantly lower only after 4 weeks of therapy ($P = 0.038$; Fig. 5C).

These observations show that a significant decrease in ESAT-6 response preceded the significant drop in lung CFU, suggesting that the ESAT-6 response is very sensitive to INH-mediated killing.

Treatment with RIF at 25 mg/kg daily for a month was more efficient in reducing lung CFU over time than treatment with INH ($P < 0.0003$; Fig. 6A), which is in agreement with RIF generally being recognized as more active during the chronic phase of infection in the mouse model (15, 20, 25). When the T-cell response was tested 2 weeks after RIF therapy (Fig. 6B), we detected a significant drop following ESAT-6 stimulation, which was confirmed at 4 weeks with CFP-10 stimulation as well ($P = 0.009$ and $P = 0.011$, respectively; Fig. 6C). This mirrored the reduction of lung bacterial load in response to
RIF treatment. Overall, treatment with either INH or RIF caused a CFU reduction, which is reflected by a drop in the number of specific IFN-γ-secreting cells in response to stimulation with RD-1 antigens, with the ESAT-6 response being the most sensitive to therapy.

DISCUSSION

In this study, we have shown that the efficacy of isoniazid and rifampin in the TB rat model was comparable to what has been reported in the mouse. We have then used this rat model to compare the ELISpot assay readout before and after treatment and evaluate whether there is a link between drug-mediated killing and T cell response. A direct correlation between ELISpot assay and CFU counts was not established in this study, as we had a limited set of data points for only one drug dose. However, the reduction of bacterial counts within the lung translated into a reduction of peripheral T cell response to ESAT-6 and CFP-10 stimulation, with the ESAT-6 response being more sensitive to treatment. The apparently less sensitive and slower CFP-10-mediated response appears to be in agreement with results reported in several clinical studies, where PBMCs from active TB cases responded more specifically to ESAT-6 (19, 31, 36). While the ESAT-6 response was shown to be dependent on CD4+ T cells (7, 23), it is not clear how T cells react to CFP-10 epitopes (14, 45). In our rat model,
analysis of cell recruitment into the lungs has shown that CD4+/IFN-γ+ T cells seem to be recruited rapidly within 2 weeks postinfection, which could favor an early response to ESAT-6 (A. Singhal, unpublished data). Therefore, drug-mediated killing of M. tuberculosis in rat lung could be reflected in the early peripheral T cell response to ESAT-6. We also detected a specific T cell burst after 2 days of INH treatment. This rapid and transient increase could potentially be exploited as an indicator of drug-mediated killing in good responders. Accordingly, a clinical study by Wilkinson et al. (43) revealed an increase in T cell response immediately after the onset of treatment, likely as a result of antigen release following bacillary killing and lysis (8, 39). The ELISpot assay has also been used to investigate B cell response following TB chemotherapy, where a rapid change of antibody production only a few days after the beginning of treatment was indicative of bacillary killing and antigen release (33). We observed that the number of peripheral IFN-γ SFCs as well as the circulating levels of IFN-γ in serum was reduced at 1 month postinfection. The recruitment of IFN-γ-positive T cells to the site of infection might be responsible for the apparent reduction of T cell response which we observed in the blood (27, 29). However, regulatory T cells which develop during active disease may also prevent the specific IFN-γ SFC response to RD-1 antigens (7, 21). Direct stimulation of pulmonary immune cells in our assay should confirm whether there is a concomitant increase in lung-specific IFN-γ SFC numbers, as recently shown in the monkey model (22). As the RD-1 antigens are secreted by the bacilli, a decrease of bacterial load due to drug-mediated killing would translate into a decrease of T cell stimulation and thus fewer IFN-γ SFCs specific to ESAT-6/CFP-10. The reactivity of effector T cells would then decrease as a result of antigen withdrawal as the level of production of IFN-γ from a single immune cell decreased after treatment. It has been shown that IFN-γ production after 24 h of stimulation fades upon treatment and correlates with the development of memory T cells (10). Cytokine production might also change after treatment, as recently suggested (24). Further studies would help to evaluate the impact of treatment on different T cell populations and on the role of newly described polyfunctional T cells (44). During the chronic phase of infection, the ESAT-6 response was significantly affected after 2 weeks of INH therapy, while a significant drop in lung CFU was seen only 4 weeks later. A recent study challenged the theory of static equilibrium—i.e., no killing, no growth—during the chronic phase of infection and showed that (i) M. tuberculosis does replicate during this stationary phase and (ii) the apparently static number of bacteria found in the lungs is thought to be a balance between growth and immune-mediated killing (9). In light of these observations, the replicating subpopulation of bacteria could be rapidly affected by INH therapy, causing a decrease in the ESAT-6 response, while the total lung CFU count is only slightly affected. In this context, the ESAT-6 response could be used to predict drug efficacy, as it precedes a significant decrease in the numbers of lung CFU. While the TB mouse model is by far the most convenient and widespread experimental tool, mice do not develop organized, necrotic, and hypoxic lesions (6, 35, 37). It is only recently that the rat has been investigated as a potentially predictive model of TB infection (4, 34). Our group has recently shown that Wistar rats can form organized granulomas. We have also observed that the Wistar rat can develop subclinical manifestations of human disease with no detectable CFU in the lung, where bacilli can be reactivated upon immunosuppression (32). Furthermore, we tested the hypothesis that during the chronic phase of infection the Wistar rat could develop hypoxic lesions that may favor dormancy and phenotypic drug tolerance. We did observe a pimonidazole staining within rat granulomas, indicative of low oxygen tension (12). Therefore, the Wistar rat could be a relevant preclinical model to test new chemical entities and evaluate drug response through new innovative biological assays based on the ELISpot assay concept.

In summary, we have shown that the efficacy of INH and RIF in the Wistar rat model of TB infection is comparable to what is seen in the mouse model and that a reduction of the ESAT-6 response accompanies CFU reduction in the lung. Such an assay could reduce the timelines of preclinical drug testing in animal models and could be used to evaluate how future treatment-shortening drugs would affect the assay readout. Importantly, our results suggest that the rat model is a useful tool to modify and optimize the ELISpot assay and to develop a predictive biomarker of drug response to be tested in clinical studies.

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