Simultaneous Measurement of Antigen-Stimulated Interleukin-1β and Gamma Interferon Production Enhances Test Sensitivity for the Detection of Mycobacterium bovis Infection in Cattle

Gareth J. Jones,*† Chris Pirson,† R. Glyn Hewinson, and H. Martin Vordermeier

TB Research Group, Veterinary Laboratories Agency—Weybridge, New Haw, Addlestone, Surrey, KT15 3NB, United Kingdom

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Bovine tuberculosis (TB) is a zoonotic disease caused by the bacterial pathogen Mycobacterium bovis, which in some developed countries continues to pose a major economic and animal health problem for the farming community. Although the transmission of M. bovis infection from cattle to humans has been dramatically reduced following the introduction of milk pasteurization and the “test and slaughter” of tuberculous cattle, the zoonotic potential of bovine TB still remains a concern in countries with insufficient or no control policies (5, 9, 12). Furthermore, this may not be a problem for developing countries only: a recent study in San Diego, CA, reported that M. bovis accounted for 45% of all culture-positive pediatric TB cases from 1994 through 2005, potentially through the consumption of imported unpasteurized dairy products (20).

The generation of a T helper type 1 (Th1)-biased cell-mediated immune (CMI) response is of vital importance in the control of mycobacterial infections, particularly through the production of gamma interferon (IFN-γ) and its subsequent activation of macrophage microbicidal pathways (1, 18). The importance of this cytokine is highlighted in mouse studies, where animals deficient in IFN-γ fail to control infection with a sublethal Mycobacterium tuberculosis challenge (4). Furthermore, this induction of a CMI response, as characterized by IFN-γ production, has been exploited in the development of diagnostic assays for mycobacterial infection in different host species, including cattle (26). The Bovigam IFN-γ assay, an in vitro test that detects IFN-γ production in bovine whole blood stimulated with antigens such as purified protein derivative from M. bovis (PPD-B), has been incorporated into the bovine TB control programs of numerous countries as an ancillary test to the standard tuberculin skin test (reviewed in reference 6). This test also has several practical advantages, including (i) avoidance of repeat visits to farms to read test results, (ii) flexibility in setting appropriate cutoffs, (iii) more opportunity for rapid repeat testing, (iv) an opportunity to use a range of M. bovis-specific antigens, (v) a quantitative readout that can be automated, and (vi) a protocol that is easy to standardize and control for quality in the laboratory, in contrast to the skin test, performed on the farm under sometimes difficult conditions.

A potential disadvantage of the Bovigam IFN-γ assay, however, is that it relies solely on a single cytokine readout for the detection of M. bovis infection. Other readouts for bovine cellular immune responsiveness to M. bovis infection have been described, including tumor necrosis factor alpha (TNF-α) and nitric oxide (25). Recently, Chegou et al. demonstrated that for accurate diagnosis of human patients at differing stages of tuberculosis disease, simultaneous measurement of at least three different host analytes was required (2). Thus, in the study presented here, we aimed to identify additional host...
cytokines that may be useful as candidates for inclusion in diagnostic tests for *M. bovis* infection in cattle. To this end, we utilized a novel multiplex system to simultaneously compare the levels of IFN-γ, interleukin 1β (IL-1β), IL-4, IL-10, IL-12, macrophage inflammatory protein 1β (MIP-1β), and TNF-α in whole-blood cultures from TB reactor animals or TB-free controls following stimulation with *M. bovis*-specific antigens.

**MATERIALS AND METHODS**

**Cattle.** All animals were housed at the Veterinary Laboratories Agency (VLA) at the time of blood sampling. Procedures were conducted within the limits of a United Kingdom Home Office License under the Animal (Scientific Procedures) Act of 1986 and were approved by the local ethical review committee.

(i) **TB reactors.** Heparinized blood samples were obtained from 35 naturally infected, single intradermal comparative tuberculin test (SICTT)-negative animals from BTB-free herds located in regions of England, by government veterinarians of the Animal Health Agency. A detailed postmortem examination of these TB reactor animals revealed visible TB lesions in all but 3 animals, confirming the presence of active disease.

(ii) **Uninfected controls.** Heparinized blood samples were obtained from 26 SICTT-negative animals from BTB-free herds located in regions of England where TB is not endemic.

**Stimulation of whole-blood cultures.** Heparinized blood samples were stimulated with either 10 μg/ml bovine tuberculin (PPD-B; VLA, Weybridge, United Kingdom), 1 μg/ml staphylococcal enterotoxin B (SEB; Sigma-Aldrich, United Kingdom), 5 μg/ml ESAT-6/CFP-10 peptide cocktail (23), or RPMI 1640 alone as a negative control (NIL antigen). Whole-blood cultures were incubated at 37°C in the presence of 5% CO2 for 24 h, after which plasma supernatants were harvested and stored at −80°C until required.

**Cytokine multiplex assay.** Simultaneous detection of IFN-γ, IL-1β, IL-4, IL-10, IL-12, MIP-1β, and TNF-α was performed as previously described (3). Briefly, multiplex 96-well plates were commercially prespotted (Meso Scale Discovery [MSD], Gaithersburg, MD) with the following capture antibodies: anti-IFN-γ (all from MSD), and Sulfo-Tag streptavidin (MSD) in MSD dilution buffer. Plates were blocked with MSD assay buffer, after which whole-blood culture supernatants or standards were added for 2 h. The following standards were used (with the highest concentration given in parentheses): IFN-γ (100 ng/ml; Endogen), IL-1β (20 ng/ml; MSD), IL-4 (2 ng/ml; MSD), IL-10 (30 μg/ml; IAH, Compton, United Kingdom), IL-12 (1,000 U/ml; IAH), TNF-α (5 ng/ml; Endogen), and MIP-1β (10 ng/ml; MSD). Plates were then washed and incubated for a further 2 h with a cocktail of biotinylated secondary detection antibodies (for IFN-γ, IL-1β, IL-4, IL-10, IL-12, MIP-1β [all from MSD], and TNF-α [Endogen]) and Sulfo-Tag streptavidin (MSD) in MSD dilution buffer. After a final wash, MSD buffer T was added, and the luminescence signal was measured on the MSD 6000 plate reader (MSD). Antigen-specific results are expressed as background-subtracted values (e.g., the antigen-induced cytokine concentration minus the cytokine concentration with the NIL-antigen control).

**Bovigam IFN-γ test.** Whole-blood aliquots were cultured as described above. IFN-γ levels in the plasma supernatants were quantified by using the Bovigam enzyme-linked immunosorbent assay (ELISA) kit (Prionics AG, Switzerland) according to the manufacturer’s instructions.

**RESULTS**

To identify the cytokines that may be useful as candidates for inclusion in diagnostic tests for *M. bovis* infection in cattle, we compared the levels of IFN-γ, IL-1β, IL-4, IL-10, IL-12, MIP-1β, and TNF-α in whole-blood cultures from TB reactor animals or TB-free controls following stimulation with *M. bovis*-specific antigens (PPD-B or ESAT-6/CFP-10). Quantification was performed using a novel luminescence-based multiplex assay, allowing for the simultaneous measurement of the seven cytokines in the same sample. As shown in Table 1, PPD-B induced significantly greater production of IFN-γ, IL-1β, and TNF-α in TB reactor animals than in controls. Although the difference did not reach statistical significance, PPD-B-induced production of IL-10, IL-12, and MIP-1β also tended to be greater in TB reactor animals. On the whole, the ESAT-6/CFP-10 peptide cocktail induced lower levels of cytokine production than PPD-B. However, the same three cytokines (IFN-γ, IL-1β, and TNF-α) were produced to significantly greater levels in TB reactor animals than in controls. Again, although the difference did not achieve statistical significance, ESAT-6/CFP-10-induced IL-12 and MIP-1β levels tended to be higher in TB reactor animals.

**Statistical analysis.** Receiver operator characteristic (ROC) curves were analyzed, and cutoff levels for differentiating between groups were determined, using GraphPad Prism 5 software, while the Mann-Whitney U test was performed using GraphPad InStat 3 software (both from GraphPad Software, Inc.).

**TABLE 1. Median levels of antigen-induced cytokines**

<table>
<thead>
<tr>
<th>Cytokine (unit of measurement)</th>
<th>PPD-B − NIL</th>
<th>ESAT-6/CFP-10 − NIL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median cytokine level (range) in:</td>
<td>Median cytokine level (range) in:</td>
</tr>
<tr>
<td></td>
<td>TB reactors (n = 35)</td>
<td>Controls (n = 26)</td>
</tr>
<tr>
<td>IFN-γ (ng/ml)</td>
<td>16.01 (0.79–69.99)</td>
<td>0.84 (0.83–9.94)</td>
</tr>
<tr>
<td>IL-1β (ng/ml)</td>
<td>1.91 (−9.35–25.44)</td>
<td>0.31 (−0.16–0.99)</td>
</tr>
<tr>
<td>TNF-α (ng/ml)</td>
<td>2.00 (−2.593–13.472)</td>
<td>0.95 (−4.47–4.55)</td>
</tr>
<tr>
<td>IL-10 (U/ml)</td>
<td>5.93 (0.65–23.64)</td>
<td>2.54 (0.86–5.21)</td>
</tr>
<tr>
<td>IL-12 (U/ml)</td>
<td>26.67 (33.9–199.4)</td>
<td>13.38 (0.14–93.31)</td>
</tr>
<tr>
<td>MIP-1β (ng/ml)</td>
<td>11.04 (520–4296)</td>
<td>8.07 (4.82–64.27)</td>
</tr>
<tr>
<td>IL-4 (ng/ml)</td>
<td>0.01 (−9.35–25.44)</td>
<td>0.01 (−0.17–3.4)</td>
</tr>
</tbody>
</table>

* For comparison of levels in TB reactors and controls by the Mann-Whitney U test.

Vol. 17, 2010

IL-1β AIDS IFN-γ IN DETECTING *M. BOVIS* INFECTION 1947
Using the data from the ROC curve analysis, it was possible to establish the relative sensitivities of *M. bovis* antigen-induced IFN-γ, IL-1β, and TNF-α for the detection of *M. bovis*-infected animals. A predetermined specificity of 96% was chosen, since this most closely represented the specificity of the Bovigam IFN-γ test, an *in vitro* laboratory-based assay currently used in the United Kingdom as an ancillary test to the SICTT (6). At this level of specificity, the proportions of PPD-B-induced IFN-γ, IL-1β, and TNF-α responders were 80%, 54%, and 32%, respectively (Table 2). Although IL-1β or TNF-α performed less well than IFN-γ when analyzed alone, we next investigated whether IL-1β and/or TNF-α could be used as a readout system to complement IFN-γ. When the different cytokines were used in combination, an animal was considered test positive if the response of at least one of the cytokines in that combination was greater than the cutoff value. When IFN-γ, IL-1β, and TNF-α were used in parallel, the

FIG. 1. Significant areas under the curve (AUC) for PPD-B-induced IFN-γ, IL-1β, and TNF-α production in ROC curve analysis. ROC curves show the accuracies of PPD-B-induced cytokines at distinguishing between TB reactor animals and uninfected controls. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$ (ROC curve analysis for AUC).

FIG. 2. Significant areas under the curve (AUC) for ESAT-6/CFP-10 peptide cocktail-induced IFN-γ, IL-1β, and TNF-α production in ROC curve analysis. ROC curves show the accuracies of ESAT-6/CFP-10-induced cytokines at distinguishing between TB reactor animals and uninfected controls. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$ (ROC curve analysis for AUC).
In this study we hypothesized that the diagnostic test sensitivity for *M. bovis* infection could be improved if different cytokines, or a combination of cytokines, were used as an alternative readout to IFN-γ. Our results showed that, in addition to IFN-γ levels, IL-1β and TNF-α levels were also significantly elevated in cultures from TB reactor animals following stimulation with either PPD-B or the ESAT-6/CFP-10 peptide cocktail. When analyzed individually, neither cytokine proved to be as sensitive a readout as IFN-γ for the detection of *M. bovis*-infected animals, irrespective of the stimulating antigen. For responses induced by the ESAT-6/CFP-10 peptide cocktail, the use of all three readouts (IFN-γ, TNF-α, and IL-1β) in parallel increased the proportion of *M. bovis*-infected animals detected by 11% but also resulted in a specificity decrease of 14%. However, the use of only IFN-γ and IL-1β in parallel resulted in a 5% increase in sensitivity without a corresponding loss of specificity. The results for PPD-B were similar, although the loss of specificity was more pronounced, even when only IFN-γ and IL-1β were used as readout systems. This loss in specificity may be due to lipid and/or carbohydrate components of PPD-B driving a more nonspecific response characterized by TNF-α and IL-1β production from monocytes, an effect that is unlikely to be prevalent when one uses a cocktail of synthetically synthesized peptides. In addition, PPD-B also contains many nonspecific proteins, lipoproteins, and glycolipids that are shared with environmental non-tuberculous mycobacteria. Thus, taken together, there is a greater potential for variability with PPD-B responses, which may contribute to the decreased specificity.

In experimentally infected cattle, cytokine gene expression profiles following restimulation with PPD-B or ESAT-6/CFP-10 have been shown to fluctuate over time (22). Indeed, IFN-γ responses peaked 30 days postinfection, while TNF-α responses were maintained at later time points (unfortunately, IL-1β was not investigated). Although this issue is beyond the scope of our study, it should be noted that the time relative to initial infection may impact the cytokine response and the accuracy of the various readouts described here.

The proinflammatory cytokine IL-1β plays a critical role in the host defense against mycobacterial infections. It has been suggested that IL-1β, in concert with TNF-α, plays an important role in driving IFN-γ-dominated immune responses and the formation of granulomas (10, 19). Recently, a reduction in bovine PPD-induced IL-1β production was shown to be associated with impaired cell-mediated delayed-type hypersensitivity responses following repeated short-interval skin testing in cattle (3). Genetically modified mice deficient in either IL-1β or IL-1R are highly susceptible to *M. tuberculosis* infection, exhibiting increased mortality and greater mycobacterial growth in lung tissue (8, 11, 16). Studies with both mice (13, 14) and cattle (7) have shown that macrophages release IL-1β upon infection with a mycobacterium, a response that appeared to be closely associated with the presence of mycobacterial region of difference 1 (RD1) (13, 14). Furthermore, *M. tuberculosis* culture filtrate proteins have long been known to induce IL-1β production by human monocytes (24). Indeed, the mycobacterial protein ESAT-6, a substrate for the ESX-1 secretion system encoded by RD1, is in itself sufficient for IL-1β production by macrophages (17). However, given that these interactions involve the innate immune response, the question of why we observed greater levels of *M. bovis* antigen-induced IL-1β in TB reactor animals than in controls remains. Recently, Mas-

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**TABLE 2. Abilities of PPD-B-induced cytokines to distinguish between TB reactor animals and controls**

<table>
<thead>
<tr>
<th>Cytokine detected</th>
<th>Cutoff (ng/ml)</th>
<th>Sensitivity (%) (95% CI)</th>
<th>Specificity (%) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ alone</td>
<td>&gt;8.33</td>
<td>80 (63–92)</td>
<td>96 (80–100)</td>
</tr>
<tr>
<td>IL-1β alone</td>
<td>&gt;0.98</td>
<td>54 (37–71)</td>
<td>96 (80–100)</td>
</tr>
<tr>
<td>TNF-α alone</td>
<td>&gt;4.17</td>
<td>32 (17–51)</td>
<td>96 (80–100)</td>
</tr>
<tr>
<td>IFN-γ, IL-1β, or TNF-α</td>
<td>As above</td>
<td>89 (73–97)</td>
<td>88 (70–98)</td>
</tr>
<tr>
<td>IL-1β or TNF-α</td>
<td>As above</td>
<td>89 (73–97)</td>
<td>92 (75–99)</td>
</tr>
<tr>
<td>IFN-γ or TNF-α</td>
<td>As above</td>
<td>80 (63–92)</td>
<td>92 (75–99)</td>
</tr>
<tr>
<td>IL-1β or TNF-α</td>
<td>As above</td>
<td>66 (48–81)</td>
<td>92 (75–99)</td>
</tr>
</tbody>
</table>

*a Data for 35 TB reactor animals and 26 control animals were analyzed.

*b (Cytokine response to PPD-B) – (cytokine response to the NIL antigen).

*c 95% CI, 95% confidence interval.

**TABLE 3. Abilities of ESAT-6/CFP-10-induced cytokines to distinguish between TB reactor animals and controls**

<table>
<thead>
<tr>
<th>Cytokine detected</th>
<th>Cutoff (ng/ml)</th>
<th>Sensitivity (%) (95% CI)</th>
<th>Specificity (%) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ alone</td>
<td>&gt;2.72</td>
<td>69 (51–83)</td>
<td>96 (80–100)</td>
</tr>
<tr>
<td>IL-1β alone</td>
<td>&gt;0.45</td>
<td>54 (37–71)</td>
<td>96 (80–100)</td>
</tr>
<tr>
<td>TNF-α alone</td>
<td>&gt;4.11</td>
<td>32 (17–51)</td>
<td>96 (80–100)</td>
</tr>
<tr>
<td>IFN-γ, IL-1β, or TNF-α</td>
<td>As above</td>
<td>80 (63–92)</td>
<td>82 (75–99)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>As above</td>
<td>74 (57–87)</td>
<td>96 (80–100)</td>
</tr>
<tr>
<td>IFN-γ or IL-1β</td>
<td>As above</td>
<td>77 (60–90)</td>
<td>92 (75–99)</td>
</tr>
<tr>
<td>IL-1β or TNF-α</td>
<td>As above</td>
<td>63 (45–79)</td>
<td>92 (75–99)</td>
</tr>
</tbody>
</table>

*a Data for 35 TB reactor animals and 26 control animals were analyzed.

*b (Cytokine response to ESAT-6/CFP-10) – (cytokine response to the NIL antigen).

*c 95% CI, 95% confidence interval.
ters et al. have demonstrated that IFN-γ stimulation drives increased IL-1β production in human cells (15). Thus, we speculate that the greater levels of IFN-γ observed in whole-blood cultures of TB reactor animals following stimulation with *M. bovis* antigens (Table 1) may provide a positive feedback loop resulting in amplification of IL-1β production by blood monocytes.

In order to investigate the diagnostic potential of IFN-γ and/or IL-1β measured in the novel multiplex system in relation to the standard IFN-γ release assay, the Bovigam IFN-γ test was also performed on a subset of the animals (19 TB reactors and 18 uninfected controls). For logistical reasons, the multiplex cytokine responses to PPD-A were not measured in this study, so an analysis was performed comparing PPD-B or ESAT-6/CFP-10 responses to cytokine production by unstimulated cultures (PPD-B ⨉ NIL and ESAT-6/CFP-10 ⨉ NIL) (Table 4). For a predetermined specificity of 94%, ROC curve analysis of the Bovigam data revealed a sensitivity of 58% for PPD-B ⨉ NIL responses. When the cutoffs detailed in Table 2 for PPD-B ⨉ NIL responses were applied to this subset of animals, IFN-γ only or IFN-γ in combination with IL-1β readouts as measured by the MSD system resulted in sensitivities of 63% or 79%, respectively. A similar analysis was performed for the ESAT-6/CFP-10 ⨉ NIL responses, revealing sensitivities of 95%, 58%, and 63% for Bovigam, MSD (IFN-γ only), and MSD (IFN-γ or IL-1β) responses, respectively. Although these systems were studied with a subset of animals, these results suggest that the measurement of PPD-B-induced IFN-γ production using the multiplex system is at least as sensitive as the Bovigam ELISA at detecting *M. bovis*-infected animals. Thus, these results again highlight the potential for improved diagnostic sensitivity when IL-1β is measured simultaneously.

Unlike PPD-B, the ESAT-6/CFP-10 peptide cocktail used in this study has been developed as a so called DIVA diagnostic reagent that is capable of distinguishing between *M. bovis*-infected cattle and uninfected animals vaccinated with bacillus Calmette-Guérin (*Mycobacterium bovis* BCG) (21, 23), which is at present the only vaccine available for bovine tuberculosis. Thus, it is particularly important to note the benefit of using the combined readout of IFN-γ and IL-1β—improved diagnostic sensitivity with no loss in specificity—following stimulation with potential DIVA reagents, such as the ESAT-6/CFP-10 peptide cocktail.

In summary, the results described here demonstrate that the use of IL-1β as an additional readout system can potentially complement IFN-γ by increasing overall test sensitivity for the detection of *M. bovis* infection in cattle. IL-1β can be measured in the same culture supernatants used to measure IFN-γ for the Bovigam IFN-γ test. Moreover, both cytokines can be easily and conveniently measured in a multiplex system, such as the MSD technology available to us. So far, however, we have only provided proof of concept with a limited number of animals. The potential sensitivity enhancement needs to be determined more precisely with a larger number of animals and a wider array of antigens.

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**REFERENCES**


8. Fremond, C. M., D. Togbe, E. Doz, S. Rose, V. Vasseur, I. Mailet, M. Jacobs, B. Ryffel, and V. F. Quesniaux. 2007. IL-1 receptor-mediated signal is an essential component of MyD88-dependent innate response to *Mycobac-


