Toward the Development of Diagnostic Assays to Discriminate between Mycobacterium bovis Infection and Bacille Calmette-Guérin Vaccination in Cattle


A scientific review of the recent sharp increase in bovine tuberculosis in Great Britain has concluded that the development of a cattle vaccine holds the best prospect for long-term disease control. It is important to develop a diagnostic test that differentiates between vaccinated and Mycobacterium bovis–infected animals, to ensure that test-and-slaughter control strategies can continue alongside vaccination. The mycobacterial antigens ESAT-6, MPB64, and MPB83 are expressed at high levels in M. bovis but are expressed at low levels or not at all in bacille Calmette-Guérin (BCG) Pasteur. Promiscuous bovine T cell epitopes of these antigens were identified and formulated into a peptide cocktail. This cocktail and a cocktail composed of recombinant forms of the 3 antigens was able to distinguish cattle infected with virulent M. bovis from those vaccinated with BCG and from those sensitized to avian tuberculosis in lymphocyte transformation and interferon-γ assays.

Bovine tuberculosis is caused by Mycobacterium bovis. It is a zoonotic disease and was the cause of ~6% of total human deaths due to tuberculosis in the 1930s and 1940s, as well as >50% of all cervical lymphadenitis cases involving children [1, 2]. The introduction of pasteurization of milk in the 1930s dramatically reduced the transmission from cattle to man, and, in 1995, only 1% of all tuberculosis isolates in England and Wales were identified as M. bovis [2]. However, human tuberculosis caused by M. bovis is still a major health issue in many developing countries [3–6].

In developed countries, bovine tuberculosis has severe implications for animal welfare, and affected farms suffer painful economic losses. In recent years, the control strategy for tuberculosis in cattle in Great Britain, based on tuberculin testing and slaughter of infected animals, has failed to prevent a sharp increase in cases of bovine tuberculosis, especially in the south-west of England. A recent independent scientific review of this problem for the government [7] concluded that the development of a cattle vaccine against M. bovis holds the best long-term prospect for tuberculosis control in British herds.

Encouraging results with BCG vaccination have been reported from New Zealand, where significant protection has been observed in BCG-vaccinated cattle subsequently challenged with M. bovis [8, 9]. However, vaccination with BCG compromises tuberculin PPD specificity (both in cattle and in humans) [10, 11]. Thus the development of cattle vaccines based on BCG (or attenuated M. bovis strains) will require the identification of specific, defined antigens that allow discrimination of M. bovis–infected animals from those that have been vaccinated.

Such candidate antigens are ESAT-6 and MPB64, which are not expressed in BCG Pasteur because of the lack of the genes encoding these proteins [12–14]. In addition, the M. bovis antigens MPB70 and MPB83 are expressed at high levels in virulent M. bovis but at low levels in BCG Pasteur [15, 16] and are strongly recognized by sera and T cells from M. bovis–infected cattle [17, 18]. It has been previously demonstrated that both ESAT-6 and MPB64, when used as skin-test reagents, discriminate between infected and BCG-vaccinated guinea pigs [19]. In addition, ESAT-6 has been shown to discriminate between cattle infected with tuberculosis and cattle sensitized by environmental mycobacteria [20].

To assess the feasibility of developing differential diagnostic tests for a live vaccine, we tested recombinant forms of ESAT-6, MPB64, MPB70, and MPB83 alone or in combination with use of peripheral blood mononuclear cells (PBMC) from M. bovis–infected, BCG-vaccinated, and Mycobacterium avium–sensitized calves. In addition, T cell epitopes from these antigens were determined, and synthetic peptides that encompass “promiscuous peptides” derived from ESAT-6, MPB64, MPB70, and MPB83 were formulated into a peptide cocktail. Our results have provided proof of principle that both protein-based and suitably formulated peptide-based cocktails can be employed to specifically diagnose M. bovis infection in field-reactor cattle without being compromised by BCG.
Materials and Methods

Cattle

We studied cattle of various breeds and ages, which we divided into 4 group groups, as follows.

**Experimental M. bovis infection.** Female calves were infected with a field strain of *M. bovis* from Great Britain (AF2122/97) by intratracheal instillation of 10⁴ cfu, as described elsewhere [8, 9, 20, 21]. Blood samples were obtained 4 months postinfection to perform the mapping experiments. Positive tuberculin skin-test reactions were observed in those animals at the time of necropsy (5 months postinfection). The necropsy revealed gross pathology typical of natural bovine tuberculosis, with visible granulomatous lesions in the lymph nodes in the head region, upper respiratory tract, and pulmonary region, as well as in the lungs. Acid-fast bacilli were observed in and *M. bovis* was isolated from the tissue samples taken.

**BCG vaccination.** Female calves aged (6–12 months) were vaccinated with BCG Pasteur by sc injection of 10⁶ cfu into the side of the neck [8, 9], followed 8 weeks later by a booster injection (same route and dose). Blood samples were taken between 3 and 5 weeks after the booster vaccination to test the diagnostic reagents.

**Controls.** Animals with stronger responses to avian PPD (PPD-A) than to mammalian PPD (PPD-M) were included as examples of sensitization with environmental mycobacteria (nonspecific reactors). Blood was also obtained from animals from herds free of tuberculosis, as negative controls.

**Field samples.** Blood samples were obtained from cattle of mixed breeds that had been designated tuberculin skin test reactors by the single intraeradical comparative tuberculin test (SICTT; field reactors). The skin tests were performed as specified (European Economic Community [EEC] directive 80/219/EEC, amending directive 64/422/EEC Annex B). Blood samples were obtained from reactor animals between 3 and 4 weeks after the tuberculin skin test, and all samples were transported by courier to our laboratory within 8 h of sampling and were processed immediately upon arrival. Routine postmortem inspections of carcasses were performed on these animals, and tissue samples were collected for histologic analysis and for *M. bovis* culture.

With the exception of table 1, all data presented are based on those obtained for confirmed reactors (i.e., *M. bovis* could be isolated from tissues and/or tuberculous lesions were found on necropsy and confirmed by histology). Table 1 also lists results from unconfirmed reactors (i.e., no visible lesions were found, and *M. bovis* could not be isolated from tissue samples).

**Antigens.** The genes that encode ESAT-6, MPB64, MPB70, and MPB83 of *M. bovis* were cloned and expressed as histidine-tagged fusion proteins in pET21d. The recombinant proteins were purified with Ni-af®nity chromatography, as described by the manufacturer (Novagen, Cambridge, U.K.). Ag85 complex antigen purified from *Mycobacterium tuberculosis* short-term culture filtrate was kindly provided by Dr. K. Huygen (Pasteur Institute, Brussels). All proteins were used at concentrations of 10 µg/mL in culture. When tested in a protein cocktail, ESAT-6, MPB64, and MPB83 were each used at concentrations of 5 mg/mL.

Synthetic peptides (for ESAT-6, 11 peptides; for MPB64, 30 pep-

Table 1. Sensitivity of diagnostic cocktails tested in single intradermal comparative tuberculin test–positive (SICTT⁺) field-reactor cattle.

<table>
<thead>
<tr>
<th>Readout system, antigens</th>
<th>Confirmed tuberculosis</th>
<th>Unconfirmed tuberculosis</th>
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<tbody>
<tr>
<td><strong>Proliferative T cell responses</strong></td>
<td></td>
<td></td>
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<tr>
<td>PPD-M &gt; PPD-A</td>
<td>16/21 (76.2)</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>Protein cocktail</td>
<td>17/21 (80.9)</td>
<td>5/8 (62.5)</td>
</tr>
<tr>
<td>Peptide cocktail</td>
<td>16/21 (76.2)</td>
<td>4/8 (50)</td>
</tr>
<tr>
<td>IFN-γ &lt; PPD-M &gt; PPD-A</td>
<td>20/21 (95.2)</td>
<td>7/8 (87.5)</td>
</tr>
<tr>
<td>Protein cocktail</td>
<td>16/21 (76.1)</td>
<td>5/8 (62.5)</td>
</tr>
<tr>
<td>Peptide cocktail</td>
<td>12/16 (75)</td>
<td>3/7 (42.8)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. of cattle positive/no. tested (%). Cpm, counts per minute; OD, optical density; PPD-A, avian PPD; PPD-M, mammalian PPD; SI, stimulation index.

* M. bovis could be cultured from tissue samples and/or presented with visible tuberculous lesions that were found on necropsy and confirmed by histology.

* No visible lesions found on necropsy and no *M. bovis* cultured from tissue samples.

Peripheral blood mononuclear cells from field reactors were isolated and cultured within 8 h of blood sampling and tested in lymphocyte transformation assays. Results were calculated as an SI: (cpm with antigen)/(cpm without antigen). Results were deemed positive for recombinant proteins and cocktails when the SI was ³3 and the signal strength of cpm with antigen was >1000. PPD-M vs. PPD-A responses: an SI (OD₄₅₀ with PPD-M)/SI (OD₄₅₀ with PPD-A) was deemed a positive response.

Blood samples from field reactors were cultured within 8 h of sampling. Whole-blood was cultured in the presence of antigens for 24 h. Results were calculated as interferon SI (ISI): (OD₄₅₀ with antigen)/(OD₄₅₀ medium control).

Peptide and protein cocktails, results were deemed positive when the ISI was ≥2. For tuberculin responses, a positive result was defined by an OD₄₅₀/PPD-M = OD₄₅₀/PPD-A value >0.05. For antigen concentrations, see Materials and Methods.

tides [16 residues long and overlapping by 8 residues]; and for MPB83, 22 peptides (20 residues long, overlapping by 10) were synthesized by solid-phase peptide synthesis, as described elsewhere [22], or by multirope peptide synthesis [23] and were used in mapping experiments at 25 mg/mL. The latter peptides were purchased from Chiron Mimotopes (Clayton, Australia). Mammalian (PPD-M) and avian (PPD-A) tuberculins were obtained from the Tuberculin Production Unit at the Veterinary Laboratories Agency–Weybridge and used in culture at concentrations of 10 mg/mL.

Composition of Peptide Cocktail

The peptide cocktail was composed of the following peptides (sequences in the letter codes): from ESAT-6, peptides 1, 3, 8 (1–16, MTEQQWNNFAIEAAS; 17–32, AİQGNVTSHİLLDE-G; 57–72, KWDATATELNŁQLNL); from MPB83, peptide 21 (195–214, GLVCGGVHANTAVYMİDTV); from MPB64, peptides 12 and 14 (89–104, APYLİNİSATYQSAI, 105–120, PPR-GTQAVVLKVYQNA); and from MPB70, peptide 11–16, MKV-KNTIAATSFAAAG. Ten µg/mL of each peptide was used in in vitro assays for proliferation, IFN-γ, or IL-2 production (see below).

Lymphocyte Transformation Assay

PBMC were isolated from heparinized blood by gradient centrifugation (Histopaque-1077; Sigma, Poole, UK) and were cultured (2 × 10⁶/well in 0.2-mL aliquots) in RPMI 1640 supple-
Figure 1. Recognition of mycobacterial proteins. Blood obtained from 10 single intradermal comparative tuberculin test-positive field reactors with confirmed bovine tuberculosis (Mycobacterium bovis group), from 5 calves vaccinated with BCG Pasteur (BCG group), and from 5 negative controls (negative group) were incubated with recombinant mycobacterial proteins and avian PPD (PPD-A) and mammalian PPD (PPD-M) at concentrations of 10 μg/mL for 24 h. Presence of IFN-γ in culture supernatants was determined with use of the BOVIGAM ELISA kit (CSL, Melbourne, Australia). Results are expressed as composite mean optical density (OD)450± SE within each group, with background OD450 values subtracted.

The amount of IL-2 in the same supernatants was determined by its ability to sustain proliferation of lymphoblasts generated by stimulation with concanavalin A, as described elsewhere [28].

Results

Recognition of recombinant mycobacterial antigens. Recombinant forms of the putative M. bovis–specific antigens ESAT-6, MPB64, and MPB83 induced IFN-γ only in PBMC from field-reactor cattle. Conversely, PBMC from animals vaccinated with BCG and from nonreactors (negative controls) did not recognize these antigens. These results are presented in figure 1, which depicts the mean IFN-γ responses (± SE) of 10 SICTT-positive field reactors from which M. bovis was cultured after necropsy (confirmed reactors), as well as from 5 BCG-vaccinated cattle and 5 negative controls. Four of 10 field reactors recognized MPB64, and 7 of 10 recognized ESAT-6 and MPB83 (individual data not shown). Comparison of proliferative responses confirmed these findings (data not shown). Purified Ag85 complex antigens, which are expressed by both M. bovis and BCG, were strongly recognized by field reactors and BCG-vaccinated animals (figure 1).

Consequently, a specific protein cocktail containing ESAT-6, MPB64, and MPB83 was formulated and tested for its ability to differentiate between M. bovis infection and BCG vaccination (described below).

Identification of promiscuous peptide epitopes of ESAT-6, MPB83, and MPB64 recognized by T cells from M. bovis–infected cattle. Since peptides are cheaper and easier to pro-
duce and standardize than recombinant proteins, our next objective was to define peptides that could be recognized promiscuously in the context of multiple BoLA class II haplotypes (also described as permissive recognition). For an operational definition, we stipulated that a peptide had to be recognized by the majority of *M. bovis*-sensitized cattle tested to be considered as promiscuously recognized. Overlapping peptides were synthesized to identify epitopic areas of ESAT-6, MPB83, and MPB64 and were tested with PBMC either from calves experimentally infected with *M. bovis* (2 calves) or from field reactors (4 animals). The results of these experiments, with peptides derived from ESAT-6 and MPB83, are shown in figure 2. Several peptides were recognized by more than half of the animals tested (peptides 1, 3, 8, and 9 from ESAT-6 and peptides 20, 21, and 22 from MPB83), whereas a number of peptides were recognized in a more restricted manner by individual cattle. As predicted, PBMC from BCG-vaccinated, environmentally sensitized cattle (i.e., nonspecific tuberculin reactors with higher PPD-A responses than PPD-M responses) or from cattle that responded to neither PPD-M nor PPD-A did not recognize any ESAT-6-, MPB83-, or MPB64-derived peptides (data not shown).

It was encouraging that peptides which induced proliferative responses in field reactors also induced IFN-γ and IL-2, as is shown for ESAT-6-derived peptides in figure 3. Peptides that were recognized strongly in a promiscuous manner were selected for inclusion in a diagnostic peptide cocktail containing peptides 1, 3, and 8 from ESAT-6 and peptide 21 from MPB83, as well as 2 peptides derived from MPB64 and 1 from MPB70. The sequences of these peptides are listed in Materials and Methods.

### Immune responses induced by peptide and protein-based diagnostic reagents

Both protein and peptide cocktails were tested with PBMC from *M. bovis*-infected SICTT-positive field reactors (5 animals), BCG-vaccinated cattle (5 animals), and 3 cattle with an immune response biased toward PPD-A (nonspecific reactors) or no tuberculin response (negatives). The results of this experiment are shown in figure 4, which details

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**Figure 2.** Identification of promiscuous epitopes from ESAT-6 and MPB83. Peripheral blood mononuclear cells isolated from 2 cattle experimentally infected with *Mycobacterium bovis* (black bars and grey bars) and from *M. bovis* field reactors (other bars) were incubated with synthetic peptides derived from ESAT-6 and MPB83 (each at a concentration of 25 mg/mL) for 6 days. Results are expressed as the stimulation index (SI; see text) of triplicate determinations (SI > 2.5, positive). MPB83 peptides 1, 2, 6, 7, 8, 9, 10, and 17 were not recognized (not shown).
Figure 3. Peptide-induced T cell proliferation (A), IFN-γ (B), and IL-2 (C) responses. Peripheral blood mononuclear cells were isolated from a typical field reactor and were incubated with ESAT-6-derived synthetic peptides (25 mg/mL each) in a lymphocyte transformation assay. In parallel, whole-blood cultures were performed, and peptide-induced IFN-γ and IL-2 production were measured after 24 h of culture. Results are expressed as mean counts per minute (cpm) or as mean optical density (OD) 450 of triplicate determinations. Kcpm, kilo cpm; D = (cpm antigen) – (cpm with medium only).

Discussion

The development of diagnostic reagents capable of differentiating between infection and vaccination is necessary for the development of a vaccine against tuberculosis in cattle [7] so that existing test-and-slaughter control strategies can continue alongside vaccination. Such differential diagnosis would also be of benefit to human vaccination regimens so that individuals who, despite vaccination, contract tuberculosis and require chemotherapy can be identified. BCG vaccination has been shown to compromise both tuberculosis skin testing in cattle (and humans) and in vitro assays that use tuberculin, including the IFN-γ–based ELISA kit [26, 29, 30].

The general trend in the diagnosis of bovine tuberculosis in cattle points to a preference for blood-based tests, since, in contrast to the tuberculin skin test, they require only a single farm visit. Moreover, it has been suggested that the IFN-γ assay can be used in conjunction with the SICHT-positive to reduce the time between consecutive tests of inconclusive-reactor animals [31]. Such blood-based assays are also being developed for other species, including humans [32].

Only a subset of infected animals will respond to any one antigen, and the immunodominance profiles recognized by an infected individual varies over the course of infection [18, 33]. It is therefore unlikely that single antigens will be able to identify the vast majority of infected animals. Moreover, formu-
Figure 4.  Proliferative T cell responses induced by protein and peptide cocktails. Peripheral blood mononuclear cells were isolated from 5 
*Mycobacterium bovis*-infected cattle (*M. bovis* group), 5 BCG-vaccinated cattle (BCG group), and 3 nonspecific tuberculin responders (nonspecific group) were stimulated in lymphocyte transformation assays with the recombinant protein cocktail composed of ESAT-6, MPB64, and MPB83 (5 mg/mL each), and with the peptide cocktail (10 mg/mL of each peptide), as well as with mammalian PPD (PPD-M) and avian PPD (PPD-A; 10 mg/mL). Results for individual animals are expressed as mean counts per minute of triplicates. Kcpm, kilo cpm; \( \Delta \text{cpm} = (\text{cpm with antigen}) - (\text{cpm with medium only}) \).

In the present study, several breeds of cattle from herds in different parts of the United Kingdom were used to define promiscuous epitopes, and >75% of field reactors tested responded to the peptide cocktail. It is therefore highly likely that we have defined truly promiscuous epitopes within the antigens mapped in this study. However, we have not formally demonstrated the association of these peptides with multiple BoLA class II haplotypes, a task which will require the development of suitable assays for cattle.

It is interesting that comparison of the sequences of the promiscuous bovine epitopes identified from ESAT-6 (p1–16, p17–32, and p57–72) with the previously described corresponding promiscuous epitopes recognized by humans and mice showed a remarkable overlap in epitope-specificity, particularly in the recognition of the N-terminal peptides (humans: p1–20, p1–30, [39]; mice p3–15 and p50–70 [40]). This is consistent with earlier data obtained for other mycobacterial antigens, which also indicated a considerable overlap in the promiscuous epitopes recognized by human and murine T cells [36]. Rationally designed peptide cocktails have already been shown to induce responses in PBMC from human tuberculosis patients and BCG-vaccinated donors with high sensitivity [41].

In the United Kingdom, ~50% of tuberculin skin test reactors will have no visible lesions at slaughter, and ~20% of reactors will be killed without subsequent confirmation of disease by
M. bovis culture [7]. It is likely that these unconfirmed reactors are composed of different groups of animals: (1) those with true false-negative reactions due to a high degree of sensitization by environmental mycobacteria and (2) animals at relatively early stages of infection in which visible lesions have not yet developed and which consequently have lower bacterial burdens not detectable by the standard culture techniques.

It is noteworthy that the defined diagnostic cocktails described in this article displayed a higher degree of sensitivity in reactors with confirmed bovine tuberculosis than in unconfirmed cases, whereas the comparative analysis of PPD-M and PPD-A detected confirmed and unconfirmed reactors in almost equal proportions. This could indicate that the defined reagents have a higher specificity, and it is possible that application of these reagents could result in fewer false-positive responses, even among nonvaccinated cattle. Since environmental exposure, as attested by high in vitro responses against PPD-A, is frequently observed (H. M. V., unpublished observation), the use of “M. avium–specific” antigens in parallel with M. bovis–specific antigens could help define such environmentally sensitized animals.

In conclusion, this study demonstrates that diagnostic cocktails based on either recombinant proteins or peptides derived from antigens expressed in M. bovis but not in BCG can distinguish between vaccinated and infected individuals in blood-based assays. We predict that the inclusion of other such antigens will result in increased sensitivity of the assay without compromising specificity. The formulation of such antigen cocktails is now under investigation in our laboratory.

Acknowledgements

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References

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