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Enhancement of the Sensitivity of the Whole-Blood Gamma Interferon Assay for Diagnosis of *Mycobacterium bovis* Infections in Cattle▽

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In this study, we determined if the sensitivity of the currently available in vitro test to detect bovine tuberculosis could be enhanced by adding the following immunomodulators: interleukin-2 (IL-2); granulocyte-macrophage colony-stimulating factor (GM-CSF); antibodies neutralizing IL-10 and transforming growth factor β (TGF-β); mono-methyl-L-arginine, which blocks nitric oxide production; and L-methyl-tryptophan, which interferes with the indoleamine dioxygenase pathway. Blood was obtained from uninfected control cattle, experimentally infected cattle, cattle responding positively to the skin test in tuberculosis-free areas (false positives), and cattle naturally infected with *Mycobacterium bovis* from New Zealand and Great Britain. Gamma interferon (IFN-γ) responses to bovine purified protein derivative (PPD-b), avian purified protein derivative, and a fusion protein of ESAT-6 and CFP-10 were measured. Mono-methyl-L-arginine, L-methyl-tryptophan, or an antibody neutralizing TGF-β had minimal impact on IFN-γ production. IL-2 and GM-CSF promoted IFN-γ release whether antigen was present or not. In contrast, adding an antibody against IL-10 enhanced only antigen-specific responses. In particular, addition of anti-IL-10 to ESAT-6/CFP-10-stimulated blood cultures enhanced the test sensitivity. Furthermore, whole blood cells from field reactors produced substantial amounts of IL-10 upon stimulation with PPD-b or ESAT-6/CFP-10. Testing "false-positive" cattle from tuberculosis-free areas of New Zealand revealed that addition of anti-IL-10 did not compromise the test specificity. Therefore, the use of ESAT-6/CFP-10 with anti-IL-10 could be useful to detect cattle potentially infected with tuberculosis, which are not detected using current procedures.

The majority of cattle infected with *Mycobacterium bovis* develop a delayed-type hypersensitivity response, which is manifested as a positive response to intradermal injection with purified protein derivative (PPD) (13). This skin test, or an in vitro correlate, the whole-blood gamma interferon (IFN-γ) test (BOVIGAM; Prionics, Zurich, Switzerland), is used to screen for *M. bovis* infections and forms the cornerstone of the test-and-slaughter approach to tuberculosis control in New Zealand and other countries (reviewed in reference 11). Unfortunately, a proportion of cattle fail to develop an in vivo or in vitro responsiveness to PPD, despite being infected with tuberculosis (so-called anergic animals) (20). The presence of such a subset of cattle sometimes necessitates the slaughter of whole herds harboring such tuberculosis-infected cattle that escape standard testing. Based on data accumulated in human tuberculosis disease, we hypothesized that the failure of these cattle to develop a response to PPD is based in part on the release by "suppressive regulatory" T cells of immunosuppressive cytokines, such as interleukin-10 (IL-10) and transforming growth factor β (TGF-β), that block the release of the key cytokines (such as IFN-γ) that are involved in PPD responsiveness (4, 6, 7, 15, 22). Many infectious diseases, particularly chronic ones, such as tuberculosis, determine the emergence of a cascade of cytokines and cells that regulate the ongoing immune response. Cytokines that drive an efficient immune response are released by a subgroup of T cells that render animals more resistant to tuberculosis. Concomitantly, the developing immune response is also characterized by the emergence of cytokines that have anti-inflammatory activities, with IL-10 and TGF-β being two prototypical examples (6). This arm of the immune response is activated to prevent the continuous exuberant expression of proinflammatory mediators that irreversibly damage the infected tissues of infected animals. We hypothesized that anergic cattle are cattle in which that anti-inflammatory component has been recruited and prevents the expression of markers of immunity to tuberculosis, such as bovine PPD (PPD-b) responsiveness. Recent data in a bovine model of tuberculosis infection has suggested that there is an appreciable release of IL-10 associated with disease progression, strengthening the case for the involvement of IL-10 in PPD anergy (36). Recent work by Buza et al. has shown the potential of anti-IL-10 to augment production of antigen-specific IFN-γ in animals infected with *Mycobacterium avium* subsp. *paratuberculosis* (9). Similarly, it has been demonstrated that certain cells release significant levels of TGF-β in the course of bovine tuberculosis (18).

The failure to develop a response to PPD may also be linked to an enhanced activation profile of monocytes or macrophages, notably the release of high levels of reactive nitrogen and/or oxygen intermediates or prostaglandins (30). These molecules have the ability to reduce T-lymphocyte proliferation and/or cytokine release. An additional pathway that may lead to unresponsiveness in this system is the degradation of the amino acid tryptophan via the activation of an indoleamine-dioxygenase pathway in activated monocytes/macro-

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phages or dendritic cells, which oxidizes tryptophan to form kynurenine (14). The net effect of the activation of this pathway is to make tryptophan unavailable for proliferating T cells, leading to immunosuppression. Some evidence has been presented in mouse models of infection suggesting that the biological "recycling" of tryptophan leads to T-cell anergy (14). Another mechanism potentially involved in blunting a T-cell response includes an elevated release of the bactericidal and antiproliferative moiety nitric oxide (NO) by activated macrophages (5).

Finally, it is possible that the deficiency of a particular factor that promotes cellular responsiveness is responsible for in vitro anergy (25, 31). It was therefore deemed that an additional hypothesis to be tested should include the supplementation of cell cultures with key cytokines, such as IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF), which may restore the responsiveness of cells from tuberculosis-infected animals to PPD.

The aim of this study was to test a range of immunomodulators that enhance IFN-γ or antibodies against factors that block the optimal release of IFN-γ. This research is focused on enhancing the sensitivity of the whole-blood IFN-γ test for bovine tuberculosis diagnosis.

MATERIALS AND METHODS

Experimentally infected cattle. In initial preliminary experiments, cattle experimentally infected with *M. bovis* were used to develop and expand the methodologies, allowing the identification of optimal doses and regimens of immunomodulators to enhance the responsiveness of immune cells to PPD. Nine Friesian cross female calves, approximately 9 months old, were obtained from tuberculosis-free accredited herds from an area of New Zealand where both farmed and feral animals were free of tuberculosis. Prior to the experiments, the cattle tested negative for reactivity to PPD from *M. bovis* (PPD-b) in the whole-blood IFN-γ assay (29). The calves were challenged intratracheally with 5×10^3 CFU of virulent *M. bovis* as previously described (8). The cattle were grazed on pasture in a high-security containment unit. These animals tested positive in the comparative cervical skin test at 14 weeks after challenge. Blood samples for IFN-γ assay were collected 8 days after the skin test was read, and the animals were killed and necropsied 17 weeks after challenge. The nine challenged cattle all had gross tuberculous lesions in the lungs and pulmonary lymph nodes. In addition, *M. bovis* was cultured from the lesions, using techniques for bacterial isolation described previously (8). Animal ethics approval was granted for all animal experiments by the local ethics committees (Wallaceville Animal Ethics Committee and Veterinary Laboratory Agency [VLA], Weybridge, Surrey, United Kingdom Committee).

Naturally infected cattle from New Zealand. Six naturally infected cattle were identified on the basis of their responsiveness to a PPD skin test and in vitro IFN-γ responsiveness to PPD-b. On necropsy, these animals all had lung or lymph node lesions typical of bovine tuberculosis.

Cattle from Great Britain. Heparinized blood was obtained from skin test- and IFN-γ test-positive cattle from herds with multiple cases of culture-confirmed bovine tuberculosis. These cattle were held at VLA Weybridge under a project license granted by the United Kingdom Home Office under the Animal (Scientific Procedures) Act of 1986. Infection was confirmed by postmortem examination and culture.

False-positive reactors. Twenty-seven cattle were classified as false positive in the tuberculin test, based on their positive responsiveness in a caudal-fold tuberculin test but negative responsiveness in the ESAT-6/CFP-10 IFN-γ test, and were from tuberculosis-free regions of New Zealand.

Tuberculin skin testing. A caudal-fold skin test was carried out by injecting a 0.1-ml volume containing 0.1 mg PPD-b (AgriQuality, Upper Hutt, New Zealand) intradermally in the caudal fold of the tail. Skin fold thickness was measured using calipers prior to injection and 72 h later. A positive delayed-type hypersensitivity response was recorded as any increase in skin fold thickness. A comparative cervical tuberculin skin test was carried out by injecting 0.1-ml volumes containing 0.05 mg avian PPD (PPD-a) and 0.1 mg PPD-b (AgriQuality) intradermally at separate sites on the neck. The skin fold thickness was measured prior to injection and 72 h after injection for both PPDs. A positive response was recorded when the response to PPD-b was ≥4 mm more than that for PPD-a (New Zealand procedure) and >4 mm more than that for PPD-a (European Union procedure).

TABLE 1. Culturing of blood with antigens and immunomodulators

Antigen	Immunomodulator
PPD-a, PPD-b, or ESAT-6/CFP-10	PBS[a]
PPD-a, PPD-b, or ESAT-6/CFP-10	Anti-IL-10 antibody[b]
PPD-a, PPD-b, or ESAT-6/CFP-10	Anti-TGF-β antibody[c]
PPD-a, PPD-b, or ESAT-6/CFP-10	MMLA[d]
PPD-a, PPD-b, or ESAT-6/CFP-10	Tryptophan[e]
PPD-a, PPD-b, or ESAT-6/CFP-10	IL-2[f]
PPD-a, PPD-b, or ESAT-6/CFP-10	GM-CSF[g]
PBS	PBS[h]

[a] Positive control.
[b] Neutralizing antibodies against IL-10 (1 μg/ml) (examines the role of endogenous IL-10 in PPD-b responsiveness).
[c] Neutralizing antibodies against TGF-β (10 μg/ml) (examines the role of TGF-β in PPD-b responsiveness).
[d] 5 mg/ml (examines the role of NO in PPD-b responsiveness).
[e] 5 mg/ml (examines the role of tryptophan degradation in PPD-b responsiveness).
[f] 1 to 10 U/ml (examines whether deficiency of IL-2 is involved in PPD-b responsiveness).
[g] 10 to 100 ng/ml (determines whether GM-CSF can enhance sensitivity to PPD-b).
[h] Negative control.

Modified IFN-γ release in response to PPD-a, PPD-b, and ESAT-6/CFP-10. Heparinized blood was obtained from each animal and transported to AgResearch or VLA Weybridge overnight at ambient temperature. The blood cultures were set up 18 to 24 h after blood collection and were cultured with PPD-a or PPD-b (24-μg/ml final concentration; Prionics, Schlieren-Zurich, Switzerland), ESAT-6/CFP-10 (4-μg/ml final concentration; Statens Serum Institute, Copenhagen, Denmark), or phosphate-buffered saline (PBS), with and without added immunomodulators as shown in Table 1. The IFN-γ assay was performed as follows. PPDs or ESAT-6/CFP-10 and the various immunomodulators were dispensed at 25 μl/well into sterile flat 96-well tissue culture plates. Two hundred and fifty microliters of whole blood was dispensed per well of the 96-well plates. The plates were incubated at 37°C and 5% CO_2 for 20 h and then centrifuged at 1,800 rpm for 15 min at room temperature. One hundred microliters of plasma from each well was collected and stored frozen at −20°C.

Neutralizing antibodies were purchased from Serotec (Oxford, United Kingdom). Our group had already validated the neutralizing activities of the antibodies used under the conditions described in different facets of our research activities. The optimal quantities of antibodies added to the various bovine cell cultures have been defined elsewhere (12; M. Denis, unpublished data). The chemicals used (mono-methyl-L-arginine [MMLA] and L-methyl-tryptophan) were purchased from Sigma International (Saint Louis, MO); these products were cell culture grade, and the products had no toxicity or side effects on cells in vitro in control experiments. The bovine cytokines used here were produced in our laboratory (35). The recombinant cytokines were pure and were determined not to be contaminated by endotoxin. Our preliminary results identified the optimal doses of materials that were to be used in future experiments. All experimental conditions were tested in duplicate, and IFN-γ levels from the plasma samples were measured with the BOVIGAM test. Positive and negative controls were included in all plates, and there was very little variation between plates. The samples in the IFN-γ enzyme-linked immunosorbent assay (ELISA) were run in duplicate. Color change in the ELISA reaction was measured as optical density (OD) at 450 nm, and the results were expressed as OD units. For the diagnosis of bovine tuberculosis in New Zealand, the cutoff for the standard IFN-γ test was as follows: PPD-b OD − PPD-a OD ≥ 0.100 OD units. The cutoff for the ESAT-6/CFP10 IFN-γ assay was as follows: ESAT-6/CFP10 OD − PBS OD ≥ 0.040 OD units.

Determination of IL-10 by ELISA. Heparinized blood was collected, and peripheral blood mononuclear cells (PBMC) were prepared as described previously (28) and suspended in RPMI 1640 (Gibco, Paisley, United Kingdom) supplemented with 10% fetal calf serum (Sigma), penicillin-streptomycin, 2-mercaptoethanol, and nonessential amino acids (all from Sigma) as described elsewhere (28). A total of 2×10^5 PBMC/well in 96-well plates were incubated for 48 h at 37°C in a humidified incubator with a 5% CO_2 atmosphere in the presence of PPD-b (10 μg/ml; VLA Weybridge, Addlestone, United Kingdom),

TABLE 2. Adding MMLA, tryptophan, or antibodies to TGF-β does not consistently modify PPD-b-driven IFN-γ release by PBMC of cattle experimentally infected with *M. bovis*

Animal[a]	IFN-γ release driven by[b]:				
	PPD-b	PPD-b + MMLA	PPD-b + anti-TGF-β	PPD-b + tryptophan	PBS
1	1.56	1.51	1.88	1.61	0.05
2	3.18	3.20	3.07	3.19	0.06
3	2.86	2.73	3.03	2.68	0.09
4	1.48	1.49	1.90	1.41	0.06
5	2.10	1.91	2.49	1.95	0.06
6	0.40	0.37	0.51	0.36	0.05
7	0.76	0.75	1.06	0.74	0.11
8	3.66	3.59	3.20	3.58	0.14
9	1.85	1.70	1.77	1.63	0.07
Uninfected animal A[c]	0.05	0.06	0.06	0.05	0.05
Uninfected animal B[c]	0.11	0.10	0.04	0.12	0.05

[a] Animals 1 to 9 were infected with 5×10^3 CFU of *M. bovis* by the intratracheal route, and blood was sampled 15 weeks after infection.
[b] Results are expressed in OD units for duplicate wells.
[c] Uninfected cattle used as controls.

recombinant ESAT-6 and CFP-10 proteins (5 μg/ml each protein; a kind donation from M. Singh, GBF, Braunschweig, Germany), or staphylococcal enterotoxin B (1 μg/ml; Sigma). Supernatants were harvested after 48 h and stored at −80°C until they were analyzed for IL-10 and IFN-γ production by ELISA. IFN-γ was determined using the BOVIGAM kit (see above), whereas IL-10 was determined by the following method. MAXISORP flat 96-well black plates (Nunc, Denmark) were coated with 50 μl monoclonal antibody (MAb) CC318 (Serotec, United Kingdom) at 4 μg/ml in coating buffer and incubated overnight at room temperature. The plates were blocked with PBS-casein at 1 mg/ml and incubated with the culture supernatant. The plates were washed, and the detection MAb, CC320-biotin (Serotec, United Kingdom), was added at 1 μg/ml. The substrate was Super Signal ELISA femto maximum sensitivity (Pierce, Illinois). The relative light unit value was read on a Victor 1420 luminometer (Perkin-Elmer, Massachusetts). The results were expressed as antigen-specific responses minus the background medium control (ΔIL-10 and ΔIFN-γ).

Statistical analyses. The statistical analyses of the effects of different treatments on IFN-γ responses was performed using the paired *t* test. *P* values of less than 0.05 were regarded as being significant.

RESULTS

Impacts of various immunomodulators on IFN-γ release in response to PPD in cattle experimentally infected with *M. bovis*. Table 2 shows the impacts of adding MMLA, L-methyltryptophan, and a neutralizing antibody against TGF-β on the PPD-b-driven release of IFN-γ in nine experimentally infected cattle. All of the cattle showed a substantial release of IFN-γ in response to PPD-b alone. However, these three immunomodulators had minimal impacts on the release of IFN-γ from blood cells of infected cattle, and no significant increases in IFN-γ responses were observed.

Effects of IL-2 or GM-CSF on IFN-γ release. As seen in Table 3, adding GM-CSF or IL-2 enhanced the responses to both PPD-b and PPD-a in experimentally infected cattle but also led to marked IFN-γ release in one uninfected cow. Both IL-2 and GM-CSF in the absence of antigen also induced the release of significant amounts of IFN-γ in most cattle tested. Attempts to reduce the amounts of bovine cytokines to levels that would enhance PPD responsiveness without inducing IFN-γ release in the absence of antigens were inconclusive, as some cattle responded to very low concentrations of cytokines and responsiveness varied widely between cattle (data not shown). Variability across animals is a hallmark of diagnostic procedures for mycobacterial infections.

Effects of a neutralizing antibody against IL-10 on IFN-γ release. Table 4 shows that a neutralizing antibody against IL-10 enhanced the response to PPD-a and PPD-b in experimentally infected cattle. The addition of anti-IL-10 significantly increased responses to PPD-a ($P < 0.001$) and to PPD-b ($P = 0.006$). Anti-IL-10 antibody added alone (without antigen) did not result in significant release of IFN-γ by the blood cells of the cattle tested. Next, we examined the potential of neutralizing antibodies against IL-10 to enhance IFN-γ release by blood cells of naturally infected cattle. As shown in Table 5, anti-IL-10 markedly enhanced the PPD-b-driven IFN-γ release in four out of six naturally infected cattle (>0.100 OD unit increase).

Given that anti-IL-10 antibody enhanced both the PPD-a and the PPD-b responses in infected cattle, we elected to study

TABLE 3. Adding IL-2 or GM-CSF enhances IFN-γ release in response to antigens but also stimulates IFN-γ release without antigens in experimentally infected and uninfected cattle

Animal[a]	IFN-γ release driven by[b]:								
	PPD-b	PPD-b + GM-CSF	PPD-b + IL-2	PPD-a	PPD-a + GMCSF	PPD-a + IL-2	IL-2 only	GMCSF only	PBS
1	1.56	2.99	2.55	1.01	3.08	2.42	0.75	2.74	0.05
2	3.18	2.97	3.31	2.21	3.01	3.15	0.48	3.10	0.06
3	2.86	3.17	3.29	0.51	3.35	2.12	0.68	3.15	0.09
4	1.48	3.41	3.37	0.42	3.47	2.27	0.79	3.33	0.06
5	2.10	3.56	3.34	0.70	2.83	2.65	1.88	3.34	0.06
6	0.40	3.45	1.51	0.18	3.38	1.24	0.66	2.59	0.05
7	0.76	3.54	2.36	0.48	3.28	2.15	0.92	3.25	0.11
8	3.66	3.41	3.77	2.17	2.59	3.41	0.07	2.60	0.14
9	1.85	3.79	3.58	0.59	3.34	2.67	1.25	3.34	0.07
Uninfected animal A[c]	0.05	2.28	0.13	0.06	2.45	0.15	0.14	2.45	0.05
Uninfected animal B[c]	0.11	0.04	2.00	0.16	0.04	1.83	1.50	0.04	0.05

[a] Animals 1 to 9 were infected with 5×10^3 CFU of *M. bovis* by the intratracheal route, and blood was sampled 15 weeks after infection.
[b] Results are expressed in OD units for duplicate wells.
[c] Uninfected cattle used as controls.

TABLE 4. Neutralizing antibodies against IL-10 enhance PPD-b- and PPD-a-driven IFN-γ release, and do not induce IFN-γ release without antigen in experimentally infected cattle

Animal[a]	IFN-γ release driven by[b]:					
	PPD-b	PPD-b + anti-IL-10	PPD-a	PPD-a + anti-IL-10	Anti-IL-10 only	PBS
1	1.56	2.98	1.01	2.30	0.06	0.05
2	3.18	2.96	2.21	3.12	0.06	0.06
3	2.86	3.27	0.51	3.10	0.07	0.09
4	1.48	3.29	0.42	3.53	0.06	0.06
5	2.10	3.48	0.70	3.47	0.07	0.06
6	0.40	1.70	0.18	1.69	0.06	0.05
7	0.76	3.43	0.48	3.56	0.12	0.11
8	3.66	3.65	2.17	3.54	0.13	0.14
9	1.85	3.69	0.59	3.61	0.07	0.07
Uninfected animal A[c]	0.05	0.06	0.06	0.07	0.06	0.05
Uninfected animal B[c]	0.11	0.041	0.16	0.04	0.04	0.05

[a] Animals 1 to 9 were infected with 5×10^3 CFU of *M. bovis* by the intratracheal route, and blood was sampled 15 weeks after infection.
[b] Results are expressed in OD units for duplicate wells.
[c] Uninfected cattle used as controls.

the potential of anti-IL-10 to enhance the IFN-γ response to the tuberculosis-specific antigens ESAT-6/CFP-10. The anti-IL-10 enhanced the response in all of the infected cattle ($P = 0.001$) and had no impact on the response of uninfected cattle (Table 6). We titrated the amounts of anti-IL-10 antibody required to enhance the IFN-γ response in this test and found that anti-IL-10 antibody amounts of 0.1 μg/ml were sufficient to enhance the ESAT-6/CFP-10 IFN-γ response in experimentally infected cattle (data not shown).

Impact of anti-IL-10 on spontaneous release of IL-10 in false-positive cattle from tuberculosis-free areas testing positive for PPD. False-positive cattle were tested for their responsiveness to anti-IL-10 alone to ascertain if the antibody alone or in the presence of ESAT-6/CFP-10 could lead to spontaneous IFN-γ release. Twenty-seven cattle that were identified as false positives, i.e., that showed a positive skin test reaction but were located in a tuberculosis-free area of New Zealand, were tested for their responsiveness to the ESAT-6/CFP-10 plus anti-IL-10. Only one animal showed a positive response, which was just above the cutoff of 0.040 OD units, to ESAT-6/CFP-10 in the presence of anti-IL-10, whereas the other 26 did not respond to the ESAT-6/CFP-10–anti-IL-10 preparation. The addition of anti-IL-10 to cultures containing either PBS or ESAT-6/CFP10 did not result in a significant increase in IFN-γ responses. Some representative results are shown in Table 7.

TABLE 5. Adding a neutralizing antibody against IL-10 enhances the PPD-b-driven IFN-γ release in some naturally infected cattle

Animal[a]	IFN-γ release driven by[b]:		
	PPD-b	PPD-b + anti-IL-10	PBS
1	0.29	0.41	0.06
2	2.58	3.52	0.07
3	3.81	3.85	0.11
4	0.84	2.27	0.08
5	3.28	3.37	0.07
6	3.15	3.68	0.09

[a] Animals 1 to 6 had typical tuberculosis lesions at necropsy.
[b] Results are expressed in OD units for duplicate wells. Insufficient blood was obtained to test for responsiveness to PPD-a.

TABLE 6. Adding a neutralizing antibody against IL-10 enhances the ESAT-6/CFP-10-driven IFN-γ release in most experimentally infected cattle

Animal[a]	IFN-γ release driven by[b]:			
	PBS	Anti-IL-10 only	ESAT-6/CFP-10	ESAT-6/CFP-10 + anti-IL-10
1	0.06	0.06	0.46	0.73
2	0.06	0.06	2.10	3.02
3	0.07	0.07	1.08	1.87
4	0.06	0.06	0.46	1.89
5	0.06	0.07	0.83	2.29
6	0.08	0.06	0.14	0.59
7	0.12	0.12	0.40	1.00
8	0.05	0.13	2.05	3.20
9	0.08	0.07	0.74	3.16
Uninfected animal A[c]	0.06	0.06	0.06	0.06
Uninfected animal B[c]	0.08	0.04	0.07	0.05

[a] Animals 1 to 9 were infected with 5×10^3 CFU of *M. bovis* by the intratracheal route, and blood was sampled 15 weeks after infection.
[b] Results are expressed in OD units for duplicate wells.
[c] Uninfected cattle used as controls.

Anti-IL-10 enhances the sensitivity of IFN-γ in field reactors from Great Britain. Field reactor cattle were selected from farms with persistent culture-confirmed bovine tuberculosis in Great Britain to assess the reproducibility of the anti-IL-10 treatment in a different cattle population. In the first experiment, blood from 31 such cattle was stimulated with PPD-a, PPD-b, or the ESAT-6/CFP-10 fusion protein in the absence or presence of IL-10-neutralizing antibodies. The results are shown in Fig. 1, and they confirm the New Zealand data sets, as significant increases in IFN-γ responses after stimulation with all three antigens were observed after IL-10 neutralization (Fig. 1, *P* values between 0.005 and 0.0002). No

TABLE 7. Adding a neutralizing antibody against IL-10 in "false-positive" cattle does not induce IFN-γ release with or without antigens

Animal[a]	IFN-γ release driven by[b]:			
	PBS	PBS + anti-IL-10 (no antigen)	ESAT-6/CFP-10	ESAT-6/CFP-10 + anti-IL-10
1	0.174	0.091	0.166	0.122
2	0.059	0.095	0.048	0.046
3	0.071	0.073	0.069	0.07
4	0.041	0.081	0.053	0.038
5	0.035	0.079	0.032	0.031
6	0.043	0.085	0.035	0.041
7	0.046	0.089	0.047	0.06
8	0.112	0.092	0.0126	0.117
9	0.082	0.087	0.085	0.093
10	0.044	0.039	0.044	0.086

[a] Representative data from 10 cattle that were false positive in the tuberculin skin test. The IFN-γ response of animal 10 was positive for ESAT-6/CFP-10 + anti-IL-10 compared to PBS (ESAT-6/CFP-10 + anti-IL-10 OD − PBS OD > 0.040).
[b] Results are expressed in OD units for duplicate wells.

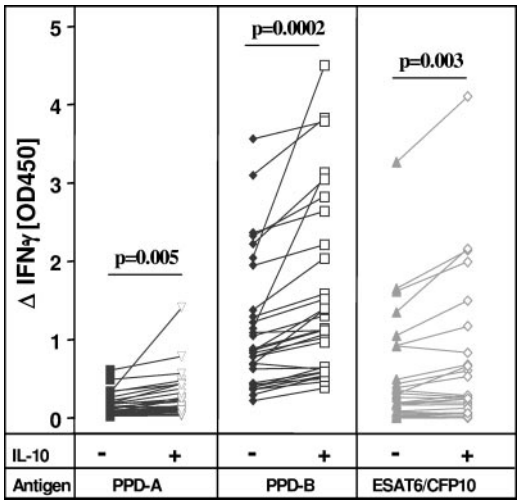

FIG. 1. Modulation of IFN-γ production by anti-IL-10 in naturally infected cattle from Great Britain. Blood samples from 31 field reactor cattle from herds with culture-confirmed bovine tuberculosis were stimulated as described in the text for New Zealand cattle with PPD-a, PPD-b, and the ESAT-6/CFP-10 fusion protein in the absence (−) or presence (+) of MAb neutralizing bovine IL-10. Antigen-specific IFN-γ responses, determined by BOVIGAM ELISA, are shown, with medium control values subtracted (ΔIFN-γ).

increased IFN-γ was observed in cultures without antigens (not shown), thus confirming the antigen-specific effect of anti-IL-10 treatment. The specificity of the anti-IL-10 treatment was further confirmed by treating a subset of 10 blood samples with an isotype control antibody. This treatment had no effect on IFN-γ production (mean PPD-b-induced IFN-γ ± standard deviation [SD] without antibody, 1.091 ± 0.929; with isotype control antibody, 1.068 ± 0.943; mean PPD-a-induced IFN-γ ± SD without antibody, 0.166 ± 0.140; with isotype control, 0.149 ± 0.137; and mean ESAT6/CFP10 fusion protein-induced IFN-γ ± SD without antibody, 0.326 ± 0.328; with isotype control, 0.318 ± 0.335).

We next determined whether the IL-10 in these cultures was present in the plasma of the cattle tested before blood culture or whether it was produced during the culture period. Plasma was removed from heparinized blood samples by centrifugation, and the pellets containing red and white blood cells were resuspended in RPMI 1640, followed by culture with PPD-b, PPD-a, or ESAT6/CFP10 in the presence or absence of anti-IL-10. Anti-IL-10 treatment was able to enhance IFN-γ production in these cultures to the same degree as in the control cultures containing plasma, thus demonstrating that IL-10 was produced during the whole-blood incubation period (data not shown). Further, PPD-b- and ESAT-6/CFP10-specific release of IL-10 (Fig. 2A) and IFN-γ (Fig. 2B) were determined using PBMC isolated from 24 field reactor cattle. The data demonstrated that significant amounts of IL-10 were produced alongside strong IFN-γ responses after both ESAT-6/CFP10 and PPD-b stimulation (Fig. 2A).

DISCUSSION

In this work, we sought to examine experimental maneuvers that enhance the sensitivity of the BOVIGAM assay for experimentally infected cattle, as well as naturally infected cattle. Uninfected cattle were used as negative controls to assess the specificity of the measurements. We tested nine experimentally infected cattle. These cattle had been infected with *M. bovis* (5×10^3 CFU by the intratracheal route) 15 weeks prior to testing. Six naturally infected cattle were also tested. In addition, 31 field reactor cattle were studied in Great Britain. In both naturally infected and experimentally infected cattle from New Zealand, it was found that modifying the BOVIGAM test by supplementing the media with tryptophan or MMLA had little impact on the PPD-b-driven IFN-γ response. Similarly, adding a neutralizing antibody against TGF-β to PPD-stimulated cultures had minimal impact on IFN-γ production. Only results for PPD-b responses are shown in Results, as PPD-a responses were not affected by these modulators (data not shown). It was therefore concluded that the release of TGF-β or NO or activation of the indoleamine dioxygenase pathway is not involved in masking responsiveness to PPD in vitro in bovine cells. In separate studies, we have observed that both NO and TGF-β are produced and released during the course of bovine tuberculosis by blood cells responding to PPD-b (M. Denis and B. M. Buddle, unpublished observations). This suggests that these mediators are present during the in vitro IFN-γ BOVIGAM but do not have a major impact on IFN-γ release.

Supplementing the cultures with IL-2 or GM-CSF enhanced the IFN-γ production by most experimentally infected cattle, but this increase occurred for both PPD-a and PPD-b. GM-CSF and IL-2 did not appreciably enhance the PPD-b re-

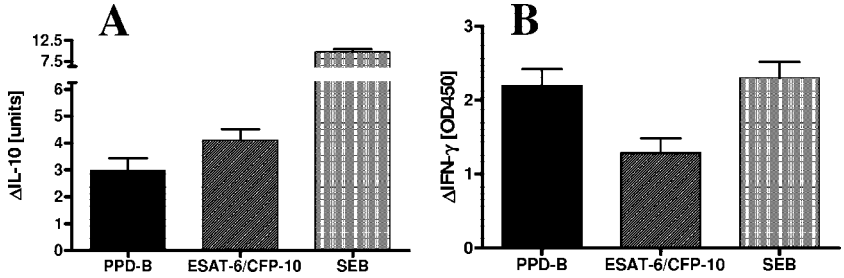

FIG. 2. Antigen-specific IL-10 and IFN-γ production in field reactors from Great Britain. PBMC, prepared from 24 naturally infected cattle, were incubated with PPD-a and PPD-b (10 μg/ml), a cocktail of ESAT-6 and CFP10 proteins (5 μg/ml each), or staphylococcal enterotoxin B (SEB) (1 μg/ml). Supernatants were collected after 48 h of incubation and assessed for antigen-specific IL-10 (A) or IFN-γ (B) production. The results are expressed as mean antigen-specific responses plus standard errors of the mean with background (medium control) values subtracted (ΔIL-10 and ΔIFN-γ).

sponses in cattle that had very high basal PPD-b responses (3 out of 10 animals). In addition, most unstimulated blood cultures supplemented with IL-2 and GM-CSF released significant levels of IFN-γ. Therefore, adding GM-CSF and IL-2 (at the doses tested here) to PPD-stimulated cultures enhances the sensitivity but decreases the specificity of the assay. Titration of cytokines to find an optimal level that would stimulate a PPD response without spontaneous IFN-γ release gave inconclusive results (data not shown).

When cells from infected cattle were stimulated with PPD-b in vitro, neutralizing IL-10 enhanced the IFN-γ release, showing that endogenous IL-10 is involved in down-regulating the IFN-γ release. It was shown that IL-10 neutralization had no significant impact in a group of naturally infected cattle with very high PPD-b responses (>3.0 OD units), suggesting that the PPD-b response in these cattle was maximal. Moreover, this increase occurred for both the responses to PPD-a and PPD-b, suggesting that the use of antigens unique to *M. bovis* should be considered.

Based on these observations, we measured the ESAT-6/CFP-10-driven IFN-γ release in whole blood of infected cattle and control uninfected cattle in the presence of the immunomodulators listed above. ESAT-6 and CFP-10 are dominant IFN-γ-inducing antigens of tuberculous mycobacteria, and they are specific to tuberculosis, being absent from most environmental nontuberculous mycobacteria (33). We found that blocking IL-10 markedly enhanced the response to ESAT-6/CFP-10 in all nine of the experimentally infected cattle, but not in the two uninfected cattle used as controls.

Studies performed on field reactor cattle in Great Britain provided additional evidence that neutralizing IL-10 led to a substantial enhancement of the IFN-γ release by whole blood cells in response to ESAT-6/CFP-10. In addition, study of the whole blood cells from field reactors showed that substantial IL-10 was produced in response to both PPD-b and ESAT-6/CFP-10.

False-positive cattle are sometimes identified in tuberculosis-free regions of New Zealand; specifically, some cattle known to be tuberculosis free respond positively to PPD-b in both the tuberculin test and BOVIGAM. In those cases, the blood cultures of the cattle are retested with the tuberculosis-specific antigens ESAT-6/CFP-10. If the currently available BOVIGAM test is to be modified, that approach must not compromise the ability of the test to uncover false positives. It is currently believed that these false positives arise because a subset of blood cells known as NK cells respond to mycobacterial antigens without any prior exposure (24). Our data presented here suggest that ESAT-6/CFP-10 with anti-IL-10 do not compromise the ability of this "specific antigen" to identify false positives. We did not find that neutralizing IL-10 led to a significant IFN-γ release by control uninfected cattle or in false positives from New Zealand in response to either ESAT-6 or CFP-10.

However, preliminary screening of large groups of tuberculin skin test-negative cattle has revealed that some cattle that produced IFN-γ in response to PPD-a also produced IFN-γ responses to ESAT-6/CFP-10, which were further amplified by the addition of anti-IL-10 (Denis and Buddle, unpublished). We are currently investigating this phenomenon to identify the mechanism(s) involved in these anomalous results in high-PPD-a-responding animals. This may be caused by exposure of these animals to environmental mycobacteria that express ESAT-6/CFP-10 (3, 34).

Although our data are strongly suggestive that IL-10 is produced by antigen-specific T cells, IL-10 could have been produced by monocytes/macrophages. The definition of the subset producing this cytokine was beyond the scope of this study and needs to be determined in future experiments. It is tempting to speculate that these cells belong to one of the IL-10-producing populations of regulatory T cells that have been described in other species (reviewed in reference 1). It is interesting, however, that despite the positive modulation of antigen-specific IFN-γ production after neutralizing IL-10, significant and strong IFN-γ responses occurred alongside IL-10 responses (Fig. 2A and B). It is therefore also possible that IL-10 is produced by Th1 cells, as described recently (2, 17), to limit the collateral damage caused by strong inflammatory responses, although this could also result in a curtailed Th1 response and failure to eliminate the pathogen.

In conclusion, neutralizing IL-10 enhances the sensitivity of the in vitro IFN-γ test for bovine tuberculosis and may offer an attractive option for testing anergic animals in the field. However, more work is required to fully ascertain the specificity of this procedure.

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