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Use of Recombinant ESAT-6:CFP-10 Fusion Protein for Differentiation of Infections of Cattle by *Mycobacterium bovis* and by *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis*

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Immunological diagnosis of *Mycobacterium bovis* infection of cattle is often confounded by cross-reactive responses resulting from exposure to other mycobacterial species, especially *Mycobacterium avium*. Early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) are dominant gamma interferon (IFN-γ)-inducing antigens of tuberculous mycobacteria, and they are absent from many environmental nontuberculous mycobacteria. Because *M. avium* exposure is the primary confounding factor in the diagnosis of *M. bovis*-infected animals, in vitro responses to a recombinant ESAT-6:CFP-10 (rESAT-6:CFP-10) fusion protein by blood leukocytes from cattle naturally exposed to *M. avium* or experimentally challenged with *Mycobacterium avium* subsp. *avium* or *Mycobacterium avium* subsp. *paratuberculosis* were compared to responses by *M. bovis*-infected cattle. Responses to heterogeneous mycobacterial antigens (i.e., purified protein derivatives [PPDs] and whole-cell sonicates [WCSs]) were also evaluated. Tumor necrosis factor alpha (TNF-α), IFN-γ, and nitric oxide responses by *M. bovis*-infected cattle to rESAT-6:CFP-10 exceeded ($P < 0.05$) the corresponding responses by cattle naturally sensitized to *M. avium*. Experimental infection with *M. bovis*, *M. avium*, or *M. avium* subsp. *paratuberculosis* induced significant ($P < 0.05$) IFN-γ and nitric oxide production to WCS and PPD antigens, regardless of the mycobacterial species used for the preparation of the antigen. Responses to homologous crude antigens generally exceeded responses to heterologous antigens. Nitric oxide and IFN-γ responses to rESAT-6:CFP-10 by blood leukocytes from *M. bovis*-infected calves exceeded ($P < 0.05$) the corresponding responses of noninfected, *M. avium*-infected, and *M. avium* subsp. *paratuberculosis*-infected calves. Despite the reported potential for secretion of immunogenic ESAT-6 and CFP-10 proteins by *M. avium* and *M. avium* subsp. *paratuberculosis*, it appears that use of the rESAT-6:CFP-10 fusion protein will be useful for the detection of tuberculous cattle in herds with pre-existing sensitization to *M. avium* and/or *M. avium* subsp. *paratuberculosis*.

Mycobacterium bovis, a member of the *Mycobacterium tuberculosis* complex, has a wide host range compared to those of other species in this disease complex, is infectious to humans, and is the species most often isolated from tuberculous cattle. Control of *M. bovis* in cattle is particularly difficult due to the presence of wildlife reservoirs, such as white-tailed deer, European badgers, and brush-tailed possums. Recently, there has been an increase in the prevalence of *M. bovis* infection in the United States. Detection of tuberculous cattle in Michigan, California, Texas, and New Mexico has resulted in the loss of the tuberculosis (TB)-free designation for these states (or portions thereof) and subsequent economic losses from increased TB testing costs and hindrances of interstate shipment of livestock from these zones. The demand for improved diagnostic capabilities is again being realized and emphasized with the increased TB testing in the United States. Tests currently approved for the detection of bovine TB include measurement of delayed-type hypersensitivity responses (i.e., skin testing) to purified protein derivative (PPD) antigens and an in vitro assay for gamma interferon (IFN-γ) produced in response to PPD stimulation (i.e., Bovigam; Biocor Animal Health, Omaha, Nebr.). A major limitation of these tests is the cross-reactivity of *M. bovis* PPD (PPDb) with responses induced by exposure to related bacteria, especially *Mycobacterium avium* subsp. *avium* (referred to hereafter as *M. avium*) and *Mycobacterium avium* subsp. *paratuberculosis*.

Early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) are IFN-γ-inducing antigens of tuberculous mycobacteria. ESAT-6 and CFP-10 are cosecreted proteins that naturally form a tight 1:1 complex upon export (14). Genes for these proteins are absent in many environmental, nontuberculous mycobacteria as well as in the TB vaccine

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strain, *M. bovis* BCG. Use of ESAT-6 and/or CFP-10 for the stimulating antigen in IFN-γ-based TB assays enhances the specificity over the specificity of the standard test using PPDb as the stimulating antigen (3, 4, 11, 12, 16). Unfortunately, *esat-6* and *cfp-10* are present in a subset of nontuberculous mycobacteria, most notably *Mycobacterium kansasii*, *Mycobacterium marinum*, *Mycobacterium leprae*, and *Mycobacterium smegmatis* (6, 7, 8; N. C. Gey van Pittius, R. M. Warren, and P. D. van Helden, Letter, Infect. Immun. **70:**6509-6510, 2002). Of particular relevance for diagnostic issues concerning bovine TB in the United States, *esat-6* and *cfp-10* have been reported to be present in *M. avium* and *M. avium* subsp. *paratuberculosis* (8). A search of the complete genome of *M. avium* subsp. *paratuberculosis* strain K10 (genome accession no. NC_002944) and *M. avium* strain 104 (http://www.tigr.org), however, did not reveal any sequences with similarity to *esat-6* or *cfp-10*. Considering this discrepancy, the objective of the present study was to determine whether a recombinant ESAT-6:CFP-10 (rESAT-6:CFP-10) fusion protein can be used to differentially detect *M. bovis*-infected cattle from cattle infected with *M. avium* or *M. avium* subsp. *paratuberculosis*.

MATERIALS AND METHODS

Animals, challenge procedures, and bacterial culture. Cattle were obtained from TB-free herds. The study consisted of two experiments. In the first experiment, groups consisted of Hereford cattle from a herd with a history of widespread exposure to *M. avium* ($n = 8$) and *M. bovis*-challenged crossbred beef cattle ($n = 8$). Calves in this portion of the study were 10 months of age at the time of sample collection. Sixty percent (30 of 50) of adult cows and 78% (32 of 41) of preweaned calves in the herd of origin of the *M. avium*-exposed calves had positive IFN-γ responses to *M. avium* PPD (i.e., IFN-γ response with *M. avium* PPD [PPDa] stimulation minus IFN-γ response with no stimulation equals ≥0.05 optical density unit) (Bovigam; Biocor Animal Health). Calves in the *M. bovis*-challenged group were tested and confirmed negative for *M. bovis* and *M. avium* exposure prior to challenge (Bovigam; Biocor Animal Health). The *M. avium*-exposed cattle were housed outdoors, whereas *M. bovis*-infected cattle were housed within a biosafety level 3 confinement facility. For *M. bovis* infection, the challenge inoculum, 2.4×10^6 CFU in 0.2 ml of phosphate-buffered saline (PBS) (0.15 M, pH 7.2), was instilled directly into the tonsillar crypts of anesthetized cattle as previously described for inoculation of white-tailed deer (10). The *M. bovis* strain used for the challenge inoculum (95-1315, U.S. Department of Agriculture, Animal Plant and Health Inspection Service designation) was originally isolated from a white-tailed deer in Michigan (15). Inoculum consisted of mid-log-phase *M. bovis* grown in Middlebrook 7H9 medium supplemented with 10% oleic acid-albumin-dextrose complex (Difco, Detroit, Mich.) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, Mo.). Lesions typical of *M. bovis* infection were detected in *M. bovis*-inoculated animals, and infection was confirmed by isolation of *M. bovis* from tissues.

In the second experiment, groups consisted of nonchallenged calves ($n = 4$), *M. avium*-challenged calves ($n = 4$), *M. avium* subsp. *paratuberculosis*-challenged calves ($n = 3$), and *M. bovis*-challenged calves ($n = 4$). Calves (males, castrated at ~3 months of age) were obtained from Holstein herds free of TB and *M. avium* subsp. *paratuberculosis* at 2 to 4 days of age and housed indoors in temperature-controlled rooms (18 to 24°C) (biosafety level 3 facility for *M. bovis*-infected calves). *M. bovis* (strain 95-1315) and *M. avium* (strain 3988; bovine isolate) for challenge inoculum were grown in Middlebrook 7H9 medium (National Animal Disease Center, Ames, Iowa) supplemented with 10% oleic acid-albumin-dextrose complex plus 0.05% Tween 80 (Sigma Chemical Co.). Medium for *M. avium* subsp. *paratuberculosis* (strain K10; cattle isolate) was additionally supplemented with 2 mg of mycobactin J (Allied Monitor Inc., Fayette, Mo.) per liter. Challenge inoculum (~10^4 CFU of *M. bovis*, ~10^{10} CFU of *M. avium,* or ~10^8 CFU of *M. avium* subsp. *paratuberculosis*) was instilled directly into the tonsillar crypts of sedated calves as described previously (19). Calves in *M. avium* subsp. *paratuberculosis*- and *M. avium*-challenged groups received four weekly doses of inoculum for a total dose of ~4×10^{10} CFU of *M. avium* or ~4×10^8 CFU of *M. avium* subsp. *paratuberculosis*.

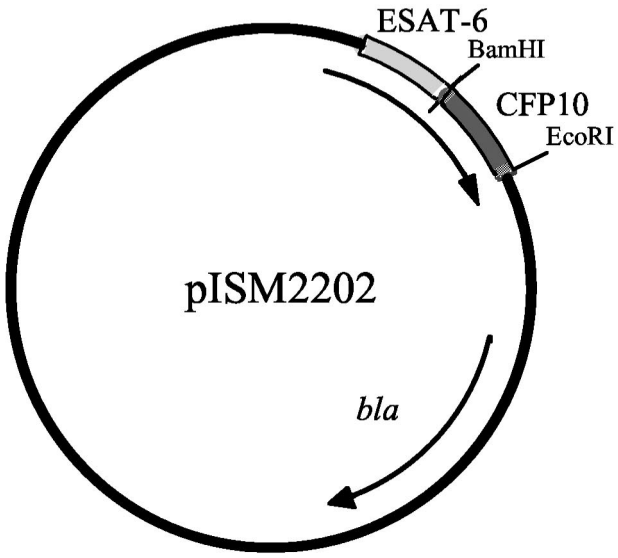
Cell culture and antigens. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coat fractions of samples of peripheral blood collected in 2× acid-citrate-dextrose (5). Individual wells of 96-well round-bottom microtiter plates (Falcon; Becton-Dickinson, Lincoln Park, N.J.) were seeded with 2×10^5 PBMC in a total volume of 200 μl per well. The medium was RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 100 U of penicillin per ml, 0.1 mg of streptomycin per ml, 1% nonessential amino acids (Sigma), 2% essential amino acids (Sigma), 1% sodium pyruvate (Sigma), 50 μM 2-mercaptoethanol (Sigma), and 10% (vol/vol) fetal bovine serum. Wells contained medium plus 5 μg of PPDb (CSL Ltd.) per ml, 5 μg of PPDa (CSL Ltd.) per ml, 20 μg of *M. bovis* strain 95-1315 whole-cell sonicate (WCS) per ml, 20 μg of *M. avium* strain 3988 WCS per ml, 20 μg of *M. avium* subsp. *paratuberculosis* strain K10 WCS per ml, and 10 μg of rESAT-6:CFP-10 per ml or medium alone (no stimulation). Antigens consisting of sonicates of the challenge strains were prepared as described previously (17). After incubation of PBMC cultures for 48 h at 37°C in 5% CO_2, culture supernatants were harvested and stored at −80°C until thawed for analysis.

Cloning and expression of rESAT-6:CFP-10 fusion protein. CFP10 was amplified from *M. bovis* genomic DNA with primers CFP10F (5′-AAGGATCCATGGCAGAGATGAAGACC) and CFP10R (5′-AAGAAGAATTCGAAGCCCATTTGCGAGGA) to incorporate BamHI and EcoRI restriction sites. The PCR products were ligated into pCR2.1 (Invitrogen, Carlsbad, Calif.) and transformed into *Escherichia coli* TOP10F′ chemically competent cells [*mcrA* Δ(*mcrCB-hsdSMR-mrr*) (ϕ80 *lacZ*Δ*M15*) Δ(*lac*)*X74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG* F′(Tet^r)]. Plasmid DNA was isolated using Qiaspin miniprep system (Qiagen, Valencia, Calif.) and screened by DNA sequencing. The appropriate plasmid was designated pISM2204. The expression vector pISM404 (pTrcHisB:ESAT-6) (9) and pISM2204 were digested with BamHI and EcoRI. The digested DNA was gel purified, ligated, and transformed into *Escherichia coli* DH5α cells [*supE44* Δ*lacU169* (ϕ80 lacZΔ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*]. Recombinant plasmids were sequenced to confirm the insertion, and the final plasmid was designated pISM2202 (Fig. 1).

rESAT-6:CFP10 protein was purified from a 200-ml 2× yeast tryptone ampicillin (50 μg/ml) culture, induced to an optical density at 600 nm of 0.6 with 1 mM isopropyl thiogalactoside for 4 h at 37°C. Cells were lysed in 20 ml of lysis buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 8 M urea) and then sonicated. Proteins were purified on 10 ml of Ni-nitrilotriacetic acid resin (Sigma) using the Biologic HR chromatography system (Bio-Rad, Hercules, Calif.). The resin was washed with 10 ml of water and then with 25 ml of lysis buffer, and the cell lysate was added. Nonspecific proteins were washed through the column with 60 ml of lysis buffer and then with 30 ml of lysis buffer containing 10 mM imidazole. The protein was eluted with 30 ml of lysis buffer containing 200 mM imidazole. Fractions were collected every 5 ml and analyzed by Western blotting against the six-histidine tag (6X His Tag; Clontech, Palo Alto, Calif.) (9). Fractions were then dialyzed overnight at 4°C in PBS and quantified by the Bradford assay.

IFN-γ assay. Heparinized blood samples were dispensed in 1.5-ml aliquots into individual wells of a 24-well plate (Falcon 353047; Becton Dickinson and Company, Franklin Lakes, N.J.). Blood cultures were incubated for 48 h. Plasma was harvested and stored at −80°C. IFN-γ concentrations in stimulated plasma (i.e., whole-blood assay) or supernatants were determined using a commercial enzyme-linked immunosorbent assay (ELISA)-based kit (Bovigam; Biocor Animal Health). Absorbencies of standards and test samples were read at 450 nm using an ELISA plate reader (Molecular Devices, Menlo Park, Calif.). In addition, IFN-γ concentrations in supernatants (three pooled replicate samples) from PBMC cultures were analyzed. Concentrations (in nanograms per milliliter) in test samples were quantified by comparing absorbencies of test samples with absorbencies of standards within a linear curve fit. Duplicate samples for individual treatments were analyzed.

Griess reaction assay. The amount of nitrite, the stable oxidation product of nitric oxide (NO), in culture supernatants is a correlate of the amount of NO produced by cells in culture. Nitrite was measured using the Griess reaction (13) performed in 96-well microtiter plates (Immunolon 2; Dynatech Laboratories, Inc., Chantilly, Va.). Culture supernatant (100 μl) was mixed with 100 μl of Griess reagent (0.5% sulfanilamide; Sigma) in 2.5% phosphoric acid (Mallinckrodt Chemicals, Inc., Paris, Ky.) and 0.05% *N*-(1-naphthyl) ethylenediamine dihydrochloride (Sigma). The mixture was incubated at 21°C for 10 min. Absorbencies of test and standard samples at 550 nm were measured using an automated ELISA plate reader. Samples were diluted in culture medium (RPMI 1640 medium with 2 mM L-glutamine and 10% fetal bovine serum [vol/vol]). Absorbencies of standards and test samples were converted to nanograms of nitrite per milliliter by comparing them to absorbencies of sodium nitrite (Fisher Chemicals, Fair Lawn, N.J.) standards within a linear curve fit. Assays were run on three sets of pooled triplicates for each treatment.

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1   ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT
1   Met Gly Gly Ser His His His His His His Gly Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly
67  CGG ACT CTG TAC GAC GAT GAC GAT AAG GAT CTC ATG ACA GAG CAG CAG TGG AAT TTC GCG GGT ATC
23  Arg Thr Leu Tyr Asp Asp Asp Asp Lys Asp Leu Met Thr Glu Gln Gln Trp Asn Phe Ala Gly Ile
133 GAG GCC GCG GCA AGC GCA ATC CAG GGA AAT GTC ACG TCC ATT CAT TCC CTC CTT GAC GAG GGG AAG
45  Glu Ala Ala Ala Ser Ala Ile Gln Gly Asn Val Thr Ser Ile His Ser Leu Leu Asp Glu Gly Lys
199 CAG TCC CTG ACC AAG CTC GCA GCG GCC TGG GGC GGT AGC GGT TCG GAG GCG TAC CAG GGT GTC CAG
67  Gln Ser Leu Thr Lys Leu Ala Ala Ala Trp Gly Gly Ser Gly Ser Glu Ala Tyr Gln Gly Val Gln
265 CAA AAA TGG GAC GCC ACG GCT ACC GAG CTG AAC AAC GCG CTG CAG AAC CTG GCG CGG ACG ATC AGC
89  Gln Lys Trp Asp Ala Thr Ala Thr Glu Leu Asn Asn Ala Leu Gln Asn Leu Ala Arg Thr Ile Ser
                                                                    BamHI
331 GAA GCC GGT CAG GCA ATG GCT TCG ACC GAA GGC AAC GTC ACT GGG ATG TTC GCA GGA TCC ATG GCA
111 Glu Ala Gly Gln Ala Met Ala Ser Thr Glu Gly Asn Val Thr Gly Met Phe Ala Gly Ser Met Ala
397 GAG ATG AAG ACC GAT GCC GCT ACC CTC GCG CAG GAG GCA GGT AAT TTC GAG CGG ATC TCC GGC GAC
133 Glu Met Lys Thr Asp Ala Ala Thr Leu Ala Gln Glu Ala Gly Asn Phe Glu Arg Ile Ser Gly Asp
463 CTG AAA ACC CAG ATC GAC CAG GTG GAG TCG ACG GCA GGT TCG TTG CAG GGC CAG TGG CGC GGC GCG
155 Leu Lys Thr Gln Ile Asp Gln Val Glu Ser Thr Ala Gly Ser Leu Gln Gly Gln Trp Arg Gly Ala
529 GCG GGG ACG GCC GCC CAG GCC GCG GTG GTG CGC TTC CAA GAA GCA GCC AAT AAG CAG AAG CAG GAA
177 Ala Gly Thr Ala Ala Gln Ala Ala Val Val Arg Phe Gln Glu Ala Ala Asn Lys Gln Lys Gln Glu
595 CTC GAC GAG ATC TCG ACG AAT ATT CGT CAG GCC GGC GTC CAA TAC TCG AGG GCC GAC GAG GAG CAG
199 Leu Asp Glu Ile Ser Thr Asn Ile Arg Gln Ala Gly Val Gln Tyr Ser Arg Ala Asp Glu Glu Gln
                                                  EcoRI
661 CAG CAG GCG CTG TCC TCG CAA ATG GGC TTC GAA TTC GAA GCT TGG CTG TTT TGG CGG ATG AGA GAA
221 Gln Gln Ala Leu Ser Ser Gln Met Gly Phe Glu Phe Glu Ala Trp Leu Phe Trp Arg Met Arg Glu
727 GAT TTT CAG CCT GAT ACA GAT TAA
243 Asp Phe Gln Pro Asp Thr Asp •••
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FIG. 1. Map of pISM2202 and sequence of ESAT-6:CFP10 fusion protein.

TNF-α assay. Tumor necrosis factor alpha (TNF-α) was measured using a capture ELISA (protocol and reagents provided by L. Babiuk, Veterinary Infectious Diseases Organization, Saskatoon, Saskatchewan, Canada). TNF-α assays were performed in Immunolon II microtiter plates (Dynatech Laboratories, Inc.). Reagents consisted of a capture antibody (mouse ascitic fluid anti-bovine-TNF-α, immunoglobulin G [IgG] fraction), detection antibody (rabbit anti-bovine-TNF-α, IgG fraction), recombinant bovine TNF, biotinylated goat anti-rabbit IgG (Zymed Laboratories, Inc., South San Francisco, Calif.), horseradish peroxidase-conjugated streptavidin-biotinylated complex (Amersham Corporation, Arlington Heights, Ill.), substrate (H_2O_2 at a concentration of 0.1% [vol/vol]), and dye (2,2′-azinodi-ethylbenzothiazoline-sulfonic acid). Internal standards consisted of recombinant bovine TNF-α diluted serially in PBS (pH 7.2, 0.01 M) supplemented with Tween 80 (0.1% [vol/vol]) and gelatin (0.5% [vol/vol]) (PBST-g). Positive and negative controls and test samples were also diluted serially in PBST-g. Capture antibody was diluted (1:1,000 [vol/vol]) in carbonate buffer (pH 9.6, 0.01 M), and detection antibody was diluted in PBST-g (1:1,500 [vol/vol]). Biotinylated goat anti-rabbit Ig was diluted 1:10,000, and horseradish peroxidase-conjugated streptavidin-biotinylated complex was diluted 1:2,000 in PBST without gelatin. Intervening washes were done with PBST without gelatin. Enzyme substrate and indicator dye were diluted in citrate buffer. Incubations were at room temperature with the exception of capture antibody in carbonate buffer, which was incubated at 4°C. Absorbencies of standards and test samples was read at 405 and 490 nm using an ELISA plate washer and reader (Dynatech MR7000). TNF-α concentrations (in nanograms per milliliter) in test samples were determined by comparing the absorbencies of test samples with the absorbencies of standards within a linear curve fit.

Delayed-type hypersensitivity responses (i.e., skin testing). Nine months after challenge, calves were tested for in vivo responsiveness to mycobacterial antigens using a modified, comparative cervical skin test as described previously (18). Animals were injected with 100 μl (1 mg/ml) each of PPDb, PPDa, and *M. avium* subsp. *paratuberculosis* PPD number 0202 (National Veterinary Services Laboratory, Ames, Iowa) intradermally. Data are presented as skin thickness (in millimeters) 72 h after injection of PPD minus preinjection skin thickness.

Statistics. Data were assessed for normality prior to statistical analysis. Arithmetic and $\log_{10}$-transformed data were analyzed as a split plot with repeated-measure analysis of variance using Statview software (version 5.0; SAS Institute, Inc., Cary, N.C.). The statistical model included effects of treatments (infection type and recall antigen), time (months relative to establishment of infection), and

TABLE 1. Comparison of IFN-γ responses by PBMC from *M. bovis*-infected cattle to responses by cattle naturally sensitized to *M. avium*

Group	IFN-γ response[a] to recall antigen:		
	PPDa	PPDb	rESAT-6:CFP-10
M. bovis-infected (6 mo postinfection)	17.3[b] (3.0)	55.2[c] (9.7)	29.1[c] (6.1)
M. avium-exposed (natural exposure)	7.5 (3.0)	−2.4 (1.1)	−2.9 (0.7)

[a] Values are the mean IFN-γ responses (in nanograms per milliliter) ($n = 8$) of PBMC to antigen stimulation minus their response to medium alone after 48 h. Values in parentheses are the standard errors of the means.
[b] Significantly different from the response by *M. avium*-exposed cattle ($P < 0.05$).
[c] Significantly different from the response by *M. avium*-exposed cattle ($P < 0.01$).

the interaction of treatment and time on IFN-γ, TNF-α, and NO production by whole blood or enriched PBMC cultures. Scheffe's test was applied when effects ($P < 0.05$) detected by the model were significant ($P \leq 0.05$).

RESULTS

Responses to rESAT-6:CFP-10 fusion protein by cattle naturally sensitized to *M. avium*. Natural exposure of cattle to *M. avium* was determined by detection of recall IFN-γ responses to PPDa. Seven of eight calves exposed to *M. avium* had positive IFN-γ responses to PPDa (i.e., IFN-γ response with PPDa stimulation minus IFN-γ response with no stimulation equals ≥0.05 optical density unit). IFN-γ responses to PPDa may also be indicative of *M. avium* subsp. *paratuberculosis* infection; however, fecal samples from cattle exposed to *M. avium* and adult members of their herd were negative for *M. avium* subsp. *paratuberculosis* growth using standard *M. avium* subsp. *paratuberculosis* culture techniques (19). IFN-γ responses by PBMC from *M. avium*-exposed calves to PPDa (7.5 ng/ml ± 3.0 ng/ml) exceeded ($P < 0.01$) parallel responses to PPDb (−2.4 ng/ml ± 1.1 ng/ml) (Table 1). Responses of *M. bovis*-infected calves to PPDa exceeded ($P < 0.05$) those of *M. avium*-exposed calves, indicating vigorous responses induced by *M. bovis* infection and the cross-reactivity of PPDs. As expected, PPDb-induced IFN-γ production by *M. bovis*-infected calves (55.2 ng/ml ± 9.7 ng/ml) exceeded ($P < 0.01$) responses elicited by PPDa (17.3 ng/ml ± 3.0 ng/ml). IFN-γ responses of *M. bovis*-infected calves to rESAT-6:CFP-10 also exceeded ($P < 0.01$) those of calves exposed to *M. avium*. Most importantly, stimulation of PBMC from *M. avium*-exposed calves with rESAT-6:CFP-10 did not elicit a significant IFN-γ response (Table 1). TNF-α and NO responses to PPDa, PPDb, and rESAT-6:CFP-10 were qualitatively similar to IFN-γ responses (Tables 2 and 3).

TABLE 2. Comparison of TNF-α responses by PBMC from *M. bovis*-infected cattle to responses by cattle naturally sensitized to *M. avium*

Group	TNF-α response[a] to recall antigen:		
	PPDa	PPDb	rESAT-6:CFP-10
M. bovis-infected (6 mo postinfection)	0.316 (0.084)	1.370[b] (0.524)	0.790[b] (0.285)
M. avium-exposed (natural exposure)	0.164 (0.048)	0.056 (0.052)	0.038 (0.095)

[a] Values are the mean TNF-α responses (in nanograms per milliliter) ($n = 8$) of PBMC to antigen stimulation minus their response to medium alone after 48 h. Values in parentheses are the standard errors of the means.
[b] Significantly different from the response by *M. avium*-exposed cattle ($P < 0.05$).

TABLE 3. Comparison of NO responses by PBMC from *M. bovis*-infected cattle to responses by cattle naturally sensitized to *M. avium*

Group	NO response[a] to recall antigen:		
	PPDa	PPDb	rESAT-6:CFP-10
M. bovis-infected (6 mo postinfection)	196.1[b] (46.1)	403.3[b] (86.4)	415.7[b] (98.4)
M. avium-exposed (natural exposure)	49.1 (9.1)	−4.5 (9.9)	−19.0 (10.1)

[a] Values are the mean NO responses (in nanograms per milliliter) ($n = 8$) of PBMC to antigen stimulation minus their response to medium alone after 72 h. Values in parentheses are the standard errors of the means.
[b] Significantly different from the response by *M. avium*-exposed cattle ($P < 0.01$).

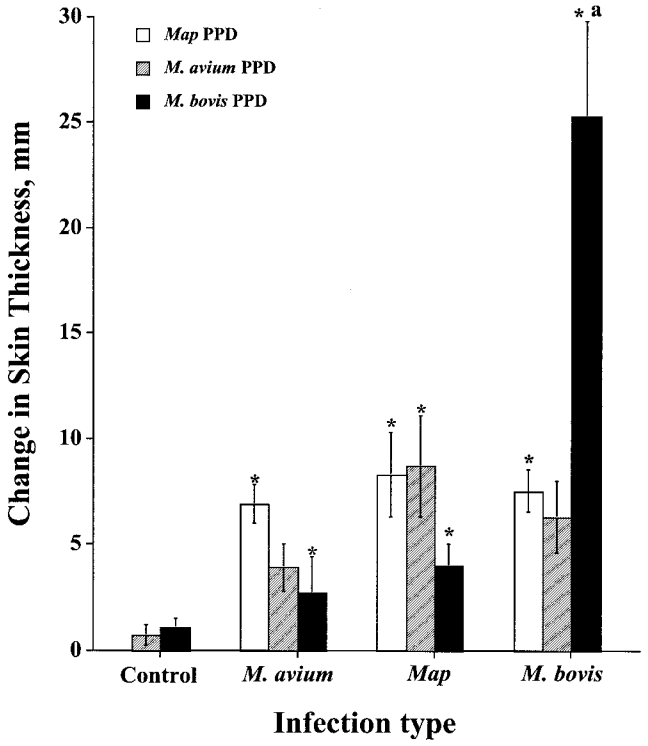

FIG. 2. Delayed-type hypersensitivity responses elicited by PPDs upon experimental infection. PPDs from *M. avium, M. avium* subsp. *paratuberculosis* (*Map*), and *M. bovis* were used. Each value is the mean change (in milliliters) in skin thickness ± standard error of the mean (error bar) 72 h after injection of PPDs. Skin tests were performed approximately 9 months postchallenge. Values that were statistically significantly different ($P < 0.05$) from the response by control cattle (asterisk) are indicated. The response to PPDb by *M. bovis*-infected cattle that is statistically significantly different ($P < 0.05$) from concurrent responses to *M. avium* subsp. *paratuberculosis* PPD and PPDa is also indicated by the letter a.

TABLE 4. IFN-γ responses to crude mycobacterial antigens upon experimental infection

Infection status	IFN-γ response[a] to recall antigen (WCS):		
	M. bovis strain 1315	*M. avium* strain 3988	*M. avium* subsp. *paratuberculosis* strain K10
Noninfected	−0.7 (0.4) A	−1.0 (0.4) A	−0.8 (0.4) A
M. bovis-infected	60.0[b] (2.5) C	15.2 (0.9) B	9.2 (0.7) B
M. avium-infected	13.1 (2.4) B	29.6[b] (2.9) C	16.8 (2.5) C
M. avium subsp. *paratuberculosis*-infected	17.0 (5.2) B	22.7 (7.5) BC	30.9 (11.6) BC

[a] IFN-γ responses (whole-blood assay) were unaffected ($P > 0.05$) by the length of postinfection period. For this reason, values are the mean IFN-γ responses (in nanograms per milliliter) (i.e., response to 20 μg of antigen per ml) minus their response to medium alone after 48 h) during the period spanning 4 to 7 months postinfection. Treatment groups consisted of four animals except for the *M. avium* subsp. *paratuberculosis*-infected group, which had three animals. Values in parentheses are the standard errors of the means.

[d] Letters A to C indicate that the treatment means for a specific type of stimulation (i.e., vertical comparisons) differ ($P < 0.05$). The same letter indicates that the IFN-γ responses were not significantly different ($P < 0.05$).

[b] Significantly different from the responses by the other cattle infected with *M. bovis* or *M. avium* (i.e., horizontal comparisons) ($P < 0.05$).

Sensitization upon intratonsillar challenge. Because the dose and duration of natural exposure to *M. avium* are not amenable to determination or control, an experimental model of *M. avium* infection was developed by our group for comparison to responses induced by experimental *M. bovis* infection (19). At 9 months postchallenge, cutaneous delayed-type hypersensitivity responses to PPDs were detected (Fig. 2). With the exception of PPDb-induced responses by *M. bovis*-infected calves, responses to the various PPDs were similar, indicating cross-reactivity. Recall IFN-γ and NO responses by *M. bovis*-infected calves to *M. bovis* WCS exceeded ($P < 0.05$) concurrent responses to *M. avium* and *M. avium* subsp. *paratuberculosis* WCSs and responses induced by experimental *M. avium* and *M. avium* subsp. *paratuberculosis* infection to *M. bovis* WCS (Tables 4 and 5). Likewise, IFN-γ and NO responses by *M. avium*-infected calves to *M. avium* WCS exceeded ($P < 0.05$) concurrent responses to *M. bovis* WCS and *M. avium* subsp. *paratuberculosis* WCS and responses by noninfected calves to *M. avium* WCS (Tables 4 and 5). Responses of *M. avium*- and *M. avium* subsp. *paratuberculosis*-infected calves to *M. avium* and *M. avium* subsp. *paratuberculosis* WCSs did not differ, reflecting the many conserved antigens of these two closely related subspecies. Analysis of IFN-γ levels in PBMC supernatants and whole-blood cultures revealed similar effects of antigen stimulation and infection status (data not shown).

TABLE 5. Nitric oxide responses to crude mycobacterial antigens upon experimental infection

Infection status	NO response[a] to recall antigen (WCS):		
	M. bovis strain 1315	*M. avium* strain 3988	*M. avium* subsp. *paratuberculosis* strain K10
Noninfected	−106 (53) A	−49 (51) A	−63 (43) A
M. bovis-infected	451[b] (46) C	184 (21) B	97 (35) AB
M. avium-infected	79 (17) B	185[b] (20) B	55 (14) AB
M. avium subsp. *paratuberculosis*-infected	87 (52) AB	213 (61) B	278 (117) B

[a] NO responses by PBMC were unaffected ($P > 0.05$) by the length of postinfection period. For this reason, values are the mean NO responses (in nanograms per milliliter) (i.e., response to 20 μg of antigen per ml minus their response to medium alone after 72 h) during the period spanning 5 to 7 months postinfection. Treatment groups consisted of four animals except for the *M. avium* subsp. *paratuberculosis*-infected group, which had three animals. Values in parentheses are the standard errors of the means.

[a] Letters A to C indicate that the treatment means for a specific type of stimulation (i.e., vertical comparisons) differ ($P < 0.05$). The same letter indicates that the NO responses were not significantly different ($P < 0.05$).

[b] Significantly different from the responses by the other cattle infected with *M. bovis* or *M. avium* (i.e., horizontal comparisons) ($P < 0.05$).

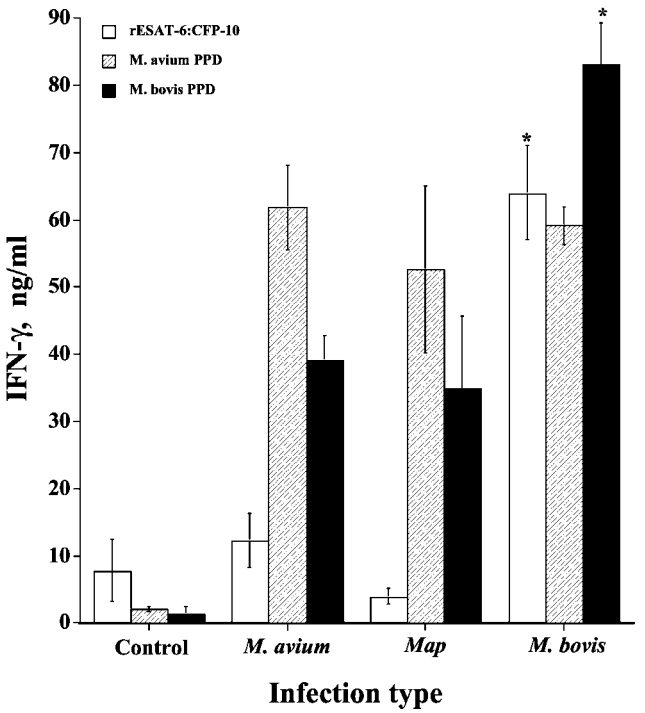

FIG. 3. IFN-γ responses upon experimental infection. IFN-γ responses (whole-blood assay) were unaffected ($P > 0.05$) by length of postinfection period. For this reason, each value represents the mean IFN-γ response (in nanograms per milliliter) (i.e., the response to antigen minus the response to medium alone after 48 h) during the period spanning 4 to 7 months postinfection ± standard error of the mean (error bar) ($n = 4$). The responses by *M. bovis*-infected animals that differ significantly ($P < 0.05$) from the responses by control animals and by *M. avium*- and *M. avium* subsp. *paratuberculosis* (*Map*)-infected animals for the particular antigen stimulation are indicated (asterisk).

Differential diagnosis using rESAT-6:CFP-10 fusion protein. Differential IFN-γ responses to PPDa and PPDb are currently used for *M. bovis* diagnosis (Bovigam; Biocor Animal Health). For this reason, responses to rESAT-6:CFP-10 were compared with responses to PPDa and PPDb (Fig. 3 and 4). IFN-γ responses of *M. bovis*-infected calves to PPDb exceeded ($P < 0.05$) those of noninfected and *M. avium*- and *M. avium* subsp. *paratuberculosis*-infected calves (Fig. 3). IFN-γ and NO responses to PPDb, however, were induced by *M. avium* and *M. avium* subsp. *paratuberculosis* infection. Responses of these calves to PPDb exceeded ($P < 0.05$) those of noninfected calves (Fig. 3 and 4). NO responses of *M. bovis*-infected calves to PPDb exceeded ($P < 0.05$) those of noninfected and *M. avium*-infected calves (Fig. 4). IFN-γ and NO responses of *M.*

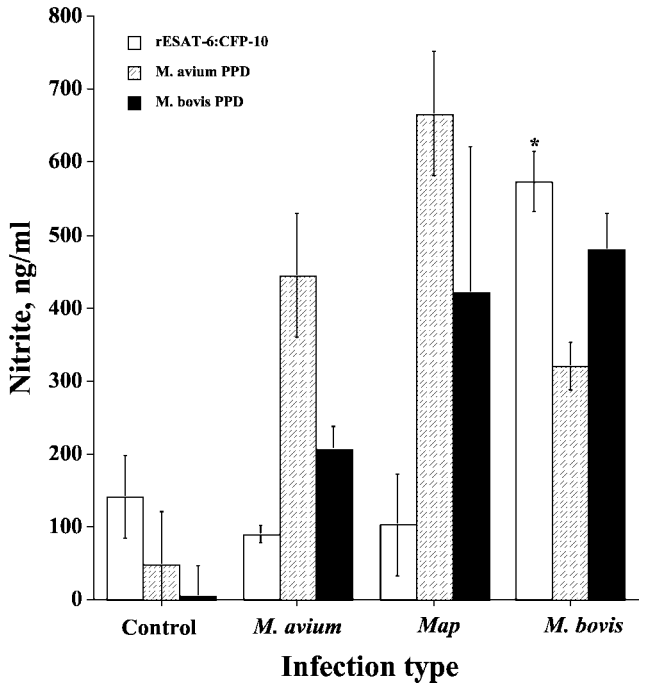

FIG. 4. Nitric oxide responses upon experimental challenge. NO responses by PBMC were unaffected ($P > 0.05$) by length of postinfection period. For this reason, each value represents the mean NO response (in nanograms per milliliter) (i.e., the response to antigen minus the response to medium alone after 72 h) ± standard error of the mean (error bar) during the period spanning 5 to 7 months postinfection ($n = 3$). The response by *M. bovis*-infected animals that differs significantly ($P < 0.05$) from the responses by control animals and by *M. avium*- and *M. avium* subsp. *paratuberculosis* (*Map*)-infected animals for the particular antigen stimulation is indicated (asterisk).

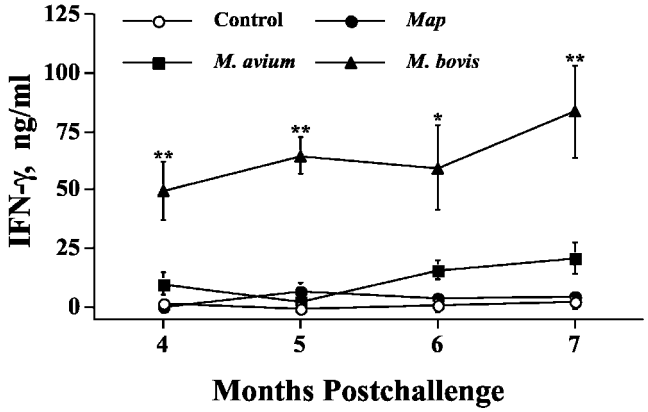

FIG. 5. Longitudinal IFN-γ response to rESAT-6:CFP-10 fusion protein. Values indicate the mean responses to rESAT-6:CFP-10 stimulation (48 h) (in nanograms per milliliter) minus the response to medium alone ± standard error of the mean (error bars). Responses by *M. bovis*-infected animals that were significantly different ($P < 0.05$) from the responses by control animals and by *M. avium*- and *M. avium* subsp. *paratuberculosis* (*Map*)-infected animals are indicated (**). The response by *M. bovis*-infected animals that was significantly different from the responses by control animals ($P < 0.05$), *M. avium*-infected animals ($P = 0.08$), and *M. avium* subsp. *paratuberculosis*-infected animals ($P < 0.05$) is indicated (*).

bovis-, *M. avium*-, and *M. avium* subsp. *paratuberculosis*-infected calves to PPDa were comparable and exceeded ($P < 0.05$) those of noninfected calves. IFN-γ and NO responses of *M. bovis*-infected calves to rESAT-6:CFP-10 exceeded those of noninfected and *M. avium*- and *M. avium* subsp. *paratuberculosis*-infected calves (Fig. 3 and 4). IFN-γ and NO responses by *M. avium*- and *M. avium* subsp. *paratuberculosis*-infected calves to rESAT-6:CFP-10, in contrast to stimulation with PPDb, did not exceed ($P < 0.05$) those of noninfected calves. IFN-γ responses of *M. bovis*-infected calves to rESAT-6:CFP-10 were not different ($P > 0.05$) at 4, 5, 6, and 7 months postinfection (Fig. 5).

DISCUSSION

Searches of publicly available bacterial genome sequences (partial or complete) have indicated that the ESAT-6 gene cluster (including *esat-6*, *cfp-10*, and flanking secretory apparatus genes) is of ancient origin, present in a subset of mycobacteria, and highly conserved (8). Proteins encoded by this gene cluster evoke potent T-cell responses that have been utilized in TB diagnostic tests (2–4, 11, 12, 16). In particular, use of these antigens enhances the specificity of IFN-γ-based tests over standard PPDs as the eliciting agent. However, humans infected with *M. leprae*, *M. marinum*, or *M. kansasii* exhibit recall IFN-γ responses to ESAT-6 and/or CFP-10, indicating that T-cell responses to these two proteins are not invariably specific for infection with *M. tuberculosis* complex mycobacteria (1, 6, 7; Gey van Pittius et al., letter). The reported presence of *esat-6* and *cfp-10* in *M. avium* and *M. avium* subsp. *paratuberculosis* is of particular concern for TB diagnosis in cattle (8). Recent studies have indicated that cattle with responses to PPDa resulting from natural exposure do not exhibit recall IFN-γ responses to ESAT-6 and CFP-10 antigens (11, 16). The dose and agents inducing the response to PPDa, however, are impossible to characterize under conditions of natural exposure. Additionally, natural exposure may result in inhibitory interactions due to exposure to multiple species of mycobacteria and/or repeated doses of antigen that induce tolerance.

In the present study, responses to ESAT-6:CFP-10 were not induced upon experimental inoculation of calves with doses of *M. avium* or *M. avium* subsp. *paratuberculosis* that clearly sensitized these animals to the respective mycobacterium. The challenge inoculum was delivered to neonatal calves housed in environmentally controlled rooms thereby limiting confounding interactions due to exposure to other mycobacteria. *M. bovis*-infected calves exhibited responses to rESAT-6:CFP-10 equal to those induced by stimulation with PPDb. These findings indicate that rESAT-6:CFP-10 should prove useful for the specific diagnosis of bovine TB, even under conditions of high exposure to *M. avium* or *M. avium* subsp. *paratuberculosis*.

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