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W. R. Waters  
*United States Department of Agriculture, Agricultural Research Service, National Animal Disease Center, Ames, Iowa*

M. V. Palmer  
*United States Department of Agriculture, Agricultural Research Service, National Animal Disease Center, Ames, Iowa, Mitchell.Palmer@ars.usda.gov*

D. L. Whipple  
*United States Department of Agriculture, Agricultural Research Service, National Animal Disease Center, Ames, Iowa*

M. P. Carlson  
*University of Nebraska Veterinary Diagnostic Center, Lincoln, Nebraska*

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Diagnostic Implications of Antigen-Induced Gamma Interferon, Nitric Oxide, and Tumor Necrosis Factor Alpha Production by Peripheral Blood Mononuclear Cells from Mycobacterium bovis-Infected Cattle

W. R. Waters,1* M. V. Palmer,1 D. L. Whipple,1 M. P. Carlson,2 and B. J. Nonnecke3

Bacterial Diseases of Livestock Research Unit1 and Periparturient Diseases of Cattle Research Unit,2 United States Department of Agriculture, Agricultural Research Service, National Animal Disease Center, Ames, Iowa 50010, and Toxicology Laboratory, University of Nebraska Veterinary Diagnostic Center, Lincoln, Nebraska 68583-09072

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Bovine tuberculosis in the United States has proven costly to cattle producers as well as to government regulatory agencies. While in vivo responsiveness to mycobacterial antigens is the current standard for the diagnosis of tuberculosis, in vitro assays are gaining acceptance, especially as ancillary or complementary tests. To evaluate in vitro indices of cellular sensitization, antigen-induced gamma interferon (IFN-γ), nitric oxide (NO), and tumor necrosis factor alpha (TNF-α) responses by blood mononuclear cells from Mycobacterium bovis-infected cattle were quantified and compared. Using an aerosol model of infection, two doses of each of two strains of M. bovis (95-1315 and HC-2045T) were used to induce a range of IFN-γ, NO, and TNF-α responses. Infection-specific increases in NO, but not in IFN-γ or TNF-α, were detected in nonstimulated cultures at 48 h, a finding that is indicative of nonspecific activation and spontaneous release of NO. The infective dose of M. bovis organisms also influenced responses. At 34 days postinfection, IFN-γ, NO, and TNF-α responses in antigen-stimulated cells from cattle receiving 10^5 CFU of M. bovis organisms were greater than responses of cells from cattle infected with 10^6 CFU of M. bovis organisms. The NO response, but not the IFN-γ and TNF-α responses, was influenced by infective strains of M. bovis. The TNF-α, NO, and IFN-γ responses followed similar kinetics, with strong positive associations among the three readouts. Overall, these findings indicate that NO and TNF-α, like IFN-γ, may prove useful as indices for the diagnosis of bovine tuberculosis.

First described by Robert Koch in 1891, the tuberculin skin reaction has been the principal means of tuberculosis diagnosis for both humans and domestic animals (23). For cattle, the caudal fold skin test (CFT) is the primary approved test for tuberculosis within the United States. The CFT relies on in vivo reactivity to Mycobacterium bovis purified protein derivative (PPDb) injected intradermally into a fold of skin at the base of the tail. Cattle classified as reactors or suspect with this test are often retested by using the comparative cervical skin test, while technically more challenging than the CFT, provides an added ability to distinguish M. bovis-infected cattle were quantified and compared. Using an aerosol model of infection, two doses of each of two strains of M. bovis (95-1315 and HC-2045T) were used to induce a range of IFN-γ, NO, and TNF-α responses. Infection-specific increases in NO, but not in IFN-γ or TNF-α, were detected in nonstimulated cultures at 48 h, a finding that is indicative of nonspecific activation and spontaneous release of NO. The infective dose of M. bovis organisms also influenced responses. At 34 days postinfection, IFN-γ, NO, and TNF-α responses in antigen-stimulated cells from cattle receiving 10^5 CFU of M. bovis organisms were greater than responses of cells from cattle infected with 10^6 CFU of M. bovis organisms. The NO response, but not the IFN-γ and TNF-α responses, was influenced by infective strains of M. bovis. The TNF-α, NO, and IFN-γ responses followed similar kinetics, with strong positive associations among the three readouts. Overall, these findings indicate that NO and TNF-α, like IFN-γ, may prove useful as indices for the diagnosis of bovine tuberculosis.

The proven, practical application of an IFN-γ-based assay for tuberculosis diagnosis is not surprising considering the robust cell-mediated response generated by tuberculosis complex mycobacteria. Indeed, IFN-γ is crucial for effective host defense during tuberculosis (8, 15, 18, 25, 30). Other readouts of mycobacterial immunity, especially cellular reactivity, may also have diagnostic application. Two essential components of tubercular host defense include nitric oxide (NO) and tumor necrosis factor alpha (TNF-α) (1, 13, 14, 16, 17, 21). Stimulation of inducible nitric oxide synthase in macrophages and subsequent generation of reactive nitrogen intermediates are potent mechanisms for mycobacterial killing (6, 7, 9, 13, 21). Mycobacterium-induced TNF-α and IFN-γ secretion by T cells and/or macrophages from infected individuals is responsible for an antimycobacterial defense mediated by reactive nitrogen intermediates (17, 33). TNF-α is also necessary for containment of the infection (i.e., granuloma formation). Mice deficient in TNF-α or TNF-α receptor are highly susceptible to fatal mycobacterial infections and fail to develop organized granulomas (11, 17, 19). NO and TNF-α, like IFN-γ, are readily produced by mycobacterium-induced PBMC from M. bovis-infected cattle.
bovis-infected cattle (24, 38), thus demonstrating their potential as diagnostic readouts for *M. bovis* infection.

The objective of the present study was to quantify and compare mycobacterium-specific IFN-γ, NO, and TNF-α production by PBMC from *M. bovis*-infected cattle. An aerosol model of *M. bovis* infection using two dosages of each of two strains of *M. bovis* was used to initiate variable responses for comparisons. Isolated PBMC were used for recall stimulation studies because this population produces vigorous IFN-γ, NO, and TNF-α responses when stimulated with mycobacterial antigens. Responses were evaluated for effects of challenge dose and strain, kinetics, and associations.

Twenty crossbred cattle of approximately 9 months of age and obtained from herds with no history of tuberculosis were housed at the National Animal Disease Center, United States Department of Agriculture, Animal Research Service, Ames, Iowa, according to the Association for Assessment and Accreditation of Laboratory Animal Care International and institutional guidelines. At the initiation of the study, all animals were tested and confirmed negative for *M. bovis* and *M. avium* exposure by using a commercially available assay (Bovigam; CSL Limited, Parkville, Victoria, Australia) for detection of IFN-γ responses to in vitro mycobacterial antigen stimulation. The animals were housed in temperature- and humidity-controlled rooms (1 to 2 animals per room) within a biosafety level 3 confinement facility. Negative airlight exited the building through HEPA (high efficiency particulate air) filters, ensuring that air from the animal pens was pulled towards a central corridor and through HEPA filters before exiting the building. Airflow velocity was 10.4 air changes per h.

The strains of *M. bovis* used for the challenge inoculum were strain 95-1315 (United States Department of Agriculture Animal Plant and Health Inspection Service designation), originally isolated from a white-tailed deer in Michigan (31), and strain HC-2045T, originally isolated from a Holstein cow in Texas. Inoculum consisted of mid-log-phase *M. bovis* cells grown in Middlebrook’s 7H9 medium supplemented with 10% oleic acid-albumin-dextrose complex (Difco, Detroit, Mich.) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, Mo.) as previously described (2). The challenge inoculum consisted of either \(10^5\) (\(n = 5\) for each of the two strains) or \(10^7\) (\(n = 5\) for each of the two strains) CFU in 2 ml of phosphate-buffered saline (PBS). The culture was restrained, and the challenge inoculum was delivered by nebulization into a mask covering the animals’ nostrils and mouths (26).

Nineteen of the twenty cattle challenged with *M. bovis* had typical tuberculous lesions with *M. bovis* organisms cultured from affected tissues. Restriction fragment length polymorphism patterns of *M. bovis* organisms isolated from tissues matched the challenge inoculum strain. Tracheobronchial and mediastinal lymph nodes and lungs were the most commonly affected tissues. Lung lesions were distributed diffusely among all lobes, consistent with aerosol exposure to droplet nuclei of <5 μm. Lesions were more severe and disseminated in cattle receiving the higher challenge dosage (i.e., \(10^5\) CFU), regardless of the challenge strain. Although it is difficult to determine the actual number of tuberculous lesions per animal, lesions were detected in more sites (i.e., organs, lymph nodes, lung lobes, etc.) in cattle receiving \(10^5\) CFU of strain HC-2045T than in those receiving \(10^5\) CFU of strain 95-1315. The numbers of lesion sites did not differ between challenge strains for cattle receiving \(10^5\) CFU. Detailed descriptions of gross, histologic, and bacteriologic findings are presented elsewhere (26).

PBMC were isolated fromuffy coat fractions of peripheral blood collected in 2× acid citrate dextrose (5). The 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 supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 100 units 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. The wells contained medium plus 5 μg per ml of PPDb (CSL Limited) 5 μg per ml of PPDa (CSL Limited) 10 μg per ml of *M. bovis* strain 95-1315 whole-cell sonicate (WCS) 10 μg per ml of *M. bovis* strain HC-2045T WCS or 1 μg per ml of PWM or medium alone (no stimulation) per ml. The WCS antigens were prepared from 4-week *M. bovis* cultures grown in Middlebrook’s 7H9 medium supplemented with 10% oleic acid-albumin-dextrose complex. Bacilli were pelleted, sonicated in PBS, further disrupted with 0.1 to 0.15 mm glass beads (Biospec Products, Bartlesville, Okla.) in a bead beater (Biospec Products), and then placed on ice. The preparation was centrifuged, and the supernatant was harvested and filtered (0.22 μm).

After incubation of PBMC cultures for 48 h at 37°C in 5% CO₂, the supernatants were harvested and stored at −80°C until thawed for analysis.

Nitrite is the stable oxidation product of NO, and the amount of nitrite within culture supernatants is indicative of the amount of NO produced by cells in culture. Nitrite was measured by using the Griess reaction (29) performed in 96-well microtiter plates (Immunolon 2; Dynatech Laboratories, Inc., Chantilly, Va.). Nitrite concentrations in the supernatants were determined by high-performance ion chromatography (HPIC). Briefly, macromolecules were separated from the aqueous portion of the sample by centrifugation through a 30,000-Da molecular mass cutoff filter. The microfiltrate was collected and injected directly into an ion-exchange high-pressure liquid chromatography system. A 4.1-by 250-mm strong anion-exchange column with a 10-μm inside diameter was used. The mobile phase consisted of an aqueous solution containing 5.28 g of NaH₂PO₄·H₂O, 43.46 g of Na₂HPO₄·7H₂O, 2.40 g of NaCl, and 100 ml of acetonitrile per liter. Nitrate was detected by absorbance at 214 nm; nitrite was detected by absorbance at 530 nm after a postanalytical coupling reaction with an aqueous solution containing 100 ml of 85% o-phosphoric acid, 40.00 g of sulfanilamide, and 2.00 g of N-(1-naphthyl)ethylenediamine dihydrochloride per liter. Ions were quantitated against their respective standard curves.

A commercial enzyme-linked immunosorbent assay (ELISA)-based kit (Bovigam; CSL Limited) was used for determination of IFN-γ concentrations in culture supernatants. Duplicate samples for each individual treatment were analyzed. Each treatment represented three pooled replicate samples. TNF-α was measured by using a TNF-α capture ELISA (protocol and reagents were provided by L. Babiuk, Veterinary Infectious
Diseases Organization, Saskatoon, Saskatchewan, Canada). The assays were performed in Immunolon II microtiter plates (Dynatech Laboratories, Inc., Chantilly, Va.). Reagents consisted of a capture antibody (mouse ascites anti-TNF-α, immunoglobulin G [IgG] fraction), a detection antibody, recombiant bovine TNF-α (IgG fraction), biotinylated goat anti-rabbit IgG (Zymed Laboratories, Inc., South San Francisco, Calif.), horseradish peroxidase-conjugated streptavidin-biotinylated complex (Amersham Corporation, Arlington Heights, Ill.), substrate (H₂O₂ at 0.1%, vol/vol), and dye (2,2'-azinodiethylbenzothiazoline-sulfonic acid). Internal standards of serially diluted rabbit anti-bovine TNF-α were prepared in PBS (pH 7.2, 0.01 M) with Tween 80 (0.1%, vol/vol) and gelatin (0.5%, vol/vol) (PBST-g). The 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 were dye diluted in citrate buffer. All incubations were at room temperature with the exception of that of capture antibody in carbonate buffer, which was incubated at 4°C. The absorbance of the standards and test samples was read at 405 and 490 nm using an ELISA plate washer and reader (Dynatech MR7000). TNF-α concentrations (nanograms per milliliter) in the test samples were determined by comparing the absorbancy of the test samples with the absorbancy of standards within a linear curve fit.

The data were assessed for normality prior to statistical analysis. Arithmetic and log₁₀-transformed data were analyzed as a split plot with repeated-measure analysis of variance (ANOVA) using Statview software (version 5.0; SAS Institute, Inc., Cary, N.C.). The statistical model included the effects of treatment (i.e., challenge strain and challenge dose), time (days relative to challenge), and the interaction of treatment and time on nitrite, IFN-γ, and TNF-α concentrations in supernatants from PBMC cultures. Fisher’s protected-least significant difference test was applied when significant effects (P < 0.05) were detected by the model. Pearson’s product-moment correlations were computed between nitrite production measured using Griess and HPIC assays, as well as between concentrations of nitrite, IFN-γ, and TNF-α in culture supernatants.

To validate the Griess reaction assay in our culture system, supernatants (49 samples) were evaluated for nitrite by HPIC at an accredited toxicology laboratory (i.e., University of Nebraska Veterinary Diagnostic Center) and by the Griess reaction (i.e., at the National Animal Disease Center). Samples included supernatants from PBMC cultures stimulated with medium alone, PPDa, PPDb, and PWM. Blood samples were collected at prechallenge (day 0) and at 34 days, 68 days, and 124 days postchallenge. For the analysis, supernatants representing a predicted wide range of responses were included. As demonstrated in Fig. 1, results from the two assays had a strong positive linear association (r = 0.94, P < 0.0001; y = 0.57x + 70.87), suggesting that both the Griess and HPIC assays generated similar results. The strongest associations between the Griess and HPIC assays were observed for samples from stim-
ultated cultures (for PPDb, $r = 0.92, y = 0.45x + 87$; for PPDb, $r = 0.96, y = 0.55x + 72$; for PWM, $r = 0.90, y = 0.48x + 95$), which generated a wider range of responses than those from nonstimulated cultures (for medium alone, $r = 0.18, y = 0.11x + 72.5$), in which there was a minimal response.

Nonspecific activation of circulating PBMC may alter spontaneous production of IFN-γ, nitrite, or TNF-α in nonstimulated cultures. Indeed, *M. bovis* BCG infection or intraperitoneal lipopolysaccharide injection of C3H/HeJ mice induces a five- to sixfold increase in serum nitrite or nitrate levels, resulting from increased production of NO by macrophages (32). Levels of IFN-γ in supernatants from 48-h nonstimulated PBMC cultures (0.07 ± 0.006 [mean ± standard error of the mean (SEM)] for samples throughout the study) were unaffected ($P > 0.05$) by time relative to infection or by the dosage or strain of *M. bovis* challenge (data not shown). Likewise, TNF-α levels in supernatants from nonstimulated cultures were unaffected ($P > 0.05$) by the dosage or strain of inculm. However, less ($P < 0.01$) TNF-α was detected in supernatants from nonstimulated cultures collected at 68 days postchallenge (0.91 ± 0.05) than in those collected at 0 days (1.82 ± 0.25), 34 days (1.71 ± 0.21), and 124 days (2.15 ± 0.31) postchallenge, regardless of dosage or the strain of *M. bovis*. In contrast to results for IFN-γ and TNF-α, increases in nitrite relative to prechallenge levels were detected at 34 days, 68 days, and 124 days postchallenge (Fig. 2) (combined data including both dosages and strains; $n = 20$) in 48-h supernatants from nonstimulated cultures. Furthermore, increases ($P < 0.05$) in spontaneous nitrite production upon infection were detected for each of

### TABLE 1. Longitudinal IFN-γ, nitrite, and TNF-α responses of blood mononuclear cells to stimulation with PPDb

| Prechallenge or day after challenge | Mean responses ± SEM$^a$| IFN-γ (OD) | Nitrite (ng/ml) | TNF-α (ng/ml) |
|-----------------------------------|------------------------|------------|----------------|---------------|
| Prechallenge                      | 0 ± 0 A                 | –10 ± 5 A  | –0.34 ± 0.09 A |               |
| 34                                | 0.84 ± 0.26 B           | 129 ± 41 C | 0.58 ± 0.29 B  |               |
| 68                                | 0.93 ± 0.22 B           | 101 ± 24 BC| 1.28 ± 0.38 C  |               |
| 124                               | 0.55 ± 0.19 B           | 59 ± 23 B  | –0.03 ± 0.16 A |               |

$^a$ Blood mononuclear cells were isolated immediately prior to challenge (day 0) and at 34 days, 68 days, and 124 days after challenge and were cultured for 48 h with medium alone or with 5 μg of PPDb per ml. IFN-γ and TNF-α concentrations were quantified by ELISA, and nitrite concentrations were quantified by the Griess reaction. Data were analyzed by repeated-measure ANOVA with $P$ values of 0.001, 0.001, and <0.0001 for IFN-γ, nitrite, and TNF-α, respectively. Values followed by different letters are significantly different ($P < 0.05$).

### TABLE 2. IFN-γ, nitrite, and TNF-α responses of blood mononuclear cells from cattle infected with *M. bovis* to soluble *M. bovis* antigens$^b$

| Stimulant (conc) | Mean responses ± SEM$^a$ | IFN-γ (OD) | Nitrite (ng/ml) | TNF-α (ng/ml) |
|-----------------|--------------------------|------------|----------------|---------------|
| HC-2045T WCS (10 μg/ml) | 0.84 ± 0.24 A | 125 ± 39 A  | 1.34 ± 0.38 A  |               |
| 95-1315 WCS (10 μg/ml)| 0.12 ± 0.04 B | 45 ± 20 B  | 0.21 ± 0.09 B  |               |
| PPDb (5 μg/ml)   | 0.24 ± 0.06 B           | 41 ± 20 B  | 0.15 ± 0.11 B  |               |
| PPDb (1 μg/ml)   | 0.93 ± 0.22 A           | 101 ± 24 A | 1.28 ± 0.38 A  |               |
| PWM (1 μg/ml)    | 2.91 ± 0.29            | 137 ± 21  | 2.60 ± 0.30   |               |

$^a$ Blood mononuclear cells were isolated from cattle 68 days postchallenge. IFN-γ and TNF-α concentrations in culture supernatants were quantified by ELISA, and nitrite concentrations were quantified by the Griess reaction. Values followed by different letters are significantly different ($P < 0.05$).

$^b$ Values represent mean (± SEM, $n = 20$) responses to stimulation (i.e., antigen or PWM) minus the response to medium alone. Responses to PWM are presented to indicate a general responsiveness of the cell population to polyclonal stimulation (i.e., a positive control), and these responses were not compared to the responses of antigen-stimulated cells.
FIG. 4. Effects of challenge strain on nitrite responses. Blood mononuclear cells collected immediately before challenge (day 0) and at 0 days, 34 days, 68 days, or 124 days after being challenged with M. bovis strain 95-1315 (closed triangles, n = 10) or M. bovis strain HC-2045T (open boxes, n = 10) were cultured with 5 μg of PPDb per ml, 5 μg of PPDa per ml, 10 μg of M. bovis strain 95-1315 WCS per ml, or 10 μg of M. bovis strain HC-2045T WCS per ml. Nitrate concentrations in 48-h culture supernatants were quantified by the Griess reaction. The P values for the effects of the strains are indicated on each graph.

The challenge dosage of M. bovis administered influenced responses with greater (P < 0.01, n = 10; 34 days postchallenge) levels of IFN-γ, nitrite, and TNF-α detected in supernatants from PPDb-stimulated cultures from cattle receiving 10^5 CFU of M. bovis organisms than in those receiving 10^3 CFU of M. bovis organisms (Fig. 3). Dosage effects were not detected at 0 days, 68 days, or 124 days postchallenge. Increases (P < 0.01, n = 20) in IFN-γ, nitrite, and TNF-α levels, regardless of dose or strain of inoculum, were detected in supernatants from PPDb-stimulated cultures at 34 days and 68 days postchallenge in comparison to those of prechallenge responses (Table 1). PPDb-induced IFN-γ and nitrite levels were also increased (P < 0.05) at 124 days postchallenge in comparison to those of prechallenge responses (Table 1). IFN-γ, nitrite, and TNF-α responses to either PPDa or HC-2045T WCS exceeded (P < 0.05) the respective responses to either PPDa or 95-1315 WCS (Table 2), regardless of the challenge strain. In general, challenge dosage, duration of infection, and type of antigen used for stimulation affected IFN-γ, nitrite, and TNF-α responses similarly.

The antigen-specific IFN-γ and TNF-α responses of cattle infected with strain HC-2045T did not differ (P > 0.05, repeated-measure ANOVA; 0 to 124 days postchallenge, n = 10) from those of cattle infected with strain 95-1315. In contrast, nitrite responses to PPDb, PPDa, HC-2045T WCS, and 95-1315 WCS of PBMC from HC-2045T-infected cattle exceeded (P < 0.05) those of 95-1315-infected cattle (Fig. 4). While clear differences in disease severity among animals receiving equivalent doses of the two strains were difficult to determine, it did appear that cattle receiving the HC-2045T strain had slightly more severe cases of the disease than did cattle receiving the 95-1315 strain. Lesions were detected at more sites in cattle receiving 10^5 CFU of HC-2045T than in cattle receiving 10^5 CFU of 95-1315, likely impacting the nitrite response.

Both IFN-γ and TNF-α are known to induce NO production (10, 17). Associations among these three responses by cattle PBMC to M. bovis antigens, however, are not clear. To evaluate these associations, differential responses to mycobacterial antigens of all M. bovis-infected cattle (n = 20) at 0 days, 34 days, 68 days, and 124 days postchallenge were evaluated by linear regression analysis (Table 3). In general, strong positive associations between all three readouts (i.e., IFN-γ, nitrite, and TNF-α) were detected with antigen-specific responses. Poor to no associations were detected with these responses to no stimulation or to PWM.

| Stimulant (concn) | Correlation coefficient (r) | IFN-γ vs nitrite | IFN-γ vs TNF-α | Nitrite vs TNF-α |
|-------------------|-----------------------------|-----------------|----------------|-----------------|
| Medium alone (no stimulant) | 0.36** | NS | NS | NS |
| HC-2045T WCS (10 μg/ml) | 0.54*** | 0.61**** | 0.47**** | |
| 95-1315 WCS (10 μg/ml) | 0.67*** | 0.35** | 0.24* | |
| PPDa (5 μg/ml) | 0.70*** | 0.73*** | 0.54**** | |
| PPDa (5 μg/ml) | 0.46**** | 0.41**** | 0.34** | |
| PWM (1 μg/ml) | 0.37*** | NS | 0.24* | |

* Asterisks indicate significant correlations at a P of < 0.05 (*), < 0.01 (**), < 0.001 (***), or < 0.0001 (****).
In vitro-bad cellular immune assays are gaining wide acceptance for use in tuberculosis diagnosis. Of relevance to cattle, an IFN-γ assay (in conjunction with skin testing) was recently approved for use in tuberculosis diagnosis. Other readouts of bovine cellular immune responsiveness (e.g., TNF-α and NO), however, have not been critically analyzed or compared to the IFN-γ response. In the present study, TNF-α and NO responses upon M. bovis infection followed similar kinetics, as did IFN-γ responses (Fig. 3 and 4; Table 1). The relative magnitude of each of these responses to variable antigens was consistent (Tables 1 and 2), and recall IFN-γ, TNF-α, and NO responses to crude M. bovis soluble antigens were clearly associated (Table 3). Thus, evaluation of TNF-α and NO responses, like that of IFN-γ responses, may prove useful for diagnosis of bovine tuberculosis. The nonspecific production of nitrite in PBMC cultures from infected cattle (Fig. 2) may be problematic for development of a useful NO-based diagnostic assay of infection. However, the NO response to M. bovis antigens generally exceeded the response to medium alone, thereby providing antigen specificity (Tables 1 and 2). As with the IFN-γ assay, adaptation of NO and TNF-α assays to a whole-blood format and usage of recombinant antigens will be necessary to enhance the practicality and specificity of these assays.

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REFERENCES

1. Bean, A. G., D. R. Roach, H. Briscoe, M. P. France, H. Korner, J. D. Sedgwick, and W. J. Britton. 1999. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphocytes. J. Immunol. 162:3504–3511.

2. Bolin, C. A., L. D. Whipple, K. V. Khanna, J. M. Risdahl, P. K. Peterson, and T. W. Mollitor. 1997. Infection of swine with Mycobacterium bovis as a model of human tuberculosis. J. Infect. Dis. 176:1559–1566.

3. Budde, B. M., T. J. Ryan, J. M. Pollock, P. Andersen, and G. W. de Lisle. 2001. Use of ESAT-6 in the interferon-gamma test for diagnosis of bovine tuberculosis following skin testing. Vet. Microbiol. 80:37–46.

4. Budde B. M., N. A. Parlane, D. L. Keen, F. E. Albewld, J. M. Pollock, K. Lightbody, and P. Andersen. 1999. Differentiation between Mycobacterium bovis BCG-vaccinated and M. bovis-infected cattle by using recombinant mycobacterial antigens. Clin. Diagn. Lab. Immunol. 6:1–5.

5. Burton J. L., and M. E. Kehrli. 1997. Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc. Natl. Acad. Sci. USA 94:5243–5248.

6. Massengill C. E. 2002. Report of the committee on tuberculosis. United States Animal Health Association Annual Meeting. Pat Campbell and Associates, Richmond, Va.

7. Monaghan, M. L., M. L. Doherty, J. D. Collins, J. F. Kazda, and P. J. Quinn. 1994. The tuberculin test. Vet. Microbiol. 40:111–124.

8. Nonnecke, B. J., W. R. Waters, M. R. Foote, R. M. Fowler, R. L. Horst, and B. L. Miller. Interferon-γ and NO secretion by mononuclear leukocytes from peripheral blood of young and adult cattle vaccinated with Mycobacterium bovis BCG: modulation by 1,25-dihydroxyvitamin D3. Int. J. Vitam. Nutr. Res. in press.

9. Ottenhoff, T. H., D. Kummaratane, and J. L. Casanova. 1998. Novel human immunodeficiencies reveal the essential role of type-1 cytokines in immunity to intracellular bacteria. Immuno. Today 19:491–494.

10. Palmer, M. V., W. R. Waters, and D. L. Whipple. 2003. Aerosol delivery of virulent Mycobacterium bovis to cattle. Tuberculosis 82:275–282.

11. Pollock, J. M., and P. Andersen. 1997. The potential of the ESAT-6 antigen secreted by virulent mycobacteria for specific diagnosis of tuberculosis. J. Infect. Dis. 175:1251–1254.

12. Pollock, J. M., R. M. Girvin, K. A. Lightbody, R. A. Clements, S. D. Neill, B. M. Budde, and P. Andersen. 2000. Assessment of defined antigens for the diagnosis of bovine tuberculosis in skin-test reactor cattle. Vet. Rec. 146:659–665.

13. Rajaraman, V., B. J. Nonnecke, S. T. Franklin, D. C. Hammell, and R. L. Horst. 1998. Effect of vitamins A and E on nitric oxide production by blood mononuclear leukocytes from neonatal calves fed milk replacer. J. Dairy Sci. 81:2728–3285.

14. Roark, G. A., J. Steele, M. Ainsworth, and B. R. Champion. 1986. Activation of macrophages to inhibit proliferation of Mycobacterium tuberculosis: comparrison of the effects of recombinant gamma-interferon on human monocytes and murine peritoneal macrophages. Immunology 59:333–338.

15. Schmitt, S. M., S. D. Fitzgerald, T. M. Cooley, C. S. Bruning-Fann, L. Sullivan, D. Berry, T. Carlson, R. B. Minnis, J. B. Payne, and J. Sikarskie. 1997. Bovine tuberculosis in free-ranging white-tailed deer from Michigan. J. Wildl. Dis. 33:749–758.

16. Stuehr, D. J., and M. A. Marletta. 1985. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. Proc. Natl. Acad. Sci. USA 82:7738–7742.

17. Thoma-Uzynski, S., S. Stenger, O. Takeuchi, M. T. Ochoa, M. Engele, P. A. Sappino, G. E. Grau, P. F. Piguet, and P. Vassalli. 1995. Tumor necrosis factor receptor p55 gene-deficiency results in impaired functions by gamma interferon, interleukin-4, and tumor necrosis factor. Infect. Immun. 63:2123–2131.

18. Van Pinxteren, L. A., P. Andersen, J. L. Casanova. 1997. Identiﬁcation of the ESAT-6 antigen for the diagnosis of tuberculosis. J. Infect. Dis. 175:1251–1254.

19. Vassalli, A. P. Sappino, G. E. Grau, P. F. Piguet, and P. Vassalli. 1989. The inducing role of tumor necrosis factor in the development of bacillary granulomas during BCG infection. Cell 56:731–740.

20. Velderschot, K., C. Manca, R. Colangeli, A. Heijbel, A. Williams, and M. L. Gennaro. 1998. Use of Mycobacterium tuberculosis complex-specific antigen cocktails for a skin test specific for tuberculosis. Infect. Immun. 66:3606–3610.

21. Waldschek, I., E. A., and S. H. E. Kaufmann. 1990. Activation of tuberculosis macrophage functions by gamma interferon, interleukin-4, and tumor necrosis factor. Infect. Immun. 58:2675–2677.

22. Fly, J. L., C. A. Scanga, K. E. Tanaka, and J. Chan. 1998. Effects of aminoguanidine on latent murine tuberculosis. J. Immunol. 160:1796–1803.

23. Fly, J. L., M. M. Goldstein, J. Chan, K. J. Triebold, K. Pfeffer, C. J. Lowenstein, R. Schriever, and B. R. Bloom. 1995. Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. Immunity 2:561–572.

24. Fly, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom. 1993. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. J. Exp. Med. 178:2249–2254.

25. Kindler, V., A. P. Sappino, M. E. Grau, P. F. Piguet, and P. Vassalli. 1989. Inducing role of tumor necrosis factor in the development of bacillary granulomas during BCG infection. Cell 56:731–740.

26. Ewing, Don Robinson, Norm Lyon, and Wayne Varland for animal care.
37. Wang, C. H., H. C. Lin, C. Y. Liu, K. H. Huang, T. T. Huang, C. T. Yu, and H. P. Kuo. 2001. Upregulation of inducible nitric oxide synthase and cytokine secretion in peripheral blood monocytes from pulmonary tuberculosis patients. Int. J. Tuberc. Lung Dis. 5:283–291.
38. Waters, W. R., B. J. Nonnecke, T. E. Rahner, M. V. Palmer, D. L. Whipple, and R. L. Horst. 2001. Modulation of Mycobacterium bovis-specific responses of bovine peripheral blood mononuclear cells by 1,25-dihydroxyvitamin D₃. Clin. Diagn. Lab. Immunol. 8:1204–1212.
39. Wilcke, J. T., B. N. Jensen, P. Ravn, A. B. Andersen, and K. Haslov. 1996. Clinical evaluation of MPT-64 and MPT-59, two proteins secreted from Mycobacterium tuberculosis, for skin test reagents. Tuber. Lung Dis. 77:250–256.
40. Wood, P. R., L. A. Corner, J. S. Rothel, C. Baldock, S. L. Jones, D. B. Cousins, B. S. McCormick, B. R. Francis, J. Creeper, and N. E. Tweddle. 1991. Field comparison of the interferon-gamma assay and the intradermal tuberculin test for the diagnosis of bovine tuberculosis. Aust. Vet. J. 68:286–290.
41. Wood, P. R., and J. S. Rothel. 1994. In vitro immunodiagnostic assays for bovine tuberculosis. Vet. Microbiol. 40:125–135.