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Immune Responses Induced in Cattle by Virulent and Attenuated *Mycobacterium bovis* Strains: Correlation of Delayed-Type Hypersensitivity with Ability of Strains To Grow in Macrophages

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Comparison of immune responses induced in cattle by virulent and attenuated strains of *Mycobacterium bovis* will assist in identifying responses associated with resistance or susceptibility to disease. Four strains of *M. bovis*, one which is virulent in guinea pigs (WAg201) and three which are attenuated in guinea pigs (an isoniazid-resistant strain [WAg405], ATCC 35721, and BCG) were compared for their abilities to induce immune responses in cattle and to grow in bovine lung alveolar macrophage cultures. Extensive macroscopic lesions were found only in cattle inoculated with the virulent *M. bovis* strain. Strong antibody responses to *M. bovis* culture filtrate, as well as persistently high levels of gamma interferon and interleukin-2 released from purified protein derivative (PPD)-stimulated peripheral blood lymphocyte cultures, were observed in the cattle inoculated with the virulent strain compared to those inoculated with the attenuated strains. All cattle inoculated with the virulent strain or two of the attenuated strains (WAg405 and ATCC 35721) elicited strong delayed-type hypersensitivity responses to PPD in skin tests, while animals inoculated with BCG induced only a weak response. The three strains which produced strong skin test responses proliferated well in bovine alveolar macrophages and induced high levels of proinflammatory cytokine mRNAs compared to BCG. Our study showed that skin test responsiveness to PPD correlated with the ability of the strains to grow in alveolar macrophages rather than to their pathogenicity in cattle.

Tuberculosis in humans remains a major health problem worldwide and causes an estimated 3 million deaths per year. Tuberculosis in cattle is a major economic problem in many countries, and its transmission to humans is a small but significant cause of human morbidity and mortality. The main causative organisms for humans and cattle, respectively, are the closely related species *Mycobacterium tuberculosis* and *Mycobacterium bovis*. Cattle are similar to humans in exhibiting a wide range of responses to tuberculosis infection (20). If the initial infection is not contained by the animal, it may give rise to progressive pulmonary disease. In tuberculous cattle, a spectrum of immune responses can develop, but it is not known which responses are clearly associated with resistance or susceptibility to disease. A comparison of immune responses induced by virulent and attenuated strains would contribute to an improved understanding of the pathogenesis of tuberculosis and could greatly help the rational development and testing of new vaccines.

Attention has also been directed at identifying the genetic basis of mycobacterial virulence with the aim of determining how the organism survives host defenses to cause disease (6, 15). It has been shown that expression of the *katG* gene in *M. bovis* and *M. tuberculosis* is associated with virulence and persistence in guinea pigs and mice (16, 25). In the *M. bovis* study, a virulent *M. bovis* strain (WAg201) was exposed to increasing concentrations of isoniazid (INH). An INH-resistant strain (WAg405) which had lost catalase activity and also had a mutation in the *inhA* gene was produced. In marked contrast to the parent, this catalase-negative strain was attenuated for guinea pigs. Integration of a functional *katG* gene into this attenuated strain restored full virulence (25). Another gene that affects the virulence of a member of the *M. tuberculosis* complex is that for the principal sigma factor, *rpoV*. Collins et al. (6) found that the virulence of the attenuated *M. bovis* ATCC 35721 strain could be restored by the addition of a wild-type copy of the *rpoV* gene. The principal sigma factor is essential to the expression of a wide range of different genes, and it has not yet been established which of them in *M. bovis* ATCC 35721 is affected to cause the loss of virulence. Similarly, although three major chromosomal deletions have been recently identified in BCG, the cause of its attenuation has not yet been established (17).

In this work, the immune responses induced by these three attenuated strains (WAg405, ATCC 35721, and BCG) in a natural host for *M. bovis* were compared to those induced by a virulent *M. bovis* strain (WAg201) by using an experimental model of tuberculosis in cattle (3, 4). The responses were also compared to the ability of the *M. bovis* strains to grow in bovine alveolar macrophage cultures and to express cytokine mRNAs. The immune responses induced by the virulent strain could be distinguished from those induced by the attenuated strains by the strong antigen-specific serum antibody response and the persistently high levels of gamma interferon (IFN-γ) and interleukin-2 (IL-2) released from purified protein derivative (PPD)-stimulated whole-blood cultures. However, the delayed-type hypersensitivity (DTH) responses to PPD correlated with the ability of the *M. bovis* strains to grow in macro-

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phage cultures and to induce proinflammatory cytokines rather than to their pathogenicity in cattle.

MATERIALS AND METHODS

Animals. Twenty female Friesian-cross cattle, approximately 15 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 experiment the cattle tested negative for reactivity to bovine PPD in the whole-blood IFN-γ assay. The cattle were grazed in a high-security isolation unit which contained a series of small separate fields.

Bacterial strains and growth conditions. The virulent *M. bovis* strain, WAg201, is a New Zealand bovine isolate and was pathogenic in guinea pigs, in which it caused extensive visible tuberculous lesions in the spleen, liver, and lung (25). The INH MIC for the INH-resistant daughter strain, WAg405, was 64 μg/ml, and the strain was shown to be attenuated in guinea pigs, causing no visible tuberculous lesions (25). *M. bovis* ATCC 37521 (TMC403), obtained from the American Type Culture Collection, was also shown to be attenuated in guinea pigs (6). *M. bovis* BCG Pasteur 1173P2 was used in this study as it had been used in previous trials in cattle (4, 5). The WAg405 strain was sensitive to H_2O_2 at concentrations of 50 μM or greater, whereas the other strains were not sensitive at levels of up to 200 μM. Strains were cultured in Tween albumin broth containing Dubos broth base (Difco Laboratories, Detroit, Mich.) supplemented with 0.006% (vol/vol) alkalinized oleic acid, 0.5% (wt/vol) albumin fraction V, and 0.25% (wt/vol) glucose. The solid medium used for *M. bovis* culture was Middlebrook 7H11 (Difco) supplemented with 0.5% (wt/vol) albumin, 0.2% (wt/vol) glucose, and 1% (wt/vol) sodium pyruvate.

Inoculation of cattle and necropsy procedure. Groups of cattle (four per group) were inoculated intratracheally with 10^6 CFU of the WAg201, WAg405, ATCC 37521, or BCG strain. The inoculation procedure was carried out as described previously (4). Briefly, an 80-cm endotracheal tube containing a fine cannula was inserted per os into the trachea of an anesthetized animal. A 1.5-ml inoculum containing an *M. bovis* strain was injected through the cannula and flushed out with 2 ml of saline. Four nonchallenged cattle served as controls. Following inoculation of the *M. bovis* strains, all of the groups of cattle were kept in separate fields to minimize any cross-infection between groups. Blood samples were collected from the animals at 0, 1, 2, 4, 10, and 15 weeks after the *M. bovis* inoculation. All cattle were killed by use of a captive bolt and severance of the carotid artery and necropsied 15 weeks after inoculation. Procedures for identifying macroscopic tuberculous lesions, processing for histopathology, and bacterial culture have been described previously (4). Samples from pulmonary lymph nodes (left and right bronchial and anterior and posterior mediastinal) were collected from all of the animals for bacterial culture. Additional samples were collected from any tuberculous lesions observed in other lymph nodes or organs.

Antibody ELISA. The *M. bovis* culture filtrate was prepared from *M. bovis* AN5, the strain used for the production of bovine PPD for skin testing in New Zealand. The culture filtrate was diluted to 3 mg/ml in carbonate buffer (pH 9.6) and 100 μl was added to each well of 96-well enzyme-linked immunosorbent assay (ELISA) plates (Maxisorb; Nunc, Roskilde, Denmark). The plates were incubated overnight at 4°C, and the antibody ELISA was carried out as described previously (26). Sera were stored at −20°C until tested. Results were expressed as absorbance indices, calculated by expressing the value found for the test serum as a fraction of the binding of a strong positive reference serum (5) multiplied by 100.

IFN-γ and IL-2 assays. Heparinized blood was dispersed in two 1.5-ml aliquots, and 100 μl of either phosphate-buffered saline (PBS) or bovine PPD (200 μg/ml; supplied by CSL Ltd., Parkville, Victoria, Australia, in the IFN-γ assay kit) was added to the blood in separate wells. After incubation at 37°C for 24 h, the plasma supernatants were harvested and their IFN-γ levels were measured by using a sandwich ELISA kit (CSL Ltd.) as described previously (21). Results were expressed as optical density (OD) indices (OD for the bovine PPD sample/OD for the PBS sample).

IL-2 in the plasma supernatants from the whole-blood cultures was assayed by a bioassay as described previously (9). Triplicate wells containing 10^4 concanavalin A (ConA)-stimulated 4-day lymphoblasts were incubated in 200 μl of supplemented RPMI 1640 medium with 1:10 dilutions of supernatant. After incubation for 24 h, 0.25 μCi of [^{3}H]thymidine was added to each well, and cultures were harvested 16 h later. The amount of tritiated thymidine incorporated was determined by using a liquid beta-scintillation counter (LS600 IC; Beckman Instruments, Fullerton, Calif.). The results were expressed as stimulation indices, defined as mean counts per minute for the bovine PPD plasma supernatant/mean counts per minute for the PBS plasma supernatant. Addition of a monoclonal antibody against bovine IL-2 to the ConA-stimulated lymphoblasts immediately before addition of the plasma supernatants has been shown to block the IL-2 bioactivity.

Intradermal test. Animals were inoculated intradermally with a 0.1-ml volume containing 0.1 mg of bovine PPD (Ministry of Agriculture and Forestry, Central Animal Health Laboratory, Upper Hutt, New Zealand; the PPD used for intradermal testing of cattle in New Zealand) in the right side of the neck 15 weeks after the *M. bovis* inoculation. DTH responses were expressed as the difference between the skin thickness at the time of inoculation and that 72 h later.

TABLE 1. Presence of macroscopic tuberculous lesions and number of animals culture positive following inoculation of cattle with *M. bovis* strains

M. bovis strain	Total no. of lymph nodes with lesions[a]	No. of cattle with lesions in:		No. of cattle culture positive[b]
		Lung	Lymph nodes	
WAg201	36	3	4	4
WAg405	0	0	0	0
ATCC 35721	3	0	2	3
BCG	0	0	0	0
Noninfected	0	0	0	0

[a] Pulmonary, head, and mesenteric lymph nodes in each animal were examined for the presence of lesions as described in Materials and Methods.

[b] *M. bovis* isolated from lymph nodes with lesions or macroscopically normal pulmonary lymph nodes.

Preparation and infection of bovine alveolar macrophages. Alveolar macrophages were obtained by lavage from freshly excised lungs of three *M. bovis*-free cattle and cultured in 96-well microtiter plates in supplemented RPMI containing 10% normal bovine serum as described previously (1). Quadruplicate wells containing approximately 1×10^5 macrophages per well were infected with 5×10^5 CFU of each *M. bovis* strain at a multiplicity of infection (MOI) of 5:1. A proportion of the macrophages were stimulated sequentially with recombinant bovine IFN-γ (50 U/ml) and lipopolysaccharide (LPS) (0.1 μg/ml; *Escherichia coli* 026:B6; Sigma Chemical Co., St. Louis, Mo.) for 20 and 5 h, respectively, prior to infection (2). The recombinant bovine IFN-γ used in this study was produced in the yeast *Pichia pastoris*. Briefly, the cDNA encoding the mature bovine IFN-γ (amino acids 24 to 166) was isolated by reverse transcription-PCR (RT-PCR) of RNA prepared from ConA-stimulated bovine lymphocytes. A construct which placed the mature IFN-γ protein, with a six-histidine tag at the N terminus, in frame with the yeast MF-α signal sequence was made in the expression vector pPIC9 (Invitrogen Corp., Carlsbad, Calif.). Recombinant bovine IFN-γ was produced by methods described previously (24) and purified by immobilized metal-chelating affinity chromatography with Talon (Clontech Laboratories Inc., Palo Alto, Calif.). For the determination of nitric oxide (NO) production and macrophage-derived cytokine mRNA expression, alveolar macrophages were cultured in 24-well plates, and 5×10^5 cells/well were infected with the *M. bovis* strains at an MOI of 5:1.

Assessment of mycobacterial growth and production of NO and cytokine mRNAs. Assessment of mycobacterial growth was determined by addition of [^{3}H]uracil to 48-h macrophage cultures and reincubation for 18 h. The cultures were harvested onto glass fiber filters, and radioactivity was counted in a liquid beta-scintillation counter as described previously (2). Cultures which had been formalin fixed on glass coverslips were stained with hematoxylin-eosin and Ziehl-Neelsen solutions and examined by light microscopy for the presence of intracellular bacilli. NO generated by infected macrophages was assayed by measurement of nitrate in 48-h culture supernatants with the Griess reagent (11).

Expression of tumor necrosis factor alpha (TNF-α), IL-1β, and IL-6 mRNAs in macrophages was determined by RT-PCR at 24 h after infection. RNA was extracted from macrophages and reverse transcribed into cDNA as previously described (2). PCR primers for the cytokines and the constitutively expressed γ-actin gene were as described previously (1). The levels of TNF-α mRNA were quantified by competitive PCR with a competitive template. This was constructed by introducing an 83-bp deletion into the cDNA amplified by the TNF-α-specific primers. Serial dilutions of the competitive template were added to PCR mixtures containing 40 ng of each cDNA to be quantified. Following amplification for 27 cycles, the reaction products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide.

Statistical analysis. Analyses of antibody, IFN-γ and IL-2 responses, incorporation of [^{3}H]uracil, and TNF-α mRNA expression were undertaken by using analysis of variance on $\log_e$-transformed data, while the analysis of skin test responses was by analysis of variance on raw data.

RESULTS

Pathological and bacteriological findings. The cattle were killed and examined at post mortem 15 weeks after inoculation. Tuberculous lesions, consisting of multiple small foci 3 mm in diameter or a large consolidated lesion up to 100 mm in diameter, were found in the lungs of three of the four cattle inoculated with the virulent WAg201 strain (Table 1). Lesions were also found in the pulmonary and head lymph nodes of all four animals in this group and in the mesenteric lymph nodes

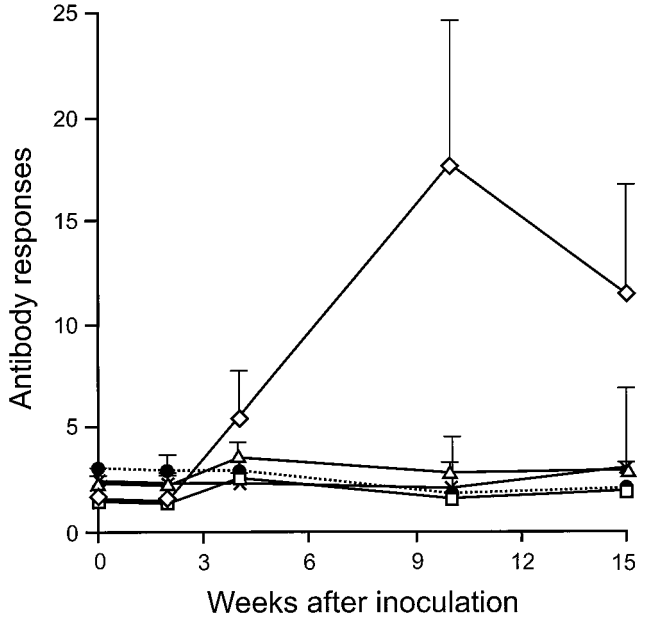

FIG. 1. Antibody responses to *M. bovis* culture filtrate. Mean antibody responses of animals ($n = 4$) inoculated with *M. bovis* WAg201 (◇), WAg405 (□), ATCC 35721 (△), or BCG (✕) or of noninfected animals (●) are shown. Data are expressed as absorbance indices ± standard errors.

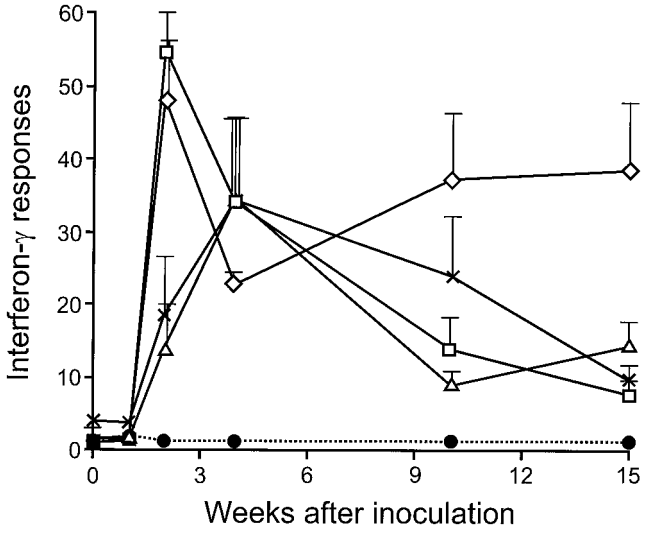

FIG. 2. IFN-γ released from bovine PPD-stimulated peripheral blood lymphocytes from animals ($n = 4$) inoculated with *M. bovis* WAg201 (◇), WAg405 (□), ATCC 35721 (△), or BCG (✕) or from noninfected animals (●). Data are expressed as mean OD indices ± standard errors.

of one animal. These lesions ranged in size from 3 to 50 mm in diameter. The ATCC 35721 strain produced small lesions in pulmonary lymph nodes in two of four animals (ranging from 2 to 3 mm in diameter), but no lesions were observed in their lungs. *M. bovis* was recovered from all of the lesions found in the lungs and pulmonary lymph nodes of the four animals inoculated with WAg201 and the pulmonary lymph nodes of three of the four ATCC 35721-inoculated animals. Tuberculous lesions were not observed in and *M. bovis* was not cultured from the WAg405-inoculated, BCG-inoculated, or nonchallenged animals.

Development of antibody responses during infection. Cattle inoculated with the virulent WAg201 strain produced a strong antibody response to *M. bovis* culture filtrate by 10 weeks after inoculation (Fig. 1). In contrast, antibody responses to *M. bovis* culture filtrate were very low in animals inoculated with either *M. bovis* WAg405, ATCC 35721, or BCG and in the nonchallenged group. The mean antibody responses observed in the WAg201 group were significantly greater than those in the other groups at 10 and 15 weeks after inoculation ($P < 0.05$).

Development of T-cell responses during infection. T-cell responses were measured by the release of IFN-γ and IL-2 from whole-blood cultures stimulated with bovine PPD and by DTH responses to bovine PPD. Cattle infected with each of the four strains of *M. bovis* produced strong IFN-γ and IL-2 responses which were significantly greater than those for the nonchallenged group from 2 weeks onwards ($P < 0.05$) (Fig. 2 and 3). The mean IFN-γ and IL-2 responses for animals inoculated with WAg405, ATCC 35721, and BCG gradually declined by 4 weeks after inoculation but remained elevated in cattle infected with the virulent *M. bovis* WAg201 strain. The mean IFN-γ response for the WAg201-inoculated group at 15 weeks after inoculation was significantly greater than those for the ATCC 35721-inoculated, BCG-inoculated, and nonchallenged groups ($P < 0.05$). For the IL-2 responses, the mean response for the WAg201-inoculated group at 10 weeks after inoculation was significantly greater than those for all of the other groups ($P < 0.05$).

DTH responses to bovine PPD in infected animals were measured by the intradermal test at 15 weeks after inoculation. Animals inoculated with the WAg201, WAg405, or ATCC 35721 strain gave strong DTH responses (Fig. 4). There were no significant differences between the mean DTH responses of these three groups, but the means were significantly greater than those for BCG-inoculated and nonchallenged groups ($P < 0.05$).

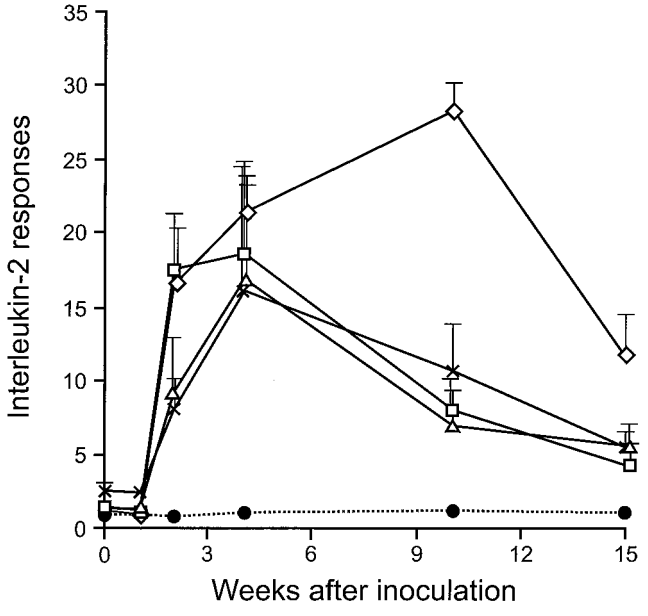

FIG. 3. IL-2 released from bovine PPD-stimulated peripheral blood lymphocytes from animals ($n = 4$) inoculated with *M. bovis* WAg201 (◇), WAg405 (□), ATCC 35721 (△), or BCG (✕) or from noninfected animals (●). Data are expressed as mean stimulation indices ± standard errors.

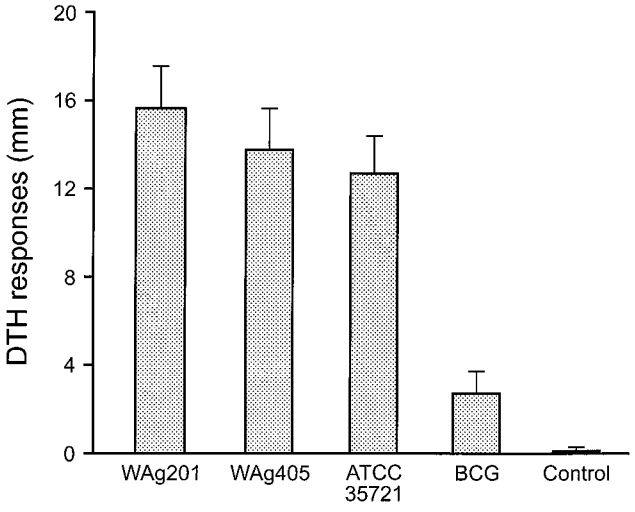

FIG. 4. DTH responses at 15 weeks after inoculation in animals ($n = 4$) inoculated with *M. bovis* WAg201, WAg405, ATCC 35721, or BCG or in noninfected animals. Results are expressed as mean differences between the skin thickness at the time of inoculation and that 72 h later (± standard error).

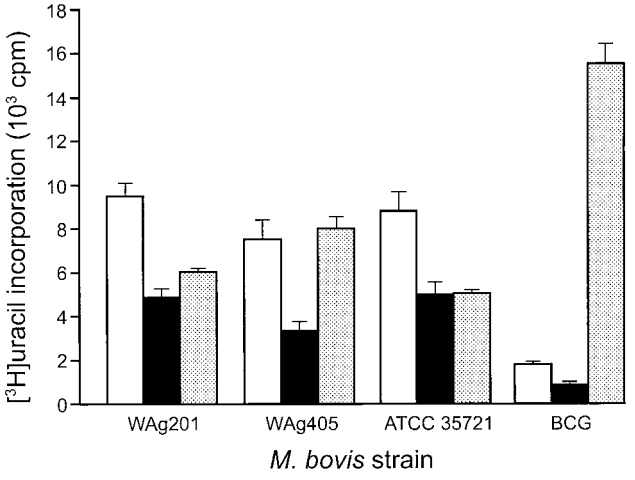

FIG. 5. Growth of *M. bovis* strains in bovine alveolar macrophages and in culture medium. Growth of *M. bovis* strains was determined by measurement of [^{3}H]uracil incorporation by bacteria at 48 h after infection of unstimulated macrophages (▭) or macrophages stimulated with IFN-γ and LPS (■) or after 48 h of incubation in medium (▨). The results are the means from experiments conducted with macrophages from three animals (± standard errors). The mean [^{3}H]uracil uptake by noninfected macrophages was 450 cpm.

Growth of *M. bovis* strains in alveolar macrophages is related to their ability to induce DTH in cattle and not to the disease status. Since the different strains varied in virulence for cattle, we examined their abilities to grow within bovine alveolar macrophages, to induce cytokine mRNA expression, and to stimulate NO production. Metabolic labelling with [^{3}H]uracil was used as an indirect measure of growth of mycobacteria in macrophages. Acid-fast staining showed that the bacilli were intracellular following infection of confluent monolayers, and therefore the uracil counts reflected intracellular growth of bacteria. The WAg201, WAg405, and ATCC 35721 strains grew well within the unstimulated macrophages, with only minor differences in their ability to incorporate uracil (Fig. 5). All of these strains also grew well in macrophages infected at an MOI of 1:1, although uracil uptake was proportionally lower than that observed at an MOI of 5:1 (data not shown). Prior activation of macrophages with IFN-γ and LPS resulted in a significant reduction in growth ($P < 0.01$). The growth of *M. bovis* BCG within both unstimulated and stimulated macrophages was significantly suppressed compared to that for the other three strains ($P < 0.05$). All strains were capable of growth when cultured in supplemented RPMI containing 10% normal bovine serum, with *M. bovis* BCG exhibiting a higher rate of [^{3}H]uracil uptake than the other three strains (Fig. 5).

Unstimulated macrophages infected with *M. bovis* produced moderate amounts of NO during the 48-h period. Stimulation of the macrophages with IFN-γ and LPS markedly increased NO production, but no differences between macrophages infected with the different strains were observed (Table 2). Induction of TNF-α, IL-1β, and IL-6 mRNAs in unstimulated macrophages infected with the WAg201, WAg405, and ATCC 35721 strains was greater than that observed in macrophages infected with BCG (Table 2). The levels of TNF-α mRNA, quantified by competitive PCR, were significantly higher in macrophages infected with WAg201, WAg405, or ATCC 35721 than in those infected with BCG ($P < 0.05$). No significant differences between the levels of TNF-α, IL-1β, and IL-6 mRNAs in the stimulated macrophages infected with the different strains were observed, but for the BCG-infected macrophages TNF-α mRNA levels were higher in the stimulated than in the unstimulated macrophages ($P < 0.05$). A comparison of the results from competitive PCR with the qualitative measurement of cytokine expression showed that TNF-α transcript levels of 5×10^6 copies/μg of total RNA were equated with a strong signal intensity (designated +++) on an ethidium bromide-stained agarose gel, while levels of 1×10^6 copies/μg of total RNA were equated with a weak signal (designated +).

TABLE 2. NO production and cytokine mRNA expression in bovine alveolar macrophages infected with *M. bovis* strains

M. bovis strain	Unstimulated macrophages				Macrophages stimulated with IFN-γ and LPS			
	NO (μM)[a]	TNF-α[b]	IL-1β[c]	IL-6[c]	NO (μM)	TNF-α	IL-1β	IL-6
WAg201	3.4 ± 1.3	6.4 ± 0.8	+++	+++	14.3 ± 4.6	3.7 ± 1.1	+++	++
WAg405	3.2 ± 1.2	5.6 ± 0.8	+++	+++	15.0 ± 2.4	4.0 ± 0.8	+++	++
ATCC 35721	3.9 ± 2.0	4.8 ± 1.4	+++	+++	16.8 ± 2.7	3.1 ± 0.8	+++	++
BCG	3.0 ± 1.4	1.1 ± 0.3	+	+	16.1 ± 2.4	4.0 ± 1.6	+++	++

[a] NO in macrophage culture supernatants was determined at 48 h after infection. Results are means of determinations with macrophages from three animals ± standard errors.

[b] TNF-α mRNA was determined at 24 h after infection by quantitative competitive RT-PCR and is shown as copies (10^6) of TNF-α mRNA per microgram of total cellular RNA. Results are means of determinations with macrophages from three animals ± standard errors. The TNF-α mRNA level in noninfected unstimulated macrophages was 7.8 (±1.0) × 10^4 copies/μg of total RNA, and that in noninfected stimulated macrophages was 3.6 (±1.2) × 10^6 copies/μg of total RNA.

[c] Determined at 24 h after infection by qualitative RT-PCR. +, weak signal; ++, moderate signal; +++, strong signal.

DISCUSSION

Three attenuated *M. bovis* strains with widely varied histories were chosen for this study. BCG has been investigated for many years in a wide range of different animal hosts, and its attenuated nature in these animals is well known; ATCC 35721 was isolated by Karlson in 1950 and found by him to be attenuated in guinea pigs and rabbits; and WAg405 was produced in this laboratory (25). Previously, both WAg405 (25) and ATCC 35721 (6) were shown by us to be attenuated in guinea pigs. In this study all three of these strains were shown to be attenuated in cattle. Apart from ATCC 35721, lesions were not observed in cattle inoculated with these strains, and the lesions that were observed with ATCC 35721 were small and limited in distribution compared with those with the wild-type strain WAg201.

Inoculation of cattle with the virulent strain WAg201 induced an antigen-specific antibody response, and persistently high levels of IFN-γ and IL-2 were released from antigen-stimulated whole-blood cultures. In contrast, inoculation of cattle with ATCC 35721, WAg405, or BCG induced no antibody response but induced IFN-γ and IL-2 responses which peaked at 2 to 4 weeks after inoculation and rapidly declined.

In other studies on the experimental infection of cattle with virulent *M. bovis*, cell-mediated responses as measured by lymphocyte proliferation and release of IFN-γ were seen early in all infected animals, and the appearance of antibody responses correlated with the severity of disease (12, 18). The persistence of high IFN-γ responses in cattle which have developed tuberculous lesions from either experimental (3, 18) or natural (26) infections and the transient increase in IFN-γ responses in cattle infected with attenuated *M. bovis* strains support the view that an active infection is required for induction of strong IFN-γ responses in cattle. This situation appears to be different from that for humans, where *M. tuberculosis* antigen-induced production of IFN-γ is often depressed in peripheral blood mononuclear cells of patients with active pulmonary tuberculosis compared to healthy tuberculin reactors (14, 22, 23). In human tuberculosis the immunosuppression of cell-mediated immune responses may be associated with production of transforming growth factor β, as neutralizing antibody to transforming growth factor β was shown to significantly increase PPD-stimulated production of IFN-γ in tuberculosis patients but not in contacts (13). In experimental *M. bovis* infection studies with cattle, increasing the challenge dose decreased the interval before antibody was detected and increased the number of lesions and their distribution, while little or no antibody was detected in tuberculous animals infected naturally (18).

In the present study, there was no correlation between the induction of DTH responses to bovine PPD and the virulence of the inoculated strain. Dissociation between IFN-γ and DTH responses has been seen in studies with IFN-γ-knockout mice, where the loss of the ability to secrete IFN-γ had a devastating effect on protective immunity against *M. tuberculosis* but did not appear to be essential for induction of a strong DTH response (7). The ability of the *M. bovis* strains in the present study to induce a DTH response correlated with their growth and persistence in bovine alveolar macrophage cultures. BCG was the only *M. bovis* strain which was not able to persist in macrophages, although this strain grew well in culture medium alone. Activation of the macrophages by pretreatment with IFN-γ and LPS partially controlled the growth of all of the strains in the macrophage cultures, but the relative differences between the strains in terms of their growth and persistence in the stimulated cultures were the same as for the unstimulated cultures.

The induction of proinflammatory cytokine TNF-α, IL-1β, and IL-6 mRNAs from the unstimulated macrophage cultures was associated with the growth and persistence of *M. bovis* strains within the macrophages, with a very low induction observed in the BCG-infected macrophages. The levels of proinflammatory cytokine mRNAs in the stimulated macrophage cultures which were infected with *M. bovis* were similar for all strains, with TNF-α and IL-6 responses being slightly lower than those observed in the unstimulated cultures infected with either WAg201, WAg405, or ATCC 35721. These lower responses may have arisen from the partial control of the growth of *M. bovis*. The increase in cytokine mRNA expression in the stimulated cultures infected with BCG is more likely to have arisen from the combined effects of stimulation with IFN-γ and LPS.

It has been considered that the pathology resulting from infection with virulent *M. tuberculosis* strains resulted from the ability of the strains to induce macrophages to synthesize and secrete cytokines (8). There was no indication from our study that production of proinflammatory cytokines from infected macrophages correlates with the virulence of the strain.

There was no association between the growth of the different *M. bovis* strains within macrophages and the levels of NO generated. However, NO production was markedly enhanced when the macrophages were stimulated with IFN-γ and LPS and was associated with a partial control of growth of all strains. This is in agreement with previous observations that increased induction of NO in stimulated macrophages was associated with a partial restriction of growth of virulent *M. bovis*, although blocking of NO production with N^G-monomethyl-L-arginine had no affect on the amount of growth (2). In an earlier study (1), the kinetics of the growth of BCG and *M. bovis* in bovine alveolar macrophages was investigated by using various MOIs with culture for 1 to 4 days. Infection of macrophages by virulent *M. bovis* consistently resulted in enhanced bacterial metabolism and induction of proinflammatory cytokines and reduced the viability of macrophages compared with BCG-infected macrophages (1, 2).

A comparison of the growth rate of the avirulent H37Ra *M. tuberculosis* strain with that of the virulent H37Rv strain in macrophages showed that H37Ra and H37Rv grew at similar rates in human monocyte-derived macrophages, whereas H37Rv grew more rapidly in murine macrophages (19). However, in a subsequent study using a very low MOI (one live *M. tuberculosis* organism per 80 cells) and with cultures observed over a 10-day period, H37Rv grew more rapidly than H37Ra in human monocyte-derived macrophages (27).

There is considerable evidence that the ability to overcome the antibacterial effects of peroxide that is produced in infected macrophages contributes to the virulence of the *M. tuberculosis* complex (10). In the present study, although WAg405 was sensitive to H_2O_2, its growth rate even when the macrophages were activated with IFN-γ and LPS was comparable to that of its parent catalase-positive strain WAg201. This indicates that in these short-term macrophage cultures, the lack of an active catalase/peroxidase is not important for optimum growth of *M. bovis*. Nevertheless, WAg405 is avirulent in both guinea pigs and cattle, so conditions for multiplication and survival of the organisms in macrophages in vivo appear to be different from those in vitro.

We have identified that the production of both a strong sustained cellular immune response, as characterized by high levels of IL-2 and persistently high levels of IFN-γ, and a strong antibody response was associated with disease. In contrast, the induction of a DTH response in cattle did not correlate with the pathological status of the animals but rather was correlated with the ability of the strain to multiply in bovine

alveolar macrophages and to induce a proinflammatory cytokine response. The marked differences observed between BCG and the other two attenuated *M. bovis* strains raise the possibility that attenuated strains which are more immunogenic than BCG can be developed, and protection studies using recently attenuated strains are being planned.

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