Mycobacterium bovis-Infected Cervine Alveolar Macrophages Secrete Lymphoreactive Lipid Antigens

FRANK E. ALDWELL,^{1*} BRIDGET L. DICKER,² FERNANDA M. DA SILVA TATLEY,³ MARTIN F. CROSS,^{4†} SIMON LIGGETT,¹ COLIN G. MACKINTOSH,⁴ AND J. FRANK T. GRIFFIN¹

Disease Research Laboratory, Department of Microbiology,¹ and Cancer Genetics Laboratory, Department of Biochemistry,³ University of Otago, Dunedin, Department of Molecular Medicine, University of Auckland School of Medicine, Auckland,² and AgResearch Invermay, Mosgiel,⁴ New Zealand

Received 30 June 2000/Returned for modification 6 September 2000/Accepted 26 September 2000

Tuberculosis is caused by intracellular bacteria belonging to the genus *Mycobacterium*, including *M. tuberculosis* and *M. bovis*. Alveolar macrophages (AMs) are the primary host cell for inhaled mycobacteria. However, little is known about the mechanisms by which infected AMs can process and present mycobacterial antigens to primed lymphocytes and how these responses may affect ensuing protection in the host. In the present study, we sought to determine whether AMs from a naturally susceptible host for *Mycobacterium bovis* (red deer) could produce and secrete soluble immunoreactive antigens following mycobacterial infection *in vitro*. Confluent monolayers of deer AMs were infected with either heat-killed or live virulent *M. bovis* or *M. bovis* BCG at a multiplicity of infection of 5:1 and cultured for 48 h. Culture supernatants were collected, concentrated, and tested for the presence of mycobacterial antigens in a lymphocyte proliferation assay by using peripheral blood mononuclear cells from *M. bovis*-sensitized or naive deer. Supernatants derived from macrophages which had been infected with live bacilli stimulated the proliferation of antigen-sensitized, but not naive, lymphocytes. Supernatants derived from uninoculated AMs or AMs inoculated with heat-killed bacilli failed to stimulate lymphocyte proliferation. The lymphoproliferative activity was retained following lipid extraction of the supernatants, which were free of amino groups as determined by thin-layer chromatography. These results demonstrate that mycobacteria which are actively growing within AMs produce lipids which are secreted into the extracellular milieu and that these lipids are recognized by lymphocytes from mycobacterium-primed hosts. We suggest that mycobacterial lipids are released from AMs following aerosol infection *in vivo* and that they play an important role in the early immune response to tuberculosis.

The rational design of improved diagnostic tests and effective vaccines against tuberculosis requires an improved understanding of the immune response to infection, particularly during the initial stages, about which little is known. Central to understanding immunity to tuberculosis is the interaction between mycobacteria and the cell ultimately responsible for their destruction, the macrophage. To survive and multiply within the host, *Mycobacterium bovis* must adapt to the hostile intracellular environment of the macrophage. A feature of mycobacteria is their ability to resist the microbicidal activities of macrophages (27). Following phagocytosis, virulent mycobacteria reside within phagosomes and are thought to avoid microbicidal activity by limiting lysosomal fusion and subsequent acidification of the phagosomal vacuole (11, 27). The subsequent intracellular growth and replication of mycobacteria are confined primarily to phagosomes, at least during the initial stages of infection. It is arguably these preliminary stages of intracellular residence that determine mycobacterial survival and whether the bacilli are able to successfully replicate.

In hosts which are susceptible to infection, virulent mycobacteria are able to replicate within macrophages. During the critical initial stages of intracellular survival and growth, it is possible that mycobacteria will generate molecules which can be recognized by the host's immune surveillance (1, 17, 23). In

turn, lymphocyte-mediated responses generated by antigens derived from the early stages of intracellular mycobacterial growth have the potential to contribute to immune protection by the host or, conversely, to mechanisms of pathogenesis. However, the ability of infected macrophages to generate immunoreactive molecules remains uncertain, particularly during the early stages of infection.

While intracellular growth and replication of mycobacteria are confined primarily to phagosomes, at least during the initial stages of infection, there is recent evidence in mice and humans for stimulation of T cells which recognize antigens released into the cytosol (13, 18, 20, 23, 28). More recently, a subclass of T cells which recognizes mycobacterial lipids presented by the nonclassical major histocompatibility complex (CD1) has been identified (25, 30). Data from these publications suggest that some mycobacterial products are able to escape the phagosome and enter the cytosol and that nonpeptide mycobacterial antigens are capable of being recognized by T cells.

In the present study, we sought to determine whether *M. bovis* actively replicating within alveolar macrophages (AMs) *in vitro* released antigens into the extracellular milieu. For these studies, we used AMs derived from red deer (*Cervus elaphus*). Red deer are a naturally susceptible host for *M. bovis* (16, 21), and large numbers of AMs can be isolated from the lungs of deer for experimental purposes. We demonstrate that AMs infected with *M. bovis* *in vitro* generate lipid molecules that can stimulate proliferation of lymphocytes derived from *M. bovis*-infected deer. These molecules are secreted into the extracellular environment soon after infection and may there-

* Corresponding author. Mailing address: Department of Microbiology, University of Otago, P.O. Box 56, Dunedin, New Zealand. Phone: 64 34797710. Fax: 64 34772160. E-mail: frank.aldwell@stonebow.otago.ac.nz.

† Present address: MHRC, Institute of Food Nutrition & Human Health, Massey University, Palmerston North, New Zealand.

fore be important determinants of immunity against tuberculosis *in vivo*.

MATERIALS AND METHODS

Bacteria. The two strains of mycobacteria used were *M. bovis* BCG Pasteur 1173P2 and virulent *M. bovis* strain 83/6235, which was originally isolated from a tuberculous lesion in a brushtail possum and has been used in previous macrophage infection studies (3, 7). The strains were grown to mid-log phase in Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) supplemented with Tween 80, 0.006% (vol/vol) alkalized oleic acid, 0.5% (wt/vol) albumin (fraction V), and 0.25% (wt/vol) glucose. Bacteria were washed twice in phosphate-buffered saline (PBS) and stored in frozen aliquots at -70°C . Prior to use in infection experiments, both strains were thawed and sonicated for 75 s at 40 W in a sonicating water bath (Branson, Shelton, Conn.).

Animals. Red deer (*Cervus elaphus*) were treated as follows. A group of five deer were challenged via the intratracheal route with 100 to 500 CFU of a virulent *M. bovis* strain (MES/89, isolated from a field case of tuberculosis in deer) (21). For some experiments, a second group of three deer served as nonsensitized controls. *M. bovis*-inoculated deer were grazed separately from the control deer at a quarantined deer farm. Blood samples were collected from the jugular vein into heparinized Vacutainer tubes.

Preparation of AMs. Lung-derived macrophages were obtained from freshly excised lungs of healthy deer by methods previously described for preparing bovine AMs (35). Briefly, 1 to 2 liters of sterile PBS was introduced into the trachea, and the lung lobes were gently massaged for 5 to 10 min. Lavage fluid was collected, and cells were washed twice in warm PBS. Cells were resuspended at $5 \times 10^5/\text{ml}$ in RPMI supplemented with 2% normal deer serum and 50 U of penicillin G per ml (supplemented RPMI). For production of macrophage supernatants (MSs), 50 ml of cell suspension was seeded into 175-cm² tissue culture flasks (Falcon). For assessment of intracellular growth, 100 μl of AM suspension was added per well of flat-bottomed 96-well tissue culture microtiter plates (Nunc). Cells were allowed to adhere for 2 h, after which nonadherent cells were removed by gentle washing with warm PBS. Adherent cell populations routinely comprised over 95% macrophages, as determined by microscopic examination following Giemsa and nonspecific esterase staining.

Assessment of mycobacterial growth. Intracellular growth of *M. bovis* BCG and virulent *M. bovis* was determined by metabolic labeling with tritiated uracil as described previously (2, 35). AMs in 96-well microtiter plates were infected with BCG or *M. bovis* at a multiplicity of infection (MOI) of 5:1. Briefly, cultures were pulsed with 1.0 μCi of [³H]uracil (Amersham, Sydney, Australia) per well at 6, 24, 48, and 72 h postinfection. After a further 24-h incubation, cultures were heated to 80°C for 30 min, allowed to cool, and harvested onto glass fiber filters (Whatman, Inc., Clifton, N.J.) with an automated cell harvester (Cambridge Technology, Inc., Watertown, Mass.). The amount of [³H]uracil incorporated was determined with a liquid β -scintillation counter (Beckman LS6000 IC; Beckman Instruments, Fullerton, Calif.).

Infection of macrophages and harvesting of supernatants. Flasks, each containing approximately 2×10^7 AMs, were inoculated with 10^8 live or heat-killed *M. bovis* BCG or virulent *M. bovis* cells. Noninoculated flasks served as controls. After incubation for 4 h at 37°C in a 5% CO_2 -95% air atmosphere, monolayers were washed three times with warm PBS to remove extracellular bacteria. The supernatant from the third wash was centrifuged at $2,000 \times g$, and the pellet was resuspended and examined microscopically for the presence of acid-fast bacilli. After the final wash, 100 ml of RPMI containing 50 U of penicillin G per ml (a concentration which was previously determined not to inhibit intracellular growth of *M. bovis* or BCG) was added to the flasks, which were incubated for a further 48 h at 37°C in a 5% CO_2 -95% air atmosphere.

Aliquots of serum-free MS were collected from tissue culture flasks and filtered through 0.20- μm -pore-size filters (Acrodisc, Gellman Sciences, Mich.). Filtered supernatants were concentrated 15-fold on an Amicon YM 3 membrane (Amicon, Danvers, Mass.) and further filtered through 0.20- μm -pore-size filters to yield a final volume of 50 to 60 ml per liter of supernatant. The protein concentration in the concentrated supernatant fractions was estimated by the Bradford method (Bio-Rad Laboratories, Richmond, Calif.). Supernatant fractions were standardized on the basis of protein estimation, aliquoted, and stored at -70°C for use in lymphocyte proliferation assays (LPAs). *M. bovis* culture filtrate (CF) for use in LPAs and Western blotting was prepared from *M. bovis* grown to mid-log phase in Middlebrook 7H9 broth, filtered through 0.20- μm -pore-size filters, and concentrated 15-fold on an Amicon YM 3 membrane.

LPA. LPAs were carried out as previously described (9) with antigen-sensitized lymphocytes obtained from *M. bovis*-infected deer at 5 weeks postinoculation. Briefly, peripheral blood lymphocytes (PBLs) were isolated from heparinized venous blood samples by centrifugation on Ficoll-Conray. Cells were washed and resuspended at a concentration of $2.5 \times 10^6/\text{ml}$ in RPMI 1640 (GIBCO, Grand Island, N.Y.) supplemented with 2 mM glutamine, gentamicin (80 $\mu\text{g}/\text{ml}$), and 10% pooled normal deer serum. Aliquots of 100 μl were dispensed into 96-well flat-bottom microtiter trays (Nunc). Fifty microliters of concentrated MS fractions (protein estimation, 20 to 100 $\mu\text{g}/\text{ml}$), lipid-extracted MS fractions (100 $\mu\text{g}/\text{ml}$), mycolic acid from *M. tuberculosis* (100 $\mu\text{g}/\text{ml}$) (Sigma), lipid-extracted *M. bovis* CF (100 $\mu\text{g}/\text{ml}$), or medium alone was added to wells in triplicate. After incubation for 72 h, 1 μCi of [³H]thymidine (Amersham, Buckinghamshire,

England) was added to each well. The cultures were incubated for a further 18 h, and the cells were harvested onto glass fiber filters with a cell harvester (Cambridge Technology, Inc.; series 2800). Radioactivity was measured with a scintillation counter (Wallac 1205 Betaplate).

Lipid extraction and analysis of antigens. Concentrated supernatant fractions were extracted by standard lipid extraction procedures (5). Supernatant (0.2 volume) was mixed with chloroform-methanol (2:1 [vol/vol]) and stirred for 4 h at room temperature. The aqueous phase (containing proteins, salts, and other polar molecules) was separated from the organic phase (mostly lipids), and the organic phase was dried in a rotoevaporator (Buchi, Flawil, Switzerland). Lipid extracts were weighed, resuspended at 100 $\mu\text{g}/\text{ml}$ in PBS-Tween (PBST) at 40°C , and stored at 4°C for use in thin-layer chromatography (TLC) analysis and LPAs. Lipid fractions of MS were loaded into silica gel TLC plates (Whatman grade 3MM; Sigma). TLC plates were developed twice in chloroform-methanol (2:1 [vol/vol]). Lipid extracts were visualized by staining with iodine crystals (29), and ninhydrin spray (Sigma) was used to stain lipids with free amino groups (26). Lipid-extracted MSs were incubated with excess chymotrypsin (10-fold excess by weight; Sigma) or proteinase K (200 $\mu\text{g}/\text{ml}$; Boehringer Mannheim) for 1 h at 37°C , followed by addition of Bowman soybean inhibitor (20-fold excess by weight; Sigma) and were further tested for lymphoproliferative responses in *M. bovis*-infected deer.

SDS-PAGE and Western blotting. *M. bovis* CF, mycolic acid, concentrated MS from *M. bovis*- or BCG-infected AMs, and lipid-extracted MS from *M. bovis*- or BCG-infected AMs were prepared as described above. Bovine purified protein derivative (PPDB; CSL, Melbourne, Australia), *M. bovis* CF, mycolic acid, and lipid-extracted MS were used at a concentration of 100 $\mu\text{g}/\text{ml}$, and concentrated MSs were used at 20 to 100 $\mu\text{g}/\text{ml}$.

For silver staining, analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 10 to 20% gradient gels (16 by 16 by 0.075 cm) under reducing conditions as described by Laemmli (19). For calibration, low-molecular-weight standard markers (Bio-Rad) were run in parallel with the samples. The gels were visualized by silver staining.

For Western blotting, antigens were separated electrophoretically by SDS-PAGE (8% polyacrylamide), as described by Laemmli (19), with a Mini-Protein 3 (Bio-Rad). Antigens were transferred electrophoretically to Trans-Blot transfer medium (pure nitrocellulose; Bio-Rad), with a Mini Trans-Blot transfer cell with buffer (25 mM Tris HCl [pH 8.3], 192 mM glycine, 20% methanol), for 1.5 h at 100 V at 4°C . A rabbit polyclonal antibody against *M. bovis* sonicate (a gift from J. Pollock, Stormont, Northern Ireland) used at a 1:500 dilution provided the primary antibody. Following incubation with affinity-purified goat anti-rabbit peroxidase-conjugated immunoglobulin G (Gibco, Grand Island, N.Y.) at a dilution of 1:5,000, development was carried out as recommended in the ECL (enhanced chemiluminescence) Western blotting detection system kit (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom).

RESULTS

Confirmation that *M. bovis* is growing within deer AMs. Isolation of AMs from deer lungs yielded 5×10^8 to 10×10^8 mononuclear cells, with lymphocytes accounting for less than 1% and neutrophils accounting for less than 4% of lavage cells. Adherent cells were greater than 97% macrophages, as determined by esterase staining and morphology following Giemsa staining. Light microscopic examination of acid-fast-stained slide chamber cultures at 6 h following infection showed that at an MOI of 5:1, 80 to 90% of AMs were infected with one or more acid-fast organisms (AFOs) per cell. Following washing and staining, all AFOs were intracellular, and extracellular or pericellular bacilli were not observed. In order to determine that supernatants were collected from AMs which contained actively growing bacilli, metabolic labelling with [³H]uracil was used as an indirect measure of intracellular growth of BCG and *M. bovis*. We have previously shown that this assay correlates with intracellular growth of *M. bovis* in macrophages (2, 35). Figure 1 shows that [³H]uracil uptake by BCG-infected AMs remained low (<3,000 cpm) between 24 and 96 h postinfection. In contrast, [³H]uracil uptake by virulent *M. bovis* increased markedly between 24 and 96 h postinfection (800 to 13,800 cpm). AMs inoculated with heat-killed BCG or *M. bovis* elicited [³H]uracil counts of 100 to 500 cpm. The mean [³H]uracil uptake by uninfected AMs for all time points was 470 cpm. At 48 h postinfection, intracellular bacteria were metabolically active, and AMs remained fully intact. This time point was chosen for collection of MS for use in LPAs.

FIG. 1. Intracellular growth of *M. bovis* and BCG in deer AMs. Macrophages were infected with *M. bovis* (◇) or BCG (□) at an MOI of 5:1. Uninoculated AMs (△) served as controls. Growth of bacilli was determined by pulsing AMs with [³H]uracil and measuring uptake of [³H]uracil by bacteria at the times indicated. The mean [³H]uracil uptake by uninoculated AMs for all time points was 470 cpm. Each value is expressed as the mean of quadruplicate determinations from experiments conducted with AMs from five deer (± standard errors).

AM-derived supernatants stimulate lymphoproliferative responses in PBLs from *M. bovis*-infected deer. Supernatants from BCG- or *M. bovis*-infected AMs which had been concentrated 15-fold elicited lymphoproliferative responses in PBLs derived from *M. bovis*-infected deer. The magnitude of these responses was titratable and was stronger for *M. bovis*-infected AMs than for BCG-infected AMs (Fig. 2). In contrast, supernatants from *M. bovis*- or BCG-infected AMs did not stimulate the proliferation of lymphocytes derived from uninfected deer. This experiment suggests that AMs infected with replicating BCG or *M. bovis* secrete antigens which are recognized specifically by *M. bovis*-infected deer. Based on these results, we chose a 1:2 dilution of concentrated MS for further studies.

FIG. 2. Titration of macrophage supernatants on deer PBLs. Lymphocyte proliferation responses to PBLs derived from *M. bovis*-sensitized deer were determined by incubating MS generated from *M. bovis* (◇) or BCG-inoculated (□) or uninoculated (△) AMs. The mean [³H]thymidine uptake by PBLs incubated with MS from uninoculated AMs for all time points was 550 cpm. Each value is expressed as the mean of triplicate determinations from three experiments conducted with PBLs from five *M. bovis*-sensitized deer (± standard errors).

FIG. 3. Lymphocyte proliferation responses to MS fractions. Concentrated AM supernatant fractions generated from control supernatants (RPMI or uninoculated MS), *M. bovis*- or BCG-inoculated MS, or heat-killed *M. bovis* or BCG MS were incubated with PBLs from *M. bovis*-sensitized (□) and nonsensitized (■) deer. Each value is expressed as the mean of triplicate determinations from three experiments conducted with PBLs from five *M. bovis*-sensitized and three control animals (± standard errors).

Supernatants derived from AMs infected with live, but not killed, *M. bovis* or BCG stimulate antigen-specific lymphoproliferative responses. To determine whether lymphoproliferative antigens were derived from AMs infected with live BCG or *M. bovis*, but not killed bacteria, supernatant fractions from AMs that were uninfected, infected with live *M. bovis* or BCG, or inoculated with heat-killed *M. bovis* or BCG were incubated with PBLs from *M. bovis*-sensitized and nonsensitized deer. Supernatants derived from AMs infected with live BCG or *M. bovis* stimulated lymphoproliferative responses in PBLs derived from *M. bovis*-infected deer. Proliferative responses were stronger for supernatants derived from *M. bovis*-infected AMs than for BCG-infected AMs. In contrast, supernatants derived from AMs which had been inoculated with heat-killed BCG or *M. bovis* failed to generate lymphoproliferative responses in PBLs from *M. bovis*-infected deer (Fig. 3). Lymphoproliferative activity in *M. bovis*-sensitized PBLs was not detected in supernatants derived from noninfected AMs. This suggests that supernatants derived from AMs infected with live bacteria are required for stimulation of lymphoproliferative responses.

Lymphoproliferative antigens are present in lipid extracts of MS. Supernatants derived from BCG- and *M. bovis*-infected AMs and *M. bovis* CF were subjected to chloroform-methanol extraction to determine whether lymphoproliferative antigens could be separated by extraction procedures selective for lipids. Figure 4 shows that lipid-extracted MS derived from AMs infected with live BCG or *M. bovis*, but not uninoculated control MS, mycolic acid, or lipid-extracted *M. bovis* CF stimulated lymphoproliferative responses in PBLs from *M. bovis*-infected deer, but not uninfected controls. The proliferation responses were considerably stronger for lipid extracts from *M. bovis*-infected compared to BCG-infected AM supernatants. To test whether these extraction procedures were selective for lipids, AM-derived supernatants were run on TLC plates and stained with iodine to identify lipids or with ninhydrin to detect amino acids. Figures 5 and 6 show that lipid-extracted MS stained with iodine, but not ninhydrin, indicating that the lymphoproliferative antigens were nonproteinaceous. Lipid extracts which were incubated with excess chymotrypsin or proteinase K for 1 h at 37°C followed by addition of Bowman soybean inhibitor retained lymphoproliferative activity (data not shown), further

FIG. 4. Lymphoproliferative responses to lipid-extracted MS fractions. Lipid-extracted supernatant fractions from uninoculated (control) AMs, *M. bovis*- or BCG-inoculated AMs, mycolic acid (*M. tuberculosis*; Sigma), or lipid-extracted *M. bovis* CF were incubated with PBLs from *M. bovis*-sensitized (□) and non-sensitized (■) deer. Each value is expressed as the mean of triplicate determinations from three experiments conducted with PBLs from five *M. bovis*-sensitized and three nonsensitized animals ($\pm$ standard errors).

suggesting that proliferative responses in lipid-extracted MS were not due to protein antigens.

Silver staining does not detect proteins in lipid-extracted MS. MSs were run on SDS-PAGE and silver stained in order to establish whether lymphoproliferative responses were due to protein antigens. Silver staining of supernatants from uninfected AMs or AMs infected with live or heat-killed BCG or *M. bovis* revealed no major differences in protein expression in MS (data not shown). To determine if proteins could be detected following lipid extraction of MS, we compared unex-

FIG. 5. TLC of lipid-extracted MS. Lipid-extracted supernatants were run on silica gel and stained with iodine. Lipid-extracted supernatant from uninoculated AMs is shown in lane 1. Lanes 2 and 3 show iodine staining of lipid-extracted supernatants from AMs inoculated with live *M. bovis* and BCG, respectively. Supernatant from *M. bovis*-inoculated AMs which had not been lipid extracted is shown in lane 4. Lipid-extracted *M. bovis* culture filtrate is shown in lane 5, and lane 6 shows lipid-extracted PPDB.

FIG. 6. Ninhydrin stain. Staining of fractions with ninhydrin shows that PPDB (lane 1) and *M. bovis* culture filtrate (lane 2) stain for amino groups, whereas lipid-extracted *M. bovis* and BCG AM supernatants, mycolic acid, and uninoculated AM supernatants (lanes 3, 4, 5, and 6, respectively) do not stain.

tracted MS and unfractionated mycobacterial proteins with lipid-extracted MS and mycolic acid by using the silver stain. Figure 7 shows that *M. bovis* culture filtrate, *M. bovis* or BCG-infected MS, and PPDB (lanes 1, 3, 4, and 7) stained positive for protein bands, whereas mycolic acid and lipid-extracted *M. bovis* or BCG-infected MS (lanes 2, 5, and 6) did not stain.

FIG. 7. Silver staining of AM supernatants. Concentrated MS or mycobacterial antigens were run on SDS-PAGE. The silver stain shows multiple protein bands in crude *M. bovis* CF (lane 1) and PPDB (lane 7), while live *M. bovis*- and BCG-inoculated AM supernatants contain fewer bands (lanes 3 and 4, respectively). Mycolic acid- or lipid-extracted *M. bovis* and BCG AM supernatants (lanes 2, 5, and 6, respectively) do not stain. Lane 8 shows molecular mass standards in kilodaltons.

FIG. 8. Western blot with rabbit anti-*M. bovis* polyclonal antibody. Western blotting of *M. bovis* fractions run on SDS-PAGE shows that rabbit polyclonal antibody against *M. bovis* sonicate reacted with crude *M. bovis* culture filtrate (lane 1), live *M. bovis*- and BCG-inoculated AM supernatants (lanes 3 and 4, respectively), and PPDB (lane 7), but not mycolic acid or lipid-extracted *M. bovis* and BCG AM supernatants (lanes 2, 5, and 6, respectively).

These results show that lipid-extracted *M. bovis*-specific lymphoproliferative antigens were not detected by silver staining, suggesting that lymphoproliferative antigens were not proteins. Based on these results, we decided to test lipid-extracted MS for the presence of *M. bovis*-specific antigens by Western blotting.

Mycobacterial antigens from BCG or *M. bovis* MS, but not lipid-extracted supernatants, are detected by Western blotting. To further demonstrate that lymphoproliferative antigens in MS were derived from mycobacteria, we used a rabbit polyclonal antibody generated against sonicated *M. bovis* to detect *M. bovis*-specific antigens by Western blotting. Figure 8 shows that supernatants derived from AMs infected with live *M. bovis* or BCG (lanes 3 and 4) are detected by the anti-*M. bovis* antibody. This antibody also recognized *M. bovis* culture filtrate antigens and PPDB (lanes 1 and 7), but not mycolic acid (lane 2) or lipid-extracted MS (lanes 5 and 6). This suggests that the rabbit antibody recognizes BCG and *M. bovis* protein antigens in MS, but not antigens derived from lipid-extracted BCG or *M. bovis* MS. While these observations support the hypothesis that lymphoproliferative protein antigens in BCG- or *M. bovis*-infected MS are derived from mycobacterial sources and not macrophages, they also show that lymphoproliferative lipid antigens are not recognized by polyclonal antibody against *M. bovis* sonicate.

Statistical analysis. Analyses of data for [³H]uracil incorporation assays and LPAs were undertaken by analysis of variance of raw data.

DISCUSSION

AMs are generally regarded as efficient defense cells, generating a range of microbicidal responses following phagocytosis, such as reactive oxygen and nitrogen intermediates (12). Mycobacteria that reside within the intracellular environment must therefore resist such microbicidal mechanisms in order to survive and replicate. It has been previously considered that, at least during the early stages of infection, virulent mycobacteria reside exclusively within intracellular phagosomal vacuoles and therefore have little communication with the cytosol or the extracellular environment (14, 22, 27). However, recent studies have indicated that CD8⁺ T cells are active during mycobac-

terial infection of macrophages in vitro, suggesting that some antigenic material can exit the phagosome and enter the endogenous (cytosolic) antigen-processing mechanism (20, 34). Previously, investigators have hypothesized that such mycobacterial antigens are leaked or expelled from the host phagosome (24) and that these antigens may be key elements in the sensitization of mycobacterial reactive T cells. More recently, lipid-containing moieties of the mycobacterial cell wall have been shown to actively traffic from the macrophage phagosome for uptake by bystander macrophages (6).

The results of our study have furthered this paradigm, and we have demonstrated here that actively metabolizing intracellular mycobacteria produce antigenic molecules which are subsequently excreted or secreted into the extracellular environment. It is highly likely that these molecules are products of mycobacterial metabolism and/or replication, since no secretory material could be detected in supernatants derived from macrophages exposed to killed mycobacteria in our study. However, it is also possible that recognition of the mycobacterial antigens by lymphocytes is dependent on processing and presentation by macrophages. This result is significant and provides evidence that during the early stages of intracellular mycobacterial replication, molecules are trafficked out of the phagosome and into the extracellular environment, indicating that the mycobacterial phagosome is not a discrete entity.

That the lipid molecules identified in MS were derived from mycobacterial sources was confirmed by immunolabelling with an anti-*M. bovis* antiserum. While we have no further evidence as to the identity of these molecules, it is most likely that they are derived from the lipid-rich cell wall of *M. bovis*. A distinguishing characteristic of mycobacteria is the presence of a thick waxy cell wall (8, 10), and up to 80% of the biomass of mycobacteria may comprise cell wall components. Outer cell wall components, such as mycolic acids and lipoarabinomannan, may be shed as the bacteria divide in macrophages and may be released from phagosomal compartments (10, 27). Furthermore, lipoarabinomannan has been shown to be transported from the mycobacterial vacuole into lysosomal compartments within viable macrophages (36). Recent studies have shown that mycobacterial cell wall components may enter the CD1 antigen presentation pathway and be presented to CD1-restricted T cells (24, 33), or they may be released by exocytosis following cell lysis (25).

Extracellular production of mycobacterial lipids due to lysis of macrophages is unlikely, since a low MOI was used, and at the time point when supernatants were collected, this MOI has been shown not to affect the integrity of alveolar macrophages (2). Mycobacterial lipids which were not derived from macrophages, such as mycolic acid and those prepared from *M. bovis* CF, failed to stimulate proliferative responses. We therefore conclude that mycobacterial lipid antigens, detected in the supernatants of live *M. bovis*-infected AMs, are most likely to be cell wall derivatives of intracellular bacterial cell replication.

Molecules secreted or excreted by infected host cells have the possibility to stimulate immune recognition in the host. Many of the key mycobacterial antigens recognized by the acquired immune response have been shown to be exported or secreted from living bacteria (4), while recent studies have indicated that mycobacterial lipids in particular can stimulate CD1-restricted responses in immunologically primed lymphocyte populations (31–33). Significantly, we have demonstrated here that lipids secreted by *M. bovis*-infected deer AMs in vitro are recognized by lymphocyte populations from deer which were experimentally infected with *M. bovis*. An advantage of using a large animal model such as red deer (which are naturally susceptible to infection with virulent *M. bovis*) is that

mononuclear cell populations can be derived from animals sensitized to mycobacteria during natural infection.

An intratonsillar infection model has been established for red deer, and the ensuing patterns of pathogenesis and immune reactivity in diseased animals closely mimic the patterns observed in naturally infected farmed deer (9, 16). In this context, we have demonstrated here that lymphocytes derived from the peripheral blood of deer infected with *M. bovis* recognize lipid molecules derived from *M. bovis*-infected AMs. It is known that deer produce strong lymphocyte reactivity against proteinaceous mycobacterial antigens during tuberculosis infection (15) and that these responses are due to interleukin-2-dependent CD4, CD8, and $\gamma\delta$ T-cell subsets (9); however, this is the first report of lymphocyte responses being directed against lipid extracts. This novel antigen recognition may provide a valuable diagnostic tool for determining mycobacterial infection in farmed animals and furthermore opens up the possibility that lipid antigen-based immunoassays may be developed as a diagnostic tool for infection in veterinary species and humans.

In summary, this is the first report of the detection of mycobacterial lipid antigens released from host macrophages which are capable of stimulating specific lymphoproliferative responses. Our results suggest that *M. bovis* growing in macrophages secretes lipids which can escape the phagosome, enter the cytosol, and subsequently be released into the extracellular milieu. Furthermore, these extracellular lipids are capable of stimulating lymphocyte proliferation responses in vitro. Mycobacterial antigens released by AMs in vivo following aerogenic infection of the lungs may be important in controlling the early immune response to tuberculosis. Identification of macrophage-induced mycobacterial antigens will assist in understanding the mechanisms by which virulent mycobacteria cause disease. Immune recognition of lipids produced by *M. bovis* growing in macrophages suggests that mycobacterial antigens other than proteins are capable of stimulating proliferation of sensitized lymphocytes. We are currently characterizing the lipid antigens and the lymphocyte subsets which recognize them.

ACKNOWLEDGMENTS

We thank Janelle McKenzie, Ngaire Chinn, Chris Rodgers, and Rob Labes for assistance with organizing and processing deer blood samples.

This work was supported by a grant from the New Zealand Animal Health Board.

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Editor: W. A. Petri, Jr.