Members of the 30- to 32-Kilodalton Mycolyl Transferase Family (Ag85) from Culture Filtrate of *Mycobacterium avium* subsp. *paratuberculosis* Are Immunodominant Th1-Type Antigens Recognized Early upon Infection in Mice and Cattle

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The characterization of protective antigens is essential for the development of an effective, subunit-based vaccine against paratuberculosis. Surface-exposed and secreted antigens, present abundantly in mycobacterial culture filtrate (CF), are among the well-known protective antigens of *Mycobacterium tuberculosis* and *Mycobacterium bovis*. Culture filtrate, prepared from *Mycobacterium avium* subsp. *paratuberculosis* ATCC 19698 grown as a surface pellicle on synthetic Sauton medium, was strongly and early recognized in experimentally infected B6 *bg/bg* beige mice and cattle, as indicated by elevated spleen cell gamma interferon (IFN-γ) secretion and lymphoproliferative responses of peripheral blood mononuclear cells, respectively. Strong proliferative and ex vivo IFN-γ responses against antigen 85 (Ag85) complex (a major protein component from *M. bovis* BCG culture filtrate) could be detected in cattle as early as 10 weeks after oral *M. avium* subsp. *paratuberculosis* infection. Synthetic peptides from the Ag85A and Ag85B components of this complex were strongly recognized, whereas T-cell responses were weaker against peptides from the Ag85C protein. A promiscuous T-cell epitope spanning amino acids 145 to 162 of Ag85B (identical sequence in *M. bovis* and *M. avium* subsp. *paratuberculosis*) was identified in experimentally infected cattle. Finally, young calves, born from cows with confirmed paratuberculosis, demonstrated proliferative responses to purified, recombinant Ag85A and Ag85B from *M. avium* subsp. *paratuberculosis*. These results indicate that the *M. avium* subsp. *paratuberculosis* Ag85 homologues are immunodominant T-cell antigens that are recognized early in experimental and natural infection of cattle.

On the basis of DNA-DNA hybridization and numerical taxonomy analysis, the mycobacterial species *Mycobacterium avium* is subdivided into three subspecies, *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum*, which share extensive sequence identity (8). Nevertheless, these subspecies can be differentiated from each other on the basis of host range, mycobactin dependence, and the presence of specific insertion elements (17). *M. avium* subsp. *paratuberculosis* causes Johne's disease, a severe gastroenteritis in ruminants, with a significant impact on the agricultural economy, particularly the dairy industry (17). In the Belgian cattle population, paratuberculosis prevalence was determined by a serological survey, conducted from December 1997 to March 1998. This approach resulted in an estimated true herd prevalence of *M. avium* subsp. *paratuberculosis* infection of 6% (6). Dairy cattle usually start fecal shedding at 2 years of age and develop clinical symptoms around 4 years of age. Infection with *M. avium* subsp. *paratuberculosis* is commonly acquired early in life via the fecal-oral route through the ingestion of contaminated colostrum, milk, water, or feed (46) and possibly through intrauterine transmission (42). *M. avium* subsp. *para-*

tuberculosis is extremely robust, and bacteria were reported to survive up to 250 days in water and feces and on pastures (27).

Cell-mediated immune responses seem to control the initial infection for a sustained period of time, and clinical symptoms only appear in cows after a number of years, often after the first or second calving, possibly because of enhanced intracellular multiplication of *M. avium* subsp. *paratuberculosis* organisms caused by alterations in the hormonal milieu (15). Decreased cell-mediated responses are likely related to a loss of antigen-specific CD4+ T cells, which is most prominent in the ileum lesions from symptomatic animals (24). Also, Khalifeh et al. demonstrated that transforming growth factor β and interleukin-10 (IL-10) mRNA levels are higher in cows that have progressed to the clinical stage of the disease, compared to subclinically infected or healthy cows (23). It is not clear for the moment, whether this reflects a shift from a Th1- to a Th2-biased immune response or rather the development of a regulatory T-cell circuit (10). Vaccines consisting of whole killed or attenuated live *M. avium* subsp. *paratuberculosis* bacilli can provide partial protection by delaying fecal shedding and reducing the number of clinically affected animals, but they do not protect against infection. In the context of bovine tuberculosis (*M. bovis*) control and eradication programs, it is worth mentioning that animals immunized with these paratuberculosis vaccines develop positive reactions in the tuberculin skin test (the reference bovine tuberculosis detection method), and therefore paratuberculosis vaccination is subject to approval by

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the veterinary services. Moreover, because of the impact on trading of living animals, farmers are reluctant to use paratuberculosis vaccination (25). It is clear that the development of an efficient paratuberculosis subunit vaccine, which would not interfere with tuberculosis detection, would offer a solution.

The precise *M. avium* subsp. *paratuberculosis* antigens that induce a protective immune response are poorly defined. Secreted and surface-exposed cell wall proteins are major antigens recognized by the protective immune response against *M. tuberculosis* and *M. bovis*, and immunization with whole-culture filtrate, a rich source of extracellular proteins, can protect mice and guinea pigs to some extent against subsequent challenge with the tubercle bacillus (1, 39). A major fraction of the secreted proteins in culture filtrates of *M. tuberculosis* and *M. bovis* BCG is represented by the antigen 85 (Ag85) complex, a 30- to 32-kDa family of three proteins (Ag85A, Ag85B, and Ag85C) (51) that all possess an enzymatic mycolyl transferase activity, required for the biogenesis of cord factor (trehalose-dimycolate) (5), and that are encoded by three paralogous genes (*fbpA*, *fbpB*, and *fbpC*) located in distinct regions of the bacterial genome (9). Healthy individuals infected with *M. tuberculosis* or *Mycobacterium leprae* (28) and BCG-vaccinated mice (19) show strong T-cell proliferative and IFN-γ responses against Ag85A, and both Ag85A and Ag85B are promising candidates for future tuberculosis vaccines (3, 20, 22, 33). Members of the Ag85 family are found in all mycobacteria, and sequence comparison indicates that the Ag85 gene family arose by duplication of an ancestral gene, before the emergence of the actually known mycobacterial species (9).

The genes encoding the three Ag85 components from *M. avium* subsp. *paratuberculosis* have been sequenced, and at the protein level, a 99% sequence identity with *M. avium* was found, with a single amino acid residue difference for each protein (13): serine/proline in position 155 of the mature Ag85A protein, serine/asparagine in position 120 of the mature Ag85B protein, and isoleucine/threonine in position 284 of the mature Ag85C protein in *M. avium* subsp. *paratuberculosis* and *M. avium*, respectively. In comparison to the mature protein sequences of *M. bovis* (which differ from those of *M. tuberculosis* by only one residue in position 100 of the mature Ag85B protein), the *M. avium* subsp. *paratuberculosis* 85A protein sequence (map 0216) shares 82% identity, the *M. avium* subsp. *paratuberculosis* 85B protein sequence (map 1609c) shares 86% identity, and the *M. avium* subsp. *paratuberculosis* 85C protein sequence (map 3531c) shares 87% identity (13). Recently, it has been suggested that the Ag85B component together with GroES could be associated with the heat resistance of *M. avium* subsp. *paratuberculosis* (45).

Little is known so far about the immune recognition of culture filtrate antigens and of Ag85 homologs, in particular, during *M. avium* subsp. *paratuberculosis* infection. In experimentally infected American bison (*Bison bison*) inoculated with *M. avium* subsp. *paratuberculosis* organisms, it was shown that Ag85 was a major antigen produced during infection that could be detected in the serum by Western blot analysis with Ag85-specific monoclonal antibodies (MAbs) (30). However, for serodiagnosis of paratuberculosis, the sensitivity of an Ag85-based enzyme-linked immunosorbent assay was clearly lower than that of a 35-kDa-based assay (43). Here, we report on culture filtrate- and Ag85-specific T-cell responses in mice

and cattle experimentally infected with *M. avium* subsp. *paratuberculosis* ATCC 19698 through the intravenous and oral route, respectively. Our results indicate that Ag85 homologs are immunodominant T-cell antigens in *M. avium* subsp. *paratuberculosis* infection, inducing strong proliferative and IFN-γ responses. Their vaccine potential for bovine paratuberculosis remains to be determined.

MATERIALS AND METHODS

Bacteria and antigens. *M avium* subsp. *paratuberculosis* ATCC 19698 was purchased from the American Tissue Culture Collection and grown on solid Löwenstein-Jensen medium. Subsequently, cultures were maintained in liquid 7H9 medium supplemented with 10% oleic acid-albumin-dextrose-catalase and mycobactin J (Allied Laboratories Inc. and Synbiotics Europe, respectively) (2 μg/ml), and (after an intermediate 6-month passage on solid potato soaked in synthetic, protein-free Sauton medium supplemented with mycobactin J) bacteria were grown as a surface pellicle on Sauton medium. *M. avium* subsp. *paratuberculosis* was grown for 4 weeks at 39°C, culture filtrate (CF) was separated from the bacteria, and CF proteins were precipitated by 80% saturated ammonium sulfate. Precipitate was extensively dialyzed against phosphate-buffered saline. Bacteria were stored as concentrated pellets in Sauton medium with 20% glycerol at −70°C. The number of bacteria was determined by plating on mycobactin J-supplemented Middlebrook 7H11 medium and visual counting of the CFU after 8 weeks of incubation at 39°C. Enumeration of *M. avium* subsp. *paratuberculosis* organisms in spleen from infected mice was performed by plating serial dilutions of organ homogenate on the same 7H11 medium. *M. bovis* BCG and *M. tuberculosis* H37Rv culture filtrates were prepared from 2-week-old cultures on synthetic Sauton medium as described previously. Native 30- to 32-kDa Ag85 was purified from BCG CF using sequential chromatography on phenyl-Sepharose, DEAE Sephacel, and Sephadex G75 (11). Bovine purified protein derivative (PPD-B) from *M. bovis* Vallée and avian purified protein derivative (PPD-A) from *M. avium* (strain 42) were kindly given by J. Nyabenda from the WIV-Pasteur Institute, Brussels, Belgium.

SDS-PAGE. Ammonium sulfate-concentrated CF from *M. avium* subsp. *paratuberculosis* was analyzed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels stained with silver nitrate. Ag85 homologues were visualized by Western blot analysis, using Ag85-specific MAb TD17.4 (21).

Preparation of genomic DNA from *M. avium* subsp. *paratuberculosis*. Genomic DNA of *M. avium* subsp. *paratuberculosis* ATCC 19698 was prepared as described previously by Tanghe et al. for *Mycobacterium ulcerans* (47). Briefly, bacteria were lysed with lysozyme, pronase, and SDS. After phenol-chloroform-isoamyl alcohol extraction and RNase A treatment, DNA was precipitated with ethanol, and the pellet was resuspended in Tris-EDTA buffer. DNA was analyzed by agarose gel electrophoresis, and the purity was evaluated by spectrophotometry. DNA was kept at −20°C until use.

Cloning and purification of recombinant mature Ag85A and Ag85B from *M. avium* subsp. *paratuberculosis*. The *fbpA* and *fbpB* genes encoding the mature Ag85A (304 residues) and Ag85B (290 residues) proteins were amplified by PCR from *M. avium* subsp. *paratuberculosis* genomic DNA using primers derived from sequences of *M. avium* (36, 37). The primers used were 5′-CGGGATCCGCGT TCTCGCGCCCCGGTCTGCCGGTGGA-3′ (forward 85A), 5′-CCCAAGCTT GGGTTAGGTGCCCTGGCCGTTCCCGGC-3′ (reverse 85A), 5′-CCCAAG CTTGGGTTTTCGCGTCCGGGCCTTGCC-5′ (forward 85B), and 5′-CCCAAG CTTGGGTTATCCGCCGCCGCCCGGGGA-3′ (reverse 85B). These primers were designed with BamHI and HindIII sites for Ag85A cloning in pQE-80L (QIAGEN) vector and with only the HindIII site for Ag85B cloning in this vector due to the presence of an internal BamHI site in the *fbpB* gene. Genes were amplified without the mycobacterial signal sequence. *fbpA* was obtained by PCR (Expand Polymerase; Roche) with 30 cycles of amplification (10 cycles at 94°C for 20 s, and 67°C for 30 s, 72°C for 1 min; 5 cycles at 94°C for 40 s, 67°C for 30 s, and 72°C for 1.25 min; 5 cycles at 94°C for 1 min, 67°C for 30 s, and 72°C for 1.5 min; 10 cycles at 94°C for 1.20 min, 67°C for 30 s, and 72°C for 2.15 min). The same protocol was used for *fbpB* amplification except that the hybridization temperature was 68°C. Amplified fragments were purified by agarose gel separation followed by purification using a QIAkit PCR kit (QIAGEN). Purified genes were ligated into a pQE-80L (QIAGEN) expression vector predigested with BamHI/HindIII for 85A cloning and by HindIII for 85B cloning (T4 DNA ligase; Roche). Ligations were electroporated into *Escherichia coli* DH5α cells, and positive clones were screened on LB-ampicillin medium and confirmed by restriction enzyme digestion. The integrity of cloned sequences was checked by

sequence analysis. Plasmids containing either the 85A or 85B sequence were transformed into Top-10F′ *E. coli* (Invitrogen) for expression. Recombinant proteins were purified by affinity chromatography on an immobilized nickel-nitrilotriacetic acid column, according to standard procedures. As a result of the cloning procedures, recombinant Ag85A and Ag85B proteins were both 317 amino acid (aa) residues long, including the six NH$_2$-terminal histidines.

Peptide synthesis. Peptides spanning the entire sequence of the mature BCG Ag85A sequence (295 aa) were synthesized as 20-mer peptides overlapping by 10 residues, with the exception of the 19-mer spanning aa 35 to 53 and the carboxy-terminal peptide spanning aa 275 to 295. Peptides spanning the entire mature BCG Ag85B sequence (285 aa) were synthesized as 18-mer peptides overlapping by nine residues, with the exception of a 21-mer peptide spanning aa 240 to 260 and the carboxy-terminal 15-mer peptide spanning residues 271 to 285. Peptides spanning the entire mature BCG Ag85C sequence (294 aa) were synthesized as 20-mer peptides overlapping by 10 residues, with the exception of two 18-mer peptides (aa 31 to 50 and 41 to 60, residues 33 and 34 lacking in Ag85C) and the carboxy-terminal 14-mer peptide (aa 281 to 294) (14).

Mice. C57BL/6OlaHsd-bg (B6 *bg/bg*) mice (age 8 to 12 weeks) were bred at the animal facilities of the WIV-Pasteur Institute from breeding couples originally obtained from Harlan (The Netherlands).

Cattle. All animals used in this study were from the Friesian-Holstein breed and originated from herds officially free of bovine tuberculosis. Five 2- to 3-week-old calves originated from paratuberculosis-free herds. Two calves born from cows suffering from clinical paratuberculosis (as confirmed by Pourquier serology and by *M. avium* subsp. *paratuberculosis*-positive fecal and postmortem organ cultures) were monitored for 47 weeks.

Experimental infection of mice and cattle. Mice were infected intravenously in a lateral tail vein with 3×10^5 (peptide mapping) or 10^6 CFU of *M. avium* subsp. *paratuberculosis* ATCC 19698, grown in Middlebrook 7H9 medium supplemented with oleic acid-albumin-dextrose-catalase and mycobactin J. Five 2- to 3-week-old calves (animals 6074, 6075, 7782, 7783, and 7785) were infected by the oral route with 10 mg (10^8 CFU) of *M. avium* subsp. *paratuberculosis* (ATCC 19698) cells per day for 10 consecutive days. These animals were subclinically infected during the 2-year follow-up period and excreted *M. avium* subsp. *paratuberculosis* sporadically in their feces. They did not show any seroconversion against either protoplasmic or lipoarabinamannan antigens and did not present any clinical signs. The evolution of ex vivo IFN-γ production in response to PPD-A and PPD-B in these animals will be reported in another paper (unpublished data). Animals were tuberculin tested 125 weeks postinfection, and the reactions were read 72 h later: with PPD-A, one animal (7785) scored positive, two animals scored doubtful (6074 and 7782), and two animals scored negative (6075 and 7783). The five animals scored negative in skin tests with PPD-B.

Mouse spleen cell cytokine production. At indicated time points, infected mice were killed by cervical dislocation; spleens were removed aseptically and homogenized by gentle disruption in a loosely fitting Dounce homogenizer. Spleens from three to six mice per group were analyzed individually or pooled, as indicated. Spleen cells were washed and resuspended at 4×10^6 white blood cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin, streptomycin, 5×10^{-5} M 2-mercaptoethanol, and indomethacin (1 μg/ml; Sigma). Cells were cultured in a humidified CO$_2$ incubator in round-bottom 96-well microplates. A volume of 180 μl of cells was added to 20 μl of the respective antigens (19). PPD-B, PPD-A, and CF were used at final concentrations of 25 μg/ml (each); synthetic peptides at were used at a concentration of 10 μg/ml (each); and native Ag85 from BCG and recombinant *E. coli*-derived Ag85A and 85B proteins from *M. avium* subsp. *paratuberculosis* were used at a concentration of 5 μg/ml (each). Culture supernatants from at least three wells were collected and pooled after 24 h (IL-2) and 72 h (IFN-γ) and stored at −20° until testing.

Proliferation assays in infected cattle. Blood was collected on heparin by venipuncture and stored for 24 h at room temperature in order to decrease background proliferation of unstimulated cells. Proliferative responses were analyzed using a whole-blood assay in 10% autologous plasma. Briefly, heparinized blood was diluted 1:10 in RPMI 1640 medium supplemented with 5×10^{-5} M 2-mercaptoethanol. A volume of 180 μl of cells was mixed with 20 μl of antigen in 96-well round-bottom microwell plates, and cultures were incubated in a humidified CO$_2$ incubator for 7 days. After 6 days, cells were pulsed overnight with tritiated thymidine (0.4 μCi/well) and collected on a Titertek Cell Harvester. Radioactivity recovered on the filters was counted in a Betaplate Liquid Scintillation Counter, and results were expressed as mean cpm ± standard deviation (SD) of triplicate cultures.

Cultured (in vitro) IFN-γ production in infected cattle. Heparinized blood was stored for 24 h at room temperature as described for proliferation assays. It was then centrifuged for 10 min at 1,500 rpm, and plasma was replaced by the same volume of RPMI 1640 medium supplemented with 10% fetal calf serum,
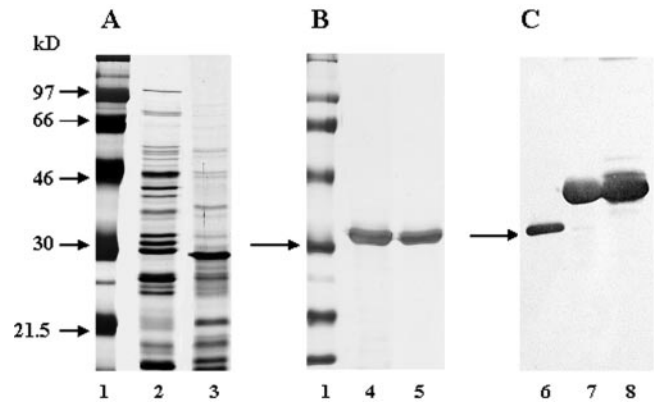

FIG. 1. (A) Comparative SDS-PAGE (12%) of culture filtrates from *M. avium* subsp. *paratuberculosis* ATCC 19698 (lane 3) and *M. tuberculosis* H37Rv (lane 2) grown as surface pellicles on synthetic Sauton medium. Proteins were visualized by silver nitrate staining. Molecular weight markers are shown in lane 1. (B) Silver nitrate staining of purified, recombinant *E. coli*-derived antigen 85A (lane 4) and Ag85B (lane 5) from *M. avium* subsp. *paratuberculosis* separated by 12% SDS-PAGE. (C) Western blot analysis of CF from *M. avium* subsp. *paratuberculosis* (lane 6) and of recombinant Ag85A (lane 7) and Ag85B (lane 8) from *M. avium* subsp. *paratuberculosis* using Ag85-specific MAb TD 17-4. The arrows indicate the position of the 30-kDa molecular weight marker (A and B) and of the native Ag85B protein in *M. avium* subsp. *paratuberculosis* CF (C).

penicillin, streptomycin, and 5×10^{-5} M 2-mercaptoethanol in order to reduce background IFN-γ levels in unstimulated cells. Leukocytes were counted in a Coulter Counter and whole-blood-cell cultures were adjusted to 10^6 white blood cells/ml in complete RPMI 1640 medium. A volume of 180 μl of cells was mixed with 20 μl of antigen (concentrations as above) in 96-well round-bottom microwell plates, and cultures were incubated in a humidified CO$_2$ incubator. After 6 days of culture, supernatants from at least three wells were pooled for each antigen and stored at −20° until testing.

Bovine ex vivo IFN-γ production. Tests were performed as previously described (49). Heparinized blood was collected from the jugular vein, and 1-ml aliquots were incubated without antigen or with purified Ag85 from *M. bovis* BCG at a final concentration of 5 μg/ml. Cells were incubated for 18 h at 37°C in a humidified 5% CO$_2$ incubator in 1-ml tubes. After centrifugation, plasma supernatants were collected and stored at −20°C until testing.

Mouse IL-2 assay. IL-2 activity was quantified using a bioassay, measuring the uptake of tritiated thymidine by the murine IL-2-dependent CTLL-2 cell line as described previously (19). Each sample was tested in duplicate. IL-2 levels are expressed as mean cpm. In this assay, a standard IL-2 preparation (21) of 600 pg/ml yielded ±15,000 cpm, and the assay detection limit was 30 pg/ml. IL-2 values were considered positive when they were at least twofold higher than control cpm values.

Mouse IFN-γ assay. IFN-γ activity was quantified in a sandwich enzyme-linked immunosorbent assay using coating antibody R4-6A2 and biotinylated detection antibody XMG1.2 (both from Pharmingen). The detection limit was around 10 pg/ml.

Bovine IFN-γ assay. Bovine IFN-γ was determined using a Bovine IFN-γ EASIA kit (catalogue no. KBC1232; BioSource Europe S.A., Nivelles, Belgium). For the day 6 in vitro IFN-γ culture supernatants, optical density values were converted to pg/ml, using a standard curve of recombinant bovine IFN-γ (Serotec) at an initial concentration of 20 ng/ml.

RESULTS

Preparation of culture filtrate from *M. avium* subsp. *paratuberculosis* ATCC 19698 grown as a surface pellicle on synthetic Sauton medium. *M. avium* subsp. *paratuberculosis* ATCC 19698 was adapted to grow as a surface pellicle on synthetic, protein-free Sauton medium supplemented with mycobactin J. Comparison of the 4-week-old *M. avium* subsp. *paratuberculosis* CF with the protein profile of a 2-week-old *M. tuberculosis*

TABLE 1. IFN-γ production in spleen cell cultures from B6 *bg/bg* mice infected intravenously with 1×10^6 CFU of *M. avium* subsp. *paratuberculosis* ATCC 19698

Antigen[a]	Amt of IFN-γ (pg/ml) in culture[b]		
	Uninfected (3)	5 weeks p.i. (6)	10 weeks p.i. (4)
Medium	78 ± 21	56 ± 54	41 ± 29
CF *M. avium* subsp. *paratuberculosis*	85 ± 42	7,703 ± 2,354	17,292 ± 10,136
CF *M. bovis* BCG	71 ± 25	8,479 ± 1,834	18,381 ± 8,037
PPD-A	164 ± 34	7,594 ± 2,214	16,760 ± 8,437
PPD-B	155 ± 16	4,757 ± 889	10,946 ± 2,892
Ag85 (BCG)	99 ± 41	5,465 ± 1,662	10,417 ± 2,481
rAg85Aptb	151 ± 18	6,510 ± 1,785	13,937 ± 7,462
rAg85Bptb	126 ± 49	ND	7,490 ± 4,731

[a] C57BL/6 *bg/bg* mice, uninfected or infected with *M. avium* subsp. *paratuberculosis* were either unstimulated (medium) or stimulated as follows: CF and PPD, 25 μg/ml; Ag85, 5 μg/ml. rAg85Aptb and rAg85Bptb are recombinant antigens from *M. avium* subsp. *paratuberculosis*.

[b] Data represent mean IFN-γ (pg/ml) levels/group ± SD of mice tested individually; the number of animals tested/group is given in parenthesis. ND, not determined.

H37Rv CF by SDS-PAGE indicated that in the region of the 30- to 32-kDa mycolyl transferase (Ag85), one protein of approximately 30 kDa was strongly expressed in the *M. avium* subsp. *paratuberculosis* CF. Trypsin digestion and matrix-assisted laser desorption ionization–time of flight analysis demonstrated that this band corresponded to the Ag85B homolog of *M. avium* subsp. *paratuberculosis* (I. Georis and R. Wattiez, personal communication). In *M. tuberculosis* CF, three protein bands can be detected in this region, corresponding to Ag85B, Ag85A, and Ag85C (18) (Fig. 1A). Recombinant His-tagged Ag85A (map 0216) and Ag85B (map 1609c) were purified by affinity chromatography on a nickel-nitrilotriacetic acid column and visualized by silver staining (Fig. 1B) and by Western blot analysis using the Ag85-specific MAb TD 17-4 (Fig. 1C).

Immune response against culture filtrate from *M. avium* subsp. *paratuberculosis* ATCC 19698 in mutant B6 *bg/bg* mice infected intravenously with *M. avium* subsp. *paratuberculosis*. Although the mouse is not a target species for Johne's disease, the low cost, the availability of genetically defined inbred strains, and a range of immunological tools make this animal species a very attractive model for pilot studies.
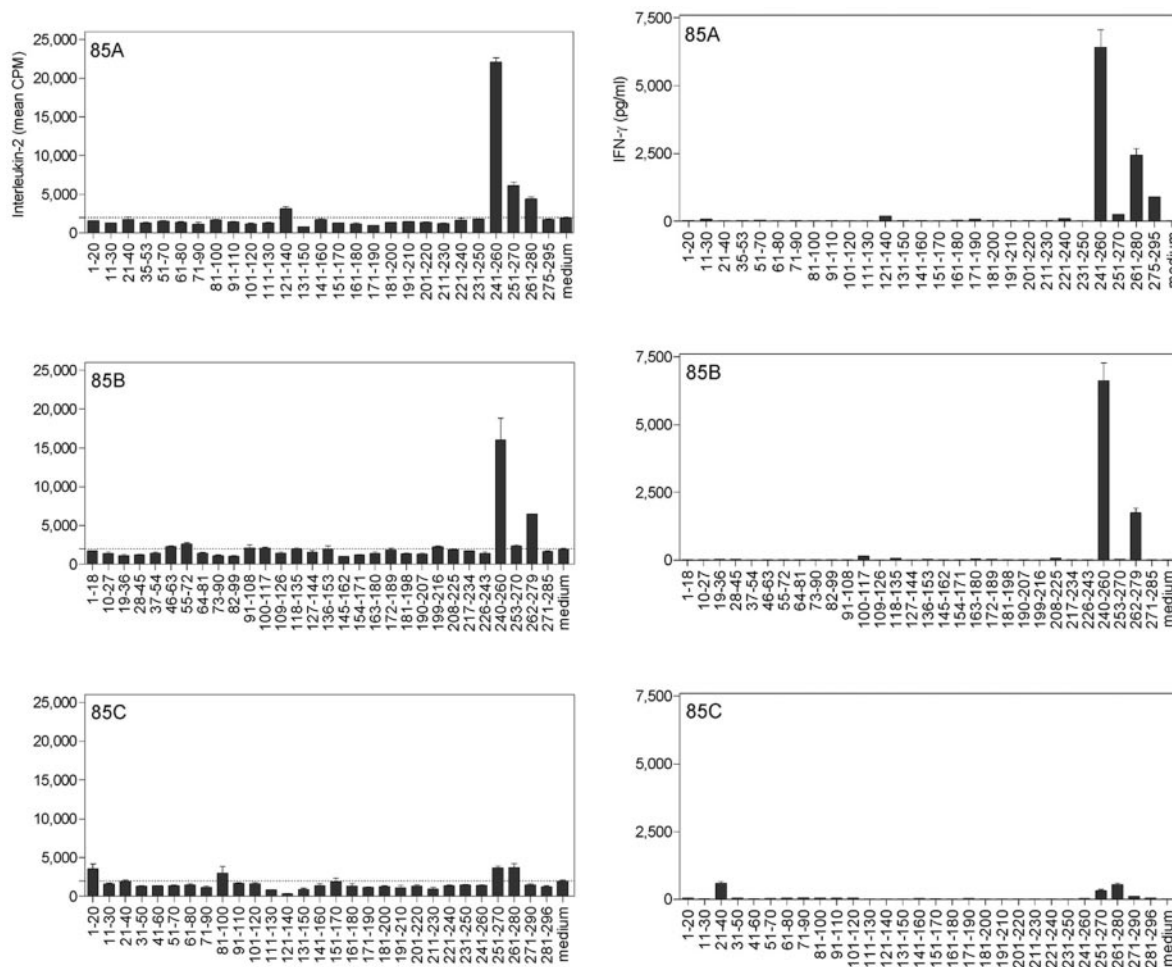

FIG. 2. Characterization of cross-reactive T-cell epitopes of Ag85A, Ag85B and Ag85C in *M. avium* subsp. *paratuberculosis*-infected B6 *bg/bg* mice, using synthetic peptides spanning the mature sequences of Ag85A, Ag85B, and Ag85C from *M. bovis* BCG. IL-2 (left) and IFN-γ (right) production was analyzed in 24-h and 72-h culture supernatants, respectively, of spleen cells from a pool of five animals infected intravenously with 3×10^5 CFU of *M. avium* subsp. *paratuberculosis* and stimulated with overlapping synthetic peptides (10 μg/ml).

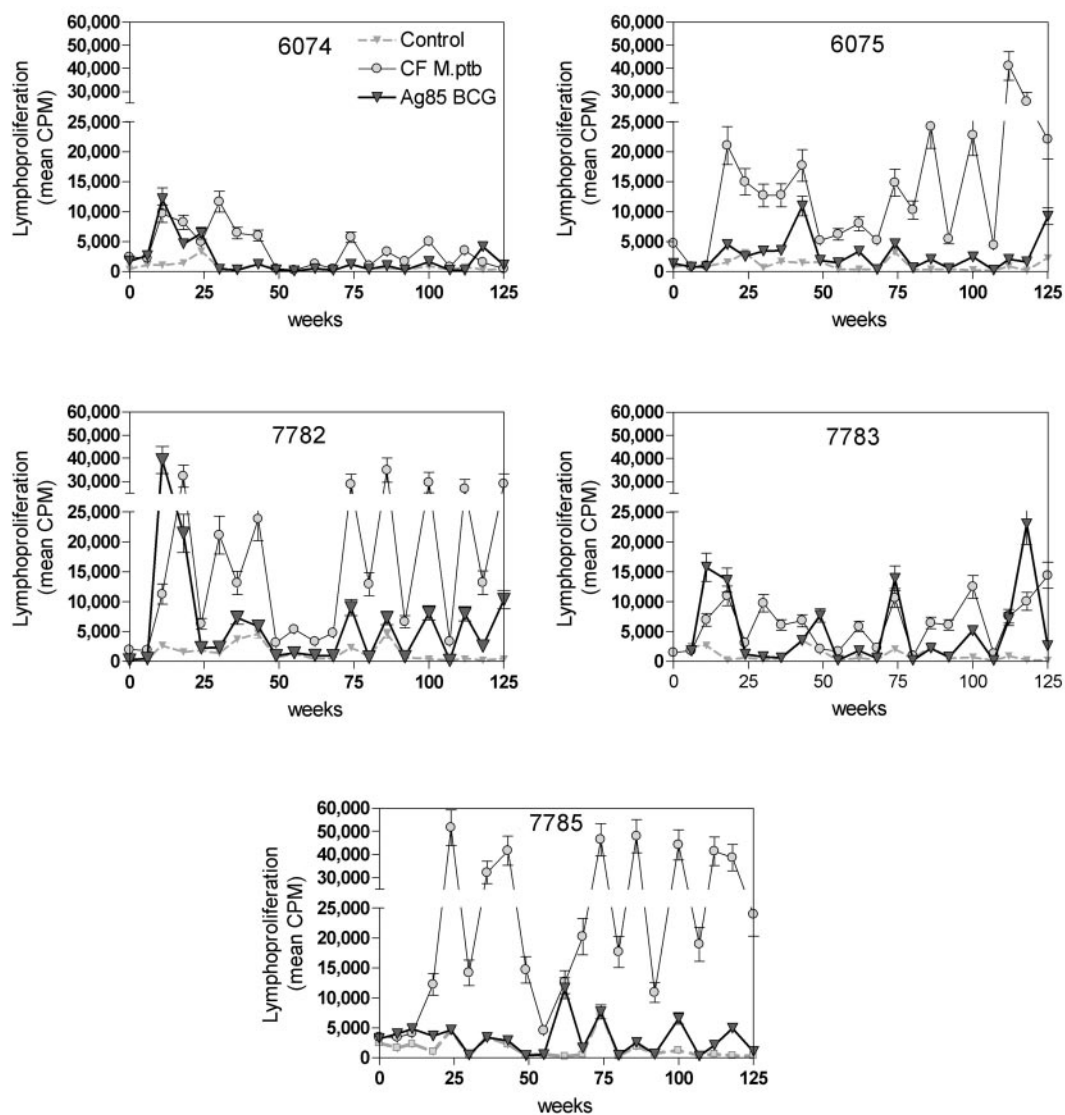FIG. 3. Bovine proliferative responses (mean cpm ± SD) of unstimulated PBMCs (Control) or of PBMCs stimulated with culture filtrate from *M. avium* subsp. *paratuberculosis* ATCC 19698 and Ag85 from *M. bovis* BCG following oral infection with 10^9 CFU of *M. avium* subsp. *paratuberculosis* ATCC 19698 in five animals, as analyzed over a follow-up period of 125 weeks postinfection.

Inbred mice with a C57BL/6 background are more susceptible to *M. avium* subsp. *paratuberculosis* than inbred CBA mice (7). We therefore decided to perform an experimental infection in mutant B6 *bg/bg* beige mice. The mutant beige strain is deficient in NK cell activity and displays impaired chemokine response and neutrophil recruitment upon *M. avium* infection (2, 16). Beige mice have also been reported to be more susceptible to *M. avium* subsp. *paratuberculosis* infection (44). As shown in Table 1, spleen cells from beige mice infected intravenously with 10^6 CFU of *M. avium* subsp. *paratuberculosis* produced significant levels of IFN-γ in response to culture filtrate from *M. avium* subsp. *paratuberculosis* and from *M. bovis* BCG, to PPD from *M. avium*, and to native Ag85 purified from *M. bovis* BCG CF. IFN-γ levels in response to bovine PPD were lower than in response to PPD-A, although the difference was not statistically significant. More importantly, strong IFN-γ responses

were detected following stimulation with recombinant *E. coli*-derived Ag85A and Ag85B proteins from *M. avium* subsp. *paratuberculosis*. IFN-γ responses could be detected in spleen cell cultures as early as 5 weeks postinfection (p.i.), and these responses further increased at week 10 p.i. Spleen cell IFN-γ responses of naive B6 *bg/bg* mice were very low following stimulation with purified Ag85. Bacterial infection persisted in the spleen during this period: 6.04 ± 0.12 $\log_{10}$ CFU ($n = 6$) at week 5 and 5.49 ± 0.11 $\log_{10}$ CFU ($n = 4$) at week 10. Intravenous injection with a similar dose of *M. bovis* BCG resulted in a rapid decline in spleen CFU counts between month 1 and month 2 (4.84 ± 0.22 [$n = 3$] versus 3.47 ± 0.49 [$n = 4$]), confirming that *M. avium* subsp. *paratuberculosis* displays a certain virulence in beige mice.

Homologues of the Ag85 complex in CF from *M. avium* subsp. *paratuberculosis* are immunodominant T-cell antigens in experimentally infected mice. In view of the high sequence
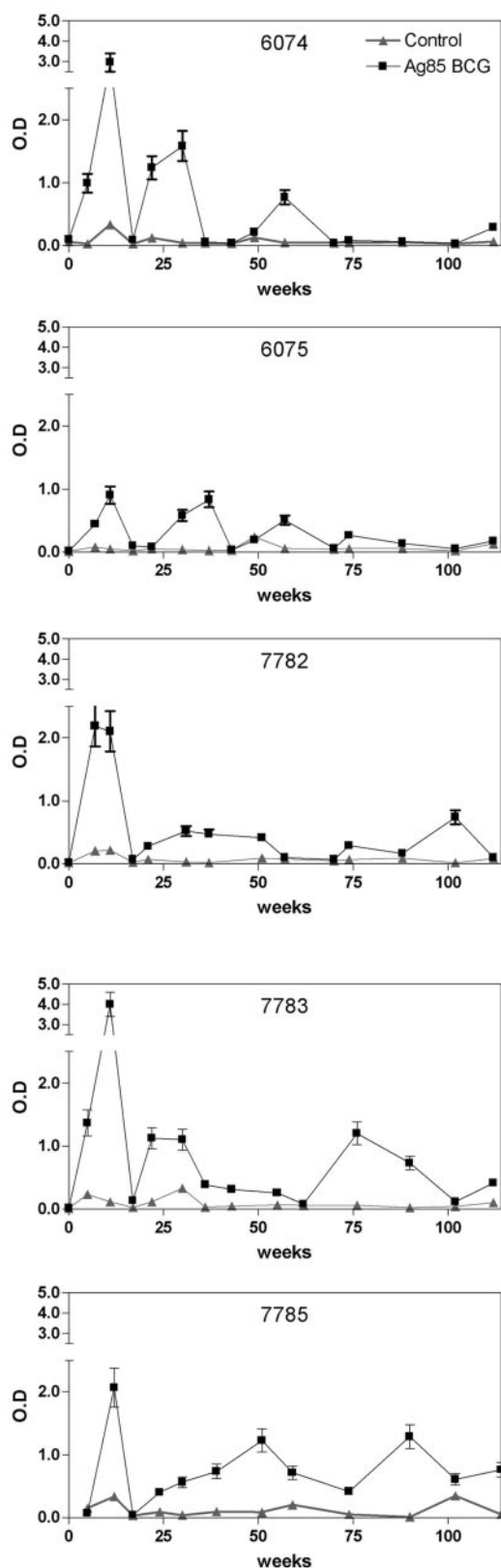
FIG. 4. Ex vivo IFN-γ production in unstimulated cultures (Control) or in cultures stimulated with Ag85 of whole blood following oral infection with 10^9 CFU of *M. avium* subsp. *paratuberculosis* ATCC 19698 in five animals, as analyzed over a follow-up period of 125 weeks postinfection. Data are represented as optical density values (mean ± SD).

homology reported for the *M. avium* subsp. *paratuberculosis* and *M. bovis* BCG Ag85 proteins, we sought to characterize the $H-2^b$-restricted, cross-reactive T-cell epitopes of Ag85 in *M. avium* subsp. *paratuberculosis*-infected mice, using synthetic overlapping peptides spanning the entire mature sequence of the three Ag85 components from *M. bovis* BCG. As shown in Fig. 2, spleen cells of B6 *bg/bg* mice infected intravenously with 3×10^5 CFU of *M. avium* subsp. *paratuberculosis* showed strong IL-2 (Fig. 2, left panels) and IFN-γ (Fig. 2, right panels) responses against certain peptides of Ag85A and Ag85B from BCG. These peptides were the same as those we have previously identified in $H-2^b$ haplotype B6 mice vaccinated with *M. bovis* BCG, infected with *M. tuberculosis*, or vaccinated with DNA encoding the *M. tuberculosis* Ag85 components (14, 21). Thus, strong IL-2 and IFN-γ production was observed in response to peptide 25 (spanning aa 241 to 260) and peptide 27 (aa 261 to 280) of Ag85A and to peptide 27 (aa 240 to 260) and peptide 30 (aa 262 to 279) of Ag85B. IL-2 responses against peptides from Ag85C were absent in spleen cell cultures from *M. avium* subsp. *paratuberculosis*-infected mice, whereas some IFN-γ was detected in response to peptide 3 (aa 21 to 40) and peptides 26 to 27 (aa 251 to 280). However, these IFN-γ levels were about 10-fold lower that the IFN-γ responses induced by the Ag85A or Ag85B peptides.

Proliferative responses against culture filtrate from *M. avium* subsp. *paratuberculosis* ATCC 19698 and Ag85 following oral infection with *M. avium* subsp. *paratuberculosis* ATCC 19698 in cattle. In view of the strong reactivity of experimentally infected mice against culture filtrate from *M. avium* subsp. *paratuberculosis* and its Ag85 components, we decided to analyze whether a similar reactivity could also be induced by an experimental infection of a target species for Johne's disease, i.e., *Bos taurus*. As *M. avium* subsp. *paratuberculosis* infection is naturally acquired in young calves through ingestion of contaminated colostrum and milk, we infected five 2- to 3-week-old calves by the oral route with 10 mg (10^8 CFU) of *M. avium* subsp. *paratuberculosis* ATCC 19698 cells per day for 10 consecutive days and monitored the animals for 125 weeks.

As shown in Fig. 3, proliferative responses against culture filtrate from *M. avium* subsp. *paratuberculosis* could be detected as early as 10 weeks p.i. in all five animals. Three animals (6074, 7782, and 7783) also demonstrated a proliferative response to Ag85, which, at this time point, was comparable in magnitude to the *M. avium* subsp. *paratuberculosis* CF-specific response. Between weeks 50 and 70 postinfection, proliferative responses to *M. avium* subsp. *paratuberculosis* CF declined but subsequently reappeared in four animals out of five. Animal 6074 reacted only weakly to CF in this second period; animal 7785 showed the strongest responses. Consistent Ag85-specific proliferative responses were detected in animals 7782, 7783, and 7785 from week 70 onwards. As reported by Koo et al. (26), cattle lymphoproliferative responses fluctuated considerably over time irrespective of background responses in unstimulated cells.

Ex vivo IFN-γ production in response to Ag85 following oral infection with *M. avium* subsp. *paratuberculosis* ATCC 19698 in cattle. All five animals demonstrated an early peak of Ag85-specific IFN-γ at about 10 weeks of infection (Fig. 4). After a sharp decline around week 20, IFN-γ responses reappeared and could be detected throughout the whole follow-up period

TABLE 2. Bovine lymphoproliferative responses at week 114 after oral infection with 10^9 CFU of *M. avium* subsp. *paratuberculosis* ATCC 19698

Antigen	Proliferative response (cpm) in animal[a]				
	6074	6075	7782	7783	7785
Medium	143 ± 20	335 ± 25	157 ± 23	133 ± 38	174 ± 45
CF *M. avium* subsp. *paratuberculosis*	276 ± 34	19,670 ± 3,374	15,790 ± 468	7,536 ± 390	27,835 ± 2,190
CF BCG	148 ± 48	1,350 ± 86	970 ± 46	2,098 ± 180	4,232 ± 233
PPD-A	184 ± 6	23,141 ± 2,203	10,347 ± 804	7,706 ± 126	34,255 ± 2,210
PPD-B	112 ± 7	1,662 ± 106	1,277 ± 143	11,123 ± 893	12,673 ± 143
Ag85 (BCG)	205 ± 11	1,131 ± 79	667 ± 24	1,194 ± 71	1,608 ± 74

[a] Mean cpm ± SD of proliferative responses of whole blood diluted 1:10 and cultured in triplicate for 7 days in the absence (medium) or presence of the various antigens. [^{3}H]thymidine was added to the cultures on the evening of day 6 and cells were harvested after overnight culture.

(albeit with some variation) in animals 7783 and 7785. Weak Ag85-specific IFN-γ responses were detected in animal 7782, whereas in animals 6074 and 6075 ex vivo IFN-γ levels were undetectable from week 70 onwards.

Proliferative and cultured IFN-γ responses at 114 weeks following oral infection with *M. avium* subsp. *paratuberculosis* ATCC 19698. A more detailed representation of the proliferative responses against CF from *M. avium* subsp. *paratuberculosis* and *M. bovis* BCG and from PPD-A, PPD-B, and Ag85 (from BCG) as tested on week 114 p.i. is shown in Table 2. Animal 6074 did not react to any of the antigens, whereas the four other animals showed a positive proliferative response following stimulation with *M. avium* subsp. *paratuberculosis* CF and PPD-A. Responses to PPD-B were two- to sevenfold lower than to PPD-A in animals 6075, 7782, and 7785 and of comparable magnitude in animal 7783. Responses to CF from BCG were lower than to CF from *M. avium* subsp. *paratuberculosis* in animals 6075, 7782, 7783, and 7785. Purified Ag85 induced positive proliferative responses in all animals except animal 6074. As for the responses to *M. avium* subsp. *paratuberculosis* CF and PPD-A, the highest Ag85-specific proliferation was observed in animal 7785. IFN-γ production was also assessed at week 114 p.i. in 6-day culture supernatants (Table 3). In animal 6074, IFN-γ responses to *M. avium* subsp. *paratuberculosis* CF and PPD-A hardly exceeded background levels (400 pg/ml). In contrast, the other animals demonstrated IFN-γ levels ranging between 2,000 and 13,000 pg/ml in response to *M. avium* subsp. *paratuberculosis* CF and PPD-A. IFN-γ responses to PPD-B were low in animals 6074 and 6075 but detectable in the three other animals. Finally, a significant IFN-γ response to Ag85 was detected in two animals, i.e., 7783 and 7785.

Permissive proliferative responses against Ag85B$_{145-162}$ peptide. Analysis of peripheral blood mononuclear cell (PBMC) responses of an 11-month-old bull, infected by the intravenous route with 10^8 CFU of *M. avium* subsp. *paratuberculosis*, using synthetic peptides spanning the entire sequence of mature Ag85A, Ag85B, and Ag85C from BCG, indicated a strong and sustained proliferative response against a peptide from BCG Ag85B spanning amino acids 145 to 162 (Ag85B$_{145-162}$) (data not shown). We therefore examined the responses against this Ag85B$_{145-162}$ peptide in the five orally infected animals at different time points after infection. Proliferative values with a stimulation index higher than 2 were considered as positive. As shown in Table 4, at week 13 p.i., only one animal (6074) reacted to the peptide. Subsequently, this animal did not react to the peptide any longer, in line with its overall nonresponsiveness to *M. avium* subsp. *paratuberculosis* CF and Ag85 observed throughout the entire follow-up period. All other animals showed significant responses to the Ag85B$_{145-162}$ peptide on at least two of the four subsequent time points tested. Again, animal 7785 ranked as the strongest reactor, with the highest stimulation index and cpm values. Proliferative responses were also observed against the corresponding peptide region of Ag85A and of Ag85C (data not shown).

Early proliferative T-cell responses against recombinant Ag85A and Ag85B from *M. avium* subsp. *paratuberculosis* in naturally infected calves. In order to find out whether similar, strong proliferative responses against Ag85 could also be observed in natural *M. avium* subsp. *paratuberculosis* infection, we examined two calves born from cows suffering from clinical paratuberculosis. Animal 3702 was 10 weeks of age at onset of the study, and animal 007 was 8 weeks old; both

TABLE 3. Bovine IFN-γ responses at week 114 after oral infection with 10^9 CFU of *M. avium* subsp. *paratuberculosis* ATCC 19698

Antigen	IFN-γ production (pg/ml) in animal[a]				
	6074	6075	7782	7783	7785
Medium	387 ± 78	521 ± 104	395 ± 79	394 ± 79	239 ± 48
CF *M. avium* subsp. *paratuberculosis*	852 ± 170	2,386 ± 477	3,902 ± 780	1,855 ± 371	13,240 ± 2,648
PPD-A	574 ± 115	5,648 ± 1,130	3,976 ± 795	2,823 ± 565	13,294 ± 2,659
PPD-B	440 ± 88	690 ± 138	1,133 ± 227	1,831 ± 366	4,547 ± 910
Ag85 (BCG)	394 ± 79	648 ± 130	440 ± 88	721 ± 144	1,787 ± 357

[a] IFN-γ production in 6-day culture supernatants of whole blood diluted to 10^6 white blood cells/ml and cultured in the absence (medium) or presence of the various antigens. Supernatants from three individual wells were pooled for the assay. Values are the means ± SD.

TABLE 4. Permissive proliferative responses against Ag85B$_{145-162}$ peptide of bovine whole blood PBMCs after oral infection with 10^9 CFU of *M. avium* subsp. *paratuberculosis* ATCC 19698

No. of weeks p.i.	Proliferative response (cpm) in animal[a]				
	6074	6075	7782	7783	7785
13	1,888 ± 97 (4.57)	293 ± 33 (1.14)	271 ± 30 (0.89)	279 ± 60 (0.82)	309 ± 39 (0.97)
28	323 ± 18 (0.94)	814 ± 234 (1.62)	2,777 ± 509 (4.32)	3,760 ± 45 (6.96)	3,713 ± 453 (15.41)
59	414 ± 98 (1.38)	2,390 ± 354 (1.76)	12,909 ± 1,391 (5.12)	1,263 ± 133 (1.02)	6,420 ± 291 (4.09)
82	859 ± 65 (1.19)	3,233 ± 252 (6.97)	1,167 ± 227 (7.39)	495 ± 101 (1.65)	9,694 ± 1,013 (3.61)
114	251 ± 39 (1.25)	1,285 ± 75 (3.84)	1,238 ± 86 (7.88)	2,293 ± 111 (17.24)	6,241 ± 442 (35.86)

[a] Proliferative responses (mean ± SD of quadruplicate cultures) as detected after 7 days of culture of PBMCs (whole blood diluted 1:10) stimulated with Ag85B$_{145-162}$ peptide (10 mg/ml). Stimulation indices compared to mean cpm values of unstimulated cells are given in parentheses.

calves were monitored for a period of almost 1 year. As shown in Fig. 5, positive T-cell responses against recombinant Ag85A and Ag85B from *M. avium* subsp. *paratuberculosis* could be detected very early in these animals. Ag85-specific responses in animal 3072 waned at week 14 of follow-up and remained subsequently very low, except for one time point at week 28 (when the animal also reacted strongly to PPD-B). Proliferative responses to PPD-A were higher than to PPD-B but lower in magnitude than to the recombinant Ag85 (rAg85) proteins. Animal 007 showed a different lymphoproliferative profile. Positive responses to rAg85 (particularly the rAg85B component) fluctuated but could be detected throughout the entire year of follow-up. Around week 28, responses to PPD-A became positive. These responses were more or less comparable to those against PPD-B and much stronger than in animal 3702.

DISCUSSION

The current vaccines against *M. avium* subsp. *paratuberculosis* are based on killed or live attenuated, whole-bacteria preparations. Although these vaccines provide partial protection, by reducing fecal shedding and reducing the number of clinically affected animals in a herd, they do not protect against infection. Animals vaccinated with these whole-bacteria vaccines develop positive tuberculin skin reactions and are of lesser economic value, because trading of PPD-positive animals is not permitted (EU Council Directive 64/432/EEC) (25). Moreover, such animals may develop observable granulomas at the injection site, which can be a problem at culling. An efficient subunit vaccine that would not interfere with bovine tuberculosis diagnosis would be very valuable in the management of paratuberculosis but requires the characterization
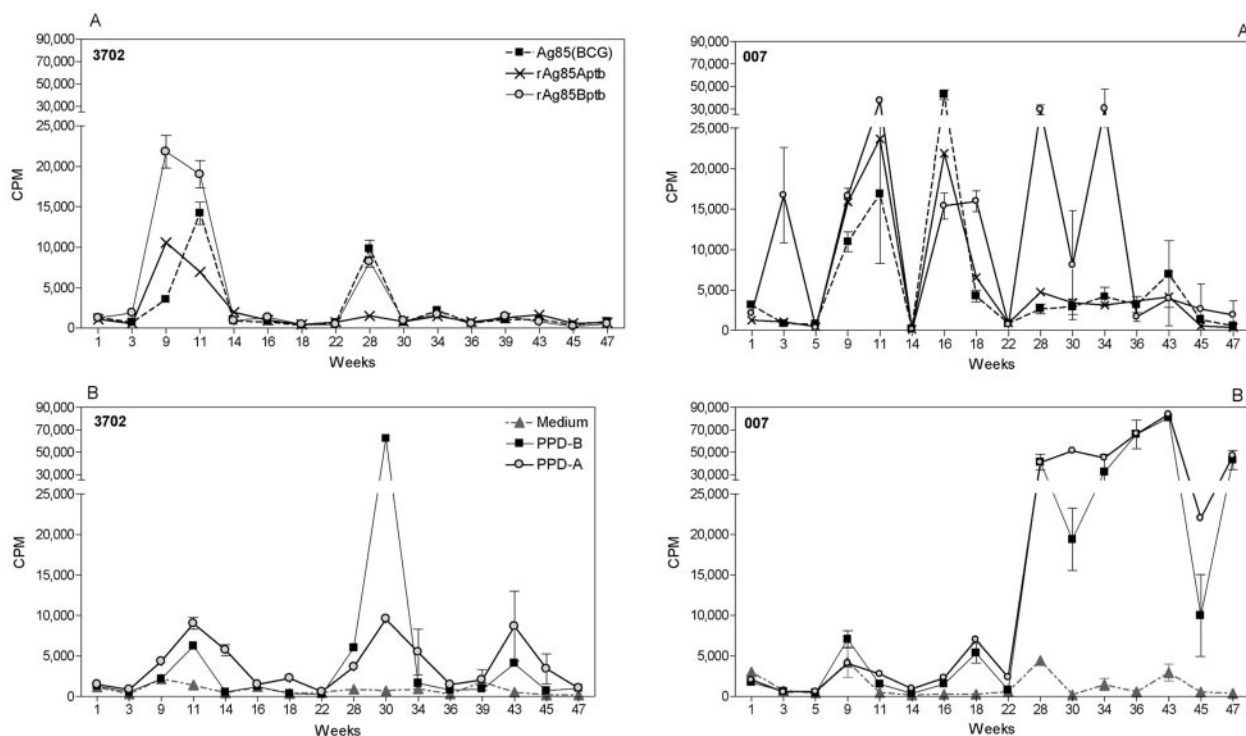

FIG. 5. (A) Proliferative response to recombinant Ag85A and recombinant Ag85B proteins from *M. avium* subsp. *paratuberculosis* and to native Ag85 from BCG in two calves. (B) Proliferative responses to PPD-B and PPD-A in two calves born from cows with confirmed Johne's disease. Responses of unstimulated cells are shown (broken lines and black triangles). Data represent mean cpm ± SD of quadruplicate cultures of whole-blood PBMCs diluted 1:10 and stimulated for 7 days with the respective antigens.

of immunodominant and protective antigens. A number of immunogenic proteins of *M. avium* subsp. *paratuberculosis* have been described (17), but they have mostly been analyzed for diagnostic purposes (4, 38), and little is known about their vaccine potential. Recently, Nagata et al. reported on the expression cloning of three recombinant, IFN-γ-inducing antigens from *M. avium* subsp. *paratuberculosis*. Two of these proteins were members of the PPE protein family (35).

Immunization with mycobacterial culture filtrate proteins can protect mice and guinea pigs to some extent against subsequent challenge with *M. tuberculosis* and *M. bovis* (1, 39). Here we have analyzed the immunogenicity and the antigenic composition of CF derived from *M. avium* subsp. *paratuberculosis* ATCC 19698. First we adapted *M. avium* subsp. *paratuberculosis* isolate to grow as a surface pellicle on synthetic Sauton medium supplemented with mycobactin J. This protein-free medium enabled us to separate the CF proteins in 30 fractions with decreasing molecular weight by whole-gel electroelution following SDS-PAGE.

Culture filtrate from *M. avium* subsp. *paratuberculosis* was strongly recognized in experimentally infected B6 *bg/bg* mice and cattle, as indicated by spleen cell IFN-γ secretion and elevated lymphoproliferative responses of PBMCs, respectively. Animals reacted strongly to electroeluted CF fractions containing the Ag85 homologues from *M. avium* subsp. *paratuberculosis* (data not shown) and against recombinant *E. coli*-derived Ag85A and Ag85B from *M. avium* subsp. *paratuberculosis*. Cross-reactive responses were observed against Ag85 purified from *M. bovis* BCG CF and against synthetic peptides from the Ag85A and Ag85B components of this complex. Responses against synthetic peptides from Ag85C were much lower. As the Ag85C homologues from *M. avium* subsp. *paratuberculosis* and *M. tuberculosis* share the highest percentage of identical amino acids, this suggests that low responsiveness to the Ag85C component is probably caused by low expression levels, rather than by low immunogenicity. Low response levels to the Ag85C component have also been described in tuberculosis (14, 32). B6 *bg/bg* mice experimentally infected with *M. avium* subsp. *paratuberculosis* reacted against the same immunodominant Ag85A and Ag85B epitopes as C57BL/6 mice vaccinated with *M. bovis* BCG or infected with *M. tuberculosis*, i.e., regions spanning aa 241 to 260 and 261 to 280 (14, 21).

We also infected five 2- to 3-week-old calves with *M. avium* subsp. *paratuberculosis* ATCC 19698 by the oral route and monitored the animals for 2 years (125 weeks). CF-specific proliferative responses could be detected at 10 weeks postinfection in all five animals. These responses were sustained for about 50 weeks and then declined and reappeared from about week 70 onwards to remain stable until the end of the 125-week follow-up period in four out of five animals. Proliferative responses against Ag85 were also detected very early in infection with magnitudes as high or even higher than responses against whole CF in three out of five animals. Ex vivo IFN-γ responses to Ag85 showed an early peak in all five animals around week 10 p.i. After a sharp decline around week 20, ex vivo Ag85-specific IFN-γ responses reappeared in three out of five animals. On the whole, the evolution of day 7 proliferative and ex vivo IFN-γ responses to Ag85 correlated well. This early recognition of Ag85 in *M. avium* subsp. *paratuberculosis* infection is reminiscent of a household contact study of leprosy

patients, in whom early T-cell reactivity against Ag85 complex (from *M. bovis* BCG) could be detected before reactivity against whole *M. leprae* bacilli or lepromin (29). CF proteins such as Ag85 are produced by actively replicating, live mycobacteria. In the phagosome of antigen-presenting cells, these proteins can be secreted and processed very early upon infection, before actual destruction of the bacteria releases antigenic components from the cytoplasm (18). This may explain the very early recognition of Ag85 components by the immune system and also the confirmed vaccine potential of this protein family.

In 2003, Bannantine and his colleagues reported on T-cell responses to PPD-A and whole *M. avium* subsp. *paratuberculosis* in three newborn calves, infected experimentally with ±1.6 × 10^7 CFU of *M. avium* subsp. *paratuberculosis* given into the tonsillar crypts (50). Positive immune responses were detected beginning around week 26 after infection. Also, in a more recent report, Koo et al. used flow cytometry to analyze proliferative responses against PPD and soluble *M. avium* subsp. *paratuberculosis* extract in orally infected calves (26). Persistent proliferative responses were again detected much later in this study (24 weeks after infection). It is probable that the higher infectious dose we used (10^9 CFU) is the reason for the more rapid appearance of reactive T cells observed in this study.

Ag85 epitope mapping in a young bull showed that one peptide spanning aa 145 to 162 of Ag85B from *M. bovis* BCG induced strong proliferative T responses after intravenous infection. The sequence of this Ag85B peptide is identical in *M. bovis* BCG and in *M. avium* subsp. *paratuberculosis* (13). Responses to this Ag85B peptide were also detected in the four orally infected calves that reacted against whole Ag85 protein. This peptide was not recognized prior to infection, nor was it recognized in animal 6074, which was nonreactive to Ag85. It has previously been shown that peptides from Ag85A and Ag85B are recognized in a permissive way by PBMCs from healthy PPD-positive human volunteers, with a majority of subjects reacting to a limited number of the same peptides, irrespective of their HLA haplotype (28, 40, 48). Interestingly, the peptide region spanning aa 141 to 160 from Ag85A was strongly recognized by 90% of these subjects (28), and we have identified in this same region a CD4$^+$ and a CD8$^+$ H-2^d-restricted epitope (shared with the Ag85B component) and a CD4$^+$ H-2^b-restricted epitope in, respectively, BALB/c and C57BL/6 mice (12, 14). With respect to bovine tuberculosis, Lightbody et al. have reported on the permissive recognition of a T-cell epitope of *M. bovis* Ag85B in 7/13 (54%) tuberculous cattle of mixed breed, but the peptide identified in that study spanned aa 61 to 70 (YYQSGLSIVM) of the mature Ag85B protein (31).

Finally, strong proliferative responses against recombinant Ag85A and Ag85B from *M. avium* subsp. *paratuberculosis* could also be detected around 3 to 4 months of age in two young calves born from cows suffering from culture-confirmed Johne's disease. Although natural infection has not been formally proven so far in these two young animals, the positive proliferative responses to PPD-A would suggest that *M. avium* subsp. *paratuberculosis* infection was indeed transmitted by the diseased mothers to their offspring. Also, the results generated with the laboratory ATCC 19698 strain of *M. avium* subsp. *paratuberculosis* seem to be relevant to natural paratuberculo-

sis infection, but a more detailed analysis and follow-up of the immune responses in naturally infected animals and bacterial culture will be required to confirm their infection status.

In conclusion, we have shown that experimental *M. avium* subsp. *paratuberculosis* infection of cattle and mice elicits a rapid and strong T-cell response against CF proteins in general and against mycolyl transferase Ag85A and Ag85B proteins in particular. We have also provided (preliminary) evidence that the Ag85 homologues are recognized very early in natural *M. avium* subsp. *paratuberculosis* infection. More work is needed to elucidate the protective potential of the antigen 85 family for the development of a new, subunit-based vaccine. It is interesting here that in a context of vaccination, the Ag85B component of *M. avium* subsp. *paratuberculosis* can elicit strong immune responses in mice when administered either as recombinant protein in Ribi adjuvant (34) or as a DNA vaccine (41). Also, mice and cattle vaccinated with an experimental vaccine consisting of whole, irradiated *M. avium* subsp. *paratuberculosis* ATCC 19698 in adjuvant showed significant proliferative and IFN-γ responses to recombinant Ag85A and Ag85B from *M. avium* subsp. *paratuberculosis* (Rosseels et al., unpublished data).

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