Alkyl Hydroperoxide Reductases C and D Are Major Antigens Constitutively Expressed by *Mycobacterium avium* subsp. *paratuberculosis*

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Antigens characteristic for *Mycobacterium avium* subspecies *paratuberculosis* were identified by crossed immunoelectrophoresis (CIE) and by absorbing out cross-reactive antigens by using a polyclonal and polyvalent *Mycobacterium avium* subspecies *avium* antiserum. Two antigens were present in *M. avium* subsp. *paratuberculosis* and not detected in *Mycobacterium avium* subsp. *avium*. They were identified as antigens 17 and 20 in a CIE reference system for *M. avium* subsp. *paratuberculosis* antigens. Purified antigen 20 was identified as alkyl hydroperoxide reductase C (AhpC) while the N-terminal part of purified antigen 17 showed 80% homology with alkyl hydroperoxide reductase D (AhpD) of *Mycobacterium tuberculosis*. AhpC had a nonreduced mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis corresponding to a molecular mass of 45 kDa and is probably a homodimer linked with disulfide bridges in its native form. AhpD had a mobility corresponding to 19 kDa. Monospecific rabbit antiserum against AhpC and AhpD reacted with 9 strains of *M. avium* subsp. *paratuberculosis* but not with 20 other mycobacterial strains except for a *Mycobacterium gordoniae* strain, against which a weak cross-reactive band was produced. Goats experimentally infected with *M. avium* subsp. *paratuberculosis* had strong gamma interferon (IFN-γ) responses toward both AhpC and AhpD, and they also had antibodies against AhpC. The ability of AhpC and AhpD to induce IFN-γ production shows that these proteins potentially could be used in future vaccines or in diagnostic assays. These results further show that AhpC and AhpD are immunologically important proteins which are constitutively and highly expressed in *M. avium* subsp. *paratuberculosis* without the bacteria being submitted to oxidative stress and that the specificities of antigens can be a matter of different levels of protein expression in various species as well as distinct structural differences.

*Mycobacterium avium* subsp. *paratuberculosis* causes a chronic granulomatous infection of the intestines characterized by persistent diarrhea and emaciation in ruminants. The bacterium has also been proposed as an etiologic agent of Crohn’s disease in humans (8, 34). Paratuberculosis in ruminants has a long incubation time and most animals remain subclinically infected. The immune responses in paratuberculosis resemble the immune responses towards other mycobacteria such as *Mycobacterium leprae*, *Mycobacterium bovis* and *Mycobacterium tuberculosis* (5, 14, 28). Protective immunity is characterized by strong Th1-cell responses, while animals with fulminating disease have strong antibody responses and weak cellular responses.

The diagnosis of paratuberculosis in living ruminants is based on several tests, and the detection of antibodies by a complement fixation test or enzyme-linked immunosorbent assay and the cultivation of feaces are routine laboratory methods. The culture is confirmed to be *M. avium* subsp. *paratuberculosis* by the identification of the IS900 element by PCR analysis. The PCR method has also been used directly on feaces, but so far this method has not shown sufficient sensitivity for diagnostic use (44). Both cultivation of feaces and antibody assays have a low sensitivity, particularly in the early stage of the infection (11, 38). This is strongly related to the finding that animals with minimal disease have low antibody responses but elicit strong Th1-cell responses as determined by the antigen-specific stimulation of cells. These responses can be measured by the gamma interferon (IFN-γ) enzyme immunosassay which originally was designed for the diagnosis of tuberculosis in cattle (Bovigam; CSL, Parkville, Australia) (6, 33, 39, 47).

The specificity of tests for cellular immunity relies on the qualities of the antigen used in the assays. Tests may be improved by selecting antigens or epitopes that are characteristic of *M. avium* subsp. *paratuberculosis.* Although several antigens in *M. avium* subsp. *paratuberculosis* have been identified (1, 3, 24, 29, 43), only a few of these antigens have been further characterized, including antigen A (a member of the Ag 85 complex), antigen D (7, 41, 45), lipoarabinomannan (42), and the A36 complex, with a 34-kDa antigen which was reported to be species specific (13). The antibody responses in infected cattle against some of these antigens have been investigated, but few reports concerning cellular immune responses against purified antigens are available (13, 21, 23).

The close genetic relationship between *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* is well established (35, 49), and it is a major challenge to differentiate between infections caused by the two organisms. The two bacteria produce different disease complexes; *M. avium* subsp. *paratuberculosis* causes a chronic inflammation in the intestines of ruminants while *M. avium* subsp. *avium* is pathogenic for birds and can cause disease in immunocompromised individuals. Even though infections with *M. avium* subsp. *avium* or other related mycobacteria usually do not cause disease in ruminants, such infections can give a high number of false-positive results in immunologic diagnostic testing for paratuberculosis.
in animals. The close genetic relationship between *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* resembles that between *M. bovis* and *M. tuberculosis*. In the latter pair, there are striking examples of proteins expressed in large quantities by *M. bovis* that are expressed only in small amounts by *M. tuberculosis* and in small amounts by *M. avium* subsp. *avium* could be expected to exist. Proteins expressed in large amounts by *M. avium* subsp. *paratuberculosis* and in small amounts by *M. avium* subsp. *avium* would be valuable for the diagnosis of the disease and may also be important in the pathogenesis of paratuberculosis.

A comparison of *M. bovis* and *M. tuberculosis* recently revealed 11 regions (encompassing 91 open reading frames) of *M. tuberculosis* H37Rv that were absent from one or more virulent strains of *M. bovis*. These potential open reading frames encode proteins that are thus species specific for *M. tuberculosis* (4). It is a distinct possibility that species-specific antigens also exist in *M. avium* subsp. *paratuberculosis* that are not represented by homologous genes in *M. avium* subsp. *avium*. The aim of this work was to identify species-specific antigens of *M. avium* subsp. *paratuberculosis* or antigens that show major quantitative differences.

**MATERIALS AND METHODS**

**Strains and antisera.** *M. bovis* AN5, *M. tuberculosis* H37Rv, and *Mycobacterium bovis* BCG Moreau were obtained from the National Hospital, Oslo, Norway. Reference strains of 13 mycobacterial species, 16 clinical isolates belonging to the *M. avium*-Mycobacterium intracellulare complex, and 14 other related bacterial species (Table 1) were obtained from the National Veterinary Institute, Oslo, Norway. The strains used for the purification of antigens were *M. avium* subsp. *paratuberculosis* strain 2E and *M. avium* subsp. *avium* strain D4. Polyclonal, polyvalent rabbit antiserum against *M. avium* subsp. *paratuberculosis* strain 2E (batch B312) and *M. avium* subsp. *avium* strain D4 were obtained from Dako, Glostrup, Denmark. Monospecific rabbit antiserum was made by immunizing rabbits with precipitation lines formed between purified proteins and polyclonal *M. avium* subsp. *paratuberculosis* antiserum. The lines were cut out from crossed immunoelectrophoresis (CIE) gels and mixed with Freund’s incomplete adjuvant, and two rabbits were immunized with each antigen (25).

**Bacterial culture and antigen preparation.** *M. avium* subsp. *paratuberculosis* strains were cultivated as surface pellicles on liquid synthetic Reid’s medium with mycobactin J (2 μg/ml) (Allied Monitor, Fayette, Missouri) until there was sufficient growth at 37°C, and the other mycobacterial species were cultivated as surface pellicles on liquid synthetic Sauton medium for 4 weeks at 37°C. *M. avium* subsp. *avium* strain D4 and *M. intracellulare* strain MNC 72 were cultivated on both Reid’s and Sauton medium. Corynebacterium pseudotuberculosis, Corynebacterium renale, Corynebacterium flavescens, Corynebacterium vitatum, Nocardia asteroides, Arcanobacterium pyogenes, and Rhodococcus equi were cultivated aerobically on brain heart infusion broth on a shaker for 2 to 4 days at 37°C. Harvested bacteria were washed three times in phosphate-buffered saline (PBS) and suspended in PBS at a concentration of 200 mg/ml. The bacteria were kept on ice and sonicated for 20 × 1 min. Sonicated samples were centrifuged at 20,000 × g for 15 min, and the supernatant was filtered (pore size, 0.22 μm) to remove residual particulate material.

**Absorption of *M. avium* subsp. *paratuberculosis* or *M. avium* subsp. *avium* antigens.** *M. avium* subsp. *paratuberculosis* or *M. avium* subsp. *avium* antiserum (800 μl; 45 mg of immunoglobulins [Ig]1/ml) was adsorbed to a HiTrap protein G column (Pharmacia, Uppsala, Sweden). Approximately 2 mg of sonicated proteins of *M. avium* subsp. *paratuberculosis* 2E and *M. avium* subsp. *avium* strain D4 was subsequently applied to the column. Bound IgG-antigen complexes were eluted with glycine-HCl buffer (pH 2.7). The primary efficient and eluate fractions were collected. Between each step, the column was washed with 0.2 mM Na phosphate buffer (pH 7.0). The protein content in the output was monitored by measuring optical density (OD) at 280 nm.

CIE. CIE with intermediate gels was performed with 1% agarose gels on 5- by 7-cm glass plates according to standard procedures (10). The first dimension was run at 8 V/cm for approximately 1 h until Evan’s blue albumin indicator had moved 3 cm. The second dimension was run at 3 V/cm overnight. In the reference plate, 10 μg of *M. avium* subsp. *paratuberculosis* sonicate proteins was used as an antigen, and 100 μl of *M. avium* subsp. *paratuberculosis* antiserum was incorporated into the top gel. The intermediate gel contained 0.9% NaCl or 100 μl of *M. avium* subsp. *avium* antiserum. After second-dimension electrophoresis, the plates were washed and pressed four times, followed by Coomasie brilliant blue (CBB) staining.

**SDS-PAGE with immunoblotting.** The antigens were separated under reducing or nonreducing conditions by horizontal sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in precast gradient Excel gel in an 8 to

<table>
<thead>
<tr>
<th>Bacterial species (no. of isolates)</th>
<th>Strain</th>
<th>Source(s) of isolate</th>
<th>Detection of AhpC and AhpD</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. avium subsp. <em>paratuberculosis</em> (1)</td>
<td>2E</td>
<td>Goat; cattle</td>
<td>3+</td>
</tr>
<tr>
<td>M. avium subsp. <em>paratuberculosis</em> (1)</td>
<td>ATCC 19698b</td>
<td>Goats</td>
<td>3+</td>
</tr>
<tr>
<td>M. avium subsp. <em>paratuberculosis</em> (7)</td>
<td>Clinical isolates</td>
<td>Pig</td>
<td>3+</td>
</tr>
<tr>
<td>M. avium subsp. <em>avium</em> (1)</td>
<td>D4</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>M. avium subsp. <em>avium</em> (4)</td>
<td>Clinical isolates</td>
<td>Goats</td>
<td>-</td>
</tr>
<tr>
<td>M. intracellulare (1)</td>
<td>MNC 72</td>
<td>Clinical isolates</td>
<td>-</td>
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<tr>
<td>M. intracellulare (5)</td>
<td>Clinical isolates</td>
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<td>+/−</td>
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<td>-</td>
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<td>MNC 115</td>
<td></td>
<td>-</td>
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<tr>
<td>M. smegmatis (1)</td>
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<td>-</td>
</tr>
<tr>
<td>M. bovis (1)</td>
<td>AN5</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>M. bovis BCG (1)</td>
<td>Moreau</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>M. tuberculosis (1)</td>
<td>H37Rv</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>A. pyogenes (3)</td>
<td>Clinical isolates</td>
<td>Cattle; sheep; sheep</td>
<td>-</td>
</tr>
<tr>
<td>R. equi (1)</td>
<td>Clinical isolate</td>
<td>Foal</td>
<td>1+</td>
</tr>
<tr>
<td>R. equi (2)</td>
<td>Clinical isolate</td>
<td>Foal</td>
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<td>N. asteroides (1)</td>
<td>Clinical isolate</td>
<td>Cattle</td>
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<td>C. pseudotuberculosis (2)</td>
<td>Clinical isolates</td>
<td>Goat; unknown</td>
<td>-</td>
</tr>
<tr>
<td>C. renale (3)</td>
<td>Clinical isolates</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>C. flavescens (1)</td>
<td>ATCC 10340</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>C. vitatum (1)</td>
<td>ATCC 10234</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

*The band strength was evaluated visually (see examples of Western blots in Fig. 1), −, no visible band; +/−, barely visible band; 1+, weak band; 2+, moderate band; 3+, strong band.

*American Type Culture Collection, Rockville, Md.

*Mycobacteria Nocardia Collection, Statens Serum Institut, Copenhagen, Denmark.*
18% concentration (Pharmacia), using a Multiphor II unit 2117 (Pharmacia). After separation, the gel was stained with CBB or the proteins were transferred to a nitrocellulose membrane (pore size, 0.2 μm) by electroblotting at 0.8 mA/cm² for 1 h or by diffusion blotting (32). The membranes were blocked with PBS containing 2% bovine serum albumin and 1% gelatin and incubated with serum overnight. Bound antibodies were recognized by horseradish peroxidase-labelled anti-rabbit or anti-goat Ig. As a substrate, 3,3’-diaminobenzidine was added to visualize the bound antibodies.

**Molecular masses of AhpC and AhpD by SDS-PAGE.** To determine the molecular masses of these antigens, the precipitation lines were cut out from CIE gels and dissolved in SDS buffer (31). The samples were then sonicated for 15 s and incubated at 37°C overnight before they were subjected to SDS-PAGE and electroblotted onto a nitrocellulose membrane as described above.

**Purification of AhpC and AhpD.** The purification procedures for AhpC and AhpD were developed as a combination of ion-exchange chromatography, hydrophobic interaction chromatography, and gel filtration using a Gradi Frac system equipped with a P-50 pump, a UV monitor, and a conductivity monitor (Pharmacia). Fractions were analyzed by SDS-PAGE with diffusion blotting (32). One milliliter of heparinized blood was dispensed into 24-well tissue culture trays (Costar). Blood samples were stimulated with purified AhpC (2.5 μg/ml) and incubated at 37°C for 3 weeks. The supernatants were removed as described. The collected fractions were investigated by SDS-PAGE with Western blotting (Fig. 2B). Cross-reactive precipitation lines were pulled down into the intermediate gel. However, several precipitation lines remained in the same position in the top gel, demonstrating virtually no antibody reactivity against these antigens in anti-M. avium subsp. avium. The most distinct of these precipitation lines showed a characteristic “inward-feet reaction” indicating a weak cross-reactivity with M. avium subsp. avium.

**Preparative isolation of M. avium subsp. paratuberculosis-specific antigens.** The CIE experiment indicated that a crude preparation of M. avium subsp. paratuberculosis-specific antigens could be made by a one-step affinity chromatographic procedure. Cross-reactive antigens in the M. avium subsp. paratuberculosis sonicate were removed as described. The collected fractions were investigated by SDS-PAGE with Western blotting. Peptides with molecular masses of 18, 31, 34, and 70 to 80 kDa in the primary effluent fraction of M. avium subsp. paratuberculosis reacted with the polyclonal M. avium subsp. paratuberculosis antiserum and not with the polyclonal M. avium subsp. avium antiserum in Western blotting (Fig. 2).

**RESULTS**

Identification of antigens characteristic for M. avium subsp. paratuberculosis with CIE. A reference plate was made as described previously and several precipitation lines were seen (Fig. 1A). To identify antigens highly expressed by M. avium subsp. paratuberculosis but not by M. avium subsp. avium, a polyclonal and polyvalent M. avium subsp. avium antiserum was incorporated into the intermediate gel on a reference plate.
There were no bands of corresponding molecular masses in the primary effluent of the *M. avium* subsp. *avium* sonicate. Several bands in the 22- to 25-kDa region could be detected in the primary effluent of *M. avium* subsp. *paratuberculosis* with both of the polyclonal antisera. The reactions were stronger and there were more bands detected with *M. avium* subsp. *paratuberculosis* antisera. The bands that still could be detected with *M. avium* subsp. *avium* antisera were separated in the first dimension. Polyclonal *M. avium* subsp. *paratuberculosis* antiserum was incorporated into the intermediate gel. (B) The primary effluent fraction of *M. avium* subsp. *paratuberculosis* antisera was separated in the first dimension. Polyclonal *M. avium* subsp. *paratuberculosis* antiserum was incorporated into the top gel while polyclonal *M. avium* subsp. *avium* antiserum was incorporated into the intermediate gel.

![CIE of crude preparation of specific *M. avium* subsp. *paratuberculosis* antigens.](image)

There were no bands of corresponding molecular masses in the primary effluent of the *M. avium* subsp. *avium* sonicate. Several bands in the 22- to 25-kDa region could be detected in the primary effluent of *M. avium* subsp. *paratuberculosis* with both of the polyclonal antisera. The reactions were stronger and there were more bands detected with *M. avium* subsp. *paratuberculosis* antisera. The bands that still could be detected with *M. avium* subsp. *avium* antisera were separated in the first dimension. Polyclonal *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* antisera can be explained by inadequate absorption or reaction with Ig fractions that did not bind to the protein G column.

**Identification of AhpC and AhpD.** The primary effluent fraction was also tested in CIE (Fig. 3A). Two distinct precipitation lines were seen in addition to several weak lines. *M. avium* subsp. *avium* antisera was then put in the intermediate gel to identify cross-reactivity with this antisera (Fig. 3B). The *M. avium* subsp. *avium* antisera did not affect the position of the two distinct precipitates, but one of these lines gave a characteristic inward-feet reaction as also seen in Fig. 1B, indicating that weakly cross-reactive antibodies were present in the *M. avium* subsp. *avium* antisera. The migration distance and the shape in the first dimension of this line, as well as the weak cross-reactivity, resemble antigen 20 as described by Gunnarson and Fodstad (24). They also described another antigen (antigen 17) in the proximity of antigen 20 that seemed to be specific for *M. avium* subsp. *paratuberculosis*. They found that three *M. avium* subsp. *paratuberculosis* antisera reacted with antigen 17 and antigen 20, and that *M. avium* subsp. *avium* and BCG antisera did not. These observations correspond very closely with our results. The N-terminal amino acid sequences described later show that antigen 20 is homologous to AhpC and antigen 17 is homologous to AhpD, so we have used these names.

**Molecular masses of AhpC and AhpD.** The precipitation lines representing AhpC and AhpD were prepared for SDS-PAGE as described above to determine the molecular masses of the peptides. In Western blotting with *M. avium* subsp. *paratuberculosis* antisera, AhpC was seen as broad band at about 45 kDa and a broad band at about 24 kDa. AhpD migrated with an apparent molecular mass of 19 kDa. However, by gel filtration on Superdex 75, AhpC could be detected in the void volume fraction, indicating a molecular mass larger than 70 kDa and that AhpC was part of a larger complex. To investigate the connection between the different bands in AhpC, a sample was separated under reducing and nonreducing conditions by SDS-PAGE with subsequent staining with CBB. Distinct differences could be observed. When purified AhpC was separated under nonreducing conditions, the most distinct band was seen at 45 kDa and a weaker band was seen at 24 kDa. Under reducing conditions, the peptide at 24 kDa became dominant and only a faint band was detected at 45 kDa (Fig. 4). These results indicate that AhpC is linked with disulphide bridges and that it exists as a homodimer in its native form. AhpD migrated as a single band under reducing and nonreducing conditions.

**Purification and purity of AhpC and AhpD samples.** The yield from one run of 140 mg of crude protein was approximately 1 mg of AhpC and 300 μg of AhpD. The purified antigens were tested by SDS-PAGE with CBB staining and Western blotting with polyclonal antisera against *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium*. Only the bands representing AhpC and AhpD could be detected in the representative samples by Western blotting with the *M. avium* subsp. *paratuberculosis* antisera and by CBB staining. The purified AhpC seemed to contain some degradation products, since two peptides could be seen at 23 to 24 kDa. These peptides were inseparable throughout the purification procedure, and the clear signal obtained by N-terminal sequencing
of AhpC suggests that these are degradation products of the same protein.

To identify the purified proteins in CIE, AhpC and AhpD were put in the intermediate gels of two different CIE plates with the primary effluent fraction of \textit{M. avium} subsp. \textit{paratuberculosis} separated in the first dimension and polyclonal antibodies were characterized by CIE and Western blotting. In \textit{AhpC} and \textit{AhpD} was made in rabbits and the resulting antisera against purified \textit{M. tuberculosis} subsp. \textit{paratuberculosis} aligned with \textit{M. avium} subsp. \textit{paratuberculosis} AhpC and AhpD, respectively. Arrows indicate the positions of amino acids.

![Image](http://iai.asm.org/download/http://iai.asm.org/download/)

**Fig. 5.** Primary N-terminal amino acid sequences of AhpC and AhpD from \textit{M. avium} subsp. \textit{paratuberculosis} aligned with \textit{M. tuberculosis} AhpC and AhpD, respectively. Arrows indicate the positions of amino acids.

**Interspecies cross-reactivity of polyclonal AhpC and AhpD antibodies.** Several mycobacterial and other related bacterial species were tested by SDS-PAGE with Western blotting with the specific antisera against AhpC and AhpD (Fig. 6 and Table 1). The antisera against AhpC reacted with all the \textit{M. avium} subsp. \textit{paratuberculosis} strains at 23 and 24 kDa. A broad band at 43 to 45 kDa could be seen in some of the \textit{paratuberculosis} strains but was most prominent in strain 2E. The antisera against AhpD also reacted with all the \textit{M. avium} subsp. \textit{paratuberculosis} strains, showing a distinct band at 19 kDa. In addition, one or two faint bands at 41 kDa could be seen in these strains. \textit{M. avium} subsp. \textit{avium} (D4) and \textit{M. intracellularur} (MNC 72) did not react with AhpC and AhpD antisera when cultivated on Reid’s or Sauton medium, showing that there is no difference in expression of these proteins due to differences between Reid’s and Sauton media. The two antisera did not react with any of the other mycobacterium strains, except for producing faint bands in \textit{Mycobacterium gordonae} (invisible in copy) that migrated with corresponding molecular masses, showing that AhpC and AhpD were expressed in very low amounts by this species. The AhpC antisera reacted with one \textit{R. equi} strain and the AhpD antisera reacted with \textit{C. flavescens}, also giving bands at the expected positions of AhpC and AhpD.

**IFN-γ production.** To test the immunogenicity of the two purified proteins, heparinized blood from experimentally infected goats was stimulated with AhpC, AhpD, and PPD. Cells from all the infected animals produced IFN-γ when stimulated.
TABLE 2. IFN-γ responses toward different antigens in experimentally infected goats (corrected OD values)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Antigen (conc.)</th>
<th>OD stimulated</th>
<th>OD nonstimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>705</td>
<td>AhpC (2.5 µg/ml)</td>
<td>0.07</td>
<td>0.50</td>
</tr>
<tr>
<td>711</td>
<td>AhpD (2.5 µg/ml)</td>
<td>0.06</td>
<td>1.50</td>
</tr>
<tr>
<td>718</td>
<td>AhpC (2.5 µg/ml)</td>
<td>0.43</td>
<td>2.5</td>
</tr>
<tr>
<td>708‡</td>
<td>AhpD (2.5 µg/ml)</td>
<td>0.01</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Animal 708 is a healthy noninfected control.

with AhpC, AhpD, or PPD while cells from the uninfected control did not (Table 2). The results are given in corrected OD values (ODantigen-stimulated − ODnonstimulated) representing the specific elevation of IFN-γ production by the various antigens. The levels of IFN-γ in the nonstimulated samples were low in all the animals. The amount of IFN-γ production in response to the different antigens correlated positively; the animal with the highest response to PPD also had the highest response to the two purified antigens. The response was always highest when the cells were stimulated with PPD, followed by AhpC and then AhpD. The response to AhpD was relatively low compared to the response to AhpC. The response to AhpC was comparatively high, considering that 10 µg of PPD per ml was used, compared to a concentration of 2.5 µg/ml for the purified proteins. None of the antigens induced IFN-γ production in cells from the uninfected control. These results show that T-cell epitopes are present on AhpC and AhpD and that the proteins induce a specific, probably T-cell-mediated, response in peripheral blood leukocytes from animals with subclinical paratuberculosis.

**Antibody responses.** Antibody production against purified AhpC and AhpD in four experimentally infected goats and one control goat was tested 20 months postinfection by Western blotting. Sera from all the infected animals reacted with AhpC but no reaction against AhpD could be detected, showing that B-cell epitopes are present on AhpC. Antibodies against the proteins were not detected in the control animal.

**DISCUSSION**

The aim of this study was to identify species-specific proteins in *M. avium* subsp. *paratuberculosis* or proteins that are expressed in much larger amounts in this bacterium than in other closely related mycobacteria. We have identified two antigens which are homologous to AhpC and AhpD from *M. tuberculosis*. AhpC and AhpD were expressed in large amounts in *M. avium* subsp. *paratuberculosis* and could not be detected by Western blotting of other mycobacterial species except for minor quantities in *M. gordonae* when the bacilli were grown without exposure to oxidative stress. The high expression of AhpC and AhpD in *M. avium* subsp. *paratuberculosis* without the need for peroxide induction is a unique feature of this bacterium. This indicates that these enzymes may be important for the particular adaptation of this organism as an extremely slow-growing intestinal pathogen and for the virulence of *M. avium* subsp. *paratuberculosis*.

AhpC and AhpD are detoxifying enzymes that are important for protection against reactive nitric and oxidative metabolites. Expression of these proteins together with other detoxifying enzymes are controlled by the central regulator OxyR (9, 22). In gram-negative bacteria, the oxyR gene can be activated by low doses of hydrogen peroxide, which induces the production of several proteins that can protect the cell from a subsequent lethal dose of peroxide (9, 15). *oxyR* and *ahpC* homologues are also present in several mycobacterial species, but only saprophytic *Mycobacterium smegmatis* shows a similar protective response, with the induction of a novel protein synthesis when exposed to low doses of hydrogen peroxide (37). In contrast, pathogenic mycobacteria (*M. tuberculosis* and *M. avium* subsp. *avium*) seem to lack the capacity for a protective *oxyR* response of the type originally defined for *Escherichia coli* and *Salmonella*.

In *M. tuberculosis*, the *oxyR* gene is nonfunctional due to deletions and point mutations (16, 17, 37) and as a result, AhpC is usually not expressed in *M. tuberculosis*. However, some isoniazid-resistant strains that lack KatG activity have been shown to overproduce AhpC as a compensatory mechanism (36).

The *oxyR* gene appears intact in *M. avium* subsp. *avium* and the expression of AhpC increases markedly when exposed to hydrogen peroxide (37). The first report on AhpC, designated Avi3, from *M. avium* subsp. *avium* described the detection of this protein in *M. avium* subsp. *avium* but not in other mycobacteria, including *M. avium* subsp. *paratuberculosis* (2, 48). Our findings do not support these observations. We observed that AhpC and AhpD were highly expressed in *M. avium* subsp. *paratuberculosis* and not detected in *M. avium* subsp. *avium* when the bacilli were grown without exposure to oxidative stress. In the earlier studies, a monoclonal antibody made against AhpC from *M. avium* subsp. *avium* was used when screening the various mycobacterial species, and minor differences in amino acid sequence could explain the failure of this monoclonal antibody to react with *M. avium* subsp. *paratuberculosis*. However, in the present study monospecific polyclonal antisera against purified proteins was used that would have reacted with corresponding proteins in the *M. avium* subsp. *avium* strains if these proteins were expressed in this species. Another indication of the low expression of AhpC and AhpD in *M. avium* subsp. *avium* and their high expression in *M. avium* subsp. *paratuberculosis* was the strong reaction of AhpC and AhpD with the polyclonal antiserum made in rabbits against *M. avium* subsp. *paratuberculosis*. An equivalent antisera against *M. avium* subsp. *avium* had very low levels of cross-reactive antibodies against AhpC and no cross-reaction was seen with AhpD. Elseghier et al. have also shown specifically elevated antibody responses in *M. avium* subsp. *paratuberculosis*-infected mice but not in *M. avium* subsp. *avium*-infected mice towards a protein that later was identified as AhpC. This shows that AhpC is also expressed by *M. avium* subsp. *paratuberculosis* in vivo (19, 20). The N-terminal amino acid sequence from the purified AhpC of *M. avium* subsp. *paratuberculosis* is identical to the N-terminal sequence of AhpC from *M. avium* subsp. *avium*, and the homology between AhpC from *M. avium* subsp. *avium* and *M. tuberculosis* is about 90%. It is thus unlikely that major differences are present further along the peptide, considering the close genetic relationship between *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium*. We interpret our results as indicating that AhpC and AhpD are expressed in much larger amounts in *M. avium* subsp. *paratuberculosis* but we cannot exclude the possibility that differences in amino acid sequence exist between the proteins from the two species. Sequencing of the genes encoding AhpC and AhpD and the OxyR transcriptional regulator in *M. avium* subsp. *paratuberculosis* and comparison with the equivalent sequences in *M. avium* subsp. *avium* are necessary in order to clarify these questions.

The immune response against detoxifying enzymes has received little attention, but it has been shown that AhpC from *M. avium* subsp. *avium* can elicit both delayed-type hypersen-
sitivity responses and lymphocyte proliferation in immunized guinea pigs (48). Deretic et al. (18) have suggested that the silencing of oxyR in M. tuberculosis may have played a role in the evolution of this species and its adaptation to macrophage parasitism. These investigators suggest that several genes are shut down when M. tuberculosis enters the macrophage in order to avoid interactions with the immune system and thus prevent stimulation of a vigorous Th1 response, which is important for protective immunity. Interestingly, purified AhpC and AhpD from M. avium subsp. paratuberculosis elicited a strong IFN-γ response in goats with experimental subclinical paratuberculosis. Strong IFN-γ responses were produced by both antigens, especially by AhpC, indicating that these proteins might play a role in protection against paratuberculosis. AhpC also elicited B-cell responses in experimentally infected goats at a later stage of infection. The strong IFN-γ response against AhpC and AhpD shows that these proteins can potentially be used in the diagnosis of paratuberculosis with an IFN-γ-based test. However, the amount of individual variation of IFN-γ response to some proteins is large, so further testing is needed to see whether these proteins can be used for diagnostic purposes. Even though AhpC and AhpD are expressed in large amounts in M. avium subsp. paratuberculosis in vitro, the situation in vivo may differ. The presence of homologous genes makes it necessary to test animals infected with different mycobacterial species to evaluate the potential of these proteins in diagnostic assays. The ability of AhpC and AhpD to induce IFN-γ production also makes these proteins good candidates for inclusion in new vaccines against paratuberculosis.

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