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Research paper

Apa antigen of *Mycobacterium avium* subsp. *paratuberculosis* as a target for species-specific immunodetection of the bacteria in infected tissues of cattle with paratuberculosis

Giliane S. Souza[a], Ana Bárbara F. Rodrigues[b], Andrea Gioffré[c], Maria I. Romano[c], Eulógio C.Q. Carvalho[b], Thatiana L.B. Ventura[a], Elena B. Lasunskaia[a,*]

[a] *Laboratory of Biology of Recognition, Universidade Estadual do Norte Fluminense, Campos, 28013-602 Rio de Janeiro, Brazil*
[b] *Laboratory of Animal Morphology and Pathology, Universidade Estadual do Norte Fluminense, av. Alberto Lamego, 2000, Campos, 28013-602 Rio de Janeiro, Brazil*
[c] *Biotechnology Institute, CICV y A INTA-Castelar, Las Cabañas y Los Reseros, 1712 Buenos Aires, Argentina*

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ABSTRACT

Comparative genomics of *Mycobacterium* spp. have revealed conservative genes and respective proteins differently expressed in mycobacteria that could be used as targets for the species-specific immunodiagnostics. The alanine and proline-rich antigen Apa is a mycobacterial protein that present significant variability in primary sequence length and composition between members of *M. avium* and *M. tuberculosis* complexes. In this study, the recombinant Apa protein encoded by the MAP1569/*ModD* gene of *M. avium* subsp. *paratuberculosis* (Map) was used to generate a panel of monoclonal antibodies which were shown to recognize the most important veterinary pathogens of the *M. avium* complex, specifically Map and *M. avium* subsp. *hominissuis*, and which did not cross-react with *M. bovis* or *M. tuberculosis*. The produced antibodies were demonstrated to be a useful tool for the species-specific immunofluorescence or immunohistochemical detection of Map in experimentally infected cell cultures or intestinal tissues from cattle with bovine paratuberculosis and, additionally, they may be employed for the discrimination of pathogenic *M. avium* subspecies via Western blotting.

1. Introduction

Conserved mycobacterial antigens differently expressed in *Mycobacterium* spp. are a potential targets for immunodiagnosis of important veterinary infections, such as bovine tuberculosis and paratuberculosis, caused by *Mycobacterium bovis* and *Mycobacterium avium* subsp. *paratuberculosis* (Map), respectively. The alanine and proline-rich secreted Ag, Apa, is a conserved mycobacterial protein that presents significant variability in primary sequence length and composition (GenBank's protein database), as well as in post-translational modifications of the protein, in the different mycobacterial species. In a recent study, we demonstrated that the Apa protein secreted by Map is represented by a 50/60 kDa polypeptide dimer, whereas that of *M. bovis* is 45/47 kDa (Gioffré et al., 2009). These structural differences could affect immunogenic and virulence properties of the Apa Ag, which is a fibronectin-attachment protein (FAP) that contributes to the infection of target cells by bacteria (Wieles et al., 1994; Schorey et al., 1996; Secott et al., 2004). Indeed, a strong difference between the response of *M. bovis*-infected animals and that of Map-infected animals against the recombinant Apa of Map (r-Apa-Map)

* Corresponding author. Tel.: +55 22 27397128, fax: +55 22 27397178.
E-mail address: elena@uenf.br (E.B. Lasunskaia).

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showed an important inter-species difference in the immunological properties of this antigen (Gioffré et al., 2009).

The described structural differences of the Apa protein in different mycobacterial species could be used as a target for bacterial detection and identification using specific antibodies. Polyclonal serum produced against the Apa-Map protein was found to be a useful tool for immunodetection of the Apa Ag secreted by different mycobacteria (Gioffré et al., 2009); however, it was unable to differentiate mycobacterial species because of cross-reactivity. The production of monoclonal antibodies (mAbs) against distinct epitopes of the antigen could significantly increase the specificity of Map recognition and improve the currently used immunohistochemical (IHC) method of mycobacterial detection in infected intestinal tissues of paratuberculous cattle. The IHC test using commercially available polyclonal serum against Map was demonstrated to be as sensitive as the in situ hybridization test using the IS*900* probe to detect Map in intestinal tissues, and, additionally, more sensitive and much more specific than Ziehl–Neelsen staining (Delgado et al., 2009). Nevertheless, false positive results could not be completely excluded from the former tests because of the possible cross-reactivity with other mycobacterial species (Cousins et al., 1999; Martinson et al., 2008), such as *M. bovis*, the causative agent of bovine tuberculosis, including its intestinal form. Recent study of intestinal tissue samples randomly collected from slaughtered cattle in an abattoir in Pakistan and analyzed by polymerase chain reaction (PCR) using primers targeting IS*900* and IS*1311* for Map detection and primers targeting genomic DNA fragment specific for *M. bovis* demonstrated that 70% of the intestinal samples positive for acid-fast staining were infected by Map, whereas *M. bovis* was detected in almost 30% of the positive samples (Khan et al., 2010). These observations demonstrate the importance of differential diagnostics of paratuberculosis and intestinal form of tuberculosis in the cattle and necessity of methods to distinguish between the mycobacterial pathogens. The IHC is the method of choice for the specific detection of mycobacteria in situ. The specificity of bacterial recognition could be increased through the production of antibodies that are able to discriminate different species and subspecies of mycobacteria.

In the present study, we aimed to generate mAbs against the recombinant Apa protein of Map (r-Apa-Map), select those specifically able to recognize Map, but not *M. bovis,* and employ these antibodies for the immunodetection of Map in tissue samples from cattle with paratuberculosis.

2. Materials and methods

2.1. Mycobacterial antigens

Recombinant Apa protein of Map (r-Apa-Map) and whole cell lysates (WCL) obtained from bacterial cultures of Map, *M. avium* subsp. *hominissuis* (Mav), *M. tuberculosis* (Mtb) and BCG were used as a mycobacterial Ags. The r-Apa-Map protein was produced in *E. coli* as described previously (Gioffré et al., 2009). WCL Ags were obtained from the following mycobacterial strains: a field strain of Map characterized by IS*900*-PCR and its dependence on mycobactin for growth (Gioffré et al., 2009); a field strain of Mav, strain P104, isolated from infected pig (Oliveira et al., 2003) and *M. bovis* BCG vaccine strain Moreau (from Butantan Institute, Sao Paulo, Brazil). Bacterial cells from an exponential-phase growing mycobacterial cultures were subjected to Fast-Prep (Qbiogene, Solon, OH, USA) bead beater disruption in Lysing Matrix B, and resulted bacterial proteins were precipitated by acetone (Gioffré et al., 2009). Secreted proteins were obtained by filtering the culture supernatant with a 0.22 μm filtration unit Nalgene (Nalge Nunc), precipitated by acetone, resuspended in PBS and kept at −80 °C until use (Gioffré et al., 2009). Biochemically purified Mtb32/Apa *M. tuberculosis* protein (Apa-Mtb), as well as WCL Mtb H37Rv strain reagents and rabbit anti-Apa Mtb serum, were kindly provided by Dr. J. Belisle, Colorado State University (USA), according to the NIH contract HHSN266200400091C.

2.2. Hybridoma generation and Ab production

MAbs and polyclonal sera were produced using standard protocol. Briefly, 6-week-old female BALB/c mice were immunized three times intraperitoneally (i.p.) with the recombinant Apa-Map protein (40 μg per injection) at 14-day intervals. The Ag was emulsified in incomplete Freund adjuvant (Sigma, St. Louis, MO). Three weeks after the last injection, the immune mice were boosted by i.p. injection of 30 μg of r-Apa- Map and spleens were obtained four days later. Alternatively, immune sera were collected seven days later. Spleen cells were isolated from the spleens of immunized animals and fused with NS0 myeloma cells to obtain hybridomas. Ag-specific-Ab-secreting cells were detected by ELISA, cloned at least twice by limiting dilution and grown in DMEM–F12 medium supplemented with 10% FCS (Gibco, BRL, EUA) and as ascite-producing tumors in the peritoneal cavities of pristane-primed BALB/c mice. MAbs containing in hybridoma culture supernatant and ascite fluid were collected and characterized for specificity of Apa Ag recognition by Western blotting technique. The produced mAbs were immunotyped in ELISA using isotype kit I (Pierce, Rockford, IL) to define the heavy chains. All experimental protocols involving animals were approved by the Animal Care and Usage Committee of Universidade Estadual do Norte Fluminense.

2.3. Western blot and immunoprecipitation assay

The protein concentration in the mycobacterial cell extracts (WCL), culture filtrates (CFP) and purified Apa proteins were determined using the Bradford method. The antigens were resolved by 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA) using a Bio-Rad Trans-Blot Cell tank transfer unit at 150 mA for 2 h in buffer, containing 25 mM Tris–HCl (pH 8.0), 0.19 M glycine and 20% (v/v) methanol. Non-specific sites in the blot were blocked by incubation for 1 h with 5% dried non-fat powdered milk in 20 mM Tris–HCl (pH 7.5), 0.5 M NaCl buffer (TBS) at room temperature. The resulted membrane was assayed by using polyclonal murine anti-Apa-Map sera