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Interferon gamma response to *Mycobacterium avium* subsp. *paratuberculosis* specific lipopentapeptide antigen L5P in cattle

Sébastien Holbert [a,*], Maxime Branger [a], Armel Souriau [a], Bérénice Lamoureux [b], Christelle Ganneau [c,d], Gaëlle Richard [a], Thierry Cochard [a], Christophe Tholoniat [b], Sylvie Bay [c,d], Nathalie Winter [a], Jean Louis Moyen [e], Franck Biet [a]

[a] UMR1282, Infectiologie et Santé Publique (ISP-311), INRA Centre Val de Loire, F-37380 Nouzilly, France
[b] Groupement de Défense Sanitaire de la Région Centre (GDS Centre), 4 rue Robert Mallet Stevens BP 501, F-36018 Châteauroux, France
[c] Institut Pasteur, Unité de Chimie des Biomolécules, Département de Biologie Structurale et Chimie, 75015 Paris, France
[d] CNRS UMR 3523, 75015 Paris, France
[e] Laboratoire Départemental d'Analyse et de Recherche de Dordogne, 161 av Winston Churchill, 24660 Coulounieix Chamiers, France

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ABSTRACT

After *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) infection the cell-mediated immune (CMI) response indicative of early Th1 activation may be detected using interferon-gamma release assay (IGRA). Currently, the purified protein derivatives (PPDs), *i.e.*, the total extract of mycobacteria antigens are used to recall CMI responses against *Map*.

This study aimed to assess the ability of the chemically synthesized *Map* specific cell wall lipopentapeptide L5P to induce CMI response in cows infected by *Map* compared to PPD. L5P and PPD elicited an IFN-γ response in 12 and 35 animals from two *Map* infected herds respectively, but IFN-γ was not detected in the 13 cows recruited from a non-infected herd. Levels of IFN-γ detected were higher with PPD than with L5P. There was no correlation between the IFN-γ response and the humoral response to *Map* or faecal culture.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (*Map*) is the causative agent of the fatal granulomatous chronic infectious enteritis named Johne's disease (JD) or paratuberculosis. This disease is found worldwide in ruminant farming (Lombard et al., 2013; Nielsen and Toft, 2009).

Map interacts with and manipulates the host immune system. The classical response pattern to mycobacteria infection involves firstly a T helper (Th) 1 response which then switches to a Th2 response (Stabel, 2000, 2006). The IFN-γ release assay (IGRA) takes advantage of this by assessing the cell mediated immune (CMI) response to purified protein derivatives (PPDs) (Jungersen et al., 2011) or recombinant antigens selected for their specificity (Gurung et al., 2014; Hughes et al., 2013; Mikkelsen et al., 2011a). This method requires antigens capable of eliciting a sensitive and specific response, and which are currently under investigation (Mikkelsen et al., 2011b). PPDs elicit a strong response with this method but include crude undefined antigens that are shared with other mycobacterium species and which can cross-react resulting in false-positive results (Bannantine et al., 2002).

In this study a cell wall lipopeptide antigen produced specifically by the *Map* subspecies: the lipopentapeptide L5P (Biet et al., 2008; Reyrat et al., 2009), was tested to recall CMI responses against *Map*. Previous studies have shown that L5P is abundant in lipid extracts from *Map* cells and differs greatly from glycopeptidolipids (GPLs), which are characteristic of the other *M. avium* complex (*Mac*) members (Barrow et al., 1995; Ripoll et al., 2007). Unlike GPL, L5P contains a peptide core which is not glycosylated due to the absence of a free hydroxyl group, and the fatty acid in the *N*-terminal domain of the molecule is shorter, saturated and not hydroxylated (Biet et al., 2008). This specific antigen has been synthesized chemically and evaluated in different studies for its potential use in serological assays to detect *Map* infection (Biet et al., 2008; Costanzo et al., 2012; Eckstein et al., 2006; Thirunavukkarasu et al., 2013; Verdier et al., 2013).

* Corresponding author.
E-mail addresses: sebastien.holbert@tours.inra.fr (S. Holbert), maxime.branger@tours.inra.fr (M. Branger), a-souriau@orange.fr (A. Souriau), blamoureux@gdma36.fr (B. Lamoureux), christelle.ganneau@pasteur.fr (C. Ganneau), gaelle703@gmail.com (G. Richard), thierry.cochard@tours.inra.fr (T. Cochard), ctholoniat@gdma36.fr (C. Tholoniat), sylvie.bay@pasteur.fr (S. Bay), nathalie.winter@tours.inra.fr (N. Winter), jl.moyen@dordogne.fr (J.L. Moyen), franck.biet@tours.inra.fr (F. Biet).

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The present study aimed to assess the ability of chemically synthesized L5P to elicit *in vitro* CMI responses, by quantifying IFN-γ release in whole blood stimulation assays from cows recruited from non-infected, *Map* infected herds and *Mycobacterium bovis* infected herds.

2. Materials and methods

Animals included in this study were not subjected to any *in vivo* manipulation prior to stunning for industrial slaughter and therefore, no specific ethics committee authorization was required.

Cattle from three groups from four herds were included in the study. (1) 13 animals from a non-infected herd which tested negative twice for *Map* antibodies in serum and faecal culture at cow-level (Supplementary file 1). (2) 36 animals randomly recruited from two *Map* infected herds, herd infection being confirmed by *Map* culture and elimination of annual clinical cases of paratuberculosis included in the national paratuberculosis surveillance programme: herd (A) of 139 cows with a seroprevalence of 7.2% and herd (B) of 163 cows with a seroprevalence of 7.2% (Supplementary files 2 and 3). (3) 27 animals from a herd free of paratuberculosis but included in official eradication campaigns for bovine tuberculosis and subject to the official diagnostic tests for *M. bovis* (single intradermal test SIT and IFN-γ assays). Of the 83 cows analysed from this herd the 27 recruited (32.5%) were certified *M. bovis* positive based on PCR IS*6110* and spoligotyping performed on the strain isolated (Supplementary file 4).

Samples of faeces, serum and blood were collected.

Blood was collected by venipuncture in vacutainer tubes. Heparinized whole blood was used for stimulation and IFN-γ response analyses. Approximately 6 h after sampling, 225 μL of whole blood was placed in 96-well plates (Falcon®) previously loaded with 25 μL of RPMI medium 1640 1× (Gibco® by Life Technologies™) as Nil antigen and 10 μg/mL of Purified Protein Derivative Avium (PPD-A 2500 Prionics Lelystad BV PPD-A); in addition for animal samples from the non-infected and *M. bovis* infected herds, wells had also been loaded with 10 μg/mL of Purified Protein Derivative Bovis (PPD-B 3000 Prionics Lelystad BV), 5 μg/mL of L5P synthesized chemically as previously described (Biet et al., 2008) and 5 μg/mL of Pokeweed mitogen (PWM) (Sigma-Aldrich) all diluted in RPMI. After stimulating for 24 h at 37 °C and 5% CO_2, whole blood was centrifuged at 1500 rpm, plasma was collected and frozen for IFN-γ cytokine quantification using an ELISA (Bovigam 1G ® Prionics). IFN-γ responses were expressed as an S/P ratio (S/P ratio = Specific Sample OD / Positive control OD − Negative control OD); Specific Sample OD indicates that Nil antigen optical density was taken into account. The cut off value of the IFN-γ response to PPD-A, PPD-B and L5P was calculated as the 95% percentile of response to each antigen in the non-infected herd.

Serology was performed with the IDEXX *Map* Ab Test (IDEXX Laboratories, Inc.) according to the manufacturer's instructions and the L5P humoral immune response was quantified as described previously (Biet et al., 2008).

Strain isolation and identification were performed with *Map* chromosomal DNA obtained as described previously (Thibault et al., 2007). All the strains isolated were screened for the *Map* specific IS*900* insertion sequence using synthetic oligonucleotides (Sigma), as described previously (Sanderson et al., 1992). *Map* isolates were then genotyped by MLVA using the markers, Mycobacterial Interspersed Repetitive Units — Variable Number of Tandem Repeats (MIRU-VNTR), according to Thibault et al., 2007 (Thibault et al., 2007).

IFN-γ responses to PPD-A, PPD-B or L5P were compared for cows from the non-infected herd, the four categories of the *Map* infected herd and the two groups of the *M. bovis* infected herd using the non-parametric Bonferroni's multiple comparison test. Correlation analysis of IFN-γ responses to PPD-A and L5P was estimated with the Spearman's rank correlation for non-parametric correlations (GraphPad Prism version 5, GraphPad Software, La Jolla, CA, USA).

3. Results and discussion

The IFN-γ responses to chemically synthetized L5P and PPD-A antigen of animals from the non-infected, *Map* infected and *M. bovis* infected herds are shown in Fig. 1. To complete the data at the time of sampling, IGRA, serology and mycobacterial culture status were determined for each animal (Supplementary files 1, 2, 3, 4). Adult animals were recruited to avoid a response related to immune immaturity.

All animals of the non-infected herd were negative for the humoral immune response to *Map* antigens and L5P and for mycobacterial culture (Supplementary File 1). IFN-γ responses to PPDs and L5P of the non-infected herd were negative and were used to calculate cut-off values. The cut-off value of the IFN-γ response to PPD-A, PPD-B and L5P was calculated as the 95% percentile of response to each antigen in the non-infected herd. Thus, the cut-off values of the IFN-γ response to PPD-A, PPD-B and L5P were 0.060, 0.065 and 0.013 (S/P ratio) respectively represented with a dotted line (Fig. 1).

In the *Map* infected herds (herds A and B), 16 of the 36 animals analysed were positive for the humoral immune response to *Map* antigens, and nine were positive for *Map* culture with the same INMV2 genotype. These results were used to define four categories of animals (Fig. 1). Animals which were: 1) positive for faecal culture and serology (FC+ S+; n = 6), 2) negative for both tests (FC− S−; n = 16), 3) only seropositive (FC− S+; n = 11) and 4) only faecal culture positive (FC+ S−; n = 3). The median IFN-γ responses are presented in Fig. 1 and were higher for PPD-A from 0.150 to 0.940 (S/P ratio) compared to the median IFN-γ response which was below 0.0165 (S/P ratio) for L5P. According to the cut-off value, 35 of the 36 cows tested IFN-γ positive for PPD-A of which 12 also tested positive for L5P. Spearman's rank correlation for IFN-γ responses to PPD-A and L5P indicates a significant correlation (alpha = 0.05) with r = 0.6305. The non-infected herd and all categories of the *Map* infected herd showed significant ($p < 0.05$) differences in IFN-γ response to PPD-A and L5P (except category S− F+). Animals recruited from the *Map* infected herds had greater IFN-γ responses to PPD-A than the non-infected herd. This would suggest that animals have been exposed to *Map*, and was confirmed by strain identification in several animals which were subclinically infected. The IFN-γ responses to PPD-A were systematically higher than to L5P. As observed previously, a single antigen induced a weaker response than PPD (Gurung et al., 2014; Mikkelsen et al., 2011a). L5P is a lipopeptide, its structure presumably involves specific CD1 antigen presentation to T cells (Young et al., 2009) but its hydrophobicity would imply a non-specific cell membrane affinity which could hamper the expected presentation, thus partly explaining the weak IFN-γ response. Furthermore, the small antigenic peptide (5 amino acids) represented only a small part of the *Map* specific antigen (Bannantine and Paustian, 2006). A combination of lipid transport molecules (van den Elzen et al., 2005) and a selected recombinant antigen pool (Mikkelsen et al., 2011a) could enhance the IFN-γ response at the same time conserving high specificity. Using an enzyme-linked immunospot (ELISPOT) assay, a highly sensitive technique to detect cytokine release, could be an alternative way to improve response detection (Begg et al., 2009). For the animals used in the present study, the IFN-γ responses using PPD-A and L5P did not enable to define a specific status which corresponded to that obtained from the humoral response and faecal culture.

In the *M. bovis* infected herd the humoral immune response to *Map* antigens and L5P was negative for all animals. *M. bovis* culture was confirmed for 27 of the 83 animals. When comparing the IFN-γ responses induced by L5P (Fig. 1A) PPD-A (Fig. 1B) and PPD-B (Fig. 1C) of cows from the *M. bovis* infected herd, the 27 *M. bovis* positive culture cows and the 56 negative culture cows showed significantly ($p < 0.05$) different IFN-γ responses to PPD-B and showed no significant differences in IFN-γ responses to L5P and PPD-A confirming *M. bovis* infection. With a currently estimated cut-off value (0.013 S/P ratio) of the IFN-γ responses to L5P from the non-infected herd, three cows from the