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Short communication

Interferon-gamma responses in sheep exposed to virulent and attenuated *Brucella melitensis* strains

Marta Pérez-Sancho[a,b], Manuel Durán-Ferrer[c], Teresa García-Seco[a,b], Paula Macías[c,d], Nerea García[a], Irene Martínez[a], Elena Ruiz[c], Emilio Legaz[e], Alberto Diez-Guerrier[b], Sergio González[a], Lucas Domínguez[a,b], Julio Álvarez[a,f,*]

[a] *Centro VISAVET, Universidad Complutense de Madrid, Avenida Puerta de Hierro s/n, 28040 Madrid, Spain*
[b] *Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Avenida Puerta de Hierro s/n, 28040 Madrid, Spain*
[c] *Laboratorio Central de Sanidad Animal, Ministerio de Agricultura, Alimentación y Medio Ambiente, Camino del Jau s/n, 18320 Santa Fe, Granada, Spain*
[d] *Sede social Tragsa y Tragsatec, C/Maldonado 58, 28006, Madrid, Spain*
[e] *Castellana de Ganaderos Sociedad Cooperativa, C/Europa 16, 28810 Villalbilla, Madrid, Spain*
[f] *Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108, USA*

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ABSTRACT

Antibody detection is the basis of large-scale sheep brucellosis diagnosis because of its sensitivity and specificity. In contrast, information on the cellular mediated immune (CMI) response triggered after *Brucella melitensis* infection, a cornerstone in the protection against this pathogen, is more limited, particularly regarding the effect of the virulence of the infecting strain in the induced CMI reaction. Here, the interferon-gamma (IFN-γ) profiles evoked after exposure by different routes to virulent (H38) and attenuated (Rev.1) *B. melitensis* strains in 14 pregnant sheep and 87 ewe lambs, respectively, were characterized accounting for different host-related factors, and compared with their serological response and with the basal IFN-γ responses observed in 155 animals non exposed to *Brucella*. No significant differences in the IFN-γ response of Rev.1 vaccinated animals depending on the inoculation route was observed, in contrast with their serological results. Response in H38-challenged followed a similar trend although peaked later, and an effect of the abortion on the IFN-γ response was detected. This information could help to understand the interaction bacteria–host that leads to its intracellular survival and could be useful for the design of new diagnostic approaches.

Abbreviations: LPS, lipopolysaccharide; S-LPS, smooth lipopolysaccharide; RBT, Rose Bengal Test; CFT, Complement Fixation Test; FPA, fluorescence polarization assay; FPSR, false positive serological reactions; CMI, cellular-mediated immune; DIVA, differentiating infected from vaccinated animals; CJ, conjunctival; SC, subcutaneous; ID, intradermal; d.p.i., days post-infection; p.i., post-infection; PC, positive control; NC, negative control; OD_{PBS}, OD of whole blood stimulated with PBS; OD_{ag}, OD of whole blood stimulated with S99-antigen; SI, stimulation index; SDTH, skin delayed-type hypersensitivity; d.p.v., days post-vaccination; LTT, lymphocyte transformation test; p.v., post-vaccination; d.p.c., days post-challenge.

* Corresponding author at: Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, 1365 Gortner Avenue, St. Paul, Minnesota 55108, USA. Tel.: +1 612 624 9458; fax: +1 612 625 7755.
E-mail addresses: jalvarez@visavet.ucm.es, jalvarez@umn.edu (J. Álvarez).

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1. Introduction

The facultative intracellular pathogen *Brucella melitensis*, main etiologic agent of ovine brucellosis, induces a cellular and humoral immune response in sheep. The smooth lipopolysaccharide (S-LPS) of outer membrane of some *Brucella* spp. is a major antigenic factor responsible of the serological response evoked by these zoonotic pathogens after infection (Garrido et al., 2001). The S-LPS-induced response is usually strong, early detectable [as early as 14 days post-exposure (Duran-Ferrer et al., 2004; Fensterbank et al., 1985)] and long-lasting [up to 4 years even when animals are exposed to an attenuated *Brucella* strain (Alton, 1967)] depending on the route of infection, bacterial load and strain, age and physiological status of the host among other factors (Alton, 1987; Fensterbank et al., 1982; Garrido et al., 2001). The serological response has been the basis of nearly all available diagnostic techniques [Rose Bengal Test (RBT), Complement Fixation Test (CFT), ELISA, FPA (OIE, 2009)] for ovine brucellosis diagnosis although some drawbacks [such as a lower sensitivity under certain epidemiological situations (latent carriers, chronically infected animals and newborns from infected females) and the possible occurrence of false positive serological reactions (FPSR) due to the antigenic similarity of the LPS of other Gram negative bacteria, especially *Yersinia enterocolitica* O:9 (Corbel, 1985)] have limited its value in the field. In addition, a lack of correlation between the serological responses induced after vaccination and the protection achieved in immunized animals has been observed (Morgan et al., 1966), thus turning serology ineffective to monitor the protection conferred by vaccination.

Brucella proteins play a crucial role in the stimulation of the cell-mediated immune (CMI) response in the host (Oliveira et al., 2002) which is a cornerstone of the protection against *Brucella* infection (Stevens et al., 1992). In the CMI response induced after *Brucella* infection, IFN-γ [produced mainly by $CD4^+$, $CD8^+$ and $CD5^+$ cells (Suraud et al., 2008)] is a crucial Th1 cytokine (Zhan and Cheers, 1993) which may participate in the clearance of *Brucella* apparently during early phases of infection (Pasquali et al., 2001).

Measurement of the cellular mediated immune response is an alternative already in use for bovine tuberculosis diagnosis, leading to an increase of diagnostic sensitivity in infected settings in the European Union [Council Directive 64/432/EEC]. The potential usefulness of IFN-γ determination as an alternative/complementary diagnostic tool for brucellosis has been also evaluated in swine, cattle, and, to a lesser extent, in sheep (Kittelberger et al., 1997; Riber and Jungersen, 2007; Weynants et al., 1995). Although specific IFN-γ production in clinical brucellosis has been demonstrated in experimental challenges (Duran-Ferrer et al., 2004), there is a lack of knowledge on the differential IFN-γ production induced by the exposure to a field versus attenuated strain (Suraud et al., 2008), what could have implications for eventual DIVA diagnosis. Information regarding IFN-γ profile during *Brucella* infection/vaccination in sheep, especially during the clinical phase of the disease (abortion), could be useful to understand the interaction between the bacteria and the host that leads to its intracellular survival and subsequent reproductive failures (abortions/weak born) and to assess the specificity of the detection of this Th1 cytokine and its potential usefulness for diagnostic purposes.

Here, the basal IFN-γ responses observed in non-vaccinated (immunogenically naïve) ewes were determined and compared with those of vaccinated ewes and non-vaccinated animals challenged at approximately 90 days of pregnancy to determine the effect of host (age, physiological status) and pathogen (virulence, dose, route of inoculation) – related factors on the IFN-γ production. In addition, the correlation of this Th1 response with specific antibody responses was also evaluated.

2. Materials and methods

2.1. Animals and experimental design

A total of 155 ewes from three *Assaf* brucellosis-free flocks (flock 1, $n = 54$; flock 2, $n = 52$; flock 3, $n = 49$) located in Madrid, Spain, were included in the study.

In a first experiment, basal specific IFN-γ production was measured in all 155 animals when they were 3–6 months old (day 0). Twenty-five ewes were subsequently sampled 7, 14, 30, 60, 90, 120 and 180 days after the initial sampling to determine specific IFN-γ dynamics with age.

In a second experiment, 87 ewes of the initial group were immunized immediately after the initial sampling with $1–2 \times 10^9$ colony forming units of Rev.1 vaccine strain by different routes [conjunctival (CJ), $n = 30$; subcutaneous (SC), $n = 28$ and intradermal (ID), $n = 29$]. These animals, included in a study designed to measure the usefulness of the intradermal route for Rev.1 vaccination (Pérez-Sancho et al., unpublished data), were subsequently sampled 7, 14, 30, 60, 90, 120 and 180 days after the initial sampling. Sera and heparinized blood samples were collected from every animal in each sampling day.

In a third experiment 14 non-vaccinated ewes of 13–16 months were challenged conjunctivally with 5×10^7 colony forming units of *B. melitensis* strain 53H38 at approximately 90 days of pregnancy. All animals were sampled for whole blood and sera at 7, 14, 21, 28, 35 and 42 days post-infection (p.i.) and a subset where further sampled at days 49 ($n = 10$), 56 ($n = 7$) and 63 ($n = 3$) p.i.

All husbandry practices and animal procedures were authorized by the scientific and animal experiments committee of Complutense University of Madrid and the animal research committee from the Madrid Region (10/549484.9/09).

3. Sample testing

The cell-mediated immune response was assessed by antigen-specific IFN-γ detection as described before (Weynants et al., 1995) with slight modifications. Briefly, whole blood samples were transported to the laboratory within the first 6 h post-collection and divided in two aliquots of 1.5 mL. Samples were cultured overnight at 37 °C in a humidified atmosphere after the addition of 15 μL of a cell suspension of *B. abortus* S-99 prepared as