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Antibody response and antigen-specific gamma-interferon profiles of vaccinated and unvaccinated pregnant sheep experimentally infected with *Brucella melitensis*

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Abstract

It is well known that the immune response in sheep against *Brucella melitensis* is subject to individual variation, depending on diverse factors. It bears asking whether these factors (e.g. clinical disease, active infection, state of previous immunity), when affecting a group, can cause variation in the performance of different diagnostic tests. To clarify some of the circumstances in which this immune response can vary, we examine the immune-response profile of sheep protected against the clinical disease by prior vaccination with strain Rev. 1 in comparison with the profile of unprotected females showing the classical brucellosis symptoms. An experimental infection was provoked at midpregnancy under controlled conditions of both non-vaccinated ($n = 7$) and previously Rev.1-vaccinated ewes ($n = 5$). Their immune response was monitored from 7 to 9 weeks before abortion or normal birth to 30 weeks afterwards. Antibody response was assessed by classical tests (Rose Bengal test, complement fixation test (CFT)) in comparison with other diagnostic tests (indirect ELISA (iELISA), competitive ELISA (cELISA), fluorescence polarization assay (FPA), immunocapture test (ICT)). In addition, the cell-mediated immune response was indirectly evaluated by the in vitro antigen-specific release of gamma-interferon. The antibody levels and antigen-specific gamma-IFN profile of the non-vaccinated ewes having the disease and excreting the pathogen was notably high and differed significantly ($P < 0.05$ or $P < 0.01$) from those of vaccinated ewes that neither contracted brucellosis nor excreted the pathogen. In general, all the tests detect the infection in the non-vaccinated ewes with substantial effectiveness. It can be concluded that the high levels of circulating antibodies and of antigen-specific gamma-IFN are related to active *Brucella* infection. Similarly, the state of protection against the disease, but not necessarily against infection, due to a previous immunization with the Rev. 1 vaccination, appears to be responsible for a low level of detectable immune response. Nevertheless, the design of the study limits conclusions

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to pregnant ewes and cannot be extrapolated to non-pregnant ewes or rams. Likewise, the study provides no information on animals which are carriers of *B. melitensis*.

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1. Introduction

Brucellosis, caused by *Brucella melitensis*, in small ruminants is essentially an acute disease of the pregnant ewes, causing abortion in the later part of pregnancy as the main symptom (Alton, 1985). This zoonosis has great socio-economic impact worldwide, and, despite the control and eradication campaigns implemented in the second half of the 20th century, persists as a major threat to food safety and public health in vast areas of the planet (Corbel, 1989; Acha and Szyfres, 2001; Nicoletti, 2002). Although other species in the genus *Brucella* are also pathogenic for humans, *B. melitensis* infection is unquestionably responsible for the highest number of cases, and often causes the gravest clinical cases (Orduña-Domingo et al., 2001).

In recent years, many efforts have been made to develop reliable diagnostic techniques both for detection of infection in sheep as well as sero-surveillance of areas free of the disease. In this sense, most techniques that have performed well in diagnosing bovine brucellosis (Nielsen et al., 1996a,b; Pouillot et al., 1997; Saegerman et al., 1999; MacMillan and Stack, 2000; Nielsen and Gall, 2001; Godfroid et al., 2002; Nielsen, 2002) have also been evaluated to a greater or lesser degree for diagnosis in sheep and goats (Blasco et al., 1985, 1994a,b; Jiménez De Bagüés et al., 1992; Nannini et al., 1992b; Jacques et al., 1998; Marín et al., 1999; Biancifiori et al., 2000; Durán-Ferrer et al., 2002). Nevertheless, the information available on performance characteristics of the different tests is still limited and at times contradictory, and research is needed to evaluate these diagnostic tools (Garin-Bastuji et al., 1998; Garin-Bastuji and Blasco, 2000; Anon, 2001).

Ovine brucellosis does not usually result in relapses (Alton, 1985, 1990), but *B. melitensis* infection nev-

ertheless tends to become chronic. The pathogen persists in flocks through non-symptomatic carriers in a proportion that has not been well estimated (Alton, 1990). Therefore, the immune response against brucellosis develops temporally, both in the individual as well as in the flock, according to such factors as the infection type (natural or artificial), dose and strain, the state of previous immunity (whether vaccinated with *B. melitensis* strain Rev.1 or not), the gestation stage, the clinical status, and the states of massive excretion or of latent carrier (Alton, 1990; Sutherland and Searson, 1990). If this immune response is subject to individual variation, it bears asking whether the performance of a diagnostic technique applied to a group of animals could also vary according to the higher or lower incidence in this group of these determining factors of the immune response. Such variation could partly explain the occasional difficulty in reconciling information from disparate studies (Blasco et al., 1985, 1994a,b; Jiménez De Bagüés et al., 1992; Nannini et al., 1992b; Jacques et al., 1998; Marín et al., 1999), which, despite using animals with established infection (*Brucella* culture-positive), do not indicate the factor or factors that may have been important in the development of the immune response of these reference animals.

To clarify some of the circumstances in which the immune response against *B. melitensis* can vary, we examine the immune-response profile of sheep protected against the clinical disease by prior vaccination with strain Rev. 1 in comparison with the response profile of unprotected females showing classical brucellosis symptoms. In the context of an experimental infection at midpregnancy under controlled conditions, we evaluated the ability of the different diagnostic serological tests to detect the “active infection”, this term being defined as the bursting of abortion due to *Brucella* and excretion of the pathogen into the environment.

2. Materials and methods

2.1. Experimental design

A total of 12 ewes were infected conjunctivally with *B. melitensis* strain 53H38 (dose of 5×10^7 CFU) between days 73 and 88 of synchronised gestation. All the sheep were from an experimental flock that had been isolated and maintained *Brucella* free prior to the experiment, as verified by regular clinical examinations and laboratory testing. The animals were divided into two groups according to their immune status before infection: (a) group VC, five vaccinated ewes aged 4–5 years and (b) group NV, seven non-vaccinated ewes aged 3–4 years. The ewes of the VC group were vaccinated conjunctivally with the Rev. 1 strain of *B. melitensis* at 2–3 years of age (dose of 4.5×10^8 CFU), 2.5 years before being infected.

2.1.1. Sample collection and bacteriological investigation

Throughout the experiment, samples were periodically and simultaneously taken for the immunological and bacteriological studies. The blood serum was examined for anti-*Brucella* antibodies, and the whole blood was cultured in vitro to study the antigen-specific release of gamma-interferon (gamma-IFN). Samples of whole blood and vaginal swabs were checked for the presence of the pathogen by bacteriological procedures. After either birth or abortion, samples were also analysed from the vagina, milk, and placenta of the ewes, as well as from the stomach content, lung, liver and spleen of the foetuses.

For the isolation of *Brucella* spp., each specimen was seeded in two Petri dishes with Farrell's medium enriched with 5% horse serum, which were incubated 10 days at 37 °C in a humid atmosphere enriched with 5% CO₂. For the blood culture, one Roux flask was used per sample with biphasic medium (solid phase: 2.5% tryptose agar, 5% sterile horse serum; liquid phase: 2.5% tryptose broth, 2% sodium citrate). The flasks were incubated at 37 °C in a humid atmosphere enriched with 5% CO₂ for 30 days. The colonies grown were identified following the classical methodology described by Alton et al. (1988).

2.2. Immunological tests

2.2.1. Rose Bengal test

The antigen suspension was prepared from *Brucella abortus* strain S-99, as in Alton et al. (1988) and MacMillan and Stack (2000). The antigen was standardized according to the OIE International Standard anti-*B. abortus* serum (OIEISS, Veterinary Laboratories Agency, Weybridge). The test was made following two methodologies, which differ essentially in the support used for the reaction and in the incubation temperature: (a) the classical method on a flat glass plate (RBT), in which the mixture of identical volumes of serum and antigen (30 µl) is allowed to react for 4 min at room temperature under rotary agitation (Alton et al., 1988; MacMillan and Stack, 2000); and (b) a method in 96 wells of round-bottomed microtitre plates (RBTm) according to the procedure described previously (Durán-Ferrer et al., 2002). Briefly, a mixture of 25 µl of serum and 25 µl of antigen diluted just before use at 2/3 in an acid buffer (0.94 lactic acid, 0.5 M NaOH, 0.13 M NaCl, 48 mM phenol, pH 3.6) was shaken for 1 min, covered by a glass lid and warmed in an oven to 37 °C for 8 min under rotary agitation. The plates were centrifuged at $20 \times g$ for 5 min before reading.

2.2.2. Complement fixation test (CFT)

The antigen suspension of *B. abortus* strain S-99 was also standardized according to the OIEISS (50% fixation at a 1/200 dilution) in microtitre plates with 96 round-bottomed wells, according to the warm method of Alton et al. (1988). The cut-off was established at 1/4 dilution and 50% fixation.

2.2.3. Immunocapture test (ICT)

This technique of immunocapture (Brucellacapt, Vircell S.L.), was followed as previously described (Durán-Ferrer et al., 2002). Briefly, anti-ovine immunoglobulin was adsorbed onto polystyrene plates with 96 round-bottomed wells. The serum was diluted to a final volume of 50 µl in the wells themselves (final dilutions 1/40 to 1/81,920) using an acid buffer at pH 5.0. Fifty microlitres of the stained antigenic suspension of cells from the 16M strain of *B. melitensis* was added and the plates were then sealed, shaken and incubated for 24 h at 37 °C in a moist chamber. Readings were made, taking the titre of the serum to be the

highest dilution that presented complete absence of sediment. The cut-off was set at a dilution of 1/640.

2.2.4. Indirect ELISA (iELISA)

Polystyrene plates were coated with crude smooth lipopolysaccharide (S-LPS) of the *B. melitensis* strain 16 M (Díaz et al., 1981). The sera were diluted to 1:100 in Tween 20 0.01% phosphate buffer (pH 7.2). The reactivity was measured using rabbit anti-sheep IgG (H+L) horseradish peroxidase as a conjugate (Nordic Immunological Labs), hydrogen peroxide as substrate and 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) as chromogen. The reaction was stopped with 1 mM sodium azide. In all the analysis plates, a negative control serum and a strong positive control serum were included (CFT titre: 1/256). Data from the sera were calculated as a positivity percentage (%P) based of that strongly positive control serum included in each plate. The cut-off point was set at 15% P.

2.2.5. Competitive ELISA (cELISA)

The standard operating procedure, reagents, and biologicals, including positive and negative control sera were provided by the Brucellosis Center Expertise ADRI, Nepean, Ont. The procedure followed was that of Nielsen et al. (1989) and Biancifiori et al. (2000). That is, polystyrene plates were coated with smooth lipopolysaccharide plates of *B. abortus*. The sera analysed were made to compete with a mouse monoclonal antibody (M84) specific for the common (C/Y) epitope of the O-polysaccharide (OPS) portion of the S-LPS antigen. Reactivity of the mouse monoclonal antibody was detected using a goat antibody to mouse IgG, conjugated with horseradish peroxidase (Jackson Labs). Hydrogen peroxide substrate and 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) chromogen were developed for 10 min and the optical density (OD) was measured without using a stopping reagent. The results were expressed as a percentage inhibition (%Inh) against antigen (Inh (%) = $100 - [(OD \text{ test sample} / \text{mean OD of conjugate control}) \times 100]$). The cut-off was set at 27% Inh.

2.2.6. Fluorescence polarization assay (FPA)

This test has been described in detail by Nielsen et al. (1996a). The standard operating procedure, reagents, and biologicals, including positive as well as negative control sera, were provided by the Brucel-

losis Center Expertise ADRI, Nepean, Ont. Briefly, OPS hydrolysed to an average molecular weight of 22 kDa was conjugated with fluorescein isothiocyanate and used as an antigen. Sera were diluted 1:40 for testing. Background activity was measured in each serum using a fluorescence polarization analyser (Sentry FP, Diachemix Corp, WI, USA), and, after incubation for a minimum of 2 min with antigen, a second reading was made to obtain the raw parallel and perpendicular data for each sample. This data was converted to millipolarisation units (mP) by the formula $mP = ((Iv - Ih) / (Iv + Ih)) \times 1000$, where Iv is the intensity of parallel light and Ih the intensity of perpendicular light (McGiven et al., 2003). The cut-off value was set at 86 mP.

2.2.7. Gamma-interferon test (gamma-IFN test)

This test was performed according to the general methodology described previously (Rothel et al., 1990; Weynants et al., 1995) with some variations, incorporating antigens specific to *Brucella*. The blood was collected from the jugular vein, using vacuum 5 ml tubes with lithium heparin as the anti-coagulant. A total of three tubes were taken per animal. The blood was cultured and stimulated in the same tubes (sealed by their own rubber caps), and inoculated, respectively, with a saline solution (B blank, 10 µl per ml of blood), *Brucella* cytoplasmic proteins, also known as brucellin (Jones et al., 1973) [P antigen (Brucellergen, Symbiotics), 10 µl per ml of blood] and a cell suspension of *B. abortus* S-99, prepared according to the method of the complement fixation test antigen (Alton et al., 1988) but concentrated 10 times [C antigen, 10 µl per ml of blood]. The culture was incubated for 22 h (± 2) at 37 °C in a humidified atmosphere. The supernatants were harvested and stored at -80 °C until assayed for g-IFN content. The g-IFN was assayed by using an ELISA kit (Bovigam™, CSL, Animal Health, Australia) that uses specific monoclonal antibodies raised against recombinant bovine g-IFN. These monoclonal antibodies have been shown to cross react extensively with g-IFN of species other than cattle, including sheep (Wood and Jones, 2001). Results of the assay were expressed in stimulation indices (SI) by using the following formula: mean of the optical densities of cultures with antigen divided by the mean of the optical densities of control cultures (Weynants et al.,

1995). For both P and C antigen, a culture was considered to produce a significant level of g-IFN if the SI was equal or greater than 2.0 (set cut-off point). A number of samples showed an abnormally high optical density of control cultures ($OD > 0.200$) and were excluded from the data analysis.

2.3. Statistical treatment

The differences between proportions of positive reactors to the various tests among groups were evaluated using Fisher's exact test. Comparisons between data series were studied using the Wilcoxon Mann–Whitney test. The test combines and ranks the data of test results for a given sampling day from sample 1 (group NV) and sample 2 (group VC) and calculates a statistic on the difference between the sum of the ranks.

3. Results

Clinical and bacteriological findings. During the period immediately following experimental infection (days 14–42 post-infection (pi)) *B. melitensis* was isolated from the non-vaccinated animals (four of seven) by blood cultures. In the non-vaccinated group, abortion served as a specific clinical sign of brucellosis (seven of seven), and the inoculated agent was isolated (seven of seven). By contrast, the parturition in the vaccinated group was normal, and the ewes neither aborted nor excreted the pathogen (abortions: zero of five; excretion: zero of five). These differences among groups in the proportion of animals having the disease and excreting the pathogen proved statistically significant ($P < 0.01$).

B. melitensis was isolated from the foetal samples, and was also detected in vaginal excretions, from the week preceding the abortions to the fourth week afterwards. The greatest frequency of isolation occurred during the 2 weeks after abortion (seven of seven). From all the ewes in this control group that presented no agalactia as a consequence of the abortions, *Brucella* was isolated from the mammary secretion between the first and sixth week after the abortion, the highest frequency of isolation coinciding with the first to the third week (six of seven).

The non-vaccinated sheep aborted between days 27 and 51 post-infection, i.e. 4–7 weeks pi. Normal birth among vaccinated ewes occurred between days 61 and 63 (9 weeks pi).

3.1. Kinetics of antibodies and antigen-specific gamma-IFN profile

The profile of the immune response during the first few months of infection showed greater uniformity in the non-vaccinated sheep (NV group, Table 2) than in those previously vaccinated with Rev. 1, in which individual variation in this response was notable (VC group, Table 1). Nevertheless, the NV group also showed individual variability, greater for the ICT and gamma-IFN test (P antigen) techniques (Table 2). The sheep seroconverted during the first month of infection. From this point the response of the vaccinated sheep began to decline, while that of the non-vaccinated sheep maintained steady levels of antibodies and gamma-IFN (Tables 1 and 2).

The kinetics of antibody levels in the non-vaccinated ewes followed similar response patterns in all the diagnostic tests (Tables 2 and 3). In the NV group, the immune response was clearly detected in all the animals prior to abortion, with minor differences among tests in the antibody peak (Table 3). For the CFT and ICT, this peak was reached between the week preceding and following the abortion, but in the FPA, iELISA and cELISA techniques, between the first and second weeks post-abortion (Table 3). The antigen-specific gamma-IFN profile (Table 3) reached its highest values between the first and second weeks post-abortion (antigen C), or between the second and third weeks (antigen P). Afterwards, antibody titres fell slowly and gradually to the end of the experiment, some 30 weeks after the abortions, and even at this time the antibody concentrations were notably high. The gamma-IFN level fell most rapidly (Table 3).

In the group of vaccinated ewes compared with the NV group, the *Brucella* inoculation induced temporally different antibody kinetics and antigen-specific gamma-IFN profile, with significantly lower levels (Table 3). All the tests registered the peak of the antibodies within the first month pi (Table 1). Afterwards, values gradually declined to the end of the experiment without any significant surge in antibody concentrations at any time during birth or afterwards

Table 1

Antibody response and antigen-specific gamma-IFN profile of *Rev.1-vaccinated-pregnant ewes* (group VC), experimentally infected with *B. melitensis*

Ewe #	Days pi	RBT	RBTm	CFT (titre)	ICT (titre)	cELISA (%Inh)	FPA (mP)	iELISA (%P)	g-IFN-C (SI)	g-IFN-P (SI)
1	0	–ve	–ve	<2 –ve	40 –ve	16 –ve	74 –ve	9 –ve	0.8 –ve	0.8 –ve
1	14	–ve	+ve	8 +ve	640 +ve	34 +ve	96 +ve	90 +ve	35.0 +ve	1.7 –ve
1	28	+ve	–ve	8 +ve	320 –ve	30 +ve	105 +ve	93 +ve	4.1 +ve	1.2 –ve
1	63	–ve	–ve	4 +ve	320 –ve	22 –ve	108 +ve	88 +ve	3.6 +ve	1.2 –ve
1	84	–ve	–ve	4 +ve	320 –ve	15 –ve	102 +ve	68 +ve	1.8 –ve	0.9 –ve
2	0	–ve	–ve	<2 –ve	<40 –ve	0 –ve	79 –ve	17 +ve	1.0 –ve	1.0 –ve
2	14	+ve	+ve	64 +ve	5120 +ve	74 +ve	149 +ve	95 +ve	4.0 +ve	0.8 –ve
2	28	+ve	+ve	64 +ve	5120 +ve	68 +ve	157 +ve	97 +ve	3.2 +ve	2.1 +ve
2	63	–ve	+ve	8 +ve	1280 +ve	42 +ve	128 +ve	94 +ve	16.8 +ve	2.5 +ve
2	84	–ve	+ve	16 +ve	640 +ve	29 +ve	129 +ve	86 +ve	11.1 +ve	2.0 +ve
3	0	–ve	–ve	<2 –ve	40 –ve	21 –ve	81 –ve	11 –ve	1.0 –ve	0.9 –ve
3	14	+ve	+ve	128 +ve	1280 +ve	88 +ve	215 +ve	97 +ve	5.2 +ve	3.5 +ve
3	28	+ve	+ve	16 +ve	1280 +ve	55 +ve	164 +ve	91 +ve	1.2 –ve	1.3 –ve
3	63	–ve	–ve	2 –ve	160 –ve	21 –ve	90 +ve	17 +ve	1.4 –ve	0.6 –ve
3	84	–ve	–ve	<2 –ve	160 –ve	14 –ve	86 +ve	11 –ve	2.7 +ve	1.2 –ve
4	0	–ve	–ve	<2 –ve	<40 –ve	18 –ve	81 –ve	9 –ve	1.1 –ve	1.0 –ve
4	14	–ve	–ve	8 +ve	320 –ve	33 +ve	90 +ve	59 +ve	3.4 +ve	0.9 –ve
4	28	–ve	–ve	4 +ve	160 –ve	21 –ve	92 +ve	47 +ve	4.2 +ve	1.6 –ve
4	63	–ve	–ve	2 –ve	160 –ve	18 –ve	90 +ve	38 +ve	1.3 –ve	0.2 –ve
4	84	–ve	–ve	8 +ve	160 –ve	32 +ve	94 +ve	60 +ve	4.0 +ve	1.3 –ve
5	0	–ve	–ve	<2 –ve	80 –ve	24 –ve	78 –ve	52 +ve	1.2 –ve	1.0 –ve
5	14	+ve	+ve	32 +ve	640 +ve	83 +ve	114 +ve	99 +ve	4.4 +ve	0.6 –ve
5	28	+ve	+ve	32 +ve	320 –ve	10 –ve	108 +ve	106 +ve	6.8 +ve	1.8 –ve
5	63	–ve	–ve	4 +ve	320 –ve	33 +ve	95 +ve	107 +ve	1.7 –ve	0.7 –ve
5	84	–ve	+ve	8 +ve	640 +ve	47 +ve	99 +ve	108 +ve	4.0 +ve	1.5 –ve

Date of parturition: *Ewe # 1*: day 62pi; *Ewe # 2*: day 63pi; *Ewe # 3*: day 61pi; *Ewe # 4*: day 62pi; *Ewe # 5*: day 63pi. g-IFN-C: gamma-IFN test (C antigen); g-IFN-P: gamma-IFN test (P antigen).

(Table 3). In comparison with the control group, the antibody levels detected by CFT, ICT and cELISA were significantly lower (Table 3) from the second week before the births to 30 weeks afterwards (at $P < 0.05$ or $P < 0.01$). The antibody level detected by iELISA and FPA were also lower in the VC group (Table 3), but no significant differences were found until week 7–10 after parturition (at $P < 0.05$ or $P < 0.01$). Finally, the antigen-specific gamma-IFN levels were also lower, with statistical significance ($P < 0.05$ or $P < 0.01$) for most of the tests made between the second week before and 30 weeks after the births (Table 3).

In terms of the proportion of positive ewes, the NV group were negative in the different tests at the time of infection, whereas the group VC showed a residual positive (two of five) to the Rev. 1 vaccine

in the iELISA technique (Tables 1 and 2). All the ewes in the NV group were positive (seven of seven) in all the diagnostic tests, except for the gamma-IFN test (P antigen), before the brucellosis abortion. The RBTm, CFT and gamma-IFN test (C antigen) identified all the infected animals as positive 2 weeks before abortion, but RBT, ICT, FPA, iELISA, and cELISA 1 week before. In addition, the gamma-IFN test (P antigen) did not detect CMI in one of the ewes of this group, and therefore absolute positivity was not reached in the group. With the exception of this gamma-IFN test with P antigen, all the techniques continued to identify all the ewes of the group as positive up to 15 weeks post-abortion, and most of them (RBTm, CFT, ICT, FPA, iELISA, cELISA) until the end of the experiment, some 30 weeks after abortion.

Table 2

Antibody response and specific gamma-IFN profile of non-vaccinated-pregnant ewes (group NV), experimentally infected with *B. melitensis*

Ewe #	Days pi	RBT	RBTm	CFT (titre)	ICT (titre)	cELISA (%Inh)	FPA (mP)	iELISA (%P)	g-IFN-C (SI)	g-IFN-P (SI)
1	0	–ve	–ve	<2 –ve	<40 –ve	0 –ve	75 –ve	8 –ve	1.0 –ve	1.0 –ve
1	14	–ve	+ve	4 +ve	320 –ve	4 –ve	80 –ve	9 –ve	4.1 +ve	1.0 –ve
1	28	+ve	+ve	32 +ve	81920 +ve	56 +ve	93 +ve	54 +ve	0.7 –ve	0.6 –ve
1	63	+ve	+ve	128 +ve	81920 +ve	80 +ve	102 +ve	89 +ve	10.7 +ve	1.7 –ve
1	84	+ve	+ve	128 +ve	81920 +ve	74 +ve	101 +ve	93 +ve	8.1 +ve	1.5 –ve
2	0	–ve	–ve	<2 –ve	<40 –ve	3 –ve	77 –ve	4 –ve	0.7 –ve	0.7 –ve
2	14	–ve	–ve	<2 –ve	160 –ve	7 –ve	88 +ve	10 –ve	6.4 +ve	1.4 –ve
2	28	+ve	+ve	128 +ve	81920 +ve	92 +ve	225 +ve	94 +ve	7.2 +ve	1.7 –ve
2	63	+ve	+ve	256 +ve	81920 +ve	98 +ve	224 +ve	95 +ve	4.6 +ve	1.4 –ve
2	84	+ve	+ve	256 +ve	81920 +ve	99 +ve	245 +ve	81 +ve	26.4 +ve	2.4 +ve
3	0	–ve	–ve	<2 –ve	<40 –ve	3 –ve	80 –ve	9 –ve	1.7 –ve	1.5 –ve
3	14	+ve	+ve	<2 –ve	81920 +ve	13 –ve	83 –ve	21 +ve	33.0 +ve	4.7 +ve
3	28	+ve	+ve	16 +ve	81920 +ve	44 +ve	92 +ve	70 +ve	27.2 +ve	24.4 +ve
3	63	+ve	+ve	64 +ve	1280 +ve	70 +ve	93 +ve	25 +ve	16.5 +ve	16.3 +ve
3	84	+ve	+ve	128 +ve	1280 +ve	91 +ve	140 +ve	70 +ve	28.0 +ve	17.1 +ve
4	0	–ve	–ve	<2 –ve	<40 –ve	3 –ve	76 –ve	11 –ve	1.6 –ve	1.2 –ve
4	14	+ve	+ve	8 +ve	2560 +ve	27 +ve	87 +ve	15 +ve	6.2 +ve	1.1 –ve
4	28	+ve	+ve	64 +ve	81920 +ve	79 +ve	108 +ve	54 +ve	14.9 +ve	8.1 +ve
4	63	+ve	+ve	64 +ve	1280 +ve	78 +ve	119 +ve	42 +ve	27.7 +ve	11.8 +ve
4	84	+ve	+ve	64 +ve	1280 +ve	89 +ve	154 +ve	70 +ve	25.9 +ve	7.3 +ve
5	0	–ve	–ve	<2 –ve	40 –ve	0 –ve	79 –ve	11 –ve	1.5 –ve	1.1 –ve
5	14	–ve	–ve	4 +ve	320 –ve	6 –ve	79 –ve	9 –ve	10.5 +ve	1.4 –ve
5	28	+ve	+ve	64 +ve	81920 +ve	76 +ve	99 +ve	73 +ve	53.8 +ve	23.5 +ve
5	63	+ve	+ve	64 +ve	81920 +ve	85 +ve	97 +ve	74 +ve	43.9 +ve	21.1 +ve
5	84	+ve	+ve	64 +ve	81920 +ve	84 +ve	108 +ve	72 +ve	21.9 +ve	17.6 +ve
6	0	–ve	–ve	<2 –ve	<40 –ve	0 –ve	76 –ve	12 –ve	1.3 –ve	1.2 –ve
6	14	–ve	+ve	8 +ve	640 +ve	12 –ve	91 +ve	13 –ve	8.9 +ve	2.0 +ve
6	28	+ve	+ve	32 +ve	81920 +ve	51 +ve	99 +ve	73 +ve	62.3 +ve	38.4 +ve
6	63	+ve	+ve	32 +ve	81920 +ve	61 +ve	148 +ve	87 +ve	47.9 +ve	18.8 +ve
6	84	+ve	+ve	32 +ve	1280 +ve	60 +ve	172 +ve	92 +ve	21.6 +ve	3.2 +ve
7	0	–ve	–ve	<2 –ve	<40 –ve	7 –ve	78 –ve	10 –ve	1.5 –ve	0.9 –ve
7	14	–ve	+ve	<2 –ve	640 +ve	14 –ve	81 –ve	14 –ve	36.8 +ve	2.1 +ve
7	28	+ve	+ve	64 +ve	81920 +ve	94 +ve	262 +ve	87 +ve	15.0 +ve	2.4 +ve
7	63	+ve	+ve	512 +ve	81920 +ve	99 +ve	284 +ve	95 +ve	39.9 +ve	31.7 +ve
7	84	+ve	+ve	256 +ve	5120 +ve	98 +ve	269 +ve	98 +ve	49.3 +ve	30.3 +ve

Date of abortion: Ewe # 1: day 27pi; Ewe # 2: day 51pi; Ewe # 3: day 43pi; Ewe # 4: day 37pi; Ewe # 5: day 44pi; Ewe # 6: day 39 pi; Ewe # 7: day 32 pi. g-IFN-C: gamma-IFN test (C antigen), g-IFN-P: gamma-IFN test (P antigen).

In the VC group, the proportion of positive sheep was substantially lower. The highest proportion of positives was reached before parturition during the first month after infection (Table 1), when only some techniques (CFT, cELISA, iELISA, FPA, g-IFN-C) detected absolute positivity (five of five) in the group. Afterwards the number of positives gradually declined though more quickly than in the NV group. Never-

theless, these differences in the proportion of positive reactors between the two experimental groups did not have a constant statistical significance, persisting over more or less long periods, except for those of the RB techniques. The RBT modality gave significant differences among groups (at $P < 0.05$ or $P < 0.01$) from the week preceding the births/abortions to the end of the experiment. The RBTm modality marked

Table 3

B. melitensis infection at midpregnancy: immune response of Rev. 1 vaccinated ewes (group VC, normal parturition) vs. non-vaccinated ewes (group NV, showing symptoms of clinical brucellosis)

Weeks	CFT (titre)				NS	ICT (titre)				NS	Competitive ELISA (%Inh)				NS	NS	NS	NS	NS
	Group VC		Group NV			Group VC		Group NV			Group VC		Group NV						
	Med	Range	Med	Range		Med	Range	Med	Range		Med	Range	Med	Range					
-4	8	(2–16)	1	(<2–128)	NS	320	(160–2560)	320	(<40–81920)	NS	43	(22–56)	6	(0–98)	*				
-3	4	(<2–16)	16	(<2–128)	NS	320	(160–2560)	10240	(<40–81920)	NS	41	(21–43)	55	(0–92)	*				
-2	2	(2–16)	64	(4–64)	*	320	(160–1280)	81920	(320–81920)	*	30	(21–36)	76	(4–89)	*				
-1	4	(<2–8)	64	(16–64)	**	320	(160–1280)	81920	(20480–81920)	**	31	(15–39)	79	(40–94)	**				
1	4	(2–8)	32	(16–256)	**	160	(40–1280)	81920	(1280–81920)	**	22	(18–42)	77	(53–98)	**				
2	8	(4–8)	32	(16–256)	**	320	(80–1280)	81920	(1280–81920)	**	27	(20–41)	83	(58–99)	**				
3	16	(<2–16)	64	(32–256)	**	320	(80–1280)	81920	(1280–81920)	**	30	(19–39)	84	(61–99)	**				
4	8	(<2–16)	64	(32–512)	**	320	(160–640)	81920	(1280–81920)	**	29	(14–47)	86	(68–99)	**				
7	8	(<2–16)	128	(32–256)	**	160	(160–640)	2560	(1280–81920)	**	27	(24–48)	89	(61–98)	**				
10	8	(<2–8)	64	(16–128)	**	160	(160–640)	1810	(1280–5120)	**	34	(26–55)	91	(56–97)	**				
15	8	(<2–8)	64	(16–128)	**	320	(160–640)	2560	(1280–5120)	**	29	(23–46)	91	(58–99)	**				
20	4	(<2–8)	64	(32–128)	**	320	(160–640)	1810	(640–5120)	**	25	(21–41)	89	(53–99)	**				
30	4	(<2–8)	32	(32–128)	**	320	(160–640)	1810	(640–5120)	**	24	(22–50)	86	(44–98)	**				

Weeks	FPA (mP)				NS	Indirect ELISA (%P)				NS	Gamma-IFN test (C antigen) (SI)				Gamma-IFN test (P antigen) (SI)				NS	NS	NS	NS
	Group VC		Group NV			Group VC		Group NV			Group VC		Group NV		Group VC		Group NV					
	Med	Range	Med	Range		Med	Range	Med	Range		Med	Range	Med	Range	Med	Range	Med	Range				
-4	107	(91–142)	83	(75–209)	NS	88	(48–105)	9	(8–93)	*	10.3	(8.3–12.3)	8.3	(1.0–33.0)	NS	3.0	(2.6–3.4)	1.4	(1.0–4.7)	NS		
-3	107	(92–138)	94	(80–225)	NS	82	(46–95)	62	(6–94)	NS	Invalid	29.7	(0.9–42.5)	–	Invalid	2.1	(1.1–36.5)	–	–	–		
-2	99	(92–135)	99	(80–210)	NS	84	(36–100)	73	(9–87)	NS	3.1	(2.2–19.3)	29.9	(4.1–62.3)	*	1.1	(0.9–4.4)	16.4	(1.0–38.4)	*		
-1	101	(88–136)	108	(85–262)	NS	69	(22–92)	77	(54–87)	NS	10.6	(1.8–21.3)	14.9	(2.4–30.3)	NS	1.5	(0.5–3.8)	3.9	(0.7–8.1)	NS		
1	95	(90–128)	122	(86–275)	NS	88	(17–107)	80	(65–93)	NS	1.7	(1.3–16.8)	38.6	(4.4–70.3)	*	0.7	(0.2–2.5)	10.3	(1.2–32.9)	**		
2	92	(89–137)	123	(87–281)	NS	79	(21–95)	81	(72–95)	NS	2.6	(1.3–17.3)	35.6	(4.6–72.1)	*	1.2	(0.6–3.3)	13.5	(1.4–33.4)	**		
3	98	(86–126)	110	(93–284)	NS	63	(7–92)	79	(25–94)	NS	4.6	(1.6–11.5)	31.4	(2.5–55.9)	*	1.3	(0.8–2.2)	16.3	(1.3–50.2)	*		
4	99	(86–129)	119	(98–284)	NS	68	(11–108)	83	(42–95)	NS	4.0	(1.8–11.1)	16.8	(3.6–47.0)	*	1.3	(0.9–2.0)	10.9	(1.1–31.7)	*		
7	97	(85–126)	172	(100–269)	*	76	(11–126)	98	(65–105)	NS	1.7	(0.8–3.6)	25.9	(3.1–49.3)	*	0.9	(0.5–1.7)	5.4	(1.0–30.3)	**		
10	98	(83–114)	170	(100–269)	*	65	(9–98)	102	(98–121)	**	5.2	(1.0–7.8)	23.2	(2.7–78.2)	*	1.1	(0.6–1.3)	5.6	(1.0–47.6)	*		
15	98	(84–114)	170	(97–249)	NS	45	(4–93)	103	(51–109)	*	1.4	(0.7–6.6)	8.0	(1.4–33.0)	*	1.2	(0.6–1.4)	4.0	(1.0–26.8)	NS		
20	97	(80–109)	159	(93–235)	*	20	(6–61)	82	(56–120)	*	1.1	(0.9–1.5)	5.2	(1.0–36.7)	NS	1.1	(1.0–1.2)	1.8	(0.9–7.8)	NS		
30	95	(83–109)	158	(101–224)	**	40	(11–76)	82	(45–95)	NS	1.5	(0.6–2.1)	13.0	(2.9–25.6)	**	1.0	(0.5–1.4)	2.0	(1.1–13.4)	**		

Time of infection: *group VC*: week 9 before parturition; *group NV*: between week 7 and 4 before abortion. Weeks: weeks before/after parturition (*group VC*) or abortion (*group NV*); Med: median. Wilcoxon Mann–Whitney test: NS: non significant.

* Significant ($P < 0.05$).

** Significant ($P < 0.01$).

differences ($P < 0.05$) between the two groups around the time of birth (weeks -1 , 1 and 2) and from the 10th week forward (at $P < 0.05$ or $P < 0.01$).

4. Discussion

Whether pregnant or not, an ewe infected with *B. melitensis* shows serum antibodies within the first week post-infection (Alton, 1990), and these are detected massively in 2 weeks (Fensterbank et al., 1982; Nannini et al., 1992a; Verger et al., 1995). The immune response then peaks, and afterwards, depending on the course of the infection, begins to fall more or less rapidly (Alton, 1990). In our study, the non-vaccinated ewes (group NV) showed the first signs of immune response at 2 weeks after inoculation. At 3 weeks, the entire group NV were positive to any of the tests used (data not shown). By contrast, in the previously vaccinated ewes (group VC), the immune response was generally manifested 1 week beforehand (Tables 1 and 2). After reaching this first peak, the response appears to be strongly influenced by the development of the infection (Alton, 1990).

Our experiment reproduces and compares two situations that can arise in certain individuals over the course of natural infection. Different diagnostic techniques were used to study the immune response associated with each of these situations. On the one hand, we monitored vaccinated ewes which gained a certain degree of immunity from the Rev. 1 vaccination and thus underwent normal gestation without detectable pathogen excretion. On the other hand, we studied non-vaccinated ewes suffering active infection—abortion being the main symptom of brucellosis together with massive excretion of the pathogen. The artificial nature of the infection should be taken into account, however, as animals with maximum susceptibility to the pathogen of a reference strain were inoculated under controlled conditions at a dosage capable of infecting 100% of the unprotected animals. Consequently, the experimental results should be viewed with caution. In addition, the experimental design itself limits the conclusions to pregnant ewes and should not be extrapolated to non-pregnant ewes or rams.

In light of the clinical and bacteriological results, the non-vaccinated ewes should be considered a refer-

ence group for the interpretation of the immunological results, as only in these was the disease and infection demonstrated by isolation of the pathogen. According to current knowledge of brucellosis pathogenicity (Alton, 1990; Crespo-León, 1994), despite the absence of brucellosis symptoms or pathogen isolation in ewes vaccinated with Rev. 1, the possibility that these ewes might be carriers cannot be ruled out.

The results show that a non-vaccinated ewe in the period of maximum susceptibility, if infected with a massive dose of *B. melitensis*, can develop an immune response detectable by any of the serological tests used, and by the gamma-IFN test, if an appropriate antigen is used (C antigen).

This response was detected unequivocally before any sign of abortion or bacteriological excretion up to 15–30 weeks after the onset of the disease (depending on the diagnostic test used), and therefore a long time after the excretion period of the pathogen. The titres of antibodies and the antigen-specific gamma-IFN response become significantly high and persist at such levels over time. On the contrary, if the ewe is protected from the clinical disease by prior immunization with the Rev. 1 vaccine (but not necessarily from infection), even under the same environmental conditions the immune response is subject to strong individual variation and may not always be detectable by some techniques in certain animals. Moreover, the concentration of antibodies and of antigen-specific gamma-IFN was significantly low (at $P < 0.05$ or $P < 0.01$). Only some tests (iELISA, FPA) were capable of consistently detecting the immune of most these vaccinated-protected animals.

Consequently, it can be concluded that the high levels of antibodies and of antigen-specific gamma-interferon are strongly related to active *Brucella* infection, in which the pathogen is massively excreted. Similarly, the state of protection against the disease, but not necessarily against the infection, provided by the Rev. 1 vaccine (Alton, 1990), appears to determine the low level of response, except for some techniques (iELISA and FPA). This notable difference in levels of antibodies and of antigen-specific gamma-interferon must determine the diagnostic performance of the different techniques, and this was reflected, though without the necessary statistical support, by the results showing the evolution of the proportion of positive animals in vaccinated and un-

vaccinated sheep. In general, the response profile for the VC group demonstrates the difficulty in finding a reliable test to show the exposure to *B. melitensis* after vaccination with Rev. 1, although the techniques with greatest sensitivity for diagnosing brucellosis in ruminants, such as iELISA and FPA (Garin-Bastuji et al., 1998; Garin-Bastuji and Blasco, 2000; MacMillan and Stack, 2000), can detect a stronger immune response.

In the course of infection by intracellular bacteria, including *Brucella*, the level of circulating antibodies is not necessarily related to the resistance capacity of the host. However, a major role of g-IFN is recognized in cell-based protective immunity (Nicoletti and Winter, 1990; Tizard, 2000). It would thus be reasonable to expect that the degree of protection against *Brucella* infection manifested by the VC group would be accompanied by a high level of g-IFN by in vitro stimulation. However, we have unveiled a radically opposing panorama which suggests a lack of correlation between this in vitro technique and the immunity demonstrated in the animals. The same conclusion was propounded by Chukwu (1987, in Nicoletti and Winter, 1990) who also detected a low CMI detected by another in vitro technique, the lymphocyte blastogenesis assay, in cows that had been vaccinated with the B-19 strain of *B. abortus* and that proved to be protected against infection. Nicoletti and Winter (1990) proposed that the magnitude and duration of detectable immune responses should decrease in immune animals which eliminate infection sooner and, therefore, remove the antigenic stimulus for a high and protracted response.

One of the controversial points concerning the immunological diagnosis of *B. melitensis* infection in small ruminants is related to which *Brucella* species and biovars are used in the production of the diagnostic antigens (Garin-Bastuji et al., 1998). The battery of tests studied in the present work are based on different *Brucella* strains used as antigens (*B. abortus* or *B. melitensis*). This approach is consistent with the information currently available on the adequate sensitivity of the antigens made from A-dominant strains (*B. abortus* biovar 1) to detect infection by M-dominant strains (*B. melitensis* biovar 1) and vice versa, as all the strains of smooth *Brucella* species share a common epitope C/Y (Douglas and Palmer, 1988; Díaz-Aparicio et al., 1993; Blasco et al., 1994a; Garin-Bastuji et al., 1998; Jacques et al., 1998).

It is noteworthy in our experiment that the Rose Bengal methods lacked effectiveness in detecting the immune response to *B. melitensis* of the VC group. This finding is supported by Fensterbank et al. (1982), who described the absence of Rose Bengal antibodies in the immune response to *B. melitensis* of previously Rev. 1 vaccinated ewes.

In all the situations considered in the present study, the RB microtitre plate procedure (RBTm) gave a pattern similar to the RB classical plate method (RBT) in detecting infection. The practical limitations of the classical method have been repeatedly pointed out (Farina, 1985; MacMillan, 1990; Garin-Bastuji et al., 1998; Anon, 2001) as it is markedly affected by incubation temperature (hard to control in some laboratories offering routine diagnoses) and lacks repeatability in the sera of animals with low levels of antibodies. In addition, it is an unviable method for analysing haemolysed sera. The microtitre plate (Durán-Ferrer et al., 2002) procedure partly exceeds the limitations of the previous methodology, as it enables effective oven control of the incubation temperature, favours automation of the procedure, reduces subjectivity in interpreting the results, and makes it possible to increase the number of samples analysed in a given time.

The CFT has been traditionally considered the reference test for *Brucella* infection in sheep, and thus has been used to confirm the diagnosis, especially in flocks vaccinated with Rev.1 (Alton, 1990; Garin-Bastuji et al., 1998; Garin-Bastuji and Blasco, 2000). However, there are several practical drawbacks in its laboratory performance, and it is not applicable to some sera with anti-complementary activity (Alton et al., 1988; Alton, 1990; MacMillan, 1990; Sutherland and Searson, 1990). This underscores the need for new techniques that facilitate the diagnostic processes. In the present work, the CFT, cELISA, and ICT showed similar behaviour in non-vaccinated sheep with active infection. Nevertheless, the ICT marked the absence of active infection more clearly in the group of vaccinated sheep, as discussed more fully in a previous work (Durán-Ferrer et al., 2002).

The immune response detected in non-vaccinated sheep by iELISA and FPA was similar to that of the other methods studied, but demonstrated greater capability of revealing antibodies in vaccinated sheep protected against the clinical disease. Consequently, with these techniques, a vaccinated animal reacting sero-

logically in an infected environment would be less effectively distinguished from a non-vaccinated one with active infection. This shortcoming becomes important from an epidemiological viewpoint (Blasco, 2002; Durán-Ferrer et al., 2002).

Diagnostic tests based on CMI may be useful when serological ones are not valid, especially at the onset of the infection or in the chronic infection phase (Fensterbank, 1985; Alton, 1990; Nicoletti and Winter, 1990; Sutherland and Searson, 1990), but above all in suspicious cases of cross reactions with anti-S-LPS antibodies from other gram-negative bacteria, particularly *Yersinia enterocolitica* O:9 (Corbel, 1985; Pouillot et al., 1997; Godfroid et al., 2002).

In sheep, the usefulness of detection by the delayed-type hypersensitivity reaction (skin test) has been pointed out for diagnosing brucellosis, although the test gives cross reactions in animals infected by *B. ovis* (Alton, 1990; Garin-Bastuji and Blasco, 2000) and cannot be applied to animals vaccinated with Rev. 1 because of the frequent errors regarding specificity (Bercovich, 1985; Fensterbank, 1985; Blasco et al., 1994b). This method also has disadvantages related to the being an in vivo test, since the animals have to be handled twice and the results are not available for 2–3 days (Blasco et al., 1994b; Weynants et al., 1995). Furthermore, the inoculation of allergen induces an anergic state in the animal during which the test cannot be repeated (Blasco et al., 1994b).

Although other serological diagnostic techniques, such as the indirect ELISA based on detecting antibodies against cytosolic proteins (Debbarh et al., 1996a,b; Letesson et al., 1997; Cloeckeaert et al., 2001), could resolve the problem of detecting cross reactions without raising problems inherent in an in vivo test such as the skin test, the gamma-IFN technique could provide the immunological investigation with the specific information concerning the cell-mediated immunity (CMI).

The gamma-IFN test has shown a good correlation with the skin test (Rothel et al., 1990; Weynants et al., 1995), and could enable a simultaneous assessment of the immune response against different bacteria that under certain circumstances could provoke problems of diagnostic specificity (Weynants et al., 1995; Kittelberger et al., 1997). Our experiment provides the first results for evaluating the gamma-IFN test as a diagnostic technique of sheep brucellosis, and it has

demonstrated a notable parallelism between, on the one hand, the detectable CMI against active infection evidenced by this in vitro technique and, on the other, the antibody response (NV group).

Nevertheless, the response profile found did vary substantially according to the antigen used in the stimulation, being notably stronger when a complete cell antigen (C antigen) was used than when cytosolic proteins (P antigen) was used. Also noteworthy was the almost absence of a detectable cell immune response against the cytosolic proteins in the vaccinated animals not suffering the disease. Thus, in agreement with what is known concerning antibody response (Garin-Bastuji et al., 1998), the data imply an immuno-dominance of the antigens of the cell wall of *Brucella* in the detectable CMI, as well as greater individual variability in the response against the cytosolic proteins.

This test showed good overall sensitivity in detecting group infection over time in the non-vaccinated animals (complete cellular antigen), combined with good overall specificity in the vaccinated group that repelled the infection (antigen of cytosol proteins). Until the necessary studies are performed to evaluate its sensitivity and specificity for individual diagnosis, this test could offer advantages for detecting *Brucella* infection at the flock level as a complement to inconclusive serology results.

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