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Characterization of the Transitory Immune Response in Cows Immunized with RB51 and its Implication on Diagnosis Within Brucellosis Endemic Zones

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Abstract: A dairy herd with 17% Brucellosis prevalence was vaccinated or re-vaccinated with RB51. Five groups of ten cows each were classified according to their response to serologic tests. Additionally, one group with infected animals and one with animals originating from a Brucellosis free herd were used as positive and negative controls. A Radial Immunodiffusion test (RID) was used to confirm results. Indirect ELISA was carried out with LPS of *B. abortus* 2308 (LPS-S) or LPS of *B. abortus* RB51 (LPS-R) as primary antigens and anti-IgG1, IgG2, IgM and IgA as secondary antigens. When LPS-S was used as antigen, vaccinated and re-vaccinated cows that were positive to card and Rivanol tests, showed an IgG1-type response. Animals that were infected mainly showed an IgG2 type response. When LPS-R was used, none of the animals showed a significant response. Immunized cows that had contact with the wild-type strain showed humoral immune response that was detected as positive serology tests. Therefore, in endemic zones confirmation tests such as RID, Rivanol with titers above 1:100, or ELISA with LPS-S as antigen and IgG2, are essential to differentiate between animals vaccinated with RB51, which have a transitory immune response, from those that are infected.

Key words: *Brucella abortus*, Brucellosis, cows, RB51, reduced dose

INTRODUCTION

Brucella abortus is a pathogen that survives within macrophages in order to avoid a hostile environment. During the course of infection with *B. abortus* the bacteria remains within cells, thus, these are responsible for destroying the pathogen with their own tools or are destroyed so that other mechanisms can have access to *Brucella* and eliminate it, such as antibodies (Golding *et al*., 2001). In this context, cellular immune response is the key to host defense against this intracellular bacterium (Zhan *et al*., 1995). Due to is location on the cell surface, the lipopolysaccharide of *Brucella* is immunodominant, therefore IgM, IgG1, IgG2 and IgA type agglutination antibodies are generated against this antigen. Humoral response is provided by type-B lymphocytes, which have been previously stimulated by the antigen and transformed into memory cells for protective response in case of a new infection. Exposure to an antigen and production of cytokines allow B-type memory cells to produce different types of antibodies (MacMillan, 1990).

Strain RB51 of *B. abortus* is an attenuated, rough vaccine derived from the virulent strain 2308. The *wbo*A gene is interrupted by insertion of the IS711 element, this vaccine strain is incapable of producing the O polysaccharide resulting in a rough phenotype (Cheville, 2000; Vemulapalli *et al*., 2000). The lack of O-chain allows the use of this vaccine without production of antibodies that

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interfere with the serological diagnosis of the disease, therefore any animal that results positive to official tests is diagnosed as infected (Uza *et al*., 2000).

Nevertheless, this statement is valid only in circumstances present in countries that have low disease prevalence, as is the case of the United States of America, where Brucellosis has been practically eradicated and incidence is very low. Mexico is a contrasting case, where the disease is endemic with high prevalence. It has been observed that bovines without infection, either vaccinated or re-vaccinated with strain RB51, can be positive to serological tests several months after immunization causing confusion in diagnosis (Cantú *et al*., 2007; Herrera *et al*., 2007). The transitory post-vaccination response that is shown in this study is triggered by contact with wild-type strains after the primary response against *B. abortus* originating from vaccination with RB51 and animals develop a secondary response that can last for several months. The presence of these antibodies demonstrates the fact that the animals had contact with the wild-type strain.

MATERIALS AND METHODS

The objective of this study was to understand the type of antibodies present in cows vaccinated with RB51 strain, that live in Brucellosis endemic zones and showed a transitory immune response.

Animals

Work was carried out in a farm located in a Mexican dairy zone where Brucellosis is endemic with 17% prevalence. Serum samples were collected from 250 Holstein friesian cows that were seronegative to Brucellosis at the time of vaccination or re-vaccination with a reduced dose of RB51.

With conventional card and Rivanol serologic tests a group of cows (n = 50) was found to be positive to the card test and either negative to Rivanol or positive with 1:25 or 1:50 titers. Bacteriological analysis was carried out with samples from these cows and resulted negative.

Cows in the negative control group came from a herd negative to Brucellosis with 210 animals that had been followed with serological and bacteriological studies for the past six years.

The animals from the positive control group came from a herd with 500 individuals where Brucellosis had an endemic status confirmed by serological and bacteriological studies.

Strains

Strains RB51 and 2308 of *B. abortus* were used for LPS extraction, they were grown in Trypticase Soy Broth (TSB) (Difco Laboratories, Detroit, Mich. USA) for 48 h at 37°C. Vaccination and re-vaccination of all cattle was carried out with commercial presentations of strain RB51 at normal and reduced dose, respectively (Productora Nacional de Biológicos Veterinarios, Mexico).

Groups

Sera of vaccinated and re-vaccinated cows were collected two months after immunization and the groups (n = 10) were constituted as indicated in Table 1.

Antigens

The process for extracting LPS from smooth (S) and rough (R) *B. abortus* has been thoroughly described by Moreno *et al*. (1984).

The native hapten (NH) was obtained from strain 2308 of *B. abortus* through aqueous extraction and subsequent ethanol precipitation (Diaz *et al*., 1981).

Serological Tests

Card and Rivanol tests were carried out with standard protocols (Alton, 1988). Radial Immunodifussion (RID) was carried out in 0.8% agarose (Difco Laboratories, USA) with 50 μg mL^{-1} NH, 10% NaCl and glycine at 0.1 M (pH 7.8).

Table 1: Groups of serums from vaccinated and re-vaccinated cows in a dairy herd located in a Brucellosis endemic zone. Negative animals come from a Brucellosis free farm and positive controls from a dairy herd located within an endemic zone of the disease

	Group						
	Vaccinated cows			Re-vaccinated cows			
Test	1	2	3	4	5	Positive control	Negative control
Card	-	+	+	+	+	+	-
Rivanol	-	-	+○	-	+	+ *	-
Isolation of *B. abortus*	-	-	-	-	-	+ §	-

○ In order to improve the analysis, group 3 was divided into two subgroups: Group 3-A with five cows that showed Rivanol test 1:50 titers; Group 3-B with five cows that showed Rivanol test 1:100 titers or more, but were all negative to RID, *1:400 titers, §: *B. abortus* biotype 1 wild strain

Indirect ELISA (ELISA-I) was carried out using LPS-S from *B. abortus* 2308 and LPS-R from RB51 as capture antigens. Serums to be tested were diluted 1:200 in PBS; polyclonal anti-cow IgG1, IgG2, IgM and IgA antibodies specific against heavy chain produced in sheep (Bethyl Laboratories, Texas USA) were used as secondary antibodies. 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) was used as substrate (Difco). Optic density was measured at 405 nm after 15 min in a spectrophotometer for ELISA (Sigma diagnostics). Absorbance values were stated as optic density units (Tabatabai and Deyoe, 1984) and the assays were carried out independently three times.

Bacteriological Study

Milk and vaginal exudates samples were inoculated twice in TSA and Farrell media (Farrell, 1974), incubated for 10 days with 10% CO_2 at 37°C. Identification of isolated strains was performed using standard protocols (Alton, 1988).

Statistical Analysis

Whenever analysis of variance tests showed significant statistical differences between groups, multiple comparisons among groups were carried out at a 0.05 significance level.

RESULTS

The type of antibodies present in cows vaccinated with RB51 strain, that live in Brucellosis endemic zones and showed a transitory immune response, presented the following pattern: Cows from groups one and two had similar levels among them for all isotypes with the I-ELISA test carried out with LPS-S as antigen. When these groups were compared with the negative control group no significant statistical difference was found ($p \geq 0.05$).

Nevertheless, cows from group 3 had similar levels than the negative control ($p \geq 0.05$) in isotypes IgG2, IgA and IgM although isotype IgG1 had higher levels with a larger standard deviation. In order to better analyze these data, this group of animals was divided into two: group 3-A, which included animals that were positive to Rivanol with 1:50 titers and group 3-B, which included animals that were positive to Rivanol with 1:100 titers or higher, but were all negative to RID.

Subgroup 3-A showed similar IgG1 levels to the negative control group ($p \geq 0.05$); while IgG1 levels of subgroup 3-B were statistically different from those of the negative control group and subgroup 3-A ($p \leq 0.05$). Nevertheless, the results for isotypes IgG2, IgA and IgM were similar to those of the negative control group ($p \geq 0.05$).

Cows that were revaccinated in group 4 were positive to the card test with a transitory serological response, but were negative to Rivanol and RID tests. Furthermore they were not statistically different ($p \geq 0.05$) from the negative control group in terms of IgG1, IgA and IgM. Although differences could be observed in IgG2 titers ($p \leq 0.05$) the observed response was less than that of infected cows.

Brucella was not isolated in the bacteriological analysis of groups one through five as well as the negative control group. The smooth biotype 1 wild-type strain of *B. abortus* was isolated in all animals of the positive control group.

The RID test was negative in all cows of the negative control group and all animals of groups one through five indicating that there was no infection present. All animals in the positive control group were positive to the RID, card and Rivanol (1:400 titers) tests.

Cows from group 5 which were positive to the Rivanol test with 1:50 or 1:100 titers but negative to RID had a response to IgG2, IgA and IgM statistically similar to group 4 ($p \geq 0.05$) while response to IgG1 was higher and statistically different ($p \leq 0.05$).

The positive control group was positive to all tests: bacteriology, card, Rivanol (1:400 titers) and RID and in ELISA they had high IgG1, IgG2 and IgM titers, but low levels of IgA. Levels of IgG2 in this group were statistically different when compared to those of vaccinated or revaccinated groups ($p \leq 0.05$).

None of the groups, including positive control group animals, showed a statistical difference ($p \geq 0.05$) in the I-ELISA using LPS-R, when compared to the negative control group.

DISCUSSION

In order to explain the transitory post-vaccination response determined in this study, it is suggested that stimulation with RB51 strain causes a primary immune response against *B. abortus*; afterwards, due to widespread presence of field strains in endemic zones, cows develop a secondary response due to infection. Presence of these antibodies demonstrates that animals had contact with field strains, but their immune response was able to clear the infection. In this study we demonstrate that cows that show a temporary reaction to agglutination tests are not infected due to the fact that RID tests and bacteriological isolations are negative.

In another study, using 35 cows that had been followed with serological tests from three months of age and had never shown evidence of *Brucella* infection, when vaccinated with RB51 strain presented at the onset of pregnancy a transitory positive response to the card test. All animals went on to have normal deliveries and antibodies disappeared in all but 2 cows, in which infection with *B. abortus* biotype 1 was confirmed (Leal-Hernandez *et al*., 2005). Using ELISA tests in this study it was possible to quantify the different isotypes present in serum samples by using two types of antigens: LPS-R to detect immune response in cattle immunized with RB51 and LPS-S to detect immune response either as a transitory response or against wild-type smooth strains. It is well known that a primary antibody response results from an initial stimulation of the immune system, while a secondary response involves stimulation of B-type cells and the expansion of clones (Abbas *et al*., 1999). The dominant antibody class in a primary response is IgM as these are the surface antibodies that are expressed in dormant cells. By contrast, in a secondary immune response other immunoglobulin isotypes, such as IgG, IgA and IgE are increased. IgG1 titers in sera of animals vaccinated with strain S19, are up to ten times higher than those of IgG2 (Butler *et al*., 1986).

Immune response induced by vaccination with strain RB51 is mainly cellular. A Th1 type response, as well as early interferon gamma production has an important role in protection, while antibody production is less important in terms of protection conferred against Brucellosis (Stevens and Olsen, 1996; Stevens *et al*., 1994, 1995).

After an infection with wild-type *B. abortus* it is known that constant concentrations of IgM are produced from the first to the third week postinfection which can be identified by agglutination tests. Later, IgG becomes predominant, with IgG1 subclass being more abundant, with agglutination titers lasting up to 10 months. IgM titers decrease gradually while IgG and IgA titers remain high (MacMillan, 1990; Stevens *et al*., 1995).

In the humoral response against S19 vaccine, IgM titers persist longer than in a wild-type infection, while production of IgG decreases until it becomes imperceptible with conventional diagnostic tests around eight months post-vaccination (MacMillan, 1990).

RB51 vaccine does not induce antibodies against LPS-S that are detectable with official serologic tests. Due to this fact any animal that shows a positive reaction to conventional tests is considered to be infected. Nevertheless, Stevens and Olsen (1996) showed that cows that are vaccinated with strain RB51 and later infected with *B. abortus* strain 2308 produce small quantities of IgG; although no IgM levels are detectable. If these same cows are inoculated with purified LPS-S they do not produce IgG or IgM.

Olsen *et al.* (1996) observed that cattle vaccinated with RB51 do not produce antibodies against the LPS from strain 2308, although they do present non-agglutinating IgG that reacts with strain 2308 and its 20 to 84 kDa proteins. On the other hand, Cloeckaert *et al.* (2002) observed that strain RB51 produced low antigen levels similar to the M antigen. Subsequent application of RB51 vaccine could allow the possibility that some animals respond to the O-chain and be detected by conventional or sensitive tests such as ELISA.

The antibody class and subclass patterns are influenced by the type of Th cells present in the T-B cell interaction and particularly by cytokines that are secreted (Brown and Estes, 1997; Brown *et al.*, 1999; Estes *et al.*, 1994, 1995; Golding *et al.*, 2001; Jiang and Baldwin, 1993). Furthermore, IgG1 response is a functional characteristic of Th2-type response in cattle which is specifically induced by intracellular bacteria (Brown and Estes, 1997; Brown *et al.*, 1999; Estes *etal.*, 1994, 1995; Jiang and Baldwin, 1993). Interferon Gamma, a Th1-type cytokine, plays an important role in activating macrophages, as well as in controlling infection by *Brucella*, both *in vitro* and *in vivo* (Jiang and Baldwin, 1993).

In field conditions with high prevalence (39%) and low prevalence (2%), Lord *et al.* (1998) vaccinated cows with RB51 either once or twice with 5×10^9 CFU and then vaccinated with S19 strain. They found that all cows vaccinated with RB51 were negative to conventional serological tests, even in the presence of natural challenges with field strains. These field results obtained by vaccinating with RB51 by Lord *et al.* (1998) are difficult to analyze and compare with studies carried out in controlled conditions (Bagnat and Maneti, 2002; Cheville *et al.*, 1993; Olsen, 2000).

In a controlled scheme, Moriyon *et al.* (2004) observed that in this type of experiments protection provided by strain RB51 is useful under a moderate challenge, but when it increases, RB51 is inefficient at preventing infection. These same authors state that the experiment by Lord *et al.* (1998) has some errors when selecting animals that are free of *Brucella*. The animals were selected based on negative serology from herds that have been infected and not from herds that are free of Brucellosis. This method does not take into account that some young individuals can acquire latent infections that do not show in serological tests, therefore introducing bias in the selection of vaccinated groups.

Furthermore, it is important to mention that none of the animals vaccinated with strain RB51 were positive to serological tests even though they had come into contact with the wild-type strain (Moriyon *et al.*, 2004). In other controlled experiments, animals that had been vaccinated with RB51 strain and were thus protected, developed titers against *Brucella* after being challenged with virulent strain 2308 with constant or decreasing titers until parturition. Field studies are inherently less reliable and their results need to be taken with caution, especially when they openly contradict those obtained from controlled studies (Moriyon *et al.*, 2004).

During the invasion and phagocytosis process, *Brucella* triggers an immune response and, some time later, these organisms are released from infected phagocytic cells. In the initial stages of this process, bacterium faces high concentrations of IgM and low concentrations of low IgG antibodies. This activity results in the destruction of bacteria through the action of complement and the infection would remain restricted to resident phagocytic cells (Hoffmann and Houle, 1995). In the present study,

the group of infected cows had high IgG2 titers, which, according to previous studies, is correlated with infection (Lawman *et al*., 1986). This is confirmed when comparative serological tests are carried out between cattle that are carriers of *Salmonella*, those that are vaccinated and those with acute infection. By using ELISA tests with LPS-S as antigen, it has been shown that carrier individuals have high quantities of IgG2 (House *et al*., 2001).

On the other hand, high levels of IgM were detected in all groups, both by ELISA with LPS-S and with LPS-R, suggesting that the specificity of ELISA tests for Brucellosis increases when IgM is not detected (Wright and Nielsen, 1990). It is possible that this is due to IgM that is produced as a response to invariant antigens and can compete with IgG by binding to antigenic sites. IgM has great affinity for these binding sites in effect blocking IgG from reaching them and causing the subsequent reduction in sensitivity of ELISA tests to detect IgG (Reid and Copeman, 2002). The low amount of IgA in serum is normal due to the fact that this immunoglobulin is produced in mucosal lymphoid tissue (Abbas *et al*., 1999).

RID tests have a 98% sensitivity to detect infected cattle and 100% specificity in heifers two months after having been immunized with S19 strain (Diaz *et al*., 1979). In order to stimulate antibody production from infectious processes, which are capable of precipitation in the presence of HN, a prolonged stimulation is required by wild strains of *Brucella* but not from vaccination strains (Diaz *et al*., 1984). This raises the possibility of using RID tests to differentiate between infected cows and individuals that only came into contact with *Brucella* but did not develop the disease. In heifers vaccinated with strain S19, tests such as card and Rivanol have 95% specificity at 270 days post-vaccination, while RID has 100% specificity (Aparicio *et al*., 2003).

Bustamante *et al*. (2000) found that even after 32 months post-vaccination with strain S19 animals were positive to Rivanol and card tests, but tested negative with RID. In cows that are within Brucellosis endemic zones and that have been repeatedly vaccinated with strain S19, RID tests with HN as antigen are the only ones capable of differentiating between vaccinated and infected animals (González *et al*., 2006).

In conclusion it may be said that animals in Brucellosis endemic zones, can come into contact with a *Brucella abortus* wild-type strain, either before or after vaccination with RB51 strain, inducing antibodies that produce positive results in official serology diagnostic tests. We consider that diagnostic confirmation tests in endemic zones continue to be essential to differentiate between infected and vaccinated animals. In light of this, we determine that tests such as RID, Rivanol with 1:100 titers or higher and I-ELISA with LPS-S as capture antigen and IgG2 as secondary antigen, are good diagnostic confirmation tests.

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