Brucella abortus RB51 Induces Protection in Mice Orally Infected with the Virulent Strain *B. abortus* 2308

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Brucellae are gram-negative, facultative intracellular bacteria which are one of the most common causes of abortion in animals. In addition, they are the source of a severe zoonosis. In this trial, we evaluated the effect of oral inoculation of *Brucella abortus* RB51 in mice against a challenge infection with *B. abortus* 2308. First, we showed that a gastric acid neutralization prior to the oral inoculation contributed to a more homogeneous and consistent infection with both vaccine strain *B. abortus* RB51 and virulent strain *B. abortus* 2308. Successively, we assessed the clearance and the immune response following an oral infection with *B. abortus* RB51. Oral inoculation gave a mild infection which was cleared 42 days after infection, and it induced a delayed humoral and cell-mediated immune response. Finally, we immunized mice by oral inoculation with *B. abortus* RB51, and we challenged them with the virulent strain *B. abortus* 2308 by an oral or intraperitoneal route 42 days after vaccination. Oral inoculation of *B. abortus* RB51 was able to give protection to mice infected with the virulent strain *B. abortus* 2308 by the oral route but not to mice infected intraperitoneally. Our results indicate that oral inoculation of mice with *B. abortus* RB51 is able to give a protective immunity against an oral infection with virulent strains, and this protection seems to rely on an immune response at the mucosal level.

Brucellae are gram-negative, facultative intracellular bacteria which are one of the most common causes of abortion in animals. In addition, they are the source of a severe zoonosis, which is characterized in human beings by a multitude of somatic complaints, including fever, sweats, anorexia, fatigue, malaise, weight loss, and depression (15). Immune response to *Brucella* spp. have been studied mainly with mice. In this animal model, protective immunity seems to be mediated by both humoral and cellular immune responses (2). In particular, in vivo studies indicate that an immune response mediated by both CD4$^+$ and CD8$^+$ T lymphocytes is important in controlling infection. Within these subsets, gamma interferon-producing CD4$^+$ T cells play a prominent role in recovery from infection (23). Vaccination against *Brucella* infections in animals is usually performed by parenteral administration of live attenuated smooth *Brucella abortus* S19. This vaccine is effective in conferring protection against virulent strains of *B. abortus*, but it also has a number of disadvantages, including causing abortion in pregnant animals, being pathogenic for humans, and inducing a humoral response which is similar to the response after natural infection, thus hampering procedures for the detection of infected animals by serological testing. In particular, the similarity in humoral responses is of great importance in field conditions because it is impossible, with *B. abortus* as the antigen, to serologically discriminate between vaccinated and infected animals (11). Recently, a stable, lipopolysaccharide O-antigen-deficient mutant of virulent *B. abortus* 2308 was produced (16). This strain is rough, rifampin-resistant, and is pathogenicity attenuated. This variant, designated *B. abortus* RB51 is currently being used in many countries as an alternative vaccine to *B. abortus* S19 because it

does not induce antibodies to *Brucella* lipopolysaccharide O-antigens (17–21). At the same time, *B. abortus* RB51 retains the ability to confer protection against infection with pathogenic strains (5, 7, 10, 12). *B. abortus* RB51 can persist for a short period of time in the spleens of mice vaccinated by intraperitoneal injection. Vaccination induces an antibody response, which is detectable 4 weeks after vaccination, and both Th1- and Th2-cell-mediated responses (13, 14, 22). Parenteral vaccination is a practical approach for domesticated animals, but it is almost impossible for free-roaming herds in some areas of the world. In these circumstances, an oral inoculation would be a valuable tool for the immunization of large numbers of animals (4). In this study, we analyzed the course of an oral immunization of mice with *B. abortus* RB51 and its effect on the onset of protection against a challenge infection by different routes.

MATERIALS AND METHODS

***Brucella* spp. cultures.** Suspensions of *B. abortus* RB51 and *B. abortus* 2308 were used. *B. abortus* RB51 was maintained by monthly subculture on rifampin (50 µg/ml)-tryptose agar. *B. abortus* 2308 was maintained by monthly subculture on tryptose agar. Bacteria were periodically renewed from freeze-dried stock to maintain a constant level of activity and plated on tryptose agar to determine the number of CFU.

Animals. BALB/c female mice were obtained from Charles River, Milan, Italy, and used in the experiments at 12 to 14 weeks of age. They were maintained in barrier housing with filtered inflow air in a restricted-access room and in pathogen-limited conditions. They were fed a commercial diet, and water was provided ad libitum. All mice were acclimatized for a minimum of 1 week prior to experimentation. Mice were employed in experimental groups consisting of 5 animals each.

In vitro susceptibility to pH. An in vitro assay was performed to assess the susceptibility to acidity. *B. abortus* 2308 or *B. abortus* RB51 (10^{10} CFU) was incubated in 5 ml of sterile saline. The pH was adjusted as required with 1 N HCl. All liquid cultures were incubated at 37°C for 90 min. The viability of bacteria was evaluated by plating aliquots of different suspensions.

Immunization protocols. For intraperitoneal immunization with live bacteria, mice were given 2×10^8 CFU of *B. abortus* RB51 in 0.2 ml of sterile saline or 2×10^4 CFU of *B. abortus* 2308. For oral immunization, a gastric lavage needle was

TABLE 1. Effect of oral infection of *Brucella* strains
under different regimens

B. abortus strain and treatment[a]	% of seropositive animals at day after infection:	
	10	18
RB51		
NaHCO$_3$	20	80
Saline	0	0
2308		
NaHCO$_3$	80	100
Saline	0	0

[a] Five mice per groups were orally administered 200 μl of 10% NaHCO$_3$ or saline 10 min after they were infected with 200 μl of a suspension of 2×10^{10} CFU of *B. abortus* RB51 or *B. abortus* 2308.

used. Ten minutes prior to oral inoculation, mice were administered 0.2 ml of 10% sodium bicarbonate to neutralize gastric acidity or 0.2 ml of sterile saline. Mice were orally inoculated with 2×10^{10} CFU of *B. abortus* RB51 or *B. abortus* 2308.

Evaluation of oral infection. On days 10 and 18 after oral administration of *B. abortus* RB51 or *B. abortus* 2308, preceded or not by gastric acidity neutralization, mice were killed and their spleens were removed. Spleens were homogenized in 1 ml of sterile saline. Aliquots of the resulting suspensions were plated to assess the spleen colonization.

In separate sets of experiments, mice were orally inoculated with *B. abortus* RB51. Oral inoculation was preceded by gastric acidity neutralization. To evaluate the excretion of bacteria after oral exposure, fecal samples were collected daily for 3 days after inoculation. Feces (0.2 g) collected in the cages were homogenized in 1.8 ml of sterile saline. Aliquots of 0.5 ml were then inoculated in 10 ml of Todd-Hewitt broth containing *Brucella* selective supplement (Oxoid) and rifampin (50 μg/ml) and incubated at 37°C for 96 h. After incubation, *Brucella* growth was assessed by standard microbiological analysis. At 7, 15, 30, and 42 days after infection, mice were bled and killed and spleens were aseptically removed. Approximately one-third of the spleen was weighed and homogenized in phosphate-buffered saline, and an aliquot of the resulting cell suspension was plated to determine the outcome of infection. The remaining two-thirds of the spleens were weighed, minced, and used to prepare spleen cell suspensions. For lymphocyte proliferation, spleen cells were suspended at a concentration of 10^6 per ml in RPMI 1640 containing 2 mM L-glutamine, 25 mM HEPES, 10% fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (RPMI). The cells were cultured in triplicate in 96-well flat-bottom tissue culture plates (37°C, 5% CO$_2$). Spleen cells were incubated with heat-inactivated *B. abortus* RB51 at a ratio of 100 bacteria to one spleen cell. Background proliferation was determined by culturing the cells in the culture media alone. Aspecific proliferation was ascertained by culturing spleen cells isolated from uninfected mice stimulated with heat-inactivated *B. abortus* RB51 at a ratio of 100 bacteria to one spleen cell. After 7 days, the proliferation was assessed according to the procedures of the CellTiter 96 AQ$_{ueous}$ One Solution cell proliferation assay kit (Promega). The stimulation index was calculated by dividing the mean optical density obtained in the wells containing cells stimulated with bacteria by the mean optical density obtained in the wells containing unstimulated cells.

Serology. Mouse sera were tested by the complement fixation test with *B. abortus* RB51, previously deprived of anticomplementary activity as described elsewhere, or *B. abortus* 99, which is a smooth strain used as a standard antigen in Europe (1). Briefly, in 96-well round-bottom microtiter plates, 25 μl of each serum was serially diluted in Veronal buffer (Bio Mérieux) from 1:2 to 1:128 and

25 μl of previously titrated antigen was added to each well followed by 25 μl of complement (Bio Mérieux). After incubation in agitation for 30 min at 37°C, 25 μl of sensitized erythrocytes was added to each well and plates were incubated as described above. Serum titers have been reported as the end-point dilution that still gives a positive reaction by using a dilution of 1:4 showing 50% hemolysis as the threshold of the reaction.

Protection assay. Mice were orally vaccinated with *B. abortus* RB51 preceded by gastric acidity neutralization or were intraperitoneally inoculated with *B. abortus* RB51 as mentioned above. Additional mice served as unvaccinated control animals. Orally and intraperitoneally vaccinated mice and unvaccinated controls were challenged with oral (2×10^{10} CFU) or intraperitoneal (2×10^4 CFU) inoculation of *B. abortus* 2308 42 days after vaccination. Oral challenge was preceded by gastric acid neutralization. Mice were bled and killed at 18 days after challenge. Spleens were weighed and homogenized to determine the number of CFU as above described as a means for assessing the protective response induced by vaccination. Sera were used to evaluate the antibody titer against both *B. abortus* 2308 and *B. abortus* RB51. These experiments were repeated to ascertain the reproducibility of obtained data, and representative results are presented.

Statistical analysis. Differences between groups were estimated by a one-way analysis of variance. Differences were considered significant when P was ≤0.05.

RESULTS

Effect of gastric acid neutralization on the course of oral infection. First we evaluated the effect of a pretreatment to neutralize gastric acidity on the course of an oral *Brucella* infection. Mice were orally administered saline or 10% NaHCO$_3$, and 10 min later, they were orally inoculated with *B. abortus* 2308 or *B. abortus* RB51 by the oral route. Mice were sacrificed 10 and 18 days after infection, and the colonization of spleens was evaluated as a mean of assessing the influence of gastric acidity neutralization on inducing a systemic infection. As shown in Table 1, we found that neutralization of gastric acidity induced a systemic infection with both *B. abortus* 2308 and *B. abortus* RB51, even if at different degrees. In fact, *B. abortus* induced a systemic infection more promptly, being able to colonize spleens in 4 of 5 mice and in 5 of 5 mice at 10 and 18 days after oral infection, respectively. Conversely, *B. abortus* RB51 required a longer period to give a systemic infection, being able to colonize 1 of 5 mice and 4 of 5 mice at 10 and 18 days after oral infection, respectively. Oral inoculation without gastric acidity neutralization did not result in spleen colonization with either *B. abortus* 2308 or *B. abortus* RB51.

In vitro effect of acid pH on viability of bacteria. The in vitro susceptibility of *B. abortus* 2308 and *B. abortus* RB51 to acid pH is reported in Table 2. We found similar results for both *Brucella* strains. *B. abortus* 2308 and *B. abortus* RB51 were devitalized after a 90-min period in a solution at pHs of 3.3 and 3.6, respectively.

Evaluation of infection after *B. abortus* RB51 oral administration. Mice were orally inoculated with 2×10^{10} CFU of *B. abortus* RB51. Oral inoculation was preceded by gastric acidity neutralization. Five mice were killed at 7, 14, 30, and 42 days

TABLE 2. Effect of different pH on viability of *B. abortus* 2308 and *B. abortus* RB51[a]

B. abortus strain	Result at pH:					
	7.0	5.3	3.6	3.3	2.6	2.1
2308	Viable	Viable	Viable	Viable	Inactivated	Inactivated
RB51	Viable	Viable	Viable	Inactivated	Inactivated	Inactivated

[a] Five milliliters of sterile saline was inoculated with 10^{10} CFU of *B. abortus* 2308 or *B. abortus* RB51. After 90 min, aliquots of different suspensions were plated to assess the viability of bacteria.

TABLE 3. Effect of *B. abortus* RB51 at different days after oral vaccination[a]

No. of days after vaccination	Animal no.	Stimulation index	CFU/ spleen	Spleen wt (mg)	Serological titer
7	1	2.6	0	98	NEG[b]
	2	1.8	0	99	NEG
	3	1.2	0	76	NEG
	4	1.2	0	87	NEG
	5	1.3	0	98	NEG
	Mean	1.6	0	92	
15	1	1.1	0	97	NEG
	2	1.1	0	96	NEG
	3	1.2	25	109	NEG
	4	1.1	25	95	NEG
	5	1.1	0	113	NEG
	Mean	1.1	10	102	
30	1	Not done	0	82	1:8
	2	1.3	975	135	NEG
	3	1.2	75	105	1:8
	4	1.1	25	66	NEG
	5	1.2	25	113	NEG
	Mean	1.2	220	122	
42	1	1.6	0	96	NEG
	2	3.7	0	112	1:2
	3	3.0	0	127	1:8
	4	1.7	0	129	1:16
	5	5.6	0	120	NEG
	Mean	3.5	0	117	

[a] Mice were orally vaccinated and killed at different times.
[b] NEG, negative.

after oral infection, and we evaluated the bacterial colonization and the weight of spleens as well as cell-mediated and humoral immune response at each time point (Table 3). We found that the level of infection was low throughout the experiment. However, systemic infection was first evidenced at 14 days, reaching a plateau phase around 30 days after oral exposure. *B. abortus* RB51 was no longer present at 42 days after oral exposure. Spleen weights did not change greatly throughout the experiment. A slight enlargement of spleens was only observed at 42 days after oral exposure.

The onset of immunity resulted in a delay compared to microbiological patterns. In fact, 2 of 5 mice were seropositive by the complement fixation test at 30 days after infection and 3 of 5 mice were seropositive at 42 days after infection. Similarly, a cell-mediated immune response was not evident until 42 days after infection.

To assess the excretion of *B. abortus* RB51, we collected daily a significant number of fecal samples and we evaluated the presence of bacteria by using standard isolation procedures. We did not find *Brucella* excretion at any time point (data not shown).

Resistance to infection. Mice were vaccinated intraperitoneally or orally with *B. abortus* RB51 and successively challenged by oral or intraperitoneal inoculation with the virulent strain *B. abortus* 2308. Mice vaccinated both orally and intraperitoneally with *B. abortus* RB51 showed similar degrees of protection

against oral infection with *B. abortus* 2308 (Fig. 1). In contrast, mice intraperitoneally vaccinated with *B. abortus* RB51 had increased resistance to intraperitoneal infection with *B. abortus* 2308 compared to unvaccinated or orally vaccinated mice that had undergone intraperitoneal infection with *B. abortus* 2308.

After challenge infections, the weights of spleens from mice intraperitoneally vaccinated with *B. abortus* 2308 and challenged with intraperitoneal inoculation with *B. abortus* 2308 were lower than those of spleens from the other groups of mice (Table 4).

Serological tests allowed us to evaluate the antibody titers of each animal, with the ability to also discriminate between antibodies against the virulent strain *B. abortus* 2308 or the vaccine strain *B. abortus* RB51 at the time of killing. Mice killed 18 days after infection did not develop antibodies with *B. abortus* 99 as the antigen (data not shown). On the contrary, as shown in Table 4, mice vaccinated by oral or intraperitoneal inoculation with *B. abortus* RB51 showed high titers with the homologous antigen.

DISCUSSION

Brucellosis can be transmitted by several routes, but the natural infection seems to occur mainly in humans and in animals by the oral route. For this reason, the mucosal milieu is of primary importance as the initial interface between bacteria and hosts. It is in this environment that the colonization takes place and can induce a systemic infection which can cause the disease.

Mucosal vaccination can represent a useful tool for the induction of protection because it can overcome the inherent difficulty in vaccinating wild animals (4). In addition, it can induce an immune response at the site of primary infection which can determine a better influx of immunocompetent cells at the mucosal level. Previous studies reported that mice vaccinated orally with *B. abortus* RB51 were protected at a lower degree than mice vaccinated intraperitoneally against a challenge infection with a virulent strain inoculated intraperitoneally (21). Results from this study confirm these findings but indicate also that oral vaccination in mice induces a mild infection which is able to confer protection at a level comparable to that of intraperitoneal vaccination against a challenge infection by the oral route. We further demonstrated that oral inoculation of both the vaccine strain *B. abortus* RB51 and the virulent strain *B. abortus* 2308 can give more homogeneous results if preceded by gastric acid neutralization.

Previous unpublished results showed that an oral inoculation of *B. abortus* RB51 at a high dose (2×10^{10} CFU/mice) did not induce systemic reaction. We supposed that it was due to the neutralizing effect of gastric acidity since it was reported that *B. abortus* had reduced viability at pH 3.8 (9). In addition, in other experiments it was seen that *Brucella canis* is devitalized at pH 4.6 and that *Brucella suis* at pH 4.6 was still capable of slowly multiplying (8). To assess the role of gastric acidity, we first performed a set of experiments in which oral inoculation with the virulent strain *B. abortus* 2308 or with the vaccine strain *B. abortus* RB51 was preceded by gastric acidity neutralization by oral injection of 10% sodium bicarbonate. We found that this treatment was able to give a systemic infection with both strains, supporting our hypothesis. Of those, *B. abortus*
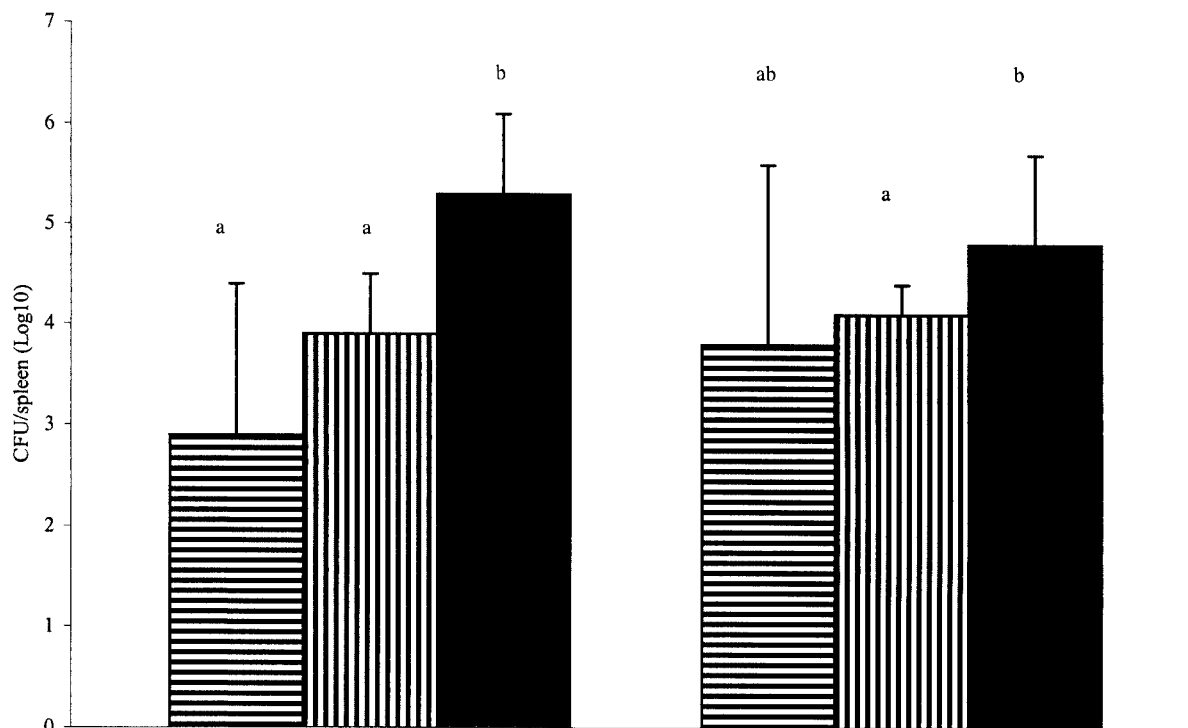
FIG. 1. Persistence of bacteria in spleens of vaccinated mice. Mice were vaccinated by oral (horizontal stripes) or intraperitoneal (vertical stripes) inoculation of *B. abortus* RB51 or were nonvaccinated (solid bars). They were challenged with oral (left side) or intraperitoneal (right side) inoculation of *B. abortus* 2308. Results are expressed as means (error bars represent standard deviations) ($n = 5$). Groups with the different letters are statistically different ($P < 0.05$).

2308 induced an earlier and heavier colonization of spleens. It is interesting that the ability of *B. abortus* 2308 to induce a systemic infection more promptly than the attenuated strain *B. abortus* RB51 can account for its virulence and can be due to a higher resistance to an acidic environment. To address this hypothesis, we tested in vitro the sensitivity to acidity of *B. abortus* 2308 and *B. abortus* RB51, and we found that they were similarly susceptible to acid stress. Even if it is difficult to make any assumption only by means of results from in vitro experiments, our finding implies that the higher virulence of *B. abortus* 2308 is not attributable to a higher resistance to acid stress than that of the vaccine strain *B. abortus* RB51. It is interesting that we did not find *Brucella* excretion at any time point. It could mean that the sensibility of our test was too low to detect excretion but can also suggest that organisms that pass through the stomach are successively inactivated by other means. Oral inoculation with *B. abortus* RB51 preceded by gastric acidity neutralization gave a mild systemic infection which induced a delayed immune response. In fact, mice gave seropositive results starting from 30 days after oral inoculation, and titers were often close to the cutoff value. Similarly, the cell-mediated immune response was even more delayed; it was not evident until 42 days after oral inoculation. These results imply that most orally inoculated bacteria are not able to overcome the natural defenses of the mucosal barrier, and few of them reach and colonize spleen cells. However, it could also show that the primary uptake of the antigen occurs at the mucosal site, and this pattern probably results in a delayed systemic response in terms of both humoral and cell-mediated response.

It is not surprising because, as already reported, low antibody titers started at 4 weeks after oral vaccination, and a transient and mild proliferative response was reported only at 8 weeks after oral vaccination (21).

To assess the efficacy of oral vaccination, we chose to use both oral and intraperitoneal challenge infection with the virulent strain *B. abortus* 2308. In fact, even if the oral challenge is not considered an efficient method to produce a consistent infection in mice (3), we found that oral challenge was able to give reproducible results when preceded by gastric acid neutralization. Hence, this approach gave us the opportunity to evaluate the efficacy of vaccination against an infection which

TABLE 4. Spleen weights and antibody titers against *B. abortus* RB51 in vaccinated and infected mice

Vaccination route	Challenge route	Spleen wt (mg)	Log antibody titer
Oral	Oral	301 ± 145	1.6 ± 0.2
Intraperitoneal	Oral	233 ± 99	1.9 ± 0.3
None	Oral	273 ± 68	Negative
Oral	Intraperitoneal	206 ± 74	ND[b]
Intraperitoneal	Intraperitoneal	151 ± 27[c]	ND
None	Intraperitoneal	284 ± 139	ND

[a] Mice were infected with *B. abortus* 2308 42 days after vaccination. Spleen weights and antibody titers were assessed at 18 days after challenge infection. Data are mean values for five animals ± standard deviations.
[b] ND, not done.
[c] Statistically different ($P \leq 0.05$).

closely resembles the natural conditions, since ruminants are mostly infected by the oral route in field conditions (6).

Mice intraperitoneally vaccinated with *B. abortus* RB51 were similarly protected against infection by the oral or intraperitoneal route. On the contrary, mice orally vaccinated with *B. abortus* RB51 were shown to be protected against an oral challenge but not against an intraperitoneal challenge. These results provide strong circumstantial evidence that oral vaccination is able to induce protection against an oral challenge but not against an intraperitoneal challenge. According to our findings, the lack of protection against an intraperitoneal challenge could be due to the observed delayed immune response, since at the time of the challenge infection, the systemic immune response was still weak.

From this scenario it is relatively easy to envisage that the vaccine-induced protection against an oral challenge is probably based on a local response of the mucosal immune system with minor involvement of systemic patterns. This finding suggests that if brucellosis is acquired by the oral route, the immune response is compartmentalized with a mucosal response which is not necessarily connected to the systemic immune response.

It is a matter of fact that the oropharynx is the main interface of *Brucella* organisms with the host (6), but the pattern of natural infection seems to be complicated and still largely unknown. In experimental conditions, in fact, it is very difficult to infect animals by the oral route with high efficacy without using particular strategies including, for example, materials such as thiabendazole paste or corn syrup which prolong and facilitate adherence between the vaccine and the oropharynx (4, 11). Here we propose an approach through which it is possible to investigate the relationship between the mucosal immune system and *Brucella* organisms. If it is true, in fact, that in natural conditions, the intestinal mucosa does not have a role in the progression of the infection, we cannot forget that the mucosal surface is a complicated system in which immune-competent cells constitute a finely regulated network which involves different organs.

In conclusion, the purpose of our study is to test whether *B. abortus* RB51 is able to give protection against a challenge infection when administered by the oral route. Since protection occurred against an oral challenge and not against a parenteral challenge, it is reasonable to suppose that the mucosal immune system is able to control oral infection with virulent strains. Moreover, although our findings suggest that the oral use of *B. abortus* RB51 is still impractical, because it requires the neutralization of gastric acidity, these data nevertheless emphasize the need for a better comprehension of the intimate mechanisms that regulate the relationship between *Brucella* organisms and the mucosal immune system. Finally, in order to make more feasible the oral use of *B. abortus* RB51, these findings highlight the need to study a combined treatment to pharmacologically reduce gastric secretions.

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