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Immunization with *Brucella* VirB Proteins Reduces Organ Colonization in Mice through a Th1-Type Immune Response and Elicits a Similar Immune Response in Dogs

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VirB proteins from *Brucella* spp. constitute the type IV secretion system, a key virulence factor mediating the intracellular survival of these bacteria. Here, we assessed whether a Th1-type immune response against VirB proteins may protect mice from *Brucella* infection and whether this response can be induced in the dog, a natural host for *Brucella*. Splenocytes from mice immunized with VirB7 or VirB9 responded to their respective antigens with significant and specific production of gamma interferon (IFN-γ), whereas interleukin-4 (IL-4) was not detected. Thirty days after an intraperitoneal challenge with live *Brucella abortus*, the spleen load of bacteria was almost 1 log lower in mice immunized with VirB proteins than in unvaccinated animals. As colonization reduction seemed to correlate with a Th1-type immune response against VirB proteins, we decided to assess whether such a response could be elicited in the dog. Peripheral blood mononuclear cells (PBMCs) from dogs immunized with VirB proteins (three subcutaneous doses in QuilA adjuvant) produced significantly higher levels of IFN-γ than cells from control animals upon *in vitro* stimulation with VirB proteins. A skin test to assess specific delayed-type hypersensitivity was positive in 4 out of 5 dogs immunized with either VirB7 or VirB9. As both proteins are predicted to locate in the outer membrane of *Brucella* organisms, the ability of anti-VirB antibodies to mediate complement-dependent bacteriolysis of *B. canis* was assessed *in vitro*. Sera from dogs immunized with either VirB7 or VirB9, but not from those receiving phosphate-buffered saline (PBS), produced significant bacteriolysis. These results suggest that VirB-specific responses that reduce organ colonization by *Brucella* in mice can be also elicited in dogs.

Brucellosis is caused by Gram-negative bacteria of the genus *Brucella*, which infect different domestic and wild animals but can also spread to humans, producing a systemic febrile illness sometimes accompanied by localized complications (1). As humans usually acquire the infection through contact with infected animals, their tissues (e.g., placental tissue), or their products (usually dairy products), prevention of the infection in host animals is an adequate strategy to prevent human brucellosis. No vaccine is currently available to prevent canine brucellosis due to *Brucella canis*. This infection, which leads to abortion, orchitis, diskospondylitis, and other health problems in dogs, has been increasingly reported in many countries (2–4) and has been shown to constitute a risk for human disease (5, 6).

Approved vaccines for use in animals for preventing brucellosis are based on live attenuated strains, including *B. abortus* S19, *B. abortus* RB51, and *B. melitensis* Rev-1. While these vaccines have reduced the virulence for animals, they still can produce disease in humans, as demonstrated by the occurrence of brucellosis cases due to vaccine strains among veterinarians and other risk groups (7–9). Live vaccines also have restricted use in animals since they can induce abortion in pregnant females. In view of these risks, many researchers have investigated alternative vaccination strategies for brucellosis, including the use of subunit vaccines based on recombinant proteins (10–12) or the use of DNA vaccination (13–15).

Brucella species are intracellular bacteria that establish their preferred replication niche in macrophages (16, 17). Due to this intracellular location, gamma interferon (IFN-γ), produced mainly by T helper 1 (Th1) and $CD8^+$ lymphocytes, has been shown to be of central importance for the control of *Brucella* infection through its ability to activate the bactericidal functions of macrophages (18). For this reason, many studies on candidate vaccines for brucellosis have focused on the induction of the immune responses leading to IFN-γ production (10, 13, 19).

One of the key virulence factors mediating the intracellular survival of different *Brucella* species is the type IV secretion system (T4SS), encoded by the VirB operon (*virB1* to *virB12* genes) (20,

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21), which has been shown to be required for *Brucella* survival *in vitro* and *in vivo* (20–23). It has been postulated that the *Brucella* T4SS mediates the secretion of virulence factors that may contribute to the ability of these bacteria to establish its replicative niche (20–22, 24).

The expression of *virB* genes is induced intracellularly in the first hours after uptake of *Brucella* by macrophages (24, 25). Since most brucellae die during the initial phase of intracellular establishment, we hypothesize that infected macrophages probably display peptides derived from VirB proteins in the context of major histocompatibility complex class II (MHC-II) molecules on the cellular surface. In this context, VirB-specific Th1 cells might recognize infected macrophages and respond with the production of IFN-γ, leading to the activation of macrophagic antimicrobial mechanisms.

The main goals of the present study were to assess whether the induction of a Th1-type immune response against VirB proteins may protect mice from *Brucella* infection and whether this type of response can be induced in the dog, a natural host for *Brucella*.

MATERIALS AND METHODS

Antigen production. *Escherichia coli* strain JM109 (Promega, Madison, WI) was used as the host for propagation of plasmids. Strain BL21(DE3) (Stratagene, La Jolla, CA) was used for expression of the recombinant proteins. Bacterial strains were routinely grown at 37°C in Luria-Bertani (LB) broth or agar, supplemented when required with 100 μg/ml of ampicillin. The plasmids pTrcHis-FusB7 A^R and pTrcHis-FusB9 A^R containing the VirB7 and VirB9 genes, respectively, with the addition of a poly(H) tail, were kindly provided by Diego Comerci, UNSAM, Argentina. Competent *E. coli* BL21(DE3) colonies were transformed with these plasmids. Ampicillin-resistant colonies containing the pTrcHis-FusB7 A^R plasmid were grown in Terrific broth medium containing 100 μg of ampicillin/ml at 37°C with agitation (160 rpm) until reaching an optical density at 600 nm (OD_{600}) of 1.0. Five milliliters of this culture was diluted to 500 ml and grown until reaching an OD_{600} of 1.0. After addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce VirB7 protein expression, bacteria were incubated for additional 4 h. Bacteria were pelleted by centrifugation (15,000 × *g*, 20 min, 4°C), suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl [pH 8.0]), and sonicated for three 1-min cycles at 4°C. After centrifugation, soluble protein was purified from the supernatant by chromatography through nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Dorking, United Kingdom).

Ampicillin-resistant colonies containing the pTrcHis-FusB9 A^R plasmid were grown in LB medium containing 100 μg of ampicillin/ml at 37°C with agitation (160 rpm) until reaching an OD_{600} of 1.0. Five milliliters of this culture was diluted to 500 ml and grown until reaching an OD_{600} of 1.0. VirB9 protein expression was induced by addition of 0.1 mM IPTG for 3 h and 0.4 mM for 1 more hour. Bacteria were pelleted by centrifugation (15,000 × *g*, 20 min, 4°C), suspended in phosphate-buffered saline (PBS), and sonicated for three 1-min cycles at 4°C. Inclusion bodies were pelleted at 20,000 × *g* for 30 min at 4°C and solubilized in a solution containing 100 mM NaH_2PO_4, 10 mM Tris-HCl, and 8 M urea (pH 8.0) at 4°C overnight with agitation. After centrifugation (20,000 × *g*, 30 min, 4°C), soluble protein was purified by chromatography through Ni-NTA agarose.

The purity of both proteins was assessed by SDS-polyacrylamide gel electrophoresis followed by Coomassie blue staining. Purified proteins were incubated with polymyxin B-Sepharose overnight at 4°C with agitation to eliminate lipopolysaccharide (LPS) contamination. The residual content of LPS in these preparations, according to the *Limulus* amebocyte assay (Associates of Cape Cod, Woods Hole, MA), was <0.25 endotoxin unit/μg protein. The protein concentrations of the antigen preparations were determined by the bicinchoninic acid method (Pierce, Rockford, IL) using bovine serum albumin as the standard.

***Brucella* strains.** *B. abortus* 544 (smooth virulent strain), and a local clinical isolate of *B. canis* were obtained from our laboratory collection. Bacteria were cultured in tryptose-soy agar (Merck, Buenos Aires, Argentina) supplemented and incubated as described previously (11, 26). *Brucella* strain manipulations were performed in biosafety level 3 facilities.

Experiments in mice. (i) Animals. Female BALB/c mice (8 to 9 weeks old), obtained from the University of La Plata, Argentina, were acclimated and randomly distributed into experimental groups. The mice were kept in conventional animal facilities and received water and food *ad libitum*. After inoculation with *B. abortus* 544, mice were kept in biosafety level 3 animal facilities. Experiments in mice were approved by our institutional animal care and use committee.

(ii) Immunization and challenge. Groups of 10 mice were immunized by the intraperitoneal (i.p.) route at day 0 with 30 μg of VirB7 or VirB9 in complete Freund's adjuvant (Sigma) and 15 days later with the same dose of antigens in incomplete Freund's adjuvant. On both occasions, another group of mice (negative control) received an equal volume of PBS with the same adjuvant. As a positive control for the protection experiments, other groups of mice were immunized once i.p. at day 0 with heat-killed *B. melitensis* H38 (8×10^8 CFU) in incomplete Freund's adjuvant or with viable *B. abortus* S19 (vaccine strain, 1.2×10^4 CFU). Blood samples were collected from mice by submandibular puncture at the time of the first immunization (day 0), at the second immunization (day 15), and at day 45. At this later time point (30 days after the last immunization), mice from each group were challenged i.p. with virulent *Brucella* organisms (4×10^4 CFU of *B. abortus* 544) or were sacrificed by cervical dislocation to perform immunity tests. All experiments were conducted at least twice. The time of challenge and the time of sacrifice postchallenge (see below) were selected on the basis of previous studies (27, 28).

(iii) Protection assessment. Protection conferred by the experimental vaccines was evaluated essentially as described previously (11, 12). Thirty days after the challenge with virulent *Brucella* organisms, mice were sacrificed by cervical dislocation, their spleens were removed aseptically and homogenized, and dilutions were plated on bacteriological agar and incubated appropriately to determine the number of *Brucella* CFU per spleen. In the case of the control group immunized with viable *B. abortus* S19, spleen homogenates were cultured in parallel under selective (0.1% erythritol, an inhibitor of S19 strain growth) and nonselective conditions in order to differentiate the CFU from the immunization strain and the challenge strain. In all cases, units of protection were obtained by subtracting the mean $\log_{10}$ CFU of the experimental group from the mean $\log_{10}$ CFU of the corresponding negative-control group.

(iv) Antibody titers by ELISA. The titers of serum immunoglobulin G of isotypes 1 and 2a (IgG1 and IgG2a) with specificity to VirB7 or VirB9 were determined by an enzyme-linked immunosorbent assay (ELISA). Polystyrene plates (Nunc, Roskilde, Denmark) were coated with purified recombinant VirB7 or VirB9 (0.5 μg/well) in PBS during 1 h at room temperature. After this incubation, plates were washed four times in PBS with 0.05% Tween 20 (PBS-T) and blocked overnight at 4°C with 200 μl/well of PBS containing 3% skim milk. Then, plates were incubated with serial dilutions (starting at 1:100) of sera for 1 h at room temperature and washed four times. Isotype-specific goat anti-mouse horseradish peroxidase conjugates (Santa Cruz Biotechnology, Santa Cruz, CA) were added (50 μl/well) at appropriate dilutions. After 1 h of incubation at room temperature, plates were washed four times, and 50 μl/well of substrate solution (200 μmol of *o*-phenylenediamine and 0.04% H_2O_2) were added to each well. After 20 min of incubation at room temperature, the enzyme reaction was stopped by the addition of 4 N H_2SO_4, and the OD was measured at 492 nm in a microplate reader (Multiskan). The cutoff value of the assay was calculated as the mean OD plus 3 standard deviations (SD) produced at a 1:100 dilution by sera obtained on day 0 from all mice. The specific titer of each serum was calculated as the reciprocal of the highest dilution that yielded an OD higher than the cutoff value.

(v) Determination of cytokine production. Spleen cells from immunized and control mice were homogenized and suspended in RPMI 1640

medium (Gibco BRL; Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml (complete medium). Cells were cultured at 5×10^6 cells/ml in duplicate with VirB7 or VirB9 (1 or 10 μg/ml), concanavalin A (ConA) (5 μg/ml) (Sigma), or complete medium alone. Cultures were incubated at 37°C in a humidified atmosphere (5% CO_2 and 95% air) for 48 h. At the end of the incubation, cell culture supernatants were collected, aliquoted, and frozen at −70°C until analysis for cytokine levels. Gamma interferon (IFN-γ) and interleukin-4 (IL-4) levels were determined by a sandwich ELISA using paired cytokine-specific monoclonal antibodies (MAbs), according to the manufacturer's instructions (BD Biosciences).

Experiments in dogs. (i) Animals. A total of 15 healthy dogs of different breeds (4 males and 11 females, ages 1 to 5 years [mean, 2.53 years]) were included in the study. Experiments in dogs were approved by our institutional animal care and use committee.

(ii) Immunization. Dogs were randomly divided into 3 groups of five animals each and were subcutaneously immunized with 1 ml of a mixture of 100 μg of VirB7 or VirB9 (or an equivalent volume of PBS) and 100 μg of QuilA adjuvant. Dogs received three subcutaneous doses of the antigens with QuilA adjuvant every 30 days, and blood samples were collected before immunization and then monthly between the first immunization and day 90 after the last immunization (150 days after the first dose) for the serological and cellular immunity studies.

(iii) Antibody titers. The titers of serum IgG specific for VirB7 or VirB9 were determined by an ELISA following the protocol described above for mice sera, but using a peroxidase-conjugated rabbit anti-dog IgG serum.

(iv) Determination of IFN-γ production. Mononuclear cells were purified from heparinized blood by the Ficoll-Hypaque method and cultured at 1×10^6 cells/ml in duplicate with VirB7 or VirB9 (1 or 10 μg/ml), ConA (5 μg/ml), or complete medium alone. Cultures were incubated at 37°C in a humidified atmosphere (5% CO_2 and 95% air) for 48 h. At the end of the incubation, cell culture supernatants were collected, aliquoted, and frozen at −70°C until analyzed for canine IFN-γ by a sandwich ELISA using paired cytokine-specific MAbs, according to the manufacturer's instructions (R&D Systems).

(v) Delayed-type hypersensitivity test. One month after the last immunization, delayed-type hypersensitivity (DTH) tests were performed as an index of cell-mediated immunity. A volume of 0.1 ml containing 10 μg of VirB7 or VirB9 was injected intradermally in the inner surface of the right thigh, and an equal volume of PBS was injected 3 cm apart as a negative control. The DTH reaction was quantified 48 h later by using a digital caliper with a precision of 0.01 mm to measure the size of the induration and erythematous area (mean of two perpendicular diameters). As described in similar studies in dogs (29), a reaction area of >5 mm was considered positive.

(vi) *In vitro* bacteriolysis assay. The assay was performed in flat-bottomed 96-well plates as described previously (30). *B. canis* suspensions were used at concentrations that did not agglutinate when mixed with sera from immunized dogs. Fifty microliters of a 1/2 dilution of heat-inactivated serum (56°C, 30 min) from each dog or a 1:1 mixture of pools of anti-VirB7 and anti-VirB9 sera was dispensed in duplicate in contiguous wells of the plate, followed by 50 μl of a *B. canis* suspension (1×10^4 CFU/ml) in PBS containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$. The mixtures were homogenized by placing the plate in an orbital shaker at 150 rpm during 90 min at 37°C to allow optimal interaction between the antibodies and bacteria. As a source of complement, 10 μl of guinea pig serum was added to each reaction, and the mixture was homogenized again in the orbital shaker at 150 rpm for 2 h at 37°C. At the end of this incubation, 25 μl of each reaction was plated in duplicate on tryptic soy agar to determine CFU numbers. Control reactions were as follows: (i) sera from immunized dogs plus bacterial suspension and heat-inactivated guinea pig serum, (ii) sera obtained from dogs before immunization plus bacterial suspension and guinea pig serum, and (iii) bacterial suspension plus guinea pig serum (no dog serum added).

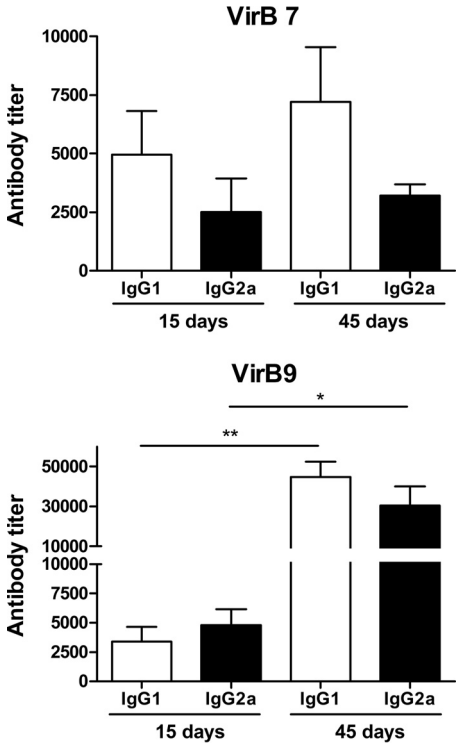

FIG 1 Serum titers of specific antibodies in mice immunized with VirB7 or VirB9. Mice were immunized with each antigen at days 0 and 15, and blood samples were collected on both occasions and also at day 45 to determine antibody titers by an indirect ELISA. Titers were calculated as the reciprocal of the last serum dilution yielding an OD value higher than the cutoff. The latter was calculated as the mean plus 3 SD of the OD values obtained with preimmunization sera diluted 1:100. The data represent the means and SD of values determined in duplicate for the 10 mice in each group. The figure shows a representative experiment of two performed with similar results. The asterisks indicate significant differences between isotypes for a given date or between dates for the same isotype (*, $P < 0.05$, and **, $P < 0.01$, ANOVA followed by Tukey's test).

Statistical analysis. CFU data were logarithmically transformed, and statistical analysis was conducted using analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. ANOVA followed by Bonferroni's test was used to analyze humoral and cellular responses of immunized animals. DTH was evaluated by one-way analysis of variance, followed by Bonferroni's multiple-comparison test. The analysis was performed using GraphPad software, version 4 (San Diego, CA).

RESULTS

Immune response of mice to immunization with VirB proteins. Titers of IgG1 and IgG2a against VirB7 and VirB9 were determined at 30 days after the last immunization with VirB proteins (i.e., at the time of challenge with virulent brucellae). As shown in Fig. 1, specific IgG1 and IgG2a antibodies against both antigens were detected. The titers of all the antibodies increased along with time after immunization, although for both isotypes, the increase only reached statistical significance for anti-VirB9 antibodies.

Splenocytes from animals sacrificed at the time of challenge were stimulated *in vitro* with VirB proteins. As shown in Fig. 2, cells from animals immunized with VirB7 or VirB9 responded to their respective antigens with significant and specific production

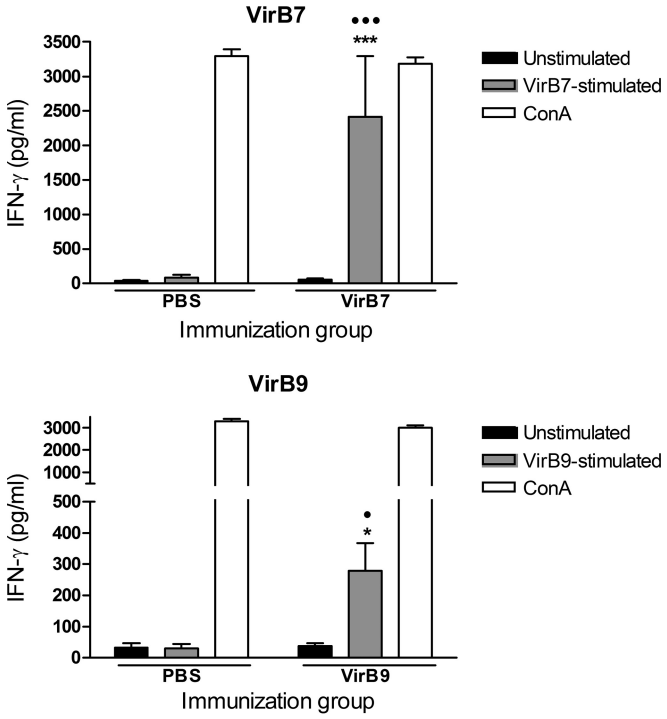

FIG 2 IFN-γ secretion by spleen cells from immunized mice in response to *in vitro* stimulation with VirB antigens. Spleen cells from VirB-immunized mice and nonimmunized mice (PBS) were stimulated during 48 h with VirB7, VirB9, or concanavalin A (ConA) or were left unstimulated (negative control). IFN-γ levels were determined in culture supernatants by an ELISA. The data represent the means and SD of values determined in duplicate for each reaction. The figure shows a representative experiment of two performed with similar results. The asterisks indicate significant differences between VirB-stimulated cells and unstimulated cells of the same group, and the dots indicate significant differences between immunization groups in the response to the same stimulus (* and ●, $P < 0.05$, and *** and ●●●, $P < 0.001$, ANOVA followed by Bonferroni's test).

of IFN-γ. In contrast, no specific secretion of IL-4 was detected in response to stimulation with any of the VirB proteins (data not shown).

Protection conferred by VirB immunization to challenge with live brucellae in mice. The production of IFN-γ (but not IL-4) by murine splenocytes in response to the *in vitro* stimulation with VirB proteins indicated that the immunization protocol used in this study induced a Th1-type specific immune response against VirB proteins. To assess the potential protection conferred by such a response, immunized mice were challenged with virulent *B. abortus* through the intraperitoneal route, and the splenic load of bacteria was determined 30 days later. Two vaccination control groups in which mice were immunized once with either heat-killed *B. melitensis* H38 or live *B. abortus* S19 were included, as these positive controls are frequently used in studies on experimental vaccines for brucellosis (12, 13, 31). As shown in Table 1, immunization with either VirB7 or VirB9 conferred a significant reduction in colonization. The splenic load of brucellae was less than 1 log lower in mice immunized with VirB proteins than in the control group (mice receiving PBS and adjuvant). No significant differences regarding splenic weights were found between the immunization groups.

Immune response of dogs to VirB immunization. In view of the protection conferred to mice by VirB immunization, which seemed to correlate with a Th1-type immune response against VirB proteins, it was deemed interesting to assess whether this type of immune response could be elicited in the dog, a natural host of *Brucella* infection. As Freund's adjuvant is not used in companion animals due to unacceptable injection site reactions (32), dogs were immunized subcutaneously with VirB proteins in QuilA adjuvant, which has been shown to induce good Th1-type responses in dogs with minimal adverse effects (29, 32).

Serum antibody levels. As shown in Fig. 3, IgG levels against VirB7 increased steadily in most dogs until day 90 (30 days after the last immunization) and showed a slight decline in the last two sampling times (120 and 150 days). Titers obtained from day 90 onward were significantly increased compared to those obtained at day 30. Antibodies against VirB9 showed an irregular but steady increase of titers until the end of the follow-up. However, only titers obtained at day 150 were significantly increased compared to those obtained at day 30, probably due to the dispersion of values obtained at each time point. At day 150, mean IgG titers against VirB7 and VirB9 were 5.6- and 18.3-fold higher than their respective titers at 30 days postimmunization.

Bacteriolytic activity of anti-VirB antibodies. Previous studies have shown that specific antibodies can bind to proteins exposed in the membrane of rough *Brucella* microorganisms (*B. canis* and *B. ovis*) (33). Moreover, antibodies against the outer membrane protein Omp31 can mediate complement-dependent bacteriolysis of *B. ovis* (34). As VirB7 and VirB9 are predicted to be associated to the outer membrane of *Brucella*, we decided to test whether the anti-VirB antibodies present in the sera of immunized dogs may mediate complement-dependent bacteriolysis of *B. canis in vitro*. The bacteria were incubated with either preimmune or immune sera from each dog and with guinea pig serum added as a source of complement before plating for CFU counting. As shown in Fig. 4, incubation with sera from the PBS control group did not reduce the CFU numbers of *B. canis* compared to those observed in reactions with preimmune sera. In contrast, incubation with sera from dogs immunized with VirB7 or VirB9 resulted in a significant reduction of CFU numbers. The involvement of complement in such a reduction was corroborated by perfor-

TABLE 1 Protection against *B. abortus* infection in mice by immunization with VirB proteins[a]

Immunogen	Splenic *Brucella* load (mean ± SD) (log CFU/spleen)	Protection (log)	Splenic wt (mean ± SD) (g)
VirB7	4.43 ± 0.44	0.87[b]	1.07 ± 0.03
VirB9	4.50 ± 0.34	0.80[c]	1.31 ± 0.22
H38	3.64 ± 0.46	1.66[d]	0.76 ± 0.04
S19	3.48 ± 0.44	1.82[d]	1.06 ± 0.05
PBS	5.30 ± 0.21	0.00	1.07 ± 0.49

[a] Mice were immunized twice (15 days apart) with VirB7 or VirB9 (the first dose with complete Freund adjuvant; the second dose with incomplete Freund adjuvant) or were immunized once with heat-killed *B. melitensis* H38 or viable *B. abortus* S19 and were challenged 30 days later with *B. abortus* 544 through the intraperitoneal route. Mice were sacrificed 30 days later to determine their splenic load of *B. abortus* 544. Splenic loads are expressed as means ± SD of log CFU per spleen. For each immunization group, protection was calculated as the difference of mean splenic load between that group and the nonimmunized (PBS) group (ANOVA followed by Dunnett's test).
[b] $P < 0.01$.
[c] $P < 0.05$.
[d] $P < 0.001$.

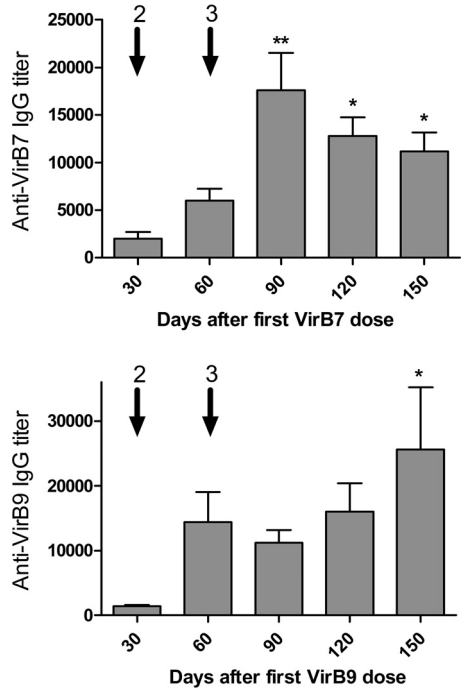

FIG 3 Serum antibody titers against VirB7 and VirB9 in immunized dogs. Specific IgG titers were determined by an indirect ELISA in serum samples obtained at monthly intervals starting at 30 days after the first antigen dose until day 150 (90 days after the last antigen dose). Titers were calculated as the reciprocal of the last serum dilution yielding an OD value higher than the cutoff. The latter was calculated as the mean plus 3 SD of the OD values obtained with preimmunization sera diluted 1:100. The data represent the means and SD of values determined in duplicate for the 5 dogs in each group. The arrows indicate the time of the administration of the second and third doses of each antigen. The asterisks indicate significant differences between titers obtained from day 60 onward compared to those obtained at day 30 (*, $P < 0.05$, and **, $P < 0.01$, ANOVA followed by Dunnett's test).

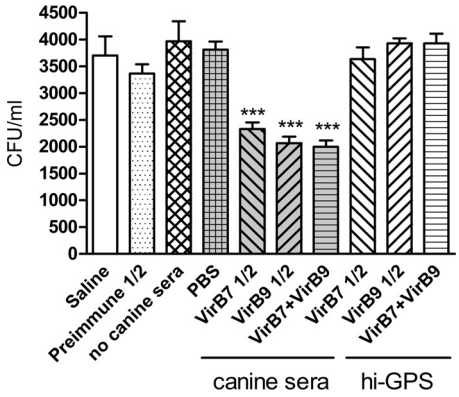

FIG 4 Complement-dependent *B. canis* bacteriolysis mediated by sera from VirB7- and VirB9-immunized dogs. Sera obtained from each dog at 30 days after the last immunization were heat-inactivated and mixed with a *B. canis* suspension. Anti-VirB7 and anti-VirB9 sera were assayed at a 1:2 dilution either when used alone or mixed (VirB7+VirB9). After proper incubation, guinea pig serum (GPS) was added as a source of complement. Two hours later, aliquots of the mixtures were plated in duplicate on agar for CFU counting. Controls included reactions performed (i) with heat-inactivated guinea pig serum (hi-GPS), (ii) with canine sera obtained before immunization, (iii) with canine sera from the control (PBS) group, and (iv) without canine serum. The data represent the means and SD of values determined in duplicate for each reaction. The figure shows a representative experiment of three performed with similar results. The asterisks indicate significant differences between CFU numbers obtained with sera from VirB-immunized animals and those from the control (PBS) group. (****, $P < 0.0001$, ANOVA followed by Dunnett's test).

mance of the same assays but with heat-treated guinea pig serum. In addition, complement did not produce any direct bacteriolytic effect on *B. canis* as demonstrated by the lack of CFU reduction when bacteria were incubated with complement alone (guinea pig serum) in the absence of dog serum. As both anti-VirB7 and anti-VirB9 antibodies produced complement-mediated bacteriolysis, we decided to test whether a 1:1 mixture of both types of sera produces a higher bacteriolysis degree than that induced by each serum alone. As shown in Fig. 4, the reduction in CFU numbers obtained with the mixture did not differ significantly from that obtained with each serum alone. It must be noted that the concentration of each serum in the mixture was the same as that present in reactions performed with each serum separately, as a 1/2 dilution was used in the latter cases.

***In vitro* cellular immune response.** The ability of peripheral blood lymphocytes from immunized dogs to secrete IFN-γ in response to the recognition of peptides derived from VirB proteins was assessed *in vitro*. As shown in Fig. 5, upon *in vitro* stimulation with VirB7 or VirB9, peripheral blood mononuclear cells (PBMCs) from immunized dogs produced IFN-γ levels significantly higher than those produced by cells from the control group. The IFN-γ levels produced specifically in response to each VirB protein were similar.

DTH tests. A positive skin test response to antigens (>5 mm) was observed in 4/5 VirB7-vaccinated dogs and also in 4/5 VirB9-vaccinated dogs at day 120 (60 days after the last vaccination) (Table 2). The intensity of the reaction recorded in dogs with a positive DTH response (median) was 9.5 mm (8.5 to 11 mm) against VirB7 and 12 mm (6.5 to 25 mm) against VirB9. The VirB9-vaccinated dog with a negative skin test result corresponded to an animal which had consistently low IFN-γ cytokine assay responses to VirB9, whereas the skin test-negative animal in the VirB7 group paradoxically showed a high *in vitro* IFN-γ response to VirB7. None of the dogs from the control group (receiving PBS plus adjuvant) exhibited a positive reaction against VirB proteins.

DISCUSSION

Since *Brucella* spp. are intracellular pathogens, which mainly reside in macrophages and dendritic cells, cellular immune responses are fundamental for protective immune responses. The IFN-γ-mediated activation of macrophages is a central component of this immune protection (18). Therefore, the identification of *Brucella* antigens that can induce a specific Th1-type response with production of IFN-γ may contribute to the development of efficacious vaccines against brucellosis. To our best knowledge, the potential usefulness of VirB proteins as vaccine candidates has not been explored.

The expression of *virB* genes is induced intracellularly after *Brucella* phagocytosis (24, 25). As most *Brucella* cells die shortly after phagocytosis, before the establishment of the intracellular replication niche, infected macrophages may display peptides derived from *Brucella* VirB proteins in the context of MHC-II. Thus, the elicitation of VirB-specific Th1 cells by specific immunization

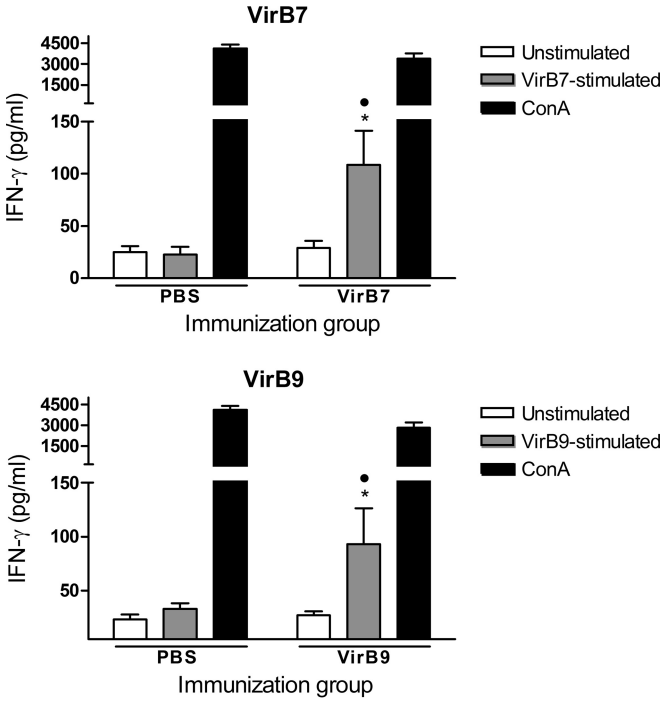

FIG 5 *In vitro* IFN-γ production by peripheral mononuclear cells (PBMCs) of dogs immunized with VirB proteins. PBMCs from dogs immunized with either VirB7 or VirB9 were obtained 1 month after the third immunization and were incubated for 48 h with the corresponding antigen or concanavalin A (ConA) or were left unstimulated to determine basal secretion. PBMCs obtained from dogs receiving PBS (control group) were treated in the same manner to determine the specificity of the measured response. IFN-γ levels were measured in culture supernatants using a commercial sandwich ELISA. The data represent means and SD of values determined in duplicate for each of the 5 dogs in each group. The asterisks indicate significant differences between VirB-stimulated cells and unstimulated cells of the same group, and the dots indicate significant differences between immunization groups regarding the response to the same stimulus. (*, $P < 0.05$, and ●, $P < 0.05$, ANOVA followed by Bonferroni's test). The figure shows a representative experiment of two performed with similar results.

may allow the recognition of infected macrophages, leading to the production of IFN-γ and the activation of macrophagic antimicrobial mechanisms.

In the present study, we have found that mice immunized with VirB7 or VirB9 mixed with Freund's adjuvant develop a Th1-biased immune response as revealed by the secretion of IFN-γ (but not IL-4) by spleen cells stimulated with these antigens. Notably, when these mice were challenged with live *B. abortus* through the intraperitoneal route, they exhibited a significant reduction of bacterial load in the spleen compared to that of the nonimmunized controls. The reduction level attained is similar to that obtained in the mouse model in previous studies with *Brucella* proteins (12). These results agree with our working hypothesis regarding the potential protective role of VirB-specific Th1 cells and strongly suggest that immunization with VirB proteins may constitute an effective approach to protect animals from *Brucella* infection.

Whereas the mouse model has been extensively used to study several aspects of *Brucella* infection and to test candidate antigens for the potential development of vaccines, it must be kept in mind that the mouse is not a natural host for *Brucella* infection. While several studies have been performed in natural hosts to test the protective efficacy of immunization with attenuated strains of *Brucella*, only a few studies have been performed with acellular *Brucella* vaccines, and only one has employed a purified antigen (30, 35). We considered a test of whether an adequate anti-VirB Th1-type response can be elicited in a natural host of *Brucella* particularly important. Dogs were chosen for this purpose for several reasons, including not only the easy handling of these animals but also the current lack of vaccines for canine brucellosis.

Canine infection by *B. canis*, which can be transmitted among dogs by venereal and oral routes, can spread rapidly within kennels, and given the limited efficacy of antimicrobials mentioned above, usually requires the castration or the euthanasia of the affected animals, with the consequent economic loss (36). Cases of human infection by *B. canis* have been increasingly reported among owners of infected dogs (37). Despite the economic and

TABLE 2 DTH reactions against VirB proteins in immunized dogs[a]

Dog	Sex	Age (yr)	Breed[b]	Immunization group	Challenge 1		Challenge 2	
					Antigen	DTH (mm)	Antigen	DTH (mm)
1	F	5	W	PBS	VirB7		VirB9	
2	M	4	G	PBS	VirB7		VirB9	
3	F	1	G	PBS	VirB7		VirB9	
4	F	5	B	PBS	VirB7		VirB9	
5	F	6	S	PBS	VirB7		VirB9	
6	M	1	B	VirB7	VirB7	11 × 11	PBS	
7	F	1	F	VirB7	VirB7	9 × 8	PBS	
8	F	2	B	VirB7	VirB7	9 × 9	PBS	
9	F	3	B	VirB7	VirB7	9 × 11	PBS	
10	M	2	B	VirB7	VirB7		PBS	
11	F	1	F	VirB9	VirB9	8 × 8	PBS	
12	F	1	F	VirB9	VirB9	20 × 12	PBS	
13	F	2	B	VirB9	VirB9		PBS	
14	F	3	B	VirB9	VirB9	7 × 6	PBS	
15	M	2	S	VirB9	VirB9	31 × 19	PBS	

[a] Dogs from the different immunization groups were intradermally injected with VirB proteins and/or an equal volume of PBS in the inner surface of the right thigh. The DTH reaction was evaluated 48 h later by measuring the size (two perpendicular diameters) of the induration. A reaction area larger than 5 mm (average of the two perpendicular measures) was considered positive.

[b] W, Weimaraner; G, golden retriever; B, beagle; F, French bulldog; S, miniature schnauzer.

health impact of canine brucellosis, no vaccines are available to prevent this disease.

Histological studies performed in dogs have shown that *B. canis*, as for other *Brucella* species, is an intracellular pathogen (38, 39). It establishes mainly in macrophages from secondary lymphatic organs and in epithelial cells from steroid-sensitive organs (prostate, testicles, uterus, and placenta). Therefore, a vaccine eliciting a specific Th1-type immune response with the production of IFN-γ is desirable. *Brucella* VirB proteins may be relevant antigens in canine brucellosis. A recent study has shown the presence of the 12 genes of the *virB* operon in more than 30 isolates of *B. canis* (40). Such an operon is functional, as *B. canis* strains carrying deletions or disruptions in such genes have reduced virulence in mice (41). Therefore, *B. canis*-infected cells are expected to present VirB-derived peptides in the context of MHC-II, and anti-VirB Th1 cells are expected to exert a protective role against *B. canis* infection.

In the present study, we found that PBMCs from dogs immunized with VirB7 or VirB9 mixed with QuilA adjuvant produce IFN-γ upon stimulation with these antigens, suggesting the elicitation of a VirB-specific Th1-type response. Moreover, an *in vivo* correlate of Th1-type response was also detected, since most immunized dogs had a positive DTH response to both VirB antigens.

While the production of IFN-γ by antigen-specific Th1 cells may have central importance for protection against *B. canis* infection, antibodies may also have a role. *B. canis* is a naturally rough strain, a character shared only by *B. ovis* within the *Brucella* genus (all the other species are naturally smooth). The molecular basis for this differential character is the lack of the O polysaccharide chain in *B. canis* and *B. ovis* LPS, which may leave outer membrane proteins (OMPs) more accessible to circulating antibodies. Notably, previous studies have shown that specific antibodies bind to OMPs of rough *Brucella* microorganisms (33). Moreover, it has been shown that antibodies against the outer membrane protein Omp31 can mediate complement-dependent bacteriolysis of *B. ovis* (34). *In vivo,* this lytic mechanism might have a protective role during the bacteremic phase of the infection before the entry of bacteria in their target cells. Notably, we found that sera from VirB-immunized dogs mediate complement-dependent bacteriolysis of *B. canis* cells. This effect was most probably mediated by anti-VirB antibodies since no bacteriolysis was observed with sera obtained from these dogs before immunization with VirB proteins. A direct effect of complement was also ruled out since no lysis was detected when bacteria were incubated with guinea pig serum in the absence of dog serum. While both anti-VirB7 and anti-VirB9 sera mediated the complement-dependent bacteriolysis, the degree of bacteriolysis did not increase when the assay was performed with a 1:1 mixture of these sera. This is probably because VirB7 and VirB9 locate very closely in the membrane of *Brucella* species, so that the binding of specific antibodies to one of these proteins may hinder the binding of antibodies to the other.

Collectively, the results obtained in the present study suggest that it may be possible to elicit an immune response potentially protective against *B. canis* infection in dogs through immunization with VirB7 and/or VirB9 mixed with QuilA adjuvant. The protocol used elicited both cellular and humoral immune responses that may mediate protective mechanisms. As both VirB proteins are present in all the *Brucella* species, a vaccine based on these antigens may also confer protection against brucellosis to other hosts.

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We declare no conflicts of interest.

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