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MEMBRANE VESICLES DERIVED FROM *BORDETELLA BRONCHISEPTICA*: ACTIVE CONSTITUENT OF A NEW VACCINE AGAINST INFECTIONS CAUSED BY THIS PATHOGEN

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ABSTRACT

Bordetella bronchiseptica, a Gram-negative bacterium, causes chronic respiratory-tract infections in a wide variety of mammalian hosts—humans included, albeit rarely. We recently designed *B. pertussis* and *B. parapertussis* experimental vaccines based on outer-membrane vesicles derived from each pathogen and obtained protection against the respective infections in mice. Here, we demonstrate that outer-membrane vesicles derived from virulent-phase *B. bronchiseptica* (OMVBbvir^{+}) protect mice against sublethal infection from different *B. bronchiseptica* strains, two isolated from farm animals and one from a human. In all infections, we observed that the *B. bronchiseptica* load was significantly decreased in the lungs of the vaccinated animals: the lung-recovered colony-forming units diminished by at least 4 logs below those detected in the lungs of non-immunized animals ($p<0.001$). In the OMVBbvir^{+}-immunized mice, we detected IgG-antibody titers against *B. bronchiseptica* whole-cell lysates along with an immune serum having bacterial-killing activity that both

recognized *B. bronchiseptica* lipopolysaccharides and polypeptides such as GroEL and OMPc and conferred an essential protective capacity against *B. bronchiseptica* infection as detected by passive *in-vivo*–transfer experiments.

Stimulation of cultured splenocytes from immunized mice by OMVBbvir$^+$ resulted in the presence of IL-5, INF-γ, and IL-17 production; indicating that the vesicles induced a mixed Th2, Th1, and Th17 T-cell–immune-response profile. We detected by adoptive transfer assay that spleen cells from OMVBbvir$^+$-immunized mice also contributed to the observed protection against *B. bronchiseptica* infection. Outer-membrane vesicles from the avirulent-phase *B. bronchiseptica* and the resulting induced immune sera were also able to protect mice against *B. bronchiseptica* infection

IMPORTANCE

Bordetella bronchiseptica, a Gram-negative bacterium, causes chronic respiratory-tract infections in a wide variety of mammalian hosts—humans included, albeit rarely. Several vaccines aimed at preventing *B. bronchiseptica* infection have been developed and used, but a safe and effective vaccine is still needed. The significance and relevance of our research lies in the characterization of the outer-membrane vesicles (OMVs) derived from *B. bronchiseptica* as the source of a new experimental vaccine. We demonstrated here that our formulation based on OMVs derived from virulent-phase *B. bronchiseptica* (OMVBbvir$^+$) is effective against the infection caused by *B. bronchiseptica* isolates obtained from different hosts: farm animals and a human. *In-vitro* and *in-vivo* characterization of humoral and cellular immune responses induced by the OMVBbvir$^+$ vaccine enabled a better understanding of the mechanism of protection necessary to control *B. bronchiseptica* infection. Here we also demonstrated that OMVs derived from *B. bronchiseptica* in the avirulent phase and the corresponding induced humoral immune response are also able to protect mice from *B. bronchiseptica* infection. This realization provides the basis for novel vaccine developments against not only the acute stages of the disease but also in stages of disease or infectious cycle where avirulence factors could play a role.

Key words

Bordetella bronchiseptica; Outer-membrane vesicles; Vaccine; Phenotypic phases

Introduction

Bordetella bronchiseptica is a Gram-negative bacterium that causes respiratory diseases in a variety of mammalian hosts [1]. Though this pathogen rarely infects humans, certain reports have indicated that *B. bronchiseptica* can infect immune-compromised patients or those with underlying respiratory diseases [2-4]. The respiratory infections caused by this zoonotic pathogen could also become chronic, though exhibiting few or no symptoms [5, 6]. The persistence of *B. bronchiseptica* in hosts seems to be facilitated through a modification of the expression of bacterial constituents mainly controlled by a two-component regulatory system encoded by the *bvgAS* locus [7, 8]. This system senses signals from the external environments, regulates the expression of hundreds of genes, and controls different phenotypic phases [9].

The prophylaxis of diseases caused by *B. bronchiseptica* is achieved through vaccination, but to date no satisfactory vaccine to confer protection in animals against the acute or chronic infection caused by *B. bronchiseptica* has been developed. Some of the current vaccines are composed of either killed wild-type bacterial strains (administered parentally) or live attenuated strains (administered intranasally) [10, 11]. Most of the vaccines containing the killed bacteria induce high serum antibody titers but do not always provide an effective protection against infection [10]. The data on the safety and efficacy of live attenuated vaccines are scarce. Moreover, this kind of vaccine is not well accepted because the strains included in the vaccines may revert to full or partial virulence since the basis of the original attenuation is still unknown. As to the acellular *B. bronchiseptica* vaccines, one is composed of the immunogenic *Bordetella* colonization factor-A protein while others contain pertactin (PRN), the outer-membrane protein that is a highly immunogenic virulence factor [12-15]. Although these vaccines appear to resolve mainly the issue related to adverse side reactions, no conclusive evidence has been garnered to support their immunogenicity [13, 16]. Therefore, the identification of appropriate bacterial components for the development of a new vaccine is still needed. In this search, the characteristic constituents of the avirulent phase could be included in such an evaluation since this phase seems to be involved in the infectious process [6, 8, 17].

In the work reported here, we investigated whether a vaccine based on OMVs derived from *B. bronchiseptica* in either the virulent or the avirulent phenotypic phase would be able to generate a protective immunity against an infection caused by *B.*

bronchiseptica. Vaccines based on OMVs against *B. pertussis* or *B. parapertussis* infections have recently been developed by our group [18-22]. The administration of OMV-based vaccines confers a complete protection against *B. pertussis* or *B. parapertussis* in mice. The protection against *B. pertussis* is long lasting and is mediated by both antibodies and $CD4^+$ T cells [20]. We have made the interesting observation that the protective capacity of OMVs obtained from a *B. pertussis* strain that expressed the avirulent phenotype was lower than that of the OMVs from virulent-phase *B. pertussis*, but the two were nevertheless protective in the mouse model used [23].

These results in combination permit the hypothesis that OMVs derived from *B. bronchiseptica* from either the virulent or the avirulent phase could constitute a suitable candidate for a vaccine against bordetellosis. In fact, the findings described here support this hypothesis since the protection experiments performed in the murine intranasal-challenge model demonstrated that the OMV-vaccine derived from virulent *B. bronchiseptica* was able to effect a significant decrease in the lung colonization of different *B. bronchiseptica* strains obtained from different hosts—*i. e.*, farm animals or a human. Furthermore, by performing *in-vitro* and *in-vivo* experiments, we detected that both a humoral response possessing killing capacity and immune splenic cells contributed to the protection induced by the OMVBbvir$^+$-vaccines. Moreover, we have also observed that protective capacity could be induced with a vaccine formulated from OMVs obtained from *B. bronchiseptica* in the avirulent phase.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bordetella bronchiseptica strain 9.73 (isolated from a rabbit) [24] and the mutant derivative strain defective in expression of the BvgA protein (blocked in avirulent phase) [25] were used throughout this study. *Bordetella bronchiseptica* strains were grown on Bordet Gengou agar medium (Difco, Houston TX, USA) supplemented with 10% (v/v) defibrinated sheep blood (blood was from Laboratorio Argentino S.A.). For challenge in the animal experiments, the strains from rabbits *B. bronchiseptica* 9.73 and *B. bronchiseptica* RB50 (Bb_{ra}RB50, kindly provided by Dr. Peggy Cotter of the University of North Carolina) and *B. bronchiseptica* AR705 (Bb_{hu}AR705, an Argentine clinical isolate obtained from a pediatric patient with cystic fibrosis) were also used.

Isolation of outer-membrane vesicles (OMVs)

To obtain OMVs from the bacterial cells, we used the method previously described by us [18, 22, 26]. The procedure stated in brief: Culture samples from the decelerating growth phase of the bacteria were centrifuged at 10,000 x *g* for 20 min at 4 ºC and the pellet obtained resuspended in 20 mM Tris-HCl, 2 mM EDTA, pH 8.5 (TE Buffer). Of the resulting pellet, approximately 1 g (wet weight) was resuspended in 5 ml of the TE Buffer. OMV release was promoted by sonication in ice-water; the cells were then removed by centrifugation at 10,000 x *g* and the OMV supernatant concentrated by ultracentrifugation at 40,000 x *g* for 3 h. The OMVs thus obtained were stored at 4 °C. Thereafter the OMVs were examined by electron microscopy after negative staining [22].

Protein assay

Protein content was estimated by the Bradford method with BSA as a standard [27].

One-dimensional electrophoresis and immunoblotting

OMV proteins were separated by sodium-dodecyl-sulfate–polyacrylamide-gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride (Immobilon P, Millipore USA) and probed with either a polyclonal anti(adenylate-cyclase hemolysin) [anti(AC-Hly)] antibody (1:300), an anti-PRN antibody (1:500), an anti-fimbriae (-FIM) antibody (1:500), an anti-flagellin (-FLA; 1:2,000) antibody, or anti-GroEL, an antibody against a protein analogous to the *E. coli* chaperonin GroEL (1:5,000) followed by incubation with anti(mouse IgG) conjugated with alkaline phosphatase at a 1:1,000 dilution. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate were used as the phosphatase substrates according to the manufacturer's protocol (Biodynamics SRL Buenos Aires Argentina). Some of the proteins present in the vesicles were identified by mass spectrometry after the initial separation by one-dimensional electrophoresis, as previously described [28, 29].

Bordetella bronchiseptica–lipopolysaccharide (LPS) electrophoresis was performed at room temperature and constant voltage. The LPS were visualized by the BioRad silver-staining technique.

Formulation of vaccines

To use the OMVs as acellular vaccines, the vesicle preparations were detoxified by mixing with aqueous formaldehyde (0.37% [v/v] and incubating at 37 °C overnight, and aluminum hydroxide (0.2 mg/ml) was then added as an adjuvant.

To prepare whole-cell vaccine (WCVBbvir^{+}), a suspension containing 2 x 10^{10} colony-forming units (CFUs)/ml of heat-killed (56 °C for 20 min) *B. bronchiseptica* 9.73 were detoxified in the same manner and were then mixed with aluminum hydroxide (0.2 mg/ml) as an adjuvant.

Expression of inflammatory markers upon systemic delivery of OMVs

In order to assess the proinflammatory capacity of the OMV-based vaccine formulation, mouse-blood samples were collected 4 h after each immunization by submandibular bleeding. Serum interleukin-6 (IL-6) was measured by enzyme-linked immunosorbent assay (ELISA) with the kit BD OptiEIA (BD Biosciences, CA USA) according to the manufacturer's instructions.

Active immunization and intranasal challenge

Four-week-old female BALB/c mice obtained from Biol SAIC, Argentina were used for all assays. As described previously [29], the immunization protocols comprised a two-dose schedule with the formulations described above over a period of 2 weeks. Two weeks after the second immunization, mice were subjected to a nasal challenge with a sublethal dose (10^{5}–10^{6} CFUs in 40 µl) of *B. bronchiseptica* strain. The lungs of the challenged mice were excised and collected for bacterial counts at 7 days after the challenge. The number of CFUs was determined as previously described [29]. At least three biological replicates were performed.

ELISA

Plates were coated with sonicated *B. bronchiseptica* (whole-cell lysates) in 0.5 M carbonate buffer, pH 9.5 in an overnight incubation at 4 ºC and then blocked with 3% (v/v) skimmed milk in blocking buffer (2 h at 37 ºC) before incubation with serially diluted mouse-serum samples (1 h at 37 ºC). The bound IgG was detected after a 2-h incubation with horseradish-peroxidase– (HRP)-conjugated goat anti-(mouse IgG) at a titer of 1:20,000 (Thermo Fisher Scientific, Buenos Aires Argentina). For measuring IgG isotypes, detection of bound antibody was determined

with HRP-labeled subclass-specific anti-(mouse IgG1) at 1:8,000 or IgG2a (1:1,000; Sigma Aldrich, USA). As substrate 1.0 mg/ ml o-phenylendiamine (OPD, Bio Basic Canada Inc) in 0.1 M citrate-phosphate buffer, pH 5.0 containing 0.1% hydrogen peroxide was used. For measuring IgG isotypes, the detection of bound antibody was determined with the peroxidase bound to subclass-specific anti–mouse-IgG1 (1:8,000) or -IgG2a (1:1,000; Sigma, Aldrich). Optical densities (ODs) were measured with Titertek Multiskan Model 340 microplate reader (ICN, USA) at 492 nm, and the OD was plotted as a function of the log of the (serum dilution)$^{-1}$. The inflection point of the curve was determined by the GraphPadPrims® software. Titers were defined as the reciprocal of the serum dilution giving an OD corresponding to the inflection point of the curve.

Bactericidal Assay

The bactericidal activity of the sera collected from mice two weeks after immunization with the OMVBbvir^{+} vaccine (*i. e.*, the immune sera) and from non-immunized mice (*i. e.*, the naïve sera) were tested *in vitro*. Both, immune and naïve sera inactivated by heat, and phosphate-buffered saline (PBS) were use as controls. Virulent *B. bronchiseptica* 9.73 were grown on Bordet Gengou agar medium and diluted to 1 x 10^5 CFUs/ml in PBS containing $MgCl_2$ 0.05 M, $CaCl_2$ 0.15 mM. Forty-five µl of serum or PBS were mixed with 5 µl of a suspension containing 500 CFUs of the bacteria. After 1 h of incubation at 37 °C, serial dilutions of the samples were spread on Bordet Gengou agar plates and incubated for 48–72 h to determine the CFUs. At least three biological replicates were performed.

Analysis of cellular response elicited by vaccination

The cellular response was analyzed as previously described [20]. The procedure stated in brief: Spleen cells from mice immunized with the OMV-based vaccine were harvested 8 weeks after the last immunization and seeded in 48-well culture plates at 10^6 per well in a volume of 500 µl of RPMI 1640 cell-culture medium supplemented with 10% (v/v) fetal-bovine serum (Invitrogen, Buenos Aires Argentina) containing 100 IU/ml penicillin and 100 µg/ml streptomycin. All the spleen cells were either stimulated with OMVs derived from *B. bronchiseptica* (5 µg/ml) or exposed to medium alone. Supernatants were removed after 72 h of

incubation at 37 °C in an atmosphere of 5% CO_2 and the production of interferon-γ (IFN-γ), IL-17, and IL-5 determined by ELISA (BD Biosciences, CA USA), according to the conditions specified by the manufacturer.

Adoptive transfer

Pooled serum (100 µl) or spleen cells (5 x 10^6) from non-immunized mice or from mice immunized with the OMV-based vaccine two weeks previously were transferred intraperitoneally to female BALB/c mice. Twenty-four hours thereafter the mice were infected with a sublethal dose (10^5–10^6 CFUs 40 in µl) of *B. bronchiseptica* 9.73 and the subsequent protection assessed by determining the CFU counts in the mouse lungs 7 days after the challenge.

In order to evaluate the contribution of $CD4^+$ T cells in the protection, adoptive transfer assays were also performed using spleen cells obtained from animals immunized with OMVBbvir+ and depleted in $CD4^+$ T cells. Depletion of $CD4^+$ T cells in vaccinated mice was performed by intraperitoneal injection with the monoclonal antibody from GK1.5 hybridoma (61.2 mg/ml) specific for CD4. The dosing schedule consisted in the administration to the donor animals of 200 µl of the antibody the day before spleens were collected for the passive immunization protocol. After spleen cells transfer, receptor animals received 2 doses of anti-CD4 antibody to ensure the depletion. Depletion of CD4+ T cells was confirmed by a reduction of at least 95% of the lymphocyte CD4 (+) population in blood and spleen by flow cytometry. Donor mice treated with control isotype (IgG2bk) were included in the assays for comparison purposes.

Statistical analysis

The data were evaluated statistically by one-way analysis of variance (ANOVA) followed by the Tukey test *post-hoc* (via the GraphPad Prism® software). Differences were considered significant at a $p < 0.05$.

RESULTS

Isolation and characterization of the OMVs obtained from *B. bronchiseptica* 9.73 grown in the virulent phase

The OMV samples obtained from *B. bronchiseptica* 9.73 grown in the virulent phase ($OMVBbvir^+$) were negatively stained and examined by electron microscopy

(Fig. 1, Panel A). The procedure was repeated at least 8 times, and in all the samples the size range (at dimensions of 50–150 nm) was both consistent from batch to batch and similar to that previously described for OMV preparations derived from *B. pertussis* [22]. To further characterize these OMVBbvir$^+$, one-dimensional electrophoresis (Fig. 1, Panel B) and immunoblotting (Fig. 1, Panel C) were performed. By this assay we could detect that the OMVBbvir$^+$ isolates contained AC-Hly, PRN, and FIM2 (Fig. 1, Panel C).

IL-6 levels after immunization

Usually after the systemic immunization of an animal, a rise in proinflammatory cytokines can be detected. The levels are related to the proinflammatory capability of the formulation employed. IL-6 is among the proinflammatory cytokines usually chosen as an indicator of this activity [30]. In our experiments, the OMVBbvir$^+$-containing formulations induced levels of IL-6 (425.54 ± 34.00 pg/ml) that were significantly lower than those detected for the *B. bronchiseptica* whole-cell vaccine (53,673.08 ± 5,987.44 pg/ml).

Protection against intranasal *B. bronchiseptica* challenge after vaccination with OMVBbvir$^+$

To evaluate the protective capability of the OMVBbvir$^+$ vaccine against *B. bronchiseptica* challenge, we used the murine model of intranasal infection. The CFUs recovered from the lungs of the OMVBbvir$^+$-immunized mice were compared with those assayed in mice immunized with the *B. bronchiseptica* 9.73 whole-cell vaccine prepared in our laboratory from wild-type bacteria in the virulent phase (WCVBbvir$^+$, Fig. 2, Panel A). Significant differences in the lung bacterial counts between the OMVBbvir$^+$-immunized animals and non-immunized mice were obtained ($p < 0.001$; Fig. 2, Panel A). The number of colonies recovered from the lungs of the OMVBbvir$^+$-immunized mice at day 7 after challenge was at least 4 logs lower than those detected in the non-immunized animals and similar to those from the WCVBbvir$^+$-immunized mice (Fig. 2, Panel A).

We next sought to investigate if the protective capability induced by the OMVBbvir$^+$ vaccine extended to strains other than *B. bronchiseptica* 9.73. To that end, we performed *in-vivo* protection assays using two other strains of *B. bronchiseptica* for challenge, one from a farm animal (Bb_{ra}RB50) and the other

306 recovered from a pediatric patient with cystic fibrosis (*Bb*$_{hu}$AR705). Mice were
307 accordingly immunized twice with the OMVBbvir^{+} formulation and then challenged 2
308 weeks after the second immunization with a sublethal dose of each *B. bronchiseptica*
309 strain:—*i. e.*, *Bb*$_{ra}$RB50 and *Bb*$_{hu}$AR705. As a negative control we used non-
310 immunized mice. For both strains used in the bacterial challenge, significant
311 differences in lung *B. bronchiseptica* colony-formation counts of more than 4 logs
312 were obtained between the immunized animals and the negative control group ($p < 0.001$; Fig. 2, Panel B).
313
314
315 **Characterization of the humoral immune response induced by OMVBbvir^{+}**
316 To characterize the immune response induced by the OMVBbvir^{+} vaccine
317 derived from *B. bronchiseptica* 9.73, titers of antibody were quantitatively measured
318 (Table 1). In comparison with the antibody levels detected in negative-control
319 animals, higher serum levels of specific IgG were found at 14 days after the OMV
320 priming (Table 1). Further tests were then conducted to determine the antibody
321 subtypes. Mice immunized with OMVBbvir^{+} produced high titers of specific IgG1
322 and IgG2a antibodies. The specific IgG1 titer was higher than that of the specific
323 IgG2a (IgG1:IgG2a = 2.2; Table 1). Examination of these IgG subclasses indicated
324 that the mice had responded to the OMVBbvir^{+} vaccination with a mixed Th1/Th2
325 profile, but with mainly a skewed Th2-type immune response.
326 To identify the main immunogenic proteins present in the OMVs, we analyzed
327 the antibody profile by immune proteomics. This analysis was achieved by examining
328 the reactivity of sera induced by the OMVBbvir^{+} vaccine against proteins from the
329 OMV derived from *B. bronchiseptica*. Fig. 3, Panel A shows protein bands detected in
330 OMVBbvir^{+} that cross-reacted with sera induced by vaccination with OMV. The
331 identity of some of those antigens was determined after the selected immunoreactive
332 bands were excised from the one-dimensional electrophoretic gels and then analyzed
333 by mass spectrometry. Bands a, and b in Fig. 3, Panel A, for example, were identified
334 as the GroEL-like protein and the outer-membrane protein OMPc, respectively. The
335 mass-spectrometric identification of the protein indicated as GroEL in particular was
336 subsequently confirmed by immunoreactivity with a GroEL-specific antibody (not
337 shown).
338 To investigate the presence of specific antibodies against the LPS in the
339 OMVBbvir^{+}-immune serum, we performed a Western-blot analysis to determine the

mobility on SDS PAGE of the purified wild-type *B. bronchiseptica* 9.73 LPS. The 3 expected bands were observed: a diffuse one (the lipid-A–KDO core O antigen) containing the O antigen (a single sugar polymer consisting of 2,3-dideoxy-di-N-acetylgalactosaminuronic acid) and the two other faster migrating bands—*i. e.*, Band A (the lipid-A–KDO core) and Band B (lipid-A–KDO) [31]. As anticipated, these LPS-associated bands were recognized by the immune serum induced by OMVBbvir$^+$ vaccination (Fig. 3, Panel B).

Since the activated components of complement had been previously reported to mediate—at least in part—the killing of *B. bronchiseptica* bacteria by direct bacterial lysis, we next performed a killing assay with immune serum [32]. For this determination, a suspension of 500 *B. bronchiseptica* 9.73 bacteria was incubated in 50 µl of 90% (v/v) serum in PBS to ensure that the serum components were not limiting. This assay revealed that the *B. bronchiseptica* 9.73 bacteria were sensitive to the immune sera (to a degree of *ca*. 50% survival), but resistant to naïve serum (*i. e.*, with 100% surviving; Fig. 3, Panel C). In control experiments using heat-inactivated sera we found 100% of bacterial survival for both immune and naïve sera.

To examine the specific role of antibodies in the control and clearance of *B. bronchiseptica*, serum from naïve or immunized animals was adoptively transferred into naïve animals 24 h before challenge with *B. bronchiseptica* (5 x 10^5 CFUs *B. bronchiseptica* 9.73). This OMVBbvir$^+$-induced serum—collected from the mice 14 days after the second dose—cleared *B. bronchiseptica* from the mouse lungs by day 7 after the inoculation (at a reduction of 3.5 logs), whereas naïve serum had no significant effect (Fig. 3, Panel D).

All the results presented here indicated that the OMVBbvir$^+$ vaccine induced a robust humoral immunity that effected an induced protection against *B. bronchiseptica*—at least partially—as a consequence of the killing activity of the serum.

Induction of a mixed Th1, Th2, and Th17 immune response by OMVBbvir$^+$ vaccination

To characterize the T-cell profile induced by the OMVBbvir$^+$ vaccine, we determined the levels of IFN-γ, IL-17, and IL-5 (markers of the respective Th1, Th17, and Th2 immune responses) produced by stimulated spleen-cell cultures (Fig. 4). Lymphocyte-proliferation assays revealed that OMVBbvir$^+$ vaccination effectively

374 stimulated lymphocyte proliferation (not shown). Two months after immunization,
375 higher concentrations of IFN-γ (5,514.48 ± 1,198.51 pg/mL; Fig. 4, Panel A) and IL-
376 17 (8,645.97 ± 1,796.29 pg/mL; Fig. 4, Panel B) were produced by spleen cells from
377 OMVBbvir$^+$-immunized mice than those present in non-immunized mice (Fig. 4). IL-
378 5 was also detected (469.14 ± 67.03 pg/mL) in the supernatants of stimulated
379 splenocytes from OMVBbvir$^+$-immunized mice (Fig. 4, Panel C). All these findings
380 strongly indicate that OMVBbvir$^+$ vaccination induces a mixed Th1-Th17-Th2
381 spleen-cell profile.

382 To investigate the role of OMVBbvir$^+$ immune spleen cells in protection, we
383 injected BALB/c mice i.p. with 5 x 10^6 intact spleen cells from nonimmunized
384 animals or from mice that had been immunized with OMVBbvir$^+$ vaccine 2 weeks
385 before. Twenty-four hours later, the mice were infected with 5 x 10^5 CFUs of *B.*
386 *bronchiseptica* 9.73 and then sacrificed 7 days later to determine the number of CFU
387 counts in the lungs (Fig. 4, Panel D). The transfer of spleen cells from immunized
388 animals, but not from the non-immunized mice, resulted in a reduction in bacterial
389 colonization of approximately 2 logs. To look specifically at the function of CD4$^+$ T
390 cells in the protection against *B. bronchiseptica* colonization; spleen cells from mice
391 immunized with OMVBbvir$^+$ and treated with anti-CD4 antibody were administered
392 to naive mice 24 h before challenge with a sublethal dose of *B. bronchiseptica*.
393 Depletion of CD4$^+$ T cells in the spleen of the donor animals, increased the counts of
394 bacteria recovered from the lungs of the recipient animals. The bacterial counts
395 detected in these animals were similar to those found in the lungs of mice that
396 received the spleen cells of naive animals (Fig. 3 Panel D).

397 All these results indicate that spleen cell-mediated immunity, and in particular
398 the CD4$^+$ T cells, plays a role in the protection against *B. bronchiseptica* induced by
399 the OMVBbvir$^+$ vaccine.

400

401 **Protection against intranasal *B. bronchiseptica* challenge after vaccination with**
402 **OMV from *B. bronchiseptica* blocked in the avirulent phase**

403 Since the avirulent phase of *B. bronchiseptica* could be involved in some stage
404 of the infectious cycle (i.e during the chronic infection), we sought to evaluate
405 whether an experimental vaccine containing the OMV derived from *B. bronchiseptica*
406 9.73 blocked in the avirulent phase (OMVBbvir$^-$) was able to confer protection
407 against *B. bronchiseptica* infection. To this end, OMVs were obtained from a mutant

previously constructed in our laboratory that was defective in the two-component signal-transduction regulatory system BvgA of *B. bronchiseptica* [25]. The characterization of OMVBbvir$^-$ evidenced no differences from the OMVBbvir$^+$ in their size distribution, as evaluated by electron microscopy (Fig. 5, Panel A); but certain changes in the electrophoretic profile were detected, even though the major components were the same as those of the OMVBbvir$^+$ (Fig. 5, Panel B, left lane). The lack of expression of the main virulence factors and the expression of the flagellin as an avirulent marker were evidenced upon immunoblotting (Fig. 5, Panel B, right lanes). After this initial characterization of the OMVs, intranasal *B. bronchiseptica* challenges were performed. In those experiments, we analyzed the effect of two previous administrations of the OMVBbvir$^-$ preparation on the subsequent colonization of the lungs of the experimental mice by the *B. bronchiseptica* strain 9.73 ($\approx 10^6$ CFUs in 40 µl; Fig. 5, panel C and D). The results were compared with those obtained for mice that had been preimmunized with the OMVBbvir$^+$ vaccine (Fig. 5, Panel C). PBS-injected mice served as a negative control. Significant differences in the lung bacterial counts between the immunized animals and the negative control group were observed ($p < 0.001$; Fig. 5). Of major significance was the observation that protection against *B. bronchiseptica* challenge was also achieved at same level by the OMVBbvir$^-$ immunization. The number of CFUs recovered from the lungs at day 7 after challenge likewise dropped by at least 4 orders of magnitude compared to that of the non-immunized mice (Fig. 5, Panel C).

For this experimental vaccine, we also evaluated the specific role of induced antibodies in the control and clearance of *B. bronchiseptica*. For that purpose, serum from naïve or immunized animals was adoptively transferred into naïve animals 24 h before a challenge with *B. bronchiseptica* (5×10^5 CFUs of *B. bronchiseptica* 9.73). As had been detected with the OMVBbvir$^+$ vaccine, the OMVBbvir$^-$-induced serum—collected from mice 14 days after the second dose—cleared *B. bronchiseptica* (by a reduction of 3.5 logs) from the lungs of the challenged mice by day 7 post-inoculation, whereas the naïve serum had no significant effect (Fig. 5, Panel D).

The results presented in this final section would clearly indicate that both the OMVBbvir$^-$ vaccine and its induced humoral immunity possessed the capability of protecting mice against an infection caused by *B. bronchiseptica*.

DISCUSSION

442 In this report we describe the development and evaluation of OMVs obtained
443 from *B. bronchiseptica* as vaccines against lung colonization. As we had previously
444 observed in the OMVs derived from *B. pertussis*, the sizes of the OMVs from *B.*
445 *bronchiseptica* 9.73 (at dimensions of 50–150 nm) were consistent from batch to
446 batch. Furthermore, the well known principal *B. bronchiseptica* surface immunogens
447 such as PRN, AC-Hly, and FIM were also detected in the OMVs through
448 immunoblotting. The OMVBbvir$^+$ thus characterized were then used in the murine
449 model to examine their safety and protective capability. In the first experiments
450 described, we performed comparisons with a whole-cell vaccine prepared in our
451 laboratory since a previous report had indicated that the same type of formulation in
452 dogs reduced the disease burden and the lesions in the vaccinated animals relative to
453 those of infected naïve controls [10]. We observed that two doses of our experimental
454 OMVBbvir$^+$ vaccine administered 2 weeks before *B. bronchiseptica* challenge fully
455 protected the BALB/c mice against the colonization of different *B. bronchiseptica*
456 strains obtained from diverse hosts—two representative ones being shown: a farm
457 animal and a human). As to the safety of these preparations, in comparison with the
458 whole-cell vaccine, we observed only a minimal rise in the levels of the
459 proinflammatory IL-6 in serum just after OMV vaccination. These results would
460 position the OMV-based vaccines above the classic cellular preparations; as
461 OMVBbvir$^+$ induces equal levels of protection, but along with adequate levels of
462 safety. Another significant and relevant result detected with our experimental vaccine
463 was that ability to induce protection against different isolates of *B. bronchiseptica*
464 obtained from diverse hosts. This result becomes especially pertinent upon
465 consideration of the diversity of genotypes already reported for *B. bronchiseptica* [33,
466 34] since this finding would indicate that the formulation of a specific OMV-based
467 vaccine for each specific isolate would be unnecessary.
468 In the present work, we also detected that the antibody titers of OMVBbvir$^+$-
469 vaccinated mice were higher on day 14 after the second dose and before *B.*
470 *bronchiseptica* challenge than those of the non-immunized control group. Moreover,
471 this OMVBbvir$^+$-vaccine–induced immune serum recognized a group of antigens that
472 included OMPc and the GroEL-like protein along with LPS. That the OMPc and
473 GroEL-like protein had been previously detected in mice immunized with OMV
474 derived from *B. pertussis* [23] was of interest to us. As discussed in a previous
475 publication, both proteins in *Bordetella* and other microorganisms have been

476 described as promising vaccine targets [35, 36]. By immunoblotting assays we also
477 detected that the OMVBbvir$^+$ was able to induce antibodies against these LPS, an
478 immunogenic bacterial component with proven protective capability [37]. Based on
479 the detection of these protection-inducing immunogens, we next sought to investigate
480 if the OMVBbvir$^+$ vaccine-induced antibodies alone were sufficient to confer
481 protection against *B. bronchiseptica.* The passive-transfer assays we therefore
482 performed demonstrated that the vaccine-induced serum reduced the number of viable
483 bacteria in the lungs by about 4 logs. This protective role of the antibodies was in
484 accordance with previous results reported by other authors [38]. In fact, protection
485 against *B. bronchiseptica* infection had been evidenced with antibodies induced by
486 either infection or vaccination [38] through mechanisms that appeared to be different:
487 whereas the immunity induced by previous infection offered significant protection
488 even in the absence of complement or the induction of the monocyte IgG receptors
489 Fc□Rs, the vaccination-induced protection required both complement and the Fc□Rs
490 [38].

491 In addition, we found that CD4$^+$ T cells also contributed to the protection
492 exhibited by the OMVBbvir$^+$-based vaccine. The protection against *B. bronchiseptica*
493 infection induced by the OMV-based vaccine seems to involve a dual mechanism
494 involving both a humoral and a cellular immune response as was evidenced by
495 adoptive transfer experiments.

496 Based on our previous results with OMVs derived from *B. pertussis* in the
497 avirulent phase, [23] and taking into account the potential role of that phase in the
498 induction of *B. bronchiseptica* the infection process (i.e chronic infection), we
499 decided to evaluate if the OMVs derived from *B. bronchiseptica* blocked in the
500 avirulent phase (OMVBbvir$^-$) were also able to induce protection. The OMVBbvir$^-$
501 formulation indeed proved to be protective, as judged by the significant decrease of 4
502 logs observed in the lung bacterial counts between avirulent OMVBbvir$^-$-immunized
503 mice and the non-immunized control group ($p < 0.001$; Fig. 5, panels C and D). The
504 detected flagella of *B. bronchiseptica* in the OMVBbvir$^-$ preparation probably
505 contributes to the protective capacity of the OMVBvir$^-$ formulation since it has been
506 described as a potent proinflammatory factor that induces chemokines, cytokines and
507 expression of the host defense gene [39]. In addition, it was demonstrated that *B.*
508 *bronchiseptica* flagellin is able to effectively signal through both human and mouse
509 the Toll-like receptor 5 [39]. The protective effect is elicited by non-replicative

avirulent components that have thus far not been reported as capable of conferring protection against *B. bronchiseptica*. To our knowledge, the only report regarding a vaccine with avirulent *B. bronchiseptica*, was done in dogs with a live, intact avirulent strain [40]. Moreover, the authors demonstrated that the vaccinated dogs were protected against colonization by as early as 48 h after immunization [40].

Considered all together, the results presented here provide clear evidence that the OMVs derived from *B. bronchiseptica* exhibit a high level of protection against *B. bronchiseptica* that is not dependent on bacterial virulence-factor expression. Moreover, we further demonstrate that the protection induced by these OMVBb-based vaccines is mediated mainly by antibodies but also by CD4+T cells.

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Table 1. Serum IgG and isotypes antibodies titers.

	Total IgG	IgG2a	IgG1	IgG1:IgG2a
OMVBbvir$^+$ immunized	259.6 ± 32.4	250.2 ± 71.9	558.7 ± 124.3	2.2
Non-immunized	9.3	ND	11.3	

LEGENDS TO THE FIGURES

Fig. 1. Panel A: Transmission-electron-microscopy image of the negatively stained preparations obtained from *B. bronchiseptica* 9.73 in the virulent phase (OMVBbvir$^+$; scale bar: 200 nm). Panel B: Analysis of OMVBbvir$^+$ by 12.5% (w/v) SDS-PAGE. The bands were visualized by staining with Coomassie brilliant blue R-250. Molecular weights in kDa are indicated on the left. Panel C: Immunoblots of OMVBbvir$^+$ and purified proteins indicating the bands binding to anti-AC-Hly–, anti-PRN–, and anti-FIM2–specific polyclonal mouse antibodies. In the figure, the sources of the samples are indicated above the panels.

687 **Fig. 2.** Effect of (i.p.) immunization with active OMVBbvir$^+$ in the mouse-intranasal-
688 challenge model. WCVBbvir$^+$ was used as a positive control. Panel A: *B.*
689 *bronchiseptica* 9.73 was used as challenge bacteria (5 x 10^5 CFUs in 40 µl). Panel B:
690 Rabbit *B. bronchiseptica* RB50 and the human clinical isolate *B. bronchiseptica*
691 AR705 were also used as challenge bacteria (5 x 10^5 CFUs in 40 µl). In all instances
692 three biological replicates were performed, with the results from a representative one
693 being presented. Results depicted are the means of four mice per group at 7 days after
694 challenge. The line indicates the lower limit of detection. In both panels, the number
695 of bacteria recovered from the mouse lungs, expressed as the $\log_{10}$means ± SEM
696 (error bars) of the CFUs per lungs, is plotted on the *ordinates* for the samples from the
697 lungs of the experimental groups indicated on the *abscissas*. The *bronchiseptica*
698 strains used for the challenges are depicted above the panels. The asterisks (*)
699 indicate significant differences at a $p < 0.001$.
700
701 **Fig. 3.** Panel A: Analysis of OMVBbvir$^+$ antigenic reactivity by 12.5% (w/v) SDS-
702 PAGE visualized by staining with Coomassie Brilliant Blue (Lane 1). Molecular
703 weights in kDa are indicated on the left. Immunoblots (Lane 2) with the OMVBbvir$^+$-
704 protein sample were probed against polyclonal antiserum obtained from mice
705 immunized with OMVBbvir$^+$. Bands a and b were identified as GroEL and OMPc
706 respectively. Panel B: Analysis of purified *B. bronchiseptica* LPS by 15% (w/v) SDS-
707 PAGE (Lane 1) and by immunoblotting with the polyclonal antiserum obtained from
708 mice immunized with OMVBbvir+ (Lane 2). The arrows indicate the locations of the
709 O antigen and the bands A and B that were recognized by the antiserum used in
710 immunoblotting in Lane 2. Panel C: *In-vitro*–killing assay of *B. bronchiseptica* with
711 immune sera derived from OMVBbvir$^+$-vaccinated animals and from non-immunized
712 mice. Three biological replicates were performed, with the results from a
713 representative one being presented. The asterisks (*) indicate significant differences at
714 a $p < 0.05$. In the panel, the percent survival of the bacteria is plotted on the *ordinate*
715 for each of the sera indicated on the *abscissa*. Panel D: Effect of passive
716 immunization with sera collected from OMVBbvir$^+$ immunized mice. *B.*
717 *bronchiseptica* 9.73 was used as the challenge bacterium (5 x 10^5 CFUs in 40 µl).
718 Three biological replicates were performed, with the results from a representative one
719 being presented. The data are the mean values from four mice per group at 7 days
720 after challenge. The line indicates the lower limit of detection. In the panel, the

721 number of bacteria recovered from the mouse lungs, expressed as the $\log_{10}$(means ±
722 SEM [error bars]) of the CFUs per lungs, is plotted on the *ordinate* for each of the
723 experimental groups on the *abscissas*. The asterisks (*) indicate significant
724 differences at a $p < 0.001$.
725
726 **Fig. 4.** Panels A–C: Cytokine production by splenocytes from immunized mice.
727 BALB/c mice were immunized with two doses of OMVBbvir$^+$ or left non-
728 immunized. Two months after the final immunization, the mice were sacrificed and
729 their cultured spleen cells stimulated with OMVBbvir$^+$ or simply incubated in the
730 culture medium (negative control). After 72 h, the concentrations of IFN-γ (Panel A),
731 IL-17 (Panel B), and IL-5 (Panel C) were determined by ELISA in the culture
732 supernatants. The results are expressed as the mean values (± the standard error) of
733 three experiments with 4 mice per group. Significant differences were analyzed for
734 each cytokine between non-immunized and immunized mice ($p \leq 0.01$, Panel A) or p
735 < 0.001 (panels B and C). In each of the three panels, the concentration of the cytokine
736 indicated on the *ordinate* in -pg.ml^{-1} that was assayed in the culture supernatant is
737 plotted for the two experimental conditions—exposure to medium or vesicles—
738 designated on the *abscissa*. Panel D: Effect of passive immunization with spleen cells
739 collected from OMVBbvir$^+$ immunized mice with or without depletion of CD4$^+$ T
740 cells. Donor animals were i.p. injected with anti-CD4 antibody or the corresponding
741 isotype control 24 h before the spleens were collected. After the i.p. injection of
742 spleen cells from OMVBbvir$^+$-immunized or non-immunized mice, as indicated on
743 the abscissa, the recipient mice were challenged with *B. bronchiseptica* 9.73 (5×10^5
744 CFUs in 40 µl). Three biological replicates were performed, with the results from a
745 representative one being presented. The data represent the means of four mice per
746 group at 7 days after challenge. The line indicates the lower limit of detection. In the
747 panel, the number of bacteria recovered from the mouse lungs, expressed as the
748 $\log_{10}$means ± SEM (error bars) of the CFUs per lungs, is plotted on the *ordinate* for
749 the samples from the lungs of the mice receiving the spleen cells from the source
750 indicated on the *abscissa*. In all four panels, the white bars indicate spleen cells from
751 non-immunized mice and the black bars those from immunized mice. The asterisks
752 (*) indicate significant differences at a $p < 0.001$.
753

Fig. 5. Panel A: transmission-electron-microscopy image of negatively stained OMVs obtained from *B. bronchiseptica* 9.73 in the avirulent phase (OMVBbvir$^-$; scale bar: 200 nm). Panel B: Analysis of OMVBbvir$^-$ by 12.5% (w/v) SDS-PAGE with visualization by Coomassie Brilliant Blue. Molecular weights are indicated (on the left side). On the right side, immunoblots of OMVBbvir$^-$ and the purified recombinant proteins AC-Hly, PRN, FLA, and FIM2, as detected by the respective specific polyclonal mouse antibodies. Panel C: Effect of active (i.p.) immunization with OMVBbvir$^-$ in the mouse intranasal challenge model. Panel D: Effect of passive immunization with sera collected from OMVBbvir$^-$-immunized mice. For panels C and D, *B. bronchiseptica* 9.73 was used as the challenge bacterium (1 x 10^6 CFUs in 40 µl). Three biological replicates were performed, with the results from a representative one being presented. The data represent the means of four mice per group at 7 days after challenge. The line indicates the lower limit of detection. In the panels, the number of bacteria recovered from the mouse lungs, expressed as the $\log_{10}$means ± SEM (error bars) of CFUs per lungs, is plotted on the *ordinates* for the lung samples from the mice receiving the immunogen—in the form of vesicles (Panel C) or serum (Panel D)—indicated on the *abscissas*. The asterisks (*) indicate significant differences at a $p < 0.001$.