Effective protection of mice against *Shigella flexneri* with a new self-adjuvant multicomponent vaccine

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Abstract

**Purpose.** The aim of this study was to develop an immunogenic protective product against *Shigella flexneri* by employing a simple and safe heat treatment-based strategy.

**Methodology.** The physicochemical characteristics of naturally produced (OMV) and heat-induced (HT) outer-membrane vesicles from *S. flexneri* were examined, including a comparison of the protein content of the products. Toxicological and biodistribution studies, and a preliminary experiment to examine the protective effectiveness of HT in a murine model of *S. flexneri* infection, were also included.

**Results.** This method simultaneously achieves complete bacterial inactivation and the production of the HT vaccine product, leading to a safe working process. The obtained HT complex presented a similar morphology (electron microscopy) and chemical composition to the classical OMV, although it was enriched in some immunogens, such as lipoproteins, OmpA or OmpC, among others. The HT formulation was not toxic and biodistribution studies performed in mice demonstrated that the vaccine product remained in the small intestine after nasal administration. Finally, a single dose of HT administered nasally was able to protect mice against *S. flexneri* 2a.

**Conclusion.** The convenient and safe manufacturing process, and the preliminary biological evaluation, support the use of the self-adjuvanted HT complex as a new vaccine candidate to face shigellosis. Further development is required, such as additional immune analyses, to evaluate whether this new subunit vaccine can be useful in achieving full protection against *Shigella*.

INTRODUCTION

Diarrhoea remains the second leading cause of death among children under 5 years old. Shigellosis is estimated to be responsible for about 170 million cases per annum and although estimates of deaths due to *Shigella* depend on the methods used, the figure may be as high as 700 000 deaths per year [1, 2].

Bacillary dysentery derives from an acute intestinal infection caused by bacteria of four species of the genus *Shigella*: *S. dysenteriae*, *S. sonnei*, *S. flexneri* and *S. boydii*. Among these, *Shigella flexneri*, and specifically serotype 2a, shows the highest rates for being endemic in developing countries. In fact, *S. flexneri* is a leading cause of childhood morbidity and mortality, particularly in developing countries, while it is the species that causes Reiter’s syndrome, as a late complication of shigellosis [3]. There is great interest in *S. flexneri* vaccine development, driven by the high disease burden and the high cost of antibiotics, in parallel with emerging antibiotic resistance [4]. However, no licensed vaccine exists.

Numerous strategies to develop vaccines against *Shigella* have been explored. The search has included live attenuated and non-living ones [5]. Within the second approach, which is safer, killed whole bacteria [6], glycoconjugates [7, 8], outer-membrane proteins (OMPs) [9], Ipa proteins [10] and multicomponent preparations, such as outer-membrane vesicles (OMVs), are being followed [11]. OMVs are naturally released by virtually all Gram-negative bacteria, and predominantly contain outer-membrane components with periplasmic compounds entrapped in the lumen [12]. Our
group investigated the potential of these OMVs derived from *S. flexneri* as a way of discovering vaccine candidates [13]. We demonstrated that the immunization of mice by the mucosal route [e.g. intranasal (i.n.) or intragastric] with OMVs derived from *S. flexneri* 2a conferred protection against a lethal dosage with the homologous strain. These data indicate that OMVs have potential for vaccine development. However, the current production of naturally released OMVs is not satisfactory, and a robust, scalable and safe production process is required to produce large batches for clinical use. Different strategies have been reported for increasing the yield of OMVs. The disruption of genes involved in the secretion of the vesicles can induce a high level of bleb shedding. The deletion of *gna33* in *Neisseria meningitidis* [14], and the modification of Tol–Pal system proteins, which are required to maintain outer-membrane integrity, are able to destabilize the cell envelope and induce the production of large amounts of vesicles [15]. In fact, the deletion of *tolR* in *E. coli* [16] and the disruption of *TolA* protein in *Shigella boydii* [17] triggered a significant increase in the production of OMVs. A different strategy is being followed with *Neisseria meningitidis* for the production of the Bexsero vaccine, which combines OMVs obtained after detergent treatment to selectively remove LPS and the inclusion of recombinant antigens designed by reverse vaccinology [18]. Alternatively, OMVs have been obtained from recombinant *N. meningitidis* strains where LPS has been genetically detoxified (lpx1-mutants), avoiding the need for detergent extraction [19]. However, these methods still require the use of cultures of virulent bacteria. Building upon the encouraging results obtained with OMVs spontaneously released during in vitro growth by *S. flexneri*, our strategy was to force the release of these vesicles through heat treatment, an approach that was used successfully with *Brucella* spp. and *Salmonella enterica* cells [20, 21]. It is suggested that the expansion of the external monolayer of the outer membrane relative to the inner monolayer induces membrane curvature, which forces the outer membrane to bleb out [22]. Accordingly, high temperatures can be used to alter the fluidic state within the membrane and produce blebbing, but can also induce bacterial inactivation via DNA through strand breaking and rRNA degradation [23].

The aim of this study was, then, to examine the physicochemical characteristics of naturally produced (OMV) and heat-induced (HT) OMVs from *S. flexneri*, including a comparison of the protein content of the products. Finally, we included toxicological and biodistribution studies, and a preliminary experiment to examine the protective effectiveness of HT in a murine model of *S. flexneri* infection.

**METHODS**

**Production of OMV and HT complexes**

A clinical isolate of *Shigella flexneri* 2a (University of Navarra Hospital, Pamplona, Spain) was used for the study concerning antigenic extract production. The strain was maintained frozen in cryobeads (Nalgene). OMVs were obtained as previously described [24]. Briefly, overnight cultures on tryptone soy agar (TSA; Biomerieux, Marcy L’Etoile, France) were used to inoculate 50 ml of tryptic soy broth (TSB; Biomerieux) and grown at 37 °C with shaking (140 r.p.m.) to log phase [optical density at 600 nm (OD_{600}) ~ 0.3]. This culture served to inoculate the final batch containing 500 ml TSB, which was incubated at 37 °C overnight (OD_{600} of ~ 1.5). The bacteria were inactivated with binary ethyleneimine [13] before the cells were removed by centrifugation (6000 g, 20 min), and then the supernatants were collected and filtered using a filter with a pore diameter of 0.22 µm. The OMVs were recovered by diafiltration through a 0.22 kD tangential filtration concentration unit (Millipore). Finally, the retentate was centrifuged (51 000 g, 2 h), lyophilized and stored at room temperature.

HT extracts were obtained as previously described [21] with some modifications. Bacterial cultures, obtained as indicated above, were heated in flowing steam (100 °C) for 15 min. After centrifugation at 6000 g for 20 min, the supernatants were filtered (0.22 µm) to eliminate whole cells. Then, these supernatants were concentrated and purified using 300 kDa tangential filtration (Millipore). The final extracts were harvested by centrifugation (57 000 g, 2 h), and the pellets (HT) were resuspended in deionized H₂O, lyophilized and stored at room temperature. To quantify the OMV and HT yields, the obtained final product amount (dry weight) was referred to the corresponding cellular pellet obtained after first centrifugation (wet cell weight).

**Charge, size distribution and visualization of OMV and HT samples**

The charge and vesicle size distribution were measured by dynamic light scattering (Malvern 4700 system). In addition, OMV and HT samples and whole cells were examined by transmission electron microscopy (Zeiss Libra 120 EETEM). Briefly, OMV and HT samples suspended in ddH₂O were laid on copper grids with a film of formvar (EMS, FF200-cu) for 30 s at room temperature. The samples were washed three times with ddH₂O and finally a negative staining was performed with 3 % uranyl acetate.

Whole bacteria were centrifuged (10 000 g, 7 min) and resuspended in 4 % glutaraldehyde in 0.1 M cacodylate. Then the cells were centrifuged (10 000 g, 7 min) and washed with cacodylate/sucrose solution. The cells were then fixed with 1 % osmium and incubated for 1 h at 4 °C. The bacteria were centrifuged, washed and incubated in 2 % agarose. Finally, the samples were incubated at 4 °C overnight, dehydrated and included in Epoxy embedding medium.

**Chemical characterization**

The total protein content was determined by Lowry’s method in the presence of 2 % SDS, with bovine serum albumin as standard (Sigma Aldrich).

Proteome analysis was performed by mass spectrometry to identify the proteins from the OMV and HT samples, using three independent batches. Briefly, 1 mg of the respective
preparations was subjected to trypsin digestion. The peptides were solubilized in 1% trifluoroacetic acid and further extracted using a C18 reverse-phase solvent (Pierce C18 Spin Tips) following the manufacturer's protocol. The extracted peptides were dried in a Speed Vac and subjected to mass spectrometry analysis. MS/MS data acquisition was performed using Analyst 1.5.2 (Sciex) and the spectra files were processed via Protein Pilot™ software (v 5.0 Sciex) using the Paragon™ algorithm for the database search and Progroup for the data grouping, and searching against the UniProtKB proteome reference. The false discovery rate was performed using a non-linear fitting method and a result group file was created that only reported results with a global false discovery rate of 1% or better. The peptide quantification was performed using Progenesis LC-MS software (Nonlinear Dynamics). The runs were aligned automatically and manually supervised. The peptide identifications were exported from Protein Pilot and imported into Progenesis LC-MS, where they were matched to the respective features. For quantification, only unique peptides were included, and the total cumulative abundance was calculated by summing the individual abundances of all the peptides assigned to each protein.

**Qualitative assessment**

The protein content of the OMV and HT extracts was compared using SDS-PAGE stained with Coomassie Blue for proteins, or silver stain for LPS after proteinase K digestion [25]. For immunoblotting, samples were transferred onto nitrocellulose membranes and incubated with sera from rabbit hyper-immunized with *S. flexneri* as previously described [24].

**Cytotoxicity and cellular activation studies**

RAW 264.7 macrophage cells (Rockville, MD, USA) were maintained at subconfluence in 95% air and a 5% CO₂ humidified atmosphere at 37°C. For routine subculture, we used complete RPMI medium supplemented with L-glutamine (Gibco) supplemented with 10% foetal bovine serum (Gibco) and penicillin (10,000 units/ml)/streptomycin (10,000 µg ml⁻¹) solution (Gibco). The MTT assay was used to measure the cytotoxicity induced by the bacterial vesicles. Briefly, RAW 264.7 macrophage cells were incubated in 96-well flat-bottom microplates (Falcon) for 24 h in complete RPMI medium. Then the cells were treated with different concentrations of OMV or HT. After a period of 2 or 24 h, a mixture of 25 µl MTT solution and 225 µl RPMI medium was added. Following 4h of incubation, the supernatants were removed and 250 µl of pure DMSO (Panreac, Spain) was added to the samples in order to dissolve the reduced MTT-formazan crystals. Finally, the absorbance was measured at 540 nm (Thermo Scientific). As a positive control, cells were treated with 0.5% Triton X-100 (Sigma-Aldrich). The results were referenced against untreated cells.

**Cell stimulation and phenotyping**

To investigate the effect of OMV and HT on cell stimulation, RAW 264.7 cells were seeded in 96-well tissue-culture plates (Falcon) and, after overnight incubation, the cells were treated with various concentrations of the bacterial vesicles (0–150 µg ml⁻¹). The cells were incubated at 37°C for 24 h and then stained with fluorescent-labelled antibodies against the following markers: CD14, MHC class II and CD40 (Life Technologies). Flow analysis was conducted on an Attune cytometer (Applied Biosystems) and assessed for the percentage of fluorescent staining and staining brightness. Free LPS from *S. flexneri* was used as a positive control.

**Mice immunization and challenge**

All mice were treated in accordance with institutional guidelines for the treatment of animals (Ethical Committee for Animal Experimentation of the University of Navarra, Spain, protocol reference CEEA 164/14). Nine-week-old female BALB/c mice (20±1 g) were separated into randomized groups of six animals and immunized with a single nasal dose of OMV or HT (20 µg). Following intranasal immunization, mice were mildly anaesthetized with a mixture of 0.2 ml of 150 mg kg⁻¹ of ketamin (Imalgene 1000, Merial) and 10 mg kg⁻¹ xylazene (Rompum, Bayer) by the intraperitoneal route. The preparations were slowly instilled by micropipette into both nares. Challenge infection was performed at week four, intranasally with a sublethal dose of 1×10⁵ c.f.u./mouse of *S. flexneri* 2a grown to logarithmic phase and suspended in 20 µl of pre-warmed PBS. After 48 h, the mice were sacrificed and the lungs were harvested and homogenized using a gentleMACS Dissociator (MACS Miltenyi Biotec), and then plated on TSA plates to determine the number of c.f.u.

Moreover, in order to elucidate the protection against a lethal infection of *S. flexneri*, the survival curves of the previous groups were established in a parallel experiment. Briefly, mice were immunized nasally with 20 µg of OMV or HT (six mice per group). The challenge infection was performed at week four, intranasally with a lethal dose of 4×10⁶ c.f.u./mouse of *S. flexneri* 2a, and the mice were then monitored for 9 days post-challenge. The percentage (%) survival was calculated for each day and compared to that for the non-immunized control group.

**Specific antibody response**

Specific IgG1 and IgG2a antibodies against OMV or HT in sera were determined by ELISA at weeks 0, 1, 2, 3 and 4 post-immunization. In brief, 96-well microtitre plates (Maxisorb; Nunc, Wiesbaden, Germany) were coated with OMV or HT (10 µg well⁻¹) in coating buffer (60 mM carbonate buffer, pH 9.6). Unspecific binding sites were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Sera from the immunized mice were diluted 1:80 in PBS with 1% BSA and incubated for 4 h at room temperature. After intensive washing with PBS Tween20 (PBS-T) buffer, the alkaline phosphatase-conjugated detection antibody, class-specific goat anti-mouse IgG1 or IgG2a (Sigma) was added and incubated for 1 h at 37°C. The detection reaction was performed by incubating the sample with H₂O₂-ABTS substrate chromogen for 20 min at room...
temperature. The absorbance was measured with an ELISA reader (Sunrise; Tecan-Austria) at a wavelength of 405 nm.

**Biodistribution**

**Technetium-99m radiolabelling of OMV and HT vesicles**

Radiolabelling of OMV and HT samples was performed by 99mTcO₄⁻ reduction with Sn⁴⁺. Sodium pertechnate was obtained by the elution of a 99Mo-99mTc generator (10 GBq Drytec; General Electric) following the manufacturer’s instructions. Several concentrations of SnCl₂·2H₂O (1.0, 0.5, 0.05 and 0.005 mg ml⁻¹) were tested and for further radiolabelling experiments we chose a higher concentration in which just a small amount of 99mTcO₄⁻ was left unreacted (1 mg ml⁻¹). We could hence ensure that virtually no 99mTc-tin colloids were produced during the radiolabelling reaction. Ten mg of OMVs or HT complex were pre-tinned with an HCl-acidified tin chloride solution, 99mTcO₄⁻ in saline was added, and then reduction carried out in a non-oxidizing atmosphere using He-purged vials and solutions. The radiochemical purity of the radio-labelled vesicles was checked by thin layer chromatography (TLC) using Whatman 3 MM strips developed with 0.9 % NaCl. The radioactivity distribution was measured and quantified using a radio-TLC system (miniGITA, Raytest GmbH, Straubenhardt, Germany). Using the optimized conditions (0.5 mg ml⁻¹ SnCl₂·2H₂O), radiolabelling proceeded with >95–97 % yield, thus avoiding the need for further purification of the radiolabelled product.

**Single photon emission computed tomography/computed tomography (SPECT/CT) studies**

BALB/c mice (n=6) received 3 µl of 99mTc-radiolabelled OMV or HT in each nostril (0.37 MBq per animal). Control studies (n=3 mice) were carried out using 0.63 MBq of free 99mTcO₄⁻ instead of 99mTc-radiolabelled bacterial vesicles. Single photon emission computed tomography/computed tomography (SPECT/CT; SYMBIA-T2 TruePoint; Siemens, Munich, Germany) studies were performed on isoflurane-anaesthetized animals at 1, 2, 4, 6, 8 and 24 h post-administration. Animals were woken up immediately after each imaging study. The SPECT acquisition protocol was set to account for radionuclide decay, increasing the acquisition time proportionally for the corresponding images according to the half-life of 99mTc (6.01 h). For quantitative analysis, data sets were exported to PMOD software (PMOD Technologies Inc., USA) and the volumes of interest (VOI) were drawn over CT images on selected regions (administration site, stomach, upper intestine), while count ratios were calculated with respect to the total animal counts and the maximum counts were normalized to the last image (24 h) for each vesicle type. After the last SPECT/CT image, the animals were euthanized with T-61 euthanasic, perfused with 10 ml saline and selected organs (liver, spleen, intestine, stomach, kidney, lungs, thymus gland, brain, thyroid, nose and blood sample) were extracted, weighed and counted in a gamma counter (1282 Compugamma CS; LKB Pharmacia, Finland) calibrated for 99mTc energy. Finally, the results were corrected for decay and expressed as the percentage of injected dose per organ (% ID/organ).

**Toxicity studies in rats**

The experimental protocols involving animals were carefully reviewed and approved by the Ethical Committee for Animal Experimentation of the University of Navarra, Spain (protocol reference CEEA/011-15). Eight-week-old female Wistar rats were purchased from Harlan (Horst, the Netherlands) and employed for an acute toxicity study. The rat model was chosen with regard to the high amount of blood needed for all of the haematological and biochemical parameters. On the day of arrival, the animals were weighed in order to ensure that body weight (BW) variation did not exceed ±20 %. They were randomly housed in groups in polycarbonate cages (Makrolon) with stainless steel covers to allow acclimatization to the environmental conditions (12 h day/night cycle, temperature 22±2 °C, relative humidity 50±20 %, standard diet from SAFE ad libitum and water ad libitum). This study was designed according to OECD guideline 425, CPMP/SWP/465/95 and WHO guidelines on non-clinical evaluation of vaccines, testing the nasal route of administration. Twenty-four female Wistar rats were randomly divided into different groups depending on formulation and dosage. The doses selected for each formulation type, OMV or HT, were 5.5, 17.5 and 55 mg kg⁻¹ for each animal. After administration, each animal was observed for a period of 48 h for signs of toxicity, mortality or changes in BW. The animals were observed individually for any clinical signs or any symptoms of toxicity at different time intervals after administration (10, 30 min, 1, 3 and 6 h) and daily for 14 days. On completion of the study, on day 14, blood samples were extracted from the retro-orbital sinus under isoflurane anaesthesia. The following haematological parameters were analysed: haemoglobin (Hb; g/dl), haematocrit (HCT, %), red blood corpuscle count (RBC), white blood corpuscle count (WBC), absolute erythrocyte indices and differential WBC. Biochemical analyses of the plasma samples were performed with a Hitachi 911 (Roche Diagnostics) analyser using the protocols obtained from Roche for the determination of the standard parameters: total protein (g/dl), albumin (g/dl), glucose (mg/dl), aspartate transaminase (AST; U/l), alanine transaminase (ALT; U/l), cholesterol, creatinine, urea (mg/dl) and total bilirubin. At the end of the study, the animals were sacrificed in a CO₂ chamber and subjected to necropsy, and various organs were collected, weighed and fixed for further histopathological examination. Tissue samples from different body organs (including heart, thymus, kidney, liver, spleen and ovaries) were taken during the necropsy for both toxicity studies, fixed in 4 % formaldehyde solution, dehydrated and embedded in paraffin. The paraffin sections (3 µm) were cut, mounted onto glass slides, and dewaxed and stained with haematoxylin and eosin (H and E) for the subsequent histopathological examination.

**Statistical analysis**

All statistical significance analyses were carried out using the parametric one-way ANOVA test (with Tukey’s post hoc test). P values of <0.05 were considered to be statistically
significant. All calculations were performed using SPSS statistical software (SPSS 15.0, Microsoft, USA).

RESULTS

OMV and HT characterization

OMV and HT preparations were obtained from controlled cultures (37°C, at 140 r.p.m. to the stationary phase, OD<sub>600</sub> of ~1.5). The transmission electron microscopy analysis of whole bacteria, either untreated or heat-treated (100°C, 15 min), showed that both release liposome-like vesicles, ranging from 20 to 250 nm. Moreover, bacteria images confirmed that heat treatment did not induce bacterial lysis (Fig. 1a, d). The corresponding purified products, OMV and HT, were characterized by their spherical bilayer shape. No bacterial debris or other structures were observed, confirming the purity of the vesicles (Fig. 1b, e). The size distribution of the purified vesicles, as determined by dynamic light scattering, indicated that the majority of OMVs (75.3 %) had a diameter of 100–150 nm, whereas a few vesicles (20 %) had a diameter of 50–100 nm. The HT vesicles showed decreased size in comparison with the control OMVs, with 56.7 % of vesicles being in the range of 100–150 nm, and 40 % having a size of 50–100 nm (Fig. 1c, f).

The corresponding yields, obtained from three independent experiments, indicated that heat treatment increased the production of membrane vesicles by around 4× compared with the control OMV recovery method (8.9±4.5 mg g<sup>−1</sup> versus 2.1±0.5 mg g<sup>−1</sup>, respectively, with respect to the cell wet weight).

From these results, a comparative characterization of the two vesicle products was performed. Quantitative analysis showed a similar protein content, 34.2±4.6 % in the case of OMV, and 32.2±4.2 % in HT. Comparative SDS-PAGE analysis of the antigenic extracts was performed after equivalent amounts of OMV and HT extracts were loaded from the same culture batch. According to the higher-density stained bands, 14 different bands were excised from the gel and identified by proteomics (Fig. 2), revealing major outer-membrane proteins, such as OmpC (40 kDa), OmpA (37 kDa), OmpW (22 kDa) or OmpX (18 kDa) in both extracts.

Western blot was also performed for OMV or HT to determine the reactivity against sera from hyperimmunized rabbits with <i>S. flexneri</i>. The highest IgG reactivity was against OmpX, OmpC/A, MipA and IpaC proteins derived from HT samples (Fig. 2).

Quantitative proteome analysis was performed on OMV and HT samples from three different purification processes. The results demonstrate that HT is enriched (<i>P</i>&lt;0.05) in outer-membrane proteins (OmpA, OmpC, IcsA, IcsP, OmpX, including lipoproteins LolB and Pal). OMV,
however, is enriched in the virulence proteins, such as VirB or VirF. OMV was enriched in ribonucleoproteins and nucleotide-binding proteins. This unexpected location of cytoplasmic proteins did not appear to occur as the result of cell death. The identification of cytoplasmic proteins in OMVs has been suggested to be related to an adsorption process [26]. No significant differences were detected between the products for Ipa proteins (Fig. 3).

In the context of vaccination, differences in the LPS content should also be taken into account, since LPS itself can contribute to the functional immunogenicity of proteins. We compared the LPS profiles in silver-stained gels after loading equivalent amounts of OMV and HT extracts (Fig. 2). The image showed the typical ladder pattern of bimodal \textit{S. flexneri} LPS, with a similar distribution for both samples. Control-free LPS is shown in the figure.

**Cell proliferation assays**

RAW 264.7 macrophage cells were treated with different concentrations of OMV or HT to evaluate their cytotoxicity compared with the control untreated cells. No significant cytotoxicity was observed after 2 or 24 h of incubation with up to 200 µg ml$^{-1}$ of any product (Fig. 4). The positive control (Triton X-100-treated cells) values were 7.75±3.5 and 8.97±7.5 %, at 2 and 24 h, respectively.

**In vitro activation of raw macrophages**

MHC class II and CD40 molecules are two major components of the immune system that are involved in antigen recognition and signal transduction. Thus, RAW 264.7 cells were incubated with 10 µg ml$^{-1}$ of either OMV or HT for 24 h and both cell surface activation markers were measured. Free LPS was used as a positive control. OMV and HT vesicles induced the expression of CD40 and MHCII on the surface of the macrophages (Fig. 5).

**Biodistribution in mice**

Biodistribution studies of $^{99m}$Tc-OMV or $^{99m}$Tc-HT in mice after nasal administration showed that the percentage of radioactivity in the intestine increased as radioactivity in the nose decreased (Fig. 6a). When both antigens were compared, HT activity was significantly higher in the intestine at 7 h post-administration (76.1±3.6 %).

The biodistribution of radiolabelled antigens in each organ/tissue after nasal administration showed greater accumulation of HT in the gastrointestinal tract after 24 h, with this being higher in the small intestine (16.53±22.48 %), whereas a small amount of OMV remained in the place of administration (0.47±0.72 %) and did not show activity in other organs after 24 h (Fig. 6b).

SPECT imaging of $^{99m}$Tc confirmed the presence of HT in the intestine after 1 h of nasal administration, and also
revealed that HT remained in the gut after 11 h 30 min (Fig. 7).

**Toxicity studies in rats**

During the acute toxicity study no mortality was observed in the different treatment groups and no dose compromised the rats’ lives by the nasal route. Detailed physical examinations conducted weekly did not demonstrate any unusual change in behaviour and no signs of toxicity were observed throughout the study. No significant differences were observed in the BW of the animals in the treatment groups compared with the controls. The haematological and biochemical parameters analysed showed normal values that did not differ from those of the control group. Finally, gross and histological pathological examination of the vital organs did not exhibit any evidence of toxicity during the animal necropsies. Thus, both vaccine candidates, OMV and HT, were found to be nasally safe at the single limit dose of 55 mg kg⁻¹ BW, respectively.

**Protection against colonization of *S. flexneri***

In order to determine whether immunization with OMV or HT was protective against colonization with *S. flexneri*, we used a pulmonary infection murine model. Mice (six per group) were immunized by the nasal route with 20 µg of OMV or HT and after 4 weeks they were infected via the intranasal route with a sublethal dose of 1 × 10⁵ c.f.u. Bacterial burdens were quantified in lungs. While all control non-immunized animals presented significant numbers of viable *S. flexneri* cells in their lungs, complete clearance of the inoculum was observed in mice that were intranasally vaccinated with HT (Fig. 8). In the case of OMV-vaccinated groups, complete clearance was achieved when the vaccine was administered by the nasal route, and there was a 2-log reduction compared to the control after oral administration. Moreover, the survival curves obtained after a lethal challenge of 4 × 10⁶ c.f.u./mouse to immunized mice showed high protection compared to the non-immunized group. All
mice (n=6) immunized with HT were able to survive after 9 days post-challenge, whereas only 50% of mice immunized with OMV survived (Fig. 9).

In order to compare the kinetics of the immune response between the different groups, the specific serum levels of IgG1 and IgG2a against OMV and HT antigens were determined by ELISA at different times post-immunization (Fig. 10). The results showed that immunization with HT elicited significant levels of serum IgG1 compared to the control mice (P<0.05) after 4 weeks of immunization, whereas OMV-vaccinated mice did not show significant IgG1 levels. Accordingly, HT-immunized mice also showed significant IgG2a levels in sera from the first to the fourth week after immunization (P<0.001), whereas mice vaccinated with OMV showed significant IgG2a levels (P<0.01) after the second and fourth weeks after immunization.

**DISCUSSION**

There is an increasing interest in developing subunit vaccines against Gram-negative bacteria based on OMVs.

These vesicles provide potential advantages, including safety, and self-adjuvant properties provided by LPS and other PRR agonists [27-30]. As a paradigm, an OMV vaccine against *N. meningitidis* has already been licensed in Europe [31]. We previously demonstrated that OMVs obtained from *S. flexneri* 2a induce a protective immune response against lethal challenge with the homologous strain in mice [24]. It is therefore worthwhile investigating methods to improve some aspects of this OMV-based vaccine. For use in vaccination, OMVs must be produced on a large scale and under safe conditions. The first option is natural production by the bacteria, but, this may not yield sufficient material. For this reason, several techniques have been developed to increase OMV yields, such as disturbing the membrane integrity using detergents, or physical shearing of bacteria to release vesicles, or milder biochemistry treatments based on oxidative stresses, treatment with chelating agents or antibiotics, inhibition of protein synthesis or inhibition of the synthesis of peptidoglycan by lysin starvation, among others [32, 33]. A paradigmatic example is the use of the surfactant sodium deoxycholate and the chelating agent EDTA to extract OMVs for the development of a vaccine against *N. meningitidis* [34]. In this case, the effect obtained was twofold, enhancing the OMV and also reducing the concentration of the toxic LPS in the resulting preparation. However, this treatment reduces the presence of immunoprotective lipoproteins [19], and also increases the concentration of cytoplasmic proteins as a result of bacterial cell lysis [35].

Additionally, with regard to the methods used for OMV production, inactivation of bacterial culture is always required. Here, we present an alternative based on heat treatment of the bacterial culture to avoid the problems associated with chemical treatment and, at the same time, generate OMVs at a higher yield. The OMV blebs from areas where the peptidoglycan links are impaired. The outer membrane is bound to the peptidoglycan layer by particular proteins, such as the lipoproteins linked covalently to peptidoglycan, the Tol-Pal proteins that link the inner and outer
membranes non-covalently through peptidoglycan, and the OmpA linked non-covalently to peptidoglycan. Thus, the deletion or overexpression of these proteins decreases membrane stability and causes hyperblebbing [36, 37]. Since the objective of this work was the use of native OMVs, no mutations were considered.

Several different methods have been used to inactivate virulent Shigella cultures for vaccine formulation, including the classical use of formalin [38]. However, incomplete inactivation may result from inadequate standardization of the dose, temperature and incubation time, and in addition, formalin treatment results in the crosslinking of proteins. Heat treatment was evaluated by Mukhopadhaya et al., who indicated that orally administered heat-killed S. flexneri can induce protection against pathogenic challenge [39]. Moreover, we previously found that the heat treatment in saline of whole Brucella or Salmonella cells rendered the release of OMVs, which are immunogenic in mice, ram and hens [21, 40]. From these precedents, in this study we evaluated the composition of heat-induced vesicles (HT) compared with naturally released OMVs (OMV) from S. flexneri cultures. HT vesicles, like OMVs, are composed of constituents of the outer membrane and periplasm [33]. As in many other cases of naturally released OMVs, the inner-membrane or cytosolic proteins constituted only a minor fraction of HT, supporting the fact that HT vesicles were not generated by cell lysis [41]. The results also demonstrated that HT is enriched (P<0.05) in specific outer-membrane proteins and lipoproteins. For instance, OmpA was significantly enriched in HT with respect to OMV, and this was one of the major quantitative differences we detected. It has been stated that the OmpA of S. flexneri is recognized by TLR-2 on B cells to induce the secretion of protective cytokines IL-6 and IL-10, and promotes increased surface expression of MHCII and CD86 on B cells [42]. Moreover, the OmpA of S. flexneri has been shown to elicit a strong Th1-mediated protective

**Fig. 6.** Biodistribution of radiolabelled OMV and HT after nasal administration in BALB/c mice. Percentage (%) of ⁹⁹mTc activity in the bowel and nose at different times after administration (a). Percentage (%) of ⁹⁹mTc activity in organs at 24 h after administration (b). The results show the mean and standard deviation of three animals (*, P<0.05). Error bars represent SD.

**Fig. 7.** Representative computer tomography SPECT of radiolabelled OMV and HT after nasal administration in BALB/c mouse. Images were obtained at different times after administration.
immunity after parenteral [43] or intranasal immunization of mice [44]. These findings support the interest in OmpA in HT as a potential vaccine product. OmpX was also enriched in HT. This protein is involved in cell adhesion [45] and contributes to the pathogenesis of Shigella [46]. Moreover, both OmpX and OmpA are regulated by PldA, and outer-membrane phospholipase A, which is associated with virulence and was also detected in both HT and OMV samples. OmpW is also enriched in HT. This protein belongs to the group of small β-barrel porins that includes OmpA and OmpX. It is involved in the colonization and virulence of Gram-negative bacteria, with a neat role in immunoprophylaxis [47, 48]. The β-barrel assembly machinery is mediated by Bam complex proteins, composed of BamA, an integral membrane protein, and lipoproteins BamB, BamC, BamD and BamE, which are localized to the inner leaflet of the outer membrane [49]. Specifically, BamC and BamE were highly enriched in HT with respect to OMV, as were other lipoproteins, such as YbjP, LoIB, MxiM and Pal. Outer-membrane lipoproteins are virulence factors that are emerging as key targets for protective immunity for Gram-negative bacteria, because of their excellent immunostimulatory properties, and because they are agonists of TLR-2, and its delivery to the host results in a potent immune response [50–54]. In this context, the removal of LPPs from the meningococcal OMVs reduced their protective capacity [55, 56]. The proteome assay also showed an increase of OmpC in HT. OmpC is a porin and a virulence factor that is required by S. flexneri for cell-to-cell spread and plaque formation [57]. Other differences between the proteomes of HT and OMV of S. flexneri were also noted. For example, Pal lipoprotein, anchored to the outer membrane which interacts with the peptidoglycan layer, is 10× poorer in OMV compared with HT. This result is consistent with the biogenesis of OMV, as being released from areas where the peptidoglycan links are impaired.

In summary, these data indicate that HT contains several virulence factors, including adhesins, invasins and invasion enzymes that might be involved in HT-mediated protection. Hence, it is important to consider the hypothetical limitations of the method used for HT preparation. Temperature can affect the antigenicity of an extract. However, our own experience with similar HT vesicles obtained from Salmonella and Brucella cells showed that antigenicity and immunogenicity were preserved in hens and rams, respectively [20, 21]. Here, using sera from hyperimmunized rabbits with S. flexneri, we showed the strong reactivity of IgG to HT proteins, including OmpA, OmpX and Ipa proteins. IpaA, IpaB, IpaC and IpaD were detected in the proteomes of OMV and HT, with no significant differences being observed between the complexes. These proteins are relevant effectors for pathogenesis, including the scrape from macrophages, the insertion of the secretion system and the subsequent intracellular invasion. In return, they represent the main immunogenic proteins for the host. Thus, a significant increase of anti-Ipa antibodies has been detected after natural or experimental Shigella infections [58]. In addition, in contrast to the antigenic heterogeneity of LPS, Ipa proteins are highly conserved [59], so the interest in Ipa proteins for vaccine development is completely justified.

Moreover, we found that OMV and HT elicited macrophage RAW cell activation without cytotoxic effects. Thus, after macrophage RAW cells were incubated with the OMV or HT vesicles, we detected elevated levels of MHC-II and the co-stimulatory molecule CD40. These results suggest that vesicles act on antigen-presenting cells to enhance adaptive immunity. This is of special relevance during vaccination. In vivo toxicity studies in rats also demonstrated the safety of this antigen. Finally, to develop an efficient vaccine to prevent Shigella infection, mucosal immunity should be considered. Therefore, in this study we assessed the mucosal immunogenicity of OMV and HT when they were

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**Fig. 8.** Protection against colonization of Shigella flexneri 2a conferred by OMV or HT. Mice were immunized with a single dose of OMV or HT from S. flexneri by the nasal route (six mice per group). A non-immunized group (control) was included. Challenge infection was performed at week 4, intranasally with a sublethal dose of $1 \times 10^8$ c.f.u./mouse of S. flexneri 2a. After 48 h, the mice were sacrificed and the number of c.f.u. per lung was determined. The results show the mean and so.

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**Fig. 9.** Survival curves of immunized BALB/c mice after S. flexneri challenge. Immunized mice (20 µg OMV or HT) and non-immunized control mice (six mice per group) were challenged intranasally with a lethal dose of $4 \times 10^6$ c.f.u./mouse at week 4 after immunization and monitored for 9 days post-challenge ($P<0.01$).
administered intranasally to mice. The results indicated that HT was even more protective than naturally released OMVs. In contrast to OMV, oral immunization with HT resulted in a predominant IgG2a response with a lower IgG1 level, indicating a marked Th1 immunity. These results are consistent with the protective activity reported here, and with the strongly pro-inflammatory Th1-Th17 cytokine profile observed during the control of S. flexneri infection [60]. At this point, it is not clear whether the higher protection conferred by HT is attributable to the enriched proteins, to the special ability of the HT liposomes to go through the mucus layer, to the smaller size, or to the lack of some immunosuppressive components present in OMV. Future research efforts should be directed towards answering this question.

In summary, although there are recognized facts and possibilities concerning the use of OMVs as vaccines, several challenges are being considered, such as the scale of production and safety. The new candidate vaccine introduced here, named HT, is an OMV-like vaccine obtained from whole bacteria by heat that circumvented the problems originating from virulence, since no live bacteria were present during vesicle recovery and purification. Furthermore, this investigation indicated that, compared with the classical OMVs, nasal immunization with HT enhanced protection efficiency, while biodistribution studies confirmed its benefits. The method for HT OMV-like isolation detailed here may provide the foundation for the generation of a safe and effective subunit vaccine for shigellosis. Further development is required, and there is a need to develop pilot-scale production and initiate clinical trials to evaluate whether this new subunit vaccine can be useful in achieving full protection against Shigella.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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