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A virosomal vaccine against candidal vaginitis: Immunogenicity, efficacy and safety profile in animal models

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ABSTRACT

A novel vaccine (PEV7) consisting of a truncated, recombinant aspartyl proteinase-2 of *Candida albicans* incorporated into influenza virosomes was studied. This vaccine candidate generated a potent serum antibody response in mouse and rat following intramuscular immunization. Anti-Sap2 IgG and IgA were also detected in the vaginal fluid of rats following intravaginal or intramuscular plus intravaginal administration. In a rat model of candidal vaginitis, PEV7 induced significant, long-lasting, likely antibody-mediated, protection following intravaginal route of immunization. PEV7 was also found to be safe in a repeated-dose toxicological study in rats. Overall, these data provide a sound basis to envisage the clinical development of this new candidate vaccine against candidal vaginitis.

1. Introduction

Infections caused by opportunistic fungi continue to be a substantial threat to the health of populations, being particularly severe in immunocompromised or otherwise debilitated hosts [1,2]. Among these infections, vaginitis caused by *Candida albicans* and related species is the most prevalent worldwide as it is estimated that one to two thirds of all women in the fertile age of life suffer from at least one disease attack [3]. While uncomplicated, acute attacks of this disease are normally cured by chemotherapy, in 4–8% of the women, a primary attack is followed by recurrences which are much less amenable to antimycotic therapy, eventually bringing the subjects into a state of chronic, non-eradicable disease which dramatically affects the quality of life and is associated with a severe economic burden [3,4].

Several factors which predispose to acute vaginitis have been reported [3], and there is increasing attention on host genetics and innate immunity assets as determinants of the recurrent disease [5,6]. Nonetheless, there is also substantial experimental and some clinical evidence that virulence factors of *C. albicans* critically contribute in causing the disease. Among these factors, members of the secretory aspartyl proteinases (Sap) family have long been considered to be major determinants of candidiasis, although with different degree of experimental evidence, particularly for systemic infection [7–13]. The Sap protein family includes at least 10 members (Sap1–10) differing in protein sequence, optimum pH for activity and biological functions [7]. Among them, Sap1–Sap3 proteins, which mostly work at acidic pH, have been strongly advocated to play a role in candidal vaginitis (reviewed in [9]). Sap2 is the most abundant form of Sap produced on protein-rich sources *in vitro* and it is consistently found to be produced *in vivo*, both under experimental and clinical settings [7,9,14,15]. Importantly, antibodies against Sap2 have been shown to exert a protective role against an experimental rat vaginal infection caused by *C. albicans* [16,17]. These results strengthen the feasibility of establishing a Sap2-based subunit vaccine against human disease.

Having this in mind, we have reported recently on the generation of a recombinant, N-terminally truncated Sap2 protein (rtSap2), and showed that rat intravaginal immunization with this protein generated anti-Sap2 IgG and IgA detectable in the vaginal fluid, and conferred a degree of protection against challenge with a vaginopathic strain of *C. albicans* [18]. Our previous observations [16,17] also showed that murine monoclonal IgM and IgG antibodies conferred passive protection in the above model [18]. The initial proof of concept was achieved with rtSap2 formulations containing cholera toxin as an adjuvant. Here, a virosomal formulation of rtSap2 was developed in order to obtain an equally potent and clinically acceptable vaccine.

Virosomes are *in vitro* reconstituted viral envelopes derived from influenza virus, devoid of viral RNA but retaining the viral

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properties with regard to target cell binding and entry, including the unique pH-dependent hemagglutinin-mediated fusion activity [19].

The antigen of interest can be displayed on the surface of virosomes in a highly immunogenic context owing to the virosomes combined action as a carrier and adjuvant, strongly promoting antigen presentation [19]. The virosome technology is established and commercially in use in vaccines against hepatitis A (Epaxal®) and influenza (Inflexal®V) [20,21]. We made use of the virosome technology to generate a highly immunogenic, protective and safe rtSap2 containing vaccine for prospective therapeutic use against chronic recurrent vulvovaginal infection by *C. albicans* (RVVC).

2. Materials and methods

2.1. Microorganisms, growth conditions and generation of recombinant, truncated Sap2

C. albicans ATCC20955 grown in Winge (0.3% yeast extract, 0.2% glucose, Difco, Becton Dickinson Microbiology Systems, MD, USA), and *Escherichia coli* M15 (nal^s, str^s, rif^s, *lac*$^-$, ara$^-$, gal$^-$, mtl$^-$, F$^-$, recA$^+$, uvr$^+$, [pUHA1]) were used as a fungal DNA source and as host strains for recombinant plasmids, respectively. The microorganisms were grown as described elsewhere [18]. Molecular cloning, expression and purification of recombinant Sap2 truncated proteinase (6His-tag-SAP2$^{77-400}$ protein, hereafter referred to as rtSap2, which has 20 amino acids less than the mature Sap2 fragment) were performed as reported elsewhere [18]. At a later stage, the rtSap2 was expressed under cGMP conditions without 6His-tag in the microbial XS system in the *E. coli* W3110 non-secreting strain (Lonza AG, Visp, Switzerland), and purified by a process including isolation of the inclusion bodies and solubilization by 8 M urea in a 80 mM Tris pH 7.4 buffer, followed by depth filtration and cation exchange chromatography. After refolding, rtSap2 was further purified by anion exchange and hydrophobic interaction chromatography before a final ultrafiltration was performed. Bulk drug substance was manufactured in accordance with ICH Q7 applicable sections. rtSap2 was obtained at a purity of ≥98% (determined by RP-HPLC and SDS-PAGE) and stored at −20 °C in a PBS pH 7.4 buffer. Protein concentration was determined by absorbance at 280 nm using an extinction coefficient $E = 0.735$ (0.1%, 1 cm). rtSap2 solution was further characterized by determination of conductivity, pH, urea content, endotoxin content (<4 EU/ml, Limulus lysate test), host cell protein content, residual DNA content and microbiological quality by using commercially available assays according to the European Pharmacopea. For Sap antigen cross-reactivity studies, recombinant Sap1–Sap6 expressed in *Pichia pastoris* were kindly provided by B. Hube (Leibniz Institute for Natural Product Research and Infection Biology, Jena, Germany).

2.2. Animals

The modalities of induction and assessment of experimental vaginal infection in oophorectomized Wistar rats were as previously described [16,22]. "Protection" was defined as more rapid clearance of the fungus from animal vagina in vaccinated animals with respect to controls.

The repeated dose toxicology study was performed in both male and female Wistar rats, substrain Crl:WI (Han), aged 6–8 weeks at start of the study.

2.3. Virosomes and virosomal vaccine formulation

Virosomes were assembled *in vitro* from synthetic lipids and purified influenza virus envelope components derived from strain A/Singapore/6/1986 (H1N1) [23]. In order to anchor the antigen rtSap2 in the lipid membrane of the virosome, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) moieties were conjugated to lysine residues present on rtSap2. The modified antigen rtSap2-DPPE was mixed with synthetic lipids and the influenza envelope components at the desired concentration prior to virosome assembly. The experimental vaccine containing rtSap2 conjugated to the lipid bilayer of the virosomes will hereafter be referred to as PEV7. Quantification of rtSap2 in PEV7 was done by ion exchange chromatography on an Agilent 1100 HPLC system by UV detection at 225 nm.

Purified heat labile toxin from *E. coli* (HLT) was purchased from Berna Biotech (Bern, Switzerland). When used for immunizations, 3 mug HLT per dose was added to the virosome formulations after particle assembly.

2.4. Rat intravaginal immunization

For active immunization, in the majority of experiments and unless otherwise indicated, groups of five rats were immunized three times by intravaginal (i.vag.) route at weekly intervals with the virosomal vaccine PEV7 and control materials. Usually control animals received empty virosomes (IRIV) or sterile saline (PBS pH 7.4). One week or 1–5 months after last immunization (as indicated in specific experiments), all animals were challenged i.vag. with 10^7 cells of *C. albicans*, and the infection was monitored by enumeration of colony forming units (CFU) present in the vaginal fluid taken from each rat, at days intervals as indicated in the specific figures [22]. Two independent experiments were carried out. For antibody measurement, samples of vaginal fluids were taken 60 min before fungal challenge by gently washing the vaginal cavity with 0.5 ml of PBS, as described elsewhere [18]. The collected fluid was centrifuged at 3500 × *g* for 15 min and the supernatant was assayed as described below.

2.5. Detection of antibodies in vaginal fluids and serum

Immunogenicity studies were performed both in mice and rats. Female NMRI mice (Bioscience Scientific Laboratories GmbH, Germany) at the age of 6–8 weeks and Wistar rats (80–100 g) were used. For serum antibody detection in mice and rats, rtSap2 protein (1 mug ml^{-1}) diluted in 0.1 M carbonate buffer pH 9.6 was used to coat polystyrene microplates (Maxisorp; Nunc/Thermo Fisher Scientific). After a blocking step with PBS containing 5% (w/v) skim milk powder and washing with PBS containing 0.05% (v/v) Tween 20, serial dilutions of the samples were distributed on the plates, using PBS containing 0.05% (v/v) Tween 20 and 0.5% (w/v) skim milk powder as dilution buffer. Bound antibodies were detected with a suitable secondary antibody conjugated to horseradish peroxidase and visualized addition of O-phenylene diamine in citrate buffer-H_2O_2. After stopping the color reaction by addition of 1 M H_2SO_4, the absorption at 492 nm (OD492 nm) was measured with an automated microreader. A reference serum was established from the pooled sera of mice immunized with PEV7 in the context of an early potency study. This reference serum was included on each plate for standardization. For analysis, the OD values of samples were expressed as % of the reference OD.

The presence of antibodies in the rat vaginal washes was assayed by a previously described enzyme-linked immunoadsorbent assay (ELISA) [16,18,22]. Unless otherwise indicated, 200 mul of native Sap2 protein (originally provided by P.A. Sullivan, Massey University, Palmerston North, New Zealand) at a concentration of 5 mug ml^{-1} in 0.2 M sodium carbonate was used as coating antigen for the detection of antibodies and was dispensed into the wells of a polystyrene micro titration plate, kept overnight at 4 °C. After three washes with Tween 20-PBS buffer, 1:2 dilutions of vaginal fluids were distributed in triplicate wells and the plates

were incubated for 1 h at room temperature. Four independent samples were analyzed. Each well was washed again with Tween 20 PBS buffer and predetermined optimal dilutions of alkaline phosphatase-conjugate and sheep anti-rat immunoglobulin IgG or IgA (obtained from Serotec Ltd.; Kidlington, Oxford, United Kingdom) were added. Bound alkaline phosphatase was detected by the addition of para-nitrophenyl phosphate solution in diethanolamine buffer and the plates were read at A 405 nm with an automated microreader (Labsystem Multiscan, MS, Finnland) blanked against air. In the absence of a "reference" immune vaginal fluid, the simple OD values were shown for vaginal antibodies in comparison with OD values of vaginal fluids from non-vaccinated animals. Vaginal fluid was considered positive for a determined antibody when the O.D. was greater than twice the value of the well coated with the same antigen and tested with the vaginal fluid of non-immunized, uninfected rats. The 1:2 diluted fluids of non-immunized rats never exceeded a reading of 0.14 [for IgA], and 0.10 [for IgG)]. The titers of antibody-positive fluids following scalar 1:2 dilution never exceeded 1::16 for IgA and 1:32 for IgG ELISA-positive vaginal fluids were confirmed by Western blotting (data not shown).

2.6. Toxicology study

A formal toxicology study was performed in Wistar rats for regulatory purposes, as part of preparatory work for the clinical testing of PEV7. The repeated dose study comprised 4 intramuscular immunizations at weekly intervals. Acute toxicity was assessed 1 day after the last immunization, and the reversibility of any adverse effects to a 2-week recovery period. Each time point was investigated in female and in male rats, in groups of 10 animals each. Virosomes with and without rtSap2 were tested in parallel in order to control for rtSap2 related effects. The rtSap2 antigen used in this study was clinical grade material expressed without the 6His-tag. The dosage per administration corresponded to the highest dose anticipated for human use, comprising 50 mug of rtSap2 and 10 mug of HA. Blood samples were collected from each animal prior to immunization and at the time point of necropsy and assessed for hematological parameters, blood chemistry, and for the presence of antibodies against rtSap2 and influenza antigens by ELISA. Upon necropsy, a pathological examination was performed, as well as histopathology on 34 organ and tissue samples from each animal, in accordance with regulatory requirements.

The study was performed by an established contract research organization (Bioscience Scientific Laboratories GmbH, Germany), in full compliance with the relevant guidelines for animal welfare, preclinical toxicological testing of vaccines, and good laboratory practice (GLP).

2.7. Statistics

Differences in ELISA values for serum and vaginal fluids antibodies, as well as in cfu numbers from of experimental rat infection by *C. albicans* were assessed by Mann–Whitney rank sum test. *P* values <0.05 two tails were taken as indicating a significant difference. When not reported in the text, the *P* values are indicated in the legend of the figures or tables.

3. Results

3.1. Immunogenicity of the virosomal rtSap2 formulation PEV7 in mice and rats

We first examined whether immunization with PEV7 (rtSap2 virosomes) generated a higher antibody response against the native Sap2 protein as compared to the immunization with rtSap2 simply mixed but not associated with virosomes. Non-immunized animals served as controls. Fig. 1A and B reports the data of NMRI mice immunized *via* intramuscular (i.m.) injection, whereas Fig. 1C shows the data of rats immunized i.vag. In mice, the immunization with PEV7 induced a much stronger serum antibody response (IgG) than immunization with the same amount of rtSap2 (20 mug/dose) co-administered but not physically associated with virosomes ($P < 0.05$; Mann–Whitney Rank sum test). Notably, the immune response against the influenza proteins integrated in the virosomes was equivalent for both animals immunized with PEV 7 and those immunized with rtSap2 mixed with virosomes, as expected (Fig. 1B). Intravaginal immunization of PEV7 in rats resulted in low but appreciable anti-Sap2 antibody levels of both IgG and IgA isotype in the vaginal fluid, as determined by ELISA (Fig. 1C). A much weaker antibody response was detected in the vaginal fluid of rats administered the mixture of rtSap2 and virosomes. No anti-Sap2 antibodies were detected in control animals. Overall, these experiments demonstrate that the physical association of rtSap2 with the virosome particle is essential for the induction of robust anti-Sap2 antibody levels, irrespective of the vaccine application route.

3.2. Cross-reactivity of anti-Sap2 antibodies with other members of the Sap protein family

In addition to Sap2, Sap1 and Sap3 are known to contribute to disease in the vaginitis model [24]. Furthermore, they have also been shown to be secreted by the fungus during clinical infection [24]. Therefore, we have examined whether the anti-Sap2 antibodies elicited in mice and rats after immunization with PEV7 could also recognize other Sap proteins. Recombinant Sap1, 2, 3, 5 and 6 expressed in *P. pastoris* and kindly provided by Prof. B. Hube were used for those experiments. Both serum and vaginal antibodies raised against rtSap2 by PEV7 immunization did also recognize Sap1, Sap3, and to a much lesser extent Sap6, but not Sap5 (Supplementary Fig. 1).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.04.069.

3.3. Protection of rats by intravaginal immunization with PEV7

We next examined whether the virosomal vaccine PEV7 conferred protection to rats experimentally challenged with *C. albicans*. For this purpose, rats received three i.vag. administrations of the vaccine, and were challenged with the fungus either 1 week (Fig. 2A) or 1–5 months (Fig. 2B) after the last immunization. An initial experiment also served to assess whether the addition of a strong mucosal adjuvant (HLT) to the virosomal formulation could enhance immunogenicity and improve the protective response. Finally, the antigen dose dependency for protection was also assessed. As shown in Fig. 2A, immunization with the virosomal vaccine conferred a substantial protection from *C. albicans* challenge as exemplified by the accelerated clearance of the fungus from the vagina and resolution of the infection at least 1 week before infection in controls (administration of empty virosomes). Importantly, no significant advantage in protection of rats was observed when adding a mucosal adjuvant such as HLT to the vaccine. ($P < 0.05$, at each day examined, between PEV7 or PV7 + HLT and controls). In addition, the mixture of plain virosomes and rtSap2 did not confer any protection to the rats, similarly to the use of virosomes only or PBS as controls, in agreement with previous data showing the absence of protection in rats immunized with rtSap2 alone [18].

No statistically significant difference was found in the dose–response experiments for the antigen dose range of 20–50 mug. Additionally, no significant difference was determined in the rate of fungus clearance and in the time to infection

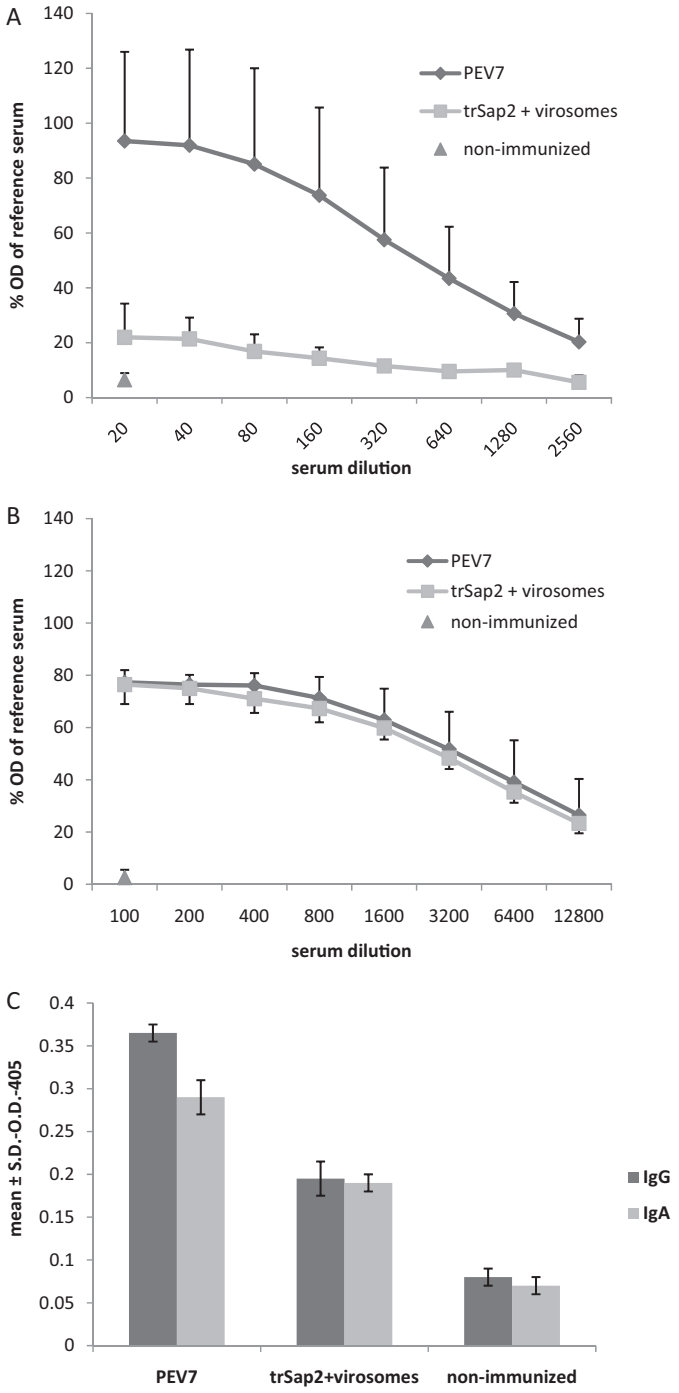

Fig. 1. Immunogenicity of the virosomal rtSap2 formulation PEV7 in mice and rats. (A and B) Anti-Sap2 and anti-influenza antibody response of NMRI mice immunized with the virosomal vaccine PEV7 (rtSap2 anchored in the lipid membrane of virosomes) or the mixture of empty virosomes and non-associated rtSap2 at equal dosage. Groups of 5 mice were immunized i.m. twice 3 weeks apart. The serum was collected 2 weeks after the last immunization and examined by ELISA for antibodies specific for Sap2 (A) and influenza (B). The data points represent the averages per group/serum dilution, and the error bars depict the standard deviations. $P < 0.05$ for the difference in titers between PEV7 and the mix virosomes plus rtSap2 (A). The P value ($P = 0.016$) between the groups was calculated by Mann–Whitney Rank sum test on the basis of the antibody titers of the individual mice ($n = 5$/group) either immunized with PEV7 (geometric mean titer: 11,763) or mixed with IRIV (GMT: 160). (C) Anti-Sap2 antibodies detected by ELISA in the vaginal fluid of rats immunized with PEV7 or the mixture of rtSap2 and empty virosomes. Administration of PBS pH 7.4 to rats served as negative control. $P < 0.05$ comparing the IgG and IgA absorbance values of the PEV7 with those of the virosomes plus rtSap2 mixture, in four independent samples.

resolution, whereas a dose of 2.5 mug of rtSap2 was significantly less efficacious, even when HLT was added (data not shown).

The persistence of vaccine-mediated protection from Candida infection was tested by challenging the rats at 1, 3 and 5 months after the last immunization with an antigen dose of 20 mug (Fig. 2B). There is some expected gradual decay of protection over time, but there was still a statistically significant protection in rats challenged with *C. albicans* 160 days after the last immunization (see the legend to the figure for *P* values at different days of CFU assessment). The late persistence was accompanied by specific vaginal IgG and IgA low levels of which were still detected after 5 months (Fig. 2C). Overall, these experiments show that a dose of 20 mug of rtSap2 in the virosomal vaccine PEV7 was sufficient to generate a persistent protection from *C. albicans* after intravaginal immunization in rats. This long lasting protective status was associated with persistence of anti-Sap2 antibodies in the rat's vagina.

3.4. Protection following intramuscular priming and intravaginal boosting

Systemic immunization may also produce substantial levels of antibodies in the mucosal compartments [25]. At the same time, direct mucosal immunization requires a high dose of antigen with possible tolerogenic effects in the absence of mucosal adjuvants. Thus, we examined whether the adoption of a prime boost i.m. and i.vag. immunization schedule could confer some advantages in terms of immunogenicity and protection as compared to the i.vag. administration alone. In these experiments, we also examined the effect on immunogenicity and protection of the i.m. vaccine administration only. As shown in Fig. 4, the three administration schedules did not cause dramatically different levels of anti-Sap2 antibodies in the rat vagina. There were slightly lower levels of IgG in the animals immunized i.vag. compared to those immunized i.m. or i.m. plus i.vag. (Fig. 3A). As expected, serum antibodies were detected in animals immunized by the i.m. and the i.m. plus i.vag. routes, but not in rats immunized intravaginally only (data not shown). In substantial accord with these immunogenicity results, all three schedules provided increased clearance of the infection as compared to the controls (Fig. 3B). However, the rats immunized *via* the i.vag. route showed some significant differences in the clearance of the fungus from vagina, particularly during the first week post-challenge. Overall, antibody levels and protection criteria did not support superiority of the i.m./i.vag. prime-boost scheme on the i.vag. only immunization scheme.

3.5. Equivalence of PEV7 with rtSap2 antigen with or without 6His-tag

For toxicity studies and for addressing phase 1 clinical trial, a cGMP preparation of the recombinant, truncated Sap2 protein lacking the 6His-tag was generated and formulated into the virosomal preparation as for PEV7. A challenge experiment was performed in order to demonstrate that lab-scale produced 6His-tagged rtSap2 material used in the pre-clinical tests and rtSap2 material without tag produced under cGMP conditions were equally protective in the rat challenge model. To this purpose, rats were i.vag. immunized with either the virosomal PEV7 displaying rtSap2 with a 6His-tag or without the 6His-tag, and the vaginal fungus counts were determined following challenge until day 28. No significant differences were detected between the protection conferred by either PEV7 vaccine formulation, with the possible exception of a small difference on day 5 (Supplementary Fig. 2). Both rtSap2 variants induced low but appreciable antibody levels, similar to those determined in previous experiments (data not shown).

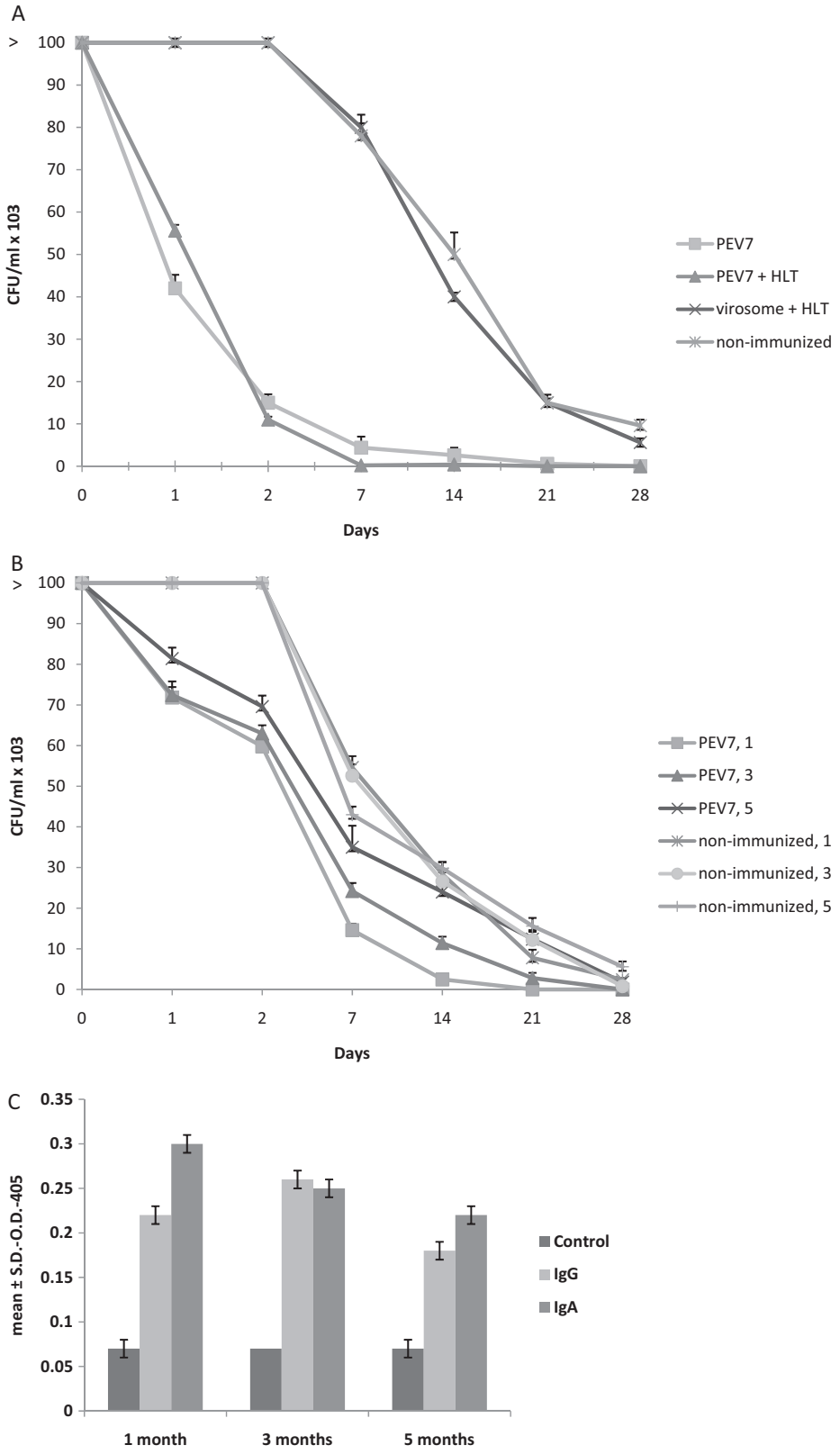

Fig. 2. Protection from *C. albicans* infection in rats by i.vag. immunization with the PEV7 and anti-Sap2 antibodies in the vaginal fluid. (A) Groups of five rats were immunized with PEV7, or the PEV supplemented with HLT, or empty virosomes supplemented with HLT as described in Section 2. Animals administered with PBS pH 7.4 served as negative control. One week from the last immunization, the vaginal fungus burden was enumerated at the days indicated. Starting from day 1, there was a statistically significant difference ($P < 0.05$; Mann–Whitney U test) at each time-point between the CFU of the animals receiving the vaccine (and also vaccine plus HLT) and those receiving the empty virosomes plus HLT or the controls. No statistically-significant CFU differences were observed between the rats immunized with the vaccine and those immunized with the vaccine supplemented with HLT. (B) Persistence of protection in animals challenged with *C. albicans* at intervals from the end of immunization, as indicated (1, 3 or 5 months, respectively). At days 1 and 2, there was a statistically-significant difference ($P < 0.05$; Mann–Whitney U test) in the vaginal Candida CFU between immunized and non-immunized (receiving empty virosomes) animals. At days 7, 14 and 21, a statistically significant difference was noticed between the animals challenged 1 and 3

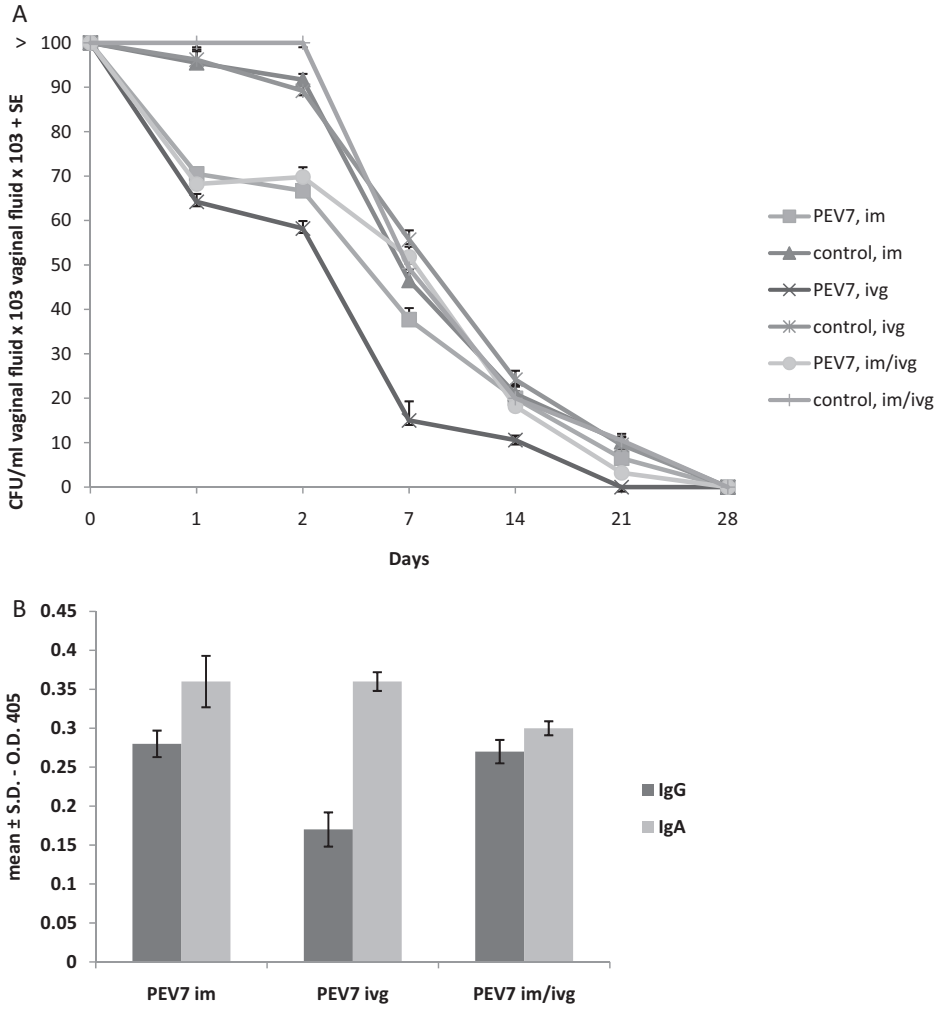

Fig. 3. Anti-*Candida* protection in rats conferred upon i.m. and/or i.vag. immunization, and anti-Sap2 antibodies in the vaginal fluids. (A) Groups of five rats were immunized i.m. or i.vag. or combined i.m. and i.vag. then challenged with *C. albicans* as described in Section 2. Other groups received empty virosomes as a control. At the indicated days, fungus vaginal burden was evaluated. On days 1 and 2 there was a statistically significant difference ($P < 0.05$, Mann–Whitney *U* test) between the vaginal CFUs of each of the three vaccinated groups and those of the corresponding control group. On day 7, the difference was significant for the i.vag. and i.m. immunized animals *versus* their controls. On day 14, only the i.vag. vaccinated group had CFUs statistically significant compared to the control. For other technical details, see Section 2. (B) Anti-Sap2 antibodies detected in the vaginal fluid by ELISA. Groups of five rats were immunized i.m. or i.vag. or combined i.m. and i.vag. with PEV7 (vaccine) or empty virosomes (control) as indicated. The OD readings of control (non-immunized animals) are not shown; all their readings were between 0.05 and 0.08, see also Fig. 1.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.04.069.

3.6. Toxicity study

The toxicological assessment of PEV7 performed in Wistar rats revealed neither functional nor behavioral vaccine-related clinical findings. Food intake and body weight gain were unaffected by the treatment, 4 vaccinations at weekly intervals at a dose of 50 mug rtSap2 per application. All hematological parameters (hemoglobin, hematocrit, red and white blood cell count, platelet count, differential blood cell count, coagulation time) and clinical biochemistry parameters (aspartate aminotransferase, alanine aminotransferase, creatine kinase, alkaline phosphatase, cholesterol, glucose, urea, creatinine, total protein, albumin, sodium, potassium) showed values well within the biological range. No differences were observed between the treatment groups, both 24 h (main study group) and 14 days (recovery group) after the last immunization.(data not shown)

As expected, the repeated intramuscular injections led to the typical histopathological changes in the muscle tissue at the administration site and in the draining lymph nodes, in similar fashion for virosomes with and without rtSap2. At the injection site, edema, mild to moderate cellular infiltrates, and muscle fiber degenerations were observed. The draining lymph nodes were enlarged and showed increased germinal center development. Interestingly, after the recovery period of 2 weeks, the histopathological changes had already regressed to minimal or mild levels. None of the other organs investigated by histopathology did reveal any treatment-related abnormalities (data not shown).

months after last immunization and the animals immunized with empty virosomes. No statistically significant difference was observed among rats challenged at different time points after immunization. For other technical details see Section 2. (C) ELISA readings of anti-Sap2 antibodies in the vaginal fluid of rats challenged as described above. These experiments were repeated once and the data shown here are those of one out of two experiments (comparable data were obtained in both). There was always a statistically significant difference ($P < 0.05$, Mann–Whitney *U* test) between IgG and IgA ELISA readings of fluids of vaccinated animals and those of controls (values not shown, always between 0.05 and 0.08: see also Fig. 1C). A statistically significant difference was also noticed between IgG and IgA ELISA reading of vaginal fluids taken from animals challenged after 1 month from immunization. No other statistically significant differences were detected.

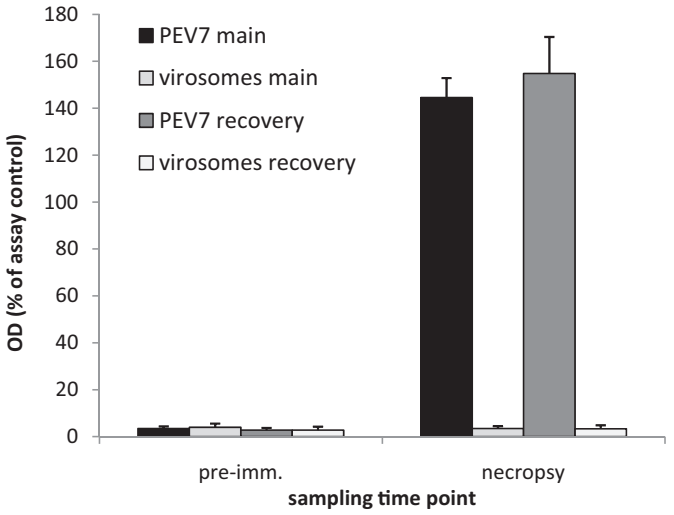

Fig. 4. Anti-Sap2 antibodies detected by ELISA in rat sera obtained from the toxicology study prior to immunization and at the time of necropsy. The animals received 4 intramuscular injections at weekly intervals, either of PEV7 containing 50 mug rtSap2 and 10 mug influenza HA per dose, or of empty virosomes containing 10 mug influenza HA per dose. The main study groups were sacrificed 1 day after the last immunization, the recovery groups 14 days after the last immunization. Each group consisted of 10 female and 10 male animals. The bars represent the mean values, the error bars the standard deviation.

The immunological analysis of the blood samples demonstrated a robust immune response against the vaccines applied, as illustrated by high antibody levels against influenza in all animals, and against rtSap2 in those animals receiving PEV7 (Fig. 4). All samples collected prior to immunization were negative for antibodies against both the influenza components and rtSap2. Both female and male rats responded equally to the vaccine administration, with regard to all clinical, pathological and immunological parameters assessed.

4. Discussion

The data reported in this article can be summarized in three main, novel findings: (1) PEV7 is immunogenic in animal models, both after intramuscular and after intravaginal application; (2) in association with this immunogenicity profile, PEV7 confers a consistent degree of protection against vaginal *C. albicans* infection in the rat model and (3) the safety profile of PEV7 in the rat model demonstrate that this vaccine candidate is acceptable for clinical trials of therapeutic vaccination against recurrent vulvovaginal candidiasis.

RVVC represents an unmet medical need that demands novel approaches to its control, among which prevention or cure by vaccination is being increasingly considered [26–28]. Hence, a number of preclinical investigations using different vaccine formulations and animal models have been carried out. In mouse models of vaginal candidiasis, a number of vaccine formulations proved to be protective against a vaginal challenge by *C. albicans*, inclusive of two different glyco-conjugates [29,30] and the recombinant Als3 protein fragment [31]. Of importance, the vaccines above greatly differ in the advocated mechanism of protection, which can be either the amplification of a Th17 CD4+ T cell responses [31] or the generation of protective anti-β-mannan or β-glucan antibodies [29,30]. In addition, these vaccines were primarily designed for immunization against systemic Candida infection, and two of them, the laminarin (β-glucan)-conjugate and the Als3 vaccine also exerted some protection against non-Candida fungi and *Staphylococcus aureus*, respectively. It is also unclear whether and to what extent the current experimental models of vaginal candidiasis are a reasonable representation of human acute vulvovaginal candidiasis and, still less, of human RVVC. Both mouse and rat models require pseudoestrus induction for successful infection, reminiscent of the role of estrogen in VVC/RVVC. On the other hand, mouse models impinge on innate immunity as a critical factor for both onset and resolution of the infection [4,32] whereas in the rat model evidence about induction of adaptive immunity and protection from infection by adaptive immune responses, in particular antibodies, has been provided [9,16,17]. It is possible that the two models represent different aspects of the same disease or even "different" diseases in consideration of the multifaceted nature of vaginal candidiasis [3]. At any rate, neither mouse nor rat models are realistic representations of RVVC and the current vaccines have been considered to be of modest appeal in terms of prospected human use [32].

Besides uncertainties in data interpretation obtained with different animal models and their relevance to the human disease, preclinical investigations performed so far generally lacked the use of human-suitable adjuvant. We have long been considering a vaccine exclusively or primarily designed to protect against vaginal candidiasis by the use of an antigen (the Sap2) that, together with its homologous members of the secreted aspartyl proteinase family of *C. albicans* (particularly Sap1 and Sap3), seems to be actively involved in the pathogenesis of candidal vaginitis. Initial suggestions that Sap2 could be indeed involved in the pathogenesis of candidal vaginitis and that antibodies against this protein could be protective against vaginal infection by *C. albicans* were provided by Cassone et al. [14] and De Bernardis et al. [33], This suggestion was confirmed and extended in different experimental models [7,10,34]. More recently, Sandini et al. [18] showed that the intravaginal administration of an N-terminally truncated recombinant Sap2 (rtSap2) adjuvanted with cholera toxin (CT) induced local anti-Sap2 antibodies and a degree of protection against *C. albicans* challenge in the rat vagina. However, this and other vaccine formulations were scarcely, if at all investigated for their local or systemic toxicity and no GMP preparations suitable to address clinical investigations were used before.

We have attempted to fill in part of these gaps by generating PEV7, a subunit vaccine based on recombinant rtSap2 [18], which is displayed on the surface of influenza virosome particles. We assessed the immunogenicity, protective efficacy and the safety of this candidate vaccine in qualified preclinical settings.

The virosomal rtSap2 preparation elicited specific antibodies which were detected in the vaginal fluids upon intravaginal and, to some extent, also intramuscular administration. In previous work with the rat model, intravaginal and intranasal routes were equally effective in generating protective anti-mannan and anti-proteinase antibodies in the vagina [35]. In the present investigations, the low but consistent IgG and IgA levels obtained by the administration of the virosomal vaccine generally matched the protective level, even in terms of duration of the vaccine protection. It should be stressed that neither experiments of passive transfer of vaginal fluid nor quantitative measurements of antibody production was attempted here, hence a strict quantitative correlation between anti-Sap2 antibodies and protection is not possible at this point. Nonetheless, a passive transfer of vaginal fluid in experiments of vaccination by rtSap2 mixed with cholera toxin [18] and the evidence that anti-Sap2 monoclonal antibodies of different format are both preventive and therapeutic in the rat vaginitis model [16,17] would suggest that at least part of the protection achieved by the PEV7 virosomal rtSap2 vaccine is attributable to specific antibodies. Of importance, the antibodies generated by the PEV7 vaccine were seen to amply cross-react with Sap1 and Sap3, the two most homologous members of the Sap family of *C. albicans*, which have been shown to be expressed in the vagina of both animals and humans during infection [15,24,36–38]. These observations indicate that

an isoenzyme shift during vaginal infection is not likely to lead to immunoevasion of the protective antibodies.

After initial negative results (data not shown), no further attempts were made to detect Sap2 – specific T cells. Various immune-effectors including CD4+, CD8+ and DC, have previously been found to play a possible role in the anti-Candida protection in the rat vaginal candidiasis model (reviewed in [9]). However, in those experiments, the animals were repeatedly challenged with live fungal cells, possibly constituting an exceptionally potent stimulation of innate and adaptive immunity. It remains quite possible, if not likely, that vaccine-induced Sap2-specific T cells provide help to anti-Sap2 antibody production, but also directly contribute to protection. The role of T cells in the context of rtSap2-mediated protection remains to be further investigated.

Details on how the anti-Sap antibodies express their protective potential remain unclear, too. Sap proteins have been particularly involved in enzymatic attack of mucosal substrates and defensive factors, promoting adherence to epithelial and endothelial cells, as well as help fungal cells evade host response [39,40]. In theory, all these activities could be neutralized by the appropriate antibodies. For instance in the rat Candida vaginitis model, antibodies can readily be shown to strongly inhibit fungus adherence to rat vaginal epithelial cells [17]. In other models, the binding of fungal cells to the vaginal epithelium is considered to be the main, decisive factor for triggering the leukocyte recruitment and inflammation [29]. Moreover, Sap2 and other Saps have recently been shown to possess a direct pro-inflammatory potential unrelated to their enzymatic activities [41]. Although these observations were made in human monocytes, an extension to epithelial cells is plausible regarding the capacity of these cells to generate proinflammatory cytokines and signals for inflammatory cell recruitment.

In summary, the preclinical evidence generated in this study, inclusive of a toxicological evaluation which is rarely found in preclinical studies of other vaccines [26,27,31] warrants progression to the clinical development of PEV7 as a vaccine against RVVC and, at the same time, suggests the need for further studies aimed at a better understanding of the immunological mechanisms underlying PEV7 protection that may help design and evaluation of clinical trial data.

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