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Effect of different baculovirus inactivation procedures on the integrity and immunogenicity of porcine parvovirus-like particles

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Abstract

We have demonstrated earlier the usefulness of recombinant porcine parvovirus (PPV) virus-like particles (VLPs) as an efficient recombinant vaccine for PPV. Here, we have demonstrated that preparations of PPV VLPs could be contaminated by recombinant baculoviruses. Since these baculoviruses can be a problem for the registration and safety requirements of the recombinant vaccine, we have tested different baculovirus inactivation strategies, studying simultaneously the integrity and immunogenicity of the VLPs. These methods were pasteurization, treatment with detergents and alkylation with binary ethylenimine (BEI). The structural and functional integrity of the PPV VLPs after the inactivation treatments were analyzed by electron microscopy, hemagglutination, double antibody sandwich (DAS)-ELISA and immunogenicity studies. Binary ethylenimine and Triton X-100 inactivated particles maintained all the original structural and antigenic properties. In addition, PPV VLPs were subjected to size-exclusion chromatography to analyze the presence of VP2 monomers or any other contaminant. The resulting highly purified material was used as the standard of reference to quantify PPV VLPs in order to determine the dose of vaccine by DAS-ELISA. After immunization experiments in guinea pigs, the antibody titers obtained with all the inactivation procedures were very similar. Triton X-100 treatment was selected for further testing in animals because of the speed, simplicity and safety of the overall procedure. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Porcine parvovirus; Virus-like particles; Viral inactivation; Vaccine; Baculovirus

1. Introduction

Porcine parvovirus (PPV) is one of the major causes of reproductive failure in pigs [1]. The infection occurs without clinical symptoms in adults; however, the virus can cross the placental barrier during the infection and cause the death of the fetuses, stillbirths and return to estrus. The virus is distributed worldwide and only a continuous vaccination policy can avoid large economic losses.

Parvovirus infections are controlled mainly by the humoral response [2]. Classical vaccines based on inactivated viruses are currently in use [3,4]. Safety considerations together with practical limitations due to poor PPV replication in vitro have justified the development of alternative vaccines. The major structural protein, VP2 is the main target for neutralizing antibodies in PPV [5,6]. When VP2 was expressed in large amounts using the baculovirus expression vector system, it assembled into virus-like particles (VLPs) similar in size and morphology to the original virions [5]. The VLPs were highly immunogenic and protected breeding sows against reproductive failure following virulent virus challenge [7]. The standard protocol for the expression and purification of PPV VLPs consists of cell lysis with a hypotonic bicarbonate buffer and separation of the VLPs from the clarified lysate by using a 20% ammonium sulfate precipitation [8].

PPV VLPs, in contrast to inactivated vaccines, do not require the propagation of infectious virus and are safer inherently. Besides their use as vaccine for PPV, these PPV VLPs have shown interesting properties as

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the ability to elicit Th and CTL responses to inserted foreign epitopes [9,10]. These characteristics extend the range of applications of PPV VLPs beyond the scope of veterinary vaccines. Moreover, many VLPs have already been described for other viruses that could be used for vaccination purposes [11–15]. A potential risk associated to these VLP-derived vaccines consists of the possible contamination with the recombinant baculoviruses. Presence of minor amounts of DNA is not an issue in veterinary vaccines. There are a number of available methods for viral inactivation of biological samples. They include ultrafiltration, alkylating agents, ultra-violet (UV) radiations, γ-radiation, thermal treatments and detergents (for a review, see [16]). Pasteurization is a simple alternative, but only acceptable if the VLPs are able to stand the thermal treatment which could be the case since PPV virions are highly thermostable (4 h at 56°C) [17], requiring up to 2 days for a complete inactivation at this temperature [18]. The non-ionic detergent Triton X-100 in combination with the solvent tri-n-butyl-phosphate (TNBP) is a good option for inactivating enveloped viruses (i.e. baculoviruses). Triton X-100 would disturb the lipidic envelope of the baculoviruses without affecting the protein–protein interactions that maintain the VP2 particle structure. Finally, the aziridine binary ethylenimine (BEI) is a highly potent alkylating agent that can selectively react with nucleophylic groups of nucleic acids but not with proteins [19]. This agent is superior in safety and antigenicity to other commonly used viral chemical inactivants, such as formalin or β-propiolactone [20]. Other inactivating strategies were generally more expensive or more laborious.

The aim of this work was to develop an optimized inactivation scheme to render a PPV-recombinant vaccine that fulfils all the safety and registration criteria. This step requires the inactivation or removal of undesirable elements, including contaminant baculovirus and non VLP structures, such as possible monomers or intermediate oligomers if any, that could cause a decline in vaccine efficacy or suppose a potential risk. We have analyzed three different baculovirus-inactivation protocols in terms of efficiency as well as characterization of the structural integrity of the treated PPV VLPs. A quantitative double antibody sandwich (DAS)-ELISA was optimized for standardization of the antigen dose. Finally, the immunogenicity of the inactivated VLPs was tested in guinea pigs.

2. Materials and methods

2.1. Viruses and cells

The *Spodoptera frugiperda* cell line Sf9 (ATCC # CRL 1711) was used to propagate the recombinant baculovirus, AcVP2PPV and to produce PPV VLPs, as described before [5]. Sf9 cells were grown in suspension or monolayer cultures at 27°C in TNM-FH medium supplemented with 5% fetal calf serum and antibiotics [21]. The baculovirus titer was calculated by a standard plaque assay [21].

2.2. Detection of recombinant baculovirus DNA

The presence of recombinant baculovirus DNA in the purified PPV VLPs was analyzed by PCR amplification with PPV VP2-specific oligonucleotides. For amplification, two PPV VP2-specific primers were used, i.e. 5′ TACAGATATTACCTATCATGC 3′and 5′ GTGTTCCTGGGTGTTGGTCTCCT 3′ in order to generate a fragment of 300 bp. PCR amplification was carried out in a total volume of 50 μl with 2.5 U of Taq DNA polymerase, 200 μM dNTPs and 200 ng of each primer. Amplifications comprised 30 cycles of denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s, and extension at 72°C for 30 s. Finally, a polishing step was carried out at 72°C for 8 min.

2.3. Inactivation of recombinant baculoviruses

In all the cases, the starting material for inactivation was PPV VLPs purified by 20% ammonium sulfate precipitation [8].

2.3.1. Pasteurization

PPV VLPs were heat-inactivated at 60°C in the presence of 20% sucrose as stabilizer. Aliquots were taken at different times from 0 to 10 h and stored at 4°C until titration of baculoviruses.

2.3.2. Inactivation by detergent

PPV VLPs preparations were incubated at 25°C in the presence of 1% Triton X-100 (Merck) and 0.3% TNBP (Merck). In order to determine the optimal conditions for the detergent treatment, a time-course experiment was carried out. Samples were removed at different times (from 0 to 8 h) and immediately dialyzed overnight against phosphate buffer saline (PBS) to wash out the detergent. The dialyzed material was stored at 4°C until required for analysis.

2.3.3. BEI inactivation

Binary ethylenimine was freshly prepared by cyclization of 0.2 M 2-bromoethylamine hydrobromide (Merck) in 0.4 M NaOH at 37°C for 2 h. The reaction was controlled by following the drop in pH linked to the formation of BEI. PPV VLPs were incubated with 10 mM BEI for 52 h at 37°C. Aliquots were taken at different times from 0 to 52 h and the residual BEI was hydrolyzed with 15 mM sodium thiosulfate. The samples were stored at 4°C until analysis.

2.4. Size-exclusion chromatography of PPV VLPs

One milliliter of partially-purified PPV VLPs (1.8 mg/ml) was loaded onto a Sephacryl S-1000 SF (Pharmacia Biotech) column, equilibrated in PBS. The sample was run at 1 ml/min flow rate and the chromatogram registered at 280 nm. Fractions of 2.5 ml were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 9% polyacrylamide gel. Homogeneous fractions were pooled and further concentrated with an Ultrafree-15 Biomax-100 filter device (Millipore).

The purity of the PPV VLPs, as well as the accurate sample concentration, was determined by amino acid analysis. Briefly, one nanomole of PPV VLPs was hydrolyzed in the vapor of 6 M HCl in vacuum-sealed ampoules. The amino acid composition and quantitation was determined by derivatization with phenylisothiocyanate and separation of the resulting phenylthiocarbamyl derivatives by reverse-phase high performance liquid chromatography (HPLC) [22].

2.5. DAS-ELISA

Microtiter plates (96 wells; LabSystems) were coated with anti-PPV monoclonal antibody (MAb) 15C5 [23] at 0.5 μg per well overnight in 0.05 M carbonate buffer (pH 9.6). Washes between consecutive steps were performed with 0.05% Tween 20 in PBS. Plates were incubated with serial dilutions of the PPV VLPs in PBS containing 0.35 M NaCl, 0.05% Tween 20 (dilution buffer) for 1 h at 37°C. After washing, we added anti-PPV biotinylated MAb 13C6 [23], 1:50 000 in dilution buffer, for 1 h at 37°C. Plates were washed and incubated with peroxidase-labeled streptavidine 1:8000 (Sigma) for 30 min at room temperature. Reaction was detected by adding 2,2′-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid; ABTS) as substrate for 10 min and stopped by the addition of 100 μl of 2% SDS. The optical density of the samples was determined at 405 nm in an ELISA reader (Bio-Tek Instruments). To calculate the capsid content in the samples, we used highly purified PPV VLPs from size exclusion chromatography, as the standard of reference.

2.6. Hemagglutination analysis

A simple approach for testing the intactness of the particle is the use of haemagglutination (HA). PPV HA titer was determined by standard titration techniques using 1% guinea-pig red cells diluted in PBS, as previously described [24].

2.7. Electron microscopy

To prepare the grids, they were placed on top of a drop of particle suspension for 10 min. Negative staining was achieved by transfer of the grids to a drop of 2% uranyl acetate solution or phosphotungstate acid for 3 min. The grids were blotted from the edge and air dried before examination by transmission electron microscopy.

2.8. Immunization of guinea pigs

Groups of five guinea pigs were used for the immunization experiments. Another group was left unvaccinated. For determination of the critical dose, animals received different doses between 0.23 and 0.0023 μg. To analyze the antibody response of the inactivated particles, an indirect ELISA was carried out. Briefly, 96-well microtiter plates (LabSystem) were coated with 0.25 μg of PPV VLPs in 100 μl of 0.05 M carbonate buffer (pH 9.6). Then, the plates were incubated with serial dilutions of the guinea pig sera in the dilution buffer (0.35 M NaCl, 0.05% Tween 20 in PBS) for 1 h at 37°C. After washing, the plates were incubated with peroxidase-conjugated protein A 1/5000 in the dilution buffer for 1 h at 26°C. Reaction was detected as in DAS-ELISA. For testing the effect of the different inactivation procedures, guinea pigs received two doses of 0.073 μg of PPV VLPs (calculated by DAS-ELISA), inactivated or not, separated by 3 weeks. The vaccine was formulated using carbopol (Carbopol 934 PH, 3×10^6 Da. BF Goodrich, USA) [25] as adjuvant at a concentration of 4 mg/ml. Vaccines were administered intramuscularly in a final volume of 0.5 ml. Animals were bled by cardiac venipuncture at days 0, 21 and 31 post-immunization.

3. Results

3.1. Detection of baculovirus contamination in purified PPV VLPs

To determine whether baculoviruses were present in the semipurified PPV VLPs after precipitation with ammonium sulfate, we performed two different analyses. First, PCR amplification and second, virus titration. Since no other baculoviruses could be present, detection of the recombinant baculovirus genome was carried out by PCR using specific oligonucleotides for the PPV VP2-inserted sequence. An amplified DNA fragment corresponding to the expected size of the PCR product was clearly observed, indicating the presence of the recombinant baculovirus in the VLP preparations (data not shown). Live baculoviruses were quantitated by plaque assay. The baculovirus titer exceeded 10^7 pfu/ml, confirming the presence of recombinant virus in the preparations of PPV VLPs. Therefore, baculovirus inactivation or removal should be accomplished in order to fulfil the safety requirements for registration and vaccine commercialization.

3.2. Baculovirus inactivation by pasteurization

Pasteurization was selected due to the thermal stability of the original PPV and the easy and simple management of the technique. Fig. 1A shows a time–course analysis of the PPV VLPs treated at 60°C, using 20% sucrose as stabilizer to reduce product damage. After 1 h incubation at 60°C, there was no detectable baculovirus. However, only 75% PPV VLPs remained intact, according to the data obtained by DAS-ELISA. The structural integrity of the remaining particles was further confirmed by electron microscopy (Fig. 1B). When samples were treated for longer times, the number of regular particles progressively declined until its complete disappearance (data not shown), in correlation with the DAS-ELISA results. The complete thermal disintegration of PPV VLPs required a prolonged incubation at this temperature, at least 10 h. However, SDS-PAGE analysis of pasteurized samples showed no evidence of VP2 degradation at different incubation times. Altogether these observations suggest that a prolonged thermal treatment causes an irreversible disassembly of the VLPs but not the breakage of VP2.

3.3. Baculovirus inactivation with detergents

PPV VLPs were treated with 1% Triton X-100 in 0.3% TNBP at 25°C in a time course experiment. At 30 min, no baculovirus was detected after the dialysis required to remove the detergent (Fig. 2A). The ability of the VLPs to agglutinate erythrocytes was preserved after treatment with Triton X-100 and dialysis. Both treated and untreated samples gave identical results by HA and DAS-ELISA as shown in Fig. 2B. Electron microscopy confirmed the physical integrity of the detergent-treated PPV VLPs. Triton X-100-treated VLPs showed icosahedral morphology indistinguishable from

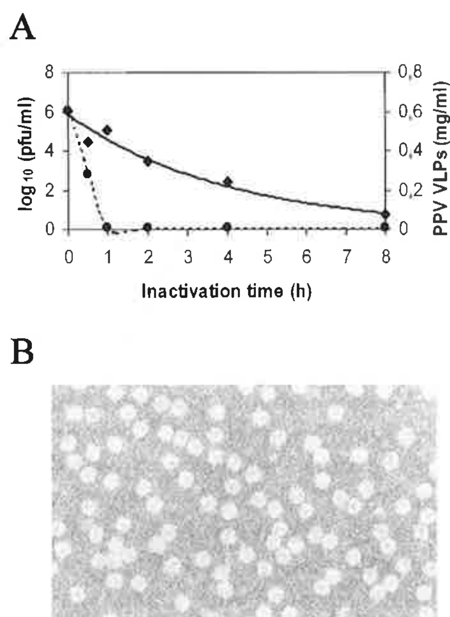

Fig. 1. Inactivation of baculoviruses by pasteurization. (A) Time course of inactivation. Virus inactivation is shown as $\log_{10}$ of the virus titer determined by plaque assay (●). The concentration of PPV VLPs was calculated by DAS-ELISA (◆); (B) electron microscopy of the capsids after pasteurization for 1 h. PPV VLPs were stained with phosphotungstic acid.

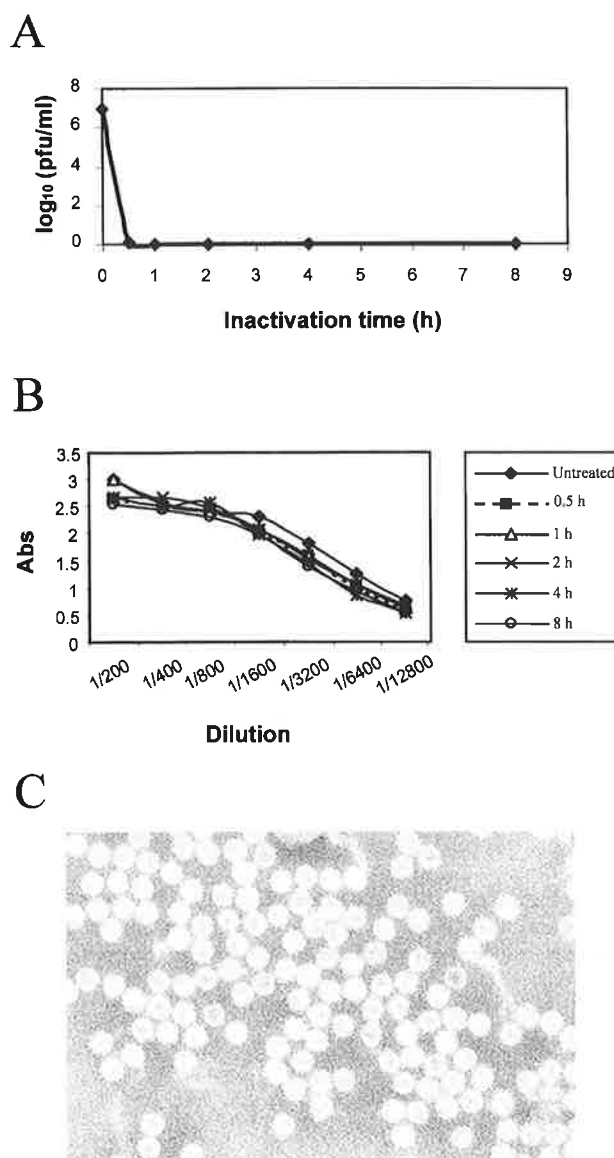

Fig. 2. Inactivation of baculovirus with 1% Triton X-100/0.3% TNBP. (A) Time course of inactivation. Virus inactivation is shown as $\log_{10}$ of the virus titer determined by plaque assay; (B) quantitation of Triton X-100 treated PPV VLPs by DAS-ELISA. PPV VLPs were incubated for different periods of time (0.5–8 h) with Triton X-100 and the amount of VLPs was estimated by DAS-ELISA and compared with untreated samples; (C) electron microscopy of PPV VLPs after 30 min inactivation. PPV VLPs were stained with phosphotungstic acid.

the original untreated sample (Fig. 2C). After two passages of the inactivated material in tissue culture, no infectious baculovirus was detected.

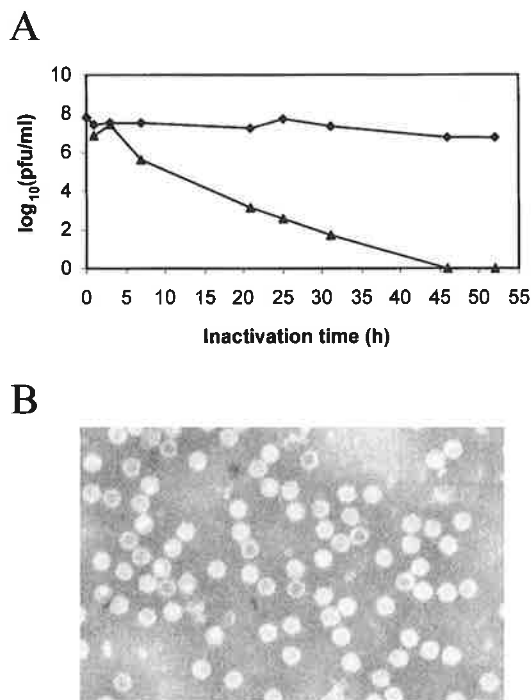

Fig. 3. Inactivation of baculoviruses with BEI. (A) Time course of inactivation with 10 mM BEI (▲) or without treatment (◆). The virus inactivation is expressed as $\log_{10}$ of the virus titer; (B) electron microscopy of the PPV VLPs after treatment with 10 mM BEI for 48 h. PPV VLPs were stained with phosphotungstic acid.

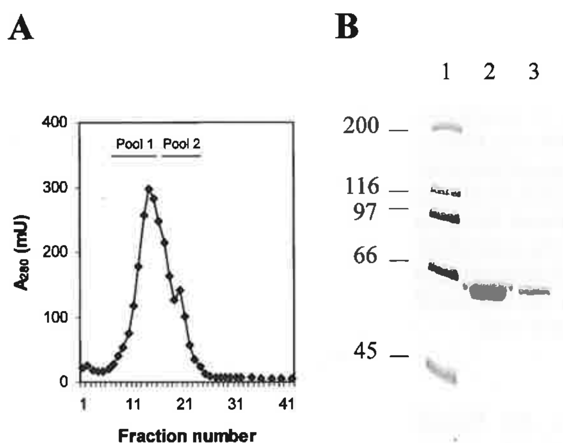

Fig. 4. PPV VLPs purification by size exclusion chromatography. (A) One milliliter of partially purified capsids (1.8 mg) was loaded onto a Sephacryl S-1000 SF column, equilibrated in PBS. The sample was run at 1 ml/min flow rate and the chromatogram was registered at 280 nm; (B) PPV VLPs-containing fractions were pooled in two groups and analyzed by Coomassie Blue staining in a 9% SDS-polyacrylamide gel. Lane 1, molecular weight markers; lane 2, pool 1; lane 3, pool 2.

3.4. BEI inactivation

The reduction of the baculovirus titer by treatment with 10 mM BEI is shown in Fig. 3A. Viral inactivation with the alkylating agent followed a first order reaction and reached the end-point (total inactivation) at 48 h. At this time, analysis by electron microscopy resulted in the detection of regular PPV icosahedral particles (Fig. 3B). Antigenicity was also preserved, according to the results obtained by HA and DAS-ELISA, as the values were similar to the untreated controls (data not shown). These results confirm the adequacy of BEI to preserve the integrity of the particles.

The absence of any residual active baculovirus was further confirmed by viral accumulation experiments. Sf9 cells were inoculated with inactivated PPV VLPs diluted 1/10 in culture medium. After 3–4 days, the supernatant was collected and used to infect fresh Sf9 cell monolayers. At the end of the third passage, the culture medium was titrated, but no baculovirus plaques were detected, indicating total inactivation of the virus.

3.5. Purification of PPV VLPs by size-exclusion chromatography

In parallel to baculovirus inactivation, a further purification step of PPV VLPs was performed by gel filtration on Sephacryl S-1000 SF (fractionation range from 5×10^5 to more than 10^8 Da). This step would allow for a complete removal of the recombinant baculoviruses as well as any VP2 monomer or intermediate VP2 oligomer left from the assembly process. The regular PPV virion contains 60 copies of VP2 (64 kDa) with an expected Mr of 3.8×10^6 Da, which is within the separation range of the matrix. The standard chromatogram registered at 280 nm is shown in Fig. 4A. A prominent peak appeared at the position expected for the VP2 capsids. No peak corresponding to VP2 monomers or oligomers was apparent. However, the VLPs-containing peak was not homogeneous, presenting a shoulder at lower retention time. We pooled the peak fractions in two (pool 1 and 2; Fig. 4A) and both presented a unique VP2 band in SDS-PAGE (Fig. 4B). When both samples were spectrophotometrically analyzed, only pool 1 gave a classical spectrum of protein with a maximum at 280 nm. Pool 2 showed a maximum absorbance at 250 nm, indicating the presence of a non-protein contaminant. However, after sample concentration with an Ultrafree-15 device, the contaminant disappeared and the sample recovered the spectral characteristics of the purified VP2.

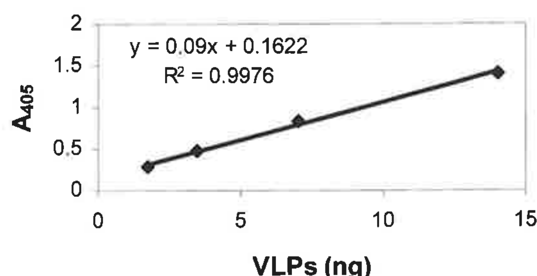

Fig. 5. Determination of PPV VLPs content by DAS-ELISA. Correlation between the amount of purified PPV VLPs (standard of reference) determined by amino acid analysis and the absorbance at 405 nm obtained by DAS-ELISA. Linear regression was calculated by using the program Excel (Microsoft).

Table 1
Comparative analysis of different methods to determine the PPV VLPs content

Sample	Densitometry [c]	Bradford[c]	Corrected Bradford[d]	DAS-ELISA
Pre2[a]	0.3	0.55	–	0.146
Pre3[a]	2.8	1.34	–	1.0
Pur 1[b]	0.927	0.43	0.675	0.659
Pur 2[b]	0.941	0.415	0.695	0.634

[a] PPV VLPs before purification by size-exclusion chromatography.
[b] PPV VLPs after purification by size-exlusion chromatography.
[c] Densitometry and Bradford were carried out using BSA as reference.
[d] Correction factor was the ratio between the value obtained by amino acid analysis of the PPV VLPs used as standard of reference and the value obtained by Bradford. It can be used only in purified samples.

According to size, intact baculovirus must elute in the void volume, before PPV VLPs. To confirm the absence of baculovirus contamination in the PPV VLPs fractions, they were titrated by plaque assays. They showed a complete absence of active virus. Moreover, the presence of inactive baculoviruses was investigated by PCR in different samples and no DNA was amplified (data not shown).

The purity of the collected VP2 samples was further confirmed by amino acid analysis. This analysis was also used to determine the accurate protein concentration of the purified VLPs. The sample showed to be homogeneous with a purity >99%.

3.6. Estimation of vaccinal dose by DAS-ELISA

To determine precisely the dose of vaccine, we used DAS-ELISA. Homogeneous PPV VLPs were used as the standard of reference for the accurate estimation of the antigen concentration in partially purified samples. By DAS-ELISA, a good linear correlation ($R^2 > 0.99$) was observed between the ELISA signal and the amount of purified PPV VLPs in a concentration interval ranging from 1 to 15 ng PPV VLPs/well (Fig. 5). Then, the DAS-ELISA was applied to several samples of PPV VLPs, purified by size-exclusion chromatography or not, and compared with other conventional methods for protein quantitation (Table 1). In general, traditional methods, as densitometry or Bradford, gave an overestimation of the amount of VLPs in less purified samples, which will be the regular antigen for vaccination purposes, as compared with the DAS-ELISA. Data from the amino acid analysis allowed establishing a correction factor for the Bradford assay, which led to more accurate estimations of VLPs in purified samples (Table 1). These results demonstrated the suitability of the DAS-ELISA to accurately estimate the VLPs concentration in the vaccine and ensure a proper standardization of the dose.

3.7. Immunogenicity of viral-inactivated VLPs

To have a useful impression of the immunogenicity of the VLPs, a critical dose was selected to apply in subsequent immunogenicity and potency studies. Therefore, a BEI-inactivated sample was tested at a series of doses (2.3–230 ng). Guinea pigs were bled at day 27 and the antibody titers were determined by indirect

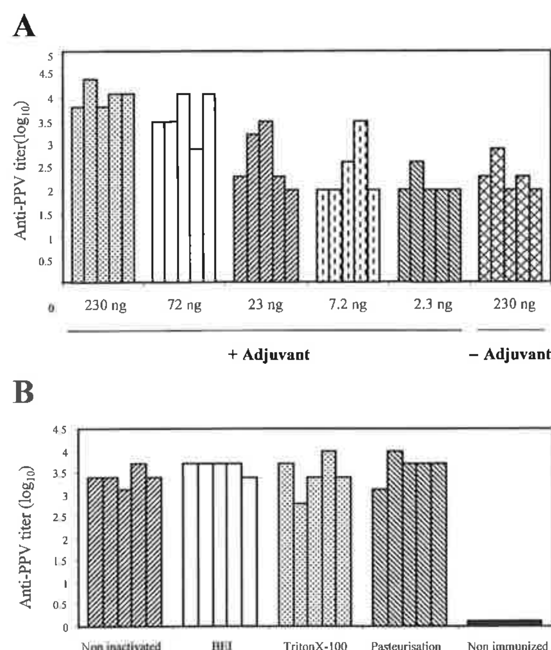

Fig. 6. Immunogenicity of PPV VLPs. (A) Determination of critical dose. Groups of five guinea pigs were immunized with different doses (250–2.3 ng) of BEI-inactivated particles in carbopol adjuvant. Sera were collected at day 28 p.i. and antibody titers against PPV VLPs were tested by indirect ELISA; (B) effect of the different treatments of inactivation. Sera from five guinea pigs immunized with either 0.073 μg of inactivated PPV VLPs, non-treated PPV VLPs or non-immunized were tested against the PPV capsids by indirect ELISA. The results are shown as the $\log_{10}$ of serum dilutions that gave an absorbance three times greater than the cut-off value.

ELISA (Fig. 6A). From the results, it appears that a dose of 73 ng of antigen represents a suboptimal level, i.e. critical dose for immunogenicity and can be used in the future for comparison of standard doses.

To check the immunogenicity of the VLPs after baculovirus inactivation, we carried out a preliminary immunization experiment in guinea pigs. Groups of five animals were twice immunized intramuscularly with 73 ng of inactivated PPV VLPs, the non-treated particles or non-immunized. VP2-specific antibodies in the guinea pig sera were evaluated by indirect ELISA. The results are shown in Fig. 6. In general, all the inactivation procedures were quite efficient in preserving the immunogenicity of the particles, rendering antibody titers between 10^3 and 10^4, similar to those obtained with the non-treated particles. BEI-inactivated VLPs induced a strong antibody response in the animals and, at the same time, less variability in the response. In contrast, Triton X-100-inactivated particles elicited a response with a slightly higher variability than BEI or pasteurization. We also tested the neutralizing ability of the guinea pig sera (data not shown). The results indicated that there was an excellent correlation between ELISA and neutralization titers, confirming the functionality of the antibodies.

4. Discussion

The registration of a recombinant vaccine for commercial use obliges to fulfil a series of safety and regulatory requirements. In particular, they have to be free of any recombinant genetic material, such as live baculoviruses. Here, we have demonstrated that partially purified PPV VLPs were contaminated by baculoviruses and required specific treatments to inactivate them. Moreover, the inclusion of a previous viral inactivation step is a prudent policy to avoid other undesired and unexpected adventitious contaminants in the vaccine.

Other VLPs expressed in insect cells have been proposed as vaccines [14,15,26,27] but no reports have dealt with baculovirus' presence and/or inactivation, which is a critical issue for vaccine development and to avoid the spread of genetically-modified microorganisms.

Therefore, we have investigated a number of protocols for the inactivation of baculoviruses, i.e. pasteurization, treatment with non-ionic detergents and DNA alkylation with BEI. Other available methods, such as photochemical inactivation by ultraviolet irradiation, were considered impractical or too expensive for large scale manufacturing processes. Pasteurization is the simplest method. It does not require specific equipment or reagents, but simple heating at moderate temperature (60°C). A short incubation time (1 h) was enough to completely inactivate baculoviruses. However, although most of the PPV VLPs remain intact, a significant amount of material ($< 25\%$) was accounted as disrupted particles after the treatment. This result was established because the MAbs employed in the DAS-ELISA did not recognize PPV VP2 in western blot [23], as they were specific for the native conformation. Therefore, pasteurization was discarded as we assume that the disassembly of the VLPs would greatly decrease the vaccine efficacy and stability.

Viral inactivation by treatment with Triton X-100 is a fast, simple and effective alternative. Since the electron microscopy and antigenicity results were excellent, this technique might represent an attractive solution that does not require the use of carcinogenic substances as BEI. However, the requirement of an additional dialysis step might eliminate the time and simplicity advantages. Dialysis does not guarantee the complete removal of the detergents, although it removes more than 95% of the material in less than 24 h. Since the required dose of antigen is extremely low, a simple and fast alternative could be the direct dilution of the inactivated material in the diluent to the final concentration. If necessary, there exist other alternatives for the complete removal of Triton X-100, such as hydrophobic resins or diafiltration.

BEI is the preferred inactivating agent for veterinary vaccines [19]. Chemical inactivation by BEI follows first-order kinetics and allows determining and controlling the reaction rate and the end-point. The excess of reagent can be easily neutralized by addition of sodium thiosulfate and does not require clearance of the chemicals. Complete inactivation of virus infectivity was confirmed by repeated passage of the virus in insect cells. Since BEI only reacts with nucleic acids, the structural and functional integrity of the VLPs was ensured. Therefore, this inactivation protocol is simple, easy to control, and preserves both structural integrity and antigenicity of the sample. However, it presents serious drawbacks as the necessity of prolonged inactivation times (48 h) and the carcinogenicity of BEI.

Other crucial issue relates to the purity of the material, as some times the presence of monomers or other intermediates has been invoked as potentially negative for the vaccine. For all these reasons, a further purification step was carried out by size-exclusion chromatography. This step yielded homogeneous PPV VLPs with a purity $> 99\%$. Remarkably, no VP2 monomer contamination was found. Even the pre-purified sample did not contain VP2 monomers because no protein eluted at the corresponding position and no protein was recovered after ultrafiltration through a 100 kDa cut-off filter, which confirms our previous indirect observations. This result suggests also that the particle assembly occurs through an 'all or none' process. Moreover, no baculoviruses were detected in the purified VLPs

preparations. However, although this strategy is efficient in removing baculovirus, it is not economically feasible for viral clearance in vaccine production.

After inactivation, a further concern would be related to the immunogenicity of the residual baculovirus. However, even in the worst scenario, animals would not receive more than 10^4 inactivated baculovirus per dose of vaccine, which, in terms of mass, would be insignificant for induction of antibodies or side effects, as hypersensitivity, even if repeated injections were necessary. On the other hand, residual baculovirus DNA from BEI or Triton X-100-inactivated VLPs was unable to regenerate recombinant baculoviruses after incubation with insect cells (data not shown). Therefore, even if minute amounts of baculovirus protein and DNA remain in the vaccine, they would not affect the safety of the vaccine.

For standardization of the vaccine dose, an appropriate method as well as an antigen standard were necessary. The DAS-ELISA presents several advantages compared with classical indirect protein determination methods, as it is highly conformation-specific, recognizing exclusively PPV VLPs, it does not require pure samples and allows proper antigen quantitation using as few as 2 ng of protein, between two or three orders of magnitude below the standard methods. As standard of reference, we used the extremely pure preparation of VLPs obtained by chromatography. This DAS-ELISA was very efficient in the estimation of exact amounts of VLPs in semi-purified preparations. In contrast, determinations made by Bradford or densitometry gave a considerable error. Therefore, our DAS-ELISA is an accurate method to specifically determine and standardize the amount of intact PPV VLPs in the vaccinal doses.

Finally, the most important pre-requisite of an inactivation and/or purification protocol is that it should not reduce significantly the immunogenicity of the sample. All the in vitro determinations performed with the inactivated samples have shown no reduction either in antibody recognition or functional hemagglutination compared with the untreated material. Most importantly, when an experimental vaccination was carried out in guinea pigs using the critical dose of PPV VLPs (0.073 μg per dose), a strong immune response was elicited. It is remarkable that despite the extremely low dose, a strong response was observed in all the cases confirming the good immunogenicity of these particles. BEI-inactivated vaccines were more consistent in eliciting a high antibody response. Regarding pasteurization, it should be taken into account that the amount of PPV VLPs used in the immunization was normalized by DAS-ELISA. Therefore, the pasteurized samples contain 25% more antigen than the others. The response elicited by Triton X-100-inactivated PPV VLPs was more variable in the induction of antibodies. However, if we take into account the advantages of Triton X-100 versus BEI, such as 30 min inactivation versus 48 h, which implies more speed and economy of the whole process, then, Triton X-100 is the best choice for vaccine production. Moreover, Triton X-100 is less toxic for the operators than BEI, well known by its carcinogenicity. Since pasteurization was discarded because the partial disassembly of PPV VLPs, Triton X-100 remained as the better choice for baculovirus inactivation and was used in the potency experiments in pigs, which are currently in progress.

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