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Mucosal or systemic administration of rE2 glycoprotein antigen loaded PLGA microspheres

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ABSTRACT

We have evaluated the ability of recombinant E2 antigen, as a surfactant free formulation of poly (D,L-lactide-co-glycolide) (PLGA) microspheres, to elicit a systemic immune response after administration by mucosal routes (oral and nasal) in comparison to intramuscular route. The sequence encoding a truncated E2 glycoprotein of the classical swine fever virus (CSFV) was expressed in insect cells following infection with recombinant baculovirus, as a His-tagged recombinant antigen. The recombinant E2 glycoprotein (rE2) antigen was co-encapsulated with rabbit serum albumin (RSA) as a protein stabilizer. rE2/RSA loaded PLGA microspheres, with a mean diameter of 4 μm were obtained by a water in oil in water solvent extraction method (w/o/w). Rabbits were immunized with 10 μg of rE2 formulated in PLGA microspheres administrated by three different routes (oral, nasal and intramuscular). After 60 days, each rabbit in all three groups was challenge with 5 μg of rE2 glycoprotein solution by intradermal administration. Blood samples were collected weekly for 90 days and specific rE2 antigen antibodies measured. This work showed that rE2 antigen loaded microspheres was able to initiate an immune response. The intradermal challenge after nasal and oral administration had a clear boost effect on the systemic immune response. Moreover, the response after nasal administration was more intense and less variable than oral route. In conclusion, these data demonstrate a high potential of rE2 loaded PLGA microspheres for their use as a mucosal subunit vaccine.

1. Introduction

The production of specific peptides and recombinant proteins in large quantities has led to increased knowledge and advances in biotechnological and pharmaceutical applications, and stimulated the delivery of therapeutic antigens over the last 10 years (Degim and Celebi, 2007). The success of a protein as a therapeutic agent depends on the development of a formulation in which the native protein structure and activity are conserved during preparation and delivery (Allison et al., 1996; Wang, 1999).

Poly (D,L-lactide-co-glycolide) (PLGA) microparticles have received much attention for delivery of vaccine antigens (Jaganathan et al., 2005; Jaganathan and Vyas, 2006; Lu et al., 2007; Pandit et al., 2007). These particles offer advantages that include protection of the antigen from degradation, controlled release, and the possibility of surface modification with ligand grafting for PLGA with carboxylic acid end groups (Brandhonneur et al., 2009). To achieve successful delivery of a protein vaccine it is necessary to preserve the structure and function of the protein encapsulated. The incorporation into microspheres of such excipients as sugars (Jaganathan et al., 2005; Perez et al., 2002; Perez and Griebenow, 2001) or proteins (Chang and Gupta, 1996; Johansen et al., 1998) assures stability, unfolding and non-aggregation of the protein upon encapsulation and release. The most commonly used method to encapsulate vaccines is the emulsion/solvent evaporation process, using classical emulsifiers such as poly(vinyl alcohol) (PVA) to stabilize the emulsion. Recently, Keegan et al. (2004) showed that a residual layer of PVA emulsifier remains coated to washed particles creating a potential barrier for efficient ligand grafting to the acid end groups of PLGA chains. Therefore, to allow ligand grafting to improve the cellular uptake of these particles, use of such excipients should be avoided.

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), a contagious and often fatal disease of swine. CSFV belongs to the genus Pestivirus in the family *Flaviviridae*. In many European countries this virus is not endemic, but outbreaks of CSFV occur periodically causing large economic losses. The currently available conventionally attenuated live CSF vaccines are safe and highly efficacious. However, following vaccination no serological discrimination between infected and vaccinated animals (DIVA

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strategy) can be made. Future vaccines should have the same efficacy as the classically live CSF vaccines but should, in addition, follow the DIVA strategy. The most immunogenic envelope protein of CSFV is E2 which induces neutralizing antibodies in pigs (Van Rijn et al., 1996). Two commercially available subunit vaccines based on baculovirus-expressed E2 glycoprotein of CSF virus have been developed: BAYOVAC® CSF Marker (Bayer Leverkusen, Germany), and Porcilis® Pesti (Intervet, Boxmeer, The Netherlands). However, the immune response obtained with these subunit vaccines is delayed compared to the conventional live attenuated CSF vaccine "Chinese strain vaccine" that establishes a strong immunity 1–2 weeks earlier.

Furthermore, these subunit vaccines are less protective compared to conventional live attenuated CSF vaccines. Indeed a single vaccination with subunit vaccines afforded reduction in morbidity and mortality following subsequent challenge with a virulent CSFV field strain at 14 days post-vaccination, but horizontal or vertical transmission was not completely prevented (Bouma et al., 1999; Uttenthal et al., 2001).

For wildlife or farm animals, oral administration of E2 encapsulated microparticles via baits or food is of interest to induce a mucosal immunisation directly at the site of infection. Mucosal immunization is an attractive alternative to parenteral immunization allowing the stimulation of humoral as well as cell-mediated responses and the simultaneous induction of systemic immunity. Hence the design of a mucosal vaccine using a controlled release microparticulate formulation of recombinant E2 glycoprotein (rE2) should be of paramount interest. Moreover, this approach should offer the possibility of discriminating CSFV infected pigs from vaccinated pigs (DIVA strategy), by detecting serum antibodies specific to a second envelope protein, the Erns glycoprotein. Indeed significant levels of antibodies are raised against Erns and E2 in infected animals (Konig et al., 1995).

The aim of this work was to evaluate the ability of rE2 glycoprotein loaded surfactant free formulation PLGA microspheres, to elicit a specific systemic immune response after administration by the mucosal route (oral and nasal). This required the production of rE2 glycoprotein in insect cells using recombinant baculovirus, maintenance of the integrity of the protein and the preparation of surfactant free PLGA microspheres.

2. Materials and methods

2.1. Chemicals

Restriction enzymes were purchased from New England Biolabs (Beverly, MA). DNA sequencing was performed using the Sequencing kit (Big Dye®) from Applied Biosciences (Warrington, UK) and DNA oligonucleotides were from Proligo Primers & Probes (Proligo, France). The pGEM®-T easy cloning vector was purchased from Promega (Southampton, UK). The expression vector pBAcPAK8 and the baculoviral genome (BacPAK6) from Clontech were used to obtain the recombinant viruses. The chromatographic columns Histrap Metal Chelating HP, HiPrep 26/10 desalting, ECL film and Anti-mouse HRP labelled antibodies and primary Anti-His tag antibody were purchased from GE Healthcare (Upsala, Sweden). *Spodoptera frugiperda* (*Sf*9) cells were maintained at 28 °C in TC100 medium (Invitrogen Carlsbad, CA) supplemented with 5% fetal calf serum.

Resomer RG 503H PLGA (lactide/glycolide ratio 50:50, inherent viscosity 0.32 dl/g, PSE MW 31 kDa, acid number 3 mg KOH/g) was obtained from Boeringer Ingelheim (Ingelheim, Germany) which presents carboxylic acid end groups. Phosphate buffered saline, phosphate buffer and rabbit serum albumin (RSA) were purchased from Sigma (Saint-Quentin Fallavier, France). And all other general chemicals and reagents were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France).

2.2. Experimental animals

The study was approved by the Committee of Laboratory Investigation and Animal Care of our institution and performed in accordance with French Ministry of Agricultures laws and guidelines for laboratory animal experiments (agreement no. B35-238-21).

In vivo experiments were performed on New Zealand white adult female rabbits (2.45–3.65 kg in weight). The animals were housed individually and maintained in animal care facilities for at least 1 week before the first immunisation. The use of rabbits as model animals in this study is appropriate as CSFV infection causes hyperthermia and induces the synthesis of specific antibodies (Chenut et al., 1999).

2.3. Production of recombinant antigen E2

For the construction of pBacPAK8-E2 CSF a reverse transcription polymerase chain reaction (RT-PCR) was performed using primers based on the E2 sequence, of CSFV, accession numbers X87939. The forward primer (5′GCG C GAA TTC AAC **ATG** GCC ACT ACT GCG TTT CTC ATT TGC 3′) was engineered to introduce an EcoRI site (underlined), start codon (bold) and a Kozak consensus sequence towards the 5′ terminus of the amplicon that should function to initiate E2 expression. The reverse primer (5′ TGT GCT CGAG *TTA* **GTG GTG GTG GTG GTG GTG** ACA AAC CTC GGC AAA GTA GTC TGT 3′) contained the recognition sequence for the restriction enzyme *Xho*I (underlined), downstream of six histidine codons (bold) and a stop codon (italic). Thus the 31 C-terminal trans-membrane amino acid sequence of native E2 was replaced by a six histidine-tag sequence. Amplicons were initially cloned into pGEM-T easy (Promega) and then subcloned into pBacPAK8 using standard techniques.The inserted sequence was verified by DNA sequencing. To generate an E2 recombinant baculovirus, 4 μg of the pBacPAK8 truncated E2 transfer vector and 500 ng of the BacPAK6 DNA, digested with Bsu36I, were used to co-transfect 2×10^6 SF21 cells in a 60 mm dish by lipofection using Insectin-Plus™ Liposomes in serum-free media, as described in the supplier's manual. Culture supernatant was recovered after 4 days of incubation at 28 °C, when signs of infection had become apparent. Recombinant baculovirus was plaque purified as follows. Six-well plates were seeded with 1×10^6 SF21 cells per well, which were then infected for 1 h at room temperature with 10^{-2}, 10^{-3}, and 10^{-4} dilutions of transfection supernatant stock. The supernatant was removed and an overlay of 1% low melting agarose was added and allowed to set. TC100 medium was supplemented with 150 μg/ml of 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-gal). The plates were incubated until plaque formation (5–7 days) was apparent. Blue plaques were observed using an inverted microscope, and four purified recombinant baculovirus clones with the expected phenotype were recovered. The E2 expression level of the selected clones was determined by immunoblotting after cell infection.

Optimally expressing baculoviral clones were diluted five times and used to infect $3–4 \times 10^6$ cells in TC100 media in a 25 cm^2 flask. After 1 h, the supernatant was discarded, 4 ml of fresh TC100 media were added and the cells were incubated for 5 days at 28 °C. The process was repeated a second time or until the virus titre was at least 10^8 pfu/ml. Virus titration was determined by standard plaque assay (O'reilly, 1997). The virus stock was stored at −70 °C prior to use.

The optimal time to harvest cells for maximum E2 protein expression was first determined by small scale expression. *Sf*9 cells adapted to suspension culture were then scaled up via intermediate stages in spinner flasks (Cellspin, IBS Integra Bio-sciences). Cells were seeded at a density of 10^7 cells/ml and infected at a multiplicity of infection (M.O.I.) of 2–3 with virus in serum-free TG3

medium for 1 h at room temperature. The infected cultures were then diluted to a cell density of 1.6×10^6 cells/ml and poured into several spinner flasks. These flasks were then incubated at 28 °C with constant spinning at 80–90 rpm until most cells showed signs of infection in 3–4 days and cell viability was estimated as approximately 70–80%. The cells were collected, counted, and washed with PBS prior to storage at −70 °C.

In order to extract E2, frozen insect cells were re-suspended in 200 μl lysis buffer (20 mM phosphate buffer pH 7.4, 0.1% (v/v) NP40, 1× protease inhibitor cocktail, 1 U/μl DNase I, RNase I) per 10^6 cells. The cells were sonicated for three cycles of 30 s with 30 s breaks repeated three times. The lysate was then incubated for 30 min on ice and then centrifuged at 100,000 g for 60 min followed by collection of the supernatant.

Automated purification of the rE2 was performed with on an AKTA Explorer (GE Healthcare) using the Unicorn software package. The clear lysate, prepared as described above, was desalted with binding/wash buffer (20 mM phosphate buffer, 500 mM NaCl, 20 mM imidazole, 0.1% NP40, pH 7.4) and loaded at flow rates 2.5 ml/min on a Histrap HP (5 ml) column pre-equilibrated with binding buffer. The column was then washed with five column volumes and eluted with a linear gradient elution buffer made by mixing progressively water and 20 mM phosphate buffer, 500 mM NaCl, 500 mM imidazole, 0.1% NP40, pH 7.4. The protein was eluted in the fraction with 100 mM imidazole.

SDS–PAGE analysis was performed directly on insect cell materials or purified protein. Samples were mixed with reducing Lane Maker sample buffer (Pierce) supplemented with 5% of β-mercaptoethanol and heated at 100 °C for 5 min. Proteins were separated in 5–15% Tris–glycine–SDS polyacrylamide gradient gels (ready gel from BIO-RAD) using the Mini-ProteanII gel apparatus (BIO-RAD) as previously described (Laemmli, 1970). Protein standards (Gibco BRL Benchmark Protein Ladders, or Amersham, ECL dual view protein marker) were included in each analysis. For western blot analysis, proteins were electrophoretically transferred (100 V for 1 h) onto nitrocellulose membranes (Hybond™ ECL-GE Healthcare) using the mini-electro blot apparatus (BIO-RAD) as previously described (Towbin et al., 1979). Non-specific protein binding sites were blocked by incubating the membrane for 30 min at room temperature in Tris-buffered-saline (TBS) (10 mM Tris–HCl, 150 mM NaCl, pH 8) containing 0.1% gelatin (BIO-RAD), 0.1% Tween-20 and 5% (w/v) powdered milk. An anti-penta-histidine mouse monoclonal antibody (Amersham) was applied at a dilution of 1/1000 for 1 h at 37 °C in TBS, 0.1% Tween containing 5% milk. Membranes were washed three times and secondary Horse radish peroxidase-conjugated anti-mouse monoclonal antibodies (GE Healthcare) were applied at a 1:1000 dilution for 1 h at 37 °C. The membranes were finally washed and subjected to ECL detection according to the manufacturer's instructions (ECL-GE Healthcare) using the detection solution Supersignal® West Dura (Pierce).

Protein concentrations were determined using the BCA procedure with BCA and bovine serum albumin as standards (Sigma–Aldrich). A theoretical molar extinction coefficient, derived from the amino acid sequence using the Vector NTI software (Invitrogen), was applied. For CSF E2 an A280 of 1 was determined to be equal to 0.73 mg/ml.

The purification steps were monitored by SDS–PAGE, western blot and by Lab on Chip Protein 200 using the Bio Analyser 2100 (Agilent). The percentage of E2 in the purified fraction and the purity were calculated using the 2100 Expert software.

2.4. Preparation and characterization of rE2 loaded PLGA microspheres

rE2 antigen (rE2)/rabbit serum albumin (RSA) loaded PLGA microspheres were prepared using a surfactant free double-emulsion (w/o/w) solvent extraction method. Briefly, 500 mg PLGA were dissolved in 5 ml dichloromethane and emulsified with 500 μl of rE2 glycoprotein (350 μg/ml) containing RSA (50 mg/ml) in phosphate buffer saline by sonication for 30 s at 20% intensity (Vibra Cell TM, Sonics & Materials Inc., USA). In this formulation of microspheres, rE2 glycoprotein represented 0.70% of the total amount of protein. This first emulsion was poured into 100 ml of phosphate buffer with 10% mannitol and mixed for 10 min at 5000 rpm using a turbine homogenizer (Polytron, PT 3000, Kinematica AG, Lucerne, Suisse). Finally, extraction of the organic solvent was accomplished by evaporation. Microspheres were collected by centrifugation at $4500 \times g$ for 5 min, washed and lyophilized. The microspheres were stored under vacuum at 4 °C.

The average volume diameter D [4, 3] and the particle size distribution were examined by laser light scattering using a Mastersizer S (Malvern Instruments, Orsay, France). The microspheres were suspended in aqueous solution containing 0.05% Tween 20 as a wetting agent to minimize agglomeration, sonicated for 1 min and analysed in triplicate. The surface morphology of the microspheres was analysed by scanning electron microscopy (SEM) using a Jeol JSM Model 6400 electron micrograph (Jeol, Tokyo, Japan).

The zeta potential of microspheres was determined using a Malvern nano ZS (Malvern Instruments, Orsay, France). Three samples were measured at ambient temperature in distilled water.

Given the sensitivity of the method (Micro BCA), we could not quantify the release of rE2. Since rE2 and RSA have similar molecular weight (MW) (rE2 ∼75 kDa and RSA ∼60 kDa), we made the assumption that the release rates were similar and that measurement of RSA release could be a surrogate marker for rE2 release rate.

The amount of rE2/RSA encapsulated in microspheres was determined by two different methods. For the first method involving indirect quantification during the fabrication process, the microspheres were collected by centrifugation, and the supernatant was removed. The Micro BCA protein assay method was used to determine the unloaded rE2/RSA in the supernatant. The second method involved direct quantification of rE2/RSA loaded in microspheres after digestion with 1 ml of 1 M NaOH at 30 °C for 24 h using the Micro BCA protein assay method.

In vitro protein release was determined by mixing 20 mg microspheres in 2 ml of phosphate buffer at pH 7.4 with 0.2% pluriol and 0.02% of sodium azide in dialysis tubing (Spectra/Por®—MWCO: 12, 14 kDa) incubated in a 1 l of similar buffer in a closed polypropylene container at 30 °C under continuous orbital rotation. At predetermined times, the supernatant from each tube was collected by centrifugation at $8000 \times g$ for 10 min. The supernatant was frozen and stored at −20 °C until analysed. The rE2/RSA concentration in the release medium was determined by the Micro BCA protein assay.

Release experiments were performed in triplicate, the results averaged and the standard deviation (SD) calculated.

2.5. In vivo studies

The experimental design was summarized in Table 1. Briefly, the first *in vivo* study aimed to evaluate the immunogenicity of rE2 as soluble antigen alone in comparison to a subunit marker vaccine Porcilis Pestis (kindly provided by Intervet international BV) as positive control after intramuscular route (IM) inoculation at two different doses (1 and 10 μg). The experiment was performed in eight rabbits. Four rabbits received 1 or 10 μg of rE2 antigen solution. As a positive control, four rabbits were vaccinated with either 1 or 10 μg of subunit marker vaccine Porcilis Pesti.

The second study aimed to compare the immune response of rE2 formulated as microparticles by three routes of administration, intramuscular (IM), intranasal (IN) or oral (O). Three different

Table 1
Experimental design of the *in vivo* studies for the evaluation of the systemic response to E2 either in solution or as rE2/RSA loaded microspheres after intramuscular (IM), nasal (IN) and oral (O) route in rabbits (number of animals studied).

Day	Dose (μg E2)	Positive control		Negative control				
		IM		IM	IN	IM	IN	O
		rE2	Vaccine	RSA microspheres		rE2/RSA microspheres		
	0	–	–	3	3	–	–	–
D0	1	2	2	–	–	–	–	–
	10	2	2	–	–	6	6	6
D60	5	–	–	ID	ID	ID	ID	ID

ID: intradermal administration.

experimental groups were compared, 10 μg of rE2/RSA encapsulated in PLGA microspheres were inoculated in six rabbits by the IM route; six received 10 μg by IN route and the last six rabbits received 10 μg by O route. As negative controls, RSA loaded PLGA microspheres were administrated in two groups of three rabbits respectively by the IM or IN route.

After 60 days, each rabbit in the second study received 5 μg of rE2 glycoprotein solution by intradermal (ID) administration to challenge the immune response.

Blood samples were collected weekly during 90 days from the vaccination (Day 0). Sera was separated from whole blood by centrifugation (3500 × *g* for 10 min) and stored at −20 °C. Specific rE2 glycoprotein antibodies were measured using the Bommeli CHEKIT CSF ELISA kit. The positive threshold of 40% was used according to manufacturer's recommendations.

2.6. Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student *t* test. A *p* value less than 0.05 was considered as statistically significant.

3. Results and discussion

Four recombinant baculovirus clones were purified from plate lysates and amplified. Small scale cultures were prepared to test the expression of rE2 protein, to identify the clones producing most rE2 and the optimum time of rE2 expression. Little difference in expression level of rE2 protein was observed between 72 and 96 h post-infection (data not shown). The purity of the rE2 CSFV protein reached 98.4%. A contaminating protein of 122 kDa, probably the homodimer form, remained in the collected sample accounting for 1.6% of the total protein (Fig. 1). The experimental conditions allowed the production of 2 mg of protein rE2 per litre of insect-cell culture. This quantity and purity of rE2 protein allowed us to study the loading and the release of rE2 from PLGA microspheres and to set up a protocol of immunization.

The mean diameter of microspheres increased from 2.5 μm (unloaded microspheres) to 4 μm with the antigen rE2/RSA incorporation. The rE2/RSA loaded PLGA microspheres were spherical in shape with a smooth surface (Fig. 2). We noticed a zeta potential of rE2/RSA loaded PLGA microspheres of −43 ± 3 mV. The production yield of the microspheres was 85 ± 10% (w/w). It has been reported that small particles (<10 μm) are more efficient in targeting the antigen presenting cells at the mucosal surfaces (Eldridge et al., 1991) and are more readily phagocytosed than larger particles. This is reported to be necessary for stimulation of MHC class I-restricted responses (Men et al., 1999). Moreover, based on the evaluation of the total volume of particles phagocytosed, the size of 3 μm has been shown to be the most suitable for an efficient delivery to a cell line derived from rat alveolar macrophages (Hirota et al., 2007).

In a preliminary immunisation experiment after IM administration of PLGA microspheres using rE2 antigen without stabilizer, no immune response was observed. This suggested either a degradation of the rE2 antigen and/or a problem related to the release of the antigen. Since the protein is stable in contact with solvent and there is no degradation of the protein observed following electrophoresis (data not shown), the lack of immune response was attributed to an unsuitable release rate of the antigen. However, this could not be confirmed directly as we were unable to quantify *in vitro* the release of rE2 due to a low sensitivity of the method. Hence, to resolve this problem, the rE2 antigen was co-encapsulated with RSA as a potential protein stabilizer during encapsulation and subsequent release. Previously, it had been shown that porcine gelatine type A or human serum albumin can stabilize tetanus toxoid in PLGA microspheres during microencapsulation and release (Chang and Gupta, 1996). Given the assumption that E2 and RSA should behave in a similar manner as a result of their similar molecular weight,

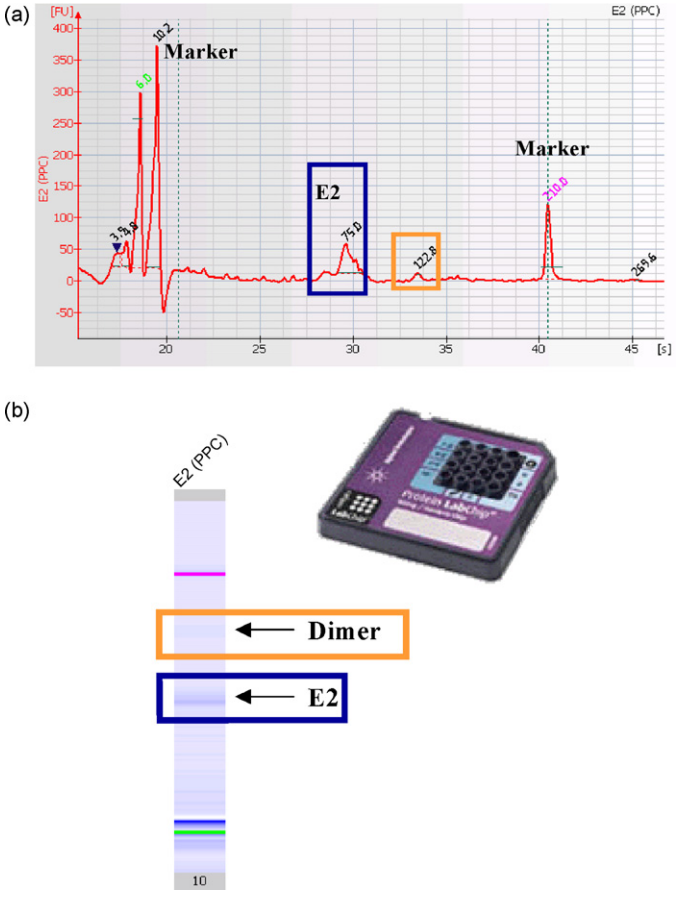

Fig. 1. Quantification and purity estimation of the rE2 protein preparation using the Bio Analyser 2100 Expert (Agilent) and LabChip Protein 200 cartridge. (A) electropherogram and (B) electrophoretic profile. Purity and protein quantification are in agreement with conventional protein quantification assay (BCA Pierce) and with the observation on a SDS–PAGE Coomassie stained gel of the eluted fractions.

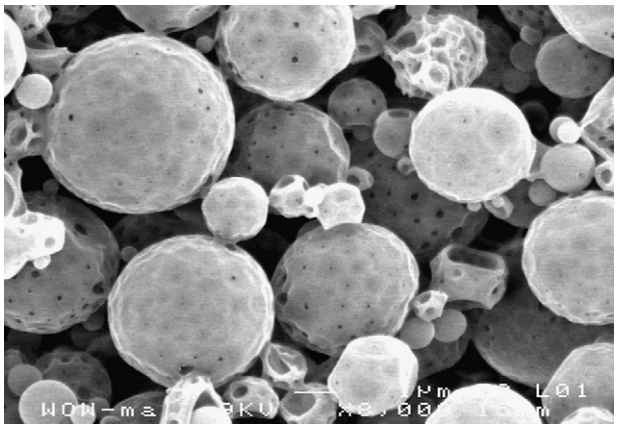

Fig. 2. Scanning electron microscopy of rE2 glycoprotein/RSA loaded microspheres prepared with PLGA RG503H polymer using water in oil in water method.

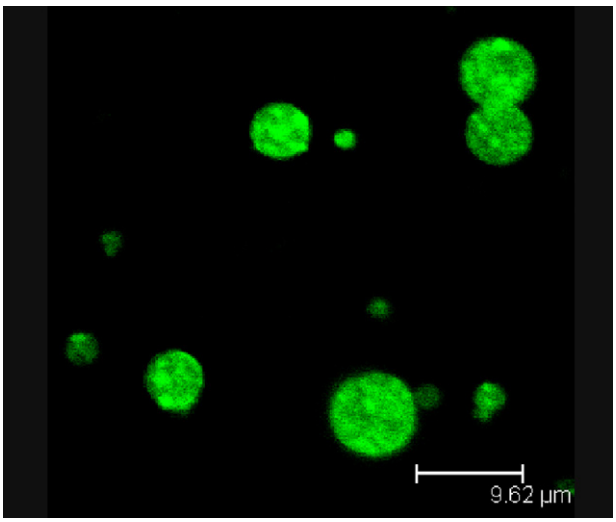

Fig. 4. Visualisation of the distribution of fluorescent molecules (BSA-FITC) loaded in PLGA microspheres.

the rE2 antigen loading was estimated to be 81 ± 6% (w/w) which is comparable to previous reports using the water/oil/water method (Azevedo et al., 2006; Ibrahim et al., 2005; Jaganathan and Vyas, 2006). Such a high loading is usually obtained by using surfactants. However, the use of such excipients can be deleterious since it can prevent a subsequent ligand grafting. In our study, we developed a formulation free of surfactant using an aqueous solution of phosphate buffer at pH 7.4 to stabilize the emulsions instead of polyvinyl alcohol which is commonly used. This surfactant free formulation was designed to permit a surface functionalization of microspheres with potential ligands as WGA, mannose and RGD after encapsulation (Brandhonneur et al., 2009). Indeed, many authors have shown that the presence of a residual layer of excipient such as PVA cannot be avoided even after suitable washing (Panyam and Labhasetwar, 2003; Sahoo et al., 2002; Scholes et al., 1999; Torche et al., 2000) and this could inhibit the covalent grafting (Keegan et al., 2004).

The *in vitro* release profile of RSA, used as a surrogate marker for E2 release, from rE2/RSA loaded microspheres did not show a burst effect (Fig. 3). RSA was slowly released from microspheres, reaching a 78.5% (w/w) cumulative release within 28 days. The lack of a burst may be attributed to the fact that the protein is homogenously distributed within the polymer matrix. Indeed confocal microscopy studies of BSA-FITC incorporated in microspheres showed the spatial distribution of BSA-FITC within the microspheres (Fig. 4).

Following IM administration, the subunit marker vaccine Porcilis Pesti induced a higher serum Ig rE2 specific antibody response compared to free rE2 glycoprotein (Fig. 5). This observation suggested that the presence of an adjuvant (i.e. liquid paraffin) in the subunit marker vaccine influenced the induction of the systemic immune response. Moreover, we observed a difference in response between immunization with 1 and 10 μg for both free rE2 glycoprotein and vaccine. This experiment clearly showed that the immunogenicity of rE2 glycoprotein was preserved during purification. The development of an immune response confirmed the lack of alteration in antigenic epitopes on rE2.

Since the response to 1 μg of rE2 was rather low, we decided to investigate the immune response induced by rE2 loaded PLGA microspheres given by different routes (oral, nasal and IM) with 10 μg of rE2 antigen.

Negative controls (RSA loaded microspheres) administered by IM or IN routes led to a percentage of inhibition lower than the threshold (<40% of inhibition) before intradermal administration (Fig. 6A and B). Seven days after intradermal administration of rE2 glycoprotein solution, specific rE2 antibodies slightly increased and

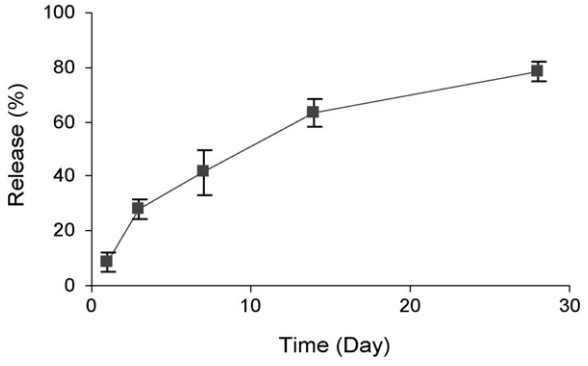

Fig. 3. Cumulative in vitro release profile of proteins (rE2/RSA) encapsulated in PLGA microspheres (mean ± SD; $n = 3$).

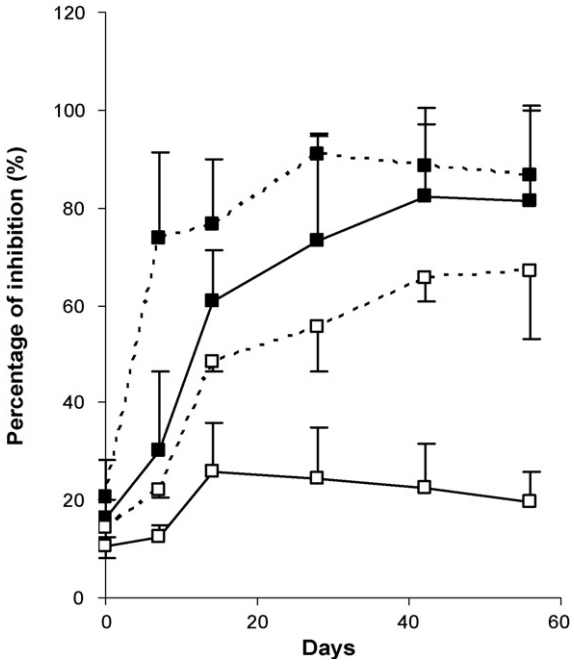

Fig. 5. Induction of total anti-rE2 immunoglobulin in rabbits immunized with rE2 (plain line) or with positive control using a subunit marker vaccine Porcilis Pesti (dashed line). IM route (dose 1: □, dose 10: ■) of rE2 protein and positive control. Data show the mean ± SD.

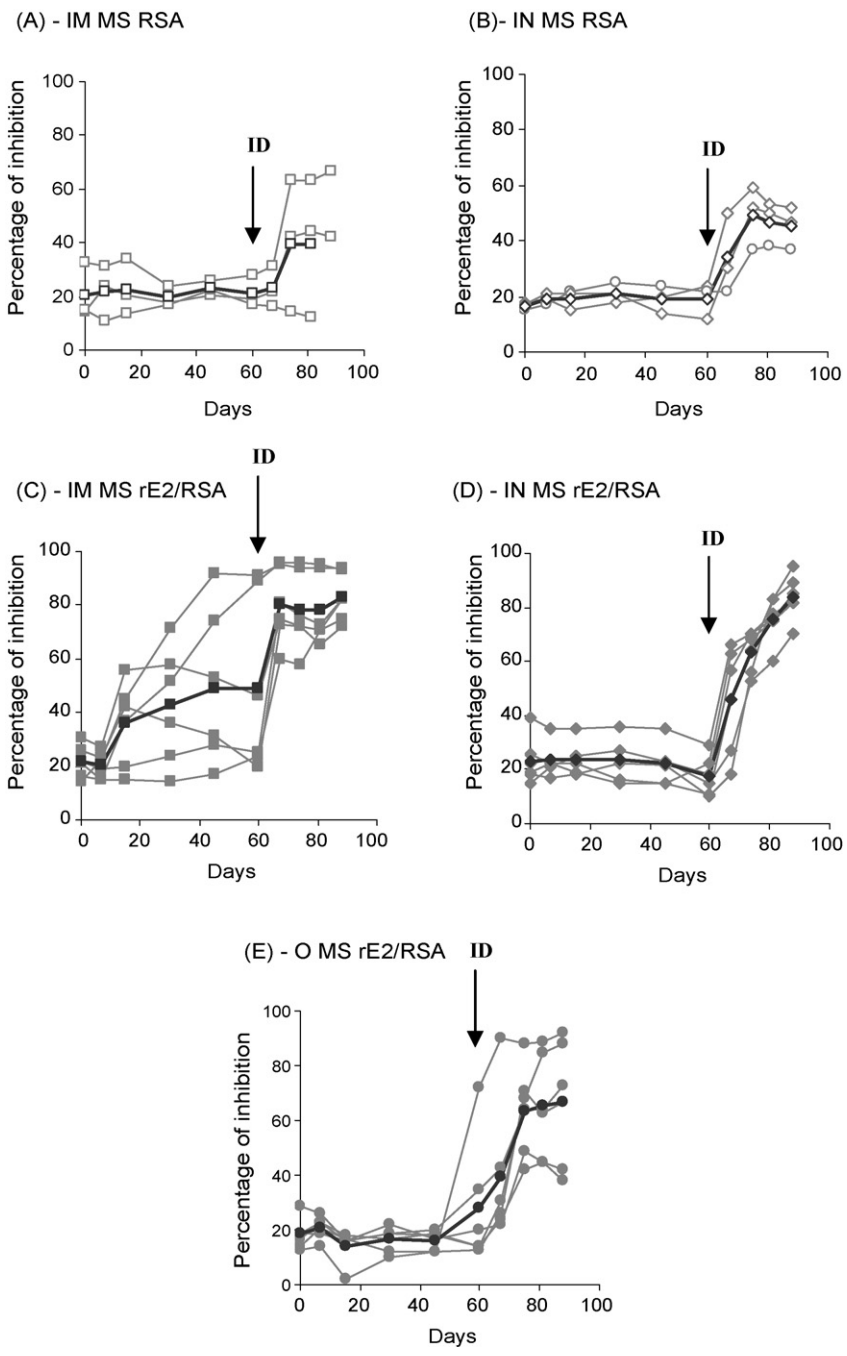

Fig. 6. Induction of total anti-rE2 immunoglobulin in rabbits immunized with RSA loaded (A and B) or rE2/RSA loaded microspheres (C–E). IM route (RSA MS: □, rE2/RSA MS: ■), IN route (RSA MS: ◊, rE2/RSA MS: ♦) and O route (rE2/RSA MS: ●). Data show individual results and in bold the mean ($n = 6$).

a plateau at a low level of specific rE2 antibodies was reached. These immune responses after IM and IN administration were very similar and not statistically different (Figs. 6 and 7). Moreover, a period of 7 days was necessary for the immune responses to be detectable. This represents a delay of approximately 10–14 days over that previously described for an E2 subunit vaccine following vaccination of naive animals (Bouma et al., 1999; Uttenthal et al., 2001).

Intramuscular administration of rE2/RSA loaded microspheres induced a highly variable level of specific rE2 antibodies occurring between 7 and 14 days post-immunization (Fig. 6C). Indeed, two animals failed to respond, two had a low response with a maximum at 30 days after immunization and two had a large immune response reaching a maximum at 40 and 60 days post-immunization. The intradermal administration 60 days after the initial vaccination increased the level of specific rE2 antibodies by 1.5 fold. This increase was obtained 7 days after intradermal administration and reached a plateau during the following 30 days of the study, demonstrating the boosting effect of the ID administration.

By nasal or oral route, the administration of rE2/RSA loaded MS did not produce specific rE2 antibodies but a priming immunization was established as demonstrated by the immediate and large increase of specific rE2 antibodies following intradermal administration (Fig. 6C and D). After oral administration, the response was quite variable reaching a plateau 14 days post-intradermal administration. However, the response after intranasal administration was higher and much less variable, increasing throughout the remaining 30 days of the study. These data showed that a better response was obtained after nasal administration of rE2/RSA loaded micro-

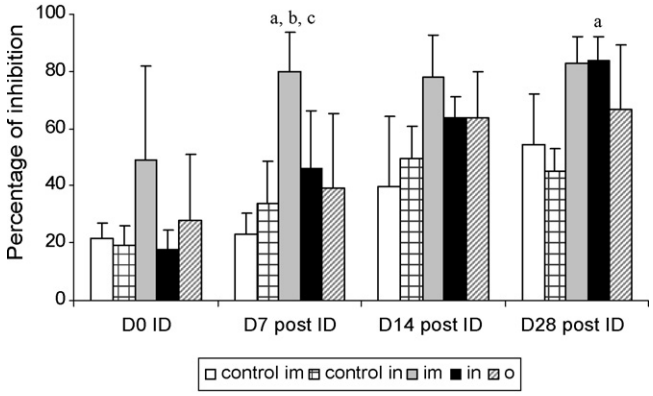

Fig. 7. Total anti-rE2 immunoglobulin in rabbits immunized with RSA loaded (control im/control in) or rE2/RSA (im/in/o) loaded microspheres at D0, D7, D14 and D28 post-intradermal administration. Data show the mean ± SD ($n = 6$).

spheres. Indeed, the response was more intense and less variable than after oral administration. This difference could result from a better accessibility to the immune cells after nasal administration. The intranasal route is an easy way for the mucosal delivery of vaccines at the infection site (Almeida et al., 1993; Yan et al., 1996). It has been shown previously (Jaganathan and Vyas, 2006) that antigens encapsulated in PLGA microspheres elicited a higher immune response after nasal administration. To explain this positive response, it was hypothesized that encapsulated antigens are sampled by specialized cells, similar to microfold cells, which overlie the nasal-associated lymphoid tissue, and are then transported to the antigen presenting cells.

The induction of systemic and local immune responses after oral and/or nasal immunization experiments have been achieved by successive administrations and not one single administration (Byrd and Cassels, 2006; Kang et al., 2006; Tafaghodi et al., 2006; Van Der Lubben et al., 2003). Moreover, it has previously been suggested (Jaganathan and Vyas, 2006) that there is a necessity of a second administration after nasal administration of recombinant Hepatitis B surface antigen loaded chitosan-PLGA microspheres. A single administration of this vaccine produced an initial response but the antibody titre did not last for a long time period. Furthermore, others (Shahin et al., 1995) have reported that a single immunization of filamentous hemagglutinin (antigen purified from *Bordetella pertussis*) was not a successful strategy for the induction of an immune response following oral administration. This could be due to an insufficient uptake of microencapsulated antigen across mucosal-associated lymphoid tissue (MALT). Less than 1–2% of an oral dose of PLGA microspheres successfully reaches the peyers patches (Eldridge et al., 1989). Indeed, the influence of diffusion barriers at the intestinal mucosal surface can limit efficient uptake of PLGA MS and thus reduce the induction of immune responses (Torche et al., 2006). The influence of a diffusion barrier is illustrated in the present work by the higher response after nasal compared to oral administration probably as a result of better accessibility to the immune cells. Hence, the effectiveness of PLGA MS to deliver antigen to MALT depends on an appropriate dose crossing mucosal barriers to release the loaded antigen.

Although previous studies demonstrated that the covalent grafting of different ligands increased their uptake by alveolar macrophages (Brandhonneur et al., 2009), the impact on the induction of immune response has not yet been shown. From the present study it is evident that a single administration of rE2/RSA loaded PLGA microparticles with subsequent intradermal administration was able to induce immune responses after nasal and oral administration in rabbits. To develop a single dose of nasal and/or oral microparticulate based vaccine, the covalent grafting of a ligand, to target relevant cells on rE2/RSA loaded PLGA microparticles surface could be a potential approach to improve efficacy. Indeed, it has been shown (Jaganathan and Vyas, 2006) that unmodified surface PLGA microspheres with or without adjuvant induced a weaker immune response compared to alum-adsorbed or modified surface PLGA microspheres.

In conclusion, the present work has shown that nasal or oral delivery of recombinant E2 of the classical swine fever virus using surfactant free PLGA microspheres was able to induce an immune response. A clear boost effect on the systemic immune response was seen after challenging the vaccinated animals by intradermal administration of the rE2. Furthermore, the response after nasal vaccination was more intense and less variable confirming the interest of this administration route in animals. Hence, microencapsulation of CSF E2 antigen could be an interesting approach to develop a CSF subunit vaccine for nasal and/or oral administration. Moreover this strategy could offer the possibility to discriminate CSFV infected from vaccinated pigs.

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