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Original article

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Abstract – Classical swine fever virus replicon particles (CSF-VRP) deficient for E^{rns} were evaluated as a non-transmissible marker vaccine. A cDNA clone of CSFV strain Alfort/187 was used to obtain a replication-competent mutant genome (replicon) lacking the sequence encoding the 227 amino acids of the glycoprotein E^{rns} (A187delE^{rns}). For packaging of A187delE^{rns} into virus particles, porcine kidney cell lines constitutively expressing E^{rns} of CSFV were established. The rescued VRP were infectious in cell culture but did not yield infectious progeny virus. Single intradermal vaccination of two pigs with 10^7 $TCID_{50}$ of VRP A187delE^{rns} elicited neutralizing antibodies, anti-E2 antibodies, and cellular immune responses determined by an increase of IFN-γ producing cells. No anti-E^{rns} antibodies were detected in the vaccinees confirming that this vaccine represents a negative marker vaccine allowing differentiation between infected and vaccinated animals. The two pigs were protected against lethal challenge with the highly virulent CSFV strain Eystrup. In contrast, oral immunization resulted in only partial protection, and neither CSFV-specific antibodies nor stimulated T-cells were found before challenge. These data represent a good basis for more extended vaccination/challenge trials including larger numbers of animals as well as more thorough analysis of virus shedding using sentinel animals to monitor horizontal spread of the challenge virus.

pestivirus / classical swine fever virus / E^{rns} / replicon / marker vaccine

1. INTRODUCTION

Classical swine fever (CSF) is a highly contagious and often lethal disease of pigs and wild boars characterized by fever, leukopenia, haemorrhages and abortion. The disease is classified in the list A of the Office International des Epizooties (OIE) and leads to severe economical losses worldwide [18]. The causing agent, CSF virus (CSFV), belongs to the genus *Pestivirus* within the family *Flaviviridae* [8]. Even though CSF has been eradicated in many European countries, the virus still threatens the pig industry mainly because it is prevalent in several wild boar populations in Europe [15]. In the 1990s, several outbreaks were reported in Europe, the worst being the one that occurred in 1997 in the Netherlands [27].

The existing live-attenuated vaccines efficiently protect pigs from CSF [29] but they do not allow serological

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differentiation between immunized pigs and pigs infected with a CSFV field strain. Since free trade is restricted to serologically negative pigs, application of these vaccines is prohibited in most countries of the EU. However, emergency vaccination is not excluded in case of an outbreak [1].

Animals infected with CSFV develop antibodies against the viral envelope proteins E^{rns}, E2, and the nonstructural protein NS3. E^{rns} and E2 also represent targets for neutralizing antibodies [35, 36]. Based on the serological response of CSFV-infected pigs, several attempts to obtain vaccines allowing differentiation of infected from vaccinated animals (DIVA vaccines) [33], also termed marker vaccines, have been made, such as protein/peptide vaccines, DNA vaccines and chimeric viruses (for review see [12, 34]). DIVA vaccines would be useful in the case of an epidemic to support the control of CSF but are also considered for CSF eradication, e.g. in the wild boar (EU project SSP1-501599, CSF-VACCINE&WILDBOAR).

A marker vaccine based on baculovirus-expressed CSFV glycoprotein E2 has been registered [19, 31] but clinical tests with this subunit vaccine have shown that protection from disease is not complete and that horizontal as well as vertical spreading of the virus is not entirely prevented [6, 7, 31]. Therefore, the establishment of a marker vaccine that is both, safe and efficacious in protecting pigs from CSF is highly demanded.

Recently, experimental CSF marker vaccines representing virus replicon particles (VRP), also referred to as defective virus particles or pseudo-infectious particles, have been described [16, 32, 37]. VRP are infectious virions that contain subgenomic RNA with specific deletion(s) in at least one of the genes encoding the viral structural proteins. Such RNA replicates and expresses the encoded viral proteins in the host cell. However, due to the defect in at least one of the envelope proteins it cannot generate progeny VRP. Thus, VRP are non-transmissible, which is one of the criteria for a safe vaccine. In addition, they allow differentiation of VRP-vaccinated from infected animals based on the absence of antibodies against the deleted protein(s) or epitope(s) in the VRP. CSF-VRP with deletions in either the E^{rns} or the E2 gene have been produced in complementing cells constitutively expressing the respective structural proteins [16, 32, 37]. Both types of marker vaccines were shown to be protective to some degree although no antibody responses were detected after vaccination. However, protection against intracellular pathogens such as viruses relies not only on antibodies but also on cell-mediated immunity (CMI) [14]. To this point, VRP of the alphavirus Venezuelan equine encephalitis virus [20] as well as self-replicating pestiviral RNA [24] have been shown to be potent inducers of CMI.

Here we addressed the question whether CSF-VRP vaccines are able to elicit a cellular immune response and whether this response correlates with the degree of protection. Earlier findings suggested that the major epitopes of E2 are required to induce immunity after intradermal vaccination [16, 32]. Thus, we constructed a CSF-VRP that lacks the E^{rns} gene but maintains the E2 gene. This VRP, CSF-VRP A187delE^{rns}, represents a negative marker vaccine that does not induce antibodies against E^{rns} consistently found in CSFV-infected pigs.

2. MATERIALS AND METHODS

2.1. Cells and viruses

SK-6 swine kidney cells (provided by Dr M. Pensaert, Faculty of Veterinary Medicine, Ghent, Belgium) were propagated in Earle minimal essential medium (EMEM) supplemented with 7% horse

serum (EMEM-HS). The porcine kidney cell line PK-15 (American Type Culture Collection, Manassas, VA, USA) was maintained in Dulbecco minimum essential medium (DMEM) supplemented with 5% horse serum. CSFV vA187-1 was derived from plasmid pA187-1 [25] containing the cDNA consensus sequence of CSFV strain Alfort/187. CSFV strain Eystrup was obtained from H.-J. Thiel (Justus-Liebig-Universität, Giessen, Germany).

2.2. Establishment of SK-6 and PK-15 cell lines expressing CSFV glycoprotein E^{rns}

The expression plasmid pCMVsigE^{rns}, containing the selectable marker gene Neo which provides resistance to the antibiotic geneticin (G418), was constructed by replacing the EGFP gene of pEGFP-N1 (Clontech Laboratories, Mountain View, CA, USA) with a cassette composed of the E^{rns} gene including the N-terminal signal sequence (sig) encoded by the 54 3'-terminal nucleotides of the core gene [26]. The sigE^{rns} sequence was amplified by PCR from pA187-1. Then the PCR-fragment was verified by DNA sequencing before it was used for the construction of pCMVsigE^{rns}.

SK-6 and PK-15 cells were transfected with *Afl* III-linearized pCMVsigE^{rns} DNA essentially as described before [16]. Selection for stable SK-6(E^{rns}) and PK-15(E^{rns}) transformants was initiated 72 h post transfection by addition of 0.5 mg/mL of G418 to the cell culture medium. Resistant colonies were screened for E^{rns} expression by immunostaining with monoclonal antibody (mAb) 01-03 (Bommeli Diagnostics, Liebefeld, Bern, Switzerland). E^{rns}-positive colonies were submitted to two rounds of end-point dilutions in 96-well plates. Single cell clones were analyzed by immunostaining. The selected clonal cell lines were maintained in the presence of 0.25 mg/mL G418 for further passaging. Production of VRP was carried out in the absence of G418.

2.3. Construction of the CSFV E^{rns} deletion mutant

Deletion of the complete E^{rns} gene (nucleotides 1175 to 1855 of the CSFV vA187-1 genome) encoding 227 amino acids was obtained by PCR-based site-directed mutagenesis, essentially as described before [16]. Thus, a PCR fragment containing the E^{rns} deletion was used to replace the corresponding region in the full-length cDNA clone pA187-1 [25] to obtain the mutant pA187delE^{rns} (Fig. 1). Details on the construction of all plasmids can be obtained upon request.

Run-off in vitro transcription of *Srf* I-linearized pA187delE^{rns} was carried out as described before [25]. The specific infectivity of the RNA, expressed as infectious units (IU) per microgram of RNA, was determined in an infectious centre assay as previously described for in vitro synthesized CSF-VRP RNA [16].

2.4. Rescue and characterization of E^{rns}-deleted CSF-VRP

For recovery of CSF-VRP, the mutant RNA was electroporated into either SK-6 or PK-15 cells expressing E^{rns} as described before [16]. Two days after electroporation the cells were submitted to two cycles of freeze and thawing and the cell lysate clarified by centrifugation at $1000 \times g$ for 10 min. For titration of VRP, SK-6 cells seeded in 96-well plates were infected with tenfold dilutions of the clarified supernatant. The titer expressed in $TCID_{50}$/mL was determined by staining the cells with mAb C16 [11] directed against viral protein NS3. The supernatant was stored as master seed at –70 °C.

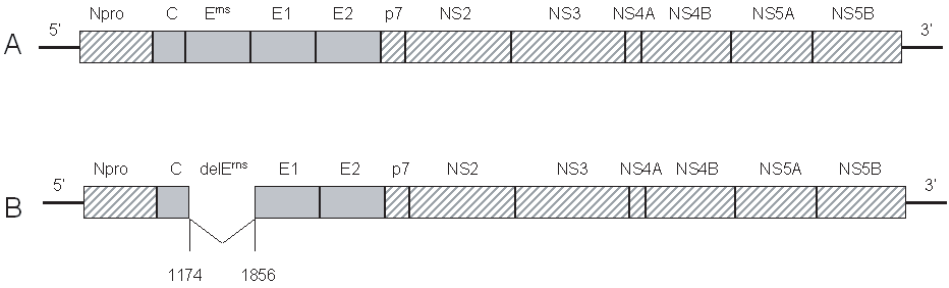

Figure 1. Schematic representation of the VRP A187delE^{rns} genome. (**A**) A187-1, genome of CSFV strain Alfort/187 [25]. The grey boxes show genes for the structural proteins and the hatched boxes genes for the nonstructural proteins. The untranslated regions of the genome are drawn as straight lines. (**B**) A187delE^{rns} genome lacking the entire E^{rns} coding sequence. Nucleotide numbers refer to the numbering of the CSFV Alfort/187 genome.

To confirm that the E^{rns} deletion in the CSF-VRP genome was stable in complementing cells, the respective region was amplified by RT-PCR from total RNA obtained by Trizol (Invitrogen, Carlsbad, CA, USA) extraction of cell cultures infected with passage 9 of VRP A187delE^{rns}. RT-PCR was performed with Expand reverse transcriptase (Roche Diagnostics, Rotkreuz, Switzerland) and *Taq* DNA polymerase (Promega, Madison, WI, USA). The primers used were PR1 (nucleotides 6454 - 6434 of the CSFV Alf/187 genome) for cDNA synthesis and ML1 (nucleotides 992 - 1022) / MR3 (nucleotides 1956 - 1940) for PCR. The latter two primers map in the flanking regions of the E^{rns} gene on the viral genome and therefore are not expected to anneal to E^{rns} DNA contained in the complementing cells. The amplified DNA fragment was characterized by agarose gel electrophoresis and sequencing.

2.5. Vaccination and challenge experiment

2.5.1. Preparation of the vaccine

For production of VRP A187delE^{rns}, 10^6 SK-6(E^{rns}) cells seeded in a 25-cm^2 tissue culture flask were infected at an m.o.i. of 0.5 with VRP rescued from transfected cells and passaged once in SK-6(E^{rns}) cells. Infected cultures were incubated for 3 days at 37 °C before they were lysed by two cycles of freeze and thawing. VRP contained in the clarified supernatant were titrated and used for vaccination.

For the mock preparation, 6×10^6 SK-6(E^{rns}) cells seeded in a 75-cm^2 flask were incubated for 3 days at 37 °C and lysed by two cycles of freeze and thawing. The clarified supernatant was used as a mock vaccine.

2.5.2. Experimental vaccination and challenge

Nine-week old littermates of specified pathogen-free (SPF) pigs from the in-house breeding unit were used for experimental vaccination. Groups of 2 to 4 animals were housed separately until one day before challenge when they were brought together in one stable. Pigs were vaccinated either orally with 10^7 TCID$_{50}$ VRP A187delE^{rns} in 10 mL EMEM or intradermally (i.d.) with the same amount of VRP contained in 0.5 mL clarified SK-6(E^{rns}) cell lysate that was applied at five different spots on the neck of the pigs

in portions of 0.1 mL each. Control pigs were mock-vaccinated either orally or i.d. with clarified lysate of SK-6(E^{rns}) cells. No adjuvant was used in any of the immunizations performed.

On day 23 post vaccination (p.v.) the animals were challenged oronasally with 5×10^3 $TCID_{50}$ of the highly virulent CSFV Eystrup.

2.5.3. Clinical monitoring and sample collection

The pigs were examined daily for clinical signs of CSF and body temperature. In order to allow a semi-quantitative judgment of the disease severity, a scoring list for the assessment of seven CSF-relevant criteria in vaccination and challenge studies was developed. This list was a modified version of an earlier scoring system used for the evaluation of clinical scores (CS) in virulence studies [17]. The new scoring system for vaccination studies included 6 parameters and their respective scores used before (liveliness, body shape, walking, skin, appetite, defecation) [17]. Fever was added as an additional parameter (≤ 3 successive days > 40 °C = score 0; 4 successive days > 40 °C = score 1; 5 successive days > 40 °C = score 2; 6 successive days > 40 °C = score 3). Scores of each of the seven parameters were determined daily and the total CS was calculated by adding the single scores. Thus, the theoretical maximum CS was 21. When the animals reached a CS of 12 or higher, they were killed for animal welfare reasons. The new scoring system also defined four parameters (massive lameness/inability to walk; massive hemorrhages in skin/epistaxis; bloody diarrhea; six successive days > 40 °C), each of them being a criterion for immediate slaughtering of the affected animal irrespective of the total CS.

EDTA blood samples (obtained from an ear vein or the vena jugularis) and saliva samples collected with cotton swabs (Salivette, Sarstedt AG, Sevelen, Switzerland) were taken on days 0, 9, 14, and 21 p.v., and daily post challenge (p.c.) for 10 consecutive days, then twice weekly until the experiment was terminated. For quantification of peripheral blood leukocytes (PBL) an aliquot of EDTA blood was diluted in TÜRK solution (Merck, Darmstadt, Germany) and PBL counted in a Neubauer counting chamber. Blood samples for ELISPOT assays were collected on the day of vaccination, day 21 p.v. and day 19 p.c., and diluted 1:3 in Alsever solution.

Serum samples were collected from the vena jugularis on day 0, 9, 14, 21 p.v., and twice weekly p.c. Surviving pigs were slaughtered 19 days p.c.

Whenever a pig was slaughtered, the tonsils were collected, minced into small pieces and resuspended in EMEM (containing 25 mmol/L HEPES pH 7.2, penicillin, streptomycin and fungizone) at a final concentration of 10% (v/v). Following centrifugation for 15 min at 20 800 × *g*, the supernatant was collected and stored at –70 °C.

2.5.4. Detection of infectious virus

For detection of infectious challenge virus, EDTA blood and tonsil homogenates each were diluted separately in EMEM (1:5) and used to inoculate SK-6 cells. The inoculum was left for 16 h on the cells, and then replaced by EMEM-HS. The presence of infectious virus was determined 48 h after inoculation by immunostaining of the cell monolayers for NS3 antigen with mAb C16.Virus present in EDTA blood on days 4 and 7 p.c. was quantified by titration in SK-6 cells.

2.5.5. Detection of viral RNA

Total cellular RNA was extracted from the EDTA blood samples by using the NucleoSpin® Multi-96 Virus kit

(Macherey-Nagel, Düren, Germany) on a Freedom EVO robot (Tecan, Maennedorf, Switzerland). Real-time RT-PCR was performed as described elsewhere [13]. Fifty cycles of amplification were carried out.

2.5.6. Detection of CSFV-specific antibodies

Analysis of sera and saliva samples for E2-specific antibodies was performed using an indirect ELISA as described before [21]. For detection of E^{rns}-specific serum antibodies, a commercial liquid blocking ELISA was used (CHEKIT-CSF-MARKER, Bommeli Diagnostics). CSFV-specific neutralizing antibodies in sera were determined in a neutralizing peroxidase-linked assay (NPLA) [28] as described previously [16]. Titers were expressed as the reciprocal of the highest neutralizing serum dilution.

2.5.7. ELISPOT assay

The ELISPOT assay was used to quantify IFN-γ secreting cells. Peripheral blood mononuclear cells (PBMC) from 50 mL Alsever blood were isolated by density centrifugation across a Ficoll-Paque (0.77 g/L; Amersham Biosciences, Otelfingen, Switzerland) as described by Carrasco et al. [4]. The assay was performed as described by Balmelli et al. [2] and the results are expressed as the number of IFN-γ secreting cells/10^6 PBMC.

3. RESULTS

3.1. Construction and characterization of the CSF-VRP A187delE^{rns} vaccine

E^{rns} deletion mutant pA187delE^{rns} was constructed from the CSFV cDNA clone pA187-1 by deleting the entire sequence coding for the glycoprotein E^{rns} (Fig. 1). The respective A187delE^{rns} RNA was obtained by in vitro transcription and used to transfect SK-6 cells. The specific infectivity of the A187delE^{rns} RNA, determined by infectious centre assay, was 10^7 IU/μg RNA. Immunostaining of the transfected cells at 24 h post transfection was positive for NS3 but negative for E^{rns} as expected (data not shown).

For packaging of A187delE^{rns} RNA into virus particles SK-6 and PK-15 cell lines constitutively expressing E^{rns} were established. After two rounds of single cell cloning, SK-6- and PK-15-derived cultures expressed E^{rns} in 100% of the cells. Both cell lines, SK-6(E^{rns}) and PK-15(E^{rns}), maintained expression of E^{rns} and supported the packaging of E^{rns}-deleted replicon RNA after 40 passages.

Electroporation of either SK-6(E^{rns}) or PK-15(E^{rns}) cells with A187delE^{rns} RNA, resulted in VRP titers of 10^6 $TCID_{50}$/mL to 10^7 $TCID_{50}$/mL. In contrast, in SK-6 or PK-15 cells electroporated with the same RNA no infectious virus particles were detected, demonstrating that packaging of the viral RNA did not occur in the absence of E^{rns} protein. Also, infection of SK-6 cells with VRP and subsequent titration of the cell lysates in SK-6 cells confirmed that the VRP were unable to generate infectious progeny virus. Furthermore, no infectious virus was observed after 10 passages of the VRP in complementing cells. Analysis of the VRP RNA contained in extracts of these cells by RT-PCR followed by sequencing of the PCR products showed that the deletion in the VRP genome had been maintained (data not shown). Thus, no indication for recombination of the replicon RNA with E^{rns} mRNA produced in the respective cell lines was obtained.

Titration of VRP A187delE^{rns} showed that after three passages the titers remained stable at a level of approximately 10^7 $TCID_{50}$/mL in SK-6(E^{rns}) cells and 10^6 $TCID_{50}$/mL in PK-15(E^{rns}) cells (data not shown). Consequently, SK-6(E^{rns}) cells

were used in all subsequent experiments, notably for the production of VRP for vaccination trials. To exclude that the VRP used as vaccine contained infectious virus, the respective VRP preparations were passaged once in SK-6 cells. As expected, no infectious particles were found after this passage.

3.2. Protective effect of VRP A187delE^{rns} against challenge infection

In order to assess the ability of VRP A187delE^{rns} to protect pigs against CSF and to characterize both, the humoral and the cellular immune response, a vaccination and challenge experiment was performed. Three SPF pigs each were immunized either orally (VRP, oral) or intradermally (VRP, i.d.) with 10^7 $TCID_{50}$ of VRP A187delE^{rns} per pig. Two pigs each were mock-vaccinated with SK-6(E^{rns}) cell lysate, either by the intradermal or the oral route and served as controls (mock, oral; mock, i.d.). Twenty-three days after one single immunization all pigs were challenged oronasally with CSFV Eystrup.

The vaccination did not affect the health of the animals in any measurable way (Figs. 2A to 2C), but one of the intradermally vaccinated pigs (#845) was lost on day 9 p.v. during blood collection. Elevated body temperature was observed the day before vaccination and the day of challenge (Figs. 2A to 2C), which can be explained by the stress of moving the pigs into the high-security facility and by putting them together in one stable the day before challenge. After challenge, the two remaining intradermally vaccinated pigs (#843 and #844) did not show any signs of disease other than a slight rise in body temperature of approximately 1 °C compared to values before challenge (Fig. 2A). In contrast, the orally vaccinated pigs and the mock-vaccinated pigs developed fever of up to 42 °C and showed clinical signs typical for CSF (Figs. 2B and 2C). The reasons for the subfebrile temperatures of the intradermally vaccinated pigs could be both the challenge infection as well as a rise in temperature of the environment caused by the febrile animals. The clinical picture was particularly severe for pigs #840 (VRP, oral), #846 (mock, oral), #848 and #849 (mock, i.d.), while animals #841, #842 (VRP, oral) and #847 (mock, oral) showed a slightly milder course of the disease. On day 7 p.c., animal #842 started to recover (body temperature < 40 °C, no clinical signs), whereas all control animals and the two other orally vaccinated pigs had to be sacrificed due to severe CSF clinical signs (fever for 6 successive days and/or CS values ≥ 12) (Figs. 2B and 2C).

All animals showed a drop in the PBL count on days 2, 3 and 4 after challenge (Tab. I). The values returned to physiological values by day 5 for the intradermally vaccinated animals and by day 9 for pig #842 (VRP, oral). Leukopenia was milder in the intradermally immunized animals when compared to both the orally immunized animals and the control animals.

At necropsy of the diseased animals sacrificed on day 7 p.c. several gross lesions characteristic for CSF, such as infarcts of the spleen, petechiae on kidneys and intestines, and hemorrhagic tonsils were detected. No gross lesions were observed upon pathological examination of the surviving pigs after termination of the experiment on day 19 p.c.

3.3. Detection of challenge virus in vaccinated pigs

EDTA blood samples and the tonsil homogenates were tested for the presence of infectious virus in cell culture (Fig. 3). All blood samples of the intradermally vaccinated animals were negative for infectious virus. For one of the orally vaccinated animals (pig #842) infectious

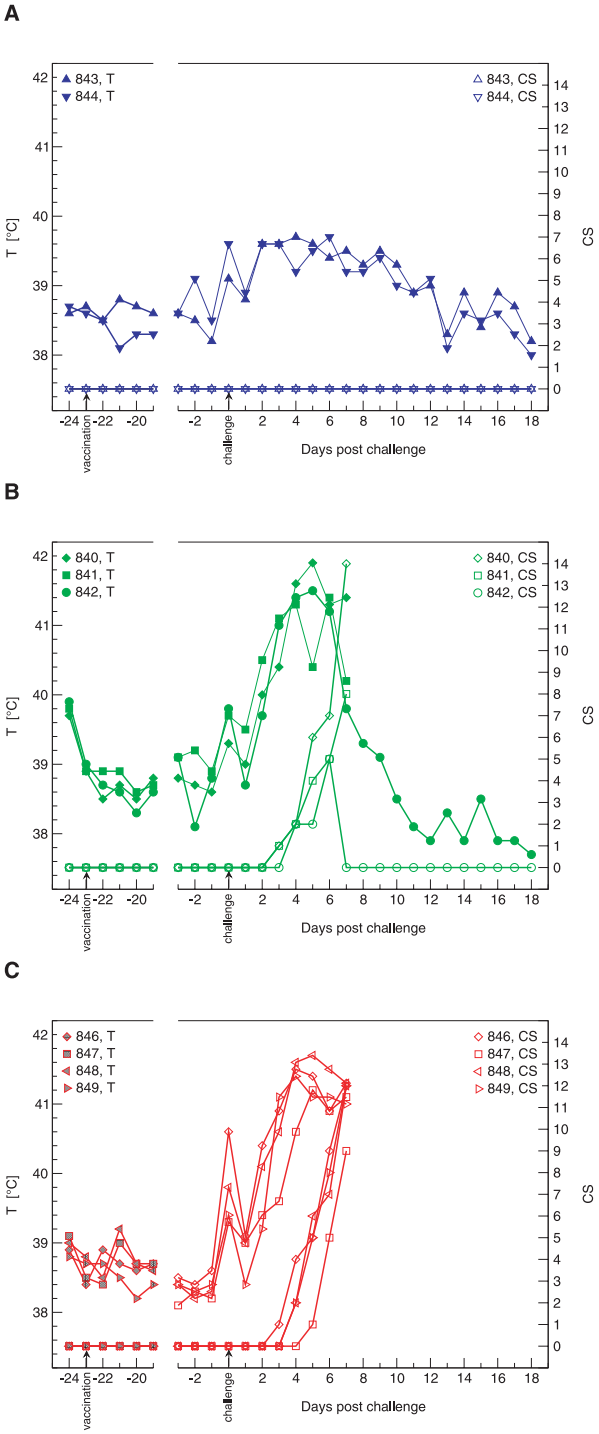

Figure 2. Vaccination and challenge experiment with VRP A187delE^{rns}. The pigs were vaccinated with 10^7 $TCID_{50}$ VRP A187delE^{rns} either intradermally (**A**) or orally (**B**) or mock-vaccinated with SK-6(E^{rns}) cell lysate (**C**) either orally (#846, #847) or intradermally (#848, #849). Twenty-three days after vaccination, all pigs were challenged oronasally with 5×10^3 $TCID_{50}$ of highly virulent CSFV Eystrup. All animals except the animals in panel A and one animal of panel B (#842) had to be sacrificed on day 7 post challenge. Clinical score (CS, empty symbols) and body temperature (T, solid symbols) are indicated. (A color version of this figure is available at www.edpsciences.org.)

Table I. Peripheral blood leukocyte (PBL) counts.

Vaccinated with	Vaccination route	Pig #	Days post challenge									
			1	2	3	4	5	6	7	8	9	10
VRP[a]	Oral	840	–	–	++[c]	++	++	+++	+++†			
		841	–	–	++	++	++	++	+++†			
		842	–	–	++	++	++	++	++	+	–	–
VRP	i.d.	843	–	+	+	++	–	–	–	–	–	–
		844	–	+	+	++	–	–	–	–	–	–
Mock[b]	Oral	846	–	+	+++	+++	+++	+++	+++†			
		847	–	++	++	++	++	+++	+++†			
Mock	i.d.	848	–	–	++	++	++	++	+++†			
		849	–	++	++	++	++	++	+++†			

[a] 10^7 $TCID_{50}$ VRP A187delE^{rns}.
[b] SK-6(E^{rns}) cell lysate.
[c] Leukopenia: + < 75%; ++ < 50%; +++ < 25% of PBL when compared to values prior to challenge.
†: Sacrificed due to severe CSF clinical signs.

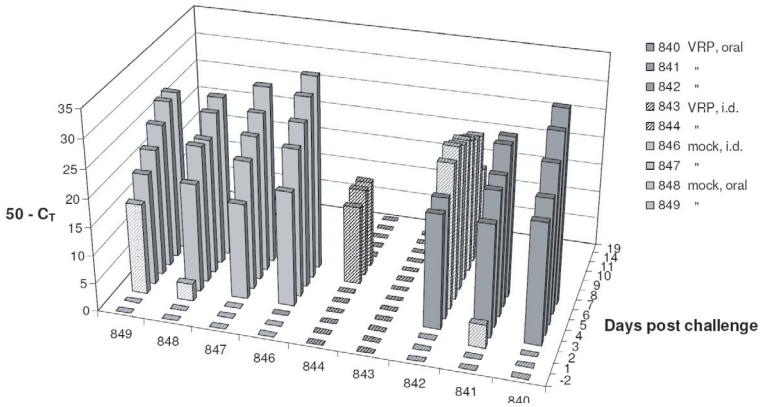

Figure 3. Detection of infectious virus and CSFV RNA in EDTA blood. The presence of infectious virus was determined in SK-6 cells. The results of TaqMan real-time PCR analysis are expressed as 50-C_T to obtain a positive correlation between C_T value and the amount of detected RNA. Hatched bars: viral RNA positive, infectious virus negative. Solid bars: viral RNA and infectious virus positive.

virus was isolated on days 3 and 4 p.c. The two other orally vaccinated pigs and all mock-vaccinated animals were viremic from day 3 p.c. (day 2 for pig #846) until they were slaughtered on day 7. Virus titers determined on day 4 p.c. were $10^{1.9}$ $TCID_{50}$/mL EDTA blood for pig #842 and ≥ 1 $\log_{10}$ higher (up to $10^{4.3}$ $TCID_{50}$/mL) for the other viremic animals. On day 7 p.c., titers reached up to $10^{5.9}$ $TCID_{50}$/mL EDTA blood. In the tonsils of the pigs sacrificed on day 7 p.c., infectious virus was found but not in the animals slaughtered on day 19 p.c. (data not shown).

In addition, the EDTA blood samples were analyzed for CSFV RNA by RT-PCR

Table II. Detection of CSFV-specific neutralizing serum antibodies.

Vaccinated with	Vaccination route	Pig #	Days post vaccination				Days post challenge[d]		
			0	9	14	21	4	7	11
VRP[a]	Oral	840	< 10[c]	nd	nd	< 10	nd	< 10	na
		841	< 10	nd	nd	< 10	nd	< 10	na
		842	< 10	< 10	< 10	15	< 10	266	800
VRP	i.d.	843	< 10	< 10	20	14	28	> 900	> 900
		844	< 10	14	94	35	60	> 900	> 900
Mock[b]	Oral	846	< 10	nd	nd	< 10	nd	< 10	na
		847	< 10	nd	nd	< 10	nd	< 10	na
Mock	i.d.	848	< 10	nd	nd	< 10	nd	< 10	na
		849	< 10	nd	nd	< 10	nd	< 10	na

[a] 10^7 $TCID_{50}$ VRP A187delE^{rns}.
[b] SK-6(E^{rns}) cell lysate.
[c] Results of NPLA are expressed as the reciprocal of the serum dilution neutralizing 100 $TCID_{50}$ of CSFV strain Alfort/187. Titers < 10: negative; ≥ 20: positive.
[d] On day 23 post vaccination pigs were challenged with CSFV Eystrup.
nd: not determined; na: not available.

(Fig. 3). CSFV RNA was found in the blood of all animals except for pig #843. For animal #844, low amounts of viral RNA were found from day 6 to day 8 p.c., whereas all other animals were positive for CSFV RNA from day 3 p.c. to the day they were sacrificed (pigs #840, 841 and the control animals) or until the end of the experiment (pig #842). These pigs showed a steady rise in the viral RNA except for pig #842, for which the viral RNA dropped after day 6 p.c.

3.4. Analysis of antibody response

Two weeks p.v. the two intradermally vaccinated pigs had developed neutralizing antibodies and anti-E2 antibodies detected by ELISA (Tab. II and Fig. 4A). The antibody levels remained stable up to the challenge when a distinct booster effect was observed that reached a maximum level 7 days p.c., and remained on this plateau thereafter. For the orally vaccinated animal that survived the challenge (pig #842) the neutralization titer before challenge was slightly below the cut-off and no anti-E2 antibodies were detected by ELISA. However, neutralizing antibodies (Tab. II) as well as anti-E2 antibodies (Fig. 4A) were observed by day 7 p.c. indicating a booster effect. None of the other animals developed neutralizing or anti-E2 antibodies.

As expected, no E^{rns}-specific antibodies were detected in any animal before challenge (Fig. 4B). The first pig that seroconverted was the intradermally vaccinated pig #844 that tested positive for anti-E^{rns} antibodies on day 11 p.c. (Fig. 4B). Animal #842 seroconverted on day 19 while the third surviving pig (pig #843) remained negative until the end of the experiment.

Up to the day of challenge, no anti-E2 antibodies were detected in the saliva of either of the animals. However, the saliva of all three surviving pigs proved positive for anti-E2 antibodies detected by ELISA on day 7 p.c. The highest values were obtained for the intradermally vaccinated pigs #843 and #844 on day 11 p.c. (data not shown).

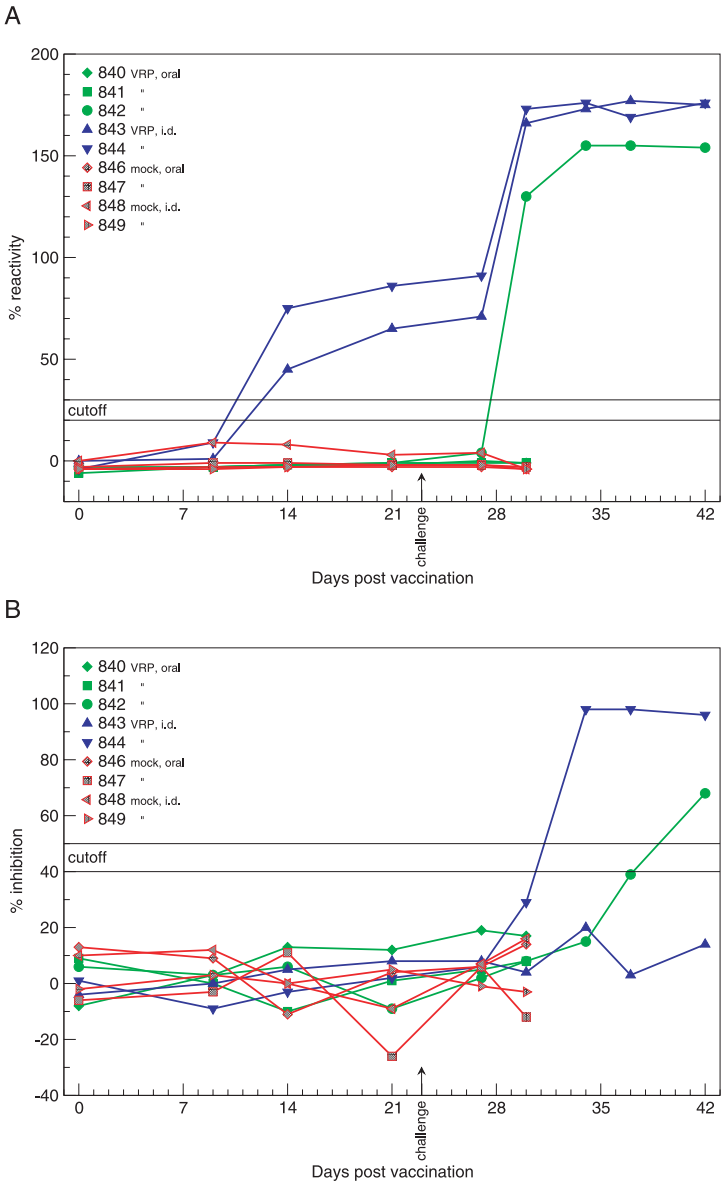

Figure 4. Detection of CSFV-specific serum antibodies. (**A**) Anti-E2 antibodies, determined by CSFi-ELISA. Reactivity < 20%, negative; 20–30%, questionable; > 30%, positive. (**B**) Anti-E^{rns} antibodies, determined by CSFm-ELISA. Inhibition < 40%, negative; 40–50%, questionable; > 50%, positive. (A color version of this figure is available at www.edpsciences.org.)

3.5. Cell-mediated immune response

Before vaccination, no IFN-γ secretion of T-cells upon stimulation with either CSFV Eystrup or SK-6 cell lysate (mock) was observed for any of the animals (Fig. 5). On day 21 p.v. (2 days before challenge) the two intradermally vaccinated pigs but neither the orally vaccinated nor the control animals showed an IFN-γ response upon stimulation of their T-cells with CSFV Eystrup, and, although at a lower level, upon stimulation with SK-6 cell lysate. Nevertheless, it is important to note that these T-cell responses (mock- and Eystrup-stimulated) were only found with intradermally VRP-vaccinated animals while intradermal vaccination with mock antigen did not induce any detectable T-cell response. On day 19 p.c., T-cells of all three surviving pigs secreted IFN-γ upon stimulation with either CSFV Eystrup or SK-6 cell lysate. T-cell activation was the strongest and most CSFV-specific for pig #843. For the clinically diseased pigs no ELISPOT assay could be performed on the day of slaughtering due to severe leukopenia.

4. DISCUSSION

Vaccines based on virus replicon particles have several advantages compared to subunit vaccines and to live-attenuated viruses. As opposed to subunit vaccines they encode all the B- and T-cell epitopes of the virus except for the ones located on the deleted protein(s). Furthermore, VRP vaccines like live-attenuated vaccines are able to infect host cells, to replicate their genome therein and to express the encoded viral proteins. Therefore they are expected to elicit a broad humoral and cellular immune response including an antiviral cytotoxic T-cell response [20, 23]. Another advantage with respect to immunogenicity is their "natural" tropism and uptake by antigen-presenting cells [5] (O. Bauhofer, unpublished). As demonstrated in this and other studies [20], VRP do not require particular formulations to support this process. At the same time, VRP are non-transmissible, both horizontally and vertically, since no infectious progeny is produced upon infection of cells excluding the emergence of virulent virus from the vaccine. Consequently, VRP vaccines are considered safer than live-attenuated viruses.

Only two CSFV proteins, E^{rns} and E2, have been shown to elicit specific antibodies in infected animals and at the same time, to be dispensable for replication of CSFV genomic RNA [22]. Thus, CSF-VRP being considered as a negative marker vaccine require partial or complete deletion of either of these two envelope proteins. CSF-VRP A187delE^{rns} has a complete deletion of the sequence encoding the 227 amino acids of E^{rns}. This is in contrast to a similar VRP described by Widjojoatmodjo et al. [37] where the sequence coding for residual N- and C-terminal amino acids of E^{rns} was maintained. We found that the replicon A187delE^{rns} was packaged as efficiently as a replicon containing the coding sequence for each of the six terminal amino acids of E^{rns} (data not shown). This indicates that the C to E1 fusion is processed as efficiently as the authentic C to E^{rns} fusion presumably by cellular signal peptidase [26].

Recently, we have suggested for VRP A187-E2del370 carrying a complete deletion of the E2 gene that the dose delivered to the vaccinee might be critical for successful immunization [16]. Thus, we used SK-6 cells stably expressing E^{rns} for packaging of A187delE^{rns} since the VRP titers obtained were approximately one $\log_{10}$ higher than in PK-15 cells.

In the immunization and challenge experiment, each pig was inoculated with 10^7 $TCID_{50}$ VRP A187delE^{rns} either intradermally or orally. Intradermal vaccination

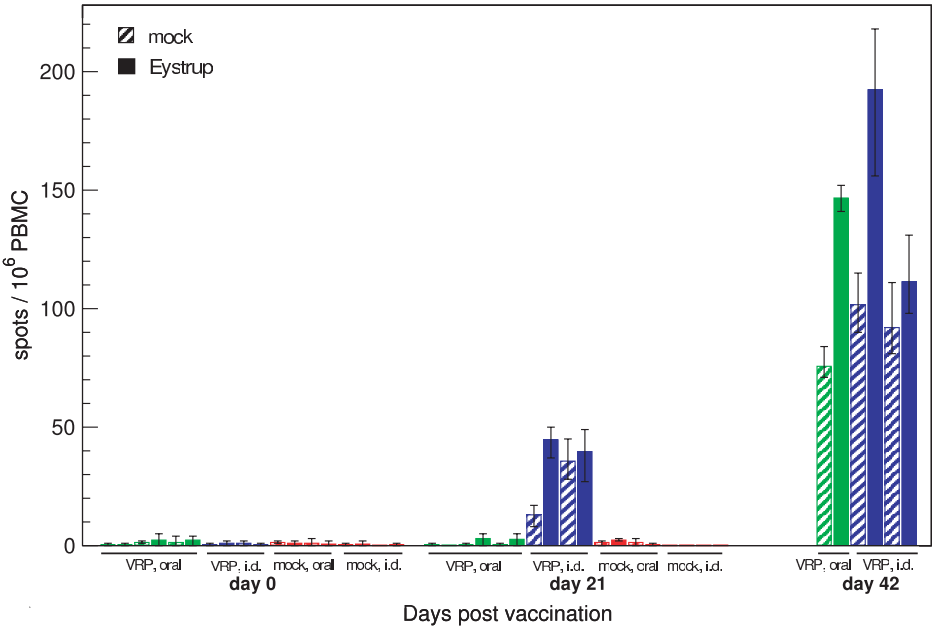

Figure 5. Determination of cell-mediated immune response by IFN-γ ELISPOT assays. PBMC were stimulated with CSFV Eystrup (solid boxes) or SK-6 cell lysate (hatched boxes), respectively. The results are presented as numbers of IFN-γ spots per million PBMC. Mean values of three independent assays carried out with each sample are given. Error bars represent the minimal and the maximal values obtained. (A color version of this figure is available at www.edpsciences.org.)

induced protection from CSF accompanied by only mild clinical signs after challenge, whereas the orally vaccinated animals got severely ill and only one out of three recovered. All control animals developed lethal CSF independently of the route of application of the lysate derived from SK-6 cells expressing E^{rns}. This demonstrates that the E^{rns} contained in the lysate of SK-6(E^{rns}) cells had no protective effect.

The virological and immunological findings were in agreement with the clinical outcome. In all pigs except the intradermally vaccinated ones, infectious virus could be isolated after challenge. Furthermore, the intradermally vaccinated pigs were positive for both, neutralizing and anti-E2 antibodies detected by ELISA before challenge, while the orally vaccinated and the control pigs did not seroconvert. Anti-E^{rns} antibodies occurred only after challenge in one of the intradermally vaccinated pigs (#844) (see below) and in the surviving orally vaccinated pig (#842).

Analysis of the cellular immune response by IFN-γ ELISPOT assay before challenge revealed specific T-cell responses only in the two intradermally vaccinated animals. The T-cells could also be stimulated with SK-6 cell lysate, which was not unexpected since the applied vaccine represented a crude lysate of VRP-infected SK-6(E^{rns}) cells, containing a multitude of cellular antigen. Interestingly, SK-6 specific T-cell stimulation was not observed for the two intradermally mock-vaccinated animals, demonstrating that the SK-6(E^{rns}) cell lysate on its own was not able to efficiently stimulate the immune system. A likely explanation for this would

be that VRP provide the "danger signal" (for instance the double stranded RNA formed during their replication) to the immune system required for efficient induction of a specific immune response [10]. As a consequence, specific immune responses would then be directed against both components of the vaccine, i.e. the SK-6(E^{rns}) cell lysate and the VRP.

Leukopenia was found in all animals after challenge, but it was less pronounced and of shorter duration in the intradermally vaccinated animals than in the orally vaccinated and control animals (Tab. I). The shorter duration of leukopenia in the intradermally vaccinated pigs can be explained by the immune response mounted prior to challenge.

There are two diverging findings for the two intradermally vaccinated pigs: (i) In pig #844 CSFV RNA was detected in the blood between days 6 and 8 p.c., while the blood of pig #843 remained negative for viral RNA (Fig. 3). (ii) Only pig #844 produced anti-E^{rns} antibodies upon challenge while pig #843 did not seroconvert (Fig. 4B). It would appear that the challenge virus in pig #843 was never able to spread from the site of initial local infection (i.e. the tonsils and adenoids), while in pig #844 a less limited replication and virus dissemination resulting in the induction of anti-E^{rns} antibodies occurred. The efficient priming of the anti-E^{rns} response can be explained by the presence of a secondary T-helper response as well as by efficient antigen presentation mediated by immune complexes.

This is the first study to show both, specific antibody response and T-cell activation with a CSF-VRP after one single intradermal vaccination and before challenge. Furthermore, the vaccinees showed no significant increase in body temperature and only temporary leukopenia. Besides, these animals did not show any clinical signs of CSF after challenge infection. In contrast, van Gennip et al. [32, 37] did not observe an antibody response prior to challenge using a similar E^{rns}-deleted VRP, and the pigs, although protected against lethal challenge infection, showed fever for three days p.c. These differences could be explained by the 10- to 100-fold higher VRP dose we used for immunization. With respect to the route of vaccine administration, our data confirm the findings of van Gennip et al. [32] demonstrating that E^{rns}-deleted CSF-VRP are protective after intradermal vaccination. These authors did not observe any protection after intranasal vaccination, which correlates with our finding, where only one out of three orally vaccinated animals survived. Maurer et al. [16] reported partial protection of pigs against challenge with highly virulent CSFV after oronasal immunization with E2-deleted CSF-VRP. These authors suggested that E^{rns}, rather than E2, might be critical for inducing protection via the oronasal route. Alternatively, the poor and inconsistent protection after oronasal immunization could be due to the fact that only a minor fraction of the vaccine applied might reach the sites where infection followed by induction of an immune response does occur. On the contrary, by intradermal vaccination, the vaccine is deposited directly into a compartment prone to deal with foreign antigen and that contains resident dendritic cells.

Although obtained with a small number of animals, the data presented here for intradermal vaccination of pigs with VRP A187delE^{rns} are promising towards the development of a safe and efficacious CSF vaccine. For differentiation of infected from immunized animals, commercially available anti-E^{rns} antibody ELISA developed as companion diagnostic tests for E2 subunit vaccines could be used [9]. Nevertheless, comprehensive future work is needed, before E^{rns}-deleted VRP can be considered for instance for emergency vaccination in case of a CSF outbreak. Also, intradermal application has to be

simplified in order to offer a practicable way of immunization. Currently new tools are being developed for convenient, needle-free intradermal vaccination, for example Vet Jet™ (Merial Limited, Duluth, GA, USA) or PowderJect ND5.2™ delivering system (PowderJect vaccines, Inc., Middleton, USA). An improvement of VRP vaccines can also possibly be achieved by inclusion of genes encoding cytokines or chemokines in the replicon [30].

Further studies with CSF-VRP should include larger numbers of animals as well as sentinel animals to monitor horizontal spreading of the challenge virus. Also, the ability of CSF-VRP to prevent vertical transmission of challenge virus has to be studied. If CSF-VRP prove capable of preventing vertical transmission, a corresponding BVD-VRP marker vaccine could be designed as intrauterine infection leading to persistently infected calves and ultimately mucosal disease is the key problem in BVDV infection [3].

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