Comparison of the effects of RNase-negative and wild-type classical swine fever virus on peripheral blood cells of infected pigs

Martina von Freyburg, Andreas Ege, Armin Saalmüller and Gregor Meyers

Bundesforschungsanstalt für Viruskrankheiten der Tiere, Paul-Ehrlich-Str. 28, D-72076 Tübingen, Germany

Elimination of the RNase activity of classical swine fever virus (CSFV) glycoprotein E\textsuperscript{Rns} was previously shown to result in virus attenuation. Specific reduction of B cell numbers in the peripheral blood, a typical symptom of CSFV infection in pigs, was not detected on infection with the RNase-negative mutant C-H346\textsuperscript{D} [Meyers et al. (1999). J Virol 73, 10224–10235]. The present report shows that this feature is restricted to this specific virus mutant, and does not represent a general property of RNase-negative CSFV. The effects induced by infection with two other RNase-negative and wild-type (wt) CSFV strains on the composition of peripheral blood cells have been further analysed. For all viruses, not only general leukopenia but also a reduction of different subsets of leukocytes (T-lymphocytes, monocytes and granulocytes) was detected. Similar to the results with B-cells, no significant differences with regard to changes in cell number were determined for RNase-negative mutants and wt virus during the initial phase of infection. Later, the values returned to pre-infection levels for the mutants, but stayed at low levels in the wt virus-infected animals. A major difference was reflected in the virus load of the infected animals, which was dramatically higher for pigs infected with wt CSFV, so that reduction of the virus load represents a further marker for attenuation resulting from RNase destruction. Attenuation was also detectable for the RNase-negative mutant C-W300G, which showed rapid reversion to the wt sequence within the infected pig. The prevention of fatal disease after infection with C-W300G is apparently determined during the short time between infection and reversion, as the virus revertant reisolated from infected pigs was shown to be virulent when used for infection in a follow-up study. Reversion of C-W300G was also detected in tissue culture during passage on swine testis epithelioid cells and porcine transformed kidney (MAX) cells, whereas the mutation was stable when SK6 or 38A1D cells were tested.

INTRODUCTION

Classical swine fever (CSF), formerly named hog cholera, represents an economically important disease of pigs in many countries worldwide. CSF is a severe disease that results in high morbidity and mortality of infected swine (Moennig & Plagemann, 1992; Thiel et al., 1996). Acute CSF is characterized by pyrexia and leukopenia. Diseased animals show anorexia and often diarrhoea and, in the later stages, central nervous disorders and haemorrhages in the skin, mucosa and internal organs. The disease often leads to death of infected animals, but recent CSF outbreaks in Europe resulted predominantly from viruses apparently inducing milder, chronic forms of the disease (Moennig & Plagemann, 1992; Thiel et al., 1996; Floegel-Niesmann et al., 2003). During acute CSF pigs show general immunosuppression. A characteristic feature detected early after infection of swine is a dramatic decrease of peripheral B- and T-cells (Susa et al., 1992; Summerfield et al., 1998). Moreover, granulocytopenia and induction of apoptosis of bone marrow haematopoietic cells were detected (Summerfield et al., 2000, 2001a, b).

The causative agent of CSF, classical swine fever virus (CSFV), is classified as a species of the genus Pestivirus within the family Flaviviridae. Three other pestivirus species are known that are predominantly found in ruminants: two types of bovine viral diarrhoea virus (BVDV-1 and BVDV-2) and border disease virus of sheep (Heinz et al., 2000).

Like other members of the family Flaviviridae, pestiviruses are small, enveloped viruses with a single-stranded RNA...
genome of positive polarity. The pestivirus RNA lacks both 5′ cap and 3′ poly(A) sequences, and contains one long open reading frame encoding a polyprotein of about 4000 amino acids, which encompasses all virus proteins arranged in the order NH₂–N³⁺–C–E₃–E₁–E₂–p7–NS₂–NS₃–NS₄A–NS₄B–NS₅A–NS₅B–COOH. The polyprotein gives rise to 11 or 12 final cleavage products by co- and post-translational processing involving cellular and virus proteases (Lindenbach & Rice, 2001). Protein C and the glycoproteins E₃ and E₂ represent structural components of the pestivirus virion (Thiel et al., 1991). E₂ and, to a lesser extent, E₃ are targets for antibody neutralization (Donis et al., 1988; Weiland et al., 1990, 1992; Paton et al., 1992; van Rijn et al., 1993). E₃ lacks a typical membrane anchor and is secreted in considerable amounts from infected cells. A highly unusual feature of this protein is its RNase activity, which was first identified by characteristic sequence motifs and then proven by enzymic tests with the purified protein (Schneider et al., 1993; Hulst et al., 1994; Windisch et al., 1996). The function of this enzymic activity in the virus life cycle is presently unknown. Experimental destruction of the RNase by site-directed mutagenesis resulted in a virus that has growth characteristics in cell culture equivalent to those of wild-type (wt) virus (Hulst et al., 1998; Meyers et al., 1999; Meyer et al., 2002). In animal experiments RNase-negative mutants of CSFV were found to be attenuated. The degree of attenuation varied for different mutations from completely apathogenic to low pathogenic, characterized by somewhat milder disease signs that vanished rapidly around day 10 post-infection (p.i.) (Meyers et al., 1999). Similarly, a highly pathogenic BVDV-2 isolate was considerably attenuated by an RNase mutation that also resulted in rapid recovery of the animals and clearance of the virus (Meyer et al., 2002). These results support a model in which the RNase activity of E₃ interferes with the immune response of the animal host. However, the mechanisms of immunosuppressive activity remain unclear. A completely apathogenic CSFV RNase mutant was shown to be unable to produce the B-cell depletion characteristic for CSFV infection in pigs. This finding suggests a role for RNase in the process of B-cell reduction. We describe here the results of animal experiments conducted with mildly pathogenic RNase mutants of CSFV to analyse their effects on different populations of peripheral blood leukocytes.

**METHODS**

**Cells and viruses.** Pig lymphoma cell line 38A1D was kindly provided by W. Schäfer, Max-Planck-Institut für Virusforschung, Tübingen, Germany. Swine testis epithelioid (STE) cells were obtained from R. Ahl, Federal Research Centre for Virus Diseases of Animals. SK6 cells were obtained from A. Summerfield, IVI, Mittelhäusern, Switzerland. The porcine transformed kidney (MAX) cell line was established at the Federal Research Centre for Virus Diseases of Animals (Pauly et al., 1995). CSFV mutant C-H297K (formerly named C-297-K) was described previously (Meyers et al., 1999); the wt CSFV used in our experiments was recovered from an infectious cDNA clone after restoration of an RNase mutation (C-346-d/Rs; Meyers et al., 1999). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS; tested for the absence of pestiviruses and antibodies against pestiviruses) and non-essential amino acids. Cells and virus stocks were tested regularly for the absence of mycoplasma contamination.

**Infection of cells and immunofluorescence assay.** As pestiviruses tend to be associated with host cells, lysates of infected cells were used for infection of culture cells. Lysates were prepared by freezing and thawing cells 48 h post-infection, and were stored at −70 °C. If not indicated differently in the text, an m.o.i. of about 0.01 was used for infection of cells.

For detection of infected cells in immunofluorescence assays, cells were fixed with ice-cold methanol/acetic (1:1) for 15 min, air-dried, rehydrated with PBS, then incubated with anti-CSFV mAb a18 which detects the E2 protein (Weiland et al., 1990). Bound antibodies were detected with an FITC-conjugated goat anti-mouse serum (Dianova).

**Generation of mutant C-W300G.** Restriction, cloning and other standard procedures were carried out essentially as described previously (Sambrook & Russell, 2001). Restriction and modifying enzymes were obtained from New England BioLabs, Amersham, Invitrogen and Roche.

Starting with plasmid p666 (Meyers et al., 1999), single-strand-based mutagenesis was performed according to the method of Kunkel et al. (1987) using Escherichia coli CJ236 cells (Bio-Rad), the VCMS single-strand phage (Stratagene) and oligonucleotide OI-W300G (5′-TTACATGGGATCGGGCCCGAGAAA). A cDNA fragment containing the desired mutation and no second-site changes was obtained by cleavage with Xhol and Sdal and inserted in the full-length cDNA clone pA/CSFV (Meyers et al., 1996), restricted with the same enzymes. The full-length plasmid with the desired mutation was linearized by SrfI cleavage and served as a template for transcription of a cRNA that was used for transfection of SK6 cells, as described previously (Meyers et al., 1996).

**RT-PCR.** RT-PCR was carried out with about 2 μg total cellular RNA isolated from infected cells by the Trifast method according to the supplier’s protocol (Peqlab). The RT-PCR was done with the One-Step RT-PCR kit as recommended (Qiagen). The following PCR primers were used. Upstream: 5′-CATGCGATGCCTTG-GCCCTTGCCGTTGATA (positions 10–33 of the primer correspond to nucleotides 1120–1143 of the CSFV Alfort/Tübingen genome); downstream: 5′-GGAATTCCTCAGGCATAGGCACAAAGAGGAGCTGAGTATATGGTACTTGGGCACAAACCCAGG (positions 11–30 of the primer correspond to nucleotides 1835–1854 of the CSFV Alfort/Tübingen genome).

Amplified cDNA fragments were purified by preparative agarose gel electrophoresis. Elution of DNA from the agarose gel was done with a Nucleotrap kit (Macherey Nagel). For sequencing with the upstream primer, the Big Dye Terminator Cycle Sequencing kit (Perkin Elmer Applied Biosystems) was used. Analysis of the sequencing products was done with an ABI Prism 377 DNA sequencer (Perkin Elmer Applied Biosystems).

**Determination of RNase activity.** Determination of RNase activity was carried out essentially as described before (Schneider et al., 1993; Meyers et al., 1999). If not specified, assays were conducted in a total volume of 200 μl containing 5 or 50 μl supernatant of the second centrifugation step (60 min at 4 °C and 45 000 r.p.m., TLA 45 rotor, Beckman tabletop ultracentrifuge TL100) and 80 μg poly(r)U (Pharmacia) in RNAse assay buffer (40 mM Tris/acetate, pH 6.5, 0.5 mM EDTA, 5 mM dithiothreitol). After incubation of the reaction mixture at 37 °C for 1 h, 200 μl 1 M perchloric acid and 20 mM lanthanum sulfate was added. After 15 min incubation on ice, the mixture was centrifuged for 15 min at 4 °C and 14 000 r.p.m. in an Eppendorf centrifuge. Three volumes of water...
were added to the supernatant and an aliquot of the mixture was analysed by measuring the absorbance at 260 nm using an Ultrospec 3000 spectrophotometer (Pharmacia). As a positive control, RNase A from bovine pancreas (Serva) with an activity of 85 Kunitz units (mg protein)\(^{-1}\) was used instead of the cell extract.

**Animal experiments.** For each CSFV variant, three or four piglets (German Landrace; 20–25 kg) were used. If not specified, the infection dose was 0.5 × 10\(^{10}\) to 1 × 10\(^{10}\) TCID\(_{50}\) per animal, depending on the size of the animals; two-thirds of the inoculum was administered intranasally (one-third in each nostril), one-third intramuscularly. The different groups were housed in separate isolation units. Blood was taken from the vena jugularis at the time points indicated. Coagulation was inhibited with heparin (20 IU/ml blood)\(^{-1}\) or sodium citrate (3.8% w/v).

To test the animals for the presence of virus in the blood, SK6 cells seeded in a 24-well plate were incubated with 10\(^{6}\) isolated peripheral blood leukocytes (prepared as described by Saalmüller et al., 1987) and 150 µl medium. After 1 h at 37 °C, the mixture was removed and the cells were washed twice with medium and incubated for 48–72 h at 37 °C. Infection of cells was determined by immunofluorescence.

**Isolation of peripheral blood mononuclear leukocytes (PBMC) and flow cytometric analyses (FCM).** PBMC were isolated from heparinized blood samples by Ficoll-Hypaque (Pharmacia) centrifugation (800 g, 30 min) as described previously (Saalmüller et al., 1987).

Leukocytes were stained for two-colour FCM for detection of B-lymphocytes, monocytes and granulocytes in a three-step procedure, as follows. (i) Incubation of a mixture of 100 µl PBS and 100 µl heparinized blood with mAb B-ly4 (anti-human CD21, IgG\(_{1}\); Pharmingen) and mAb 74-12-4 (IgG2b; Pescovitz et al., 1985) seeded in a 24-well plate were incubated with 10\(^{6}\) isolated peripheral blood leukocytes (prepared as described by Saalmüller et al., 1987) and 150 µl medium. After 1 h at 37 °C, the mixture was removed and the cells were washed twice with medium and incubated for 48–72 h at 37 °C. Infection of cells was determined by immunofluorescence.

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**RESULTS**

**Infection of pigs with RNase mutants H297K and W300G results in B-cell depletion**

Destruction of the RNase activity of the structural glycoprotein E\(_{\text{ms}}\) of CSFV strain Alfort/Tübingen by site-directed mutagenesis led to the recovery of attenuated virus mutants (Meyers et al., 1999). Depending on the position at which the mutation had been introduced into the protein, two types of attenuated virus were recovered. Mutants with changes affecting amino acid 346 were found to be completely apathogenic, whereas those with a change at position 297 induced disease signs similar to the wt virus during the initial phase, followed by a rapid recovery of the infected animals around day 10 p.i. In an animal experiment with one of the apathogenic mutants (C-346Δ, originally named C-346-d), the reduction in B-lymphocyte numbers typically observed for CSFV-infected animals was found to be absent (Meyers et al., 1999). To analyse further the effects of different mutations blocking RNase activity with regard to attenuation and B-cell reduction, further animal experiments were conducted with the RNase-negative mutant C-H297K and a second mutant that was generated by exchanging the tryptophan at position 300 for glycine (mutant C-W300G). The mutation in C-W300G was stable in tissue culture for at least five passages in SK6 cells. Similar to the other E\(_{\text{ms}}\) mutants tested so far, the recovered virus proved to be RNase-negative in our test system and grew as well as wt virus in tissue culture cells (not shown).

The results of the experiment conducted with C-H297K were comparable with published data (Meyers et al., 1999): viraemia was detected for 5–8 days and the pigs developed fever (Fig. 1) and mild disease signs but then recovered fully between days 10 and 13 p.i. The control animals infected with wt CSFV showed a similar course of disease in the initial phase, but exhibited severe clinical signs comprising diarrhoea, anorexia, ataxia, severe weakness and respiratory problems later on. Hyperthermia started at day 4 p.i. and persisted until the animals were put down in a moribund state at day 10 or 12 p.i. (animals #15/6 and #15/7, or #15/8 and #15/9, respectively). The wt CSFV-infected animals were viraemic from day 3 p.i. until death, whereas pigs infected with the mutant contained the virus only transiently in the blood (days 3–7, or 10 p.i.).

Similarly to C-H297K and the other mutants with changes...
affecting position #297, mutant C-W300G showed a partially attenuated phenotype in the host. The three animals infected with C-W300G developed fever for several days, but the increase in body temperature was less pronounced than that of the wt controls and body temperature returned quickly to normal values (Fig. 1). The animals also showed reduced food consumption for a few days. However, except for one animal (#7/1), no other signs of disease were detected. Animals #7/2 and #7/3 were viraemic for only 5 days (days 5–9 p.i.), whereas virus could be detected from days 6–16 in the blood of pig #7/1. Post-mortem analysis revealed that pig #7/1 suffered from an abscess located between its rectum and spinal cord. This abscess was obviously independent of the CSFV infection and probably caused the prolonged viraemia, fever and additional disease signs observed for this animal. In contrast to the animals infected with the mutant, the control animals inoculated with the wt virus developed fatal CSF with severe disease signs and continued viraemia, and had to be put down on day 16 p.i.

Analysis of the white blood cells showed a clear leukopenia for all the animals included in the two studies (Fig. 1). In contrast to the experiment with mutant C-H346Δ, the mutant that did not induce any clinical signs in previous experiments, we observed a significant decrease in B-cell numbers, not only for the animals infected with wt CSFV, but also for the two different mutants (Fig. 1). However, in comparison with the wt virus-infected pigs, the level of leukocytes and B cells started to increase again after 6 or 7 and 9 or 10 days p.i., respectively. For the reasons outlined above, animal #7/1 needed longer to restore leukocyte numbers. Taken together, the two RNase mutants tested here were different from the completely attenuated mutant C-H346Δ, as they induced a clearly detectable reduction of B-cell numbers in a range comparable to wt CSFV. The

**Fig. 1.** Key data from animal experiments with the RNase mutants C-H297K and C-W300G (■) in comparison with wt CSFV (●). Leukocyte numbers are given as $10^3$ cells (μl blood)$^{-1}$, whereas data for B lymphocytes are shown as percentages of B cells among isolated PBMC and demonstrate that the decrease exceeds the general leukopenia. Curves show mean values for four animals (left panels) or three animals (right panels). Error bars were calculated as the SEM.
recovery of cell numbers clearly correlates with the general recovery of the animals and can hardly be regarded as a primary effect of the destruction of the RNase.

**Infection with RNase-negative or wt CSFV has equivalent effects on the composition of peripheral blood cells in pigs**

To find out whether there is a difference between animals infected with RNase-negative and wt virus with regard to other leukocyte populations in the peripheral blood, a further animal experiment was conducted with mutant C-W300G and a variety of different parameters were tested. The disease signs were similar to those observed in the previous experiments. For the animals infected with the mutant, mild signs of disease, elevation of body temperature, leukopenia and reduction of B-cell numbers were observed during the first approximately 10 days p.i. followed by a sudden recovery (Fig. 2a). Blood cell viraemia was detected for up to 5 days (days 5–9 p.i.). Controls infected with the wt virus developed fatal CSF with continuous pyrexia, viraemia (from day 2 p.i. until death), anorexia, diarrhoea, ataxia, respiratory problems, leukopenia and B-cell depletion (Fig. 2a), and had to be put down on day 12 or 13 p.i. in a moribund state of health.

In addition to leukocyte and B-cell numbers, the numbers of natural killer cells, granulocytes, monocytes, TcR-γδ T cells, cytolytic T cells and T-helper cells in the peripheral blood were also determined. The latter fraction was further characterized with regard to the content of naive MHC class-II-negative versus MHC class-II-positive activated or memory cells (Fig. 2b; data not shown). No significant difference between animals infected with wt CSFV or C-W300G was found for any of the cell types tested. However, the attenuated nature of C-W300G was obvious from the recovery of cell numbers starting around days 10–12 p.i. A further parameter demonstrating the attenuation of RNase-negative mutants was observed when we determined the degree of virus infection for some of the cell types by detection of the CSFV E2 protein. Considerable numbers of virus-positive cells were detected only in animals infected with wt CSFV, with very high levels in granulocytes and monocytes (Fig. 2b). Similarly to the findings reported for C-W300G, an equivalent experiment with the attenuated mutant C-H297K did not lead to detection of virus in the different leukocyte populations in the FACS analysis (data not shown). Thus, the degree of infection of blood leukocytes appears to be a good parameter by which to judge the virulence of CSFV in the natural host.

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**Fig. 2.** Data from a second animal experiment with CSFV mutant C-W300G. As in the experiment shown in Fig. 1, data recorded for animals infected with the mutant (■, three animals) are compared with those infected with wt CSFV (△, two animals). (a) Development of body temperatures, leukocyte numbers [given as $10^9$ cells (µl blood)$^{-1}$] and B-cell numbers [expressed as numbers of CD21-positive cells (µl blood)$^{-1}$]. (b) Numbers of granulocytes, monocytes and T-helper cells (left) and degree of infection of granulocytes, monocytes and lymphocytes (right) in the course of infection with wt (△) and mutant C-W300G (■). Other details as for Fig. 1.
The number of infected peripheral leukocytes correlates with the titre of the infectious virus in blood plasma. In titration experiments, the amount of virus present in the plasma of animals infected with mutant C-H297K was so low that it could not be detected in the plasma except for a low titre (10^{2.89} TCID_{50} ml^{-1}) in one animal at day 7 p.i. In contrast, virus was detected in the plasma of wt virus-infected animals from day 5 p.i. until death, and the final titres were around 10^6 TCID_{50} ml^{-1} plasma in all animals (Table 1). Taken together, the virus load was much higher in those animals infected with the wt virus compared with one of the RNase-negative mutants. It is not clear whether this effect was due to reduced replication of the virus mutant within the animal or was a result of (enhanced) immunological control of the pathogen. Regardless of the mechanism, the results confirm once again that loss of the RNase residing in the E^{NS} protein leads to considerable attenuation of CSFV. The effects of the mutation at position 300 resemble those observed for mutants at position 297 (this report and Meyers et al., 1999) that also were not completely apathogenic but induced disease signs in the initial phase of infection followed by early recovery of infected pigs.

**Table 1. Titration of virus in plasma samples**

Tenfold dilutions of plasma samples were distributed in triplicate in 96-well plates seeded with SK6 cells and incubated for 5 days. Titres were calculated after CSFV-specific immunofluorescence staining of glycoprotein E2 and are expressed as log_{10} TCID_{50} (ml plasma)^{-1}. –, No virus could be isolated; ND, no data available.

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<th>Time (days p.i.)</th>
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*Background immunofluorescence after infection with plasma; therefore these samples were freeze–thawed and 30 µl from each well was transferred to fresh cells, incubated for 5 days and analysed as described above.
†Animal was dead.

Mutant W300G reverts rapidly to wt CSFV within the natural host and some types of tissue culture cells

Analysis of viruses recovered from infected pigs during different experiments with RNase-negative CSFV mutants showed that all tested mutations affecting positions 297 or 346 of the polyprotein were stable in the natural host. A routine check of viruses isolated from animals inoculated with C-W300G revealed that this mutant was not stable but reverted to the wt sequence. In three independent animal experiments, we were not able to recover the mutant from the blood of infected pigs. In one case (isolate from animal #13/3 on day 5 p.i.), the results were doubtful and could not be verified as the virus was lost during passage. Sequencing of RT-PCR products and RNase tests conducted with the viruses isolated from buffy coat samples revealed the presence of RNase-positive viruses with genomic RNAs encoding tryptophan at position 300. The earliest virus-positive buffy coat samples taken 5 days p.i. contained reverted viruses (Fig. 3), and we found no indication of the presence of a mixture of mutant and revertant. Similarly, nasal swabs taken during the third animal experiment with C-W300G contained only reverted virus. To test whether this result could be reproduced in tissue culture, we conducted further passage experiments. Propagation on SK6 cells did not result in a detectable change of the sequence following more than 16 passages. The same was true for viruses passaged eight times on pig lymphoma cell line 38A1D. In contrast, virus propagation in STE and MAX cells resulted in reversion being detected. The first signs of reversion were detected in an RNase test conducted after the sixth passage, and complete reversion was observed after eight passages (Fig. 4).

**Virus reisolated from animals infected with C-W300G is virulent**

After inoculation of pigs with C-W300G, only revertants were found in the blood of the infected animals, except for one doubtful sample that might have contained the original mutant. This finding appears to contradict the observation that this mutant exhibits a clearly attenuated phenotype. To make sure that the reverted virus did represent a virulent CSFV, we infected three pigs with CSFV reisolated from an animal infected with C-W300G. All three animals showed high fever and anorexia and had to be sacrificed for welfare reasons in a moribund state at days 12 (one animal) and 17 (two animals) p.i. Extended viraemia (starting from day 3 p.i.) and dramatic reduction of B-cell
numbers as a typical symptom of severe CSF in pigs were detected for all animals (Fig. 5). In the late stage of the disease, about 25% of B cells in the peripheral blood were virus-positive (not shown). Moreover, post-mortem analysis revealed typical signs of fatal CSF: bloody lymph nodes and multifocal infarcts at the margin of the spleen. These results clearly prove that the virus revertant is virulent and able to induce fatal CSF.

**DISCUSSION**

Pestiviruses are known to induce immunosuppression on infection of their natural hosts, but the molecular basis for this effect remained obscure for a long time. Only recently have indications been found for a (putative) connection between the immunosuppression and a unique gene product of pestiviruses, namely the glycoprotein E\(^{\text{rns}}\). This is an essential structural glycoprotein of the virus (Thiel et al., 1991; Widjojoatmodjo et al., 2000). It is a target for virus-neutralizing antibodies and plays an undefined role during infection of cells (Weiland et al., 1992; Hulst & Moormann, 1997). Moreover, E\(^{\text{rns}}\) displays RNase activity (Schneider et al., 1993; Hulst et al., 1994; Windisch et al., 1996). Destruction of this enzymic function has not influenced virus viability in tissue culture cells in any detectable way (Hulst et al., 1998; Meyers et al., 1999; Meyer et al., 1998).

![Fig. 3. Reversion of mutant C-W300G within the natural host. Viruses reisolated from infected animals were tested for RNase activity (a) and sequence changes at codon 300 (b).](image1)

(a) Open bars represent samples for which no infection of leukocytes was detected; hatched bars represent cases in which only few positive cells were detected in the immunofluorescence analysis or the observed weak signal probably represented background fluorescence. After blind passage these samples gave negative results in the immunofluorescence analysis. Number of animal and day of sampling (e.g. d5 means day 5 p.i.) are indicated. Animals with numbers prefixed ‘13’ were from the same experiment as shown in Fig. 2; animals with numbers prefixed ‘16’ were from the third study, conducted with C-W300G. Animal #13/1 represents a control infected with wt CSFV. (b) Reisolated viruses derived from animal #16/1. IV, Virus used for infection of animals; BC, virus isolated from buffy coat at day 5 p.i.; NS, virus isolated from nasal swab at day 10 p.i. The peaks visible below the main peaks, especially in the sequence derived from the original C-W300G virus (IV), would specify T residues, but analysis of the non-coding strand sequences clearly demonstrated that they are artefactual (data not shown). Codon 300 is underlined.

![Fig. 4. RNase tests showing the reversion of C-W300G during serial passages in two types of tissue culture cells.](image2)
of putative target cells and the enzymic activity could cause severe damage to a cell. Cytotoxicity is a well-known feature of a variety of other RNases including bovine seminal RNase (Youle & D’Allessio, 1997), and the E\textsuperscript{sm} protein was shown to induce a cytopathic effect after addition to lymphocyte cultures from different origins (Bruschke et al., 1997). Alternatively, the RNase could interfere with the host’s innate immune response. It was shown only recently for BVDV that E\textsuperscript{sm} blocks the activation of beta interferon by double-stranded RNA, and that this activity is dependent on its RNase activity (Iqbal et al., 2004).

CSFV is known to induce a specific reduction in B-cell numbers in the blood and different organs of the infected pig (Susa et al., 1992). This reduction exceeds by far the general leukopenia observed during CSF in pigs, and represents an early marker of CSFV infection. It was therefore attractive to speculate about a connection between RNase activity of E\textsuperscript{sm} and destruction of B cells. The first analyses conducted with one RNase-negative CSFV mutant (C-H346D) showed that the reduction in B-cell numbers was not detectable in animals infected with the mutant (Meyers et al., 1999). However, C-H346D was exceptional as it was completely apathogenic and did not result in substantial viraemia, whereas other RNase-negative CSFV variants induced clearly detectable disease signs and could consistently be recovered from the blood of infected pigs. It is therefore apparent that the phenotype of C-H346D is not only due to lack of the RNase activity, but results from the destruction of the RNase and an additional factor that is not further defined. We therefore repeated the analysis with other RNase-negative CSFV mutants and revealed that the absence of B-cell depletion is not a general feature of animals infected with such E\textsuperscript{sm} mutants. This result parallels experiments with equivalent BVDV mutants in cattle (Meyer et al., 2002). B-cell depletion is not observed in BVDV-infected cattle, and comparison of the B-cell numbers in the peripheral blood of animals infected with wt BVDV or RNase-negative BVDV mutants did not reveal any differences. Taken together, the data obtained for the different CSFV and BVDV mutants indicate that B-cell depletion is not induced by the E\textsuperscript{sm} RNase, and it seems likely that B cells do not represent the target of the RNase.

Based on the finding that destruction of the RNase activity of E\textsuperscript{sm} leads to virus attenuation and on the putative cytotoxic potential of the enzyme, we searched for effects on the composition of peripheral blood cells in infected pigs that could be due to the activity of the RNase. The general leukopenia induced by the two types of virus showed equivalent levels. We compared the changes induced by infection with RNase-negative or wt CSFV on granulocytes, monocytes, different T-lymphocyte subpopulations (TcR-\gamma\delta T cells, cytotoxic T cells, total T-helper cells, naïve T-helper cells, activated and memory T-helper cells) and natural killer cells (part of the data not shown). Reduction was detected for all leukocyte subpopulations, but no significant differences between animals infected

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**Fig. 5.** Key data from an animal experiment conducted with CSFV reisolated from an animal infected with W300G that had reverted to the W300 RNase-positive type. The animals \((n=3)\) were put down between days 12 and 17 p.i. in a moribund state.
with RNase-negative or wt CSFV were found during the initial phase of infection. Later, the numbers of all tested cells recovered in the animals inoculated with RNase mutants, but this effect coincides with the general recovery of the animals. It can therefore be concluded either that the RNase does not affect the peripheral blood cells in a way that changes the overall leukocyte composition or that these changes are not prominent enough to be detected in our assays. The most obvious difference observed during our analyses concerned the virus load in the blood of infected animals, which differed dramatically between wt virus and mutants. This result can certainly be regarded as further proof of the attenuation of RNase-negative viruses, and explains why wt CSFV-infected animals develop more severe disease signs. However, this difference cannot answer questions concerning the target of the RNase and the mechanism by which it increases virus virulence. Further analyses are necessary that will need to include further cell types, for example bone marrow cells, which have been shown to undergo apoptosis in response to CSFV infection in tissue culture experiments (Summerfield et al., 2000, 2001b).

The mutant C-W300G was found to revert to the wt virus in pigs soon after infection. We were not able to isolate the mutant virus from the infected animals. Even virus obtained at the earliest viraemic time-points apparently did not represent a mixture, but displayed an unambiguous sequence with TGG at codon 300. Similarly, only the revertant could be isolated from nasal swabs. Thus C-W300G virus was not secreted from the infected cells at the site of infection (two-thirds of the inoculum administered into the nose). By contrast, the mutant was stable during propagation in tissue culture in a porcine spleen cell line (SK6) and a pig lymphoma cell line (38A1D). However, similarly to the situation within the natural host, reversion occurred in STE and MAX cells. This finding cannot be explained by a tendency to restore RNase activity, as we never found any indication of reversion in a variety of other RNase-negative viruses, although the H297L and H346L mutants would also be able to revert by single base substitutions. It therefore has to be concluded that the revertants are selected by certain cell types because of an advantage concerning another feature of Erns, for example its function during infection of cells. The rapid reversion within the animal shows that the wt virus has to have a significant advantage over the mutant for propagation in the tissues or cells representing the primary replication sites of the virus. As little is known about the function of Erns as a structural protein and its possible interaction partner(s) on the surface of the host cell, discussion of the molecular basis of this finding would be premature. A more urgent question is why C-W300G is obviously attenuated although there is prompt reversion to wt virus. CSFV live vaccines are known to induce protective immunity quickly. It therefore might be that the time between infection and the presence of significant amounts of revertants is sufficient to prime the immune system in a way that allows the pig to mount a timely immune response. Alternatively, the effect of the Erns RNase might be an early event so that a certain target has to be hit once in the initial phase of the disease to interfere with the immune response. Further experiments are necessary in order to distinguish between these possibilities. It will be very interesting to elucidate the mechanism behind the obvious attenuation of RNase-negative pestiviruses, but the present report indicates that this will require alternative approaches.

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