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The role of the defective interfering particle DI9c in mucosal disease in cattle

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Summary. Mucosal disease occurs in cattle persistently infected with a noncytopathogenic strain of bovine viral diarrhoea virus (BVDVnc) following *in utero* infection. The disease can be initiated by superinfection with a cytopathogenic biotype (BVDVc) of the virus with antigenic "homology" to the persisting virus. A BVDVc isolated from a clinical case of mucosal disease has been discovered to consist of a defective interfering particle, DI9, and an associated BVDVnc helper virus. A defective virus corresponding to DI9 was recently recovered from an infectious cDNA clone and was named DI9c. To evaluate the role of DI9 in the pathogenesis of mucosal disease a two-part experimental study was carried out which included clinical, haematological, pathological and virological investigations. Eight of nine calves persistently infected with BVDVnc were experimentally inoculated with DI9c. The defective virus was propagated in cells preinfected with the same strain of virus used to persistently infect the calves *in utero*. The calves were euthanased on days 4, 7, 14, 21, 28, 40, 40 or 87 post inoculation. None of the inoculated animals developed classical mucosal disease, neither clinically nor pathologically. DI9c was not found in serum, nasal swab or tissue samples from the calves by observing cytopathogenic effect and/or using a polymerase chain reaction after reverse transcription (RT-PCR) of viral RNA. DI9c did not replicate to a detectable extent in these assays, and its participation in the pathogenesis of mucosal disease could not be proven.

Introduction

The pestivirus bovine viral diarrhoea virus (BVDV) is a worldwide pathogen of cattle responsible for economic losses due to reproductive failure, immunosuppression and fatalities associated either with an acute haemorrhagic syndrome or the highly fatal mucosal disease (MD) [25]. BVDV has a single stranded RNA genome of positive polarity, of approximately 12.3 kb and exists as two biotypes, noncytopathogenic (BVDVnc) and cytopathogenic (BVDVc). The biotypes are differentiated by the effect they have upon cultured cells *in vitro*, but they also have different roles in disease pathogenesis. An infection with a BVDVnc variant of the virus during the first trimester of gestation may lead to specific immunotolerance and a persistent infection of the foetus. Consequently as the foetus develops, the animal does not produce antibodies or cytolytic T-cells against the persisting BVDVnc virus [21]. Mucosal disease appears only in these persistently infected animals and is characterized by high case fatality with fever, anorexia, depression, watery diarrhoea and severe dehydration [2]. Extensive ulceration of the gastrointestinal tract is described to be the most prominent lesion. Virus isolation reveals both BVDVnc and BVDVc biotypes, comprising a virus pair, in animals with mucosal disease [6, 8]. The consistent feature of such a pair is the antigenic "homology" that prevents the animal from recognising the BVDVc virus. The BVDVc strain may arise spontaneously *in vivo* by mutation or recombination of the persisting virus genome and may then spread horizontally to other animals [21]. The cytopathogenic phenotype is strictly correlated with appearance of the 80 kDa non-structural protein NS3, which is expressed by BVDVnc strains as a 120 kDa NS2-3 fusion protein [11, 23]. Analysis of BVDVc strains from mucosal disease cases has revealed many different genetic strategies for the expression of the NS3 protein [17, 30].

The occurrence of defective interfering (DI) particles was first discovered in the early 1950s [33]. DI particles have been identified in most plant and animal virus systems [15, 26], and are usually a positive factor for the host animal. They are naturally occurring virus mutants that lack part of the normal viral genome, making them unable to replicate unless the cell is infected simultaneously with the normal parent or "helper" virus. Such co-infection complements the genes that are missing or defective in the DI particle. Cattle persistently infected with BVDVnc harbour the virus in tissues and serum all their life, and this virus could act as helper virus for a DI particle.

In 1988 a BVDVc called CP9 was isolated from an animal with mucosal disease [10]. Later, this strain has been analysed and found to comprise of two viral RNA molecules; a complete BVDVnc RNA genome and a subgenomic viral RNA with a deletion encompassing the whole region coding for the structural proteins and the NS2-coding region of the NS2-3 gene [31]. In this case, the presence of the truncated RNA was shown to lead to the expression of NS3 and to mediate cytopathogenicity. It was also demonstrated to significantly interfere with the replication of the helper virus RNA, thus exhibiting all the properties of a DI particle. In this case, the agent responsible for the cytopathogenic effect

in cells infected with BVDVc is the DI9 particle, with the associated BVDVnc acting as a helper virus [31]. Later on, a defective viral genome mimicking the DI9 RNA was recovered from an infectious cDNA clone (pA/BNVDV/D9) [19]. This cDNA genome was identified to represent a so-called replicon that was named DI9c [4].

The existence of DI9 as a particle dependent upon the structural proteins of a BVDVnc helper virus for propagation raises an interesting possibility for the pathogenesis of mucosal disease. An antigenically "homologous" BVDVc isolate infecting a persistently infected animal may lead to development of mucosal disease. Propagation of the DI9 should be possible in any cells infected with BVDVnc and would create a noncytopathogenic-cytopathogenic virus pair which are more than "homologous" having antigenically identical structural proteins. The question then arises, and the aim of the study was to answer, is DI9 competent to induce MD in cattle persistently infected with BVDVnc? To answer this question persistently infected calves were inoculated with DI9c.

Material and methods

Animals and study design

The study included nine calves, all experimentally infected with BVDVnc strain Pe515nc *in utero*. After birth they were confirmed to be persistently infected with this virus strain. They were all born small and appeared unthrifty but otherwise healthy. Eight of the calves were inoculated with DI9c. The study composed of two different experiments (Experiments A and B) and included clinical, haematological, pathological and virological examinations. The animal experiments were approved by the Norwegian Animal Research Authority and the Home Office of the UK.

Experiment A composed of 6 of the calves and was done at the Norwegian School of Veterinary Science, Oslo, and involved virological, haematological and sequential pathological analysis. The calves were of Norwegian Red breed and were included in another study [28]. The calves were between $3\frac{1}{2}$ and 8 months of age when five of them were inoculated with DI9c. The calves were housed together, but isolated from other animals, and euthanised sequentially on days 4, 7, 14, 21 or 87 post inoculation. One calf was not inoculated with DI9c as a control, housed separately from the inoculated calves, and euthanised at five months of age. Experiment B included three calves and was conducted at the Royal Veterinary College, London. Virological and haematological parameters were followed after challenge with DI9. These calves were of mixed Friesian breed and housed isolated from other animals when they were inoculated with DI9c at the age of 5 months.

Inoculations and inocula

The subgenomic DI9c RNA was generated by in vitro transcription of the DI9c plasmid [29, 4]. Briefly, T7 RNA polymerase was used to transcribe 1 ug DNA template (MegaScript kit, Ambion). RNA was purified by LiCl precipitation, resuspended in DEPC-treated water and transfected into foetal bovine lung (FBL) cell cultures preinfected with BVDVnc strain Pe515nc. Cytopathogenic virus was recovered following passage in culture. The culture was expanded and the amount of DI9c measured by a plaque-forming assay. The inoculum contained 4.4×10^4 plaque forming units per millilitre (pfu/ml) (Experiment A) or 7.5×10^4 pfu/ml (Experiment B) of DI9c. The calves were inoculated with a total of 10 ml

inoculum, 5 ml were injected in the jugular vein and 5 ml sprayed intranasally, divided with 2.5 ml in each nostril.

Clinical examination

In both experiments, veterinary staff examined all the calves from the day of infection. Recordings included temperature, faecal consistency, urine, appetite and general behaviour. If any signs of abnormality were observed, the animal was examined more thoroughly.

Pathological examinations

The calves were euthanised using pentobarbital intravenously and necropsied immediately. Representative tissue samples were fixed in formalin and gross lesions recorded. Fixed tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin for histopathological evaluation. Gut content was examined bacteriologically and parasitologically according to standard routines.

Haematological sampling and examination

Blood samples were collected into EDTA vacutainers, on days 0, 4, 7, 14, 21 and 45 post inoculation in Experiment A and approximately every second day for six weeks in Experiment B. Total leukocyte counts were assessed using an automated haematological analyser (Technicon Axon System, Technicon Industrial Systems, NY, Experiment A and Cell Dyn, Abbot, Experiment B) running an established bovine haematology profile to determine both total and differential leukocyte counts. Lymphocytes, neutrophils and monocytes were all quantified in the differential count, and where abnormal values were indicated, manual differential counts were made. Results were expressed as counts ($\times 10^9\,L^{-1}$).

Virological examination

In Experiment A, a sample of tissue was collected from the bronchial and the mesenteric lymph nodes and lymphatic tissue caudally in the nasal septum. Specimens were snap frozen in isopentane at −70 °C for cryopreservation. Blood was collected from the jugular vein two days after inoculation, centrifuged and serum stored at −70 °C.

In Experiment B nasal swab samples were taken on days 0, 3, 4, 5, 6, 7, 8, 25, 26 and 27. The swabs were suspended in transport medium containing antibiotics and antimycotics. For virus isolation the sample was clarified by low speed centrifugation and 0.5 ml was cultured with low passage foetal bovine lung cells (FBL) for 2 h. The inoculum was removed and replaced with 1 ml fresh medium and the culture continued for 5 days. To improve the chances of recovery of DI9c, samples were routinely cultured both on fresh FBL cells and on FBLs infected with Pe515nc for 5 days at 37 °C. All cultures were observed for cytopathogenic effect. After 5 days the cultures were frozen and thawed to release cultured virus, and a 0.05 ml sample of each was passaged for a second time onto fresh FBL cells. Portions of the cell lysate obtained at passage 1 and passage 2 were processed for RNA extraction and RT-PCR analysis.

Blood samples taken at days 6, 7 and 8 post inoculum in Experiment B were processed to purify buffy coat cells. A minimum of 5 ml of EDTA treated blood was collected from each animal on each sampling, the erythrocytes were then lysed with ammonium chloride lysis buffer, the sample centrifuged, the leukocyte pellet was washed twice in PBS, and finally resuspended in 1 ml of PBS. Each sample was divided into two and stored at −70 °C. To detect BVDV, each sample was thawed and the cell lysate diluted 1:4 in maintenance medium (minimum essential medium containing 2% foetal calf serum and antibiotic/antimycotic solution). Each diluted sample was seeded onto freshly prepared monolayers of FBL cells

and FBLs plus Pe515nc, prepared in 24 well tissue culture plates and then incubated for 5 days at 37 °C. All cultures were observed for cytopathogenic effect. After 5 days the plates were frozen and thawed to release cultured virus, and a 0.05 ml sample of each was passaged for a second time onto fresh FBL cells. Portions of the cell lysate obtained at passage 1 and passage 2 were processed for RNA extraction and RT-PCR analysis.

RT-PCR

RT-PCR was done similarly on all material collected for virological examinations in both experiments. Total RNA was extracted from 100 mg cryopreserved tissue, 400 µl serum (using Trizol® Reagent, Gibco BRL, Life technologies, Experiment A) or from FBL cell lysates following passage of nasal swab or buffy coat samples (using RNA Stat, Ambion, Experiment B) according to the manufacturer's instructions. To assist with the recovery of the RNA pellet from serum, 10 µl of 10.6 mg/ml tRNA (Sigma) was added as a carrier. The RNA pellets were finally resuspended in 20 µl diethyl pyrocarbonate-treated H_2O. The reverse transcription reaction used 2 µl of the RNA suspension and random hexamer primers to generate cDNA (M-MLV RT, Promega, Experiment A, and Superscript II from Gibco BRL, Life technologies, Experiment B). For amplification, 2 µl of cDNA was used with Taq DNA polymerase (Gibco BRL, Life technologies or Promega) and a programme of 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and a final extension time of 7 min at 72 °C. For the detection of BVDV DI9, the forward primer used was within the 5′-UTR, primer 324, 5′-ATGCCCWTAGTAGGACTAGCA-3′ (NADL 108–128). Three different reverse primers within the NS3 coding region were tested and compared for their sensitivity for the amplification of low levels of target DNA: oligo1 5′-GGTGTAGTCCCCCTCGGCAT-3′ (Pe515nc derived sequence, NADL equivalent 5526–5507), oligo2 5′-GACCATCCTTTCAAGTTTTTT-3′ (Pe515nc derived sequence, NADL equivalent 5909–5890), and NS3R6067 5′-GCTGGTTATCTTCTTGACCAT-3′ (Pe515nc derived sequence, NADL equivalent 6067–6047). These reactions give amplimers of approximately 0.8, 1.2 and 1.4 kb from the DI9 genome. The reverse primer NS3R6067 gave the best results with highest yields of product, and 324 + NS3R were routinely used, despite giving the longest amplimer, however many samples were also tested with 324 + oligo2. Amplification of the 5.4, 5.8 or 6 kb target sequence in the Pe515nc helper virus genome is not achieved with the reaction conditions used. RNA extracted from FBL cells infected with the Pe515nc/DI9 combination served as a positive control. By use of this positive control samples, DI9 genome could be detected in less than 200 pg of total cDNA with this method. As a control for the RNA extraction and RT reactions, cellular β-actin was also amplified with primers Act4 5′-GAGAAGCTCTGCTATGTCGC-3′ and Act2 5′-CCAGACAGCACTGTGTTGGC-3′ to produce an amplimer of 280 bp.

The amplification products were visualized on 1% agarose gels stained with ethidium bromide. To increase the sensitivity of detection of DI9 genome in the nasal swab and buffy coat samples in Experiment B, both 2 µl and 5 µl of the cDNA derived from 1st and 2nd passage FBL cells was also subjected to PCR using a Cy-5-labelled copy of the 324 primer. This allowed the amplimer to be analysed on the ALF DNA sequencer (Amersham Pharmacia) run in fragment analysis mode and detected by fluorescence.

Results

Clinical findings

Few signs were observed during the period of either experiment, as summarised in Table 1.

Table 1. Clinical and pathological outcome in 3–8 months old calves persistently infected with bovine viral diarrhoea virus and inoculated with a defective interfering particle, DI9c

	Calf no.	Euthanised on days post inoculation	Clinical findings	Pathological changes
Experiment A	1	4	temp. 40.2 °C day 4	[a]
	2	7	soft faeces days 5–7	hyperaemia in gut[a]
	3	14	none	[a]
	4	21	none	[a]
	5	87	temp. 40.2 °C day 4, diarrhoea and depression days 30–37 and 85–87	soft gut content[a]
Experiment B	6	40	none	none
	7	40	none	none
	8	28	depression, poor appetite days 27–28	some gut changes

[a]Several reactive lymph nodes, increased cellularity of the mucosa in the gut and perivascular dermatitis

Experiment A: The calf euthanised 4 days post inoculation showed increased temperature (40.2 °C) on the last day, but no other signs. The calf killed on day 7 had diarrhoea during days 6 and 7, and was moderately depressed. The calves killed on days 14 or 21 did not show any clinical signs. The fifth calf had a temperature rise on day 4 (40.2 °C), diarrhoea and decreased appetite during days 30–37, soft faeces and moderate depression with lower appetite during days 84–87, on which day the calf was euthanised.

Experiment B: One calf showed a depressed demeanour and poor appetite on day 25 and was euthanised on day 28. The other two calves showed no clinical signs and were euthanised on day 40 post inoculation.

Pathological findings

Experiment A: Gross pathological changes included several reactive lymph nodes in all calves, but less obvious in the calf killed on day 4 post infection. The changes were typically seen in the bronchial, mediastinal, mesenteric and retropharyngeal lymph nodes. Some degree of hyperaemia and soft content were observed in the intestines in the calves killed on days 4 or 87. Faeces were without parasites, except small numbers of cryptosporidium found in the calf killed on day 87.

Histological examination showed moderately reactive lymph nodes. In the lymph nodes the typical findings were a cell-rich mantel area around the germinal centre. In the gut an increased number of mononuclear cells and some eosinophilic granulocytes were observed in the mucosa. In the Peyer's patches the follicles were large and cell rich. All the calves had a perivascular infiltration of mononuclear cells in dermis, moderate infiltration of mononuclear cells in mucosas in general,

and some animals also had a moderate infiltration of lymphoid cells in other organs. The histological changes were most obvious in the two calves killed on day 7 or 14. The control calf had no gross changes but similar histological changes as the inoculated calves, but to a much less prominent extent.

Experiment B: In two calves, nos. 6 and 7, neither gross nor histological changes were observed. However, calf no. 8 had moderate depletion of lymphoid cells in the gut associated lymphoid tissues e.g. Peyer's patches and the mesenteric lymph nodes. Such depletion was not obvious in the peripheral lymph nodes. In the intestinal sub-mucosa of the jejunum and the ileum, there was focal infiltration of lymphocytes and some evidence of mild oedema. The palatine tonsil showed medullary hyperaemia with marked hyperplastic follicles in the cortex.

Haematological findings

In Experiment A, no significant haematological changes were observed. In Experiment B, the total leukocyte counts indicated a slight but sustained day-on-day decrease for all three animals between days – 3 and 21 (Fig. 1). At this point there was a precipitous drop in leukocyte number in calf 8 (killed on day

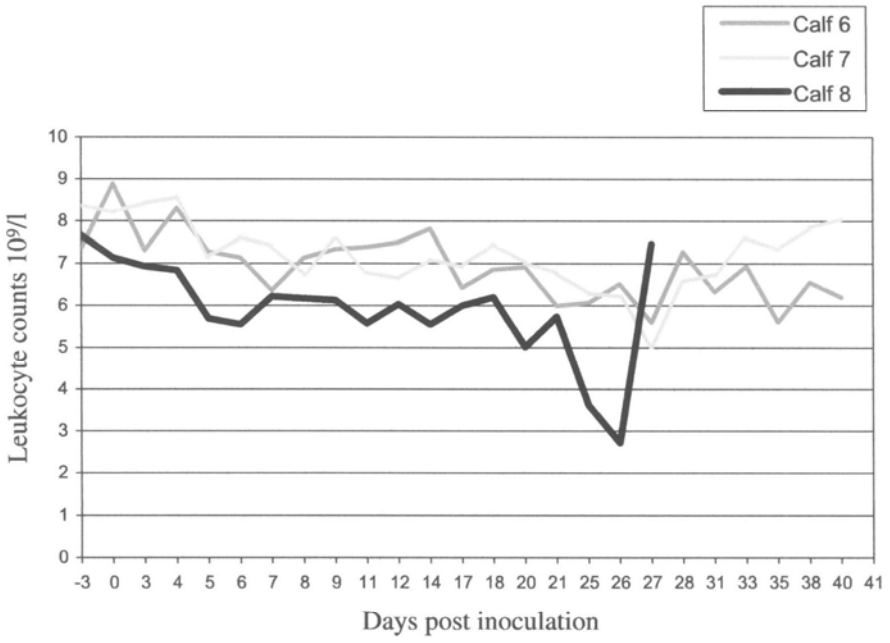

Fig. 1. Leukocyte counts in three calves persistently infected with bovine viral diarrhoea virus and inoculated with a defective interfering particle, DI9c

28), coincident with the onset of clinical signs. This drop affected lymphocyte, neutrophil and monocyte populations equally and although at day 26 neutrophil and monocyte counts were apparently recovering, the sample at day 27 contained many small and immature cells. The leukocyte counts for the other two calves were at their lowest point on day 27 but recovered with the animals showing no clinical signs.

Virological findings – Experiment B

No BVDV with cytopathogenic effect was observed in FBL cultures neither from nasal swab nor buffy coat samples. This result was the same whether or not the FBL cells were preinfected with Pe515nc.

RT-PCR

In both experiments all the cultures, tissue and serum samples examined for DI9c were consistently negative (Fig. 2). Even when more cDNA was added to a reaction or the Cy5-labelled amplimer detected using the ALF fragment analysis system (Experiment B) the samples were negative for the DI9c genome. Controls for cellular actin or for the 5′-UTR of BVDV using primers 324 and 326 were consistently positive, indicating that the RNA extraction and RT-PCR reactions were functioning as expected.

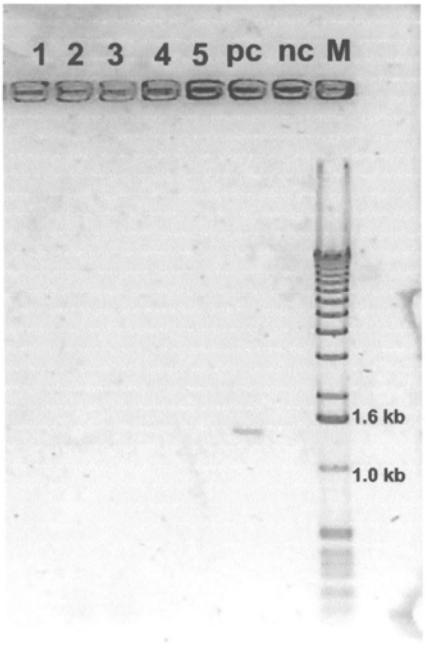

Fig. 2. RT-PCR for detection of DI9c from the mesenterial lymph node of five calves persistently infected with bovine viral diarrhoea virus (*1–5*) and inoculated with DI9c, 1% agarose gel stained with ethidium bromide. The positive control (*pc*) is the challenge inoculum, *nc* is the negative control and Marker (*M*) is a 1 kb ladder

Discussion

In this study, DI9c was not recovered in any of the eight inoculated calves despite repeated sequential sampling, as judged by the absence of cytopathogenic effects when samples were co-cultured in FBL cells and a series of negative RT-PCR reactions from cell and tissue samples. As the challenge dose was given both intravenously and intranasally, the DI9c particles should have reached many tissues where growth could have been possible. During persistent infection BVDVnc has a wide-ranging tropism, with many different cell types including most of the peripheral blood mononuclear cells showing some degree of infection [5]. Particularly high levels of virus occur in lymphoid tissues, where a widespread distribution of virus, in both T and B-cell areas, is observed [18, 27]. In bovines, antigens given intravenously are mainly removed by macrophages lining small blood vessels in the lungs, and inhaled particles should reach the draining lymph nodes near the lungs [32], therefore the inoculated virus could be expected to reach many susceptible target cells. Analysis of the actions of DI particles in animals is complicated, since the production and effects of DI particles depend strongly on helper virus, host cell type and may vary from tissue to tissue. Although the production of a high titer DI9c challenge dose had been difficult, due to the interference observed, a dose in excess of 10^5 infectious DI particles (pfu) was inoculated into each animal but it is still impossible to predict the kinetics of *in vivo* growth. This may account for the lack of a clear correlation seen between *in vitro* and *in vivo* effects by DI particles [3]. Besides, the difference between an experimental infection like the present one and a natural situation where DI particles develop in the infected tissue of an animal might be of significance.

The clinical findings in this study did not include classical signs of mucosal disease. However, two animals had a rise in temperature four days post inoculation, which could be interpreted as a consequence of the infection with DI9c. One of these calves had some periods with diarrhoea later and was moderately depressed. Another animal had a precipitous drop in the leukocyte count at day 25, co-incident with onset of clinical signs of depression and anorexia. The pathological findings in this animal are not unusual in the tissues of some persistently infected calves. The changes in lymphoid tissue in gut and lymph nodes are also difficult to interpret. It is known that clinically healthy cattle, persistently infected with BVDV, may show pathological changes and altered immune system, including immunosuppression [24]. The clinical and pathological findings could be due to the challenge with the DI9c. However, they could also be related to the persistent infection. Additionally, it could also be an early phase in a spontaneous development of mucosal disease or possibly a chronic form of the disease. Inoculation with DI9c could also cause some symptoms without being able to replicate to a sufficiently high titre to cause mucosal disease. However, it is still an unexpected observation that DI9c was not detected in serum samples collected two days post inoculation. The control animal, which was also persistently infected with the same strain but not given DI9c, did not give any clear answer to these questions, since some degree of the same pathological changes were observed.

Due to the presence of the persisting Pe515nc virus in all samples assayed, the only method to differentially detect the DI9c genome was RT-PCR. The primers used in the PCR reaction would in theory amplify sequences from both the helper virus and the DI9c genome. However, the amplification conditions chosen favour the detection of only the smaller DI9c amplimers. Three different sized targets were compared for their sensitivity by choosing primers with different targets within the NS3 coding region; surprisingly, the longer amplimers consistently gave higher yields of product. With the method used, the DI9c genome could be detected in less than 200 pg of total cDNA from FBL cells co-infected with Pe515nc and DI9c from control samples. This makes it unlikely that sensitivity of detection was the limiting factor in our results.

In another pestivirus, classical swine fever virus (CSFV), three cytopathogenic isolates have been found to include DI particles which are responsible for the cytopathic effect observed. The autonomously replicating helper viruses are non-cytopathogenic, exactly as seen in *in vitro* experiments with BVDV DI9c [20]. Acute challenge of CSFV naïve pigs with cytopathogenic isolates results in a more rapid viremia, and clinical signs are more prominent compared to animals infected with noncytopathogenic CSFV [16]. In a clinical experiment, pigs inoculated with a cytopathogenic CSFV strain which composed of a noncytopathogenic CSFV and a cytopathogenic DI particle developed typical signs of classical swine fever, as pigs inoculated with only noncytopathogenic CSFV [1]. Both helper virus and DI particles were detected in samples from the inoculated pigs. However, some of the signs in the experiment appeared delayed, indicating that the DI particles did inhibit the signs induced by virulent helper CSFV. This is in accordance with the fact that replication of DI particles generally reduces the titre of the helper virus and therefore may modify natural virus infections [3].

Short term variations in BVDV levels have been detected by ELISA and virus isolation in leucocytes from clinically healthy persistently infected cattle [7, 34]. However, it is not likely that the level of helper virus would be a limiting factor for the replication of DI9c when animals persistently infected with BVDVnc harbour the virus in most tissues. The specific immunotolerance generated by *in utero* infection of cattle with BVDVnc permits the survival of the persisting virus, and eliminates antigenic variants or quasispecies arising during viral replication although it does not prevent the generation of diversity [9, 14, 22]. Also, BVDVnc are known to replicate at constantly high rates in such animals. Desport et al. (1998) found that in persistently infected animals, analysis of viral genome sequences revealed many examples of genome recombination events [12]. Probably, most of the recombinations will result in defective genomes. Analyses of samples taken from an MD animal have proved the coexistence of cytopathogenic DIs and autonomously replicating cytopathogenic BVDV [13]. Thus, DI particles may be a by-product of replicating BVDV, more than a causing factor of MD.

In this study in animals persistently infected with BVDVnc and inoculated with DI9c, some signs and pathological changes were observed. However, it is uncertain if these findings can be related to the inoculation. DI9c was not found in any of the serum, cell or tissue samples by use of a RT-PCR reaction, and no

cytopathogenic effect was observed. It can be concluded that we failed to detect an infection with DI9c in any of the present animals, and therefore the role of defective interfering particles in the pathogenesis of mucosal disease could not be confirmed.

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