Interferon-β-modulatory sequences from the genome of porcine circovirus type 2

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Summary

Porcine circovirus (PCV) type 2 is an emerging pathogen among pigs, which has been associated with several severe disease syndromes. To date little is known of the pathogenesis and epidemiology of PCV, and how the virus affects the immune system of the host. To evaluate one possible mechanism of pathogenesis, the genome of PCV-2 was examined for content of CpG-motifs. Five 20 nucleotide long sequences from the genome were tested for their ability to induce production of IFN-α by porcine peripheral blood mononuclear cells (PBMC). One of the oligodeoxynucleotides (ODNs) proved to inhibit the IFN-α production induced by the other ODNs that were stimulatory. The inhibitory ODN (PCV-2/1) was tested against other known inducers of IFN-α and showed a variable degree of inhibitory action depending on the construct of the inducer. ODNs containing phosphorothioate backbone and poly- G- sequences seemed more resistant to inhibition. Also, the inhibitory activity of ODN PCV-2/1 differed against the viral inducers Aujeszky´s disease virus (ADV) and Sendai virus (SV), and the plasmid pcDNA3. Inhibition was most effective against ADV, moderately effective against pcDNA3, but did not affect the IFN-α production induced by SV. The variation in sensitivity to inhibition among the agents could be due to differences in target cell populations. The presence of immune modulatory sequences in the genome of PCV-2 could possibly explain parts of the pathogenesis of the virus.

Sammanfattning

Porcint circovirus (PCV) typ 2 har på senare tid uppmärksammats i växande omfattning, och har associerats med flera allvarliga sjukdomssyndrom hos gris. Man vet fortfarande mycket lite om virusets patogenes och epidemiologi, och hur det påverkar immunsystemet i värddjuret. För att undersöka en möjlig mekanism i patogenesen studerades genomet av PCV-2 med avseende på förekomst av CpG-motiv. Fem sekvenser om 20 nukleotider vardera valdes ut från genomet och deras förmåga att inducera IFN-α i perifera mononukleära blodceller (PBMC) testades. En av oligonukleotiderna (oligos) visade sig kunna hämma IFN-α produktionen som inducerats av de övriga fyra oligos. Den hämmande oligon (PCV-2/1) testades mot andra typer av kända inducerare och visade en varierande förmåga att inhibera IFN-α produktion beroende på konstruktionen av induceraren. Oligo vars kedja till en del utgjordes av fosfotioater istället för fosfodiestrar, samt innehöll upprepade G- sekvenser I 3´- änden verkade mer resistenta mot den inhibitoriska aktivitet hos PCV-2/1. Dessutom var PCV-2/1 inhiberande i varierande grad mot olika typer av virala och bakteriella inducerare. Aujeszky´s disease virus (ADV) hämmades effektivt medan plasmiden pcDNA3 bara hämmades delvis. IFN-α produktionen inducerad av Sendaivirus (SV) påverkades inte av PCV-2/1. Mekanismerna bakom denna variation är inte känd, men kan bero på vilka cellpopulationer som aktiveras av induceraren. Fyndet att PCV-2-genomet innehåller IFN-α-modulerande sekvenser kan bidra till ökad förståelse av virusets patogenes.

Introduction

Circovirus of the pig (PCV) are small, nonenveloped DNA virus containing a circular single-stranded genome (Allan and Ellis 2000). There are two types identified, PCV-1 and PCV-2. PCV-1, which was originally found in 1972 as a contaminant of the porcine kidney cell line PK-15 (Tischer et al. 1974) is believed to be apathogenic (Tischer et al. 1986; Allan et al. 1995) and spread worldwide (Allan and Ellis 2000). PCV-2 was first identified in Canada in 1991 when it was associated with an outbreak of wasting disease among SPF-pigs (Allan et al. 1998; Ellis et al. 1998; Harding et al. 1998), and was subsequently found to be a separate strain of PCV showing less than 80% nucleotide homology with PCV-1 (Meehan et al. 1998). PCV-2 has since then been associated with several severe disease syndromes in pigs, not only post weaning multisystemic wasting syndrome (PMWS), but also congenital tremors (Stevenson et al. 2001; Choi et al. 2002), porcine dermatitis and nephropathy syndrome (PDNS) (Allan et al. 2000; Rosell et al. 2000; Thomson et al. 2000) and exsudative epidermitis (Wattrang et al. 2002) have been suggested as PCV-2 related diseases. Also PCV-2 is thought to be spread widely in the world, it has been isolated and/or associated with disease in several countries in America, Europe and Asia (Allan et al. 1999; Choi et al. 2000; Fenaux et al. 2000; Mankertz et al. 2000; Mori et al. 2000; Wellenberg et al. 2000; Celer and Carasova 2002; Liu et al. 2002). In countries where serological surveys have been conducted the results indicate a high seroprevalence of antibodies to PCV-2 (Allan and Ellis 2000).

Although PMWS is the disease syndrome most often associated with PCV-2 new data suggest that the virus is involved in the development of many other diseases, maybe as a co-infection (Allan and Ellis 2000). In experimental infections PCV-2 in combination with porcine parvo virus (PPV) can produce severe disease while the two infections by themselves do not (Allan et al. 1999; Krakowka et al. 2000), the reason for this being unknown. PCV-2 has also been isolated on several occasions from pigs diagnosed with the atypical form of respiratory and reproductive syndrome caused by the PRRS virus (Ellis et al. 1999; Allan and Ellis 2000). In Sweden, a limited serum survey suggests a prevalence of PCV-2 of 96% (Linné et al. 2000) but PMWS has never been diagnosed clinically. On the other hand, PCV-2 has been associated with an outbreak of exsudative epidermitis in a Swedish SPF-herd (Wattrang et al. 2002), and the same virus, which was isolated from one affected pig, was used to experimentally induce PMWS in combination with PPV in pigs in Northern Ireland (Allan et al. 2002).

Little is known about the epidemiology and pathogenesis of PCV-2 and how it possibly interferes with other infectious microbes. Neither do we know how PCV-2 interacts with the immune system of the host and if it is capable of modulating this to its own benefit. Since the virus genome is small (1759 nt) and circular it shows similarities to plasmid DNA, which today is known to act immunomodulatory in vertebrates (Tighe et al. 1998). The immunomodulatory activity, which includes induction of cytokine production, proliferation and Ig-secretion by B-cells as well as enhanced NK-cell activity, is ascribed to the relatively high content of unmethylated CpG-dinucleotides (CpG-motifs) in

plasmids and other forms of bacterial DNA (Yamamoto et al. 1992; Sato et al. 1996; Krieg 2002). Using synthetic ODNs it has been demonstrated that alteration of the nucleotides flanking the CpG-motif affect their immune stimulatory capacity, and that optimal flanking bases vary between murine and human systems (Krieg 2002). As regards other species (Rankin et al. 2001), the identification of immune stimulatory sequences (ISS) for use in pigs (Kamstrup et al. 2001), cattle (Zhang et al. 2001; Pontarollo et al. 2002), fish (Kanellos et al. 1999; Jorgensen et al. 2001), dogs and cats (Wernette et al. 2002) is initiated. The ability of bacterial DNA to induce cytokine production (Kamstrup et al. 2001; Magnusson et al. 2001) explains at least partly the efficacy of DNA-vaccines and is currently explored in the use of synthetic oligodeoxynucleotides (ODNs) as adjuvant components. Dissection of the requirements for immune stimulatory activity has revealed DNA motifs that act inhibiting. Inhibitory motifs can resemble the stimulatory sequences, but the most efficient inhibitors contain a G-tetramer (Krieg et al. 1998; Pisetsky and Reich 2000; Zhao et al. 2000; Stunz et al. 2002). Also, the position of the inhibitory sequence in relation to a stimulatory sequence on the same strand of DNA might be of importance for the net effect of the ODN (Yamada et al. 2002).

Studies in mice and man show that CpG-DNA interacts with Toll like receptor (TLR)-9, which is expressed by B-cells and a subpopulation of dendritic cells (Krieg 2002). Interestingly, an abundance of PCV-2 antigen is found in the cytoplasm of monocytes/macrophages and dendritic cells of infected pigs but the virus does not seem to be replicating (Allan et al. 1998; Ellis et al. 1998). Since these cell types are efficient cytokine producers and play an important role in directing the immune response, they are an important target for viral evasive mechanisms. In the present study, five 20-nucleotide long sequences were selected from the PCV-2 genome and analysed for their ability to induce production of the anti-viral cytokine interferon (IFN)-α by porcine leukocytes. One out of five ODNs tested inhibited the IFN-α production induced by the others ODNs and was therefore used for inhibition studies with other synthetic and natural IFN-α inducers.

Materials and methods

ODNs and virus strands

The genome of PCV-2 (Stoon) was aquired from the GenBank (GenBank accession nr AF055392). Oligodeoxynucleotides used for induction of IFN-α were purchased from Cybergene AB (Stockholm, Sweden), and were desalted and dissolved in water. The sequences of the ODNs are given in table 1. The plasmid pcDNA3 (Invitrogen, San Diego, CA) was used in the concentration of 2.5 mg per ml culture medium. Aujeszky´s disease virus (ADV; strain Bartha, 105 ID50/ml), inactivated by 4 cycles of UV irradiation (1 Joule/cm2), was used at 100-fold dilution. Live Sendai virus (SV) propagated in eggs was used as chorioallantoic fluid in 10-fold dilution in the cell cultures (Cederblad et al. 1998).

Table 1. *Sequence of ODNs used for induction of IFN-α in poPBMC*

ODN name	Sequence 5´ to 3´
PCV-2/1	CCC CCC TCC CGG GGG AAC AA
PCV-2/2	ACT TCG GCA GCG GCA GCA CC
PCV-2/3	ACC CTG TAA CGT TTG TCA GA
PCV-2/4	CTG TGT GAT CGA TAT CCA TT
PCV-2/5	GTT TTC GAA CGC AGC GCC GA
D19	ggT GCA TCG ATG CAG ggg gg
D25	GGT GCA TCG ATG CAG GGG GG

Highlighted sequences correspond to known immune stimulatory ODNs (Kamstrup, Verthelyi et al. 2001; Magnusson, Johansson et al. 2001). All ODNs contain phosphodiester backbone except chimeric ODN D19 wich contains phosphorotioate backbone at nucleotides in lower case.

Incubation with lipofectin

ODNs and virus were pre-treated with lipofectin (Life Technologies, Paisley, UK) in medium without FCS. The lipofectin was first diluted in medium without FCS and incubated at RT for 1 h before addition to ODN or virus. After 15 minutes of pre-treatment, the mix of ODN or virus and lipofectin was added to cell cultures containing PBMC suspended in growth medium as described below. When two inducers were mixed both separate and co-incubation in lipofectin was used. The final concentration of lipofectin in culture medium was 2.5 mg/ml, which has previously been evaluated as the optimal concentration for IFN-α production by Domeika et al. (manuscript).

Cell cultures and induction of IFN-α

Conventionally reared Yorkshire pigs or Yorkshire crosses, housed at the University Research Station Funbo – Lövsta, Sweden, were used at the age of 9 – 12 weeks. Within the experiments pigs from separate litters were used. Blood samples were collected from vena cava cranialis in evacuated test tubes (B-D Vacutainer, Meylan Cedex, France) with heparin (143 USP units). Peripheral blood mononuclear cells (PBMC) were purified from blood by Ficoll – Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient and suspended in growth medium, i. e. RPMI 1640 medium (Biowhitaker, Verviers, Belgium) with 20 mM HEPES buffer, supplemented with L-glutamine (2 mM), penicillin (200 IU/ml), streptomycin (100 mg/ml), 2-mercaptoethanol (5x10-5M) and 10% foetal calf serum (FCS; Myoclone,; Life Technologies, Paisley, UK). Flat-bottomed 96 well plates (Nunc, Roskilde, Denmark) were used for induction. 0.1 ml of the inducer were added in triplicate cultures with 5x106 PBMC/ml in a total volume of 0.2 ml per well. After 20 h incubation in 37oC, 7% CO2 in air, supernatants were pooled and stored at –20oC until further analysis.

Detection of IFN-α

To qantify IFN-α in cell culture supernatants a dissociation-enhanced lathanide fluoro-immunoasssay (DELFIA) was used as previously described (Artursson et al. 1995). This is based on two mAbs directed against porcine IFN-α. The results are

given as units per ml (U/ml), or as percentage inhibition calculated as mean value ± SEM for four animals if not otherwise stated.

Results

IFN-α inducing capacity of ODNs from the genome of PCV-2

The genome of PCV-2 (Stoon, GenBank accession nr AF055392) was analyzed for the presence of potentially immunoregulatory sequences. Five 20 nucleotide long sequences were chosen and designated PCV-2/1 – PCV-2/5 respectively (Table 1). PCV-2/1 contained sequences of C-and G-repeats, PCV-2/2 represents a binding site for replication proteins, and PCV-2/3 – 5 each had six bases corresponding to previously identified CpG-motifs known to induce cytokines (Kamstrup et al. 2001; Magnusson et al. 2001). The ODNs were synthesized and tested for their IFN-α inducing capacity in cultures of PBMC obtained from four pigs. Three different concentrations were tested (5, 10 and 25 µg/ml), with or without pre-treatment with lipofectin. For comparison, plasmid DNA pre-treated with lipofectin (lipofected pcDNA3) was included as IFN-α inducer.

Regardless of concentration, pre-treatment of the ODN with lipofectin was necessary to achieve IFN-α production. PCV-2/4 and PCV-2/5 were the strongest inducers, but also PCV-2/2 and PCV-2/3 induced IFN-α production (Fig. 1). The IFN-α induction increased with concentration of the ODN and 25 µg per ml consistently induced the highest concentrations of IFN-α. At this concentration, ODNs PCV-2/4 and PCV-2/5 induced similar levels of IFN-α as lipofected pcDNA3 (pig 1: 316 U/ml, pig 2: 653 U/ml, pig 3: 386 U/ml, pig 4: 368 U/ml). Although the IFN-α producing capacity varied between pigs, their responses to the various inducers showed the same internal relationship. One of the ODNs, PCV-2/1, did not induce IFN-α production by PBMC from any of the pigs tested, regardless of pre-treatment with lipofectin or not (data not shown). Therefore, the possible neutralizing activity of ODN PCV-2/1 was assessed.

PCV-2/1 inhibits the IFN-α induction by stimulatory ODNs selected from the PCV-2 genome

To study if PCV-2/1 only was unable to induce IFN-α production or if it also affected the IFN-α production, poPBMC were exposed to PCV-2/1 in combination with the other ODNs from the PCV-2 genome. Initially, equal amounts of PCV-2/1 (25 µg/ml) and each of the other four ODNs were incubated together in lipofectin before addition to the cultures. In all cases except for ODN PCV-2/5, presence of PCV-2/1 abolished the IFN-α production (data not shown). The induction of IFN-α by PCV-2/5 was markedly decreased when mixed with PCV-2/1 at equal concentrations and totally abolished when PCV-2/5 was tested in combination with PCV-2/1 at a three fold higher concentration (Fig 2).

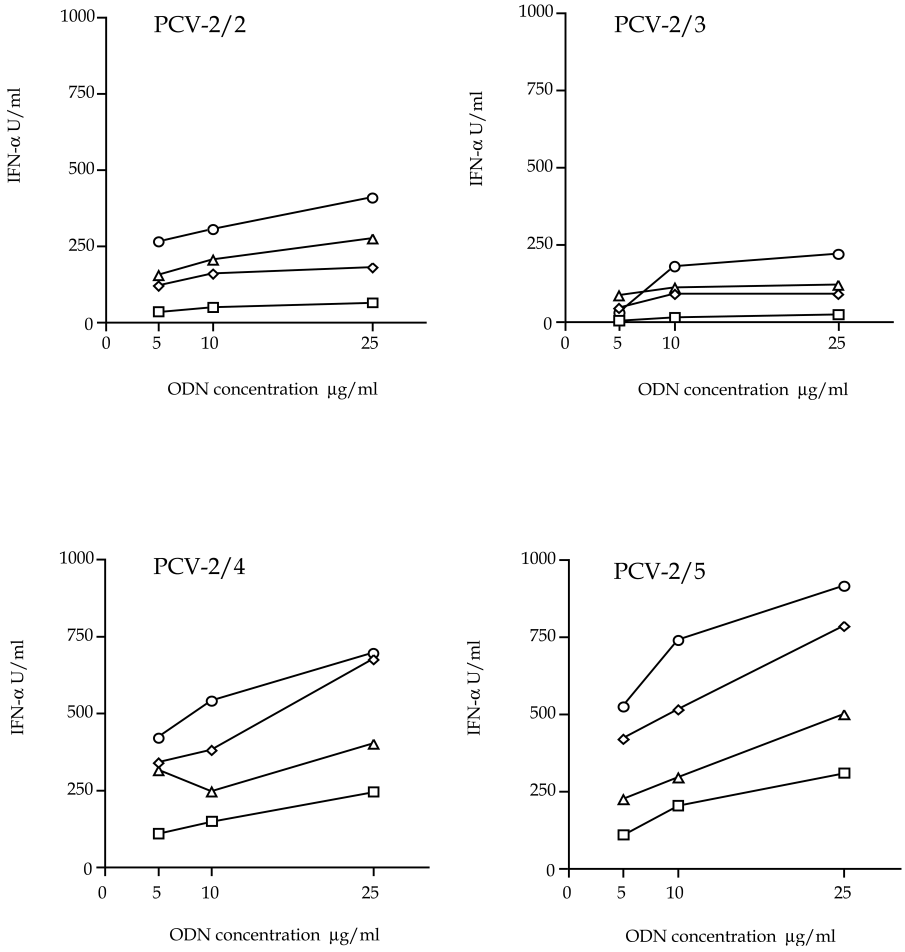

Figure 1. *IFN-α inducing capacity of ODNs selected from the PCV-2 genome (PCV-2/2 – 5). The ODNs were 20 nucleotides long and tested at concentrations of 5, 10 and 25 mg/ml. PBMC from pig 1 (□), 2 (◇), 3 (○) and 4 (△) were used for all ODNs, and all samples were pre-treated with lipofectin. Results are expressed as units per ml (U/ml).*

PCV-2/1 partially inhibits the IFN-α induction by some non-related ODNs

Previous studies in the pig have shown that some ODNs induce IFN-α production without pre-treatment with lipofectin (Domeika et al, manuscript). Therefore, the effect of mixing PCV-2/1 with two such ODNs (the phosphodiester ODN D25 or the phophodiester/phosphorothioate chimera ODN D19), consisting of a central CpG motif and G-repeats in their 5´and 3´ends, was tested in the presence or absence of lipofectin (Fig. 3). Pre-treatment of ODNs with lipofectin was conducted separately during 15 minutes before the samples were mixed. PCV-2/1 was used in two concentrations, 25 and 75 µg/ml. The IFN-α production induced

by the phosphodiester ODN D25 was partially inhibited by PCV-2/1. The higher concentration of PCV-2/1 seemed to be slightly more inhibitory, but there was no evident effect of the pre-treatment with lipofectin. On the other hand, the IFN-α inducing capacity of the chimerial form (D19) was not clearly inhibited by PCV-2/1 at any of the concentrations tested, and no obvious effect of pre-treatment with lipofectin was observed. Thus, the IFN-α producing capacity in response to some ODN constructs is retained in the presence of PCV-2/1.

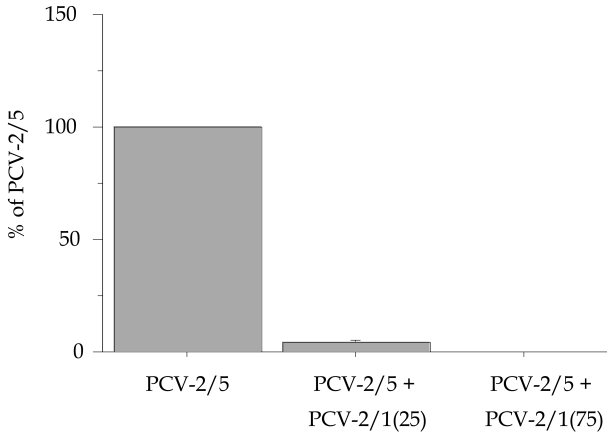

Figure 2. *Inhibitory activity of PCV-2/1 on the IFN-α inducing capacity of ODN PCV-2/5. PCV-2/1 was used in two concentrations, 25 mg/ml (25) and 75 mg/ml (75). Results are expressed as percentage of the induction in the absence of PCV-2/1.*

PCV-2/1 inhibits the IFN-α induction by ADV and pcDNA3 but not SV

Virus are the best known inducers of IFN-α and the effect of PCV-2/1 was therefore studied using two viral preparations, inactivated ADV and live SV. For comparison, lipofected pcDNA3 was used as a source of bacterial DNA (Fig 4). As in the previous experiment PCV-2/1 was tested in two different concentrations, in the presence and absence of lipofectin. In the absence of PCV-2/1 SV induced mean levels of IFN-α of 109 ± 28 U/ml, ADV 390 ± 149 U/ml and pcDNA3 952 ± 245 U/ml. When mixed with live SV, PCV-2/1 did not clearly affect the levels of IFN-α produced, and there was no effect of pre-treatment with lipofectin. The IFN-α production of ADV on the other hand was clearly inhibited by PCV-2/1 in both concentrations, with and without lipofectin. PCV-2/1 in combination with pcDNA3 resulted in a partial inhibition of IFN-α production by the plasmid, the higher concentration of PCV-2/1 being most effective. Since pcDNA3 does not induce IFN-α production in the absence of lipofectin, only lipofected samples were tested.

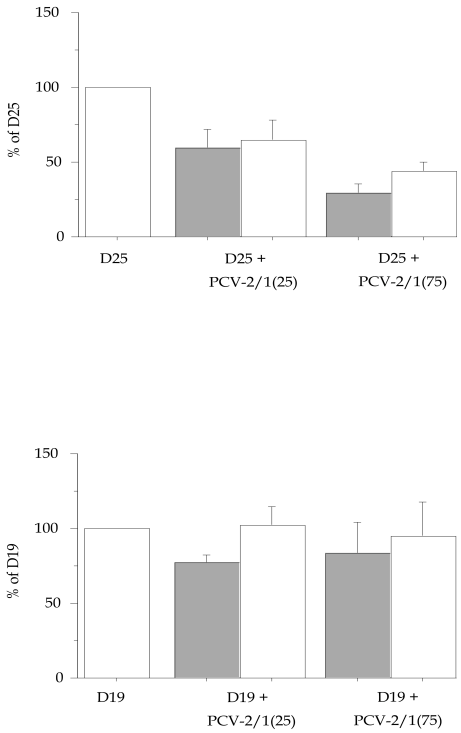

Figure 3. *Inhibitory activity of PCV-2/1 on the IFN-α inducing capacity of ODNs D25 and D19 respectively. PCV-2/1 was used in two concentrations, 25 mg/ml (25) and 75 mg/ml (75). Samples were tested in the presence and absence of lipofectin, grey bars showing lipofected samples, white bars showing non-lipofected samples. Results are expressed as percentage of the induction in the absence of PCV-2/1.*

Discussion

The genome of PCV-2 was demonstrated to contain several nucleotide sequences with the ability to modulate the immune response of porcine PBMC. In this study four out of five oligodeoxynucleotides (ODNs) selected from the genome of PCV-2 acted stimulatory via induction of IFN-α production by porcine PBMC, while one ODN (PCV-2/1) showed inhibitory activity. This ODN contained sequences of C- and G- repeats and proved to inhibit the IFN-α production induced by the other ODNs from the genome of PCV-2 completely. It also showed a variable degree of inhibitory action on other known inducers of IFN-α.

All ODNs selected from the PCV-2 genome were synthesized with phosphodiester backbone and four of the ODNs induced IFN-α production comparable to that induced by other natural inducers, provided the ODNs were pre-treated with lipofectin. One ODN (PCV-2/1) did not induce IFN-α production and was found

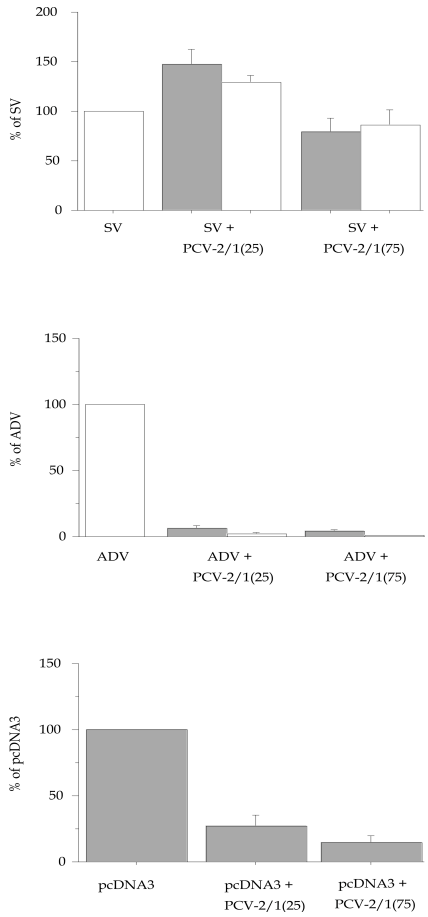

Figure 4. *Inhibitory activity of PCV-2/1 on the IFN-α inducing capacity of SV, ADV and the plasmid pcDNA3 respectively. PCV-2/1 was used in two concentrations, 25 mg/ml (25) and 75 mg/ml (75). Samples were tested in the presence and absence of lipofectin, grey bars showing lipofected samples, white bars showing non-lipofected samples. Results are expressed as percentage of the induction in the absence of PCV-2/1.*

to inhibit the IFN-α production by the other ODNs. The inhibitory activity of PCV-2/1 was therefore tested against other constructs of known IFN-α inducing ODNs. The ODN D19, containing nuclease resistant phosphorotioate backbone, was not inhibited by PCV-2/1, while the phosphodiester form of the same ODN (D25) was partially inhibited. Phosphorothioate backbone thus seems to make the ODN more resistant to the inhibitory activity of PCV-2/1. A special feature of the ODNs D19 and D25 was their ability to induce IFN-α production in the absence of lipofectin in contrast to the ODNs selected from the PCV-2 genome. Lipofectin is believed to protect the ODN from nuclease activity, but is also thought to aid the uptake of ODNs unspecifically into cells by encapsulating the ODN and react with

the cell membrane (Xu and Szoka 1996). The ability to induce IFN-α in the absence of lipofectin could therefore be explained by the presence of the poly- G-tails located at the 3´ ends of these ODNs. It has previously been shown that G-repeats surrounding the CpG-motif affect the stimulatory activity (Pisetsky 1999). The exact mechanism of this is not known, although, the poly- G- tails are thought to facilitate cellular uptake of the ODN by mediating binding to scavenger receptors (Kimura et al. 1994). Thus the ODNs D19 and D25 were able to induce IFN-α production without pre-treatment with lipofectin while the ODNs selected from the PCV-2 genome were not. This difference could at least partly explain the difference in inhibitory capacity of the ODN PCV-2/1. In this context it is interesting to note that PCV-2/1 could exert it´s inhibitory effect in the absence of lipofectin.

When mixed with different viral and bacterial agents the inhibitory activity of PCV-2/1 varied considerably. The IFN-α production induced by live Sendai virus (SV) was not inhibited while the induction by Aujeszky´s disease virus (ADV) was almost abolished. Both viruses are efficient inducers of IFN-α in the absence of PCV-2/1. Two of the four pigs tested responded considerably higher to ADV than to SV, but the other two pigs showed no pronounced difference in IFN-α production induced by the two viral preparations (data not shown). One possible explanation to the differences in response to PCV-2/1 among microbial agents could be a variation in the type of cell producing IFN-α. Among human PBMC two populations of IFN-α/β producing cells are recognized, monocytes and cells of plasmacytoid dendritic cell (PDC) origin, the natural interferon producing cells (NIPC) (Colonna et al. 2002). In the pig cells with many similar characteristics of NIPC have been demonstrated after induction with transmissible gastroenteritis virus (TGEV) or ADV (Artursson et al. 1992; Nowacki et al. 1993; Nowacki and Charley 1993). When human PBMC are exposed to SV, monocytes in addition to NIPC produce IFN-α, whereas only NIPC respond to Herpes Simplex virus (HSV) (Magnusson et al. 2001). The difference in target cell populations may be one explanation why the induction of IFN-α by SV is not affected by PCV-2/1 while the induction by ADV is greatly reduced. Also, the production of IFN-α induced by plasmid DNA (pcDNA3) was clearly reduced upon addition of PCV-2/1. pcDNA3 has previously been shown to induce IFN-α production in human NIPC (Vallin et al. 1999). Thus the inhibitory activity of PCV-2/1 could be more pronounced for IFN-α production by PDC than by monocytes.

IFN-α, which is primarily produced by PDC, is an important cytokine with anti-viral properties that also contributes to the specific immune response to viral infections (Le Bon and Tough 2002). The PDC therefore compose a potentially important strategic target for viral evasion. To date the site of replication of PCV is unknown, but viral DNA accumulates in the cytoplasm of DCs and macrophages (Allan and Ellis 2000). The role of other cytokines involved in the immune response to CpG DNA has not been assessed in this paper, and further studies are needed to evaluate the effect of PCV-2/1 on other important actors of the immune response.

Today the importance of PCV-2 infection on the general health and immune status of pigs is largely unknown. Immunosuppression is debated by some to be

one of the characterisics of PCV-2, and a circovirus related to PCV, Chicken anemia virus (CAV), causes severe immunosuppression in chicken (Rosenberger and Cloud 1998). As in the case of PCV, the pathogenesis of CAV is not fully understood, but the virus has been shown to interfere with the transcription of IFN-a and IFN-g mRNA (Ragland et al. 2002). The genome of CAV has a high content of sequences with striking similarities to the inhibitory ODN PCV-2/1. These sequences may play a role in the pathogenesis of CAV, as well as PCV, and could possibly explain the suggested immunosuppressive effect of the virus. The mechanisms of this action are not yet understood and therefore further studies are needed to evaluate this hypothesis.

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