Identification of a sequence from the genome of porcine circovirus type 2 with an inhibitory effect on IFN-α production by porcine PBMCs

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Porcine circovirus type 2 (PCV-2) has been identified as the causal agent of postweaning multisystemic wasting syndrome and has been associated with several other disease syndromes in pigs. To date, however, little is known regarding the mechanism(s) underlying the pathogenesis of PCV-2-induced diseases and the interaction of the virus with the host immune system. In the present study, oligodeoxynucleotides (ODNs), with central CpG motifs selected from the genome of PCV-2, were demonstrated to modulate the immune response of porcine PBMCs. Four of the five ODNs tested were demonstrated to act in a stimulatory manner via induction of IFN-α production, whereas only one of the five ODNs showed inhibitory activity. Also, this inhibitory ODN was demonstrated to completely inhibit IFN-α production induced by the other stimulatory ODNs and showed a variable degree of inhibitory action on other known inducers of IFN-α. Although no single common characteristic among resistant or susceptible inducers could be identified, the presence of immune modulatory sequences in the genome of PCV-2 may represent an underlying mechanism of the pathogenesis of PCV-2-associated diseases.

INTRODUCTION

Porcine circoviruses (PCV) are small, non-enveloped DNA viruses containing a circular, ss genome (Allan & Ellis, 2000). Two types, PCV-1 and PCV-2, have been identified and exhibit less than 80% nucleotide sequence identity (Meehan et al., 1998). PCV-1 was identified originally as a contaminant of the porcine kidney cell line PK-15 (Tischer et al., 1974) and is believed to be apathogenic (Allan et al., 1995; Tischer et al., 1986). PCV-2 was isolated first from an outbreak of wasting disease among high health status pigs (Allan et al., 1998; Ellis et al., 1998; Harding et al., 1998) and has been demonstrated to be the causal agent of post-weaning multisystemic wasting syndrome (PMWS) (Allan et al., 1998; Ellis et al., 1998; Kennedy et al., 2000; Krakowka et al., 2000, 2001). Since then, PCV-2 has been associated with several other severe disease syndromes in pigs, such as congenital tremor (Choi et al., 2002; Stevenson et al., 2001), porcine dermatitis and nephropathy syndrome (Allan et al., 2000; Rosell et al., 2000) and exudative epidermitis (Wattrang et al., 2002). Serological surveys demonstrate that PCV-2 is spread throughout most pig populations and antibodies reactive to a PCV-2-like virus have been present in the pig population for at least 30 years (Walker et al., 2000). In addition, examination of archived tissue samples has revealed that PMWS occurred sporadically prior to the epidemic outbreaks observed during the last 5 years (Sandvik et al., 2001). Experimental reproduction of PMWS has shown that coinfection with other viruses, such as porcine parvovirus (Allan et al., 1999; Kennedy et al., 2000), or nonspecific immune activation (Krakowka et al., 2001) is required for the development of PMWS in 100% of inoculates.

The small (1759 nt), circular genome of PCV-2 is analogous to plasmid DNA, which is known to exhibit immunostimulatory activity in vertebrates (Tighe et al., 1998). This immunostimulatory activity includes induction of cytokine production, proliferation and immunoglobulin secretion by B-cells as well as enhanced NK-cell activity and is ascribed to the relatively high content of unmethylated CpG dinucleotides in plasmids and other forms of bacterial DNA (Krieg, 2002; Sato et al., 1996; Yamamoto et al., 1992). An unmethylated CpG dinucleotide flanked by two 5’ purines...
and two 3' pyrimidines was referred to initially as a stimulatory CpG motif (Sato et al., 1996). Using synthetic oligodeoxynucleotides (ODNs), it has been demonstrated that alteration of the flanking nucleotides affects the immune stimulatory capacity. However, optimal flanking bases vary between species and with the immune parameter studied (Klinman et al., 2002; Krieg, 2002; Mutwiri et al., 2003). Furthermore, dissection of the requirements for immune stimulatory activity (CpG-S) has revealed DNA motifs that neutralize (CpG-N) or inhibit immune stimulation. The sequence of inhibitory motifs can resemble those of stimulatory motifs but the most efficient inhibitors contain a G tetramer or repeated clusters of G sequences (Krieg et al., 1998; Pisetsky & Reich, 2000; Stunz et al., 2002; Zhao et al., 2000). In addition, the position of the inhibitory sequence in relation to a stimulatory sequence on the same strand of DNA is considered of importance with respect to the net effect of the ODN (Yamada et al., 2001b). All ODNs contain a phosphodiester backbone, except chimeric ODNs D19 and 2216, which contain a phosphorothioate backbone (nucleotides in lower case). All CG dinucleotides are underlined. References are selections of publications where the ODNs have been tested previously.

Studies in mice and man suggest that CpG DNA interacts with Toll-like receptor 9, which, in the human system, is expressed by B-cells and a subpopulation of dendritic cells (Krieg, 2002). PCV-2 has been demonstrated to accumulate in the cytoplasm of monocytes/macrophages and dendritic cells of infected pigs in the absence of any evidence of active virus replication (Allan et al., 1998; Ellis et al., 1998). Since these cell types are efficient cytokine producers and play an important role in directing the host immune response, they are important targets for virus evasion.

In this study, the genome of PCV-2 was examined for CpG content and five sequences, each 20 nt long with central CpG motifs, selected for their similarity to known stimulatory and inhibitory ODNs, were analysed for their ability to modulate the production of the antiviral cytokine IFN-α by porcine leukocytes.

**METHODS**

**ODNs.** The genome of PCV-2 (Imp. 1010 Stoon) was acquired from GenBank (accession no. AF055392) and sequences 20 bases long were selected for synthesis together with a number of reference ODNs used previously for studies on IFN-α induction. All ODNs (Table 1) were purchased desalted and dissolved in water (Cybergene). To hybridize, equimolar amounts of sense and antisense ODNs were mixed and heated to 95°C for 5 min and incubated subsequently for 30 min at room temperature, as described earlier (Magnusson et al., 2001b).

**Vertebrate DNA, plasmid DNA and virus preparations.** Salmon sperm DNA (Sigma) was dissolved according to the manufacturer’s instructions and used as a source of vertebrate DNA. The plasmid pCDNA3 (Invitrogen) was propagated in Escherichia coli and purified using the EndoFree Plasmid Mega kit (Qiagen). Aujeszky’s disease virus (ADV, strain Bartha; kindly supplied by the section of Veterinary Virology, Swedish University of Agricultural Sciences) was purified from the supernatants of PK-15-infected cells by centrifugation (2600 g for 30 min at 4°C) and inactivated by four cycles of UV irradiation (1 J cm⁻²). Sendai virus (SV, strain Cantell, kindly supplied by H.-L. Kauppinen, Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) was propagated in embryonated hens’ eggs and harvested with the chorioallantoic fluid.

**Lipofection.** Salmon sperm DNA, ODNs, plasmid DNA and viruses were, where indicated, pretreated with lipopectin (Life Technologies). For lipopectin treatment, lipopectin (10 μg ml⁻¹) was incubated for 1 h at room temperature in RPMI 1640 medium (BioWhittaker) before mixing with an equal volume of medium containing salmon

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

Sequences in bold correspond to known immune stimulatory ODNs (Kamstrup et al., 2001; Magnusson et al., 2001b). All ODNs contain a phosphodiester backbone, except chimeric ODNs D19 and 2216, which contain a phosphorothioate backbone (nucleotides in lower case). All CG dinucleotides are underlined. References are selections of publications where the ODNs have been tested previously.

<table>
<thead>
<tr>
<th>ODN</th>
<th>Sequence (5′→3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV-2/1</td>
<td>CCCCCCCTCCCGGGGGGAAACAA</td>
<td>Kamstrup et al. (2001)</td>
</tr>
<tr>
<td>PCV-2/2</td>
<td>ACTTCGGCAGCGGCAAGCACC</td>
<td>Kamstrup et al. (2001)</td>
</tr>
<tr>
<td>PCV-2/3</td>
<td>ACCCTGTAAACGTGGTTGTCAGA</td>
<td>Sato et al. (2001)</td>
</tr>
<tr>
<td>PCV-2/4</td>
<td>CTGTCGTGATCGATACCATTT</td>
<td>Sato et al. (2001)</td>
</tr>
<tr>
<td>PCV-2/5</td>
<td>GTTCGCGCAAGGGCGGCGCA</td>
<td>Sato et al. (2001)</td>
</tr>
<tr>
<td>PCV-2/1 C</td>
<td>TGGTCGCCGCCGGGGGGGGGGGG</td>
<td>Sato et al. (2001)</td>
</tr>
<tr>
<td>D19</td>
<td>ggTGCATCGAGCGGAGGGGG</td>
<td>Kamstrup et al. (2001)</td>
</tr>
<tr>
<td>D25</td>
<td>GGTGCATCGATCGAGGGGGGG</td>
<td>Kamstrup et al. (2001)</td>
</tr>
<tr>
<td>H</td>
<td>TTTTCGAATCCGGAATGATG</td>
<td>Sato et al. (1999)</td>
</tr>
<tr>
<td>I</td>
<td>ATTCATCTCGAATTGGAAAA</td>
<td>Magnusson et al. (2001)</td>
</tr>
<tr>
<td>2216</td>
<td>ggGGGACGATCTGGAGGGGG</td>
<td>Krug et al. (2001)</td>
</tr>
</tbody>
</table>

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sperm DNA, ODN, plasmid or virus. Samples were pretreated for 15 min and were then added to cell cultures, as described below. When cells were cocultured with two types of inducers, these were incubated with lipofectin either individually or together before addition to cell cultures. In all cases, the final concentration of lipofectin was 2.5 μg ml⁻¹ in medium.

Cell cultures and induction of IFN-α. Blood samples were collected from conventionally reared Yorkshire pigs or Yorkshire crosses, aged 8–12 weeks and housed at the University Research Station Funbo, Lövsta, Uppsala, Sweden. Blood was collected from crosses, aged 8–12 weeks and housed at the University Research Station Funbo, Lövsta, Uppsala, Sweden. Blood was collected from conventionally reared Yorkshire pigs or Yorkshire crosses, aged 8–12 weeks and housed at the University Research Station Funbo, Lövsta, Uppsala, Sweden. Blood was collected from conventionally reared Yorkshire pigs or Yorkshire crosses, aged 8–12 weeks and housed at the University Research Station Funbo, Lövsta, Uppsala, Sweden.

RESULTS

Detection of IFN-α. IFN-α in cell culture supernatants was quantified by a dissociation-enhanced lanthanide fluoro-immunoassay (DELFIA), as described previously (Artursson et al., 1995). DELFIA, which is based on two mAbs directed to porcine IFN-α, had a detection limit of 0.3 U IFN-α ml⁻¹. The concentration of IFN-α was determined by comparison with a laboratory standard of natural porcine IFN-α and the results are given as U IFN-α ml⁻¹. Results from experiments in which ODNs were cocultivated are given as percentage, calculated from the formula: 100 × (U IFN-α ml⁻¹ in cultures with both inducers)/(U IFN-α ml⁻¹ in cultures with a single inducer). All values are the mean ± SEM for four animals.

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RESULTS

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

The genome of PCV-2 (Imp. 1010 Stoon, GenBank accession no. AF055392) was analysed for the presence of potentially immunoregulatory sequences. Five sequences, each 20 nt long, were chosen and designated PCV-2/1 to PCV-2/5 (Table 1). PCV-2/1 was chosen due to the presence of sequences with GC repeats, PCV-2/2 because it represents the binding site for the replication initiator Rep protein identified in PCV-1 (Mankertz & Hillenbrand, 2001) and PCV-2/3 to -2/5 because of the presence of six bases corresponding to Cpg motifs identified previously as having known cytokine-inducing capacity (Kamstrup et al., 2001; Magnusson et al., 2001a; Sato et al., 1999). ODNs were synthesized and tested for their IFN-α-inducing capacity in cultures of porcine PBMCs. Three different concentrations of ODNs were tested (5, 10 and 25 μg ml⁻¹), with or without pretreatment with lipofectin. For comparison, plasmid DNA pretreated with lipofectin (lipofected pcDNA3) was included as a positive control IFN-α inducer.

Regardless of the concentration used, pretreatment of ODNs with lipofectin was necessary to achieve IFN-α production. ODNs PCV-2/4 and PCV-2/5 were the strongest inducers but ODNs PCV-2/2 and PCV-2/3 also induced IFN-α production (Fig. 1). IFN-α induction increased with concentration of the respective ODN and 25 μg ODN ml⁻¹ consistently induced the highest amounts of IFN-α. At this concentration, ODNs PCV-2/4 and PCV-2/5 induced similar levels of IFN-α as lipofected plasmid DNA (pcDNA3: pig 1, 316 U IFN-α ml⁻¹; pig 2, 653 U IFN-α ml⁻¹; pig 3, 386 U IFN-α ml⁻¹; pig 4, 368 U IFN-α ml⁻¹). Although the overall IFN-α-producing capacity varied between individual pigs, their responses to the various inducers showed the same internal relationship. However, of particular significance was that one of the ODNs, PCV-2/1, did not induce IFN-α production by PBMCs from any of the pigs tested (<0.3 U IFN-α ml⁻¹), regardless of pretreatment or not with lipofectin. Consequently, the possible inhibitory activity of ODN PCV-2/1 was studied further.

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

To study whether PCV-2/1 was merely unable to induce IFN-α production or if it also inhibited the IFN-α production induced by the other ODNs selected from the PCV-2 genome, porcine PBMCs were exposed to PCV-2/1 in combination with the other ODNs. Equal amounts of PCV-2/1 (25 μg ml⁻¹) and each of the other four ODNs were incubated together with lipofectin prior to addition to culture. In all cases, the presence of PCV-2/1 counteracted IFN-α production (<0.3 U IFN-α ml⁻¹ culture supernatant).

To study whether the inhibitory effect of ODN PCV-2/1 observed was due to competition between nucleic acid preparations for incorporation into liposomes, the effects of ODN PCV-2/1 were compared to that of salmon sperm DNA. Liposomes consisting of ODN PCV-2/5 (25 μg ml⁻¹) or salmon sperm DNA (50 μg ml⁻¹) alone induced 251 ± 34.4 and 12.2 ± 4.8 U IFN-α ml⁻¹, respectively. Liposomes made up of both PCV-2/5 (25 μg ml⁻¹) and salmon sperm DNA (25 μg ml⁻¹) induced 74.4 ± 10.4 U IFN-α ml⁻¹, whereas no IFN-α (<0.3 U ml⁻¹) was induced by liposomes consisting of ODN PCV-2/5 (25 μg ml⁻¹) in combination with ODN PCV-2/1 (25 μg ml⁻¹). Thus, the decrease in IFN-α production can be explained only in part by a reduced concentration of ODN PCV-2/5 in the liposomes and a specific inhibitory effect of ODN PCV-2/1 is indicated.

In the following experiment, PCV-2/5 and PCV-2/1 were pretreated with lipofectin separately before addition to the cultures. PCV-2/5 induced 363 ± 192 U IFN-α ml⁻¹ in the absence of PCV-2/1. When mixed with an equal concentration of PCV-2/1 (25 μg ml⁻¹), the induction of IFN-α by PCV-2/5 was decreased to 13 ± 6 U IFN-α ml⁻¹ and induction was abolished totally (<0.3 U IFN-α ml⁻¹ culture supernatant) when PCV-2/5 was tested in combination with PCV-2/1 at a threefold higher concentration.
Thus, no obvious difference between individual or combined incubation with lipofectin was observed and, in subsequent experiments, inducers needing pretreatment with lipofectin were incubated separately.

**PCV-2/1 partially inhibits IFN-α induction by some nonrelated ODNs**

To study further the inhibitory capacity of PCV-2/1, three additional ODNs with the ability to induce IFN-α production in the absence of lipofectin (Domeika, 2003) were synthesized. These ODNs consist of a central Cpg motif and G repeats in their 5’ and 3’ ends (phosphodiester ODN D25 or the phosphodiester/phosphorothioate chimeras ODN D19 and 2216, Table 1). According to previous results, ODNs D19 and D25 were used at the concentration 25 μg ml⁻¹, and ODN 2216 at 5 μg ml⁻¹, concentrations that are optimal for IFN-α induction in porcine PBMCs (Domeika, 2003). The IFN-α induction of these ODNs following cocultivation with PCV-2/1 (25 and 75 μg ml⁻¹) in the absence of lipofectin is shown as percentage of these values (Fig. 2).

In the absence of PCV-2/1, D25 induced 478 ± 144 U IFN-α...

**Fig. 1.** IFN-α-inducing capacity of ODNs selected from the PCV-2 genome (PCV-2/2 to -2/5). ODNs were 20 nt long and tested at concentrations of 5, 10 and 25 μg ml⁻¹. PBMCs from the same four pigs were used for all ODNs and all samples were pretreated with lipofectin. Results are expressed as U IFN-α ml⁻¹ for pig 1 (□), pig 2 (○), pig 3 (●) and pig 4 (△).

**Fig. 2.** Effect of PCV-2/1 on the induction of IFN-α by various ODN constructs. ODNs H, I, H-I and H Poly-G were pretreated with lipofectin, while D25, D19 and 2216 were not. PCV-2/1 was used at two concentrations, 25 μg ml⁻¹ (white bars) and 75 μg ml⁻¹ (grey bars). ODN PCV-2/1 was pretreated with lipofectin when used in combination with ODNs H, I, H-I and H Poly-G but was used neat in combination with ODNs D25, D19 and 2216. Results are expressed as percentage induction of IFN-α by the ODN in the absence of PCV-2/1 (control) (mean value ± SEM, n = 4). See Table 1 for a description of the ODNs.
PCV-2/1 inhibits ODNs that require pretreatment with lipofectin

ODN H and its antisense counterpart ODN I (Table 1) have been used previously as efficient IFN-γ inducers in both ss and ds forms, provided they are pretreated with lipofectin (Magnusson et al., 2001b). Modification of ODN H by addition of a poly(G) sequence at the 3' end increases its IFN-γ-inducing capacity but does not circumvent the need for lipofectin (Domeika, 2003). To study the influence of poly(G) sequences on the inhibitory effect of PCV-2/1, these lipofected ODNs were used to induce IFN-γ in porcine PBMCs alone or in combination with lipofected PCV-2/1 (Fig. 2).

In the absence of PCV-2/1, ODN H induced a mean value of 398 ± 150 U IFN-γ ml⁻¹; ODN I induced 313 ± 126 U IFN-γ ml⁻¹ and the ds form H–I induced 716 ± 248 U IFN-γ ml⁻¹. When any of these three ODNs were pretreated separately with lipofectin and mixed with PCV-2/1 at equal concentrations (25 µg ml⁻¹), induction of IFN-γ was abolished totally. ODN Hpoly(G) (25 µg ml⁻¹) induced a mean value of 1124 ± 254 U IFN-γ ml⁻¹ in the absence of PCV-2/1. After pretreatment with lipofectin, it was mixed with PCV-2/1 at two different concentrations (25 and 75 µg ml⁻¹). At the lower concentration, IFN-γ production was reduced and at the higher concentration it was abolished totally. Thus, PCV-2/1 seemed to efficiently inhibit ss as well as ds ODNs that required pretreatment with lipofectin to induce IFN-γ. PCV-2/1 could also inhibit ODN H after modification by addition of a poly(G) sequence to its 3’ end.

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

Viruses are well known inducers of IFN-γ and the effect of PCV-2/1 was, therefore, studied using two different virus preparations: inactivated ADV and live SV. For comparison, lipofected pcDNA3 was used as a DNA IFN-γ-inducing positive control. As in the previous experiments, PCV-2/1 was tested at two different concentrations (Fig. 3). In the absence of PCV-2/1, SV induced mean values of 109 ± 28 U IFN-γ ml⁻¹, ADV induced 390 ± 149 U IFN-γ ml⁻¹ and pcDNA3 induced 952 ± 245 U IFN-γ ml⁻¹. When mixed with live SV, no consistent effect of PCV-2/1 on the levels of IFN-γ produced was observed. In contrast, however, IFN-γ production induced by ADV was inhibited clearly by PCV-2/1 at both concentrations. PCV-2/1 in combination with pcDNA3 resulted in a partial inhibition of IFN-γ production induced by the plasmid, with the higher concentration of PCV-2/1 being most effective. Since pcDNA3 does not induce IFN-γ production in the absence of lipofectin, only lipofected samples were tested. Pretreatment of the virus inducers with lipofectin did not influence the inhibitory effect of PCV-2/1 (data not shown).

PCV-2/1 in a ds form is also inhibitory

To be able to assess the inhibitory activity of the ds form of PCV-2/1, the complementary strand to PCV-2/1 (PCV-2/1 C) was synthesized and the two ss ODNs were hybridized to form a ds form (PCV-2/1 ds). Both ss forms as well as the ds form were tested at 25 µg ml⁻¹ against ADV, SV and pcDNA3. The results using the ss complementary strand, PCV-2/1 C, or PCV-2/1 ds were similar to those obtained with ss PCV-2/1. No inhibition of SV-, a clear inhibition of ADV- and a partial inhibition of pcDNA3-induced IFN-γ production were observed (Fig. 4). Although a great individual variation was observed, inhibition by the complementary strand was equal to inhibition by ODN PCV-2/1.

In summary, these in vitro results indicate that parts of the PCV-2 genome have the capacity to modulate the IFN-γ response induced by some CpG-S ODNs and by some other viruses or bacterial DNA.

DISCUSSION

Using the criteria suggested by Krieg et al. (1998), examination of the genome of PCV-2 revealed a total of
Four of the ODNs induced IFN-α production at levels similar to that induced by plasmid DNA. Thus, one of the presumed inhibitory ODNs (PCV-2/2) did induce IFN-α production despite the presence of two inhibitory motifs, CGG, spaced by 3 nt. This unexpected capacity could be due to the positioning of the motifs in relation to each other, to the flanking nucleotides and the lengths of the entire ODN. Alternatively, this CGG motif might simply not be an inhibitory motif for IFN-α induction in pigs. The fifth ODN (PCV-2/1), which was selected due to its content of CG repeats did not induce detectable levels of IFN-α and, of particular significance, was demonstrated to inhibit IFN-α production induced by the other ODNs representing parts of the PCV-2 genome. The inhibitory activity of PCV-2/1 was demonstrated further against two unrelated ODNs (H and its complementary strand I), which lost their IFN-α-inducing capacity in the presence of PCV-2/1. Thus, PCV-2/1 inhibited almost completely the IFN-α induction by phosphodiester ODNs, in both their ss and their ds forms.

In a previous study on IFN-α induction by plasmid DNA, lipofected salmon sperm DNA was used as a ‘neutral’ control DNA preparation that was shown not to induce IFN-α production in porcine cells (Magnusson et al., 2001a). Liposomes formed with equal amounts of salmon sperm DNA and ODN PCV-2/5 were less efficient inducers of IFN-α than liposomes containing only ODN PCV-2/5, but the presence of salmon sperm DNA in the liposomes did not inhibit IFN-α-inducing capacity, as demonstrated in the presence of ODN PCV-2/1. Thus, the inhibitory effect of ODN PCV-2/1 could not be explained by competition between inert and stimulatory DNA in the liposomes. It is notable that the inhibitory motif identified was also inhibitory as a ds form, representing the replicative form of the viral DNA. Indeed, the complementary sequence (PCV-2/1 C) showed a similar, or even higher, inhibitory capacity. However, further studies are needed to compare and evaluate the inhibitory effects of ds and ss forms of the ODNs.

Most CpG phosphodiester ODNs need preincubation with lipofectin to induce IFN-α in cultures of human and porcine PBMCs (Magnusson et al., 2001b). In addition, to protect against nucleases, lipofectin also facilitates the cellular uptake of incorporated molecules (Xu & Szoka, 1996). Exchange of phosphodiester to phosphorothioate nucleotides increases resistance to nucleases (Stein et al., 1988) and two ODNs with a chimeric backbone (ODN D19 and ODN 2216) both induced IFN-α efficiently without preincubation with lipofectin. Of particular significance, ODN PCV-2/1 was unable to inhibit IFN-α production induced by ODN D19 but reduced ODN 2216-induced levels of IFN-α by 50 % or more, both in the presence and in the absence of lipofectin. The discrepancy in inhibitory effect on ODN 2216 and D19 could be explained by the fact that the two ODNs have different concentration optima for IFN-α induction (Domeika, 2003) and ODN 2216 was used at a fivefold lower concentration (5 µg ml⁻¹) than ODN D19 (25 µg ml⁻¹). Nevertheless, these results indicate that PCV-2/1 activity is not dependent on lipofectin to exert its inhibitory effect. IFN-α induction by ODN D25, which contains the same base sequence as ODN D19 but is constructed with a phosphodiester backbone, was only inhibited partially by PCV-2/1. This ODN is known to activate the genes for IL-6, IL-12 and TNF-α in porcine PBMCs (Kamstrup et al., 2001) and contains a poly(G) sequence, which, in other species, has been demonstrated to mediate uptake via scavenger receptors (Dalpke et al., 2002; Peiser et al., 2002). However, the addition of a poly(G) sequence to ODN H did not substitute the need for pretreatment with lipofectin to induce IFN-α production by this ODN and reduced only marginally the inhibitory effect of PCV-2/1. Thus, no single ODN characteristic could

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Image: Fig. 4. Effect of ds and ss forms of PCV-2/1 on IFN-α induction by SV, ADV and pcDNA3. pcDNA3 was pretreated with lipofectin, while SV and ADV were not. PCV-2/1 (white bars), PCV-2/1 C (grey bars) and PCV-2/1 ds (black bars) were used at a concentration of 25 µg ml⁻¹. ODN PCV-2/1 was pretreated with lipofectin when used in combination with pcDNA3 but was used neat in combination with ADV and SV. Results are expressed as percentage induction of IFN-α by the inducer in the absence of PCV-2/1 (control) (mean value ± SEM, n = 4).
explain to what level PCV-2/1 inhibited the IFN-\(\alpha\) induction by other ODNs. General modifications of the ODNs, such as addition of a poly(G) sequence or conversion to a phosphorothioate backbone, are known to increase the immune stimulatory activity of the ODN even in the absence of a CpG motif (Hartmann et al., 1996; Krieg, 2002), seemed, however, to counteract the inhibitory effect of PCV-2/1.

One possible explanation to the variable effect of PCV-2/1 could be that particular cell types are activated to produce IFN-\(\alpha\) by the different ODNs. In human PBMCs, two populations of IFN-\(\alpha/\beta\)-producing cells are recognized: monocytes and cells of plasmacytoid dendritic cell (PDC) origin, the natural IFN-producing cells (NIPCs) (Colonna et al., 2002). In the pig, cells with many characteristics similar to NIPCs have been demonstrated after induction with transmissible gastroenteritis virus (TGEV) or ADV (Artursson et al., 1992; Nowacki et al., 1993; Nowacki & Charley, 1993). When human PBMCs are exposed to SV, monocytes in addition to NIPCs produce IFN-\(\alpha\), whereas only NIPCs respond to herpes simplex virus, pcDNA3 (Vallin et al., 1999) or CpG-ODN (Magnusson et al., 2001b). In particular, ODN 2216 is a potent inducer of IFN-\(\alpha\) that activates PDC/NIPC cells selectively (Krug et al., 2001). In support of this hypothesis, the induction of IFN-\(\alpha\) by SV was not affected by PCV-2/1, while the induction by ADV was reduced substantially. In addition, the production of IFN-\(\alpha\) induced by plasmid DNA (pcDNA3) was reduced clearly upon addition of PCV-2/1. Current phenotyping of porcine cells suggests that cells responding with IFN-\(\alpha\) production at exposure to TGEV (A. Summerfield, Institute of Virology and Immunopathology, Mittelhäusern, Switzerland, personal communication) as well as to ODN 2216 and pcDNA3 (K. Domeika, M. Magnusson, M.-L. Elorenta, L. Fuxler, G. V. Alm and C. Fossum, unpublished results) constitute a subset of dendritic cells with great similarities to human NIPCs. In contrast, porcine monocyte-derived dendritic cells produce IFN-\(\alpha\) following exposure to SV but are nonresponsive to ADV and plasmid DNA (Johansson et al., 2003). Thus, the inhibitory activity of PCV-2/1 seems to be more pronounced for IFN-\(\alpha\) production by PDCs than by monocytes.

In addition to its direct antiviral effect, IFN-\(\alpha\) contributes to the specific immune response to virus infections (Le Bon & Tough, 2002). Therefore, PDCs comprise a potentially important strategic target for virus evasion. To date, the site of replication of PCV-2 is unknown but the virus accumulates in the cytoplasm of dendritic cells and macrophages in the absence of any evidence of active virus replication (Allan & Ellis, 2000). In this study, the demonstration of IFN-\(\alpha\)-inducing as well as inhibitory motifs in the genome of PCV-2 could, therefore, contribute to the understanding of the pathogenesis of PCV-2-associated syndromes, such as PMWS.

In general, viral DNA appears to have evolved in the direction towards either lower CpG content or a ratio between CpG-S and CpG-N that is biased to substantially more CpG-N motifs (Karlin et al., 1994; Krieg et al., 1998; Sun et al., 1997). Thus, the evolution of some viruses seems to aim at avoidance of immune activation via CpG. One such example is adenovirus type 2, which has substantially more CpG-N motifs than adenovirus type 12. This renders its genome inert or even inhibitory for cytokine induction and has been suggested to contribute to the establishment of a persistent adenovirus infection (Krieg et al., 1998). Interestingly, another member of the family Circoviridae, chicken anaemia virus (CAV), causes severe immunosuppression in young chickens (Rosenberger & Cloud, 1998). As in the case of PCV-2, the pathogenesis of CAV is not understood fully but the virus has been shown to interfere with the transcription of IFN-\(\alpha\) and IFN-\(\gamma\) 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, therefore, play a role in the pathogenesis of CAV and could possibly explain the immunosuppressive effect of both of these viruses. The mechanisms of this action are not understood yet and further studies are needed to evaluate this hypothesis.

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