Immunostimulatory DNA – studies on the importance of secondary structure formation and CpG-motifs for IFN-α induction/inhibition using ODNs related to the genome of porcine circovirus type 2

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Abstract

Nucleotide sequences containing CpG-motifs are recognized as immunomodulators in pigs among other species. Phosphodiester oligodeoxynucleotides (ODNs), such as ODN H, have been demonstrated to be potent inducers of interferon-alpha (INF-α) in porcine blood mononuclear cells (poPBMC) provided they are pre-treated with Lipofectin®. In the present study the IFN-α inducing activity of ODN H was demonstrated to be altered after addition of poly-G sequences at the 5' and/or 3' end and by increasing the number of nucleotides (nt) in the base-pairing sequence of the ODN. Alterations that facilitated the formation of secondary structures reduced the need for pre-treatment with Lipofectin®. Furthermore, deliberate destruction of secondary structures by heat-treatment of ODN 2216 reduced the IFN-α inducing capacity of that ODN. Thus, results suggest that the stimulatory activity of the ODNs is dependent on secondary structure formation. Other ODNs are recognized as potent inhibitors of the INF-α production in poPBMC. The ODN PCV2/1 corresponding to a 20 nt long sequence from the genome of PCV2, has been identified as such an inhibitory ODN. Further, it was demonstrated that the inhibitory capacity of ODN PCV2/1 not depend on the presence of a central CpG-motif. Further, results from the *in vitro* studies show a significant difference between ODN PCV2/1 found in the genome of PCV2 from healthy pigs in Sweden and ODN PCV2/1S1, which predominates in pigs from farms with PMWS in Sweden. However, the biological significance of these findings remain to be elucidated.

Sammanfattning

Nukleotidsekvenser i mikrobiellt DNA som innehåller CpG-motiv kan fungera som immumodulatorer hos bl.a. gris. För att efterlikna detta *in vitro* kan man använda sig av oligodeoxynukleotider (ODN). Vissa fosfodiester ODN, såsom ODN H, har visat sig kunna aktivera vita blodkroppar så att de producerar interferon-alfa (IFN-α) förutsatt att oligodeoxynukleotiderna förbehandlats med Lipofektin®. Denna studie visar att den IFN-α inducerande förmågan hos ODN H kan förändras genom tillsats av poly-G sekvenser till 5' och/eller 3' änden samt genom en ökning av antalet nukleotider i den basparande sträckan. För att undersöka hur den stimulatoriska aktiviteten påverkas av denaturering utfördes studier på ODN 2216, som har förmågan att baspara med sig själv. Resultaten visade på en minskning av den IFN-α inducerande aktiviteten efter denaturering vilket tyder på att den stimulatoriska aktiviteten hos en ODN är beroende av formation av sekundärstrukturer. Andra ODN har visat sig kunna inhiberar IFN-α produktionen från vita blodkroppar, till dessa hör ODN PCV2/1 som motsvarar en 20 nt lång sekvens som återfinns i genomet hos porcint circovirus typ 2 (PCV2) hos friska grisar i Sverige. *In vitro s*tudien visar att det centrala CpG-motivet inte är nödvändigt för den inhiberande förmågan hos ODN PCV2/1. Studien visar dock på en signifikant skillnad mellan den IFN-α inhiberande kapaciten hos ODN PCV2/1 och ODN PCV2/1S1, som dominerar bland grisar från svenska besättningar med PMWS och saknar det centrala CpG-motivet. Det kvarstår dock att klarlägga den bilogiska relevansen av dessa fynd.

Introduction

Porcine circoviruses (PCV) are small, nonenveloped viruses (Allan *et al.,* 2000) that were first identified as a contaminant of the continuous pig kidney cell line PK/15 in 1974 (Tischer *et al.,* 1974). The virus (PCV PK/15) was shown to have a single-stranded, circular DNA genome of 1.76 kb and was classified into the animal virus family, *Circoviridae*. The viron DNA has four conserved open reading frames (ORFs) and is encapsulated by a single viral protein, coded for by ORF 2 (Meehan *et al.,* 1998; Mankertz *et al.,* 2004).

Today two genotypes of PCV have been isolated from pigs, PCV1 that corresponds to the circovirus first described in PK15 cells (Tischer *et al.,* 1974), and PCV2 (Meehan *et al.,* 1998). Isolates of PCV2 are highly homologous (> 96%) while the homology between PCV1 and PCV2 isolates is roughly 62% (Allan *et al.,* 1999b). PCV1 does not produce clinical disease at experimental infection in pigs and is considered apathogenic (Krakowka *et al.,* 2000). PCV2 on the other hand is now recognised as a porcine pathogen and is generally accepted as the causal agent of postweaning multisystemic wasting syndrome (PMWS). The clinical signs of PMWS include wasting or unthriftiness, enlarged lymph nodes, dyspnea, diarrhea and pallor or icterus (Ellis *et al.,* 1998). At necropsy, lesions in liver, kidneys and lymph nodes are common findings and a criteria for the diagnose PMWS is that PCV2 is demonstrated in the leisons (Allan *et al.,* 1998; Ellis *et al.,* 1998; Kennedy *et al.,* 2000; Krakowka *et al.,* 2000, 2001).

Although PCV2 is the infectious agent that always is present at PMWS, also other factors i.e. other infectious agents or nonspecific immune modulators appear to be essential for full development of clinical disease (Allan *et al.,* 1999a; Kennedy *et al.,* 2000; Krakowka *et al.,* 2001). PMWS was first recognised in a Canadian herd in 1991 and has since then been described in several countries within and outside Europe. PMWS was reported for the first time in Sweden in December 2003 and is now spreading rather slowly through Swedish pig herds. A pilot study in Sweden has shown that 96% of randomly selected porcine sera contained antibodies to PCV2 (Linné *et al.,* 2000), which confirms that the virus infection is widespread among Swedish pigs. PCV2 has also been associated with several other severe diseases in pigs such as porcine dermatitis and nephropathy syndrome (PDNS) (Allan *et al.,* 2000) and during the last years four cases of PDNS have occurred in Sweden.

The majority of pigs are however persistently infected with PCV2 without showing any signs of clinical disease, and in vitro studies have identified cells of the monocyte/macrophage lineage, including dendritic cells, as the primary targets for PCV2 infection (Gilpin *et al.,* 2003; Vincent *et al.,* 2003). Although large amounts of infectious virus can accumulate in these cells the virus does not seem to be replicating (Gilpin *et al.,* 2003). The fact that PCV2 lacks its own DNA polymerase forces the virus to depend on the host cell for its replication. Thus, cells that regularly undergo division and therefore have an active polymerase are most likely to be favourable for replication of the virus. Nevertheless, monocytes/macrophages and histiocytes, sometimes in the form of multinucleated giant cells, are the predominant cell types that accumulate in the lymph nodes when these become depleted of lymphocytes at the late stages of PMWS (Rosell *et al.,* 1999, Segalés *et al.,* 2004). It is therefore intriguing to speculate that PCV2 in one way or another modulates the normal activity of the cells that harbours the virus. In that context it is notable that PCV2 antigen also seems to be acquired by the subpopulation of dendritic cells, referred to as natural interferon producing cells.

Natural interferon producing cells (NIPCs) represent 0.2%-0.8% of peripheral blood mononuclear cells in human and mice. These cells are specialised in rapid (within 24 h) secretion of massive amounts of type I IFNs (IFN-α and -β) following a viral infection. NIPC produce 100-1000 times more type I IFN than for instance monocytes/macrophages following exposure to Herpes simplex virus (Liu, 2005). A corresponding celltype that respond with IFN-α production at exposure to a porcine herpes virus, Aujeszky's Disease Virus (ADV) has been identified in the pig (Artursson *et al.,* 1995). This porcine NIPC constitute approximately 0.3% of poPBMC and as its human counterpart it also responds with IFN-α production at exposure to DNA containing immunostimulating sequences (Domeika *et al.,* 2004, Guzylack-Piriou *et al.,* 2004) The immunostimulatory effect of e.g. bacterial DNA is ascribed to the relatively high content of unmethylated CpG dinucelotides in certain base contexts (Sato *et al.,* 1996; Krieg, 2002). To characterise immunostimulatory DNA motifs (IS-DNA), plasmid DNA (Nelson *et al.,* 1999; for review see Pisetsky, 1996) or synthetic oligodeoxynucleotides (ODNs) have been extensively used (for review see Agrawal *et al.,* 2002).

Plasmid DNA and ODN 2216 are both believed to interact via their CpG motifs with Toll-like receptor 9 (TLR 9). This interaction results in an induction of cytokine production, proliferation and/or immunoglobulin secretion and an enhanced NK-cell activity (Sato *et al.,* 1996; Krieg, 2002). The importance of TLR 9 for CpG DNA-mediated effects is supported by studies on TLR 9-deficient ($TLR9^{-/-}$) mice, that are resistant to the lethal effect of DNA containing unmethylated CpG dinucleotides (Hemmi *et al.*, 2000). It has also been suggested that both stimulatory and inhibitory CpG-motifs exist and act on the same cells (Yamada *et al*., 2002). Analysis of the PCV2 genome in search for potentially immunoregulatory sequences revealed a nucleotide sequence (PCV2/1) that inhibited the IFN-α production induced by stimulatory ODNs selected from the PCV2-genome and by some other well-known IFN inducers i.e. ADV, pcDNA3 and ODN 2216, respectively (Hasslung *et al.,* 2003). Comparison of the sequence coding for ORF 2 of PCV2 in material obtained from pigs raised in healthy and diseased farms in Sweden have shown that the corresponding sequence appears in two different variants, here designated PCV2/1 and PCV2/1S1. PCV2/1 has only been found in samples from healthy pigs whereas PCV2/1S1 predominates in pigs from farms with PMWS.

The present study was undertaken to further test the importance of CpG motifs, and formation of secondary structure for the IFN-α inducing capacity of short DNA sequences using poBMC. Further, the effect of natural occurring alterations in the regions of the PCV2 genome containing the IFN-inhibitory sequence was tested in vitro.

Materials and methods

Purification of poPBMC and induction of IFN-α

Conventionally reared pigs housed at the Research Station Funbo, Lövsta, Uppsala, Sweden and specific pathogen free (SPF) pigs housed at Hagalund, Sala, Sweden were used. Serum samples from all pigs were tested and found negative for presence of IFN-α. Peripheral blood mononuclear cells (PBMC) were purified from heparinized blood by Ficoll-Paque (Amersham Biosciences AB, Uppsala, Sweden) density gradient centrifugation. The PBMC were 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

U/ml), streptomycin (100 μg/ml), 2-mercaptoethanol ($5x10^{-5}$M), with 10% foetal calf serum (FCS, Myoclone®, Invitrogen Life Technologies). The cells were seeded in flat-bottomed 96 well plates (F96 Cert, Nunc, Roskilde, Denmark) or in tissue culture flasks (Nunclon^{TM}Sureface, Nunc, Roskilde, Denmark). For induction of IFN-α production various ODNs as specified below were added in triplicate cultures with $5x10^6$ poPBMC per ml in a total volume of 0.2 ml. After 20h incubation at 37^{o}C, 7% CO_2 in air, culture supernatants were pooled and stored at -20^{o}C until further analysis. As a control, lipofected plasmid DNA (pcDNA3; Invitrogen, San Diego, CA) was used at a final concentration of 2.5 μg per ml.

ODNs

Ten oligodeoxynucleotides (ODNs) were purchased from Cybergene AB (Stockholm, Sweden), desalted and dissolved in water. The ODNs were synthesised in various scales and delivered at stock concentrations given in Table 1. The nucleotide sequences of the ODNs are given in Table 1 and their theoretical ability to form double strands (IDT Scitools, Oligo Analyzer 3.0) is depicted in Figure 1.

Table 1. Sequence of ODNs used for inhibition/induction of IFN-α in poPBMC and stock concentration for each ODN

ODN	Sequence (5´→ 3´)[a]	Stock conc. (μg/ml)[b]	Final conc. (μg/ml)[c]
PCV2/1	CCC CCC TCC CGG GGG AAC AA	2430;2171;1907	25
PCV2/1S	GCC CCC TCC GGG GGG AAG AA	2148	25
PCV2/1S1	GCC CCC TCC TGG GGG AAG AA	2422	25
HB1G5’	GGG GGG TAT TTC GAA ATA GG	2574	25
HB1G3’	GGT ATT TCG AAA TAG GGG GG	2716;640;634	25
HB1G5’3’	GGG GGG TAT TTC GAA ATA GGG GGG	1980	25
HB4G5’	GGG GGG TTC GAA GG	2960	25
HB4G3’	GGT TCG AAG GGG GG	3039	25
HB4G5’3’	GGG GGG TTC GAA GGG GGG	2317	25
2216	ggG GGA CGA TCG TCg ggg gG	4171; 3268	5

[a] All ODNs are built up by a phosphodiester backbone except from the chimeric ODN 2216 which has phosphorotioate backbone at nucleotides in lower case. Underlined sequences correspond to CpG-motifs. [b] Stock concentration, the concentration that the ODNs was delivered at. Several different stocks of ODNs PCV2/1, HB1G3’ and 2216 were used. [c] Final concentration used per ml culture medium

When indicated, one of the ODNs (2216) was diluted to a final concentration of 5 μg per ml growth medium and denatured by heating to approximately 100 °C for 5 min in a heating block followed by rapid cooling of the sample on ice water. Denaturation was estimated by measurement of the A260 values by spectrofotometric analysis (Nanodrop® ND-1000 Spectrophotometer) prior to and after heat treatment of the ODN. All ODNs were tested for presence of endotoxin using the Limulus Amebocyte Lysate (LAL) QCL-1000® test kit (Cambrex Bio Science Walkersville, Inc, Walkersville) and were found to contain less than 0.2 EU per ml.

HB1G5'
dG: -19.41 kcal/mole

```
5'-GGGGGGTATTTCGAAATAGG-3'
         ||||||||||||
    3'-GGATAAAGCTTTATGGGGGG-5'
```

HB1G3'
dG: -19.41 kcal/mole

```
    5'-GGTATTTCGAAATAGGGGGG-3'
         ||||||||||||
3'-GGGGGGATAAAGCTTTATGG-5'
```

HB1G5'3'
dG: -19.41 kcal/mole

```
5'-GGGGGGTATTTCGAAATAGGGGGG-3'
         ||||||||||||
3'-GGGGGGATAAAGCTTTATGGGGGG-5'
```

HB4G5'
dG: -10,65 kcal/mole

```
5'-GGGGGGTTCGAAGG-3'
         ||||||
    3'-GGAAGCTTGGGGGG-5'
```

HB4G3'
dG: -10,65 kcal/mole

```
    5' GGTTCGAAGGGGGG 3'
         ||||||
3' GGGGGGAAGCTTGG 5'
```

HB4G5'3'
dG: -10,65 kcal/mole

```
5'-GGGGGGTTCGAAGGGGGG-3'
         ||||||
3'-GGGGGGAAGCTTGGGGGG-5'
```

2216
dG: -17.68 kcal/mole

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  5'-ggGGGACGATCGTCgggggG-3'
         ||||||||||
3'-GgggggCTGCTAGCAGGGgg-5'
```

Figure 1. Predicted self-dimer structures of ODNs HB1G5', HB1G3', HB1G5'3', HB4G5', HB4G3', HB4G5'3' and 2216, showing the self-dimer formation with the lowest dG value. The predicted secondary structures were determined using the IDT SciTools Oligo Analyzer 3.0 software (http://www.idtdna.com/SciTools.sapx).

Pre-incubation with Lipofectin®

The effect of pre-treatment of the ODNs with Lipofectin® was tested as previously described (Magnusson *et al.*, 2001a). In brief, the inducers were diluted in growth medium and when indicated incubated with

Lipofectin® (Lipofectin® Reagent, Invitrogen Life Technologies, Carlsbad, CA) for 15min before addition of PBMC to the wells. When PBMC were co-cultured with two inducers these were incubated with Lipofectin® together before addition of cells to the cultures. When present, the final concentration of Lipofectin® was in all cases 2.5 μg per ml.

Detection of poIFN-α

For quantification of INF-α a dissociation-enchanced lanthanide fluoroimmunoassay (DELFIA), which is based on two mAbs (F17 and K9-Eu) directed to poIFN-α (Artursson *et al.*, 1995) was used. The IFN-α concentration was determined by comparison with a laboratory standard consisting of natural poINF-α. Results from the experiments are given as percentage of IFN-α induction by ODN 2216 or lipofected pcDNA3.

Isolation of RNA and cytokine PCR

To study the expression of cytokines genes, RNA was isolated from cells by Trizol extraction following the manual from the manufacturer (Invitrogen, Carlsbad, CA, USA). The sample RNA was resuspended in 25μl diethylpyrocarbonate treated water (DEPC H_20) and the quantity and purity (OD ratio 260/280 nm) of RNA were determined by spectrophotometric analysis (NanoDrop® ND-1000 Spectro-photometer). Quality of the RNA was tested by agarose gel electrophoresis on a 1% TAE-agarose gel before using the RNA for cDNA production.

Complementary DNA (cDNA) was produced from the sample RNA by Reverse Transcriptase PCR (RT-PCR) according to the protocol from Invitrogen (Stockholm, Sweden) with minor modifications. In short, 1 μg of sample RNA was mixed with 4μl 10mM deoxynucleotide triphosphate-mix (dNTP)(TaKaRa Bio Inc., Shiga, Japan), DEPC H_2O and 2μl of a specially designed oligo (dT) primer (530 μg/ml). The mixture was incubated at 65 ^oC for 5 min and cooled for 2 min at 1 ^oC. During cooling of the mixture a second mixture containing 8μl 5 x first strand buffer, 4μl 0.1 M DTT and 2μl RNAse Out (Invitrogen, Carlsbad, CA, USA) was added. After 2 min incubation at 42^oC, to allow primer binding, Superscript II reverse transcriptase was added and the samples were incubated for 50 min at 42 ^oC. The enzymes were

deactivated by heating for 5 min at 75 ^{o}C and the samples were then quickly chilled to 4^{o}C. To control for detection of genomic DNA in the analysis of IFN-α and IFN-β, which lack introns, a mixture without the Superscript II reverse transcriptase was run in parallel to those with the enzyme included. All the products were stored at -20 ^{o}C until further use.

PCR amplifications of expressed cytokine genes were performed with primers designed using published porcine nucleic acid sequences selected from GenBank (Table 2). To minimise the risk of amplifying genomic DNA, the primers (Technology A/S, Aarhus, Denmark) were chosen to be located on different exons of the gene, with the exceptions of IFN-α and IFN-β, which lacks introns (Andersson M. *et al.,* 2006, Wattrang E. *et al.,* 2005, Zhu Z. *et al.,* 2006). PCR products were synthesized for the following cytokines: IL-1β, IL-6, IL-8, IL-10, IL-12 p35, IL-12 p40, IL-18, IFN-α, IFN-β, IFN-γ, TNF-α, TGF-β, MIF and GM-CSF. GAPDH and HPRT were used as housekeeping genes. The amplification of cDNAs was performed in two steps for the cytokines IFN-α, IFN-β, TNF-α and in one step for the rest of the cytokines and for the two housekeeping genes. The PCR amplification with two steps was in the case with IFN-α and IFN-β carried out using a specific forward primer (No. 3) and a common back primer (p23A) while in the case of TNF-α specific forward (Fw1) and specific backward (Bw1) primers were used. In the second step primers 1 and 2 were used together at a concentration of 10 pmol/μl. The samples were heated to 95 ^{o}C followed by a cycle consisting of denaturation for 30 s at 95 ^{o}C, annealing for 30 s and extension for 30 s at 72 ^{o}C with a final extension for 5 min at 72 ^{o}C. Annealing temperature and number of cycles used in the different amplifications are given in Table 3. The PCR products were analysed by agarose gel electrophoresis on a 1% TAE-agarose gel.

Table 2. Oligodeoxynucleotide primers used to amplify porcine cytokine and house keeping genes. Primers designated 1 and 2 were used in the single step PCR amplification as well as in the second step of the two step PCR amplification. In the multi-PCR primers designated 3 and p23A were used for amplification of IFN-α and IFN-β and primers designated Fw1 and Bw1 were used for amplification of TNF-α

Gene	PCR product (bp)	Oligonucleotide sequences (5'-3')	GenBank acces. no.
IL-1β[1]	378	(1) TGG ACC TTG GTT CTC TGA GA (2) CAT CTC CTT GCA CAA AGC TC	M86725
IL-4[1]	296	(1) TGA ACA TTC TCA CAG CGA GA (2) TCA GCT TCA ACA CTT TGA GT	L12991
IL-6[1]	666	(1) ATG AAC TCC CTC TCC ACA AG (2) CTA CAT TAT CCG AAT GGC CC	M80258
IL-8[1]	304	(1) GAC TTC CAA ACT GGC TGT TG (2) GCT GTT GTT GTT GCT TCT CA	AB057440
IL-10[1]	345	(1) GGC CCA GTG AAG AGT TTC TT (2) GAT GTC AAA CTC ACC CAT GG	L20001
IL-12 p35[1]	502	(1) CGT CAG CAA CAC ACT TCA GA (2) GCT CAT CAT TCT GTC GAT GG	L35765
IL-12p 40[1]	529	(1) TCC TGG TTT TCC CTG GTT TG (2) ACT TCT TAT ACT CCC CGA GG	U08317
IL-18[1]	323	(1) ACT TTG GCA AGC TTG AAC CT (2) TCC AGG AAC ACT TCT CTG AA	U68701
IFN-α[1]	303	(1) CAG GAA GAA GGA GTG ACA GA (2) TAG GCA GCA AGG GCA TCT AT (3) CAG AAA CCT GCA AGA CAG AC	M28623
IFN-β[1]	291	(1) TGG ATG ACC TGG AGA CAA TC (2) AAA GAG CTT CCC CTG CTT GA (3) CTT GTG GAA CTT GAT GGG CA	M86762
IFN-γ[1]	392	(1) GAC ATG TTT CAG AGG TTC CT (2) AGT CAC AGG ATA CAG GAA TC	AY188090
TNF-α[2]	286	(Fw1) ACT GAG AGC ATG ATC CGA GA (Bw1) CGG CTT TGA CAT TGG CTA CA	X57321
	156	(1) GTG CCT CAG CCT CTT CTC CT (2) GTT TGA GAC GAT GAT CTG AG	
TGF-β[4]	399	(1) GAG CCA GGA CCT TGC TGT AC (2) GAC CCG CAG AGA GGC TAT AG	Y00111
MIF[1]	251	(1) TGG TGC AGG CCA TGG GC A AG (2) GTT CCA GCC CAC ATT GGC CG	AF176246
GM-CSF[1]	301	(1) CAA GCA CTA TGA GCA GCA CT (2) GCC TGT ATC AGG GTC AAC AT	AY116504
GAPDH[1]	520	(1) ATG GTG AAG GTC GGA GTG AA (2) TCA TGA GTC CTT CCA CGA TG	AF017079
HPRT[1]	365	(1) TGC CGA GGA TTT GGA AAA GG (2) CCA AGG AAA GCA AGG TTT GC	AF143818
cDNA-primer[3]		CCT GAC CCA ACC AGT AGA CCA TTT TTT TTT TTT TTT TTT TN	
p23A		TGA CCC AAC CAG TAG ACC A (Berg *et al.*, 1999)	

[1] From Andersson *et al.*, 2006, [2] From Zhu *et al.*, 2006,[3] Verfaillie *et al.*, 2005, [4] From Wattrang *et al.*, 2005

Table 3. Annealing temperature and number of cycles used in the PCR reactions

	Nested PCR		Multi PCR	
Cytokine	Annealing temperature (^oC)	No. of cycles	Annealing temperature (^oC)	No. of cycles
IL-1β	58	30		
IL-4	55	30		
IL-6	55	25		
IL-8	60	26		
IL-10	60	30		
IL-12 p35	55	35		
IL-12 p40	60	35		
IL-18	58	35		
IFN-α	60	20	55	35
IFN-β	60	20	60	35
IFN-γ	55	35		
TNF-α	60	20	60	30
TGF-β	60	35		
MIF	65	35		
GM-CSF	55	30		
GAPDH	55	30		
HPRT	55	30		

Data and statistical analysis

All data are expressed as mean values ± SEM. Statistically significant differences, in IFN-α induction (units per ml) by various ODNs were determined using the Wilcoxon rank-sum test (Stat view 512, version 1.01, Abacus Concepts, Calabas, USA). A value of $p \leq 0.05$ was considered as significant.

Results

IFN-α production induced by various phosphodiester ODNs

To test the effect of poly-G-sequences at the 5' and/or 3' ends of ODNs with various lengths of base-pairing sequences (6nt or 12 nt), six different ODNs with the same central hexamer (5'-TTCGAA-3'), HB1G5', HB1G3', HB1G5'3', HB4G5', HB4G3', HB4G5'3' were tested for their ability to induce IFN-α production by poPBMC. All six ODNs were able to induce IFN-α production after pre-incubation with Lipofectin® but at various levels. Three ODNs (ODN HB1G5', ODN HB1G3' and ODN HB4G3') consistently induced higher levels of IFN-α (621±399, 695±457, 744±432 units per ml respectively;

meanvalue±SEM, n=6), than the other three ODNs (189±195, 46±63 and 209±299 units per ml; mean value±SEM, n=6), but in all cases the magnitude of responses varied considerably between pigs. To adjust for this individual variation in IFN-α producing capacity (Edfors-Lilja *et al.*, 1998), the plasmid pcDNA3 was included at all test occasions, and the levels of IFN-α induced by an ODN was expressed as percentage of the corresponding value induced by pcDNA3. This comparison revealed that three of the ODNs induced as much, or even more, IFN-α than plasmid DNA3 when pre-incubated with Lipofectin® (Fig. 2). Only one of the ODNs (HB1G5'), induced IFN-α without pre-incubation with Lipofectin® and these levels were consistently lower than those induced in the presence of Lipofectin®. ODNs HB4G3' and HB4G5'3' were only able to induce trace amounts of IFN-α (corresponding to 0.2% and 3.0% of the amounts induced by pcDNA3 respectively) when not pre-incubated with Lipofectin®.

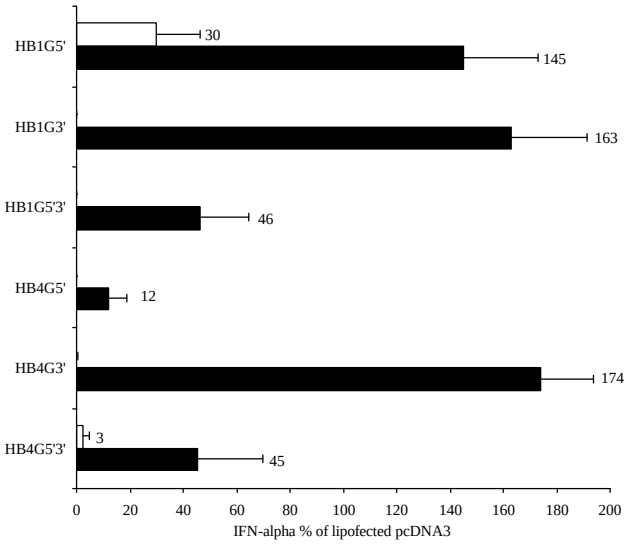


Figure 2. The IFN-α inducing capacity of the ODNs HB1G3', HB1G5', HB1G5'3', HB4G5'3', HB4G3', HB4G5', used at a concentration of 25μg/ml. The results are given as mean values ± SEM, n=6, expressed as percentage of the IFN-α levels (U/ml) induced by lipofected pcDNA3. The IFN-α production induced by pcDNA3 varied between 188-723 units IFN-α per ml for the experimental pigs. Open bars correspond to cultures induced with ODNs in the absence of Lipofectin®, and filled bars to cultures where ODNs were pre-treated with Lipofectin®.

Thus, addition of a poly-G sequence to the 3´end enhanced the IFN-α inducing capacity of the ODN, irrespectively of the length of the

central base-pairing sequence. Addition of a poly-G sequence to the 5' end also enhanced the IFN-α inducing capacity of the longer ODN but had no enhancing effect on the ODN with a short (6nt) base-pairing sequence. Addition of a poly-G sequence to both 5' and 3' ends reduced the IFN-α inducing capacity of both the 6nt and 12nt long basepairing ODNs

IFN-α production induced by ODN HB1G3' synthesised at different stock concentrations

The fact that HB1G3' was unable to induce IFN-α production without pre-incubation with Lipofectin® was contradictory to previous results using a lower stock concentration (approximately 250-640 μg per ml) of the ODN (Hasslung *et al.*, 2006). Therefore two different stock solutions (concentrations approximately 600 and 2700 μg per ml respectively) of ODN HB1G3' were tested for their IFN-α inducing capacity, both at a final concentration of 25μg per ml culture medium. Results from this comparison are summarised in Figure 3 and show that ODN prepared from the lower stock concentration induced higher levels of IFN-α than ODN kept at a higher stock concentration. This difference was most clearcut when the ODN was used in the absence of Lipofectin®. When not lipofected, IFN-α level of 41±100 units per ml was induced by ODN HB1G3' at the stock concentration of 2716 μg per ml, which corresponds to 4% of the IFN-α induction obtained with lipofected pcDNA3. HB1G3' at the stock concentration of 250-640 μg per ml not pre-incubated with Lipofectin® induced 1354±1317 units per ml, which equals 184% of the IFN-α induction obtained with lipofected pcDNA3. This difference in results by the various preparations of ODN indicates that the physical form of the ODN was altered when the ODN was kept at various stock concentrations in a way that affected its ability to induce IFN-α production.

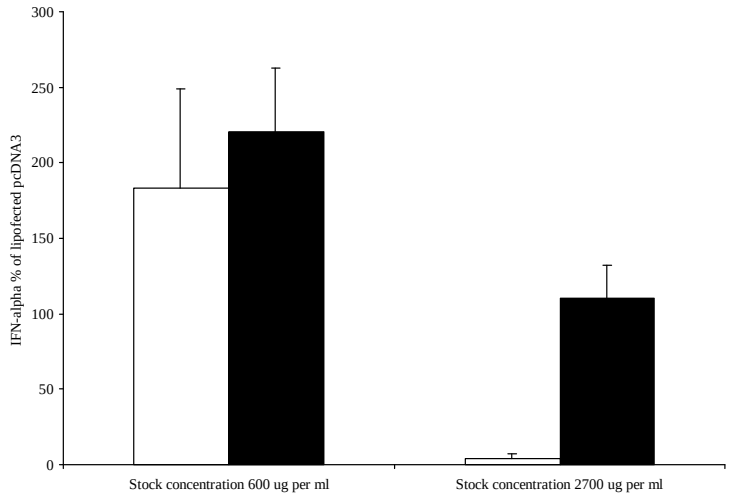


Figure 3. Production of IFN-α by porcine peripheral blood mononuclear cells (PBMC) stimulated with ODN HB1G3' (25 μg/ml), delivered at different stock concentrations (600 or 2700 μg per ml). Results are given as mean values ± SEM, n=17, expressed as percentage of IFN-α induced by lipofected pcDNA3 (996±566 units IFN-α per ml). Open bars correspond to cultures induced with ODNs in the absence of Lipofectin® and filled bars to cultures were ODNs where pre-treated with Lipofectin®.

IFN-α inducing capacity of denatured ODN 2216

To study whether self-complementary single stranded ODNs spontaneously form double strands or other multimeric forms during storage, a well-known IFN-α inducing CpG-ODN, ODN 2216, was used. This ODN has a chimeric backbone with phosphodiesters and phosphorothioates, a polyG-tail at the 3' end, and a theoretical capacity to form double strand allowing a 10 nucleotide long base-pairing sequence as depicted in Figure 1.

ODN 2216 was used for in vitro IFN-α induction un-treated or after heat-treatment/denaturation for 5 min at 100°C. The denaturation was estimated by measurements of the A260 values, which increased with approximately 20% after heat treatment. Both preparations of ODN 2216 were tested neat or pre-incubated with Lipofectin® and the response was compared to that induced by pcDNA3 in the same pig (Fig. 4).

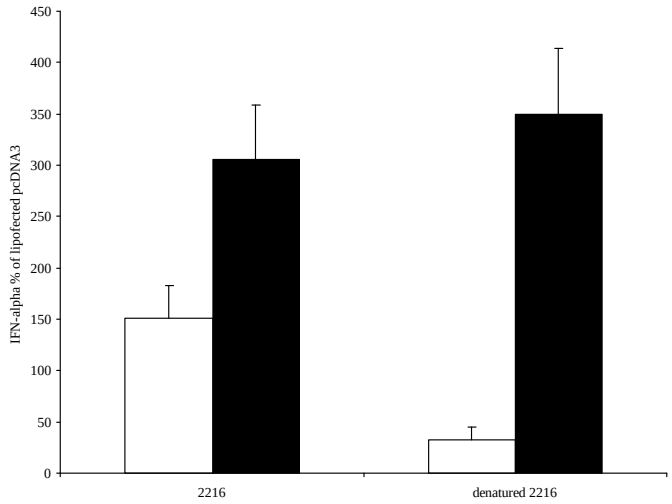


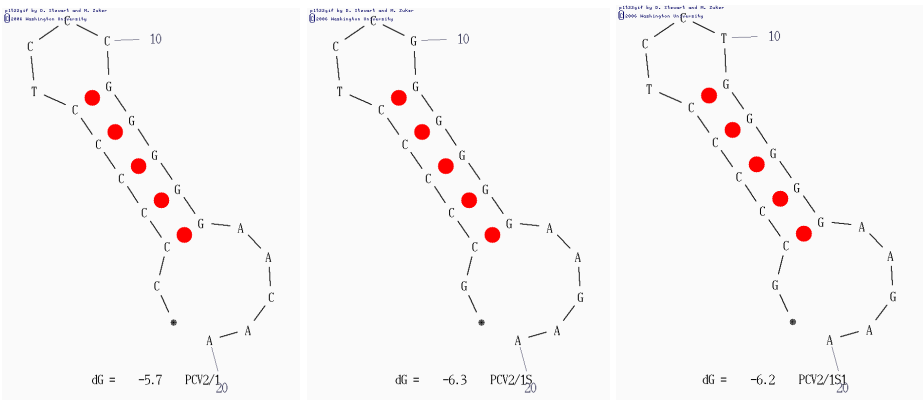
Figure 4. Alterations in the IFN-α inducing capacity of ODN 2216 before and after heat treatment. The ODN was used at a concentration of 5μg/ml and the results are given as mean values ± SEM, n=16, expressed as percentage of the IFN-α induced by lipofected pcDNA3. Open bars correspond to cultures induced with ODNs in the absence of Lipofectin® and filled bars to cultures where ODNs were pre-treated with Lipofectin®.

The heat treatment/denaturation of ODN 2216 reduced the IFN-α inducing capacity of the ODN from 151% (native) to 33% (heat-treated) of the IFN-α production induced by pcDNA3. Interestingly, the IFN-α inducing capacity of ODN 2216 could be restored after denaturation through pre-incubation of the heat-treated preparation with Lipofectin® (1837±930 units per ml), which corresponds well to the levels of IFN-α induced by the native ODN 2216 pre-incubated with Lipofectin® (1701±868 units per ml). Thus, the IFN-induction by ODN 2216 was clearly reduced by heat-treatment but the denatured preparation regained the IFN-α inducing capacity by pre-incubation with Lipofectin®. These results indicate that the IFN-α inducing capacity of the ODN is dependent on formation of multimeric structures.

Effects of minor modifications in the sequence of ODN PCV2/1 on the inhibition of IFN-α production

An ODN corresponding to a 20 nucleotide long sequence from the genome of PCV2 (Imp. 1010 Stoon) has previously been shown to inhibit the IFN-α production induced by some other ODNs including ODN 2216 (Hasslung *et al.*, 2003). Preliminary data demonstrated that

this inhibitory effect was dependent on the secondary structure of the ODN PCV2/1 (Hasslung *et al.*, 2006). To further study the influence of exchanging nucleotides within ODN PCV2/1 that not affected the hairpin formation but modified the CpG motif, two new variants of the ODN where synthesised, PCV2/1S and PCV2/1S1 (Fig. 5).



a. b. c.

Figure 5. Predicted hairpin formation of the ODNs PCV2/1, dG: -5.7 kcal/mol (a), PCV2/1S, dG: -6.3 kcal/mol (b) and PCV2/1S1, dG: -6.2 kcal/mol (c). The predicted secondary structures were determined using the IDT SciTools Oligo Analyzer 3.0 software (http://www.idtdna.com/SciTools.sapx).

When tested in cultures of poPBMC, none of the three ODNs (ODN PCV2/1, ODN PCV2/1S, ODN PCV2/1S1) were able to induce IFN-α production regardless of whether the ODNs were pre-incubated with Lipofectin® or not. The ability of the various PCV2-ODNs to inhibit IFN-α production induced by ODN 2216 was tested in cultures of poPBMC in the presence of ODN 2216 and one of the PCV2-ODNs. The IFN-α production induced by ODN 2216 without pre-incubation with Lipofectin® varied from 187 to 2084 U IFN-α/ml (950±806 units IFN-α per ml; mean value±SEM, n=6) but was decreased by all three ODNs tested (ODN PCV2/1: range <0 to 253; ODN PCV2/1S: range 3.8 to 497; ODN PCV2/1S1: range 5.5 – 468 units IFN-α per ml). A similar variation between individuals was found with lipofected ODN 2216 alone (from 334 to 1868 units IFN-α per ml; 1093±643 units IFN-α per ml; mean value±SEM n=6) and in combination with lipofected ODN PCV2/1 (0.8 – 352 units IFN-α per ml), ODN PCV2/1S(16 – 718 units IFN-α per ml) or ODN PCV2/1S1 (17 – 814 units IFN-α per ml) as shown in Figure 6.

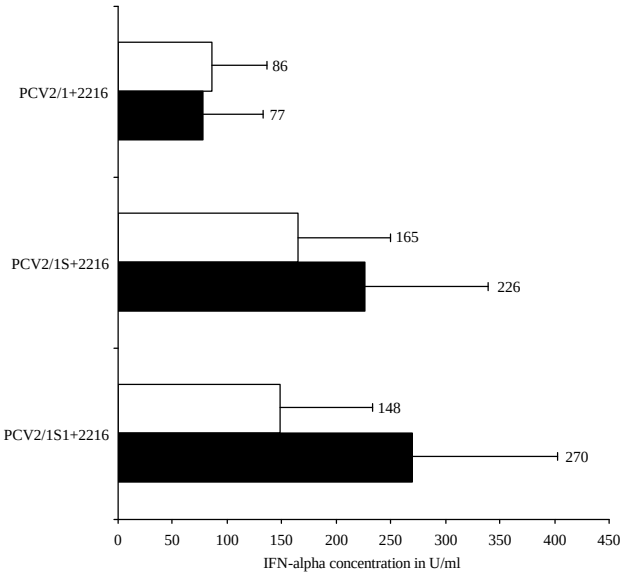


Figure 6. Inhibitory effect of ODNs PCV2/1, PCV2/1S and PCV2/1S1 on the IFN-α production induced by ODN 2216. The IFN-α production is expressed as units per ml and the results are given as mean values ± SEM, n=6. Open bars correspond to cultures induced by ODNs in the absence of Lipofectin® and filled bars to cultures where ODNs were pre-treated with Lipofectin®.

Statistical analysis showed that the concentration of IFN-α was significantly lower ($p<0.05$, Wilcoxon rank-sum test) in supernatants collected from cultures of poPBMC incubated with the combination of ODN PCV2/1 and ODN 2216 than in those incubated with the combination of ODN 2216 and ODN PCV2/1S or ODN PCV2/1S1, regardless of pre-incubation of the ODNs with Lipofectin® or not. Thus, the CpG-motif is not vital for the ODNs IFN-α inhibitory capacity since no significant difference could be seen between the inhibitory effect of ODN PCV2/1, containing a CpG-motif, and PCV2/1S1, lacking a CpG-motif. The inhibitory capacity of lipofected ODN PCV2/1S1 was consistently greater than that of ODN PCV2/1S1 not pre-incubated with Lipofectin® ($p< 0.03$).

To correct for the variation in IFN-α producing capacity between pigs the inhibitory effect was expressed as percentage of the amount IFN-α induced by ODN 2216. These results illustrated that the unmodified ODN PCV2/1 had the best inhibitory capacity compared to ODN PCV2/1S and ODN PCV2/1S1, both in the presence and absence of Lipofectin®. As can be seen in Figure 7, ODN PCV2/1 was able to inhibit 95% of the IFN-α production induced by ODN 2216 both when

pre-incubated with Lipofectin® and not, while neat ODN PCV2/1S and ODN PCV2/1S1 inhibited 87% and 90% of the IFN-α induced by ODN 2216 respectively. When lipofected, the inhibitory action of ODN PCV2/1S and ODN PCV2/1S1 on IFN-α induction obtained from ODN 2216 was slightly less than 80% and 77%, respectively. Analysis of the correlation between the concentration of IFN-α induced by ODN 2216 and the inhibitory capacity of ODN PCV2/1 revealed that the two parameters were related to each other (r = -0.93, correlation coefficient) although in all cases the inhibitory capacity of ODN PCV2/1 was more than 75% (Fig. 8).

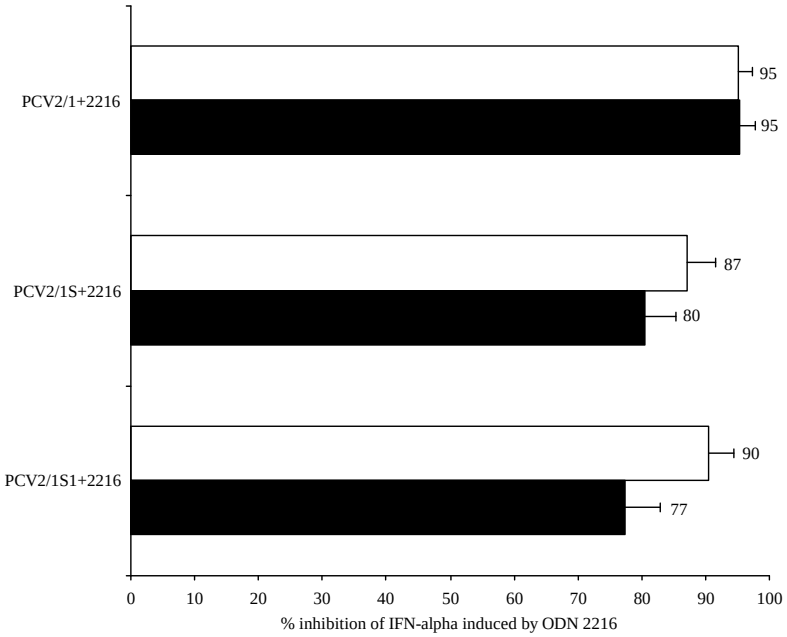


Figure 7. The effect of the ODNs PCV2/1, PCV2/1S and PCV2/1S1 on the IFN-α production induced by ODN 2216 at co-culture. The IFN-α production is expressed as percent inhibition in co-cultures compared to IFN-α production induced by ODN 2216 alone, which varied between 187-2084 units per ml. The results are given as mean values ± SEM, n=6. Open bars correspond to cultures induced with ODNs in the absence of Lipofectin® and filled bars to cultures where ODNs were pre-treated with Lipofectin®.

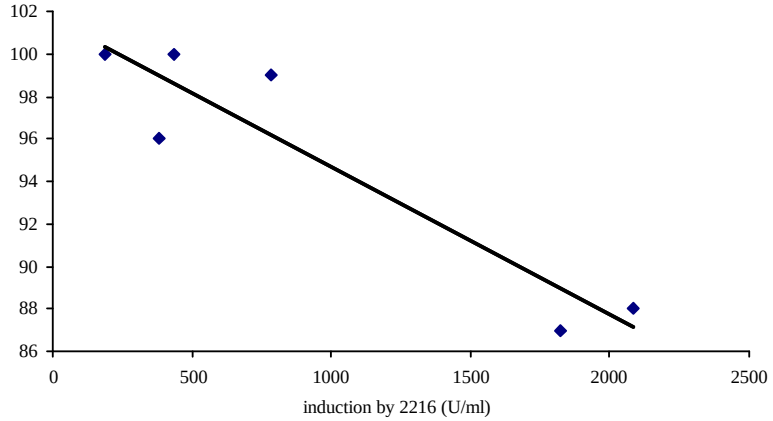


Figure 8. Correlation between the amount of IFN-α (U/ml) induced by ODN 2216 and the inhibitory capacity of ODN PCV2/1 in percent (correlation coefficient = -0.93).

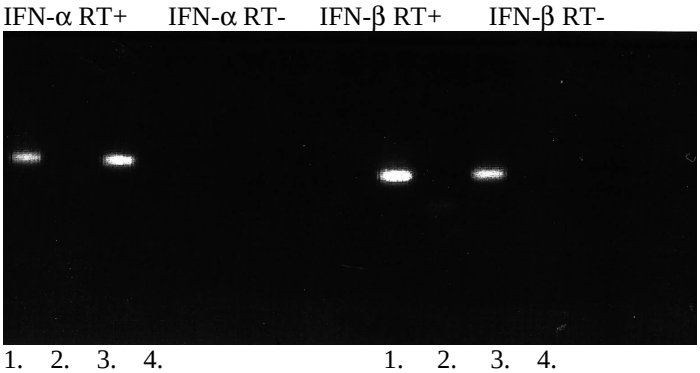
Cytokine expression at mRNA-level in poPBMC cultured with ODNs 2216, PCV2/1 or in combination of the two ODNs

It is unknown at which level ODN PCV2/1 inhibits the IFN-α induction of other CpG ODNs. To further elaborate the inhibitory function of ODN PCV2/1, the effect of ODN PCV2/1 on the expression of cytokines at messenger RNA-level was studied by RT-PCR.

The mRNA expression of the cytokines, IL-1β, IL-6, IL-8, IL-10, IL-12 p35, IL-12 p40, IL-18, IFN-α, IFN-β, IFN-γ, TNF-α, TGF-β, MIF and GM-CSF, was determined by PCR analysis of RNA isolated from poPBMC cultured in the presence of ODN 2216 or/and ODN PCV2/1 or in plain growth medium. Parallel cultures were initiated and cells for mRNA analysis were collected after 6 hours whereas culture supernatants for detection of secreted IFN-α were collected after 24 hours of incubation. The inductions were repeated with cells obtained from different pigs and in many cases mRNA for several of the cytokines were detected at PCR analysis of unstimulated PBMC. Therefore, it only became clear that transcription of for IFN-α and IFN-β were induced in cultures with ODN 2216 alone or in the combination with ODN PCV2/1. As shown in Figure 9, the levels of IFN-α determined in culture supernatants after 24 hours were considerably reduced when the cells were cultured in a mixture of ODN 2216 and ODN PCV2/1 compared to when cultured with only ODN 2216. It is notable that in one case mRNA for IFN-α was detectable in a culture

with unstimulated cells, while this signal was absent in the culture with cells from the same pig induced with ODN PCV2/1, although it is still unknown how PCV2/1 inhibits the production of IFN-α.

a.



1. culture with ODN 2216, 2. culture with ODN PCV2/1, 3. co-culture with ODN 2216 and ODN PCV2/1, 4. culture in plain medium

b.

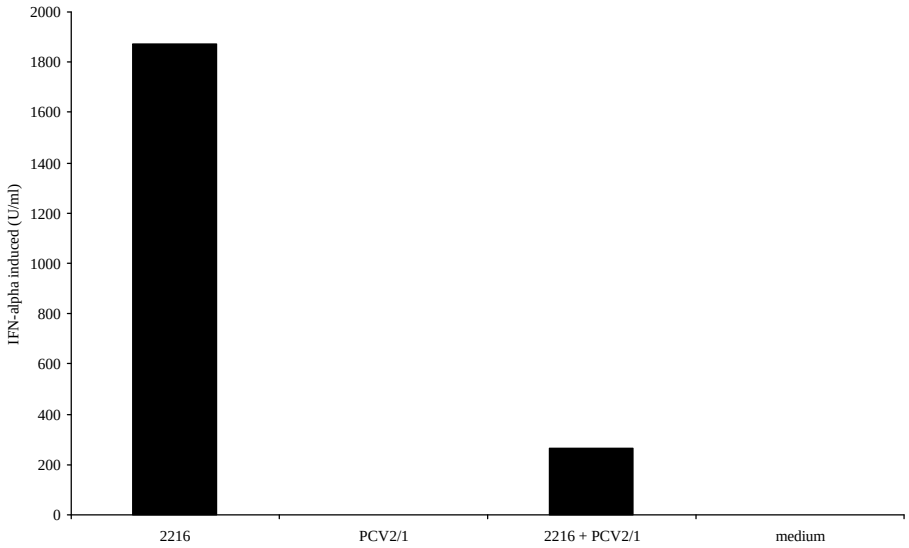


Figure 9. The IFN-α induction in parallel cultures incubated at 37°C in the presence of ODN 2216 and/or ODN PCV2/1 or in plain growth medium. Products for mRNA analysis were run on 1% agarose gel (a) and as a control a mixture without the Superscript II reverse transcriptase (RT-) was run in parallel to those with the enzyme included (RT+) for IFN-α and IFN-β. Supernatants from the corresponding 24 hours cultures were collected and analysed for presence of secreted IFN-α (b).

Discussion

In the present study, synthetic ODNs were tested for their ability to induce or inhibit IFN-α production by poPBMC in vitro. Studies of ODN H (5'-TTTTCAATTCGAAGATGAAT-3'), originally identified in serum from a patient with systemic lupus erythematosus (SLE), have shown that the ODN after pre-treatment with Lipofectin® is able to induce IFN-α in human (Magnusson *et al.,* 2001b) and porcine (Domeika *et al.,* 2004) PBMC. Further, in vitro studies have shown that the IFN-α inducing capacity of ODN H can be increased by double strand formation and/or addition of repeated guanosines (poly-G) sequences, although none of the modifications abolish the need for pre-treatment with Lipofectin® (Domeika *et al.,* 2004; Hasslung *et al.,* 2006), which also have been indicated for other phophodiester ODNs (Pisetsky, 1999a; Dalpke *et al.,* 2002). To further study the importance of double strand formation and poly-G sequences six new variant of ODN H, designated ODN HB1G5', HB1G3', HB1G5'3', HB4G5', HB4G3' and HB4G5'3', were constructed and tested for their IFN-α inducing capacity in porcine PBMC. All six of the ODNs contained the central hexamer motif present in ODN H (5'-TTCGAA-3') and in addition to this the total number of possible base pairs in a self-dimer structure was varied as well as the position of the poly-G sequences.

According to the present study neither addition of a poly-G sequence at the 5' or the 3' end of the ODNs nor increasing the total number of possible base pairs in a self-dimer structure circumvented the need for pre-treatment with Lipofectin® in order to induce IFN-α production. After pre-treatment with Lipofectin® three out of the six ODNs ODN HB1G5', ODN HB1G3' and ODN HB4G3' readily induced IFN-α, while HB1G5'3', HB4G5' and HB4G5'3' only were able to induce small amounts of IFN-α. This suggest that for the ODNs with a basepairing sequence of 12 nucleotide an addition of poly-G sequence at either the 5' or 3' end can enhance its IFN-α inducing capacity, while addition of poly-G sequences at both 5' and 3' ends decreased the amount of IFN-α induced. Although when the base pairing sequence was shorter, 6 nucleotides, addition of a poly-G sequence at the 3' end is the only modification that enhanced the IFN-α inducing capacity. The importance of an addition of a poly-G sequence to the 3' end of the ODN was in common for ODNs with both 12 and 6 nt base-pairing sequences. This enhancing effect could be explained by protection of the 3' end of the ODN from 3' exonuclease activity in cell

cultures (Clusel *et al.,* 1993). Furthermore, the increase in IFN-α production could be ascribed to the possible spontaneous formation of nanoparticels since ODNs with at least four guanosines in their poly-G sequences are known to self-associate via Hoogsteen base pairing to form quadruplex structures i.e. G-tetrads (Williamson *et al.,* 1989) which has been suggested to enhance cellular uptake via scavenger receptors and thereby contribute to the immune modulatory activity of the ODNs (Dalpke *et al.,* 2002). On the other hand, addition of a poly-G sequence at the 5´ end of the ODN has been suggested to be unfavourable since the cellular recognition of the nucleotide sequence is performed in a 5' – 3' direction (Yamada *et al.,* 2002; Kandimalla *et al.,* 2002). Nevertheless, addition of a poly-G sequence at the 5' end of the HB1G5' enhanced the IFN-α inducing capacity of this particular ODN.

Unexpected results have been described previously when testing various ODNs for biological effects. Indeed, the same ODN sequence synthesized by various companies gave diverging results when tested with poPBMC (Van der Stede *et al.,* 2005). The ODNs used in the present study have each time been ordered desalted and dissolved in water from the same company. Despite that when compared to results from previous studies of ODN HB1G3' (Hasslung *et al.,* 2006) a remarkable difference was found. In the present study, ODN HB1G3' seem to be dependent on pre-treatment with Lipofectin® to be able to induce IFN-α production in poPBMC while the previous studies have shown that addition of a poly-G sequence to the 3' end of the ODN H eliminated the need for Lipofectin® pre-treatment. These discrepancies lead us to believe that the properties of the ODN were influenced by the stock concentration in which the ODN was delivered. Comparison between results from tests performed with ODN HB1G3' delivered at different stock concentrations further supported this assumption as ODNs from the lower stock concentration (600 μg/ml) seemed to have a better IFN-α inducing capacity also in the absence of Lipofectin®. Theoretically, ODN H can form nanoparticle structures similar to what has been described for ODN 2216 (Kerkmann *et al.,* 2005).

To test the importance of base-pairing between ODNs secondary structures of ODN 2216 were denatured by heat treatment i.e. heating to approximately 100^{o}C and rapid cooling. This treatment reduced the IFN-α inducing capacity from ODN 2216, but this could be restored by treatment of the denatured ODN 2216 with Lipofectin®. Thus, the incorporation of an ODN into a larger structure which is provided by

cationic lipids mimicking the spontaneously assembled nanoparticles present among untreated ODN 2216 seems to be important for it's ability to induce IFN-α production. The importance of ODN aggregation might also explain the differences in results obtained using preparations of ODN HB1G3' delivered at various stock concentrations because it has been shown that ODNs at a high concentration form a complex network of unspecific DNA structure rather then assembled nanoparticles (Costa *et al.,* 2004). Thus, addition of poly-Guanosine sequences (poly-G sequences) to the ODNs can affect their immunostimulatory activity most likely due to formation of secondary structures. The formation of secondary structures appear to facilitate the uptake/internalisation of CpG DNA in the cells and thereby increase the IFN-α inducing capacity of the ODN (Kandimalla *et al.*, 2003; Domeika *et al.,* 2004).

ODNs have not only been described as potent IFN-α inducers, but certain ODNs instead act inhibitory on the IFN-α production induced by other ODNs, viral inducers or bacterial inducers. The genome of PCV2 has been shown to contain several nucleotide sequences with the ability to modulate the porcine immune response among them an inhibitory sequence, PCV2/1 (Hasslung *et al.*, 2003). In the present study three variants of the inhibitory sequence PCV2/1 were shown to inhibit the IFN-α production by ODN 2216 to a variable degree. The first variant, ODN PCV2/1, has only been found in samples from healthy pigs in Sweden while the second variant of the ODN, ODN PCV2/1S1, predominates in pigs from Swedish farms with PMWS. To study the significance of the central CpG-motif in the sequence a third ODN, with a nucleotide-exchange in the CpG-motif, was constructed, PCV2/1S. All three variants were used to measure their ability to inhibit IFN-α production by ODN 2216. The result presented demonstrate that the inhibitory capacity of ODN PCV2/1 was retained although minor modifications to the nucleotide sequence of the ODN were performed. Nevertheless, these modifications did result in a difference ($p<0.03$) in the degree of inhibition of the IFN-α production by poPBMC. In accordance with previous studies using neutralizing phosphorothioate ODNs (Zhao *et al.,* 2000) this study clearly demonstrate that the inhibitory capacity of the ODN PCV2/1 is not dependent on the presence of a central CpG-motif since no significant difference between the inhibition obtained from the ODN PCV2/1S1, lacking a CpG-motif, and PCV2/1S, with an introduced CpG-motif, were found.

Theoretically the nucleotide sequence of ODN PCV2/1 gives it the possibility to spontaneously form secondary hairpin structures, as depicted in Figure 6, which has been shown to be crucial to the inhibitory action of the ODN (Hasslung *et al.,* 2006). This might in fact be a way for the ODN to act in an inhibitory way on the IFN-α production induced by TLR9 agonists. Secondary structures such as hairpins in DNA can bind to transcription factors (Chu *et al.,* 1991) and they also provide a protection, which make them more resistant against degradation by endonucleases than ss DNA (Chu *et al.,* 1992), and this could prolong the inhibitory activity of ODN PCV2/1. In addition, the formation of higher-order structures might explain why the inhibition not was totally dependent on pre-treatment with Lipofectin®.

To study if ODN PCV2/1 inhibited IFN-α production before or after transcription of the gene the mRNA was determined for a number of cytokines. Even though the parallel inductions for 6 and 24 hours were repeated several times with cells obtained from different pigs no conclusive results were obtained. In most cases cytokine mRNA was detected in samples collected from PBMC grown in plain medium. Nevertheless it is clear that transcription of IFN-α and IFN-β were induced in cultures with either ODN 2216 alone or in combination with ODN PCV2/1. Analyses of supernatants collected from the 24 hours incubations showed a considerably reduction of secreted IFN-α in co-cultures with ODN 2216 and ODN PCV2/1 compared to cultures with only ODN 2216. In other systems, competition between inhibitory and stimulatory ODNs has been suggested and one theory is that binding of inhibitory ODNs to TLR9 results in a conformational change of the MyD88 binding site which interrupts the further signalling through TLR9 (Ashman *et al.,* 2005; Lenert *et al.,* 2006), which would mean that the inhibitory effect of the ODN is exerted before transcription of the mRNA. In the present study, the method used for detection of cytokine mRNA was not quantitative and therefore the amount of transcribed IFN-α and IFN-β in co-cultures with ODN 2216 and ODN PCV2/1 could not be compared to the amounts in cultures with only ODN 2216. Thus, additional studies using a quantitative method is needed to elaborate the mechanism behind the IFN-α inhibitory capacity of the ODN PCV2/1. Further, results from the *in vitro* studies show a significant difference between ODN PCV2/1 found in the genome of PCV2 from healthy pigs in Sweden and ODN PCV2/1S1, which predominates in pigs from farms with PMWS in Sweden. However, the biological significance of these findings remain to be elucidated.

Acknowledgment

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