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Characteristics of oligodeoxyribonucleotides that induce interferon (IFN)-α in the pig and the phenotype of the IFN-α producing cells

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Abstract

The immunostimulatory effects of oligodeoxyribonucleotides (ODN) containing unmethylated CpG dinucleotides (CpG-ODN) in certain base contexts have been extensively studied in man and mice. One major action is their ability to trigger production of massive amounts of interferon-α (IFN-α) by plasmacytoid dendritic cells (PDC), also referred to as natural IFN-α/β producing cells (NIPC). The present study using porcine PBMC activated by CpG-ODN or plasmid DNA revealed a considerable variation in the IFN-α production in response to various CpG-ODN constructs. Several phosphodiester ODNs, such as 5′ TTTTCAATTCGAAGATGAAT 3′ (ODN H), and the plasmid pcDNA3 all required pre-incubation with lipofectin in order to induce IFN-α. Intact unmethylated CpGs were also important, because methylation or substitution of the cytosines and CpG-inversion strongly reduced the IFN-α induction by single- or double-stranded forms of ODN H. Certain CpG-ODNs that contained flanking phosphorothioate or phosphodiester poly-G sequences were potent inducers of IFN-α without pre-incubation with lipofectin, for instance the ODN 2216 (5′ GGGGGACGATCGTCGGGGGG 3′). While poly-G sequences have been suggested to increase uptake of ODNs by cells, they did not obviate the need for lipofectin when added to the ODN H. However, they resulted in up to five-fold increases of the IFN-α levels caused by ODN H upon lipofection, indicating other enhancing effects of poly-G sequences on the induction of IFN-α. The identity of the IFN-α producing cells (IPC) stimulated by CpG-ODN or plasmid DNA was studied by means of flow cytometry using combined staining for intracellular IFN-α and surface markers. Approximately 1–3 IPC/10^3 PBMC were detected, compared to only 3 IPC/10^4 PBMC stimulated by Aujeszky's disease virus. The IPC frequencies were confirmed by detection of IFN-α mRNA positive cells by in situ hybridisation. The IPC induced by CpG-ODN or plasmid DNA had a similar phenotype, expressing CD2 and CD4 and intermediate levels of MHC class II and the myeloid marker SWC3, but not the markers of T and B cells or

Abbreviations: ADV, Aujeszky's disease virus; CpG-DNA, DNA containing unmethylated CpG dinucleotides; CpG-ODN, oligodeoxyribonucleotides containing unmethylated CpG dinucleotides; DELFIA, dissociation-enhanced lanthanide fluoroimmunoassay; DC, dendritic cells; ds, double stranded; FCM, flow cytometry; HSV, herpes simplex virus; IPC, IFN-α producing cell; IF, immunofluorescence; NIPC, natural IPC; ODN, oligodeoxyribonucleotide; PDC, plasmacytoid dendritic cell; poPBMC, porcine PBMC; RNA, ribonucleic acid; RT, room temperature; ss, single stranded; TGEV, transmissible gastroenteritis virus

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monocytes (CD3, CD21 and CD14). Consequently, porcine IPC that respond to CpG-DNA seem to correspond to the PDC/NIPC.

Keywords: Porcine; Interferon; IFN-α producing cells; Dendritic cells; CpG-DNA

1. Introduction

The type I interferons IFN-α and IFN-β are potent anti-viral cytokines that represent an important part of innate immunity (Samuel, 2001). In man and mice it has been demonstrated that IFN-α/β also affect adaptive immunity, for example, by maturation of DC and promotion of a Th1-like immune response (Belardelli and Ferrantini, 2002; Biron, 2001). Production of IFN-α can be induced by many viruses and bacteria, including components of these microorganisms such as glycoproteins, double stranded ribonucleic acid (dsRNA) and DNA containing unmethylated CpG dinucleotides (CpG-DNA). Such CpG-DNA is now recognized as a pathogen-associated molecular pattern that can induce B-cell proliferation, NK cell activation and production of cytokines, including type I IFN (for review see Krieg, 2002; Yamamoto et al., 2002).

In the pig, only a limited number of studies on CpG-DNA have been conducted using either oligodeoxyribonucleotides (ODN) that contain unmethylated CpG-dinucleotides (CpG-ODN) or plasmid DNA. It was found that the plasmid pcDNA3 induced IFN-α in porcine PBMC (poPBMC) in vitro, when pre-incubated with lipofectin (Magnusson et al., 2001a), and also when administered in vivo into subcutaneous tissue chambers (Johansson et al., 2002). Methylation of the cytosine in the CpGs inhibited the IFN-α inducing capacity of plasmid DNA in vitro (Magnusson et al., 2001a) and in vivo (Johansson et al., 2002). Other in vitro studies using ODN showed that the ability to induce IL-6, IL-12 and TNF-α mRNA in poPBMC was dependent on the presence of CpG (Kamstrup et al., 2001). However, both a CpG-ODN and the same ODN, but with the CpGs inverted to GpC, enhanced the ovalbumin-specific immune responses after intramuscular injection in pigs (Van der Stede et al., 2002). Therefore, the CpG may not be necessary for some immunostimulatory effects of ODN. In fact, the ability of certain ODNs to induce IFN-α in human PBMC is retained or enhanced if the CpG is methylated or inverted to GpC (Magnusson et al., 2001b).

It has been described that CpG-DNA is recognized by the Toll-like receptor (TLR) 9 (Bauer et al., 2001; Hemmi et al., 2000) which is expressed by plasmacytoid dendritic cells (PDC) and B cells in humans (Jarrossay et al., 2001; Kadowaki et al., 2001b). The human PDC, also referred to as natural IFN producing cells (NIPC), appear at a low frequency but produce high levels of IFN-α upon exposure to, for example, herpes simplex virus (HSV) (Cederblad and Alm, 1990; Siegal et al., 1999; Svensson et al., 1996) or CpG-ODN (Jarrossay et al., 2001; Krug et al., 2001a; Magnusson et al., 2001b). A corresponding cell type has been described in mice (Asselin-Paturel et al., 2001) and in the pig, a small population of efficient IFN-α producing cells (IPC) has been detected at stimulation of blood leukocytes by the coronavirus transmissible gastroenteritis virus (TGEV) (Nowacki and Charley, 1993) or the herpes virus Aujeszky's disease virus (ADV) (Artursson et al., 1992). After intradermal administration of ADV-infected cells, infrequent IPC were mainly localized to the regional lymph nodes (Artursson et al., 1995). The porcine blood IPC activated by TGEV were shown to resemble the human NIPC by their appearance as non-phagocytic, non-adherent non-B, non-T cells, of which some expressed CD4 and MHC class II molecules (Charley and Lavenant, 1990; Nowacki and Charley, 1993). Immunohistochemical analysis of TGEV-activated IPC in various lymphoid tissues revealed that these cells had a similar phenotype and that at least a subpopulation of splenic IPC expressed the myeloid marker SWC3 (Riffault et al., 1997, 2001). Also, a small subpopulation of SWC3+ blood cells that express CD4 but not CD14 was shown to produce IFN-α upon exposure to TGEV which further supports the existence of a porcine counterpart to the human/murine NIPC/PDC (Summerfield et al., 2003).

Induction of type I IFNs is considered important to improve the efficacy of several vaccine formulations (Cull et al., 2002; Endresz et al., 2001; Rizza et al., 2002; Tudor et al., 2001; Tüting et al., 1998) and adjuvants targeting the principal IPC, i.e. NIPC/PDC should then be advantageous. The aim of the present study was therefore to determine the importance of certain properties of ODNs for their IFN-α inducing capacity and to delineate the phenotype of the responding cells in the pig. For that purpose, a panel of ODNs that had previously shown IFN-α inducing capacity in other species, or that had been used as experimental vaccine adjuvants in pigs, were selected and analysed for IFN-α induction in porcine cells.

2. Materials and methods

2.1. Purification of poPBMC

Each experiment was carried out on PBMC obtained from at least four conventionally reared Yorkshire pigs, originating from at least two different litters. The pigs were housed at the University Research Station, Funbo-Lövsta, Sweden, and aged 9–12 weeks. Serum samples from each pig were always analysed for presence of endogenous IFN-α.

Blood samples were collected from *vena cava cranialis* in evacuated test tubes (B-D Vacutainer, Meylan Cedex, France) without additives or with heparin (143 USP units). PBMC were purified by Ficoll-Paque (Amersham Biotech, Uppsala, Sweden) density gradient centrifugation for 30 min at 500 × *g*. The PBMC were washed in PBS and diluted to 10×10^6 cells/ml in growth medium, i.e. RPMI 1640 medium (Biowhittaker, Verviers, Belgium) with 20 mM HEPES buffer, supplemented with L-glutamine (2 mM), penicillin (200 IU/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (0.05 mM) and 10% foetal calf serum (FCS; Myoclone®; Life Technologies, Paisley, UK). To allow comparison between experiments, lipofected ODN H-I (see below) was included as an inducer of IFN-α in each experiment.

2.2. Reagents used for IFN-α induction

All oligodeoxyribonucleotides were purchased desalted and dissolved in H_2O (Cybergene AB, Stockholm, Sweden). For ODNs with methylated cytosines, 5-methylcytosine was used in the synthesis. Some of the ODNs were tested in single-stranded (ss) as well as in double-stranded (ds) forms, hybridized as previously described (Magnusson et al., 2001b). The sequences of all ODNs are given in Table 1. The plasmid pcDNA3 (Invitrogen, San Diego, CA) was prepared as described (Magnusson et al., 2001a) and used at a concentration of 2.5 μg/ml culture medium. *Actinobacillus pleuropneumoniae* serotype 2, strain 200/89 (*A. pleuropneumoniae*) was heat-inactivated and used at a concentration of 3.8×10^6 CFU/ml (Wattrang et al., 1998). ADV (strain Bartha, 10^5 ID_{50}/ml) was inactivated by four cycles of UV irradiation (1 J/cm^2) and used at 100-fold dilution for IFN-α induction (Johansson et al., 2003).

2.3. Pre-incubation with lipofectin

The inducers were diluted in growth medium without FCS and, when indicated, pre-incubated with lipofectin (Life Technologies). The lipofectin was first diluted in growth medium without FCS, incubated for 1 h at room temperature (RT) and then incubated with the inducer for 15 min. After initial titrations in combination with ODNs, 5 μg lipofectin/ml was used for lipofection, giving a final concentration of 2.5 μg lipofectin/ml culture medium. The same concentration of lipofectin was used for pre-incubation of plasmid pcDNA3 and *A. pleuropneumoniae*, whereas ADV always was used neat.

2.4. Induction of IFN-α production

To determine the concentration of produced IFN-α in culture medium, 0.1 ml of the inducer was added to 0.1 ml of poPBMC (final concentration 5×10^6 cells/ml) in triplicate cultures in flat-bottomed 96-well plates (Nunc, Roskilde, Denmark). The cultures were incubated for 20 h at 37 °C and the supernatants from each triplicate were then pooled and stored at −20 °C until analysed for presence of IFN-α.

2.5. Detection of poIFN-α in culture medium

A dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA), based on two mAbs directed to poIFN-α was used for quantification of IFN-α in

Table 1
Designations and nucleotide sequences of the ODNs tested

ODN name	Sequence 5′–3′[a,b]	Reference[c]
H	TTT TCA ATT CGA AGA TGA AT	Magnusson et al. (2001b), Sato et al. (1999)
H^{A}	TTT TCA ATT AGA AGA TGA AT	Magnusson et al. (2001b)
H^{GC}	TTT TCA ATT GCA AGA TGA AT	Magnusson et al. (2001b)
H^{met}	TTT TCA ATT Me^5CGA AGA TGA AT	Magnusson et al. (2001b)
$H^{G\text{-tail }1}$	TTT TCA ATT CGA AGA TGA ATG GGG G	New modification of H
$H^{G\text{-tail }2}$	GGT TCA ATT CGA AGG GGG GG	New modification of H
$H^{G\text{-tail }3}$	GGT TTT CAA TTC GAA GAT GAA TGG GGG G	New modification of H
I	ATT CAT CTT CGA ATT GAA AA	Magnusson et al. (2001b)
I^{T}	ATT CAT CTT CTA ATT GAA AA	Magnusson et al. (2001b)
I^{GC}	ATT CAT CTT GCA ATT GAA AA	Magnusson et al. (2001b)
I^{met}	ATT CAT CTT Me^5CGA ATT GAA AA	Magnusson et al. (2001b)
A2	GCT AGA CGT TAG CGT	Klinman et al. (1996, 1999), Van der Stede et al. (2002)
$A2^{GC}$	GCT AGA GCT TAG GCT	Van der Stede et al. (2002)
2216	ggG GGA CGA TCG TCg ggg gG	Jarrossay et al. (2001), Krug et al. (2001a)
2216diester	GGG GGA CGA TCG TCG GGG GG	New modification of 2216
D19diester (D25)	GGT GCA TCG ATG CAG GGG GG	Kamstrup et al. (2001)
D19	ggT GCA TCG ATG CAG ggg gg	Verthelyi et al. (2001)
MM1	ggG GTC ATC GAT GAg ggg gG	New

[a] Relevant CpG dinucleotides, the modified CpG dinucleotides and methylated cytosines (Me^5) are underlined.
[b] Nucleotides with phosphodiester backbone are indicated with capital letters and phosphorothioate nucleotides with small letters.
[c] Selected references where the ODNs previously have been tested.

culture medium as previously described (Artursson et al., 1995). A laboratory standard (Diaz de Arce et al., 1992), consisting of natural poIFN-α was included on each plate and the results are given as laboratory units (U) per ml, mean value ±S.E.M. for four animals if not otherwise stated.

2.6. In vitro transcription of a poIFN-α cRNA probe

In vitro transcription of a biotinylated poIFN-α cRNA probe and corresponding sense control probe was performed from the plasmid vector pGEM/poIFN-α containing a 829 bp *Eco*RI–*Hpa*I fragment of the poIFN-α_1 gene. The plasmid DNA (1 μg) was linearized utilizing appropriate restriction enzyme sites present in the cloning box. The in vitro transcription was performed using biotin-16-UTP and T7-RNA polymerase or Sp6-RNA polymerase according to the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). The RNA was precipitated with 4 M LiCl and 99.5% ethanol and then dissolved in DEPC-treated H_2O. The probes (designated biotin-poIFN-α_1 and sense biotin-poIFN-α_1) were stored at −80 °C until use.

2.7. Detection of cells expressing IFN-α mRNA by in situ hybridization

PoPBMC (5×10^6 cells/ml) were stimulated in 24-well plates (Nunc) with ODN 2216 (5 μg/ml), ADV or lipofected pcDNA3. The cells were harvested after 6 h, fixed in 2% paraformaldehyde and applied to 8-well slides (Histolab Products, Gothenburg, Sweden). The slides were pretreated and then pre-hybridized for 1 h at 50 °C in the hybridization solution (Sandberg et al., 1994), containing 500 μg/ml salmon sperm DNA (Sigma-Aldrich, St. Louis, MO). They were subsequently incubated overnight at 65 °C with biotin-poIFN-α_1 cRNA probe or sense control probe (5 μg/ml) in hybridization solution containing 100 μg/ml salmon sperm DNA. The slides were then treated with RNAse A (Sandberg et al., 1994) and rinsed twice in 2× SSC for 5 min, once in 1× SSC for 10 min and once in 0.5× SSC for 30 min at RT. After a final rinse in 0.1× SSC for 20 min at 55 °C, endogenous peroxidase activity was blocked with 0.3% H_2O_2 in PBS for 20 min at RT. Any bound biotin-poIFN-α_1 probe was detected by the DAKO GenPoint signal amplification system (Dako, Glostrup, Denmark) following the manufacturer's instruction and

using the primary streptavidin–horseradish peroxidase complex at a dilution of 1:1000. The frequency of the IFN-α mRNA expressing cells was determined by examining 8000–15,000 cells per microslide well.

2.8. Detection and phenotyping of IFN-α producing cells by flow cytometry

For flow cytometry (FCM), PBMC (5×10^6 cells/ml) were stimulated in 6-ml cultures in 6-well plates (Nunc) with ODN 2216 (5 μg/ml), ADV or lipofected pcDNA3. Non-stimulated cells, incubated in plain growth medium, were used as controls. Cells for FCM analyses were cultured for 9 h, the last 4 h in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich) to increase intracellular concentrations of IFN-α. Thereafter, cells were incubated for 5 min on ice, harvested with a sterile cell-scraper and washed twice in ice-cold PBS before dilution in PBS supplemented with 1% SPF pig serum. For simultaneous detection of surface markers and presence of intracellular IFN-α, cells were first incubated with antibodies detecting surface molecules by direct or indirect immunofluorescence (IF), then fixed and permeabilized and subsequently stained for IFN-α. In brief, 5×10^6 cells were incubated for 30 min on ice in the dark in a total volume of 100 μl with mAbs detecting SWC3 (IgG1, 2.5 μg/ml, clone 74-22-15; VMRD, Pullman, WA), CD2 (IgG2a, 2.5 μg/ml, clone MSA4; VMRD), CD3 ($IgG2a^{FITC}$, 5 μg/ml, clone BB23-8E6-8C8; BD PharMingen, San Diego, CA), CD4 ($IgG2b^{FITC}$, 5 μg/ml, clone 74-12-4; BD PharMingen), CD14 ($IgG2b^{FITC}$, diluted 1:10, clone MY4; Beckman Coulter, Miami, FL), CD21 (IgG1, 5 μg/ml, clone BB6-11C9; VMRD), MHC class II (IgG2a, 1 μg/ml, clone H42A; VMRD) or isotype controls IgG1, IgG2a, $IgG2a^{FITC}$ (Dako) or $IgG2b^{FITC}$ (BD Pharmingen). After washing, cells subjected to indirect IF were stained for 30 min on ice in the dark with FITC-conjugated goat-anti mouse Ig (Dako) diluted 1:200 in PBS supplemented with 0.1% HSA, washed and fixed in 1% paraformaldehyde, whereas directly labelled cells were fixed immediately. Cells were thereafter stored at 4 °C overnight.

For intracellular detection of IFN-α, fixed cells were washed and incubated for 30 min at RT in PBS supplemented with 0.1% Tween-20 (Sigma-Aldrich) for permeabilization and 5 μg/ml mouse Ig (Caltag Laboratories, Burlingame, CA) and 10% SPF pig serum to reduce nonspecific binding. The cells were then washed and incubated for 30 min with a biotinylated mAb directed to poIFN-α (Clone F:17, Lefevre et al., 1990) or with a biotinylated mouse isotype control (Dako), both at 0.3 μg/ml in PBS supplemented with 0.1% Tween-20 and 1% SPF pig serum. The cells were then washed in PBS with 0.1% Tween-20 and 0.1% HSA and incubated for 30 min in the dark with phycoerythrin (PE)-conjugated streptavidin (Jackson Immunoresearch Laboratories, West Grove, PA), diluted 1:1000 in PBS supplemented with 0.1% Tween-20 and 0.1% HSA. After washing, 5×10^5 cells/sample were acquired and analysed in FL-1 (FITC-detection) and FL-2 (PE-detection) using a FACScan flow cytometer and the CellQuest software (Becton Dickinson, San Jose, CA). A limit distinguishing PE positive (i.e. IPC) from PE negative cells was determined using non-induced cells and the biotinylated mAb F17 and also by using induced cells stained with a biotinylated isotype control in combination with PE-conjugated streptavidin as described above. This limit was set to exclude all but 0.01% of the unstimulated cells. Cells above this limit were defined as IPC. The expression of surface molecules on IPC was analysed by comparison to IPC stained with isotype control. To further define the phenotype of the IPC, their labelling intensity for the various surface markers was related to that of the entire PBMC population.

2.9. Statistical analyses

Data are presented as mean values ±S.E.M. if not otherwise indicated. When appropriate, the significance of differences between IFN-α responses to various ODNs was determined using the Wilcoxon signed rank test (StatView 5.0, Abacus Concepts, Berkeley, CA).

3. Results

3.1. Establishment of conditions for IFN-α induction by phosphodiester ODN H-I

The IFN-α inducing capacity of the selected ODNs was tested in cultures of poPBMC. Initially, optimal

conditions were determined for the ds form of the phosphodiester ODN H-I (dsODN H-I) that previously has been evaluated as an IFN-α inducer in cultures of human PBMC (Magnusson et al., 2001b). The concentration of dsODN H-I was varied from 5 to 100 μg/ml in combination with different concentrations of lipofectin (2.5–50 μg/ml). The IFN-α production increased with increasing concentrations of dsODN H-I and reached a plateau level at 50–100 μg ODN/ml (Fig. 1A).

At all ODN concentrations tested, the highest levels of IFN-α were detected when the dsODN H-I was used with 2.5 μg/ml lipofectin. Increasing the levels of lipofectin seemed to have an inhibitory effect on the IFN-α induction. No IFN-α production was detected in culture supernatants collected from cells exposed to dsODN H-I that had not been pre-incubated with lipofectin, regardless of ODN concentration. For a closer titration, 25 μg/ml of dsODN H-I was pre-incubated with different concentrations of lipofectin ranging from 0.08 to 5 μg/ml (Fig. 1B) and again the optimal concentration of lipofectin was found to be 2.5 μg/ml. Based on these results, further IFN-α inductions were performed with 25 μg/ml of ODN, using a final concentration of 2.5 μg/ml of lipofectin, if not otherwise indicated. During these conditions, the levels of IFN-α induced by dsODN H-I were comparable to those induced by lipofected pcDNA3, lipofected *A. pleuropneumoniae* or ADV (Fig. 1C). Lipofectin by itself did not induce any production of IFN-α (Fig. 1A and B).

3.2. Modifications in the central CpG dinucleotides affect the IFN-α induction by phosphodiester ODNs

The IFN-α inducing capacity of ssODN H, and the complementary ssODN I, was compared to that of dsODN H-I, all at a final concentration of 25 μg/ml, pre-incubated or not in lipofectin. Furthermore, to study if alteration of the unmethylated CpG in these diester ODNs affected their IFN-α inducing ability, a panel of ODNs resembling ODN H and ODN I, but with modified CpGs (see Table 1) was tested. It was found that the IFN-α levels induced by ODN H or ODN I in the ss form were 2- to 10-fold lower ($P < 0.05$) than that induced by the dsODN H-I (Fig. 2). Therefore, each modification of these ODNs was also tested in the ds form. None of the ODNs shown in Fig. 2 induced IFN-α in the absence of lipofectin (data not shown).

Alteration of the CpG to ApG (ODN H^{A}), or to CpT (ODN I^{T}) essentially abolished the IFN-α levels induced both by the ss ($P < 0.05$) and ds (ds ODN H^{A}-I^{T}) forms. Inversion of the CpG to GpC (ODN H^{GC}, ODN I^{GC} and dsODN H^{GC}-I^{GC}) did not completely abolish, but strongly reduced the levels of IFN-α induced ($P < 0.05$), although the magnitude of decrease varied between individuals. Furthermore, inversion of CpG to GpC principally abolished the IFN-α production by ODN A2 (ODN A2 391 ± 165 U/ml versus ODN $A2^{GC}$ 3 ± 3 U/ml; $n = 4$), which in both forms had immunostimulatory capacity in vivo in pigs (Van der Stede et al., 2002). Methylation of the cytosine in the CpG (ODN H^{met} and ODN I^{met}) severely hampered the IFN-α inducing capacity (ODN H^{met} versus ODN H, $P < 0.05$) and substantial decreases in the IFN-α levels were also observed when the ds form (H^{met}-I^{met}) was used for induction (Fig. 2). In all cases, the magnitude of decrease in comparison with unmethylated ODNs varied between individuals. Taken together, the presence of unmethylated CpG dinucleotides strongly influences the IFN-α inducing ability of phosphodiester ODNs, when tested in cultures of poPBMC.

3.3. Comparison of the IFN-α inducing ability of phosphodiester/phosphorothioate ODNs and their corresponding phosphodiester ODNs

To study if the presence of phosphorothioate nucleotides in the backbone of an ODN affected its IFN-α inducing capacity, thioate/diester ODNs, and the corresponding ODNs with a complete phosphodiester backbone, were used (Table 1). All ODNs were tested in ss form at 5 and 25 μg/ml in the presence or absence of lipofectin.

For all ODNs tested, a large variation in the IFN-α production between pigs was observed. In general, ODNs with thioate/diester backbone (ODNs 2216 and D19) induced high levels of IFN-α, regardless of whether they were pre-incubated with lipofectin or not (Fig. 3). The IFN-α response to these ODNs, especially ODN 2216, seemed better at 5 μg ODN/ml than 25 μg ODN/ml. When the thioate/diester backbone of ODNs 2216 and ODN D19 were altered to exclusively consist of phosphodiester nucleotides,

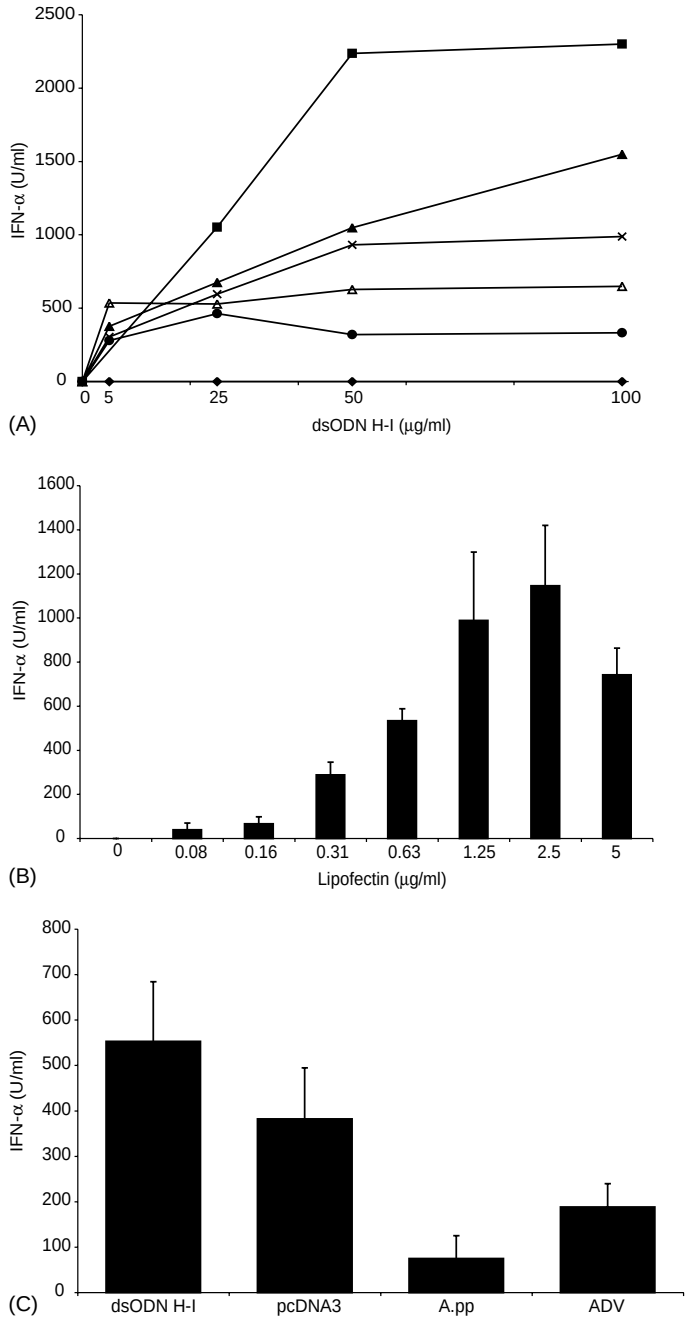

Fig. 1. Evaluation of the relative importance of lipofectin and ODN concentration in the activation of IFN-α production by dsODN H-I in cultures of poPBMC. Five to 100 μg/ml dsODN H-I was pre-incubated with 0 μg/ml (–◆–), 2.5 μg/ml (–■–), 5 μg/ml (–▲–), 10 μg/ml (–×–), 25 μg/ml (–△–) or 50 μg/ml (–●–) of lipofectin (A), or 25 μg/ml of dsODN H-I was pre-incubated with ascending concentrations (from 0.08 to 5 μg/ml) of lipofectin (B). The levels of IFN-α induced with 25 μg/ml of dsODN H-I and 2.5 μg lipofectin/ml was compared to those induced by lipofected pcDNA3, lipofected *A. pleuropneumoniae* or ADV (C). The levels of IFN-α (U/ml) in culture supernatants were determined with immunoassay after 20 h. Representative data obtained from one of three pigs tested are shown in A whereas results are shown as mean ± S.E.M. of four animals in B and C.

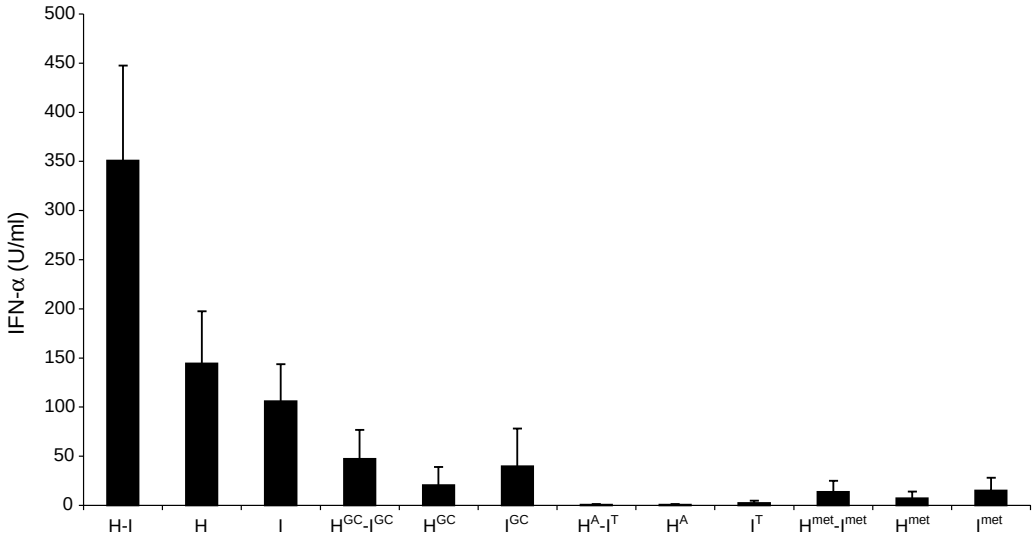

Fig. 2. Importance of unmethylated CpG motifs in ODNs for the induction of IFN-α production in cultures of poPBMC. The CpGs of the diester dsODN H-I, ssODN H or ssODN I were modified by base substitutions or by methylation of the cytosine in the CpG (see Table 1). All ODNs were used at a concentration of 25 μg/ml and in the presence of 2.5 μg/ml lipofectin. The levels of IFN-α (U/ml) in culture supernatants were determined with immunoassay after 20 h. Results are shown as mean ± S.E.M. of six animals or in the case of H^{A}-I^{T}, I^{met} and H^{met}-I^{met} four animals. No IFN-α was detected without pre-incubation of ODN with lipofectin (results not shown).

again an IFN-α production was detected regardless of pre-incubation with lipofectin. Furthermore, these diester ODNs induced similar amounts of IFN-α when tested at 5 or 25 μg/ml. Taken together, the backbone composition (thioate/diester versus diester) of these particular ODNs was not decisive for the IFN-α inducing capacity of the ODN.

3.4. Effects of poly-guanine sequences on the IFN-α inducing ability of ODNs

A common feature of all the ODNs that induced IFN-α production in the absence of lipofectin was the presence of a poly-guanine (poly-G) sequence in thioate or diester form in the 5′ and 3′ ends. In accordance, the IFN-α inducing capacity of another CpG-ODN containing a thioate/diester backbone and poly-G sequences (ODN MM1; Table 1) was independent of lipofectin and 5 μg/ml was the most efficient concentration of this ODN for IFN-α induction. The results from four pigs (mean value ± S.E.M.) showed that 5 μg ODN/ml induced 1900 ± 600 U IFN-α/ml in the presence of lipofectin and 2184 ± 554 U IFN-α/ml without lipofectin, whereas 25 μg ODN/ml induced 1122 ± 357 U IFN-α/ml and 1062 ± 405 U IFN-α/ml with and without lipofectin, respectively.

To evaluate the effects of poly-G sequences on phosphodiester ODNs, various numbers of guanines were added to the ssODN H (ODNs $H^{G\text{-tail }1}$, $H^{G\text{-tail }2}$ and $H^{G\text{-tail }3}$; see Table 1), and the modified ODNs were tested for their ability to induce IFN-α production (Fig. 4). In the absence of lipofectin, no or very low levels of IFN-α were detected in cultures stimulated by ODN H or by ODN H that contained additional guanines. However, the presence of poly-G sequences strongly increased, approximately 1.5- to 5-fold, the IFN-α inducing capacity, of ODN H when pre-incubated with lipofectin. This enhancing effect of poly-G sequences on the IFN-α induction was observed both when the ODNs were tested at 5 and 25 μg/ml. Thus, the addition of poly-G sequences does not eliminate the need for lipofectin, but seems to increase the levels of IFN-α induced by the ODN.

3.5. Analyses of the IFN-α producing cells by flow cytometry and in situ hybridization

The in vitro results using poPBMC demonstrated considerable differences in IFN-α inducing capacity

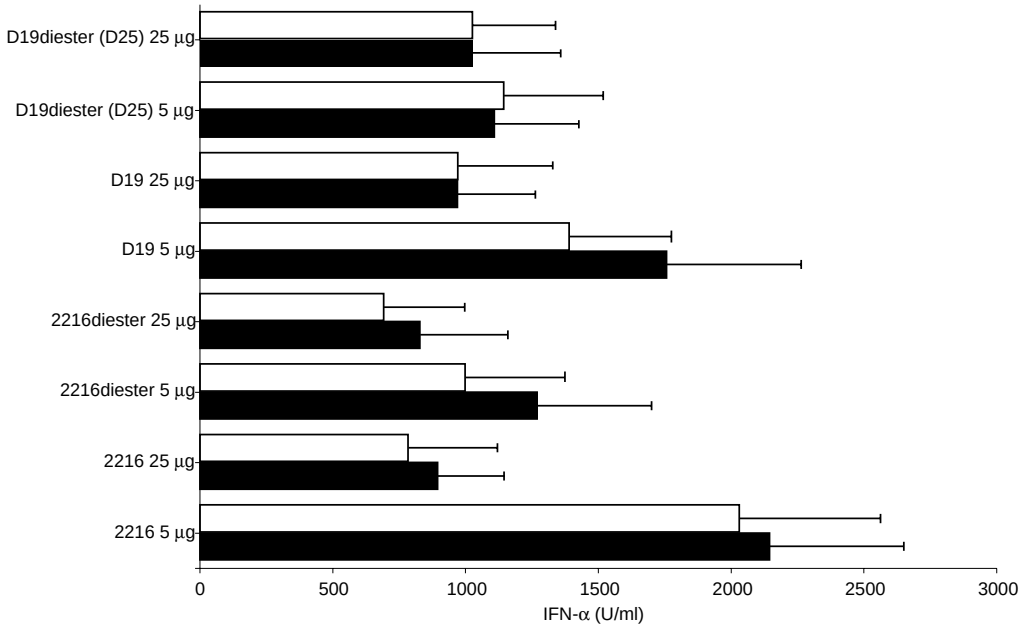

Fig. 3. IFN-α inducing ability of ssODNs containing phosphodiester nucleotides flanked by poly-G sequences in phosphorothioate or phosphodiester form. The ODNs D19, D19diester (D25), 2216 and 2216diester were tested at 5 or 25 µg/ml in cultures of poPBMC. The ODNs were pre-incubated (black bars) or not (white bars) with lipofectin. The sequences of the ODNs are described in Table 1. The levels of IFN-α (U/ml) in culture supernatants were determined with immunoassay after 20 h. Results are shown as mean ± S.E.M. of four animals. The corresponding IFN-α values for lipofected dsODN H-I were 1281± 924 U/ml using 25 µg/ml ODN and 852 ± 823 U/ml using 5 µg/ml ODN, respectively. No IFN-α was induced by dsODN H-I in the absence of lipofectin.

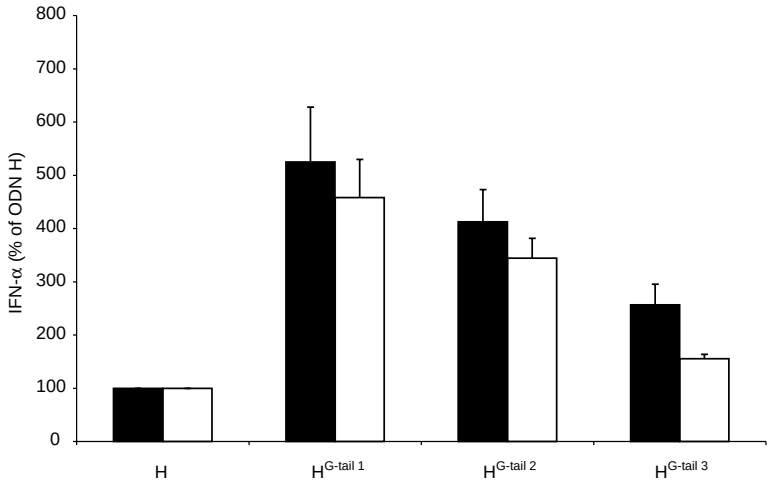

Fig. 4. Effect of addition of phosphodiester poly-G sequences to ODN H on its ability to induce IFN-α production in cultures of poPBMC. All ODNs were pre-incubated with lipofectin and tested at 25 µg/ml (black bars) or 5 µg/ml (white bars). No IFN-α was detected without pre-treatment of the ODN with lipofectin. The sequences of the poly-G-modified ODNs are described in Table 1. The levels of IFN-α (U/ml) in culture supernatants were determined with immunoassay after 20 h. Results are expressed as percent of the levels of IFN-α induced by the unmodified ODN H (% of ODN H) and represent mean ± S.E.M. of four animals.

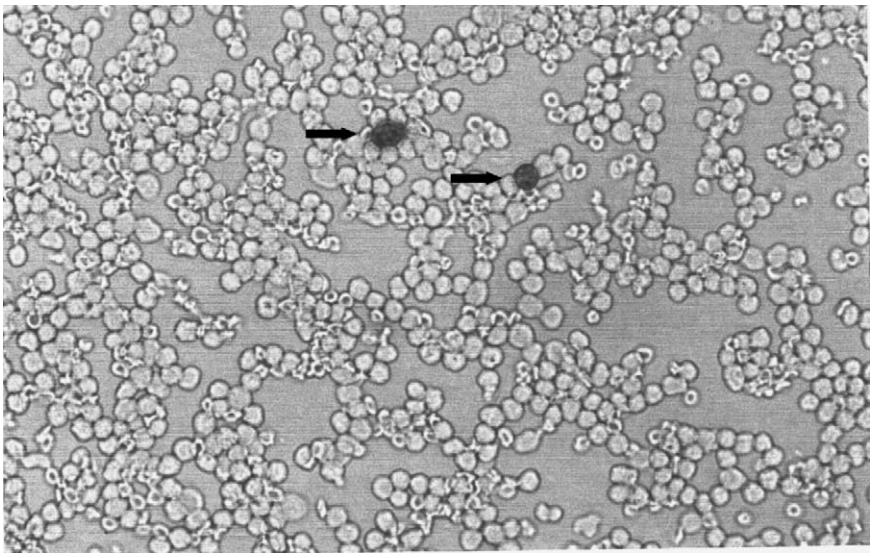

Fig. 5. Detection of cells expressing porcine IFN-α mRNA by in situ hybridization after stimulation of poPBMC with ODN 2216 for 6 h. Cells positive for IFN-α mRNA are indicated with black arrows. A representative result of four experiments is shown.

of the various inducers. Generally, the IFN-α levels induced by the most potent ODN (2216) or bacterial plasmid DNA (pcDNA3) far exceeded the levels of IFN-α induced by the virus ADV. In order to determine if the difference was due to different numbers of IPC and/or different types of IPC activated, i.e. monocytes or NIPC/PDC, the IPC responding to these various types of stimuli were characterized using FCM and in situ hybridization.

Regardless of inducer used, in situ hybridization for detection of IFN-α mRNA consistently demonstrated a uniformly strong staining of cells (Fig. 5 and data not shown) that constituted $\leq$0.1% of total poPBMC. No positive cells were detected among unstimulated PBMC, or among induced cells using the control sense biotin-poIFN-α_1 probe (result not shown). The number of IPC activated was further analysed by detection of intracellular IFN-α by FCM analysis (Fig. 6). When poPBMC were stimulated with ODN 2216 or lipofected pcDNA3, 0.17% (median value, range: 0.02–0.28%, $n = 8$) and 0.07% (median value, range: 0.02–0.13%, $n = 4$) IPC were detected, respectively. In contrast, the proportion of IPC detected at induction with ADV was only 0.02% (median value, range: 0.02–0.03%, $n = 4$). At induction with ODN 2216 or pcDNA3, the light scatter analysis showed that the major population of IPC consisted of relatively small cells with low granularity (right column, Fig. 6).

Simultaneous detection of intracellular IFN-α and surface molecules on PBMC stimulated with either CpG-ODN (2216) or bacterial plasmid DNA (pcDNA3 plus lipofectin) revealed that the majority of IPC (filled line, Fig. 7) were positive for CD2, CD4, MHC class II and SWC3, but lacked CD3, CD14 and CD21. The CD2 and CD4 expression by the IPC was similar to those of the major CD2 and CD4 positive populations among all poPBMC (shaded grey, Fig. 7). In contrast, the staining intensity for SWC3 and MHC class II on the IPC was weaker than that of the major PBMC populations expressing these molecules. The low proportion of IPC detected at induction with ADV precluded a phenotypical analysis of these cells.

4. Discussion

The importance of a central unmethylated CpG for the induction of IFN-α production by poPBMC was evaluated in the present study using the ODN H. Substituting the CpG with other bases or methylation of the CpG strongly reduced or essentially abolished the IFN-α induction. Furthermore, the IFN-α production was abolished after inversion of the CpGs to GpC

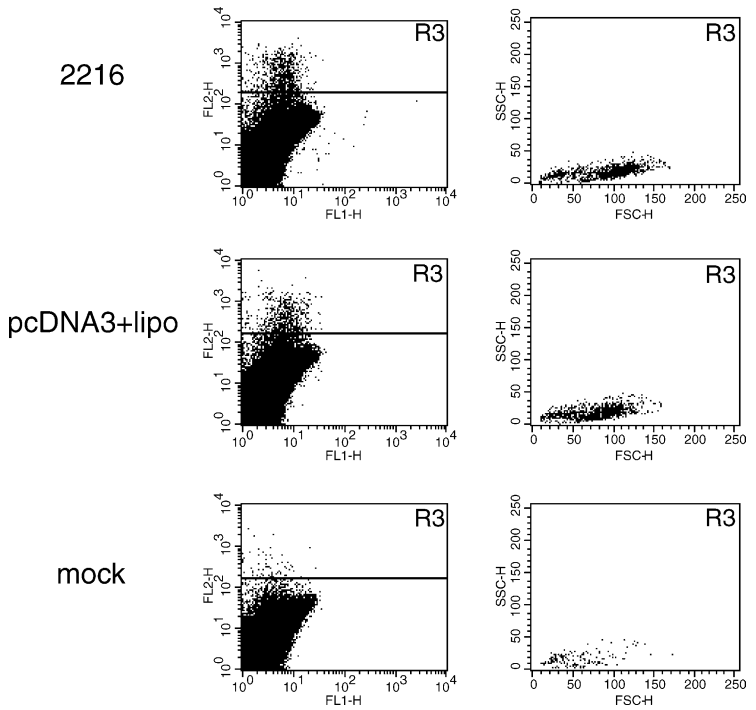

Fig. 6. Detection of porcine IFN-α producing cells by flow cytometry. PBMC were induced for 9 h and brefeldin A was added at 5 h. The cells were stained for intracellular IFN-α (PE, FL2) as described in Section 2 and analysed in FL1 and FL2 (left column). The gate for IFN-α positive cells (R3) was defined by comparison to mock-stimulated cells (mock). The FSC/SSC characteristics of the IPC as defined by the gate R3 is shown in the right column. The results from one pig stimulated with ODN 2216 (0.21% IPC) or pcDNA3 + lipofectin (0.13% IPC) or mock (0.01% IPC) are shown.

in ODN A2. However, the IFN-α inducing capacity was partly retained after inversion of the CpG to GpC in ODN H and ODN I which is in line with previous results using human PBMC (Magnusson et al., 2001b). It is in this context noteworthy that, after inversion of the CpGs to GpC, the ODN A2 retained its ability to enhance ovalbumin specific antibody production and cell proliferation when injected in pigs (Van der Stede et al., 2002), but not its protective effect at challenge with *Listeria monocytogenes* in mice (Klinman et al., 1999). Thus, the importance of CpG motifs may differ for immune parameter studied. The activity of a given ODN may also be influenced by species differences in the recognition of immunostimulatory DNA (Takeshita et al., 2001).

The induction of IFN-α by the phosphodiester ODNs H, I and A2 required pre-incubation of the ODN with lipofectin, which is in accordance with earlier results using human cells (Magnusson et al., 2001b). Lipofectin forms cationic liposomes with nucleic acids which increases cellular uptake of ODN (Bennett et al., 1992; Hartmann et al., 1998). The need for lipofection therefore suggests that cellular uptake of ODN is required for IFN-α induction, which is in line with the finding that endocytosis of CpG-ODNs is required for their immune stimulatory effect (Ahmad-Nejad et al., 2002; Häcker et al., 1998) and that stimulatory CpG ODNs interact with TLR-9, localized in intracellular compartments (Ahmad-Nejad et al., 2002).

Another possible explanation for the effect of lipofectin is protection of DNA/RNA from degradation by exonucleases (Thierry and Dritschilo, 1992) which also can be achieved by replacing phosphodiester nucleotides with nuclease-resistant phosphorothioate nucleotides (Agrawal et al., 1995; Pisetsky and Reich, 1999; Zhao et al., 1993). In the present study, CpG-ODNs with phosphorothioate nucleotides at their ends, i.e. ODNs 2216, D19 and MM1, were potent inducers of IFN-α in poPBMC even without

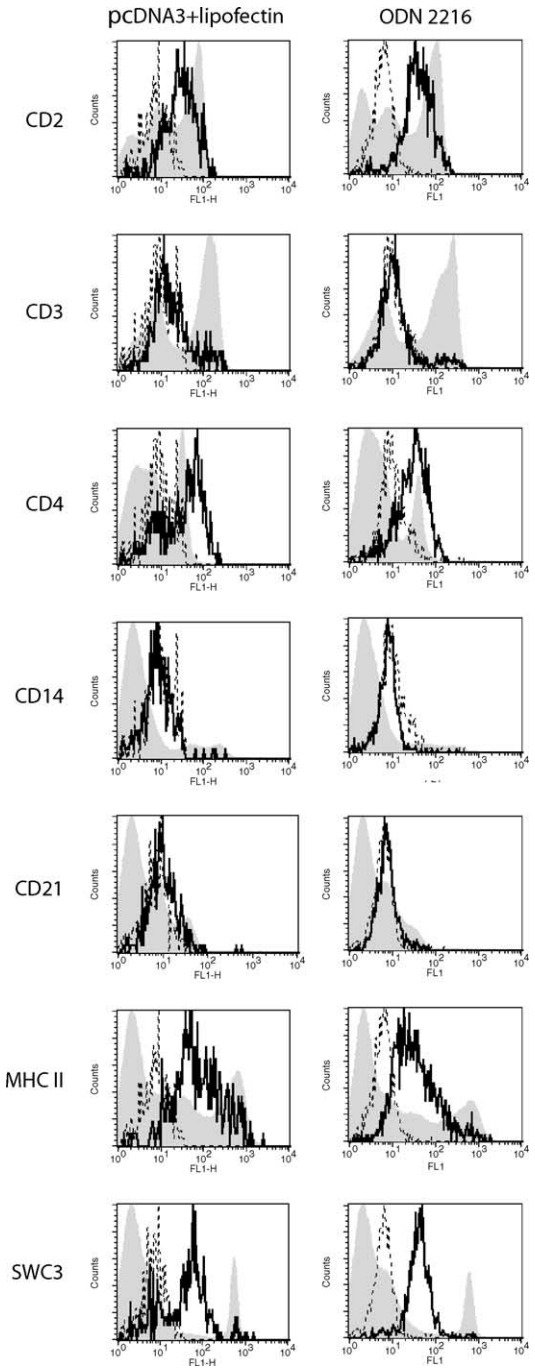

Fig. 7. Flow cytometric characterization of the phenotype of poPBMC producing IFN-α at induction with ODN 2216 or lipofected pcDNA3 by simultaneous detection of surface molecules (FITC, FL1) and intracellular IFN-α (PE, FL-2). The PBMC were harvested after 9 h (brefeldin A was added at 5 h) and stained as described in Section 2. Cells expressing IFN-α according to the gate definition described in Fig. 6 were analysed for the indicated cell surface molecule (filled line) and compared to cells expressing IFN-α stained with the corresponding isotype control (dotted line). To relate the staining intensity of IPC to that of other cells, the entire cell population stained for the indicated cell surface marker is shown in each graph (shaded grey). For each cell population, the scale is normalized to the channel with the highest number of cells. The results from the two experiments shown (ODN 2216 or pcDNA3 + lipofectin as inducer) were repeated twice with 2216 as inducer and three times with pcDNA3 as inducer with similar results.

pre-incubation with lipofectin. However, the same ODNs synthesized with a complete phosphodiester backbone also induced IFN-α without pre-incubation with lipofectin. One reason for this may be that these ODNs have flanking poly-G sequences that may form quaternary structures that enhance uptake, possibly by binding to scavenger receptors (Kimura et al., 1994; Lee et al., 2000; Pearson et al., 1993; Pisetsky, 1996). It was therefore tested if the addition of poly-G sequences could affect the IFN-α inducing ability of ODN H. The addition of guanines at the 3′ end of ODN H increased the IFN-α induction and reduced the concentration of ODN needed but did not eliminate the need for lipofectin. Thus, the exact role of poly-G sequences or phosphorothioates in the activation of IFN-α is still elusive. It is however notable that ODNs 2216, D19 and MM1, in contrast to ODN H with G-tails, all contain a 10-base long self-complementary nucleotide sequence that may form a ‘‘hair pin’’ structure. Presence of such structures can increase the stability against nucleases and seems to be important for IFN-α induction in human PDC (Cong et al., 2003), and could thus explain why ODNs 2216, D19 and MM1 induced IFN-α production in poPBMC in the absence of lipofectin. However, poly-G sequences as such can also have direct immunostimulatory effects, for example, hexamers of guanines can induce IL-1, IL-6, IL-10 and IL-12 in human PBMC (Filion et al., 2003) and small modifications of a guanine can render the single nucleotide immunostimulatory (Lee et al., 2003). Interestingly, the induction of a protective Th1 type of immunity in a model of murine leishmaniasis, using phosphodiester CpG-ODNs, was dependent on poly-G sequences (Dalpke et al., 2002; Lee et al., 2000). The use of phosphodiester ODNs instead of phosphorothioate ODNs could be beneficial to

avoid side effects such as long-lasting IFN-γ and IL-12 production, that can be harmful or even break tolerance (Segal et al., 2000). The identification of phosphodiester ODNs that were potent inducers of IFN-α production by poPBMC may therefore be valuable for future use as adjuvant components. Although a large variation was observed between individual pigs in the magnitude of IFN-α production, the different IFN-α inducers showed the same relative potency in each pig. Thus, it should be possible to predict the adjuvant activity of ODNs in pigs by comparing their IFN-α inducing capacity in poPBMC cultures.

Our estimation of the frequency of porcine IPC in the present study revealed that up to 0.3% of the PBMC responded to lipofected plasmid DNA or ODN 2216. This frequency is similar to the frequency of human NIPC/PDC that constitute less than 0.3% of PBMC (Dzionek et al., 2000; Siegal et al., 1999; Svensson et al., 1996) and the murine counterpart that represent 0.2–0.5% of cells from spleen, bone marrow or lymph nodes (Asselin-Paturel et al., 2001). In contrast, approximately 10-fold lower numbers of IPC were detected among ADV-induced poPBMC, which is in line with earlier studies using either TGEV (Nowacki and Charley, 1993) or ADV (Artursson et al., 1995) as inducer. The superior ability of CpG-ODN or lipofected plasmid DNA to induce production of IFN-α in comparison with the virus ADV may therefore be due to the increased number of cells activated.

Phenotypic analysis of the IPC that respond to ODN 2216 or lipofected plasmid DNA showed that they did not express CD21 or CD3, which implies that they are not B- or T-cells, a feature shared with human and murine NIPC/PDC. The porcine IPC were also clearly negative for the myeloid/monocytic marker CD14, but they showed an intermediate expression of SWC3 and MHC class II and a uniform expression of CD4, which is in line with earlier phenotyping of porcine TGEV-activated IPC (Riffault et al., 1997, 2001). Furthermore, a recent phenotyping of poPBMC revealed that TGEV-activated IPC were found among SWC3+ cells and that they expressed CD4 but not CD14 or T- or B-cell markers (Summerfield et al., 2003). Taken together, the IPC responding to CpG-DNA in the present study phenotypically resemble the IPC responding to TGEV.

The SWC3 is regarded as a pan-myeloid porcine marker (Summerfield and McCullough, 1997), that is present on 80–90% of peripheral blood monocytes and granulocytes, but also on 5% of lymphocytes (Thacker et al., 2001). This precludes a clear assignment of porcine IPC as myeloid or lymphoid. The hematopoietic lineage origin of murine NIPC/PDC is also unclear (Björck, 2002), whereas the human NIPC/PDC most likely are of lymphoid origin (Rissoan et al., 2002; Schotte et al., 2003). However, it has also been suggested that human NIPC/PDC can convert to myeloid-like cells (Comeau et al., 2002). The porcine IPC responding to CpG-DNA also expressed CD2, seemingly in contrast to results of early studies where depletion of CD2+ cells did not hamper the ability of poPBMC to produce IFN-α in response to TGEV (Nowacki and Charley, 1993). The reason for this discrepancy is not known. The CD2 expression by porcine IPC is however in line with the expression of CD2 on human NIPC/PDC (Comeau et al., 2002). The CD2 molecule is expressed by many different cell types including porcine T- and NK-cells (Yang and Parkhouse, 1996), but also on porcine thymic dendritic cells (Salmon et al., 2000) and human monocytic precursors of dendritic cells (Di Pucchio et al., 2003). However, the porcine IPC activated by CpG-DNA are not likely monocytic DC because monocytes or monocyte-derived porcine DC did not produce IFN-α upon treatment with CpG-DNA (Johansson et al., 2003), which is in line with the finding that human monocytes or monocyte-derived DC cannot produce IFN-α in response to CpG-DNA (Kadowaki et al., 2001a; Krug et al., 2001b; Magnusson et al., 2001b).

The present study shows that DNA in the form of ODNs containing unmethylated CpG is a potent inducer of IFN-α in porcine leukocytes, which confirm our earlier findings using plasmid DNA (Johansson et al., 2002; Magnusson et al., 2001a). Double labelling for intracellular IFN-α and various cell surface markers showed that the porcine IPC activated by immunostimulatory DNA exhibit many similarities to the human NIPC/PDC. It is thus likely that the porcine IPC that respond to the phosphorothioate CpG-ODN 2216 or lipofected plasmid DNA constitute the porcine counterpart to the NIPC/PDC earlier described in man and mice. Furthermore, phosphodiester CpG-ODNs were identified that efficiently induced IFN-α production in poPBMC. More detailed analyses of the essential interferogenic DNA structures and the way

they activate porcine NIPC/PDC may therefore result in more efficient vaccine adjuvants.

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