

Monoclonal antibodies to equine interferon- α (IFN- α): New tools to neutralize IFN-activity and to detect secreted IFN- α

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Abstract

Interferon- α (IFN- α) is a type I interferon that is secreted during the early stages of the innate immune response and is often induced upon infection with viral pathogens. IFN- α production affects multiple downstream events influencing both innate and adaptive immune responses. Here, we describe the expression of an equine rIFN- α /IgG4 fusion protein in mammalian cells. The anti-viral activity of rIFN- α /IgG4 was found to be 70-fold higher than that of a previously described IFN- γ /IgG1 as tested by bioassay. The purified rIFN- α was subsequently used for the generation of six monoclonal antibodies (mAbs) to equine IFN- α . Four of these mAbs inhibited the protective anti-viral effect of equine leukocyte IFN in bioassays. One mAb (clone 240-2) showed a high-neutralizing capacity. An ELISA was established using two anti-equine IFN- α mAbs (clones 29B and 240-2) and its analytical sensitivity for was found to be around 800 pg/ml and 3 U/ml for rIFN- α and equine leukocyte IFN, respectively. When analyzing samples with a likely dominance of IFN- α among type I IFNs, such as supernatants from equine peripheral blood mononuclear cells stimulated with CpG-oligodeoxyribonucleotides, the results obtained by ELISA and IFN bioassay showed a high agreement ($r_{sp}^2 = 0.98$). When analyzing samples likely containing a mixture of type I IFNs, such as serum and nasal secretions from virally infected horses, the ELISA only detected some of the IFN-activity recorded in the bioassay. Overall, the data showed that the new anti-equine IFN- α mAbs are valuable tools to detect native IFN- α for further characterization of the early innate immune response and anti-viral immunity in horses.

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1. Introduction

Type I interferons (IFNs) belong to a family of closely related proteins encoded by intronless genes and include IFN- α , IFN- β and IFN- ω . Their production is induced during the early stages of the innate immune response in particular to viral infections. The first discovered and most extensively studied feature of type I IFNs is their ability to inhibit viral infection of, and

Abbreviations: CHO cells, Chinese hamster ovary cells; CpG-ODN, CpG-oligodeoxyribonucleotide; DC, dendritic cell; MDBK cells, Madin–Darby bovine kidney cells; mDC, myeloid dendritic cell; ODN, oligodeoxyribonucleotide; pDC, plasmacytoid dendritic cell; TLR, Toll-like receptor; VSV, vesicular stomatitis virus.

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replication in, cells by inducing the so-called anti-viral state (Samuel, 2001). These direct anti-viral effects are however far from the sole effects mediated by type I IFNs on the immune system because they induce and affect many downstream events in both innate and adaptive immune responses. They are for instance major inducers of immature dendritic cells (DCs), favor the induction and maintenance of Th1-cells, regulate the expression of various immune mediators and MHC class I molecules, and induces cross-priming by DC (Theofilopoulos et al., 2005; Le Bon and Tough, 2007). In humans, the most potent producers of interferon- α (IFN- α) identified are plasmacytoid dendritic cells (pDCs), also termed natural IFN producing cells. Human pDCs are able to produce 10–100 times more type I IFN than other cell types (Fitzgerald-Bocarsly et al., 2008). Other cells of both non-hematopoietic and hematopoietic origin such as monocytes, were also found to produce IFN- α and virtually every cell type can be triggered to produce other type I IFNs, mainly IFN- β , in response to infection with viral or bacterial pathogens (Liu, 2005). High production of type I IFNs is often mediated by Toll-like receptors (TLRs) in response to dsRNA (TLR3), ssRNA (TLR7 or TLR8), lipopolysaccharide (TLR4), and prokaryotic unmethylated CpG-DNA (TLR9; Theofilopoulos et al., 2005). In humans, IFN- α has extensively been used for treatment of patients with some types of cancer and viral diseases (Ferrantini et al., 2007). Type I IFNs also play a pivotal pathogenic role in the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus and insulin-dependent diabetes mellitus (Theofilopoulos et al., 2005).

In the horse, earlier studies have detected IFN-activity both *in vivo* and *in vitro*. For instance, IFN-activity in serum and nasal secretions from horses was observed after experimental infections with equine herpesvirus type 1 (EHV-1) and type 4 (EHV-4) (Edington et al., 1989a, Chong and Duffus, 1992) and during natural or experimental equine influenza A2 infections (Jensen-Waern et al., 1998, Watrang et al., 2003). Anti-viral IFN-activity was also detected after *in vitro* stimulation of equine cell cultures with Newcastle disease virus, EHV-1, Aujeszky's disease virus, polyinosine-polycytidylic acid in combination with DEAE-dextran or with lectins (Yilma et al., 1982; Edington et al., 1989b; Jensen-Waern et al., 1998). In an early study of the equine type I IFN genes (Himmler et al., 1986) the sequences of four IFN- α , one IFN- β , and two IFN- ω genes were presented and based on results from Southern blotting a minimum of seven IFN- α , two IFN- β and eight IFN- ω genes were hypothesized. The four

cloned equine IFN- α genes shared a high-nucleotide sequence similarity and resulted in predicted mature proteins of 161 amino acids. The similarity between equine and human IFN- α mature proteins was 71–77% and equine *r*IFN- α 1 and *r*IFN- α 2 expressed in *E. coli* mediated anti-viral activity in both equine skin epidermis and human lung carcinoma cell lines (Himmler et al., 1986). In clinical studies, human *r*IFN- α was tested for oral treatment in horses to prevent or cure viral infection. In Standardbreds with inflammatory airway disease, *r*IFN- α was given in addition to rest. A significant lower relapse rate was observed in horses treated with *r*IFN- α compared to the placebo (Moore et al., 2004). In contrast, human *r*IFN- α prophylaxis in weanlings experimentally infected with EHV-1 did not reduce viral shedding or clinical signs of disease (Seahorn et al., 1990).

In this manuscript, we describe the production of recombinant equine IFN- α 1 (*r*IFN- α) in a mammalian expression system. Subsequently, the *r*IFN- α was used to produce monoclonal antibodies (mAbs) to equine IFN- α that were characterized in a bioassay to neutralize the anti-viral effect of equine leukocyte IFN and in an ELISA to detect native equine IFN- α in preparations of equine leukocyte IFN, supernatants from *in vitro* stimulated equine leukocytes or sera and nasal secretions from horses.

2. Materials and methods

2.1. Construction of the IFN- α expression vector and establishment of a stable transfectant

The equine IFN- α gene was amplified from the plasmid pAH50 (Himmler et al., 1986) containing the coding sequence of the equine IFN- α 1 gene (GenBank accession A33683). Primers for PCR amplification contained a *Xho*I and a *Bam*HI site for cloning of the 570 bp IFN- α cDNA into the expression vector. The PCR was performed as previously described (Wagner et al., 2005). The IFN- α cDNA was cloned into the expression vector pcDNA/IGHG4 (Wagner et al., 2008). The construct pcDNA-IFN- α /IGHG4 contained a sequence encoding an enterokinase digestion motif between the IFN- α gene and the equine IgG4 heavy chain constant region gene. The construct was used to transfect Chinese hamster ovary (CHO) cells and to generate a stable transfectant. The stable transfectant secreted the *r*IFN- α /IgG4 fusion protein as detected by ELISA and flow cytometry. All steps of the procedure were performed as previously described in detail for other IgG fusion proteins (Wagner et al., 2005).

2.2. IFN bioassay and neutralization of IFN-activity

Interferon activity was detected with a vesicular stomatitis virus (VSV) cytopathic effect inhibition bioassay on Madin–Darby bovine kidney (MDBK) cells performed as previously described for equine type I IFNs (Jensen-Waern et al., 1998; Watrang et al., 2003). In brief, confluent monolayers of MDBK cells in flat-bottomed microtiter plates were incubated with samples for 24 h at 37 °C, 7% CO₂. Afterwards, the samples were replaced by live VSV in an amount sufficient to cause complete destruction of unprotected cells over 24 h. After another 24 h of incubation, residual cells were stained with crystal violet and the IFN-activity in the samples was calculated by defining 1 unit (U) IFN as the amount protecting 50% of the cells in one well from VSV cytopathic effect. Biological activity of equine *rIFN-α*/IgG4 (85 ng/ml), *rIFN-γ*/IgG1 and *rIL-2*/IgG1 (both 500 ng/ml), respectively, was assessed in this assay by twofold serial dilutions starting at dilution 1:2. Laboratory standards of equine leukocyte IFN (Jensen-Waern et al., 1998) and human IFN- α were included on every test plate to calibrate the assay.

Monoclonal antibodies raised to purified equine *rIFN-α* (see below) were tested for capacity to neutralize IFN anti-viral activity in the bioassay. Ammonium sulfate precipitated polyclonal rabbit antibodies and a murine mAb, 3:B5, both raised to recombinant equine IFN- α 1-GST fusion protein (Watrang et al., 1999) were used as positive controls for neutralization in these assays. Two types of neutralization assays were used: a “constant IFN method” and a “constant Ab method” (Grossberg et al., 2001). In the constant IFN assay, antibody samples were titrated in twofold dilutions and mixed with an equal volume of equine leukocyte IFN standard at a concentration of 5 U/ml. In the constant Ab assay equine leukocyte IFN standard was titrated in twofold dilutions from 500 to 4 U and mixed with an equal volume of antibody samples. In both assays, IFN and mAbs were pre-incubated at 37 °C for 30 min. Then, they were added to microtiter wells with confluent layers of MDBK cells and the IFN bioassay was performed as described above. In the constant IFN assay the dilution of inhibiting antibodies where 50% of cells were protected against VSV cytopathic effect was determined as neutralizing titer. Neutralizing capacity of the antibodies in the constant Ab assay was expressed as neutralized units of IFN-activity in the leukocyte IFN preparation. The activity in the well where 50% protection was achieved in the presence of antibody was set to 1 U/ml.

2.3. Preparation of equine leukocyte IFN

Equine leukocyte IFN was prepared in accordance with previously described methods (Yilma et al., 1982; Yilma, 1986; Jensen-Waern et al., 1998). Equine peripheral blood mononuclear cells (PBMC) were purified from heparinized blood using density gradient centrifugation on Ficoll-Paque (GE Healthcare Life Sciences, Uppsala, Sweden) and resuspended in RPMI 1640 medium (Sigma Ltd., Poole, UK) supplemented with 2 mM L-glutamine, 200 IU/ml penicillin, 100 μ g/ml streptomycin and 5% (v/v) FCS (Gibco, Invitrogen Ltd., Paisley, UK), at a concentration of 5×10^6 cells/ml. The PBMC were incubated in tissue culture flasks for 2 h at 37 °C, 5% CO₂, in a humid atmosphere, and then infected with EHV-1 (strain Ab4) at 1.86×10^6 TCID₅₀/ml and subsequently cultured for a further 24 h. In some preparations, equine leukocyte IFN at 25–30 U/ml was added to the PBMC during the 2 h incubation prior to infection, so-called “priming” (Fitzgerald-Bocarsly, 2002). After 24 h of culture cells were removed by slow speed centrifugation and the pH of the supernatant was lowered to 2 using 1 M HCl. The supernatant was subsequently stored at 4 °C for 24 h. Then, the pH was restored to 7 using 2 M NaOH and the supernatant was stored at –20 °C. Acid treatment was reported to remove any IFN- γ from the preparation (Yilma et al., 1982). Among the acid stable type I IFNs that remained one would expect a predominance of IFN- α considering the inducer and cell type used. The equine leukocyte IFN preparations typically contained approximately 300 U IFN-activity/ml without priming and approximately 1000 U IFN-activity/ml with priming.

2.4. Purification of *rIFN-α*

The *rIFN-α* was purified from the IgG4 fusion protein partner as described previously (Wagner et al., 2005). Briefly, serum-free supernatant containing the IgG fusion protein was given over a Protein G column. During this step, the *rIFN-α*/IgG4 fusion protein was bound to the column via the IgG4 heavy chain which has a high affinity to Protein G. While still on the column, the *rIFN-α* was separated from the IgG4 heavy chain by enterokinase digestion. After digestion, the *rIFN-α* was eluted from the column in PBS, while the IgG4 heavy chain remained bound to the Protein G. The purified *rIFN-α* was applied to a 10–20% SDS-gel. The gel was run under non-reducing conditions and was then stained with Coomassie Brilliant Blue. A protein of approximately 24 kDa was observed corresponding to the calculated molecular weight of equine IFN- α (data not shown).

2.5. Generation of monoclonal antibodies

Purified *rIFN- α* was used to generate mAbs by immunization of a BALB/C mouse. The experiment was approved by the Cornell University Institutional Animal Care and Use Committee. The immunization procedure and the cell fusion to generate mAbs were performed as previously described in detail (Wagner et al., 2003). Clones secreting mAbs to *rIFN- α* were identified by an ELISA described previously for other equine IgG fusion proteins (Wagner et al., 2005). In brief, a polyclonal goat anti-horse IgG(H + L) antibody (Jackson ImmunoResearch Lab., West Grove, PA), was used for coating of the ELISA plates. In the next step, *rIFN- α /IgG4* fusion protein, *rIL-10/IgG4* fusion protein (Wagner et al., 2008) or Protein G purified IgG from equine serum was added. The major IgG isotypes in the Protein G purified fraction were IgG1, IgG4 and IgG7. Then, supernatants of the anti-*IFN- α* hybridomas were added, followed by incubation steps with peroxidase conjugated goat anti-mouse IgG(H + L) (Jackson ImmunoResearch Lab., West Grove, PA) and substrate. The reactivity to *rIL-10/IgG4* or purified equine IgG was used to distinguish mAbs that specifically detected the *rIFN- α* portion of the IgG4 fusion protein from those that had reactivity to the IgG4 part. Only mAbs to the *rIFN- α* were selected, grown up and cloned by limiting dilution. The murine isotypes of the anti-equine *IFN- α* mAbs were determined by ELISA (Sigma, St. Louis, MO).

2.6. ELISA for equine *IFN- α*

For further investigation of the anti-*IFN- α* mAbs by ELISA, two clones (29B and 240-2) were grown up in large scale and the mAbs were purified using Protein G. The anti-*IFN- α* 240-2 mAb was biotinylated. The assay was performed as described before for other cytokines (Wagner et al., 2006, 2008). Briefly, anti-*IFN- α* 29B was used for coating of the plates in a concentration of 5 μ g/ml. The *rIFN- α* was applied to the plates in twofold dilution ranging from 25 ng/ml to 390 pg/ml and *rIFN- α /IgG4* supernatant (85 ng/ml) was diluted ranging from 1:2 to 1:64. Equine leukocyte IFN was used in twofold dilutions ranging from 3.125 to 200 U/ml. Samples subjected to analysis (see below) were tested at different dilutions (from undiluted to 1:10) to fit into the linear range of detection of the ELISA. For detection, biotinylated anti-*IFN- α* 240-2 was used. This step was followed by incubation with streptavidin-peroxidase and substrate. The *IFN- α* content in positive

samples was calculated by linear regression onto a standard curve of equine leukocyte IFN.

2.7. Samples tested in the equine *IFN- α* ELISA

Supernatants from two types of leukocyte cultures, PBMC and monocyte-derived macrophages, were used. Equine PBMC were isolated, stimulated with different oligodeoxyribonucleotides (ODN) and cultured as previously described (Watrang et al., 2005) and supernatants were collected after 24 h of culture. In the present study, PBMC originated from 3 different horses and supernatants from 16 different PBMC cultures were tested. The supernatants were tested for IFN-activity in the bioassay described above and the activity in individual samples ranged from not detectable to 1280 U/ml.

Equine monocyte-derived macrophages or DCs were cultured in 24-well plates as described previously (Flaminio et al., 2007). On day 4 of culture, non-proliferating ultraviolet irradiated *E. coli* were added to macrophages or DCs cultures at a concentration of 10 bacteria/cell. The *E. coli* were isolated from equine feces by the Animal Health Diagnostic Center at Cornell University. The ultraviolet treatment was performed at the New York State Agricultural Experiment Station, Cornell University. In addition, cells without *E. coli* treatments were cultured as controls. The cell culture supernatants were collected before the addition of bacteria, and at 24 and 48 h after *E. coli* addition. The supernatants were centrifuged to separate cells and debris and stored at -80°C until testing.

Samples of nasal secretions and serum (obtained in collaboration with Dr. Janet Daly, Animal Health Trust, Newmarket, UK) originated from horses experimentally infected with equine influenza A2 virus. Sampling and infection procedures have been described previously (Watrang et al., 2003). These samples were tested for IFN-activity in the bioassay and the activity in individual samples ranged from not detectable to 512 U/ml for nasal secretions and from not detectable to 32 U/ml for serum samples.

2.8. Statistical analysis

The statistical analysis was performed using the GraphPad Prism software, version 5.01. A linear regression analysis was performed on the results obtained by the IFN bioassay and the ELISA. To describe the consistency in ranking Spearman rank correlations were calculated for results obtained by both tests.

3. Results

3.1. Recombinant equine IFN- α

Recombinant IFN- α was expressed in mammalian cells as an IgG4 fusion protein. The anti-viral activity of the *rIFN- α /IgG4* fusion protein was tested in an IFN bioassay and compared to that of *rIFN- γ /IgG1* and a control fusion protein (*rIL-2/IgG1*). The anti-viral activity of *rIFN- α /IgG4* (85 ng/ml) was found to be 48 U/ml. The activity of *rIFN- γ /IgG1* (500 ng/ml) was determined as 4 U/ml and *rIL-2/IgG1* (500 ng/ml) showed no anti-viral activity. Considering their initial concentrations, the biological activities of the two fusion proteins were calculated to be 5.6×10^8 U/g *rIFN- α /IgG4* and 8×10^6 U/g *rIFN- γ /IgG1*, respectively. Thus, the anti-viral activity of the *rIFN- α /IgG4* was 70-fold higher than that of *rIFN- γ /IgG1*.

To exclude that the different bioactivities observed for *rIFN- α /IgG4* and *rIFN- γ /IgG1* resulted from differences in the IgG constant regions of the fusion proteins, we also expressed the equine IFN- α gene as *rIFN- α /IgG1* fusion protein. The biological activity of the *rIFN- α /IgG4* and *rIFN- α /IgG1* fusion proteins did not differ (data not shown). The *rIFN- α /IgG4* transfectant was used to purify *rIFN- α* for monoclonal antibody production.

3.2. Monoclonal antibodies to equine IFN- α

The purified *rIFN- α* was used to produce monoclonal antibodies. Six anti-equine IFN- α mAbs detecting the IFN- α /IgG4 fusion protein were identified. Equine IL-10/IgG4 or purified IgG were not detected by any of these mAbs indicating their specificity for equine *rIFN- α* . The murine isotypes of the anti-IFN- α mAbs were determined by ELISA (Table 1). The mAbs were subsequently tested for detection and neutralization of native equine IFN- α .

3.3. Neutralization of the anti-viral effect of equine leukocyte IFN by anti-IFN- α mAbs

Individual anti-IFN- α mAbs were tested for their capacity to neutralize the anti-viral activity of equine leukocyte IFN in two types of neutralization assays; first, a “constant IFN assay” and second, a “constant Ab assay”. A clear difference in the neutralizing potential of the six anti-IFN- α mAbs was observed in both assays (Table 1). While clones 81, 89A and 150 did not neutralize the anti-viral effect of leukocyte IFN or showed only low-neutralizing capacity in either assay, clone 240-2 had high-neutralizing capacity in both assays and was able to completely neutralize all activity in the highest concentration (500 U/ml) of the leukocyte IFN preparation. Clones 177A and 29B showed clear

Table 1
Anti-equine IFN- α mAbs and their potential to neutralize leukocyte IFN mediated anti-viral effect

mAb clone	Mouse isotype (concentration ^a)	Neutralization of anti-viral activity ^b	
		Constant IFN assay (neutralizing titer)	Constant Ab assay (neutralized activity)
29B ^c	IgG1 (10.0 μ g/ml)	1:16	15 U/ml
81	IgG1 (12.8 μ g/ml)	No inhibition	No inhibition
89A	IgG1 (13.4 μ g/ml)	1:2	Not tested
150	IgG2b (8.9 μ g/ml)	No inhibition	3 U/ml
177A	IgG1 (15.3 μ g/ml)	1:8	10 U/ml
240-2 ^d	IgG1 (14.5 μ g/ml)	>1:256 ^e	>499 U/ml
Controls			
Rabbit Abs ^f	NA	1:256	>499 U/ml
3:B5 ^f	Not tested	1:128	Not tested
Cell culture medium	NA	No inhibition	No inhibition

NA, not applicable.

^a Initial concentrations of the anti-IFN- α mAbs determined by ELISA.

^b Antibodies were tested for neutralization of equine leukocyte IFN mediated protection of MDBK cells against VSV cytopathic effect in two types of neutralization assays.

^c Used as coating antibody in IFN- α ELISA.

^d Used as biotinylated detection antibody in IFN- α ELISA.

^e The anti-IFN- α mAbs were diluted from 1:2 to 1:256.

^f Positive controls; polyclonal rabbit antibodies and mAb 3:B5 were raised to recombinant equine IFN- α 1-GST fusion protein (Watrang et al., 1999).

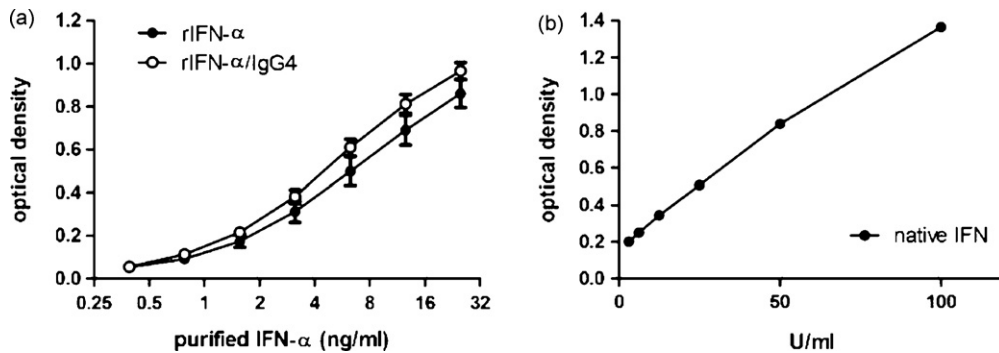


Fig. 1. Detection of IFN- α by ELISA using the anti-IFN- α mAb clones 29B and 240-2 for coating and detection, respectively. (A) Serial dilutions of *rIFN- α* and *rIFN- α /IgG4* and (B) leukocyte IFN were applied to the plates to determine the analytical sensitivity of the assay. The left graph (*rIFN- α*) represents means and SEMs of five measurements. The right graph (leukocyte IFN) shows means of duplicates.

inhibitory activity in both assays but to a lower degree than clone 240-2. Based on these assays, it is likely that the six anti-IFN- α clones recognized different epitopes on native IFN- α and binding to them corresponded to no neutralizing effect (clones 81, 89A and 150), low-neutralizing effect (clones 29B and 177A) or high-neutralizing effect (clone 240-2) on IFN- α mediated anti-viral activity.

3.4. ELISA for equine IFN- α

All six mAbs were tested for pairing in the ELISA using *rIFN- α* (data not shown). The best results were obtained with anti-IFN- α clones 29B for coating of the plates and 240-2 for detection of purified *rIFN- α* and *rIFN- α /IgG4* fusion protein (Fig. 1A). The ELISA also confirmed that the mAb clones 29B and 240-2 detected different epitopes on the *rIFN- α* . The assay was used to detect native IFN- α in the leukocyte IFN preparation (Fig. 1B). An analytical sensitivity of 800 pg/ml *rIFN- α* or 3 U/ml native leukocyte IFN and linear ranges of detection between 800–32,000 pg/ml *rIFN- α* and 3–100 U/ml native IFN, respectively, were determined for the equine IFN- α ELISA.

The ELISA was further evaluated by testing of different biological samples and the results from some of these samples were compared to the IFN-activity recorded in the bioassay. Supernatants ($n = 16$) from PBMC cultured in the presence or absence of different ODN were tested by ELISA and bioassay (Fig. 2) and the detected amounts of IFN showed a high consistency ($r_{sp}^2 = 0.98$) between the two assays. All of the samples that tested negative in the bioassay ($n = 4$) were also negative in the ELISA. Among samples that showed IFN-activity (range 12–1280 U/ml) in the bioassay only one sample (12 U/ml) was not detectable by the IFN- α ELISA. None of the supernatants from *E. coli*

stimulated monocyte-derived macrophages or dendritic cells showed detectable concentrations of IFN- α using the ELISA.

Samples of nasal secretions ($n = 10$) and serum ($n = 10$) collected from experimentally influenza A2 virus infected horses were tested by both assays (Table 2). Two samples of nasal secretions and two sera were negative for IFN-activity in the bioassay and IFN- α was not detectable by the ELISA. Six samples of nasal secretions and six sera that tested positive for IFN-activity were negative for IFN- α in the ELISA. Two samples of nasal secretions with high levels of IFN-activity in the bioassay resulted in IFN- α concentrations of 87 and 41 U/ml by ELISA. For two sera with IFN-activity in the bioassay, 6 and 7 U/ml IFN- α were detected by ELISA.

In summary, a high correlation between biological IFN-activity and the levels of IFN- α detected by the IFN- α ELISA was observed for supernatants from

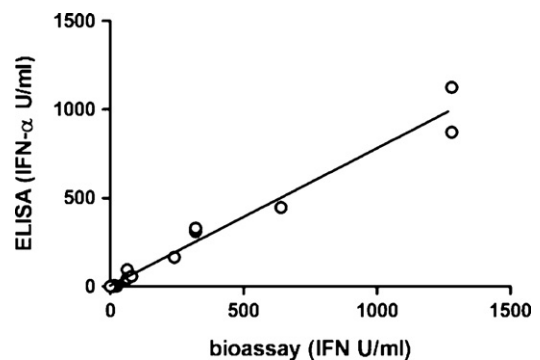


Fig. 2. Comparison of equine IFN- α detection by ELISA and IFN-activity determined by bioassay in samples of cell culture supernatants ($n = 16$) from equine PBMC cultured in the presence or absence of different ODN. The line shows the linear regression curve calculated for the data points. Spearman's rank correlation analysis resulted in a $r_{sp}^2 = 0.98$.

PBMC stimulated with ODN, while a larger discrepancy was found between results obtained by both assays when samples from influenza A2 virus infected horses were tested.

4. Discussion

The lack of available reagents to measure equine cytokines has hampered research on the immune response for many years. Individual research groups have addressed this need during the past few years by developing mAbs for equine key cytokines such as IFN- γ (Gutmann et al., 2005), IL-4 (Wagner et al., 2006) and IL-10 (Wagner et al., 2008). Other valuable reagents have become available by characterization of mAbs for other species that have proven crossreactivity to the corresponding equine cytokines, such as IFN- γ (Pedersen et al., 2002; Wagner et al., 2005; Breathnach et al., 2005; Paillot et al., 2006) and TGF- β (Desjardins et al., 2004). In addition, the efforts of international reagent initiatives, such as the BBSRC Immunological Toolbox (<http://www.immunologicaltoolbox.co.uk>), the Cytokine Center at the University of Utrecht (<http://www.cytocen.com>) and the US Veterinary Immune Reagent Network (<http://www.vetimm.com>) also target a variety of recombinant cytokines and mAbs to cytokines for various species, including the horse. These reagents are likely to become available within the next few years, and will provide valuable tools to improve immunological research and vaccine development to existing and emerging diseases in veterinary species.

In this report, we have used our previously established equine IgG fusion protein expression system (Wagner et al., 2005, 2008) to produce equine *r*IFN- α , which was subsequently tested for biological activity. The *r*IFN- α /IgG4 fusion protein showed clear bioactivity in the IFN-bioassay which is in agreement with earlier observations using *r*IFN- γ /IgG4 fusion protein in this assay (Wagner et al., 2005). The 70-fold higher anti-viral activity of the IFN- α fusion protein compared to its IFN- γ counterpart is also well in range of the 10–100-fold higher activity that has been observed for natural IFN- α and IFN- β compared to IFN- γ from other species (Farrar and Schreiber, 1993). The reaction between IFN and its receptor (see below) is highly specific. Thus, it seems unlikely that any unspecific binding between the IgG fusion partner and the receptor occurred, and the use of an IgG1 or IgG4 fusion partner was suggested to have only minor direct influence on the IFN bioassay. Because the IgG fusion partner is larger than the IFN- α or IFN- γ , the IgG

could potentially alter the binding of the IFN fusion proteins to the receptor, thereby influencing the biological effect of the IFNs. However, the equine IgG1 and IgG4 constant heavy chain regions share a very high-amino acid homology and an almost identical structure (Wagner et al., 2002). Thus, a potential influence on the IFN bioactivity is likely to be identical between IgG1 and IgG4 fusion proteins. This was confirmed by testing of a *r*IFN- α /IgG1 fusion protein in the bioassay. The anti-viral activity of this protein did not differ from the *r*IFN- α /IgG4 fusion protein.

Using the purified *r*IFN- α , six mAbs to equine IFN- α were generated. The anti-IFN- α mAbs were found to detect native IFN- α by two approaches: first, bioassays to inhibit the protective anti-viral effects of type I IFN, and second, an ELISA to detect IFN- α using a pair of the new anti-IFN- α mAbs (clones 29B and 240-2).

The anti-equine IFN- α mAbs were found to differ in their potential to inhibit the anti-viral activity of leukocyte IFN varying from complete to no neutralizing capacity. The IFN preparation used for the neutralization tests is classically known as leukocyte IFN and is composed of acid stable IFN derived from PBMC after viral induction. The type I IFN composition of human leukocyte IFN produced with different inducers has been extensively studied (Nyman et al., 1998; Tölö et al., 2001; Pestka, 2007) and shown to contain mainly a mixture of IFN- α subtypes with a predominance of a few subtypes. That the anti-viral activity of our equine leukocyte IFN preparation mainly was conferred by IFN- α was confirmed by the strong neutralization achieved by the polyclonal rabbit antibody and mAb 240-2 raised to different recombinant proteins based on the same equine IFN- α nucleotide sequence. The composition of IFN- α subtypes in the equine leukocyte IFN preparation is unknown, but it seems reasonable to assume that it contained several subtypes and that one or a few subtypes could dominate. Because mAb 240-2 effectively neutralized all anti-viral activity in the bioassays it must recognize all IFN- α subtypes present in the leukocyte IFN preparation. This neutralization pattern also suggests that mAb 240-2 binds to an IFN- α epitope that is essential for binding to the type I IFN receptor (IFNAR), or that receptor dimerization, which is required for signaling through the receptor, was inhibited after binding of the IFN- α /mAb 240-2 complex to the IFNAR2 receptor subunit. Type I IFNs, e.g., IFN- α and IFN- β , share a heterodimeric, high-affinity receptor composed of the IFNAR1 and IFNAR2 subunits that are expressed on various cell types in small numbers. The binding of IFN- α /- β to the IFNAR2 subunit initiates receptor dimerization which then leads

Table 2

Comparison of IFN-activity (U/ml) detected by bioassay and detection of equine IFN- α by ELISA (U/ml) in sera and nasal secretions from equine influenza A2 virus infected horses

Sample no.	Nasal secretions		Serum	
	IFN-activity (bioassay)	IFN- α (ELISA)	IFN-activity (bioassay)	IFN- α (ELISA)
1	<2	nd	<2	nd
2	<2	nd	<2	nd
3	8	nd	32	7
4	128	nd	32	6
5	64	nd	8	nd
6	512	87	10	nd
7	4	nd	10	nd
8	512	41	4	nd
9	8	nd	5	nd
10	16	nd	8	nd

<2 = the lower limit of detection in the bioassay was 2 U/ml since the start point of titration was at dilution 1:2. nd = not detectable, i.e., below the detection limit of 6 U/ml (due to the lowest sample dilution of 1:2) in the ELISA.

to phosphorylation of both receptor chains and signal transduction (Theofilopoulos et al., 2005).

Clones with clear inhibitory capacity of a lower potency than that of mAb 204-2, such as mAb 29B and 177A, could either bind with low affinity to an inhibitory epitope, bind to an epitope that only mediates partial inhibition or recognize only one or a few IFN- α subtypes. However, the inhibitory pattern of these mAbs in the different neutralization assays indicates that they recognize all subtypes present in the EHV-1 induced leukocyte IFN preparation. Moreover, comparison of IFN determination by bioassay and ELISA in supernatants from PBMC induced with ODN shows that IFN- α was the major source of IFN-activity in these samples and suggest that mAbs 204-2 and 29B also must detect all IFN- α subtypes induced by CpG-ODN in order to achieve this type of linear relationship between bioassay and ELISA results. That CpG-ODN stimulation may give a clear predominance of IFN- α production when type IFN induction is achieved was earlier shown for human cells (Krug et al., 2001a) and was also shown at the mRNA level for equine cells (Wattrang et al., 2005).

In contrast, the ELISA only detected IFN- α in a few serum and nasal secretion samples from influenza A2 infected horses. These samples showed high-IFN-activity in the bioassay, and only some of this was found by ELISA, while samples with lower IFN-activity in the bioassay remained negative in the ELISA. This suggested that the samples from influenza A2 infected horses were composed of various type I IFNs. These could include certain IFN- α subtypes not detected by

the ELISA or, more likely, the IFN-activity recorded in the bioassay was due to other type I IFNs, such as IFN- β . Because equine influenza A2 virus only infects the respiratory epithelium of horses (Hannant and Mumford, 1996) one would expect a predominance of IFN- β produced by epithelial cells in nasal secretions and that any IFN detected systemically would be an “overspill” of local production and thus have the same composition.

Although the functions of IFN- α are beneficial during immune responses to viruses and other infectious agents, increased levels of IFN- α have also been associated with the pathogenesis of autoimmune diseases (Baccala et al., 2007). Examples include systemic lupus erythematosus (Rönblom and Alm, 2001), insulin-dependent diabetes mellitus (Stewart, 2003; Devendra and Eisenbarth, 2004), and a wide spectrum of autoimmune manifestations after IFN- α treatment for unrelated conditions such as viral infections or cancer (Gota and Calabrese, 2003). Notably, systemic lupus erythematosus also occurs in horses (Geor et al., 1990) and the neutralizing mAb to equine IFN- α could be useful for research on and for the development of new therapeutic strategies to autoimmune diseases in horses.

In humans and mice, pDCs were found to be major natural producers of IFN- α (Siegal et al., 1999; Nakano et al., 2001; Asselin-Paturel et al., 2001; Björck, 2001; Fitzgerald-Bocarsly et al., 2008) while conventional or myeloid DC (mDC) commonly do not produce high levels of IFN- α when stimulated with non-infectious nucleic acid inducers or bacterial cell wall products. In other species, such as primates (Coates et al., 2003), pig (Guzylack-Piriou et al., 2004; Domeika et al., 2004) and rat (Hubert et al., 2004), small populations of circulation pDCs were identified as potent producers of high amounts of IFN- α . In humans, pDCs selectively expressed TLR7 and TLR9 within their endosomes, which were found to be the receptors for viral ssRNA, and viral DNA or CpG, respectively. Signaling through these receptors resulted in activation of pDCs characterized by secretion of large amounts of type I IFN and followed by their differentiation into mature DCs (Liu, 2005). In contrast, mDCs expressed TLR3 and TLR8 in their endosomes and recognition of viral dsRNA or ssRNA by these receptors triggers IL-12 secretion in mDCs. Human monocytes and mDCs also expressed TLR4 on their surfaces. Binding of bacterial LPS to TLR4 induced the production of pro-inflammatory cytokines, such as IL-1, IL-6, IL-10 and TNF- α in these cells (Liu, 2005). It should also be noted that TLR expression and cytokine production by DCs vary between humans and mice. Murine pDCs produced

both, type I IFNs and IL-12 (Krug et al., 2001b; Nakano et al., 2001) and type I IFN production was reported after stimulation of murine mDCs with LPS via TLR4 or dsRNA via endosomal TLR3 (Baccala et al., 2007).

The present results confirmed that the type I IFN-activity observed upon stimulation of equine PBMC with CpG-ODN (Watrang et al., 2005) was conferred by IFN- α . Moreover, in our experiment stimulating equine monocyte-derived macrophages and DCs with *E. coli*, no IFN- α production could be detected. This suggests that neither bacterial nucleic acids nor cell-wall products, such as LPS, were able to induce detectable IFN- α production in equine monocyte-derived DCs. This finding is in agreement with the above-mentioned cytokine expression profiles of human and porcine (Johansson et al., 2003) monocytes and mDCs after activation with bacterial LPS and nucleic acids. Thus, although it is not yet possible to distinguish between the various equine DC phenotypes, our results suggest that DC populations, TLR expression pattern and signaling pathways similar to those in humans might exist in horses. However, the detection of secreted CpG-induced IFN- α in supernatants of PBMC requires confirmation by phenotyping of the IFN- α producing cells to definitely characterize them as pDCs. This probably needs to await the development of appropriate cell surface markers for equine DCs and other cell types.

In conclusion, the new anti-equine IFN- α mAbs are valuable reagents to investigate this important cytokine and the roles of pDCs during the innate immune response of the horse. The initial observations made here on stimuli that trigger different TLR pathways and their potential to induce IFN- α production in equine cells can now be extended to immunological studies on various equine infectious diseases. The anti-equine IFN- α mAbs are available to the scientific community for research collaborations.

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