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Interferon-α Production and Tissue Localization of Interferon-α/β Producing Cells after Intradermal Administration of Aujeszky's Disease Virus-Infected Cells in Pigs

K. ARTURSSON*, M. LINDERSSON*, N. VARELA†, A. SCHEYNIUS‡ & G. V. ALM*

**Department of Veterinary Microbiology, Division of Immunology, Swedish University of Agricultural Sciences, Uppsala, Sweden*
†Department of Virology, CENSA, San Jose de Las Lajas, Cuba and
‡Department of Clinical Immunology, Karolinska Hospital, Stockholm, Sweden

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Intradermal administration of glutaraldehyde-fixed Aujeszky's disease virus (ADV) infected autologous or allogeneic cells resulted in the induction of an interferon(IFN)-α response in pigs. Using a sensitive dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA), IFN-α was detected in blood at 8 and 24 h after injection of ADV-infected cells. In parallel, by means of *in situ* hybridization, IFN-α/β mRNA containing cells were demonstrated in regional lymph nodes. Occasional IFN-α/β mRNA positive cells were also seen in injected dermal areas, but not in contralateral lymph nodes, spleen, bone marrow, blood or liver. The ability of leucocytes in whole blood cultures to produce IFN-α upon stimulation by ADV was markedly diminished 3–7 days after intradermal injection of ADV-infected cells. In contrast, cultures of purified peripheral blood mononuclear cells (PBMC) had intact IFN-α responses. Further, serum from ADV-injected pigs inhibited the *in vitro* ADV-induced IFN-α responses in PBMC from control pigs, most likely due to the demonstrated presence of anti-ADV antibodies. We suggest that the IFN-α/β producing cells in lymph nodes may participate in the development of antiviral immunity and could be equivalent to Natural IFN-α/β producing (NIP) cells.

Karin Artursson, National Veterinary Institute, Box 7073, 750 07 Uppsala, Sweden

INTRODUCTION

Viral infections usually cause prompt production of the type-I interferons (IFN), IFN-α and -β, which can directly limit the viral replication in cells by activating several intracellular mechanisms [1]. The type I IFN also have many immunomodulatory effects [1], and recently IFN-α has been implicated in promoting the development of T_h1 lymphocytes and IgG2a antibody production that are important in antiviral immunity [2, 3].

Many different cell types can produce IFN [1]. However, in human mononuclear leucocytes stimulated by RNA viruses, monocytes are the main producers of IFN-α/β [4–6]. In contrast, certain DNA viruses, such as the herpes viruses, free or glutaraldehyde-fixed and cell-associated, stimulate IFN-α/β responses in infrequent, but highly productive, mononuclear blood leucocytes. In humans, these cells lack markers typical of monocytes as well as T and B lymphocytes, but express for instance MHC class II antigens, CD4 and CD36 [4, 7–13]. They have provisionally been designated natural IFN-α/β producing cells (NIP cells) [11]. Although the NIP cells have some resemblance to antigen presenting dendritic cells, they have, in one study at least, been separated from such cells [14]. The corresponding cells in pigs, which can be triggered by free or cell-associated transmissible gastroenteritis virus (TGEV), a coronavirus, have also been well characterized [15, 16]. The same cell population appears to be stimulated by cells infected by Aujeszky's disease virus (ADV), also termed Pseudorabies virus [17].

With regard to the IFN-α/β response *in vivo*, little or no information is available regarding its cellular basis or the distribution of IFN-α/β producing cells at sites of viral infections and in lymphoid tissues. In patients with hepatitis, IFN-α producing cells were demonstrated by immunohistochemical staining in liver sections [18]. In mice injected with polyinosinic-polycytidylic acid (poly-IC), IFN-β producing cells were demonstrated in the spleens within a few hours [19]. To further study the IFN-α/β response *in vivo*,

we developed an experimental model, where specific pathogen free (SPF) pigs were injected intradermally with glutaraldehyde-fixed, ADV-infected (and thus non-infectious) autologous or allogeneic cells. Because significant levels of IFN-α were found in serum samples 8 and 24 h after the injections, we examined injected dermal tissue, regional and contralateral lymph nodes, the spleen, bone marrow and the liver for the presence of IFN-α and IFN-β mRNA expressing cells, respectively, at the corresponding time points. We also studied the ability of peripheral blood mononuclear cells (PBMC) from injected animals to produce IFN-α *in vitro*.

MATERIALS AND METHODS

Animals. A total of 16 pigs were studied. They were crossbreed gilts (Swedish Yorkshire × Swedish Landrace) of SPF origin, 12 weeks old at the start of the study. Two to three pigs were kept per room at the animal department of the National Veterinary Institute, Uppsala and all pigs were allowed 1 week's acclimatization prior to the study.

Preparation of PBMC. Blood samples were collected by jugular vein puncture in vacutainer tubes (Becton Dickinson, Grenoble, France) with or without the addition of Sodium Heparin (150 USP/10 ml blood). The blood was diluted 1 : 1 in phosphate buffered saline (PBS) and centrifuged for 30 min, at 550 × *g*, on Ficoll-Paque (Kabi-Pharmacia, Uppsala, Sweden). The PBMC were collected, washed in PBS and resuspended in complete medium, that is RPMI 1640 (Flow Laboratories, Irvine, UK) supplemented with penicillin (60 μg per ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and 5% Myoclone™ fetal calf serum (FCS; Gibco, Paisley, UK).

Virus. Aujeszky's disease virus of the Phylaxia strain (kindly provided by Dr Sandor Bélak, National Veterinary Institute, Uppsala, Sweden) was grown in porcine kidney (PK15) cells (Flow Laboratories) in Dulbecco's modification of Eagle's medium (DMEM; Flow Laboratories) supplemented as described above. The virus-containing supernatants were harvested and centrifuged for 5 min at 400 × *g* to remove cell debris. The virus titre in PK15 cells was determined to be 10^6 $TCID_{50}$ per ml.

To study the inhibitory effects of immune serum on the *in vitro* ADV-induced IFN-α response, we used purified ADV as the inducer of IFN. Cell culture supernatants, containing the virus, were clarified by centrifugation at 2000 × *g* for 30 min at 4°C. The virus was concentrated by ultrafiltration using a 300 kDa cut off tangential flow filter (Filtron Corp., Clinton, MA, USA) . The virus was pelleted by centrifugation at 85000 × *g* for 2 h at 4°C. Pellets were resuspended in PBS and stored in aliquots at −80°C. The ADV was used at a concentration of 10^3 $TCID_{50}$ per ml and was prior to use inactivated by UV irradiation, approximately 0.5 J/cm^2. The absence of infectious viral particles was confirmed in PK15 cell cultures.

Preparation of ADV-infected stimulatory cells. In experiment 1, PBMC were prepared from the experimental pigs 1 week before start of the experiment. A total of 50×10^6 autologous PBMC were incubated with ADV, 4×10^6 $TCID_{50}$, in 14 ml complete RPMI medium in 25 cm^2 culture bottles (Nunc, Roskilde, Denmark) for 2 h at 37°C and 7% CO_2 air concentration. The cells were harvested and then washed in PBS by centrifugation, fixed in 0.05% glutaraldehyde in PBS for 15 min at room temperature, washed again in 0.15 M NaCl and stored at 4°C in 0.15 M NaCl with 10% swine serum, penicillin (60 μg/ml) and streptomycin (100 μg/ml) until use. The swine serum did not contain antibodies to ADV. These cells are designated ADV-PBMC. Control cells not incubated with ADV were concurrently prepared. Before use, all stimulatory cells were washed and diluted to a concentration of 50×10^6 cells per ml in 0.15 M NaCl.

In experiments 2 and 3, confluent monolayers of PK15 cells in 225 cm^2 culture bottles (Nunc) were trypsinized, washed with PBS and resuspended at 0.4×10^6 cells per ml in DMEM complete medium containing HEPES (10 mM). The cells were incubated in slowly shaking culture bottles for 2 h at 37°C and then for another 2 h at 37°C with the addition of 8×10^4 $TCID_{50}$ ADV per ml. The cells were then washed in PBS, glutaraldehyde-fixed as in experiment 1, treated with 3% glycine in PBS for 15 min at room temperature, washed again in PBS and then treated with 0.5 J/cm^2 UV-irradiation. These cells are designated ADV-PK15 cells. Control cells were prepared in parallel, omitting the ADV. All cells were stored as described above for ADV-PBMC and the cell concentrations were adjusted to 75×10^6 cells per ml in 0.15 M NaCl prior to injection.

Before injection into the pigs, all stimulatory cell preparations were verified to be free of ADV infectious to PK15 cells and free of significant levels of endotoxin by employing a limulus assay using a chromogenic substrate (Kabi-Pharmacia, Stockholm, Sweden).

Injection of cells and samplings. In experiment 1, four pigs were injected intradermally in the flanking region of the abdomen with approximately 60×10^6 fixed ADV-PBMC and 60×10^6 control PBMC, each distributed within six marked adjacent sites. Serum samples were collected immediately prior to injections and at 8, 24, 48 and 72 h as well as 1 week after injection. Concurrently, skin biopsies were taken at cell-injection sites and immediately frozen in isopentane chilled by dry ice in acetone.

In experiment 2, six pigs were injected intradermally in the flanking region with approximately 60×10^6 fixed ADV-PK15 cells distributed within four adjacent sites in the right flanking region and 7 days later in the same manner with control PK15 cells, but in the left flank. Serum samples were collected immediately before injection and at 8, 24, 48 and 72 h after each set of injections.

In experiment 3, four pigs were injected intradermally in the flanking region with 60×10^6 fixed ADV-PK 15 cells as described in experiment 2. Two pigs were used as non-injected controls. Blood samples were taken before cell injection and then immediately before anaesthesia (see below). At 6 and 24 h after injection, two pigs, injected with ADV-PK15 cells, and one non-injected pig were anaesthetized by the use of the combination azaperone (2 mg/kg bodyweight i.m.) and methomidate (10 mg/kg body weight i.p). Skin biopsies (circular, 3 mm diameter) were taken both at the sites of injection and on the contralateral flank of the pigs. The regional subiliac lymph nodes, draining the area of injection [20], and the contralateral lymph nodes were procured, as well as small parts of the spleens and livers, before the pigs were killed. Bone marrow specimens were removed after death and immediately mixed with an equal volume of a solution containing 90% FCS and 10% DMSO. All samples were immediately frozen, as described above, and stored in liquid nitrogen.

Preparation of specimen. Cryostat sections, 6 μm thick, were prepared from collected tissues. Sections from lymph nodes, spleens and liver measured about 1 cm^2. The skin biopsies were sectioned longitudinally. Sections for *in situ* hybridization were fixed on slides pretreated with 2% gamma-aminopropyltriethoxy-silane in acetone,

for 1 min in 4% paraformaldehyde in PBS. The slides were then stored in 70% ethanol in diethylpyrocarbonate (DEPC)-treated distilled water at 4°C until hybridized.

A volume of 4 ml DEPC-treated distilled water was added to 0.9 ml of heparinized blood to lyse erythrocytes. After a 1 min incubation, 5 ml of PBS was added. The leucocytes were pelleted and treated for 5 min with 5 ml of 1% paraformaldehyde in PBS. After washing in PBS, the cells were resuspended in 0.3 ml of PBS and 25 μl were added per well of ethanol-pretreated eight-well slides with hydrophobic coating (8 mm well diameter), dried and stored in 70% ethanol at 4°C.

In situ hybridization. The cRNA probes labelled by α-^{35}S-UTP were prepared by *in vitro* transcription of DNA as described previously [17]. For detection of IFN-α mRNA, a 829-bp Eco RI-Hpa I fragment of the porcine (po) IFN-α_1 gene [21] was transcribed. For detection of IFN-β mRNA a 712-bp Eco RI-Pst I fragment of the poIFN-β gene [17] was transcribed.

The RNA-RNA *in situ* hybridizations were performed as previously described [17], that is, at high stringency for 3–4 h at 50°C with probes in 50% formamide and 2xSSC, followed by RNase treatment. The slides were covered with NTB-2 nuclear track emulsion (Eastman-Kodak, Rochester, NY, USA), and developed after 10 days exposure. The hybridized sections were stained by haematoxylin-eosin according to standard procedures. In total, two to eight sections of each specimen were microscopically examined and the mean number of labelled cells per section was estimated.

In vitro induction of IFN-α responses. At each sampling occasion in experiment 2, heparinized blood from each pig was diluted 5 × in RPMI complete medium and concurrently PBMC were prepared and diluted to a concentration of 4×10^6 cells per ml in RPMI complete medium. Quadruplicate cultures were established by adding 0.1 ml per well, of either diluted blood or purified PBMC, in flat bottom 96-well microtitre plates (Nunc). To each type of culture, 0.1 ml of ADV-PK15 cells or control PK15 cells, 0.8×10^6 per ml in RPMI complete medium, were added. The cultures were incubated for 20 h at 37°C in 7% CO_2 air concentration, and the supernatants were then harvested for IFN-α determinations.

To study the effect of immune sera on the IFN-α response, PBMC cultures from a control pig, not previously used in the experiments and free from antibodies to ADV, were established as described above, except that the FCS was excluded from the medium. The medium was supplemented with 5% of individual sera collected from the pigs in experiment 2, as indicated. The cultures were stimulated by adding 0.1 ml volumes per well of purified, UV-treated ADV diluted 200 × in complete RPMI 1640 medium without FCS. Supernatants were collected for IFN-α assays after 20 h of culture.

Measurement of antibodies to ADV in sera. Antibodies to ADV in sera were determined by an ELISA, based on the ability of serum anti-ADV antibodies to bind to ADV. In brief, 96-well microtitre plates (Immuno Plate Maxisorp; Nunc) were coated overnight at room temperature with 100 μl per well of purified ADV, protein concentration 6 μg per ml in 50 mM Tris HCl pH 9.5. Unspecific binding sites were blocked by post-coating for 1 h at 37°C with ELISA dilution buffer, that is PBS with 0.5% (w/v) BSA, 0.25% (w/v) merthiolate and 0.05% (v/v) Tween 20. After washes, serum samples diluted 500 × in ELISA dilution buffer, were added in volumes of 100 μl per well, incubated for 1 h at 37°C and washed. Bound serum antibodies were detected by incubation for 1 h at 37°C with 100 μl per well of HRP-conjugated rabbit anti-swine Ig antibodies (DAKO Corp., Glostrup, Denmark), diluted 10000 × in ELISA dilution buffer. After washing, 200 μl per well of substrate consisting of tetramethylbenzidine (1 mM) in 0.1 M pH 4.25 potassium citrate buffer containing 2 mM H_2O_2 was added. After 10 min at room temperature, reactions were stopped by adding 1 M H_2SO_4, 100 μl per well, and the absorbance was read at 450 nm in a plate spectrophotometer.

Immunoassay of IFN-α. An immunoassay for porcine IFN-α was established, based on the principle of dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) [22]. It is based on two murine MoAbs, designated K9 and F17 [23, 24], directed to independent epitopes on poIFN-α and used previously in an ELISA for poIFN-α [25]. The MoAbs were precipitated from mouse ascitic fluid by ammonium sulphate, and further purified by ion exchange chromatography (Mono S; Kabi-Pharmacia Biotechnology, Uppsala, Sweden). The K9 MoAb was labelled with Europeum lanthanide chelate according to the manufacturer's recommendations (Wallac Oy, Turku, Finland), and purified by gel filtration on a Superose 12 column (Kabi-Pharmacia Biotechnology). The Europeum-labelled MoAb was used in the assay at a concentration of 0.1 μg per ml in dilution buffer.

The F17 MoAb was treated with 50 mM HCl and diluted to 3 μg per ml in 0.2 M NaH_2PO_4 (pH 4.5) with 0.005% NaN_3, and used to coat flat bottomed microtitre plates (Immuno Plate Maxisorp; Nunc). Volumes of 0.1 ml per well were used, and plates were incubated overnight at room temperature. They were washed in PBS with 0.05% Tween 20, and postcoated for 1 h at 37°C with 0.3 ml per well of a buffer (pH 4.5) containing 6% sorbitol, 0.15 M NaCl, 0.1% BSA, 50 mM NaH_2PO_4, 0.1 mM $CaCl_2 \times 2H_2O$, 4 μM EDTA and 0.005% NaN_3. The plates were washed once in 50 mM Tris buffer (pH 7.8) with 0.15 M NaCl, 0.05% Tween 20 and 0.005% NaN_3. The samples were diluted in dilution buffer, that is, 50 mM Tris buffer (pH 7.8) with 0.15 M NaCl, 0.005% NaN_3, 0.5 mM $CaCl_2 \times 2H_2O$, 20 μM EDTA and 0.5% BSA, and 0.2 ml volumes were added per well. When serum samples were assayed, 0.5% normal mouse serum was included in the dilution buffer. The samples were incubated for 2 h while undergoing slow shaking at room temperature, and the plates were then washed three times.

Europeum-labelled K9 MoAb was then added, 0.2 ml volumes per well, plates were incubated for 1 h and washed six times. Enhancement solution (Wallac Oy), 0.2 ml per well, was added. After 20 min the fluorescence (counts per second) was measured in a DELFIA Fluorometer (Wallac Oy). The IFN-α concentrations in antiviral units [U] per ml was calculated using standard curves constructed by the use of a porcine IFN-α preparation obtained from Sendai virus-stimulated leucocytes [26].

Statistics. The paired Student's *t*-test was used to determine the significance of differences, using the STATVIEW 512+ program (Brain-Power, Inc., Calabasas, CA, USA) on a Macintosh computer.

RESULTS

IFN-α levels in serum of pigs injected with ADV-infected cells

The DELFIA developed for measurement of IFN-α in this study proved to be highly sensitive and IFN-α levels as low as 0.1 U per ml were routinely detected. In serially acquired samples from individual animals, even lower IFN-α levels produced specific signals (see below). With appropriate fitting

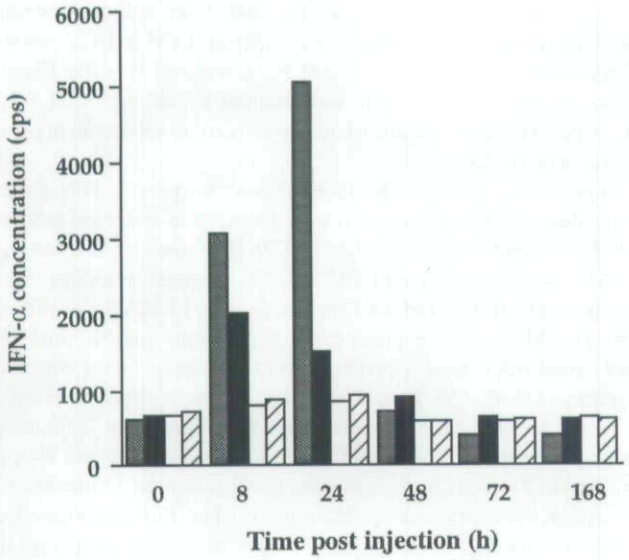

Fig. 1. Concentrations of IFN-α in sera of four pigs injected intradermally with ADV-infected, glutaraldehyde-fixed autologous leucocytes. The concentrations of IFN-α in sera are expressed as counts per second (cps) as measured by a DELFIA. The highest IFN-α response in this experiment (5067 cps), corresponds to 3.4 U of IFN-α per ml.

of standard curves in log/log plots, linear ranges up to at least 800 U per ml were obtained. Coefficients of variation for the measurements of samples throughout this range were usually below 10%.

Two separate experiments were performed to study the IFN-α levels in serum of pigs injected with ADV-infected cells. In the first (Fig. 1), four pigs were injected intradermally with 60×10^6 glutaraldehyde-fixed autologous ADV-PBMC and an identical number of control cells. Serum from all four pigs, obtained at 8 and 24 h post injection, displayed increased IFN-α concentrations measured as counts per second (cps). The two pigs with the highest IFN-α responses had maximal levels corresponding to 0.9 and 3.4 U per ml, respectively.

In the second experiment (Table 1), a group of six pigs were first injected intradermally with 60×10^6 glutaraldehyde-fixed ADV-PK15 cells and 1 week later with control PK15 cells, using the same method. Serum samples from all animals, obtained 8 and 24 h after injection with ADV-PK15, displayed increased cps-values in the DELFIA for IFN-α compared to the values for sera before injection (0 h) and at 48 h or later. The values at 8 and 24 h were significantly increased ($P = 0.03$ and $P = 0.005$, respectively), compared to 0 h values. The highest IFN-α concentration, caused by ADV-PK15, was in this experiment 1.9 U per ml serum. No evidence of IFN-α response after injection of control PK15 cells was seen.

Detection of cells producing IFN-α or -β in vivo

In experiment 1, skin biopsies obtained from the sites of injection of the fixed autologous ADV-PBMC were analysed for the presence of cells containing IFN-α mRNA by means of a ^{35}S-labelled cRNA probe applied to cryostat sections. Positive cells were found at a low frequency, only one or two per section at 24, 48 and 72 h after injection. These cells were localized adjacent to injected ADV-PBMC, which could readily be discerned in the sections. Sections of biopsies at other time points were all negative, as were sections from control injection sites receiving control PBMC. *In situ* hybridizations with a cRNA probe for human β-actin mRNA were positive, indicating little degradation of RNA. Examination of skin sections revealed little or no infiltration of inflammatory cells.

It was unlikely that the low number of IFN-α producing cells detected in the skin accounted for the significant systemic IFN-α response in the pigs. Therefore other tissues were examined in addition to skin for presence of IFN-α or IFN-β producing cells. In this experiment four pigs were injected with 60×10^6 ADV-PK15 cells and two non-injected pigs were used as controls. Two injected and one control pig were killed at each 6 and 24 h. The results of the evaluation of the collected material from skin and lymph nodes are summarized in Table 2.

Infrequent cells containing mRNA for IFN-α or IFN-β were detected in sections of skin taken 6 and 24 h after injection of fixed ADV-PK15 cells (Fig. 2a). The labelled cells appeared in the vicinity of the injected ADV-PK15 cells, easily identified at both time points. No significant

Table 1. Concentrations of IFN-α in serum[1] of pigs injected intradermally with ADV-infected, glutaraldehyde-fixed PK-15 cells (week 1) or with the corresponding dose of non-infected PK-15 cells (week 2)

Time post injection (h)	Pig number					
	5	6	7	8	9	10
Week one						
0	949	863	820	916	772	1066
8	1256	1585	968	3207	1689	2345
24	1182	2108	1445	1932	1520	2618
48	912	844	874	1102	759	1020
72	834	834	769	804	696	886
Week two						
0	756	714	776	977	783	934
8	712	729	700	823	748	802
24	722	762	695	886	829	824
48	647	808	750	822	830	872
72	742	764	702	770	832	806

[1] The IFN-α concentrations were measured by the DELFIA described in Materials and Methods and are expressed as counts per second (cps). The highest IFN-α concentration obtained in this experiment (3207 cps) corresponds to 1.9 Units of IFN-α per ml.

Table 2. Number of IFN-α and -β mRNA expressing cells[1] in skin and lymph nodes after intradermal administration of ADV-infected, glutaraldehyde-fixed PK-15 cells in pigs

Time p.i. (h)		IFN-α[1,2]		IFN-β[1,2]		Serum IFN-α (cps)[3]	
		regional	control	regional	control	0 h	6/24 h
Pig 11							
skin[4]	6	0	0	0	0	582	554
lymph node[5]	6	178	0	43	0		
Pig 12							
skin	6	0	ND	1	ND	714	1238
lymph node	6	86	0	34	0		
Pig 13							
skin	24	0	0	0	0	536	1566
lymph node	24	62	0	7	0		
Pig 14							
skin	24	2	ND	1	ND	636	979
lymph node	24	2	0	1	0		

[1] Evaluated by *in situ* hybridization of tissue sections, using ^{35}S-labelled cRNA probes specific for poIFN-α and poIFN-β mRNA, respectively.

[2] The mean number of IFN-α/β mRNA expressing cells per section is indicated.

[3] As measured in the DELFIA assay, described in Materials and Methods.

[4] Skin biopsies were taken at the site of injection (regional), which was in the flanking region, and control biopsies were taken from non-injected skin (control) in the opposite flank.

[5] Lymph node biopsies were taken from the subiliac lymph node (regional) draining the site of injection and from the corresponding contralateral lymph node (control).

ND is not done.

infiltrates of inflammatory cells were observed in the skin sections.

Larger number of cells positive for IFN-α or IFN-β mRNA were found in the subiliac lymph nodes which drain the sites of injections of ADV-PK15 cells. The IFN-α and IFN-β mRNA positive cells were found both at 6 and 24 h after injection (Fig. 2 b–d). Clusters of injected ADV-PK 15 cells were seen along the lymphonodular trabeculae in limited parts of the nodes. The IFN mRNA containing cells essentially lined such ADV-PK15 cell aggregates on the sides facing the cortex, but were also seen deeper in the cortex. In the lymph node of pig number 14, in which very few IFN mRNA positive cells were detected, no ADV-PK15 cells were found. A mean of 109 (range 62–178) IFN-α mRNA positive cells were detected per whole lymph node section from pigs 11, 12 and 13 (Table 2). The IFN-β mRNA positive cells were less heavily labelled, fewer in number and had a mean of 28 (range 7–43) positive cells per section. Examination of sections from the contralateral subiliac lymph nodes, sections of spleen and liver or smears of bone marrow or peripheral blood did not reveal any clearly positive IFN-α or IFN-β mRNA containing cells. Also, similar preparations from the two non-injected control pigs were negative.

The in vitro ADV-induced IFN-α response of PBMC and whole blood leucocytes

The six pigs injected with ADV-PK15 cells in experiment 2 were also examined for the ability of leucocytes in whole blood cultures, or as purified PBMC, to produce IFN-α when exposed *in vitro* to glutaraldehyde-fixed ADV-PK15 cells. The results are summarized in Fig. 3.

The mean concentration of IFN-α produced in cultures of leucocytes derived from pigs before the injection of ADV-PK15 cells were in PBMC cultures 497 ± 97 U/ml (Fig. 3a) and in whole blood cultures 43 ± 31 U/ml (Fig. 3b). One week later, immediately before the injection of the control PK15 cells, the corresponding IFN-α concentrations were 455 ± 162 U/ml and 2.2 ± 5.3 U/ml, respectively.

The IFN-α production in purified PBMC at 24 h after injection of the ADV-PK15 cells was significantly higher than that at 0 h ($P = 0.02$). Further, the IFN-α production at 48 and 72 h was significantly lower than that at 0 h ($P < 0.05$). During the second week, when the pigs were injected with control PK15 cells, the ability of PBMC to produce IFN-α was relatively constant. No IFN-α was produced *in vitro* when the PBMC were cocultured with control PK15 cells.

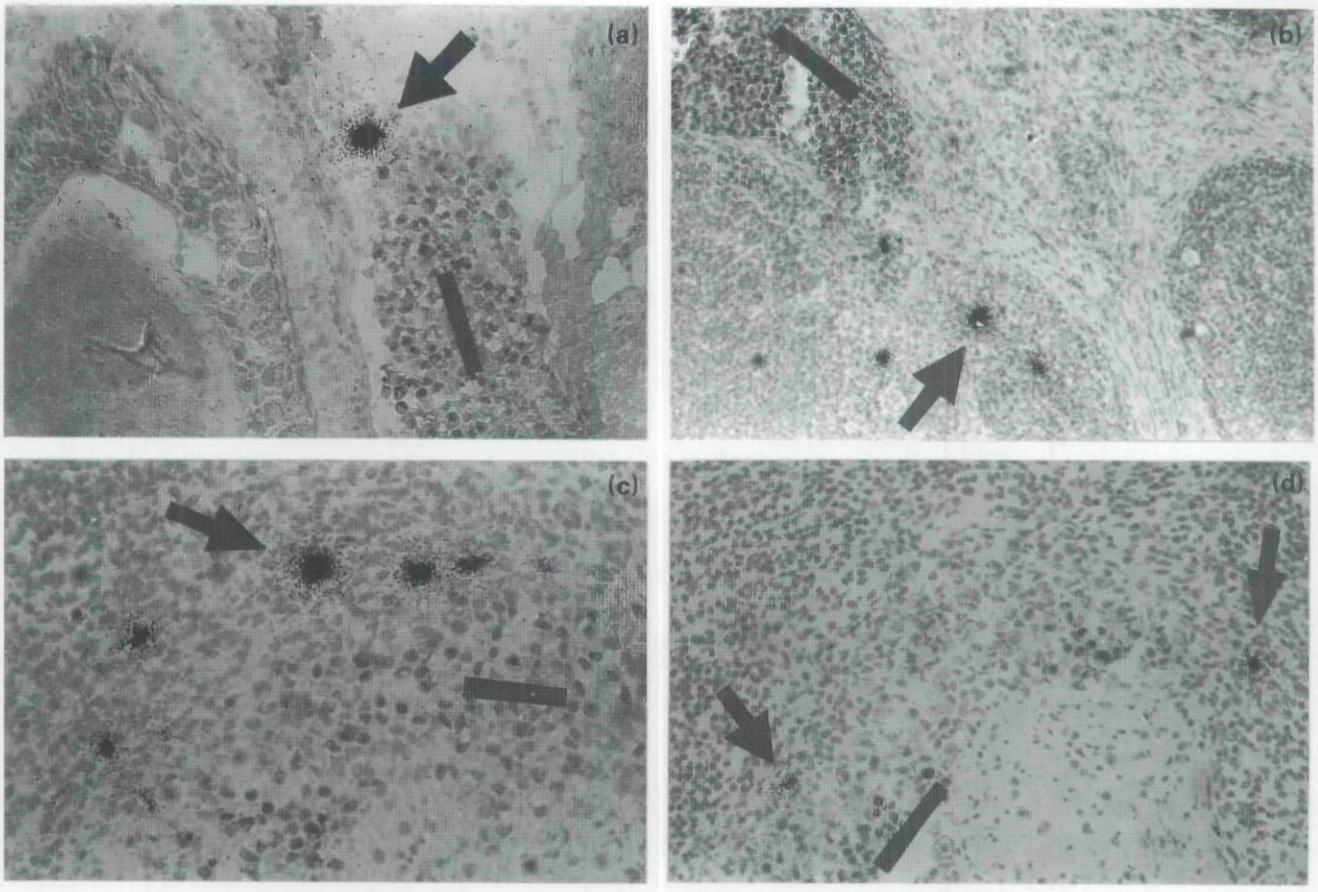

Fig. 2. Demonstration of cells expressing mRNA for poIFN-α and poIFN-β, respectively, after intradermal injection of ADV-infected, glutaraldehyde-fixed PK 15 cells. The IFN-α and IFN-β positive cells were demonstrated by *in situ* hybridization, using ^{35}S-labelled cRNA-probes. Arrows indicate the IFN mRNA positive cells and bars indicate the injected cells, which were detected both in injected skin and in the regional lymph nodes. The signals for IFN-β were always weaker than for IFN-α mRNA. A. IFN-α mRNA positive cell in skin 24 h p.i., in close association to injected, ADV infected cells ($\times$250). (B and C) IFN-α mRNA positive cells in regional lymph nodes, 6 (B) and 24 h (C) p.i., respectively. The IFN mRNA positive cells are localized adjacent to injected cells lining the trabeculae in the cortical areas of the lymph node ($\times$160 and $\times$320, respectively). D. IFN-β mRNA positive cells 6 h p.i. in a regional lymph node, localized close to injected cells in a cortical area ($\times$250).

When the induction in whole blood cultures was studied, the IFN-α responses were generally lower, about one tenth of those of purified PBMC, as would be expected from their lower number of mononuclear leucocytes. A tendency towards decreased IFN-α production ($P = 0.09$) was seen already at 8 h after the administration of infected cells. The production at 24 and 48 h was significantly lower ($P < 0.05$) than that seen at 0 h. Furthermore, immediately before injection of the control PK15 cells and at all times thereafter throughout the second week, whole blood cultures of all animals essentially failed to produce IFN-α when stimulated with ADV-PK 15 cells.

As shown in Fig. 4, antibodies to ADV were first detected in serum samples 7 days after the injection of ADV-PK15 cells and their levels further increased during the following 3 days.

Anti-ADV antibodies or other serum factors could possibly cause the differences in the IFN-α producing ability between leucocytes in whole blood cultures and as purified PBMC. Therefore, purified PBMC from a control pig, not previously used in the experiments, were cultured in the presence of 5% of the sera of each of the six pigs used in experiment 2. As shown in Table 3, sera collected 7 and 10 days after injection of the ADV-PK15 cells markedly inhibited IFN-α responses, compared to sera collected before and 3 days after injection. Thus, the suppressive effect appeared concomitantly with the first detection of anti-ADV antibodies.

DISCUSSION

The results of our study demonstrate that an intradermal injection in pigs of allogeneic or autologous cells, infected by Aujeszky's disease virus and glutaraldehyde-fixed, induces the production of IFN-α of such quantities that low but significant levels can be detected in blood within 8 h. Instrumental in the detection of such low levels of IFN-α in serum

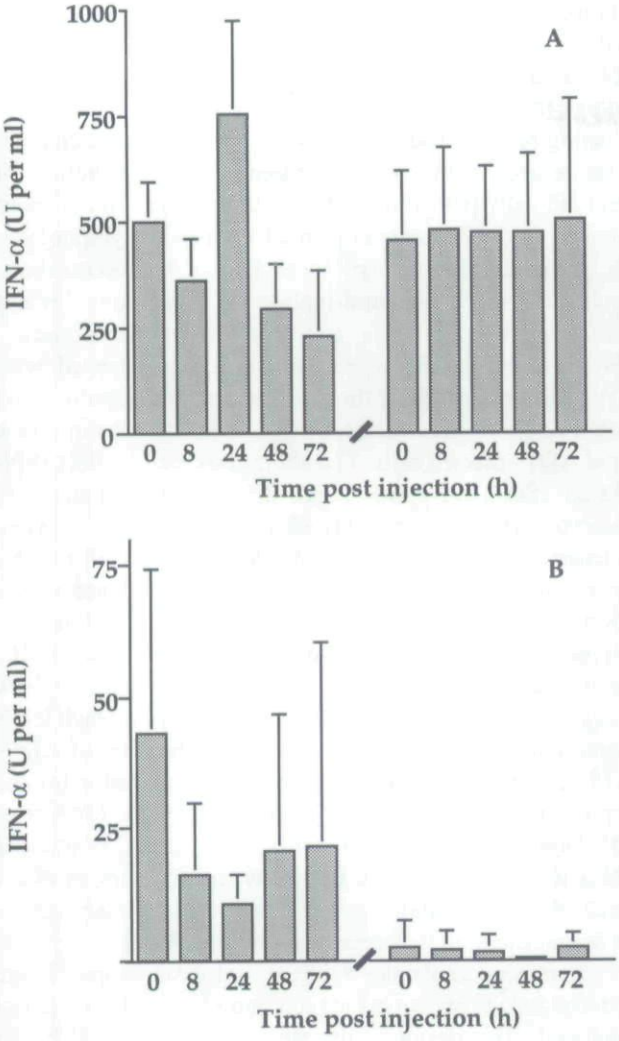

Fig. 3. *In vitro* production of INF-α, induced by ADV-infected, glutaraldehyde-fixed PK-15 cells in PBMC derived from six pigs injected with equivalent ADV-infected PK-15 cells.The *in vitro* induction of IFN-α was carried out (A) in cultures of purified PBMC or (B) in whole blood cultures, as described in Materials and Methods. Concentrations of IFN-α are expressed as units [U] per ml culture medium (means + 1 SD) for each occasion of testing. Values below the lower limit for detection (0.3 U/ml) were given this value. No INF-α, was detected in control cultures of PBMC incubated in the presence of non-infected PK-15 cells.

was the development of the highly sensitive immunoassay, using the DELFIA principle, which was able to detect concentrations at 0.1 U per ml.

Significant levels of IFN-α in blood were seen both after the administration of autologous ADV-PBMC and after the administration of allogeneic ADV-PK15 cells, but not with the non-infected control cells. Since no infectious virus was present, our results confirm *in vivo* that glutaraldehyde-fixed cell-associated virus can induce IFN-α/β, as has been demonstrated previously *in vitro* [17, 26–29].

After a single intradermal administration of ADV-infected inducer cells, maximal IFN-α levels in blood were present

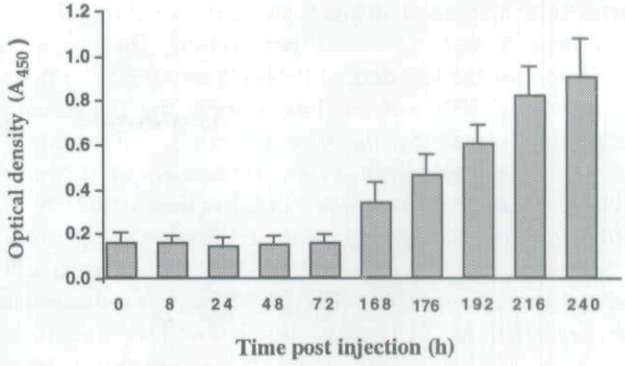

Fig. 4. Serum levels of antibodies to ADV in six pigs injected intradermally with approximately 60×10^6 ADV-infected glutaraldehyde-fixed PK-15 cells at 0 h in week 1 and 60×10^6 non-infected control cells at 0 h in week 2. Antibody levels were determined by ELISA and are expressed as absorbancies (means + 1 SD).

already at 8 h after injection and remained high at 24 h. In contrast, little or no serum IFN-α was detected at 48 h, despite presence of injected ADV-infected cells in histological sections of intradermal injection sites at this time. This discrepancy might be due to elimination of the interferogenic ADV, or perhaps more likely, active suppression caused by anti-interferogenic anti-ADV antibodies (see below).

Attempts were made to trace cells that were responsible for the production of IFN-α or -β *in vivo* in response to the intradermally injected ADV-infected cells. However, in

Table 3. The effect of sera[1] from pigs injected with ADV-infected glutaraldehyde-fixed PK-15 cells on ADV-induced production of IFN-α *in vitro*[2] by PBMC[3]

Point of time for collection of serum samples (days p.i.)	Pig number					
	5	6	7	8	9	10
0	30	16	21	26	16	21
3	21	31	26	20	23	21
7[4]	2	<2	<2	<2	<2	<2
10	<2	<2	<2	<2	<2	<2

[1] Sera were obtained prior to injection with ADV-infected, glutaraldehyde-fixed PK-15 cells (day 0) and at indicated days post injection (p.i.).

[2] The *in vitro* induction of IFN-α production was carried out as described in Materials and Methods, but the FCS was substituted with 5% serum, collected from experimental pigs number 5–10 at indicated days p.i. The amount of IFN-α produced was measured by the DELFIA described in Materials and Methods and is expressed as units per ml culture medium.

[3] Cells obtained from a control pig, verified to be free of serum antibodies to PRV.

[4] Due to the experimental design the pigs were injected with non-infected control cells on day 7.

injected skin, no more than one to two cells expressing IFN-α or -β mRNA were identified per section. One possible explanation for the low dermal IFN-α/β responses is a poor recruitment of IFN-α/β producers from the circulation, which is supported by the observed weak inflammatory response. By extrapolating the low frequencies of IFN-α/β positive cells observed in skin sections, less than 1000 IFN-α/β producing cells should be present per injection site. Assuming that these cells correspond to NIP cells and each cell produces as much as 10 U of IFN [17, 30] before production is down-regulated at 24 h after stimulation [31], the total amount of IFN-α produced can have significant local effects. However, the produced IFN-α can only account for a minor part of the IFN-α detected in serum since the half-life of IFN-α here is a matter of minutes [32].

In contrast to the dermal injection sites, relatively high numbers of IFN-α and -β mRNA containing cells were demonstrated in the regional lymph nodes 6 and 24 h after administration of ADV-PK15 cells. Based on their frequency in sections, and size of the nodes, approximately 10^5 IFN-α and 3×10^4 IFN-β mRNA positive cells were present at one time. These cells should be able to produce in the order of $1–10 \times 10^5$ U IFN-α, which could well result in the serum levels of IFN-α detected by the immunoassays. Furthermore, no IFN-α or IFN-β producing cells were observed in other tissues, including contralateral lymph nodes, spleen, bone marrow, blood and liver. It is therefore likely that most of the IFN-α/β production in the pigs actually occurred in the regional lymph nodes.

The IFN-α/β mRNA positive cells were localized near aggregates of ADV-PK15 cells in the lymph node tissue and tended to be positioned preferentially in lymphocyte-dense cortical areas and not in the medulla. The fact that each porcine lymph node consists of accumulations of several independent lymphonoduli, each with separate afferent lymph vessels [33], may explain why IFN mRNA positive cells and ADV-infected cells were only seen in parts of each subiliac lymph node. Also, the efferent lymph of porcine lymph nodes is free of lymphocytes, which instead must exit via efferent blood. This may not be possible for ADV-PK15 cells which instead may be trapped in the nodes, offering one explanation for the lack of IFN-α/β mRNA positive cells in other tissues and organs.

A reasonable hypothesis is that the IFN-α/β mRNA containing cells identified *in vivo* in the present study correspond to the NIP cells previously characterized in pigs and in humans [4, 7–13, 15, 30], but this awaits confirmation. *In vitro* studies indicate that NIP cells are the only leucocytes capable of IFN-α/β production after exposure to inducers of the type represented by glutaraldehyde-fixed ADV-PK15 cells [10, 11, 15, 17, 22, 34].

The production and very high concentration of IFN-α/β in the regional lymph nodes could well have a significant impact on early antiviral immune responses. Thus, IFN-α/β has been implicated in the regulation of immune reactions, for instance, promoting development of T_h1 cells and IgG2a production [2, 3]. Further evidence that locally produced IFN has a significant effect on a lymphoid organ are the findings that poly-IC injected intraperitoneally in mice, via induction of cells producing IFN-β, caused dramatic changes in tissue architecture of the spleen and redistribution of lymphoid cells [19]. Furthermore, IFN causes enlargement of lymph nodes by increasing their content of lymphocytes [35]. In addition, the high local IFN-α/β concentrations should efficiently block viral replication in lymph nodes and prevent negative viral impacts on early immune responses.

When purified PBMC derived from pigs injected with ADV-PK15 was studied, their IFN-α responses, induced by ADV-PK15 cells in culture, were increased 24 h after injection of the ADV-infected cells. The alterations could reflect IFN-induced effects on PBMC, like distributional changes of leucocytes [19, 35] or priming effects [1]. In contrast, when the leucocytes were tested in whole blood cultures, all animals showed decreased IFN-α responses 7 days p.i. Since it was shown that serum collected from the animals at this time suppressed ADV-induced IFN-α responses of normal PBMC, one or several suppressive serum factors may be involved. Sera from the experimental pigs in the present study had high levels of antibodies to ADV about 1 week after injection of ADV-PK15 cells. Such antibodies may well be responsible for the suppression, because both polyclonal and certain MoAbs to ADV have been shown to efficiently inhibit the ADV-induced IFN-α response by porcine PBMC *in vitro* (Artursson *et al.*, unpublished observations). Also, antibodies to other viruses can inhibit their interferogenic activity [28, 29, 36].

Since antiviral antibodies during a viral infection initially are produced and are present in high concentrations in those regional lymph nodes that obviously also are main sites for the IFN-α/β response, we suggest that the anti-interferogenic activity of antibodies is a physiologically relevant negative feedback mechanism, which limits IFN-α/β responses and might in this way influence the development of antiviral immunity.

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