Interferon-gamma response of PBMC indicates productive pseudorabies virus (PRV) infection in swine

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Abstract

In Chinese Meishan/German Landrace cross-bred swine F_2 generation interferon gamma (IFN-γ) production by peripheral blood mononuclear cells (PBMC) was determined directly ex vivo at different time points after survival of a virulent pseudorabies virus (PRV) infection. This reactivity was compared with the reactivity of naïve PBMC. Significant IFN-γ production was determined in ELISA and ELISPOT only after in vitro PBMC re-stimulation with PRV and not with the closely related bovine herpesvirus BHV-1.

The PRV-specific IFN-γ secretion from re-stimulated PBMC showed high levels 6 days after infection, before the presence of serum antibodies, and it persisted at a high level over a 3 months period. The response of a group of eight piglets infected intranasally with PRV varied. Only two animals showed the expected typical fever response. PRV specific IFN-γ production by PBMC clearly indicated that infection had occurred. Early significant IFN-γ production by primed PBMC turned out to be a reliable and specific ex vivo marker for cellular response against productive PRV infection in swine before antibody formation.
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Keywords: Swine; Pseudorabies virus infection; Interferon gamma

1. Introduction

Swine are the principal host for pseudorabies virus (PRV) infection and develop Aujeszky's disease with high morbidity and mortality as a juveniles after infection with virulent strains or field isolates (Wittmann and Rziha, 1989; Mettenleiter, 2000, 2003). Immune response against PRV have been the subject of many studies primarily in vaccine trials (for review see Zuckermann, 2000; Mettenleiter, 2000). After acute infection swine develop specific antibody and T-cell responses against PRV (Wittmann and Rziha, 1989; Kimman et al., 1994; Mettenleiter, 1996). The latter can be characterised by the activity of

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antigen specific cytolytic T-lymphocytes (CTL) and by PRV-mediated proliferation of PBMC (Kimman et al., 1995; Summerfield et al., 1996; De Bruin et al., 1998, 2000; Zuckermann, 2000). Besides the induction of type I interferons, IFN-γ production of PBMC has been reported to be a sign of cell-mediated immunity in PRV and other viral infections of swine (Mateu de Antonio et al., 1998; Zuckermann et al., 1998, 1999; Zuckermann, 2000; Suradhat et al., 2001). The interferons (IFNs) are representative cytokines in viral infections that not only play an important role as antiviral agents but are inter-connected regulators in innate and adoptive immune responses (Brinkman et al., 1993; Fearon and Locksley, 1996; Biron, 1998). Within the interferon family, IFN-γ (formerly ''immune IFN'') represents a single gene encoded cytokine that has been found to be secreted by NK cells and predominantly by activated T-lymphocytes which outlines its role in adoptive immune responses (Vilcek and Sen, 1996). Moreover, the determination of IFN-γ allows the detection and quantization of antigen specific CD8$^+$ T-lymphocytes and a efficacy detection of other cytokines such as IL-12 and IL-18 (Pittet et al., 2001; Harrington et al., 2002; Speller and Warren, 2002; Domeika et al., 2002).

We were interested in the direct ex vivo follow up of PBMC-derived IFN-γ, in context with disease resistance and survival of Meishan cross-bred swine against a virulent PRV infection (Reiner et al., 2002). To determine virus specific IFN-γ production, ELISA and ELISPOT technique were applied to monitor PBMC reactivity to in vitro PRV re-exposure at different time points after infection.

2. Materials and methods

2.1. Animals

F$_2$ generation cross-bred Meishan/German Land-race swine were obtained from the Institute of Animal Breeding and Biotechnology, University of Hohen-heim, Stuttgart, Germany (Reiner et al., 2002). All piglets were used for PRV infections at an average age of 10 ($\pm$2) weeks (between 20 and 30 kg body weight). The animals were housed and fed in groups of two or four in the isolated stables of the Federal Research Center under standardized conditions. Infection

experiments were performed by intranasal PRV spray application (spray bottle, Roth, Karlsruhe, Germany) with 10^5 p.f.u. of the highly virulent PRV strain NIA-3 (McFerran and Dow, 1975) according to the permission No. Ak. Z. 37-9185-81-4 after the German legislation for animal experiments. Starting 3 days before until 12 day after infection (p.i.) rectal temperatures were recorded daily. None of the experimental animals had PRV-specific serum antibodies before the PRV challenge. The relative resistance of cross-bred Meishan piglets to a productive PRV infection without the development of lethal neurological symptoms but accompanied by phases of fever with temperatures >40 °C has been reported earlier (Reiner et al., 2002). The development of fever was defined according to Gillespie et al. (2000) when the rectal body temperature was measured >40 °C. Only animals (in total $n = 20$) that did not show any clinical signs of neurological symptoms such as trembling, in-coordi-nation, ataxia, paralysis circling or paddling and regained physiological body temperature were selected for long-term immunological investigation. Among the experimental infection experiments one was performed with a group of eight animals showing a heterogeneous course of body temperature profiles after intranasal PRV application. Thus this group of animals was divided into three subgroups according to their body temperature course: group A with the expected rise in rectal temperature ($n = 2$); group B with a moderate rise in rectal temperature ($n = 2$); group C with no rise in rectal temperature ($n = 4$) until day 6 after PRV application (Fig. 1). To control the take of PRV infection within the subgroups B and C, the six non-febrile animals received a second PRV application but this time intramuscularly (i.m.) at day 6 following the intranasal PRV application (Table 1).

2.2. Cells and viruses

Porcine peripheral blood mononuclear cells (PBMC) were prepared as described previously (Büttner et al., 1995; Saalmüller et al., 1989) and were maintained in RPMI 1640 medium supplemented with 300 mg/l stable L-Glutamine and 10% pestivirus-free fetal bovine serum (culture medium, Biochrom, Berlin, Germany; FBS, PAA, Linz, Austria). Viability of cells was determined using trypan blue dye exclusion in light microscopy. PBMC from naïve as
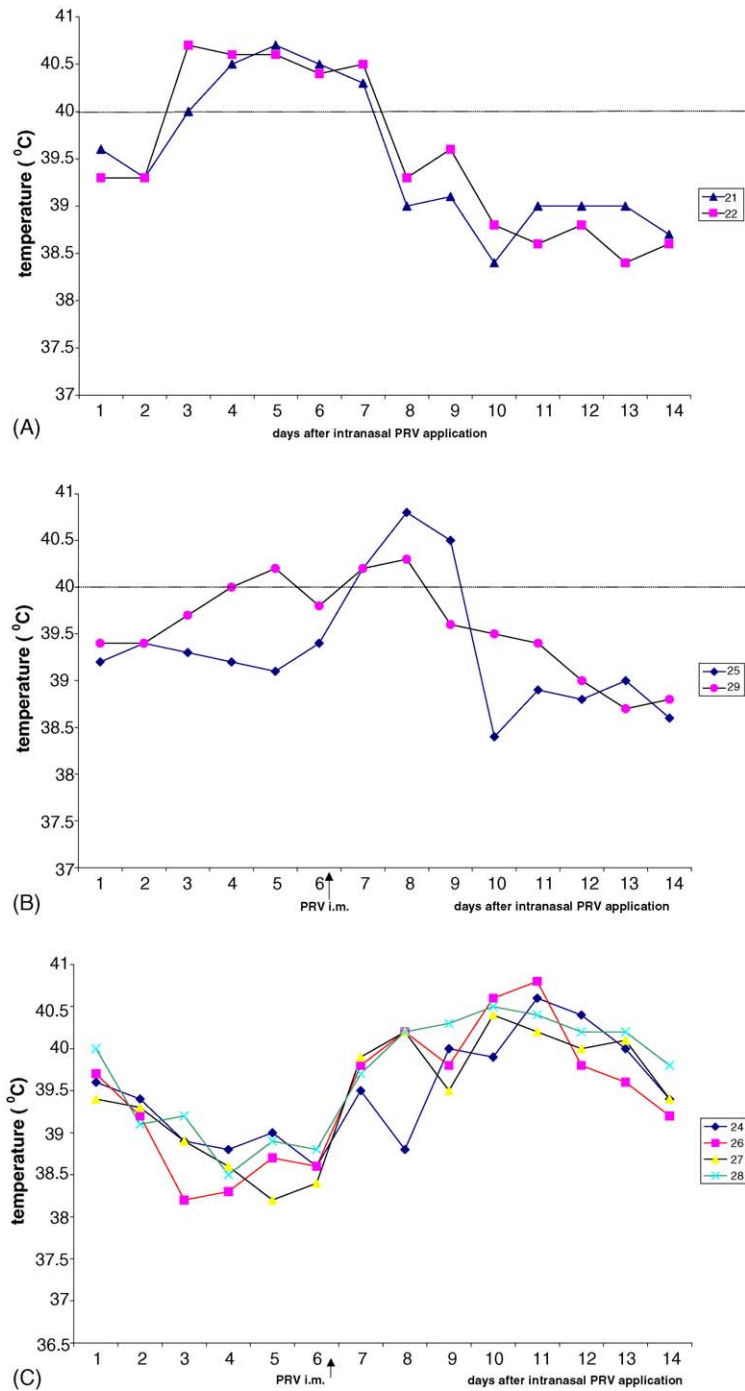
Fig. 1. Differential course of rectal body temperature in eight piglets after intranasal infection with virulent PRV strain NIA-3. (A) The course of the temperature of two animals which developed fever (>40 °C) until day 6 post infection. (B) The course of rectal temperature of two animals from which one (No. 29) showed a moderate rise in body temperature. (C) The mean temperature course is summarized for four animals which showed rather a drop in body temperature than a rise after intranasal infection. After intramuscular (i.m.) injection of the same dose of PRV in non-febrile animals (B and C) at day 6 after intranasal application (arrows) all six piglets developed fever within 6 days.

Table 1
Diverse temperature profiles, IFN-γ secretion by PBMC and antibody titres from piglets after take of intranasal PRV NIA-3 infection (group A) and failure of productive infection (groups B and C)

Days after intranasal PRV application	IFN-γ secretion (pg/ml)	PRV-specific antibody titre 1	Body temperature (°C)	Animal no.: temperature response
Group A				
6	2450	Not detectable	>40.5	21 fever
12	23450	1280	<40.0	
19	30200	2800	<40.0	
6	1838	Not detectable	>40.5	22 fever
12	17500	640	<40.0	
19	30000	2078	<40.0	
Group B				
6 i.m. injection	<40	Not detectable	<40.0	25 moderate temp. rise
12	28400	160	>40.0	
19	28400	2560	<40.0	
6 i.m. injection	<40	Not detectable	40.0	29 short-term fever
12	18900	320	>40.0	
19	32000	5120	<40.0	
Group C				
6 i.m. injection	<40	Not detectable	<40.0	24 no temperature rise
12	13892	5	>40.0	
19	7500	6400	<40.0	
6 i.m. injection	<40	Not detectable	<40.0	26 no temperature rise
12	9167	Not detectable	>40.0	
19	73600	1600	<40.0	
6 i.m. injection	<40	Not detectable	<40.0	27 no temperature rise
12	14800	Not detectable	>40.0	
19	18000	1600	<40.0	
6 i.m. injection	<40	Not detectable	<40.0	28 no temperature rise
12	12200	Not detectable	>40.0	
19	35600	1600	<40.0	

Response after second i.m. PRV application (day 6 post intranasal application) is indicated below the dotted lines and reflects a productive infection in animals from groups B and C accompanied by the development of fever (see Fig. 1). IFN-γ production and rise of body temperature in bold for day 6 post PRV application. i.m. = intramuscular.

well as PRV immune or primed animals were treated in vitro with PRV strain NIA-3 (McFerran and Dow, 1975), or BHV-1 strain Los Angeles (L.A.) (virus collection, Institute of Immunology) at a multiplicity of infection of 1 (MOI = 1).

For the production of PRV and bovine herpesvirus 1 (BHV-1) a pestivirus-free bovine kidney cell line, MDBK (ATCC, CCL22), was used and maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 5% horse serum (Gibco-Invitrogen, Paisley, UK).

The PRV strain NIA-3 was propagated and quantified in MDBK cells, stocks of 2 ml containing 10^8 p.f.u./ml were aliquoted and stored at $-70\,^{\circ}$C. The control bovine herpesvirus 1 (BHV-1) strain L.A. was prepared in the same way.

2.3. Antibody detection

For the detection of PRV-specific antibodies a commercial ELISA kit (Chekit Aujeszkytest II, Hoechst, Schleißheim, Germany) was used as prescribed by the manufacturer.

2.4. IFN-γ detection

2.4.1. ELISA

The specificity of mAb reactive with porcine IFN-γ has been described (Mateu de Antonio et al., 1998). For the determination of cell-free IFN-γ a specific ELISA was used according to the recommendation of the manufacturer (Cyto Sets Swine IFN-γ, Biosource, Amarillo, CA, USA). Cytokine concentrations were

calculated using the linear portion of the ELISA titration curve obtained using the recombinant standard of the kit. The titration of the standard was run on each plate and the concentrations of the test samples expressed in pg/ml.

2.4.2. ELISPOT assay

An ELISPOT assay was performed essentially as described by Zuckermann et al. (1998). Analysis of IFN-γ secretion by single cells was done using 96-well nitrocellulose-lined plates (MAIP 54510, Millipore, Eschborn, FRG) that were coated with 100 μl of anti-porcine IFN-γ mAb (Endogen, Bedford, MA, USA) (5 μg/ml) in PBS without calcium/magnesium (PBS-A) overnight at 37 °C. Wells were washed with PBS-A (wash buffer) and blocked for 1 h at room temperature (RT) with culture medium. Cell preparations were added at a concentration of 2×10^5/well and incubated for 48 h with ConA (5 μg/ml), PRV or BHV-1 (MOI ~ 1) in a final volume of 200 μl. After the incubation period the plate was washed and 100 μl of an anti-porcine IFN-γ serum (2.5 μg/ml) (Endogen, Bedford, MA, USA) was added and incubated for 1 h at RT. The plate was washed again and incubated with anti-rabbit alkaline phosphate conjugate (100 μl/well, Promega, Mannheim, Germany) for 1 h at room temperature. After five times washing, the wells were incubated with 100 μl of the substrate solution BCIP/NBT (Sigma Taufkirchen, Germany) for 5–10 min. The plates were finally dried and the spots per well were counted with the aid of a dissecting scope or after photographing the wells.

2.5. Statistical analysis

Comparison of mean differences in interferon production was done using the Wilkoxon rank sum test. Differences were considered significant if $P < 0.05$.

3. Results and discussion

3.1. Clinical observations and antibody detection

The majority of infected and long-term monitored animals ($n = 12$) showed a rise in rectal body temperature above 40 °C between days 3 and 8 after intranasal PRV application (not shown) in the absence of other clinical signs. Following the febrile course of productive intranasal infection PRV-specific serum antibodies were first found 12 days p.i. by ELISA reaching a plateau (mean titer: 1/2800) around 8 weeks post challenge (not shown). Thus a correlation with the development of fever and take of infection could be confirmed by the presence of PRV-specific serum antibodies around 2 weeks after experimental infection. This is consistent with the reported data from vaccination and challenge experiments as well as from survival of natural PRV field infections (Wittmann and Rziha, 1989; Kimman et al., 1994; Mettenleiter, 1996).

In the group of eight animals showing a heterogeneous course of temperature reaction, only two (group A: 21 and 22) reacted with a fever response showing a sharp rise in rectal temperature around day 4 post virus application (Fig. 1A). In this experiment the take of infection was questionable because of the failure in rectal temperature rise among the remaining six animals from which only one in group B (No. 29) reacted with short-term fever (Fig. 1B, Table 1). The two animals showing the typical fever response (group A: 21, 22) developed high titers of PRV-specific antibodies as early as 12 days after intranasal virus application (Table 1). The course of body temperature of one piglet (29) in group B showed a short-term moderate rise pointing to a PRV sensitization (Fig. 1B). Both animals had developed low titers of PRV specific antibodies at day 12 after intranasal application (Table 1). The remaining four animals (group C) did not respond with a rise of body temperature within 6 days and also did not have detectable serum antibodies at day 12 after intranasal PRV application indicating a non PRV-sensitized status (Fig. 1C; Table 1). To control the assumption of a PRV sensitization (group B) versus the complete failure of PRV take (group C) a second virus application by the intramuscular (i.m.) route was performed to avoid insecurity in local virus application. The i.m. route of second virus application induced a fever response in all animals from groups B and C (Fig. 1B and C). However, the kinetics of antibody response revealed the difference between preceding PRV sensitization versus the failure of triggering an immune response by intranasal PRV application. Whereas both animals in group B had developed serum antibody titers already 6 days after i.m.
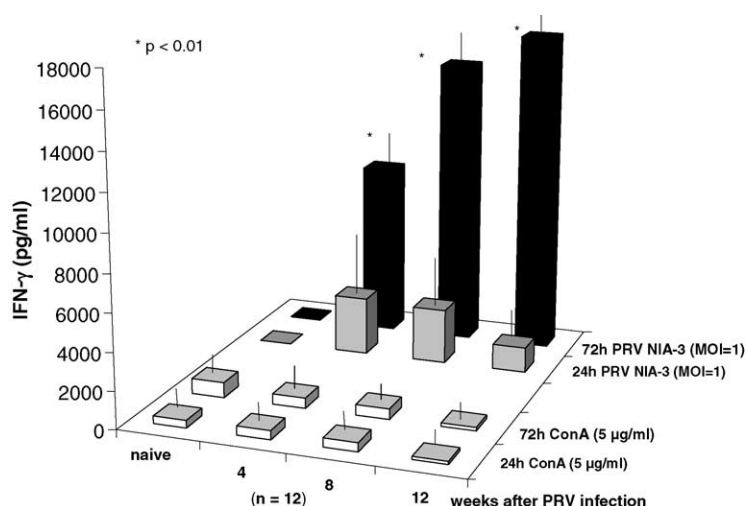
Fig. 2. IFN-γ response after in vitro (re)stimulation. PBMC from non infected (naïve) and PRV infected animals (4, 8, 12 weeks after infection) were either stimulated with ConA or with PRV. The content of IFN-γ in the PBMC supernatants was quantified after 24 and 72 h in the IFN-γ specific ELISA.

infection, the non-sensitized animals in group C needed 12 days for the formation of PRV specific serum antibodies after i.m. infection (Table 1).

3.2. PBMC-mediated IFN-γ production after PRV re-stimulation

After ConA stimulation as a positive control IFN-γ was detectable in supernatants of PBMC from naïve as well as PRV challenged swine (Fig. 2). However, after in vitro exposure to live PRV naïve PBMC never secreted IFN-γ higher than 40 pg/ml independent of a short (24 h) or longer incubation period (72 h). In contrast after PRV infection, in vitro PRV re-stimulation of PBMC from the same animals resulted in the release of high amounts of IFN-γ in the culture supernatants exceeding by far those after ConA stimulation (Fig. 2). Although the mean cell viability had decreased by 12% as compared to the beginning of in vitro culture, a significant increase ($P < 0.01$) in PRV induced IFN-γ secretion was observed after longer in vitro exposure (72 h versus 24 h) of PBMC to PRV (Fig. 2). The significantly higher IFN-γ concentrations after longer (72 h) in vitro presence of PRV as the PBMC re-stimulating agent can be explained by a promotion through type I IFN and other cytokines (Brinkman et al., 1993; Manetti et al., 1995; Saraneva et al., 1998; Taniguchi and Takaoka, 2001)

as well as by the autocrine IFN-γ mediated longevity of activated T-cells (Marrack et al., 1999; Akbar et al., 2000). A specificity of PBMC response to in vitro PRV re-stimulation was evident in PBMC supernatants tested 4 weeks after PRV infection. The mean IFN-γ concentration in the supernatants of PRV re-stimulated PBMC of 12 individuals was 5.195 pg/ml (Fig. 2) whereas the highest IFN-γ concentration after 72 h in vitro stimulation of the same cells with the closely related alpha herpesvirus BHV-1 was only 82 pg/ml (not shown). The PRV dependence of IFN-γ release was also demonstrated by the frequency of IFN-γ producing cells in IFN-γ specific ELISPOTs (Fig. 3). Stimulation of PBMC with the same MOI of bovine alpha herpesvirus BHV-1 (Fig. 3B) and medium cultivated cells (Fig. 3A) served as controls. As shown in Fig. 3B, BHV-1 stimulation of PBMC at 4 weeks after PRV infection survival only caused a slightly higher background compared to the cells incubated for 72 h in culture medium alone. In contrast, the same PRV re-stimulated PBMC regularly produced a high frequency of spots indicating a specificity in alpha herpesvirus-mediated induction of IFN-γ (Fig. 3C). Together with the identification of responding lymphocyte subtypes (Saalmüller et al., 1999) it remains to be determined which component(s) of PRV in contrast to related alpha herpesviruses such as BHV-1 are essential for the induction of IFN-γ synthesis in
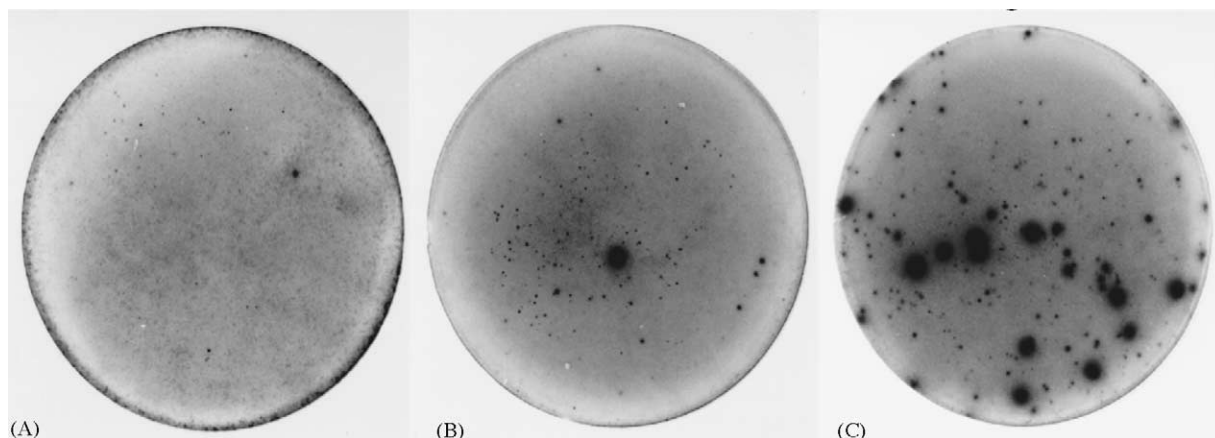
Fig. 3. Determination of antigen-specific induction of the frequency of IFN-γ producing PBMC in ELISPOT assays (2×10^5 cells/well, 72 h) either re-stimulated with PRV (C) or stimulated with the closely related alpha herpesvirus BHV-1 (B). Only slightly elevated background spot numbers compared to culture medium controls (A) are present when BHV-1 is used as the stimulating virus in PRV primed PBMC from PRV challenge surviving piglets.

antigen primed porcine PBMC. It cannot be excluded that a glycoprotein per se or even certain epitopes might be sufficient for PBMC-mediated IFN production in re-stimulation experiments as already shown with porcine cells for corona virus type I IFN inducing capacity (Charley and Laude, 1988) and recently for herpes simplex virus glycoprotein D induced IFN-γ production with murine CD4^{+} peripheral T-lymphocytes (BenMohamed et al., 2003).

The PBMC-mediated, PRV-specific IFN-γ secretion was also tested after the fortuitous diverse course of body temperature reaction following intranasal virus application in a group of eight animals (Table 1). As early as 6 days after productive infection accompanied by a strong fever response the IFN-γ secretion of PBMC was significantly higher (2450 and 1838 pg/ml) than the pre-infection level (<40 pg/ml) in the two piglets from subgroup A (Table 1). At that early time point after intranasal PRV application PBMC from the animals in groups B and C which had not reacted with a significant rise in body temperature failed to produce IFN-γ in response to in vitro PRV re-stimulation (Table 1). However, again in correlation with the development of fever, PBMC from all animals in groups B and C produced IFN-γ 6 days after the second and secure i.m. virus application (Table 1). Thus the productive PRV infection in any case was linked to a fever response and later on to the formation of serum antibodies within 12 days. The latter were

also detectable after i.m. application of PRV in the previously non-responding piglets (intranasal application group C) but with a time delay of 1 week compared to the earlier antibody detection (day 12 p.i.m. application) in sensitized animals (group B, Table 1). In addition the PBMC from obviously PRV-sensitized piglets in group B responded with a higher IFN-γ secretion early after the i.m. virus application (day 6) than the animals keeping a naïve status after the failure of intranasal virus take. Although strong individual differences in IFN-γ production exist similar to the antibody response (Hessing et al., 1995), a general rise in the IFN-γ levels in PBMC supernatants was observed later after productive PRV infection persisting at least for 12 weeks (Fig. 2). This is the first example of a direct ex vivo correlation of PBMC-mediated IFN-γ production depending on productive PRV infection of piglets accompanied by fever and followed by the presence of serum antibodies 12 days after infection. The detection of PRV-specific IFN-γ production by PBMC offers an early and reliable parameter for cellular immune response preceding the specific antibody formation (Zuckermann et al., 1998, 1999; Mateu de Antonio et al., 1998). Following our observations with sensitized and non-responding animals the IFN-γ production of PBMC to in vitro PRV (re-)stimulation may be a sensitive reaction to indicate efficient vaccination and to assess the potency of vaccines (Fischer et al., 2000;

Dufour et al., 2000; Zuckermann, 2000; Robinson and O'Garra, 2002).

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