

## Immunity to porcine rubulavirus infection in adult swine

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### Abstract

The immune response against the porcine rubulavirus was analyzed in experimentally infected adult pigs. High titers of virus neutralizing and hemagglutinating inhibitory antibodies were identified in infected animals. The antibody specificity was directed towards HN, M, and NP rubula virion proteins; immunodominance of HN proteins was demonstrated. Peripheral blood mononuclear cells from infected, but not from non-infected pigs proliferated in vitro in response to virus antigenic stimuli, showing a bell-shaped plot with the highest peak at 5 weeks post-infection. Virus-induced lymphoblasts expressed CD4<sup>+</sup>CD8<sup>+</sup> phenotype, whereas lectin-induced lymphoblasts were mainly identified as CD4<sup>+</sup>CD8<sup>-</sup> cells. Phenotype analysis of freshly prepared PBMC revealed increased number of both monocytes (PoM1<sup>+</sup>) and total T lymphocytes (CD2<sup>+</sup>) early during infection, with reduced values of B lymphocytes at 4 weeks post-infection. Decrease in CD4<sup>+</sup>CD8<sup>-</sup> blood cells was observed at 3 weeks post-infection, whereas both CD4<sup>-</sup>CD8<sup>+</sup> and CD4<sup>+</sup>CD8<sup>+</sup> cells increased 1 and 4 weeks post-infection, respectively. This work discusses the relevance of CD4<sup>+</sup>CD8<sup>+</sup> T cells in the control of porcine rubulavirus infection. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Immunity; Lymphocytes; Antibodies; Rubulavirus; Paramyxovirus; Porcine

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## 1. Introduction

Blue eye disease of swine emerged in La Piedad Michoacan, Mexico (LPM) in 1980; it was characterized by encephalitis, pneumonia and corneal opacity in suckling pigs and reproductive disorders in older pigs. Clinical signs were reproduced in piglets inoculated with a virus with hemagglutinating activity isolated in 1981 (Stephano et al., 1988). Characterization of LPM virus (LPMV) isolated from a meningo-encephalitic piglet in 1984 confirmed paramyxoviral etiology of the disease. LPMV is an enveloped RNA virus, which possesses hemagglutinating, hemolytic and syncytia-forming activities (Moreno-López et al., 1986). LPMV is constituted by six structural proteins, hemagglutinin–neuraminidase (HN), fusion (F) and matrix (M) proteins are expressed at the envelope; whereas nucleoprotein (NP), phosphoprotein (P) and large (L) proteins form the nucleocapsid (Sundqvist et al., 1990). Cloning and sequencing of M (Berg et al., 1991), HN (Sundqvist et al., 1992); P (Berg et al., 1992), F (Berg et al., 1997), and L (Svenda et al., 1997) protein genes showed that LPMV is closely related to Mumps virus and Simian virus 5, supporting the classification of porcine LPMV in the *Rubulavirus* genus of *Paramyxoviridae* family (Rima et al., 1995).

A severe neuropathological syndrome developed in 3-day-old pigs inoculated with the rubulavirus, which died or were moribund from 8 to 11 days after infection. The virus was widespread in the central nervous system (CNS) (Allan et al., 1996), but the highest titers of virus were found in the midbrain of these pigs (McNeilly et al., 1997). Inoculation of porcine rubulavirus in 17-day-old pigs induced mild respiratory and nervous signs, with restricted distribution of virus antigen to olfactory bulb and midbrain structures (Allan et al., 1996).

Naturally infected adult pigs showed decreased infertility rates in gilts, stillbirths and mummified fetuses in pregnant sows and epididymitis and orchitis in boars (Stephano, 1994). A novel strain of porcine rubulavirus (Jalisco/1992) was isolated during an outbreak in a breeding farm (Ramírez-Mendoza et al., 1997). Genital tract alterations were reproduced in 9-month-old sexually mature boars inoculated with this virus, including swelling of epididymis, temporal orchitis, reduced spermatozoa concentration and motility, as well as testis atrophy. Rubulaviral antigen was recognized at the epithelium of epididymis head at 15, 30, 45, and 70 days after infection. No infectious virus nor viral antigen was identified in the CNS of these pigs (Ramírez-Mendoza et al., 1997).

Host susceptibility to viruses depends on both expression of virus receptors and virus survival to immune surveillance. We have identified that porcine rubulavirus recognizes Neuraminic acid  $\alpha$ 2,3 Galactose (NeuAc $\alpha$ 2,3Gal) oligosaccharides, which inhibited the virus hemagglutinating activity (Reyes-Leyva et al., 1993). We found that cell culture expression of NeuAc $\alpha$ 2,3Gal, but not its isomer NeuAc $\alpha$ 2,6Gal, was a requirement for the porcine rubulavirus infection process (Reyes-Leyva et al., 1997). Furthermore, we have also identified a broad expression of NeuAc $\alpha$ 2,3Gal-glycoconjugates at CNS and respiratory tissues of newborn pigs, whereas high expression of NeuAc $\alpha$ 2,6Gal was observed in the olfactory mucosa and bulb of adult pigs, indicating that expression of specific oligosaccharide sequences seems to be correlated with tissue susceptibility to porcine rubulavirus.

Several reports suggest that porcine rubulavirus is cleared by host defenses in infected older pigs (Stephano et al., 1988; Allan et al., 1996); however, until now, no information is available on the role of immune responses in the control of porcine rubulavirus infection. This work analyzes the immune response to porcine rubulavirus in infected adult pigs.

## **2. Materials and methods**

### *2.1. Virus*

The porcine rubulavirus strain Jalisco/1992, was obtained from the Veterinary Faculty, National University of Mexico, Mexico (Ramírez-Mendoza et al., 1997). Rubulavirus was propagated in the pig kidney cell line PK-15 with MEM (supplemented with 10% fetal bovine serum, 100 U ml<sup>-1</sup> penicillin and 100 µg ml<sup>-1</sup> streptomycin) until the cultures showed maximum cytopathic effect. Then, infected cell cultures were frozen/thawed thrice and supernatants were clarified by centrifugation at 3200 rpm for 45 min at 4°C. The virus infectivity was titrated in cell cultures in 96-well microculture plates (Falcon Labware, NJ), using serial 10-fold dilutions of virus made in MEM, and the titer was expressed in TCID<sub>50</sub> ml<sup>-1</sup> (Burleson et al., 1992). For viral antigen production, the virus in the supernatants was concentrated by centrifugation at 100 000 g for 4 h at 4°C, filtered through 0.45 µm membranes, aliquoted and stored at -70°C until use. Negative antigen control was similarly prepared with non-infected cell culture supernatants (Ramírez-Mendoza et al., 1997). Protein determination was made by the method of Bradford (1976).

### *2.2. Antibodies*

Mouse monoclonal antibodies (MAb) specific for porcine cluster of differentiation (CD) molecules were purchased from VMRD (Washington, DC), these were: MAb MSA4, IgG2a anti-CD2; MAb 74.12.4, IgG2b anti-CD4; MAb 76.2.11, IgG2a anti-CD8; MAb PG130A, IgM anti Po-M1; and MAb PIg45A, IgG2b anti-IgM. Fluorescein isothiocyanate (FITC)-conjugated goat polyclonal antibodies anti-mouse IgG; phycoerythrin (PE)-conjugated rat MAb anti-mouse IgG2a, FITC-conjugated rat MAb anti-mouse IgG2b, and FITC-conjugated goat polyclonal antibodies anti-mouse IgM were purchased from Becton & Dickinson (Mountain View, CA).

### *2.3. Experimental design*

Ten 9-month-old male, York Landrace hybrid pigs were used. They were born at a 'blue eye' disease-free farm and were free of porcine rubulavirus and pseudorabies virus as assessed by the lack of specific serum antibodies in both virus neutralization and indirect immunofluorescence tests. Seven boars were intranasally inoculated with 5 ml of rubulavirus (10<sup>4</sup> TCID<sub>50</sub> ml<sup>-1</sup>) and placed individually in an isolation facility. Blood

samples for both serum and cell preparations were taken 1 week before and each week after inoculation, for 7 weeks post-infection (p.i.).

#### 2.4. Serological test

Hemagglutination inhibition (HI) and virus neutralization (VN) tests were carried out with sera from infected and non-infected pigs, according to standard procedures used for diagnosis of rubulavirus porcine diseases (Ramírez-Mendoza et al., 1996). Briefly, for the HI test, sera were heat-inactivated at 56°C for 30 min and treated with 25% acid-washed kaolin to remove non-specific inhibitors, then, sera (50 µl) were titrated by serial 2-fold dilutions in phosphate-buffer saline (PBS, pH 7.2). HI test was performed using 0.5% bovine erythrocytes (50 µl) and virus antigen (50 µl) with eight hemagglutinating units (HAU, titre=8) in U-well plates (Falcon). The HI titre was considered with the last dilution of serum that completely inhibited the eight HAU of the virus. For VN assays, sera were diluted in MEM supplemented with 4% fetal bovine sera. Serial 2-fold dilutions of sera were performed in 96-well flat bottom microtitre plates and mixed with an equal volume (50 µl) of rubulavirus containing 300 TCID<sub>50</sub>. The plates were then incubated for 60 min at 37°C. Finally, 200 µl of PK-15 cell suspension (10<sup>4</sup> cells/well) was added to each well and incubated for 72 h at 37°C. The end point was determined by HAU in the infected cells' supernatants. Equal volumes (50 µl) of supernatant fluid and 0.5% bovine erythrocytes were mixed in a U-well microtitre plate. The VN titre is considered with the last dilution of serum that completely inhibited hemagglutinin production of the virus.

#### 2.5. Western-blot assays

Virus proteins (100 µg ml<sup>-1</sup>) were separated by SDS-PAGE (Laemmli, 1970) and transferred to 0.22 µm nitrocellulose membranes (Towbin et al., 1979). Membranes were treated for 1 h at 37°C with washing buffer (WB) (0.1 M phosphate, 0.5 M sodium chloride, 5% fat-free milk, 0.5% Tween 20, pH 7.2). For immunodetection, membranes were incubated with a 1:25 dilution of the test sera, 1 h at room temperature, washed with WB, incubated with biotin-labeled rabbit anti-porcine IgG diluted 1:150, 1 h at room temperature, washed with WB, incubated with 1:3000 dilution of streptavidin–horseradish peroxidase conjugate, 30 min at room temperature, washed with WB, and finally incubated with NBT–BCIP substrate.

#### 2.6. Lymphocyte proliferation assay

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll–Hypaque 1077 (Sigma, St. Louis, MO) gradient centrifugation (density=1.067) and cultured at 1.5×10<sup>5</sup> well<sup>-1</sup> in 96-well flat bottom plates in HEPES-buffered RPMI1640 culture medium, supplemented with 5×10<sup>-5</sup> M 2-mercaptoethanol, 2 mM sodium pyruvate, 2 mM L-glutamine, 100 U ml<sup>-1</sup> penicillin, 100 µg ml<sup>-1</sup> streptomycin, 1 µg ml<sup>-1</sup> gentamycin, 10% non-essential amino acids and 10% fetal bovine serum (Kimman et al., 1993). PBMC were stimulated with 10 µg ml<sup>-1</sup> of viral antigen previously inactivated by

heating for 10 min at 90°C. Lack of infectious virus was subsequently checked by cell culture. The cells were stimulated for 5 days at 37°C, in a humidified incubator with 5% CO<sub>2</sub>. Negative antigen was used for mock stimulation. Phytohemagglutinin (PHA, 8 µg ml<sup>-1</sup>) and Concanavalin A (Con A, 1 µg ml<sup>-1</sup>) (Sigma) were used as positive controls; 1 µCi of <sup>3</sup>H-thymidine (Specific activity=6.7 Ci/mmol; New England Nuclear, Boston, MA) was added during the last 18 h of culture (Kimman et al., 1993). Cells were harvested onto glass fiber filters and radioactivity incorporated into DNA was measured in a Beckman LS6000SE SE-counter (Beckman, Fullerton, CA). Lymphoblasts obtained after stimulation of PBMC with virus or lectins were processed for double-stained flow cytometry assays, to identify expression of CD4 and CD8 molecules on these cells.

### 2.7. Single and double cytofluorometric (CF) analysis

Phenotype of freshly prepared PBMC or lymphoblasts was determined as in Summerfield et al. (1996). Briefly, for single CF analysis, cells were incubated with mouse MAb specific towards CD2 (MAb MSA4), Po-M1 (MAb PG130A) or IgM (PIg45A); followed by incubation with FITC-conjugated goat polyclonal antibodies against mouse IgG. For double CF analysis, cells were incubated with MAbs anti-CD8 (76-2-11) and anti-CD4 (74.12.4), followed by incubation with both FITC- and PE-conjugated isotype-specific rat MAbs. All incubations were carried out at 4°C for 15 min. Cells were washed with 0.1 M PBS, pH 7.2, 0.2% BSA, 0.1% NaN<sub>3</sub>. Stained cells were analyzed by cytofluorometry, (FAS Calibur Becton & Dickinson, Mountain, View, CA) as described.

## 3. Results

### 3.1. Clinical signs

Infection of sexually mature pigs with the porcine rubulavirus induced the clinical signs of reproductive disease, which consisted in epididymis and testis swelling. Neither neuropathological nor other lesions were observed in these animals, as previously described (Ramírez-Mendoza et al., 1997). The presence of circulating virus was determined by immunofluorescence assays on blood slides, and viremia was identified from 6 to 15 days p.i. (data not shown).

### 3.2. Antibody response

Induction of antibody response during rubulavirus infection in adult swine was determined by VN and HI tests. Seroconversion started at 1 week p.i. with the production of VN antibodies, which ranged from 4 to 6 log<sub>2</sub> titers during the first 4 weeks p.i. VN titers increased up to 8.5 log<sub>2</sub> at 5 weeks p.i., remaining at high titers until the end of the study. HI antibodies were identified at the second week p.i., with 5 log<sub>2</sub> titers. In contrast to the VN response, HI titers remained in a plateau throughout the study (Fig. 1).

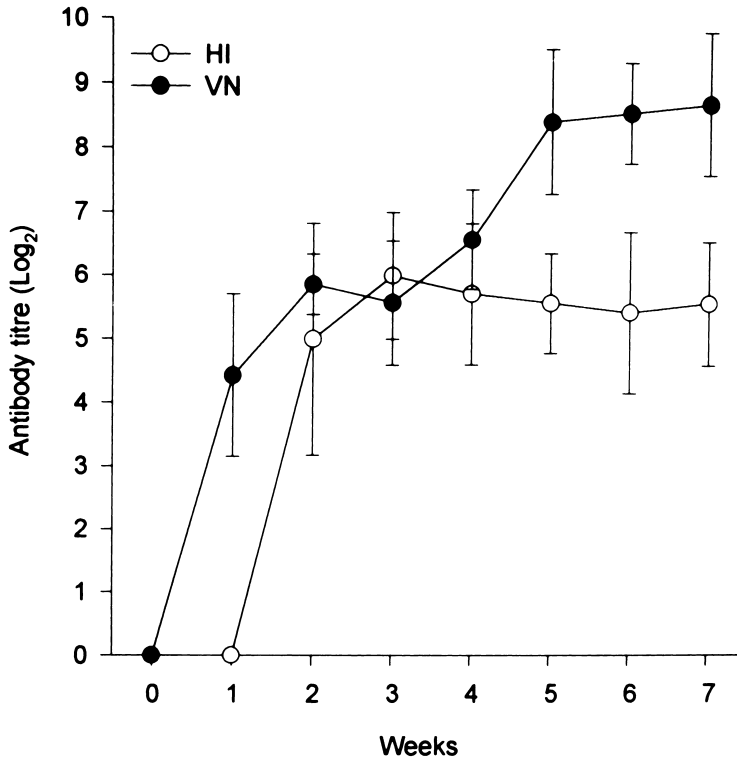


Fig. 1. Antibody response from rubulavirus infected pigs. Sera from infected pigs ( $n=7$ ) were analyzed by VN and HI assays each week after infection.

### 3.3. Antibody specificity

To characterize the specificity of antibodies induced by the virus and the time at which each viral protein antigen was recognized, rubulavirus proteins were separated by SDS-PAGE and Western blot assays were performed with sera from infected pigs, obtained weekly. These assays revealed antibody response against 68, 66, and 40 kDa antigens, corresponding to NP, HN, and M proteins. Western blot assays revealed antigenic predominance of HN glycoprotein in the antibody response triggered against rubulavirus infection, since HN glycoprotein was recognized by all infected pigs. Six of the seven infected pigs recognized HN glycoprotein at 2 weeks p.i. at 3 weeks p.i. all pigs had responded and remained seropositive hereafter (Fig. 2). Response against M protein was identified from 4 weeks p.i. by three out of the seven infected pigs. Antibodies against NP protein were identified in four pigs at 5 weeks p.i., and in six pigs at 6 and 7 weeks p.i.; one pig remained seronegative to NP. Antibodies against F, P, or L proteins were not identified in the Western blot under the conditions of these assays.

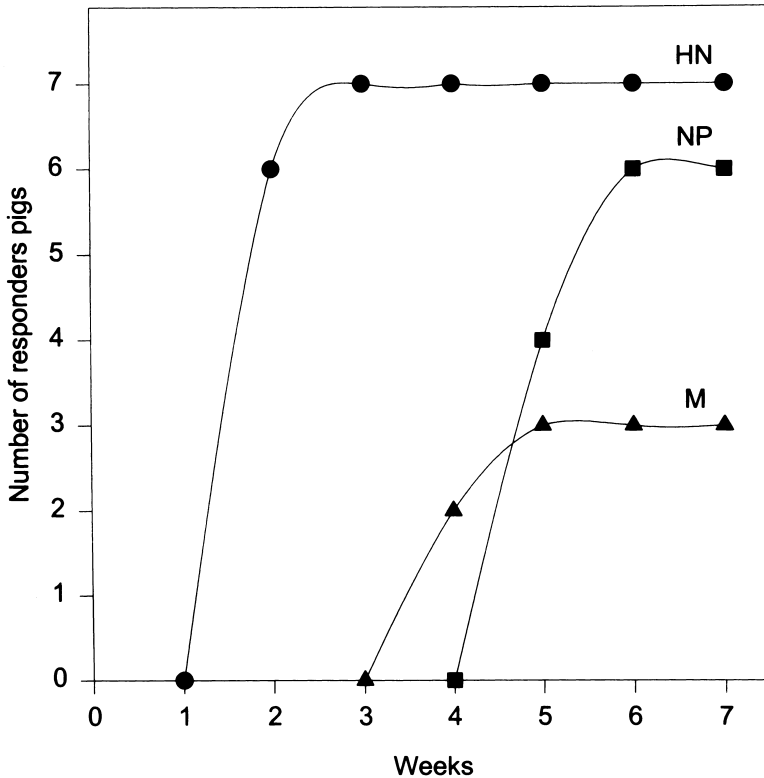


Fig. 2. Kinetics of immune response measured by the number of pigs showing specific antibody response to HN, NP, and M after infection with porcine rubulavirus. Porcine rubulavirus proteins were separated by SDS-PAGE and analyzed by Western blot assays with sera from infected pigs obtained weekly.

#### 3.4. Lymphocyte proliferation induced by lectins

The status of the cellular immune response was analyzed in lymphoproliferative assays using lectins. We prepared PBMC from control and rubulavirus-infected pigs to evaluate their capacity to proliferate in response to T mitogenic lectins. Cells were cultured in the presence of PHA or Con A lectins during 72 h and proliferation was determined by incorporation of  $^3\text{H}$ -thymidine. Results are expressed as stimulation indexes ( $\text{SI} = \text{cpm of stimulated cells} / \text{cpm of unstimulated cells}$ ). The plots of proliferative responses obtained after stimulation of PBMC with PHA or Con A (Fig. 3) were similar among infected and control pigs. PHA- or Con A-stimulated PBMC obtained from non-infected pigs showed proliferation values ranging from 40 to 80 SI throughout the study. The proliferative responses induced by both PHA and Con A in PBMC from infected pigs were irregular along time; however, three events were consistent: (a) A lack of proliferative response at 1 week p.i. ( $\text{SI} < 20$ ), which represented a significant difference of  $p < 0.005$  with respect to uninfected pigs; (b)  $\text{SI} (> 80)$  higher than those of non-infected pigs were measured at 2–5 and 7 weeks p.i.; and (c) a decline in the proliferative response at 6 weeks p.i. (Fig. 3).

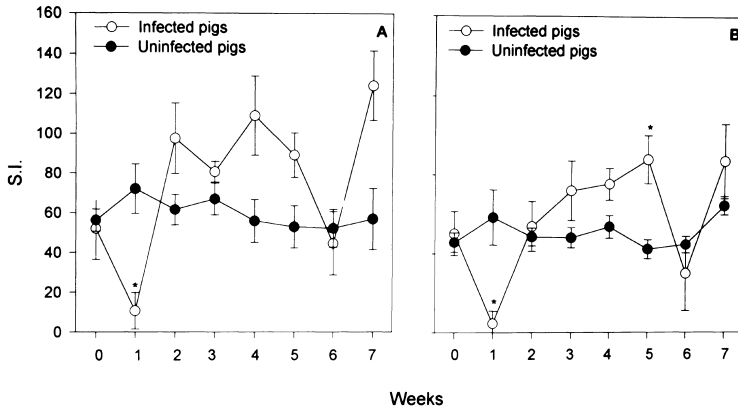


Fig. 3. Mitogen induced lymphoproliferative responses. PBMC from non-infected pigs (filled circles,  $n=3$ ) and infected pigs (open circles,  $n=7$ ), were stimulated with Con A (A) and PHA (B) lectins. Response was evaluated by SI 18 h after  $^3\text{H}$ -thymidine incorporation.  $\text{SI} = \text{cpm} \times 10^3 \text{ stimulated cells} / \text{cpm} \times 10^3 \text{ unstimulated cells}$ . The cpm in PBMC from unstimulated pigs was  $<900$ .  $^*p < 0.005$ .

### 3.5. Lymphocyte proliferation induced by viral antigen

The optimal conditions for virus-induced lymphoproliferative response were established previously, the highest SI was obtained by incubation of PBMC in the presence of  $10 \mu\text{g ml}^{-1}$  of virus antigen for 5 days (data not shown); these conditions were applied in this study. In order to identify the induction of cellular immune response through infection, PBMCs were prepared from infected and non-infected pigs and cultured in the presence of both viral or negative antigen. The immunogenicity of porcine rubulavirus antigen was confirmed by the induction of proliferative response in PBMC from infected pigs stimulated with viral-recall antigen; this response was apparent at 2 weeks p.i., showing a peak at 4 weeks p.i., when proliferation had reached a mean of  $14 \pm 2$  SI (Fig. 4). PBMC from non-infected pigs did not proliferate after incubation *in vitro* with rubulavirus antigen.

### 3.6. Virus-induced lymphoblasts expressed $\text{CD4}^+\text{CD8}^+$ phenotype

Phenotype analysis was performed to identify predominance of single positive (SP)  $\text{CD4}^+$ , (SP)  $\text{CD8}^+$ , or double positive (DP)  $\text{CD4}^+\text{CD8}^+$  T cell subpopulations proliferating in response to rubulaviral antigen. Analysis of cell phenotype was determined by double-strained flow cytometry after 5 days of viral-antigen stimuli. PHA- and mock-stimulated PBMC were used as positive and negative controls, respectively. Resting (small lymphocytes) (Fig. 5, R1) and activated cells (lymphoblasts) (Fig. 5, R2) in the same culture were independently analyzed for their phenotype based on their characteristic light scatter profile (Fig. 5, top). After this, cell phenotypes were determined by contour plots. Small lymphocytes (R1) did not reveal differences between virus or PHA stimulated PBMC (Fig. 5, middle: B and C, respectively). In virus-induced

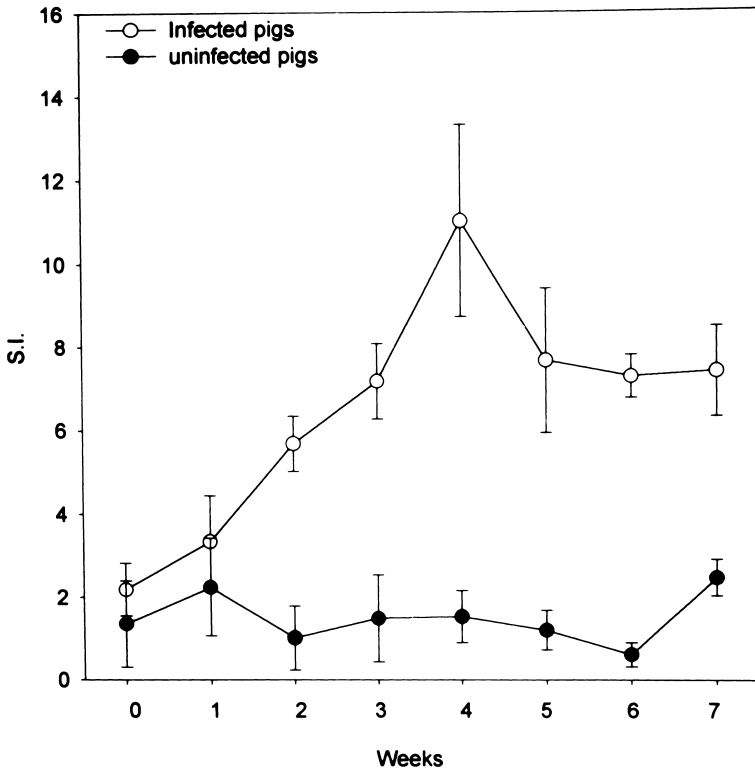


Fig. 4. Virus-induced lymphoproliferative responses. PBMC from infected (open circles,  $n=7$ ) and non-infected pigs (filled circles,  $n=3$ ) were cultured in the presence of virus antigen. SI was evaluated 18 h after  $^3\text{H}$ -thymidine incorporation.  $\text{SI} = \text{cpm} \times 10^3$  stimulated cells /  $\text{cpm} \times 10^3$  unstimulated cells. The cpm in infected pigs in the presence of mock virus, as well as in uninfected pigs in the presence of viral antigen was  $<1200$ . \* $p < 0.005$ .

lymphoblasts (R2) we identified 39% of SP  $\text{CD4}^+$ , 14% of SP  $\text{CD8}^+$ , and 21% of DP  $\text{CD4}^+\text{CD8}^+$  cells (Fig. 5, bottom: B), the two first values corresponded to an increase of 95% and 110%, while the latter, represents a reduction with respect to mock-induced lymphoblasts (Fig. 5, bottom: A). In contrast, phenotyping of PHA-stimulated PBMC revealed 57% of SP  $\text{CD4}^+$ , 22% of SP  $\text{CD8}^+$ , and 9% of  $\text{CD4}^+\text{CD8}^+$  cells (Fig. 5, bottom: C). The values of  $\text{CD4}^+$  cells correspond to an increment of 185% with respect to mock-stimulated lymphoblasts. In addition, it is noteworthy that DP cells did not proliferate in response to PHA stimuli, as occurred in virus.

### 3.7. Identification to T, B, and macrophage cells in PBMC from infected pigs

The relative values of blood mononuclear cell populations, i.e.  $\text{CD2}^+$  T cells,  $\text{IgM}^+$ , B cells and  $\text{PoM1}^+$  monocytes/macrophages were determined 1 week before and 4 weeks after infection. For cell identification, single color cytofluorometric analysis was performed in freshly prepared PBMC. Both infected and non-infected pigs showed

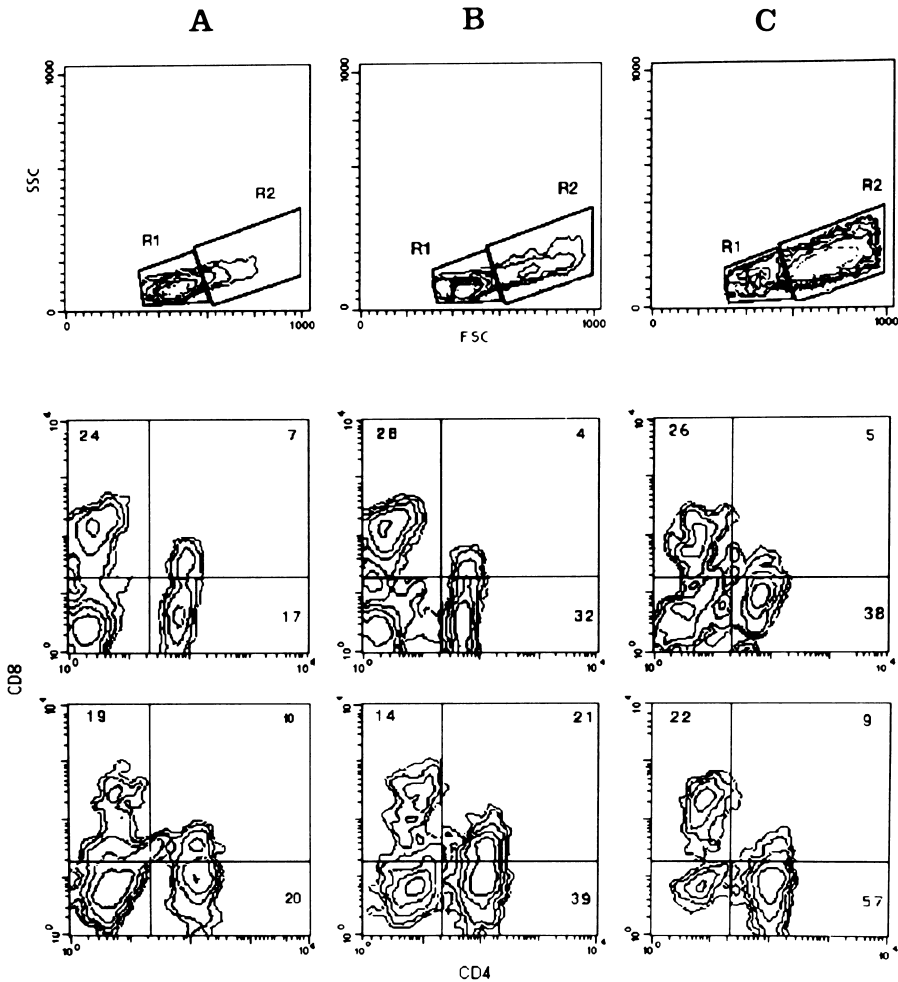


Fig. 5. Phenotyping of virus-induced lymphoblasts in PBMC from rubulavirus-infected pigs. PBMC were cultured with virus-antigen (B), PHA (C) or with mock stimuli (A); incubated with anti-CD4 (74.1.24) and anti-CD8 (76.2.11) MAb and their size analyzed (forward scatter, FCS) vs. granular contents (side scatter, SSC) (top). Resting cells were gated in region 1 (R1, middle) whereas lymphoblasts in region 2 (R2, bottom). CD4 and CD8 percentages were determined by contour plot.

similar values of CD2<sup>+</sup>, IgM<sup>+</sup>, and PoM1<sup>+</sup> cells in the blood samples obtained before infection. Virus infection induced an *in vivo* T cell proliferative response that was evidenced by the increase of CD2<sup>+</sup> cells in infected pigs, when compared with control ones (Table 1). The values of B lymphocytes IgM<sup>+</sup> in infected pigs were similar to those of non-infected pig during the first two 2 weeks p.i. however, a significant decrease in circulating B cells was observed at 3 and 4 weeks p.i. (Table 1). The values of PoM1<sup>+</sup> cell population were the most variable after rubulavirus infection. PoM1<sup>+</sup> cells increased

Table 1  
The relative values of blood mononuclear cell populations were determined by flow cytometry

Weeks	T Lymphocytes		B Lymphocytes		Monocytes	
	U	I	U	I	U	I
0	39.0±2.0	39.0±2.0	27.0±2.1	27.6±5.2	16.0±2.6	17.6±3.3
1	41.5±1.8	50.2±6.1	27.0±2.6	25.0±4.4	17.3±4.6*	32.6±5.6
2	40.3±2.5	44.7±4.3	25.6±4.0	29.5±3.2	16.5±4.9	8.24±4.4
3	39.3±3.0	32.7±3.3	27.0±2.0	19.1±3.6	16.3±2.3	7.7±4.6
4	41.0±3.0	48.7±2.7	29.0±2.7	16.0±5.0*	14.6±2.3	12.3±4.2

PBMC from infected ( $n=7$ , I) and uninfected pigs ( $n=3$ , U), were obtained weekly to determine percentages of T lymphocytes ( $CD2^+$ ), B lymphocytes ( $IgM^+$ ), and monocytes/macrophages ( $PoM1^+$ ). Flow cytometry was performed using mouse monoclonal antibodies anti- $CD2$ ; anti- $IgM$ , and anti- $PoM1$  recognized by isotype-specific rat anti-mouse antibodies (For more details, see Section 2).

\* $p<0.005$ .

up to 32.6% ( $\pm 5.6$ ) at 1 week p.i., but decreased to 8.24% ( $\pm 4.4$ ) and 7.7% ( $\pm 4.6$ )% at 2 and 3 weeks p.i., respectively; at 4 weeks p.i., monocytes returned to normal values (Table 1).

### 3.8. $CD4^+CD8^+$ T cell expression in infected pigs

For T cell-subtype identification, double-stained cytofluorometric analyses were performed in freshly prepared PBMC. Infected and control pigs showed similar values of SP  $CD4^+$ , SP  $CD8^+$ , and DP  $CD4^+CD7^+$  cells in the blood samples taken before infection (Fig. 6). The  $CD4^+CD8^-$  cells did not change at the first 2 weeks p.i. in infected and non-infected pigs; however, at 3 weeks p.i. the values in infected pigs

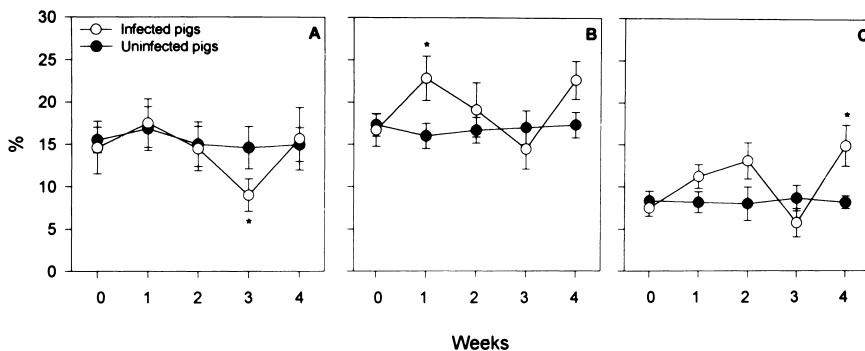


Fig. 6. The relative values of T lymphocyte subsets from blood mononuclear cells determined by flow cytometry. PBMC from infected (open circles,  $n=7$ ) and non-infected pigs (filled circles,  $n=3$ ), were obtained weekly to determine percentages of  $CD4^+CD8^-$  (A),  $CD4^-CD8^+$  (B), and  $CD4^+CD8^+$  (C), using mouse monoclonal antibodies anti- $CD4$ , and anti- $CD8$  recognized by isotype-specific rat anti-mouse antibodies. \* $p<0.005$ .

decreased to  $8.98 \pm 1.92$  ( $p < 0.005$ ), which corresponds to  $0.9 \times 10^6$  CD4<sup>+</sup>CD8<sup>-</sup> cells ml<sup>-1</sup> of blood. At the end of the experiment, this cellular population increased and showed similar numbers to those found in control-animals ( $1.6 \times 10^6$  cells ml<sup>-1</sup> of blood). An increase in CD4<sup>-</sup>CD8<sup>+</sup> cells was observed at the first week p.i., thereafter this population decreased presenting the lowest levels at the third week. The DP CD4<sup>+</sup>CD8<sup>+</sup> cells from infected pigs increased at first and second week p.i. (from  $0.8 \times 10^6$  to  $1.3 \times 10^6$  cells ml<sup>-1</sup>), although this increment does not represent a significant difference ( $p > 0.005$ ). At the third week p.i., this population of cells presented the lowest percentages in infected pigs; however, at the fourth week p.i., the percentages of CD4<sup>+</sup>CD8<sup>+</sup> in infected pigs were  $14.91 \pm 2.45$  ( $1.5 \times 10^6$  cells ml<sup>-1</sup>), whereas, in uninfected pigs, they were  $8.16 \pm 0.76$  ( $p < 0.005$ ) ( $0.8 \times 10^6$  cells ml<sup>-1</sup>).

#### 4. Discussion

The structural and biological characteristics of porcine rubulavirus have been described previously (Stephano et al., 1988; Linné et al., 1992). This virus produces acute meningoencephalitis in suckling pigs (Stephano et al., 1988; Allan et al., 1996), whereas a non-fatal reproductive syndrome is shown in infected adult pigs (Ramírez-Mendoza et al., 1997). Our group has been interested in establishing the mechanisms involved in the susceptibility and resistance to porcine rubulavirus; indeed we have identified the specificity of porcine rubulavirus towards oligosaccharide structures and their role in the infection process (Reyes-Leyva et al., 1993, 1997). In this work, we describe, for the first time, some parameters concerning antibody and T cell response during experimental infection of adult pigs. The antibody response was monitored with VN and HI tests. Serum-neutralizing antibodies were detected as early as 1 week p.i., but the highest titers were observed after 4 weeks p.i. Antibodies able to inhibit the hemagglutinating activity of the virus were detected at 2 weeks p.i., presenting low and constant titers during the experiment. Presence of specific antibodies towards rubulavirus proteins, with high titers until 1 year after the occurrence of natural infection, have been described, suggesting the relevance of antibodies in the control of infection (Stephano et al., 1988). In infections induced by other paramyxoviruses, such as parainfluenza virus type 3, the presence of serum-neutralizing antibodies has also been correlated with the resistance to infection (Chanock and McIntosh, 1990). The specificity of antibodies was further characterized by Western blot analysis, which showed that all infected pigs recognized the HN glycoprotein from 2 weeks p.i. and thereafter. Some infected pigs also recognized M and NP proteins, but at 4–6 weeks p.i., indicating that the porcine rubulavirus HN protein is the most immunogenic protein, and may provide the antigenic basis to improve diagnosis of this rubulavirus and development of vaccination programs. Work is now in progress to characterize the HN protein and its antigenic properties (Zenteno et al., 1998).

The cellular immune response was evaluated by measuring the proliferative response of PBMC to heat-inactivated virus and to lectins and subsequently determining the phenotype of proliferating lymphoblasts. An immunosuppression phase was identified in infected animals represented by low SI of lymphocytes stimulated with Con A and PHA

during the first week p.i. Stephano et al. (1988) described a high susceptibility of rubulavirus-infected animals to secondary infections, even to other paramyxoviruses (Griffin et al., 1994), which could result from the infection produced by the rubulavirus. The specific response to the rubulavirus was observed 5 days after stimulation of PBMC with inactivated virus. This response was considered positive when SI was greater than 4. The viral-recall antigen response was observed in the infected pigs from 2 weeks p.i. and thereafter. These results indicate that rubulavirus infection induces memory lymphocytes that can be reactivated in the *in vitro* assays. PBMC from uninfected pigs did not proliferate in response to viral antigen, confirming the specificity of the memory response.

Phenotype analysis of freshly prepared PBMC revealed increased values of monocytes (PoM1<sup>+</sup>) and total T lymphocytes (CD2<sup>+</sup>) during early infection, with reduced values of B lymphocytes at 4 weeks p.i. At the end of the study, we observed a reduced number of B cells (surface IgM<sup>+</sup>), probably as a result of the maturation of these cells to become antibody-producing cells, expressing surface IgG and producing high amounts of antibodies (Zinkernagel et al., 1996). The highest numbers of CD4<sup>-</sup>CD8<sup>+</sup>-positive cells were found at 1 week p.i. These T cells have been related usually to cytotoxic activity (Scott and Kaufmann, 1991), and are frequently observed at elevated percentages in several viral infections (Tripp et al., 1995). Although in this work the cytotoxic activity of CD4<sup>-</sup>CD8<sup>+</sup> cells was not evaluated, their relevance in early response to the porcine rubulavirus cannot be excluded.

Stimulation of PBMC from infected pigs with viral antigen-induced increase CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>+</sup>CD8<sup>+</sup> T lymphoblasts; in contrast, stimulation with lectins increased only CD4<sup>+</sup>CD8<sup>-</sup>, but did not affect CD4<sup>+</sup>CD8<sup>+</sup> cells, indicating that expression of the CD4<sup>+</sup>CD8<sup>+</sup> phenotype was driven by viral antigen stimulation. Interestingly, the numbers of CD4<sup>+</sup>CD8<sup>+</sup> T lymphocytes also increased in the whole blood of pigs infected with porcine rubulavirus. Although, the concentration of double-positive cells showed increased numbers during almost the whole experiment, they diminished at 3 weeks p.i., along with other T cell subpopulations. This phenomenon could be attributed to arrest of effector cells in immunocompromised tissues. Although the specific role of porcine CD4<sup>+</sup>CD8<sup>+</sup> lymphocytes has not been clearly stated, some authors suggest that this group of cells has memory functions, since these cells are able to react in an antigen-specific secondary immune response, suggesting that memory/effector cells are present within this T lymphocyte population (Summerfield et al., 1996; Zuckermann and Husmann, 1996). We suggest that the possible effector function of CD4<sup>+</sup>CD8<sup>+</sup> cells participates in the regulation of the immune response to rubulavirus in pigs. At the present, the possible effector function of CD4<sup>+</sup>CD8<sup>+</sup> in rubulavirus infections is under investigation in our laboratory.

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