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SCIENCE DIRECT
Virology 351 (2006) 249–259
VIROLOGY
www.elsevier.com/locate/yviro

Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs

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Received 22 March 2005; returned to author for revision 28 February 2006; accepted 31 March 2006
Available online 19 May 2006

Abstract

Immunization of piglets with two different European-type modified live vaccines against porcine reproductive and respiratory syndrome (PRRS) virus produced different outcomes. After vaccination, pigs became viremic (42 days), neutralizing antibodies did not develop, and frequencies of virus-specific gamma-interferon-secreting cells (IFN-γ-SC) were low. Levels of interleukin-10 (IL-10) produced by peripheral blood mononuclear cells (PBMC) seemed to inversely correlate with interferon-gamma responses. After a challenge with a virulent Spanish strain, one vaccine (V3) protected piglets against viremia while the other (V1) did not. The vaccine V3 induced the highest IFN-γ-SC frequencies. IL-2, IL-4 or transforming growth factor-beta responses were not detected at any time for neither of the vaccines. In contrast, haptoglobin rose in sera of viremic pigs after the challenge. These results indicated a strong involvement of IFN-γ, and maybe IL-10, in the development of immunity against PRRS virus.
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Keywords: Porcine reproductive and respiratory virus; Interferon-gamma; Interleukin-10; Vaccine

Introduction

Porcine reproductive and respiratory syndrome (PRRS) emerged in Europe in the early years of the 1990 decade and rapidly spread all over the continent becoming one of the major causes of economic losses for swine producers. Simultaneously, the disease emerged in USA with a similar impact on pig production. This syndrome, caused by an arterivirus (Meulenberg et al., 1994), is characterized in its reproductive form by abortions, stillbirths and weakly born piglets and, in young pigs, causes pneumonia and weight gain losses. PRRS virus (PRRSV) is a small positive-stranded polyadenylated RNA virus that contains nine open reading frames (ORFs). ORFs 1a and 1b comprise 80% of the genome and encode the RNA polymerase; ORFs 2 to 5 encode four structural glycoproteins (GP2, GP3, GP4 and GP5) and a small non-glycosylated protein called 2b (Wu et al., 2005); ORF6 encodes a non-glycosylated integral membrane protein (M) and ORF7 encodes the nucleocapside protein (N) (Conzelmann et al., 1993; Meulenberg et al., 1997; Snijder and Meulenberg, 1998). Currently, it is known that two distinct genotypes of the virus exist, the European and the American, that share only 55–80% similarity (Meng et al., 1995).

Soon after the discovery of the virus vaccines were marketed. The first ones were a modified live virus (MLV) vaccine called RespPRRS made of an American-type strain (Christopher-Hennings et al., 1997; Dee and Joo, 1997; Mengeling et al., 1996), and an inactivated vaccine called Cyblue, made of a European strain (Plana-Duran et al., 1997). From then, several vaccines, both attenuated and inactivated, have been commercialized.

Notwithstanding, none of the vaccines can claim full protection against the disease. On one hand, PRRSV, either wild type or attenuated, induces a low level of cell-mediated immunity (Meier et al., 2003) and neutralizing antibodies (NA)

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doi:10.1016/j.virol.2006.03.046

Table 1
Percentages of similarity between the PRRSV strains used in the study

	LV	V1	V3	VP21
ORF 2				
LV	–	94.6	98.0	94.6
V1	94.6	–	94.3	98.3
V3	98.0	94.3	–	93.9
VP21	94.6	98.3	93.9	–
ORF 3				
LV	–	94.2	98.3	94.0
V1	94.2	–	92.5	99.4
V3	94.2	92.5	–	93.3
VP21	94.4	99.6	93.9	–
ORF 4				
LV	–	94.2	99.0	94.5
V1	94.2	–	94.2	99.2
V3	99.0	94.2	–	93.5
VP21	94.5	99.2	94.5	–
ORF 5				
LV	–	94.9	98.9	94.5
V1	94.9	–	94.4	99.6
V3	98.9	94.4	–	94.0
VP21	94.5	99.6	94.0	–
ORF 6				
LV	–	97.7	97.7	96.9
V1	97.7	–	100	98.6
V3	97.7	100	–	98.6
VP21	96.9	98.6	98.6	–
ORF 7				
LV	–	96.6	100	96.3
V1	96.6	–	96.6	99.7
V3	100	96.6	–	96.3
VP21	96.3	99.7	96.3	–

Lelystad virus (LV, Genbank accession number *M96262*) was used as a reference.
LV = Lelystad virus, V1 = Modified live vaccine 1, V3 = Modified live vaccine 2, VP21 = wild-type Spanish PRRSV strain used for the challenge.

do not develop until a late phase of the infection (Meier et al., 2003; Vezina et al., 1996; Yoon et al., 1995). In addition, the cytokine imbalance after a vaccination or an infection suggests a possible downregulation of many cytokines (Royaee et al., 2004; Van Reeth et al., 1999). On the other hand, genetic diversity of PRRSV is thought to influence the efficacy of vaccines under field conditions (Labarque et al., 2004; Pesch et al., 2005) although some degree of cross protection exists (Mengeling et al., 2003a, 2003b). With this panorama, the development of improved vaccines needs a deeper understanding of the immunity against PRRSV.

Up to date, only one report has dealt with the evolution of the immune responses, both humoral and cellular, after vaccination with a European type PRRS-modified live vaccine (MLV) (Sipos et al., 2003). However, that study was done with only one vaccine, did not challenge the animals and did not evaluate frequencies of IFN-γ-secreting cells (IFN-γ-SC), a parameter that seems to be related to protection (Meier et al., 2003). The aim of the present study was to characterize the humoral and the cellular responses of pigs in the course of a vaccination with two European-type PRRS-MLV vaccines and after a challenge with a European PRRS wild-type virus.

Results

Virus sequencing and phylogenetic analysis

Similarity between vaccines and VP21 strain was above 90%. Highest values were obtained for ORF7 and ORF6 and lowest similarities corresponded to envelope glycoproteins (Table 1). As a whole, VP21 was closer to V1 (both are Spanish strains) than to V3. Interestingly, in ORF6 both vaccines had exactly the same sequence that differed from VP21 and LV by a change in the codon starting at position 14,402 in LV genome (cga → caa). That change would correspond in the amino acid sequence to a substitution of an arginine by a glutamine.

Detection of PRRSV

Viral isolation attempts in PAM or MARC-145 cells from blood samples taken after vaccination were negative. In contrast, all vaccinated animals were positive by nPCR at least once during the first 6 weeks PV (Table 2). However, viremia was probably low as, with a single round RT-PCR, only six samples were positive during the first 14 days PV. After the challenge, only G1 pigs were detected as positive by nPCR (4/5) at 7 days post-infection (PI). Sequencing of the nPCR amplicons showed the virus to be the VP21 strain. None of the control pigs yielded nPCR-positive results or viral isolation.

Hematology

In all three groups, hematological values remained between normal ranges in the examined period (28 days PV). However, some parameters significantly changed in vaccinated pigs at first week PV, particularly for neutrophils, monocytes and platelets ($P < 0.05$). Table 3 summarizes these results.

Humoral immune response

All pigs were seronegative to PRRSV at day 0. By using ELISA, all vaccinated animals seroconverted by day 14 PV (mean *S*/*P* ratio, 2.5 ± 0.2 for G1 and 2.4 ± 0.6 for G2) (Table 4). ELISA titers peaked at 21 and 28 days PV for G1 and G2, respectively (G1 = 3.2 ± 0.3 and G2 = 3.1 ± 0.3). After the

Table 2
Evolution of viremia in vaccinated pigs as determined by RT-nested PCR

Group	Days post-vaccination								
	0	7	14	21	28	42	63 [a]	70	91
G1	0	5	4	5	2	1	0	4	0
G2	0	5	3	3	1	2	0	0	0

[a] At 63 days post-vaccination, both groups were challenged with 10^6 $TCID_{50}$ of a wild-type Spanish PRRSV strain (VP21).

Table 3
Evolution of hematological parameters of vaccinated pigs from day 0 to day 28 post-vaccination

Parameter/ Group	Days post-vaccination			
	0	7	14	28
Total leukocytes				
G1	16.1 ± 1.8	12.6 ± 2.9[a]	15.2 ± 2.0	13.4 ± 1.9
G2	16.1 ± 2.5	15.4 ± 1.0[b]	15.7 ± 4.4	12.5 ± 1.9
C	19.1 ± 4.2	12.8 ± 1.2[a]	12.2 ± 3.1	13.3 ± 2.2
%Neutrophils				
G1	49.7 ± 6.4	36.7 ± 9.2[a]	16.6 ± 3.4	25.0 ± 5.1
G2	43.7 ± 7.1	28.4 ± 4.1[b]	21.8 ± 4.6	21.2 ± 7.0
C	53.8 ± 8.1	39.8 ± 3.9[a]	24.7 ± 6.9	20.0 ± 5.0
Neutrophils (10^3/μl)				
G1	7.9 ± 1.3	4.6 ± 1.3[a]	2.4 ± 0.7	3.3 ± 0.6
G2	6.9 ± 1.2	3.3 ± 0.6[a]	3.3 ± 0.8	2.7 ± 1.1
C	10.3 ± 3.3	6.1 ± 0.9[c]	3.0 ± 1.0	2.7 ± 0.9
%Monocytes				
G1	3.8 ± 1.2	3.2 ± 0.5[a]	6.0 ± 2.2	4.9 ± 1.4
G2	3.1 ± 1.5	6.6 ± 2.2[b]	4.9 ± 2.2	5.2 ± 1.8
C	3.2 ± 2.0	5.1 ± 0.4[b]	4.6 ± 1.4	5.1 ± 2.5
Monocytes (10^3/μl)				
G1	0.6 ± 0.2	0.4 ± 0.1[a]	0.9 ± 0.4	0.7 ± 0.3
G2	0.5 ± 0.4	0.8 ± 0.3[b]	0.8 ± 0.7	0.7 ± 0.2
C	0.7 ± 0.5	0.8 ± 0.1[b]	0.5 ± 0.1	0.6 ± 0.5
Platelets (10^3/μl)				
G1	431 ± 111	347 ± 72[a]	515 ± 110	383 ± 96
G2	434 ± 120	489 ± 43[b]	573 ± 114	465 ± 91
C	564 ± 95	422 ± 37[b]	470 ± 88	518 ± 101

Superindexes indicate statistical differences (Kruskal–Wallis test using the Conover–Iman method for multiple comparisons; $P < 0.05$). Groups with the same superscripted letter were statistically equal (a, b, or c). The remaining cells subsets did not show statistical differences.

challenge with VP21, titers did not show any significant change. With IPMA, positive results were only obtained from day 14 PV onwards. The highest mean IPMA titers were seen at day 28 PI, namely 91 days PV (log_{10} = 3.2 ± 0.1 for G1 and 3.0 ± 0.3 for G2) (Table 4).

NA were only detected after the challenge (28 days PI) and only in four vaccinated pigs (two animals/group) (Table 4). By using whole serum, the highest titer was log_{10} (titer) = 1.3 ± 0.0 (G2). Addition of fresh complement increased titers by one dilution. All unvaccinated controls remained seronegative to PRRSV in all tests throughout the study.

IFN-γ-SC responses

Frequencies of IFN-γ-SC strongly depended on the strain used for in vitro stimulation. When V1 was used, IFN-γ-SC frequencies were very low in both vaccinated groups and did not exceed 15 IFN-γ-SC/10^6 PBMC (Fig. 1). In contrast, use of V3 for in vitro stimulation produced more clear results, with mean frequencies of IFN-γ-SC that ranged from as low as 12/10^6 PBMC (G1 at 28 days PV) to as high as 125/10^6 PBMC (G2 at day 28 PI, namely 91 days PV) (Fig. 2).

In vitro stimulation with VP21 strain produced results similar to V3 but of a slightly lower intensity (Fig. 3). Taking the results obtained with the VP21 stimulus as a common reference to all groups, PRRSV-specific IFN-γ-SC were firstly detected at 7 days PV (16/10^6 PBMC for G1 and 42/10^6 PBMC for G2) rising to >60/10^6 PBMC at 14 days PV ($P < 0.05$ compared to controls). Then frequencies of PRRSV-specific IFN-γ-SC decreased in both vaccinated groups (<40/10^6 PBMC). At 63 days PV, G1 showed a mean value of 23 IFN-γ-SC/10^6 PBMC and G2 values increased up to 80 IFN-γ-SC/10^6 PBMC ($P < 0.05$). After challenge (63 days PV), a booster effect was observed in both vaccinated groups, but the increase was more evident in G1. By day 28 PI, mean values of PRRS-specific IFN-γ-SC were about 115/10^6 PBMC in both groups.

Stimulation with V1, V3 or VP21 of PBMC from control unvaccinated and uninfected pigs yielded negative results in ELISPOT with no significant background.

Cytokine and haptoglobin ELISAs

In vitro stimulation of PBMC with V1, V3 or VP21 showed no significant differences among groups for IL-2 or IL-4 in cell culture supernatants (data not shown). Regarding IL-10, when V1 was used as stimulus, PBMC of control pigs produced high levels (110–322 pg/ml) (Fig. 4). In contrast, V3 and VP21 did

Table 4
Serological evolution of PRRS-MLV vaccinated pigs by ELISA (HerdCheck, Idexx Laboratories), immunoperoxidase monolayer assay (IPMA) and viral neutralization test (VNT)

Test	Vaccine	Days post-vaccination								
		0	7	14	21	28	42	63[a]	70	91
ELISA no. of positive S/P ratio	G1	Neg	Neg	5/5 2.5 ± 0.2	5/5 3.2 ± 0.3	5/5 3.1 ± 0.3	5/5 3.1 ± 0.3	5/5 2.8 ± 0.4	5/5 3.0 ± 0.1	5/5 3.0 ± 0.1
	G2	Neg	Neg	5/5 2.3 ± 0.6	5/5 2.9 ± 0.5	5/5 3.1 ± 0.3	5/5 3.1 ± 0.2	5/5 3.0 ± 0.4	5/5 2.9 ± 0.2	5/5 2.8 ± 0.1
IPMA no. of positive (log_{10} titer)	G1	Neg	Neg	5/5 2.3 ± 0.3	5/5 2.4 ± 0.2	5/5 2.8 ± 0.1	5/5 3.0 ± 0.3	5/5 2.6 ± 1.9	5/5 2.8 ± 0.4	5/5 3.2 ± 0.0
	G2	Neg	Neg	5/5 2.4 ± 0.2	5/5 2.4 ± 0.2	5/5 2.8 ± 0.2	5/5 2.9 ± 0.1	5/5 2.7 ± 0.3	5/5 2.8 ± 0.4	5/5 3.0 ± 0.3
VNT no. of positive (log_{10} titer)	G1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	2/5 1.2 ± 0.1
	G2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	2/5 1.3 ± 0.0

Results are expressed as the mean ± standard deviation titer.
Unvaccinated animals were negative in all assays.
[a] At 63 days post-vaccination, both groups were challenged with 10^6 $TCID_{50}$ of a wild-type Spanish PRRSV strain (VP21).

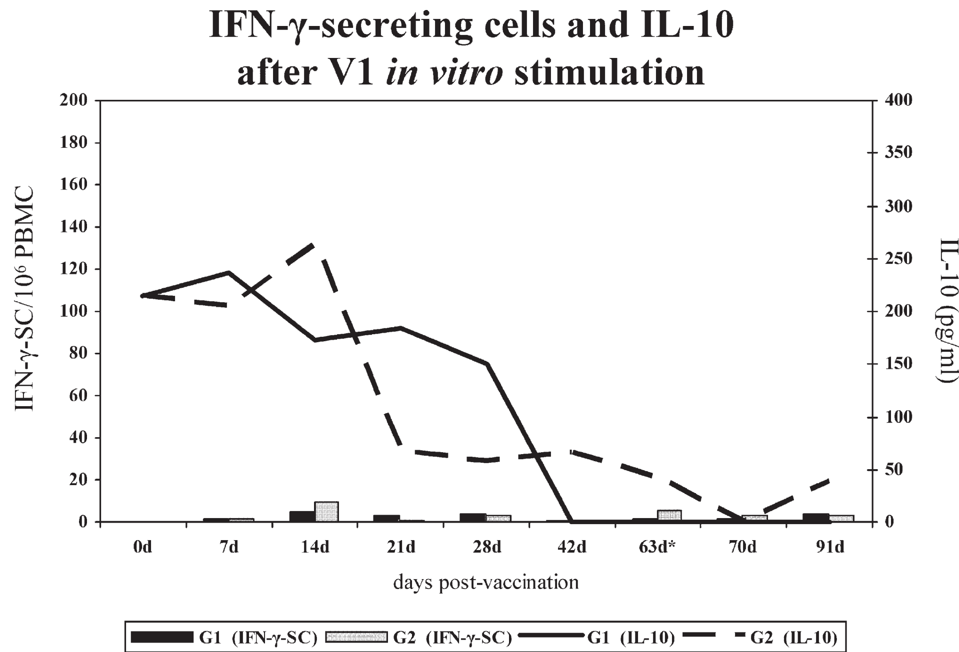

Fig. 1. The figure shows the frequency of IFN-γ-secreting cells (ELISPOT) and IL-10 levels in peripheral blood mononuclear cell culture supernatants (ELISA) after V1 in vitro stimulation in both vaccinated groups. For IL-10 results are expressed as the average of IL-10 secreted (pg/ml) (detection limit = 32 pg/ml), whereas specific-virus IFN-γ-secreting cells are expressed as the average frequency of the specific-virus IFN-γ-secreting cells by 10^6 PBMC. *At 63 days post-vaccination, both groups were challenged with 10^6 $TCID_{50}$ of a wild-type Spanish PRRSV strain (VP21).

not induce IL-10 secretion in cells of control pigs (<42 pg/ml; $P < 0.05$).

When the specific IL-10 response was examined in vaccinated pigs, stimulation of PBMC produced different results depending on the virus used. In G1 pigs, stimulation with V1 produced high levels of IL-10 until 42 days PV (150–236 pg/ml); from this date on, IL-10 could not be detected (Fig. 1). For G2 pigs, in vitro stimulation with V1 produced lower levels of IL-10 that began to decline by day 21 PV and became undetectable by day 70 PV. Stimulation of PBMC

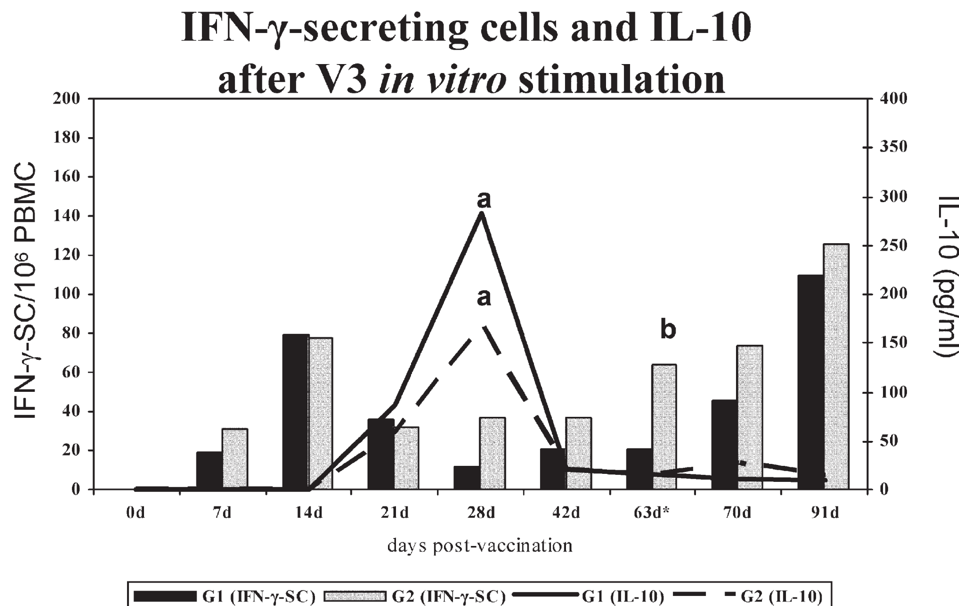

Fig. 2. The figure shows the frequency of IFN-γ-secreting cells (ELISPOT) and IL-10 levels in peripheral blood mononuclear cell culture supernatants (ELISA) after V3 in vitro stimulation in both vaccinated groups. For IL-10 results are expressed as the average of IL-10 secreted (pg/ml) (detection limit = 32 pg/ml), whereas specific-virus IFN-γ-secreting cells are expressed as the average frequency of the specific-virus IFN-γ-secreting cells by 10^6 PBMC. ([a]) G1 and G2 > Controls IL-10 levels ($P < 0.05$). ([b]) G2 > G1 IFN-γ-secreting cells ($P < 0.05$). *At 63 days post-vaccination, both groups were challenged with 10^6 $TCID_{50}$ of a wild-type Spanish PRRSV strain (VP21).

IFN-γ-secreting cells and IL-10 after wild-type Spanish PRRSV strain (VP21) *in vitro* stimulation

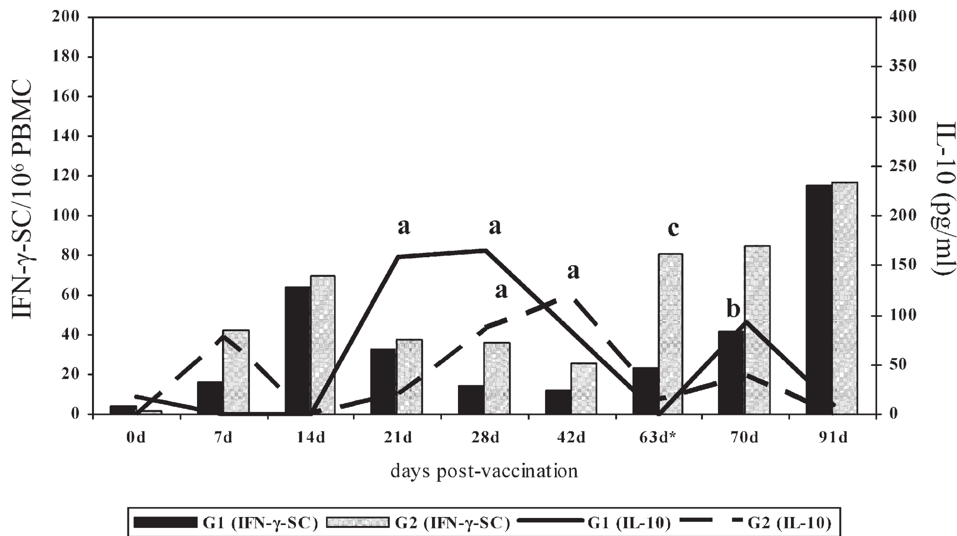

Fig. 3. The figure shows the frequency of IFN-γ-secreting cells (ELISPOT) and IL-10 levels in peripheral blood mononuclear cell culture supernatants (ELISA) after wild-type Spanish PRRSV strain (VP21) in vitro stimulation in both vaccinated groups. For IL-10 results are expressed as the average of IL-10 secreted (pg/ml) (detection limit = 32 pg/ml), whereas specific-virus IFN-γ-secreting cells are expressed as the average frequency of the specific-virus IFN-γ-secreting cells by 10^6 PBMC. ([a]) G1 and G2 > Controls IL-10 levels ($P < 0.05$). ([b]) G1 > G2 IL-10 levels ($P < 0.05$). ([c]) G2 > G1 IFN-γ-secreting cells ($P < 0.05$). *At 63 days post-vaccination, both groups were challenged with 10^6 $TCID_{50}$ of a wild-type Spanish PRRSV strain (VP21).

with V3 produced low IL-10 values in all animals, except at 28 days PV, when these levels significantly increased in both vaccinated groups (Fig. 2). Finally, use of VP21 as an in vitro stimulus yielded two IL-10 peaks in G1 pigs (between 21 and 28 days PV and 7 days PI) and one peak in G2 pigs (between 28 to 42 days PV) ($P < 0.05$, compared to control group). Interestingly, this phase of higher ability of PBMC for producing IL-10 after in vitro stimulation with the VP21 strain (between 21 and 42 days PV) corresponded to the lower IFN-γ-SC values (Fig. 3).

None of the sera yielded positive results for IL-10 and TGF-β. In contrast, serum haptoglobin levels in G1 increased

IL-10 levels in control pigs

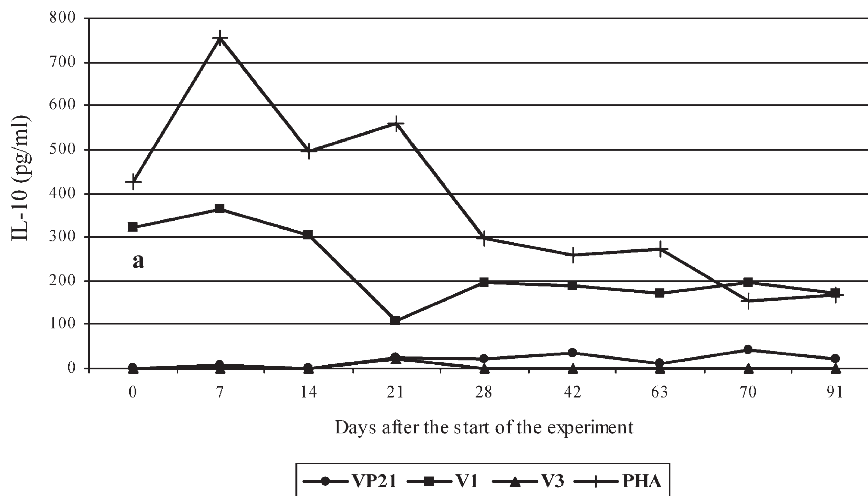

Fig. 4. IL-10 levels in peripheral blood mononuclear cells culture supernatants (ELISA) after wild-type Spanish PRRSV strain (VP21), PRRS-MLV made of a Spanish strain (V1), PRRS-MLV made of a European strain (V3) and PHA stimulation in vitro assays of unvaccinated and uninfected pigs (controls). Results are expressed as the average of IL-10 secreted (pg/ml) (detection limit = 32 pg/ml). ([a]) IL-10 levels after V1 in vitro stimulation always were significantly higher than V3 and VP21 ($P < 0.05$).

significantly at 7 days PI ($P < 0.05$), returning to normality by day 14 PI (Fig. 5).

Discussion

Several studies have reported that the immune response against arteriviruses is unique in its features (Balasuriya and MacLachlan, 2004; Cafruny et al., 1999; Meier et al., 2003). Regarding PRRSV, it seems that both, humoral and cellular, responses do not control the infection adequately. NA develop slowly and usually appear after the resolution of viremia. In addition, cell-mediated immunity also rises slowly, and virus-specific IFN-γ-SC frequencies are low compared to other viral pig diseases (Meier et al., 2003). Some authors suggested that, in PRRS, immunity might play a secondary role and stated that availability of permissive macrophages may determine the outcome of the infection (Diaz et al., 2005; Xiao et al., 2004). A similar hypothesis has been postulated for lactate dehydrogenase-elevating virus (LDV), a related arterivirus (Onyekaba et al., 1989; Van den Broek et al., 1997).

Vaccines made of MLV or inactivated PRRSV are being commercialized and have shown a relative efficacy (Mengeling et al., 2003a, 2003b; Plana-Duran et al., 1997; Van Woensel et al., 1998). Nevertheless, this is a controversial issue. Firstly, because it is unclear how well vaccines prevent the infection and how safe they are (Labarque et al., 2003; Mengeling et al., 2003a, 2003b) and, secondly, because it is thought that the genetic diversity of PRRSV can negatively affect the field efficacy of vaccines (Labarque et al., 2004; Meng, 2000).

In the present study, two European-type vaccines were examined. To assess differences in protection, it was important to evaluate the genetic similarity between them and the challenge VP21 strain. As expected, main differences between the PRRSV strains used were found in ORF3 and ORF5, which are known to be the most variable regions in the PRRSV genome (Pirzadeh et al., 1998; Forsberg et al., 2001).

Besides this, it is interesting to note that ORF6 of both vaccine viruses shared a change corresponding to the codon starting at position 14,402 in LV. This change (cga → caa) produced a substitution of an arginine by a glutamine compared to VP21 or LV. Grebennikova et al. (2004) showed that in the American-type PRRSV strain NADC-8, a change in the position 14,440 (alanine → threonine) contributed significantly to reduce viral virulence. In our case and theirs, one amino acid present in virulent strains was substituted by a polar uncharged one in the attenuated viruses. This finding supports the idea that this site in ORF6 can be related to attenuation.

After vaccination, both G1 and G2 pigs became viremic, and some animals remained so as late as 42 days PV. Sipos et al. (2003) reported 3 weeks of viremia in piglets using one of the European-type PRRSV MLV used in the present study. These extended periods of viremia could significantly contribute to the spread of vaccine virus in the pig population. This fact was reported for American-type vaccines used in Europe (Mortensen et al., 2002; Storgaard et al., 1999). These results point out the question of attenuation. Extended periods of viral replication should not be interpreted as equivalent to virulence or vice versa. As Xiao et al. (2004) suggested, in PRRS, viral replication would occur as long as permissive macrophages exist. Thus, with an attenuated PRRSV strain, that can be thought to produce less intense cytolytic cycles, viral replication should last a long time.

In our case, pigs vaccinated with V1 developed viremia when infected with VP21, while in G2 pigs virus was not detectable in blood after the challenge. Genetic similarity was higher between V1 and VP21 (98.3–99.7%) than between V3 and VP21 (92.3–98.6%). That fact shows that, in our case, the heterologous strain provided better protection than the homologous one. This apparent contradiction could be explained by

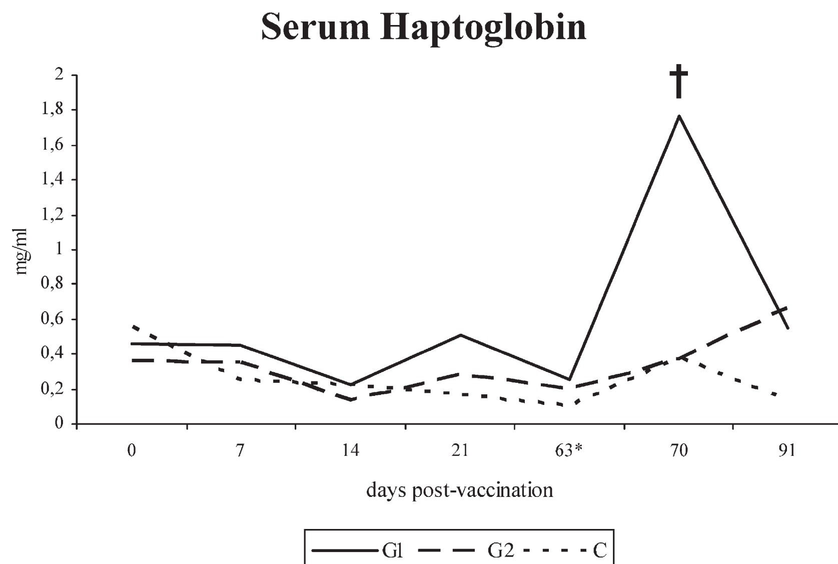

Fig. 5. Kinetics of serum haptoglobin as measured by ELISA in vaccinated and control pigs. (†)$P < 0.05$. *At 63 days post-vaccination both groups were challenged with a wild-type Spanish PRRSV strain (VP21).

the different adjuvants used with the vaccines or by differences in the ability of each vaccine strain to induce PRRSV-specific IFN-γ-SC. In addition, the capability of V1 to stimulate IL-10 responses might also have had a role.

Regarding the hematological parameters examined, we observed some changes at 7 days PV, but all values remained between the physiological ranges for pigs. This has also been reported by Sipos et al. (2003).

By using ELISA, development of specific antibodies could be seen at 14 days PV in all vaccinated pigs and titers remained high throughout the experiment. Interestingly, ELISA titers did not increase significantly after inoculation of the wild-type strain even in viremic pigs. Some authors claimed (Johnson et al., 2004) that the quantitation of the humoral response (total antibodies) measured by ELISA can serve as an indicator of the virulence of a given PRRSV strain. In the present case, using the same ELISA than Johnson et al. (2004), titers after vaccination reached a high of 3.15 (*S*/*P* ratio), similar to the values obtained with the most virulent strain used in Johnson's work. In our opinion, ELISA titers can not be used to measure virulence, at least, when European-type PRRSV strains are evaluated.

In our study, vaccinated pigs did not develop NA before the challenge. Meier et al. (2003), using a MLV vaccine made of an American-type strain, needed two doses of the vaccine to induce only in some animals low levels of NA. Although it seems that high titers of NA could play a role in the outcome of the infection (Lopez and Osorio, 2004) most experiments showed a low, delayed and sporadic development of NA (Meier et al., 2003; Mengeling et al., 1999; Vezina et al., 1996). However, as it has been observed in other arterivirus such as LDV, viremia can be cleared in absence of NA (Balasuriya and MacLachlan, 2004). In our experiment, G2 pigs achieved sterilizing immunity without the need of detectable NA.

Regarding the cellular immune response using VP21 or V3 as stimuli, levels of IFN-γ-SC rose at 14 days PV, then were erratic, and finally rose again at 63 days PV. This figure is similar to the data reported by us in a previous experiment, where unvaccinated pigs were experimentally infected with the VP21 strain (Diaz et al., 2005).

Just before the challenge, stimulation of PBMC with VP21 produced 81 IFN-γ-SC/10^6 PBMC in G2, while the same stimulation only produced 21 IFN-γ-SC/10^6 PBMC in G1. After the challenge with VP21, G1 pigs became viremic (4/5) while G2 pigs did not. Therefore, V1 vaccine demonstrated a reduced ability to induce protective immune memory compared to V3 vaccine. The remaining pig in G1 that did not develop viremia had the higher IFN-γ-SC frequency at the day of challenge (45 IFN-γ-SC/10^6 PBMC). These results add a strong evidence for the role of the IFN-γ-SC in protection against PRRSV infection in piglets. Recently, Lowe et al. (2005) observed a strong correlation of cell-mediated immunity measured by IFN-γ-SC with protection against reproductive failure in sows of commercial herds as well. As discussed later, V1 had a higher ability to induce IL-10 secretion, and this could be have impaired the development of IFN-γ-SC. However, it cannot be ruled out that the adjuvant used with V3 had an influence on that observation although some authors denied the effect of adjuvants in PRRSV immunization (Meier et al., 2003).

Our results also showed that IFN-γ-SC frequencies were extremely low when V1 was used for in vitro stimulation. This was observed even in pigs vaccinated with V1. The impairment of IFN-γ-SC responses when V1 was used as a stimulus could be explained by the ability of V1 to induce the production of high levels of IL-10 in PBMC. Since this IL-10 production was seen in control pigs, it is reasonable to think that this was an intrinsic characteristic of the V1 strain and did not involve mechanisms of the adaptative immunity. Also, PBMC are thought to be non-permissive cells for PRRSV and therefore, the IL-10 release probably did not originate from viral replication in blood cells. The explanation for this fact remains unclear but a reasonable hypothesis is that IL-10 secretion was the result of the interaction of some of the envelope glycoproteins, the most variable part of the virus, with cellular receptors in monocytes or lymphocytes. However, this is only a hypothesis to be tested in further studies on PRRSV and immunity.

Regarding IL-10 secretion after recall PRRSV stimulation, it could be seen that this cytokine peaked between days 14 and 42 PV depending on the group and the strain (V3 or VP21) used. By day 63 PV, IL-10 was practically undetectable in cell culture supernatants. Disappearance of IL-10 correlated with the resolution of the viremia after vaccination. When G1 pigs were challenged with VP21 and developed viremia, IL-10 rose again. In a previous experiment (Diaz et al., 2005) we observed that in pigs experimentally infected with VP21, viremia and cessation of IL-10 production were also correlated. These facts suggest either that replication of the virus induces IL-10 or, alternatively, that IL-10 release sustains viral replication.

In the present experiment, as seen in control pigs, the potential of IL-10 secretion of PBMC changed over time. Thus, PHA stimulation produced some 700 pg/ml at 5 weeks of age and, from 8 weeks of age on, the same stimulus only yielded some 200–300 pg/ml. This fact indicated an influence of age on the ability to secrete IL-10. Interestingly, when PBMC from vaccinated pigs were stimulated in vitro with V1, a similar pattern was seen, but production of IL-10 was lower. From 42 days PV, V1 stimulation of PBMC from vaccinated pigs produced very low or undetectable IL-10 levels. Taken together, these observations can be interpreted as a virus-specific stimulation of PBMC that took place regardless of the immune status of the pig. The explanation for that is not fully clear but from 42 days PV on, frequencies of PRRSV-specific IFN-γ-SC rose. This suggests a role for the Th1/Th2 balance in this pattern.

Results for IL-2 and IL-4 responses did not show significant differences between control and vaccinated pigs using any of the viral strains. These data support the notion that some type of immunomodulation takes place in the course of the infection or vaccination (Royaee et al., 2004; Sipos et al., 2003).

Haptoglobin was only detected in sera of G1 animals after the challenge with VP21. Those animals were the only ones that developed viremia after the challenge. In our opinion, haptoglobin release was probably linked to the tissular damage caused by the wild virus but not by the replication of an

attenuated PRRSV strain. It is known that haptoglobin is related with IL-6 release in inflammatory processes (Asai et al., 1999).

In summary, the pattern of the evolution of both the humoral and the cellular immune responses after a PRRSV vaccination is very similar to that of a PRRSV infection in piglets (Diaz et al., 2005). Vaccine V1 had the lowest capacity to induce IFN-γ-SC and, in contrast, was able to stimulate a very high IL-10 release from PBMC. After the challenge with the virulent virus, the response of pigs vaccinated with V1 resembled that of an unvaccinated pig in terms of prevention of viremia. This observation leads to the notion that IFN-γ-SC most probably is the main factor in protecting against PRRSV infection as well as that IL-10 release may hamper the development of such IFN-γ-SC. Thus, the capacity of a given PRRSV strain to induce a strong cell-mediated immunity, measured as IFN-γ-SC, may be probably modulated by the IL-10 release (Chung and Chae, 2003; Suradhat and Thanawongnuwech, 2003; Suradhat et al., 2003; Diaz et al., 2005). Once the modulation of the immune response disappears, IFN-γ-SC frequencies rose and, when a given frequency is reached, the pig will be protected (Lowe et al., 2005). Nevertheless, this is only attained at late stage. On the other hand, since the ability to produce IL-10 may change with age, this host factor may also influence the outcome of the immunization or the infection.

In addition, we saw that the protection afforded by a given vaccine against a given PRRSV strain cannot be forecasted only by a global view of the genetic similarity between the vaccine virus and the challenge virus but, maybe, can be forecasted by examining the specific ability of a given viral strain to induce IFN-γ-SC and to modulate the immune responses. In consequence, the development of an effective vaccine for piglets should take into account the balance between IL-10 induction and the development of IFN-γ-SC. These data can be of critical importance to develop new and better PRRS vaccines.

Materials and methods

Animals and housing

Thirteen healthy 3-week-old Landrace pigs were randomly selected in a high health farm belonging to the Institut de Recerca i Tecnologia Agroalimentària (IRTA, Barcelona, Spain) that has historically been free of all major pig diseases including PRRS. Animals were transported to the experimental facilities, randomly divided in three groups (G1, G2 and C) and kept during 7 days to allow adaptation to the new conditions. Before the start of the experiment, pigs were examined for antibodies against porcine circovirus type 2, Aujeszky's disease virus, porcine parvovirus, swine influenza and *Mycoplasma hyopneumoniae*. All animals were seronegative for all of the abovementioned pathogens. Also, animals were confirmed to be free of PRRSV, as determined by ELISA (Herdcheck PRRS 2XR, IDEXX Laboratories, Westbrook ME, USA).

Vaccines and virus

Animals in group G1 (n = 5) were intramuscularly (IM) vaccinated with $10^{4.0}$ $TCID_{50}$ of a MLV made of one Spanish PRRSV strain (V1) using water as a vehicle (2 ml). G2 pigs (n = 5) received $10^{4.0}$ $TCID_{50}$ of a commercial MLV made of other European PRRSV strain (V3) (2 ml, IM) with an oil-in-water adjuvant. Vaccines were diluted with the vehicles recommended by each of the manufacturers. The remaining three pigs were kept as controls (C) and received 2 ml of sterile minimal essential medium (MEM) (Sigma, Alcobendas, Spain) as a placebo.

The virulent PRRSV VP21 strain used for the challenge was isolated in porcine alveolar macrophages (PAM) from sera of naturally infected pigs during an outbreak of PRRS affecting a breeding farm located in the north of Spain in December 1991. That outbreak was characterized by abortions, stillbirths and, later on, piglet mortality. The virus was shown to be of the European genotype by sequencing of ORFs 2 to 7. Viral stock was done by passage (n = 3) in PAM and titrated by means of the immunoperoxidase monolayer assay (IPMA) according to Wensvoort et al. (1991). Before to be used, the viral stock was checked for bacterial contamination and for the presence of other viruses.

At 63 days post-vaccination (PV), pigs in groups G1 and G2 were inoculated intranasally with 2 ml viral suspension containing 10^6 $TCID_{50}$/ml of PRRSV VP21 strain diluted in sterile MEM, whilst pigs in group C only received 2 ml of sterile MEM.

Virus sequencing and phylogenetic analysis

ORFs 2 to 7 of the vaccine viruses and challenge virus were amplified by PCR using specific primer pairs (Table 5). Sequencing was done using an ABI 3100 sequencer (Applied Biosystems). Sequences were formatted and translated to amino acid sequences. Alignments were done using ClustalW software. As a reference, Lelystad virus (LV) sequence (Genbank accession number M96262) was used. Nucleotide sequences were deposited at Genbank under accession numbers DQ009657-DQ009659 (ORF2); DQ009654-DQ009656 (ORF3); DQ067250, DQ064784, DQ064785 (ORF4); DQ009647, DQ064787, DQ064788 (ORF5), DQ009651-DQ009653 (ORF6) and DQ009648-DQ009650 (ORF7).

Samples

Blood samples were collected in duplicate (heparinized and siliconized blood-collecting tubes) immediately before vaccination and then at days 7, 14, 21, 28, 42, 63, 70 and 91 PV. Sera were used for PRRSV isolation and specific RT-nested PCR (nPCR), determination of humoral responses and evaluation of haptoglobin, IL-10 and transforming growth factor-beta (TGF-β) levels. Heparinized blood samples were used for hematological evaluation. Peripheral blood mononuclear cells (PBMC) were also obtained from heparinized blood samples and used for in vitro experiments.

Table 5
Oligonucleotides used for PCR amplification of different open reading frames (ORFs) from PRRSV with the respective annealing temperatures and the number of PCR cycles

Gene specificity	Primer[a]	Oligonucleotide sequences (5′–3′)	Annealing temperature (°C)	No. of cycles
ORF2	L	CTG GCA CAG AAT TGC AGG TA	55	45
	R	GCA CAC TGA TGA GCC ATT GT		
ORF3	L	ACA ATG GCT CAT CAG TGT GC	55	35
	R	TGA AGC CTT TCT CGC TCA TT		
ORF4	L	AGC GTG ACC ATG ATG AGT TG	55	39
	R	AAA AGC CAC CAG AAG CAA GA		
ORF5	L	TGA GGT GGG CTA CAA CCA TT	55	35
	R	AGG CTA GCA CGA GCT TTT GT		
ORF6	L	GTC CTC GAA GGG GTT AAA GC	55	35
	R	CTG TCC TCC CCT AGG TTG CT		
ORF7	L	GGC AAA CGA GCT GTT AAA CG	55	35
	R	AAT TTC GGT CAC ATG GTT CC		

[a] L, left or forward primer; R, right or reverse primer.

Detection of PRRSV

Serum samples were inoculated on MARC-145 and PAM cultures and incubated at 37 °C in 5% CO_2 for 90 min. Then sera were removed, and MEM was added. Cultures were incubated for three days at 37 °C in 5% CO_2. Infection of inoculated cells was determined by IPMA (Wensvoort et al., 1991).

Besides viral isolation, sera were examined by nPCR. Briefly, total RNA was extracted from 150 μl serum by using a commercial system (Nucleospin RNA virus; Macherey-Nagel, Düren, Germany). Total RNA was transcribed, and cDNA was used in a first PCR round directed to viral ORF5 (Mateu et al., 2003). This first-round PCR had a sensitivity of about 10^2 $TCID_{50}$/ml of serum. PCR products were used in an nPCR (forward primer, 5′-TCTTGCTTCTGGTGGCTTTT-3′; reverse primer, 5′-CATGTTTGATGGTGACGAGG-3′) that produced a 499-bp amplicon. Cycling parameters for both PCRs were 94 °C for 45 s; 55 °C for 45 s; 72 °C for 45 s for a total of 35 cycles. Under these conditions, the nPCR was considered to be able to detect <10 viral copies/ml of serum.

Hematology

Hematological cell counts were done using an automated system (Advia 120, Bayer) with protocols adapted to pig blood samples.

Humoral immune response

A commercially available ELISA (Herdchek PRRS 2XR, Idexx Laboratories) was used to measure PRRSV-specific antibodies. According to the manufacturer, sample to positive control (*S*/*P*) ratios of >0.4 were considered positive. PRRSV antibody titers were also determined in sera by using IPMA (Wensvoort et al., 1991), using MARC-145 cells infected with the PRRSV VP21 strain. Tests were done in duplicate.

NA were measured by the technique described by Jusa et al. (1996) and Yoon et al. (1994). Tests were done in triplicate by using whole serum, inactivated serum and serum plus 10% fresh guinea-pig complement. Briefly, 50 μl of each serum to be tested was diluted serially from 1/20 to 1/160 in cell culture medium. Dilutions were mixed with 50 μl viral suspension containing 200 $TCID_{50}$ of the PRRSV strain VP21 (with or without complement). Virus–serum mixtures were incubated for 1 h at 37 °C and then added to MARC-145 cultures in duplicate (96-well plates) and incubated for 3 days at 37 °C in 5% CO_2. Infection of cell cultures was revealed by using IPMA.

Isolation and culture of PBMC

PBMC were separated from whole blood by density-gradient centrifugation with Histopaque 1.077 (Sigma). For PBMC cultures, RPMI 1640 medium supplemented with 10% fetal calf serum (Invitrogen, Spain), 1 mM non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 5 mM 2-mercaptoethanol (Sigma), 50,000 IU/l penicillin (Invitrogen), 50 mg/l streptomycin (Invitrogen) and 50 mg/l gentamicin (Sigma) was used. Trypan blue stain was used to assess viability.

PRRSV-specific IFN-γ-SC

Frequencies of PRRSV-specific IFN-γ-SC in PBMC were analyzed by an ELISPOT assay using commercial mAbs (Swine IFN-γ Cytosets, Biosource Europe) according to a previously reported method (Díaz and Mateu, 2005). Briefly, ELISA plates (Costar 3590, Corning, USA) were coated overnight with 8.3 μg/ml IFN-γ capture antibody diluted in carbonate–bicarbonate buffer (pH 9.6). Plates were then washed and blocked for 1 h at 37 °C with 150 μl of PBS with 1% of bovine serum albumin. After removal of the blocking solution, 5×10^5 PBMC were dispensed per well (50 μl) and stimulated with V1, V3 or VP21 strains diluted in RPMI at a multiplicity of infection (MOI) of 0.01. After 20-h incubation at 37 °C in a 5% CO_2 atmosphere, cells were removed, and the biotinylated detection antibody was added at 2.5 μg/ml (50 μl) and incubated for 1 h at 37 °C. The reaction was revealed by sequential incubation of plates with streptavidin-peroxidase (1 h) and insoluble TMB blue (Calbiochem, Spain). Unstimulated cells and phytohemagglutinin (PHA)-stimulated controls (10 μg/ml) were also included.

To calculate the PRRSV-specific frequencies of IFN-γ-SC, counts of spots in unstimulated wells were subtracted from

counts in virus-stimulated wells. Frequencies of IFN-γ-SC were expressed as responding cells in 10^6 PBMC.

Cytokine (IL-2, IL-4, IL-10 and TGF-β) and haptoglobin ELISAs

PBMC were seeded at a density of 2×10^6 cells per well (250 μl) in 96-well plates and were mock stimulated or stimulated with V1, V3 or VP21 strains (MOI of 0.01) or PHA (10 μg/ml). After 24 h of incubation at 37 °C in 5% CO_2, cell culture supernatants were collected and frozen at −80 °C until needed. Capture ELISAs were performed as reported previously (Darwich et al., 2003; Díaz and Mateu, 2005) using commercial pairs of mAbs (Swine IL-2, IL-4, and IL-10 cytosets, Biosource Europe). The cut-off point of each ELISA was calculated as the mean optical density of negative controls plus three standard deviations. Cytokine concentrations were calculated using the linear regression formula from optical densities of the cytokine standards provided by the manufacturer. IL-10 and TGF-β were also measured in sera of experimental animals by using the abovementioned ELISAs. Detection limits for both cytokines were 32 pg/ml. Serum haptoglobin levels were determined by means of ELISA (Haptoglobin assay phase range, Tridelta, Ireland) according to the manufacturer's instructions.

Statistical analysis

The regression line of cytokine standards in ELISA was calculated by using SPSS v12.0 (SPSS Inc., Chicago, USA). Statistical comparisons between groups (Mann–Whitney/Kruskal–Wallis tests) were done by using Statsdirect v2.4.1. All tests done in the study, as well as the statistical analysis, were performed blind.

Acknowledgments

We thank Núria Navarro and Mercè Giménez for technical help and assistance and Dr. J. Dominguez (INIA) for sharing mAbs used in this work. Ivan Díaz has been financially supported by Departament d'Universitats, Recerca i Societat de la Informació of the Generalitat de Catalunya. This work has been funded by the Spanish Ministry of Education and Science; project BIO-2000-054-P4-03.

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