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Isotype-specific antibody-secreting cells in systemic and mucosal associated lymphoid tissues and antibody responses in serum of conventional pigs inoculated with PEDV

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Abstract

An enzyme-linked immunospot (ELISPOT) has been developed to detect porcine epidemic diarrhea virus (PEDV)-specific antibody secreting cells (ASC) in gut associated lymphoid tissues (duodenum and ileum lamina propria and mesenteric lymph nodes) and systemic locations (spleen and blood) of conventional pigs so as to characterise the mucosal and systemic antibody response generated by the infection with PEDV. A total number of 28 eleven-day-old conventional pigs were orally inoculated with the field isolate of the PEDV strain CV-777. Diarrhea was observed in 32% of the pigs and virus shedding was demonstrated in 100% between postinoculation day (PID) 1 and 8. Serum IgG and IgA antibodies to PEDV were detected by isotype ELISA from PID 12 and 15, respectively, reaching maximum values at PID 32 (IgG) and 21 (IgA). PEDV specific IgM ASC occurred in all the tissues between PID 4 and 7, with the strongest response in the intestinal lamina propria. IgA and IgG ASC responses were evident in the intestinal lymphoid tissues from PID 21, the highest number of specific ASC corresponded to the duodenum lamina propria. In the systemic lymphoid tissues the number of IgG and IgA ASC detected were lower than in the mucosal tissues, however, in the blood, presence of IgA ASC was constantly detected from PID 14 until the end of the experiment. Memory antibody response to the PEDV was also studied by secondary in vitro stimulation of the mononuclear cells (MNC) isolated from mesenteric lymph nodes, spleen and blood. The memory B cell response was prominent at PID 21 and 25 and consisted in IgG and IgA ASC. To our knowledge, this is the first report to research into the presence and distribution of

Abbreviations: ELISPOT, enzyme-linked immunospot; PED, porcine epidemic diarrhea virus; PEDV, porcine epidemic diarrhea virus; TGE, transmissible gastroenteritis; TGEV, transmissible gastroenteritis virus; ASC, antibody secreting cells; PID, postinoculation day; MNC, mononuclear cells; GMT, geometric mean titer

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specific ASC in different locations of the systemic and the gut associated lymphoid tissues after a PEDV infection as well as the presence of memory B cells. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Porcine epidemic diarrhea virus; ELISPOT; Antibody secreting cells; Mucosal immunity

1. Introduction

Porcine epidemic diarrhoea virus (PEDV) is an important pathogen causing severe gastroenteritis with a clinical picture similar to that of transmissible gastroenteritis (TGE). PEDV has been classified in group I of the *Coronaviridae* family, which also includes transmissible gastroenteritis virus (TGEV), feline infectious peritonitis virus (FIPV) and human respiratory coronavirus 229E (HCoV229E) (Cavanagh et al., 1994; Murphy et al., 1999).

Porcine epidemic diarrhoea (PED) was first described in Great Britain in 1971 (Oldham, 1972), and nowadays the virus is widely distributed throughout Europe and Asia, although has not been detected in America. In Spain, a serological survey carried out in 1993–1994 detected antibodies against the virus in 54% of the breeding herds (Carvajal et al., 1995b). Moreover, over the last 4 years the virus has been found in a high percentage of the clinical cases of diarrhoea analysed at our laboratory. These data indicate that PEDV is currently one of the most important causes of gastroenterical disorders in pigs in Spain, which seems to be the same case in other countries (Van Reeth and Pensaert, 1994).

Clinically, the disease can appear in two forms, PED Type I, which affects only pigs older than 4–5 weeks, and PED Type II, which affects pigs of all ages. Both are characterised by profuse, watery diarrhoea, depression and anorexia. Morbidity is high, close to 100%, but mortality rate is relatively low in adult pigs (3%), whereas not in suckling piglets among which the severity of the disease increases and mortality can reach 90% (Pensaert, 1999).

The epithelium of the small intestine and the colon is the site for virus replication and although the presence of viral antigen in the mesenteric lymph nodes has been described, there is no evidence of virus replication in tissues other than in those in the gastroenteric tract. Infection of the enterocytes causes vacuolisation and finally destruction, which leads to villous atrophy and watery diarrhoea due to malabsorption.

So far, there is no effective vaccine or specific treatment available, and the only measures to control the disease are those directed to preventing the entrance of the virus on the farm (Pensaert, 1999). The development of immunological strategies in order to induce protection would be desirable, mainly those involving the protection of suckling piglets less than 2–3 weeks old. Little has been reported relating to the immunological aspects of the disease other than detection of serum antibodies against the virus in convalescent animals (Carvajal et al., 1995a; Van Nieuwstadt and Zetstra, 1991; Debouck and Pensaert, 1984). However, due to the special features of the mucosal immune system of the pigs, the presence of serum antibodies against gastroenteric pathogens is not always correlated with protection (Saif and Wesley, 1999; Saif, 1996; Saif et al., 1994; Tô et al., 1998; Ward et al., 1996) and only proves the contact with the microorganism.

In the present work, we have tried to contribute to the information on the immune mechanisms occurring after PEDV infection by making a first approach to the characterisation of the antibody response generated by the virus. Considering the fact that in the enteric infections in porcine is the local immunity which plays the main role in protection instead of the systemic immunity, as previously mentioned, the first step was to develop techniques to investigate the immune response to PEDV in different tissues. Finally, we tried to emulate a natural infection by inoculating conventional pigs with a virulent strain of PEDV and monitored the humoral response in different locations of the lymphoid system, involving gut associated lymphoid tissues as well as systemic tissues.

2. Materials and methods

2.1. Viruses and cells

The wild type isolated of the CV-777 strain of PEDV, kindly provided by Dr. Peansert (Gent, Belgium) was amplified by passages in conventional 1-week-old piglets without antibodies against PEDV. Animals were orally inoculated and sacrificed in the acute phase of diarrhoea, collecting the intestinal contents and the small intestine. The small intestine from each animal was macerated in PBS (1:2 (w/v)) and, like the intestinal contents, clarified by centrifugation at $5000 \times g$ for 20 min at 4 °C. Finally, all the fractions were pooled and stored at -70 °C.

The cell-culture adapted PEDV, strain CV-777, was propagated in Vero cells as previously described (Hofmann and Wyler, 1988). Briefly, Vero cells were grown with Eagle's minimum essential medium (Gibco, Life Technologies) buffered with bicarbonate and supplemented with 5% (v/v) foetal calf serum (Gibco), 0.04% (w/v) yeast extract (Difco, MI, USA), streptomycin (10 mg/l) and penicillin (10,000 UI/l) (Penicillin-streptomycin, Gibco). Confluent monolayers were infected by removing the growth medium and adding the viral inoculum diluted in medium without foetal calf serum but containing 10 μ l/ml trypsin (Difco).

2.2. Animals and samples

2.2.1. Standardisation of the ELISPOT

In order to standardise the ELISPOT, three conventional 4-week-old pigs were hyper-immunised and used as a source of lymphocytes primed against PEDV. Pigs were orally inoculated with a suspension of the virulent, wild type, PEDV and boosted 4 and 12 weeks later by peritoneal injection of the same inoculum concentrated by ultracentrifugation at $10\,000 \times g$ and diluted (1:1) in Freund's adjuvant (complete in the first injection and incomplete in the second) with antibiotics (gentamycin: 500 μ g/ml (Gibco), streptomycin: 20 μ g/ml, penicillin: 20,000 UI/ml (Penicillin-streptomycin, Gibco)). Serum samples were taken weekly after the first inoculation to monitorise the antibody production. Once a high serum antibody titer was reached, blood samples were collected periodically in 25% (v/v) acid citrate glucose to obtain the mononuclear cells (MNC). Finally, the pigs were sacrificed and the spleen and mesenteric lymph nodes were aseptically collected. As a negative control, blood samples from PEDV seronegative conventional pigs were collected in the same manner.

2.2.2. *Experimental design*

A total number of 28 conventional pigs, seronegative to PEDV and from a herd with no previous history of the disease, were weaned at 11–12 days of age and maintained in isolation facilities. Pigs were inoculated orally with 3 ml of the virulent CV-777, a dose previously established which assesses a high rate of infection and the effective development of the immune response but without causing mortality among the pigs. The animals were observed daily for clinical signs and rectal swabs from all of them were taken for 11 days after the inoculation. Blood samples were also collected twice a week until the end of the experiment. At postinoculation days (PID) 4, 7, 14, 21, 25 and 31, 4–5 selected pigs were euthanised by a sodic penthotal injection (Eutalender, Normon, Madrid, Spain). The small intestine (duodenum and ileum), spleen, mesenteric lymph nodes and blood were aseptically collected for isolation of MNC.

Three conventional, seronegative, pigs served as negative controls and were sacrificed without previous inoculation.

2.3. *Blocking-ELISA*

An ELISA that combines the use of two monoclonal antibodies (Mab) against the S protein of PEDV (CVI-PEDV 66.31 and 66.49) and a blocking step with rabbit-anti PEDV hyperimmune serum or gut-origin PEDV, was carried out as previously described (Carvajal et al., 1995a) to detect specific antibodies in serum or viral antigen in rectal swab samples, respectively.

2.4. *Antibody isotype ELISA*

Antibody isotypes IgG and IgA to PEDV in serum samples were detected and titrated using an indirect ELISA as previously described (de Arriba et al., 1994). Antigen was obtained from PEDV infected cell culture supernatants that were lysated, concentrated 50 times by ultracentrifugation ($100\,000 \times g$, 4°C , 2 h) and semi-purified by ultracentrifugation under the same conditions through 20% sucrose. Mock-infected cultures were given the same treatment in order to obtain a control antigen. Viral or control antigens were immunocaptured in polystyrene microtiter plates (Costar, MA, USA) previously coated with the Mab CVI-PEDV-66.31. In the next step, serial 2-fold dilutions, starting at 1:20, of each serum sample were incubated in paired wells, containing viral or control antigen. Two biotinylated Mabs 3H7 (80 ng/ml) and 6D11 (65 ng/ml) against porcine IgG and IgA, respectively (Paul et al., 1989), followed by horseradish peroxidase-conjugated streptavidin (KPL, MD, USA) and ABTS substrate were used for the detection of both isotypes of antibodies. Titres were expressed as the reciprocal of the lowest positive sample dilution and titres <20 were assigned a value of 10 for calculation of geometric mean titre (GMT).

2.5. *Isolation of MNC*

MNC were isolated from MLN, blood, spleen and the lamina propria of the small intestine (duodenum and ileum) by using modifications of previously described methods (Chen et al., 1995; Van Cott et al., 1993; Yuan et al., 1996).

Blood was collected aseptically in 25% (v/v) acid citrate glucose and the peripheral blood lymphocytes were isolated by Ficoll-Paque (Ficoll-Paque Research Grade, Pharmacia Biotech., Uppsala, Sweden) density gradient centrifugation. Lymphocytes collected from the interface were washed twice in Hanks' balanced salt solution and resuspended in enriched medium (RPMI 1640 containing 8% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential aminoacids, 20 mM HEPES, and 20 µg of ampicillin and 100 µg of gentamicin per millilitre).

Spleen, mesenteric lymph nodes and two fragments of the small intestine (one from duodenum and the other from ileum) were collected aseptically and placed in ice-cold wash medium (RPMI 1640 containing 10 mM HEPES and 200 µg of gentamicin and 20 µg ampicillin per ml). Spleen and mesenteric lymph nodes were homogenised pressing through stainless steel screens (80 mesh) of a cell collector (Cell-Selector, E-C Apparatus, FLA, USA). Cell suspensions were placed in a 30% Percoll (Pharmacia Biotech.) solution and centrifugated at $1200 \times g$ for 30 min at 4 °C. The resulting pellets were subjected to a discontinuous gradient centrifugation in Percoll: the cells were resuspended in 43% Percoll, underlined with 70% Percoll and centrifuged at $1800 \times g$ for 20 min at 4 °C. Finally, the MNC were aspirated from the Percoll interface, washed twice with wash medium and suspended in enriched medium.

Fragments of approximately 20 g of duodenum and ileum were cut in small pieces, washed twice with wash solution and twice with Hanks' balanced salt solution. In order to remove epithelial cells, the tissues were placed in Hank's balanced salt solution containing 1 mM dithiothreitol and 5 mM EDTA and vigorously shaken for 30 min. Tissues were then digested for two 31-min periods at 37 °C in gentle shaking with enriched medium containing 400 UI of Type II collagenase (Sigma, MO, USA) per millilitre and 5 mM EDTA. Digested supernatants were collected and the remaining tissues were pressed through the stainless steel 80-mesh screens. The single cellular suspensions obtained were pooled with the digested supernatants and subjected to the gradient centrifugation in Percoll as described for mesenteric lymph nodes and splenic MNC. Viability of all MNC preparations was proved by the trypan blue exclusion test, being in any case higher than 95%.

2.6. Antigen-coated plates for ELISPOT

Two different kind of plates were assayed to set up the ELISPOT, on one hand PEDV infected and fixed cell monolayers and on the other, semi-purified antigen that was immunocaptured in plates previously coated with a Mab against the S protein of the virus.

2.6.1. Infected and fixed cell monolayers

Vero cells, grown in 96-well tissue culture plates, were inoculated with the cell culture adapted PEDV as described (Hofmann and Wyler, 1988). After incubation at 37 °C in a humid incubator with 5% CO₂, plates were fixed in 80% acetone in PBS for 20 min at room temperature and stored at -20 °C. Different dilutions were tested in order to find the optimal multiplicity of infection to yield over 90% of infected cells. Control plates were mock-inoculated and treated in the same way.

Optimal antigenic expression was verified in every batch of plates by indirect immunofluorescence using the Mab 113B, directed to the S protein of the PEDV and kindly

provided by Dr. M. Ackermann (Zurich, Switzerland). Only the plates showing more than 80% of fluorescent surface were used to perform the ELISPOT.

2.6.2. *Immunocaptured antigen*

Ninety-six-well microtiter plates were coated and dilutions of viral or control antigen were immunocaptured as described for the isotype ELISA.

2.7. *ELISPOT assay*

The ELISPOT technique was based on previously published methods (Chen et al., 1995; Czerkinsky et al., 1983; Sedgwick and Holt, 1983; Van Cott et al., 1993; Yuan et al., 1996) modified and adapted by us for the detection of PEDV-specific antibody secreting cells (ASC).

Fixed cell plates were thawed and rehydrated by incubation with PBS for 5 min at room temperature while antigen captured plates were washed three times before use in the ELISPOT. Different amounts of MNC (5×10^3 , 5×10^4 and 5×10^5) from each tissue were added to duplicate wells of the fixed-PEDV infected or mock-infected cell plates or to the plates with the immunocaptured antigen (viral and control antigen). Plates were centrifuged at low speed ($50 \times g$) for 5 min and incubated at 37 °C with 5% CO₂ for different periods. In order to remove the cells and between steps, the plates were washed 5 times with 0.05% Tween 20-PBS (PBST). The antibody production was detected by using biotinylated mouse Mabs 3H7 (80 ng/ml) and 6D11 (65 ng/ml) against porcine IgG and IgA, respectively (Paul et al., 1989) and goat anti-porcine IgM serum (1:20,000, KPL) diluted in PBST. After 2 h incubation at room temperature, horseradish peroxidase-conjugated streptavidin (KPL) was added (1:20,000) and incubated for 1 h at room temperature. Finally, the spots were developed by tetramethylbenzidine (TMB) with H₂O₂ membrane peroxidase substrate system (KPL) and counted under an optic microscope. Counts were averaged from the duplicated wells at the dilutions showing less than 40 spots per well and were expressed relative to 5×10^5 MNC. Working conditions were optimised to detect the highest number of specific spots against PEDV.

2.8. *In vitro viral stimulation of MNC*

The in vitro viral stimulation technique was modified from published methods (Van Cott et al., 1993, 1994). MNC purified from spleen, blood and mesenteric lymph nodes were diluted in enriched medium containing 50 µM 2-mercaptoethanol (2ME-enriched medium) (Sigma) to 5×10^6 MNC per millilitre. 750 µl of each cell preparation were added to two consecutive wells of a 12-well tissue culture plate and stimulated with semi-purified PEDV viral antigen, prepared as described for the isotype ELISA and diluted in 750 µl of the 2ME-enriched medium. Plates were maintained in a humid incubator with 5% CO₂ for 5 days and, from the second day on, 500 µl per well of fresh 2ME-enriched medium were added. On the fifth day, MNC were harvested, rinsed twice with wash medium, suspended in 2ME-enriched medium and tested by ELISPOT (testing 5×10^2 , 5×10^3 and 5×10^4 MNC per well).

2.9. Statistical analysis

Kruskal-Wallis non-parametric analysis of variance was used to prove statistically significant differences in the number of ASC between different days. Antibody GMT were compared by means of Student *t*-test at each point in time. Significance was assessed at $p < 0.05$. For the analysis the SYSTAT for Windows v.5.03 (SYSTAT) and the spreadsheet Microsoft EXCEL v.7.0 (Microsoft) were used.

3. Results

3.1. Clinical signs and virus shedding

Pigs inoculated with the virulent isolate of the PEDV strain CV-777 exhibited moderate signs of the disease, mainly semi-liquid diarrhoea, shown in 32% of the pigs. The onset of the diarrhoea was between PID 2 and 4 and its average duration was 1.7 days. Rectal virus shedding was detected by blocking-ELISA in 100% of the PEDV exposed animals. Viral antigen was present in faecal samples from PID 1 to 8, but most of the pigs shed the virus in faeces between PID 3 and 6. The average duration of the shedding period was 5.4 days and the peak of the GMT of viral antigen in rectal swabs was at PID 5 (Fig. 1).

3.2. Antibody responses in serum to PEDV

After inoculation with virulent PEDV, seroconversion was demonstrated in 100% of the pigs by blocking ELISA. Specific antibodies were detected in 52.6% of the pigs at PID 4, in 96% at PID 7 and in 100% at PID 12.

The isotype-specific ELISA antibody titers in the serum of inoculated piglets are shown in Fig. 2. Titters of IgG increased significantly from PID 12 over the duration of the experiment, reaching the maximum at that moment (PID 32, GMT 2650). IgA serum

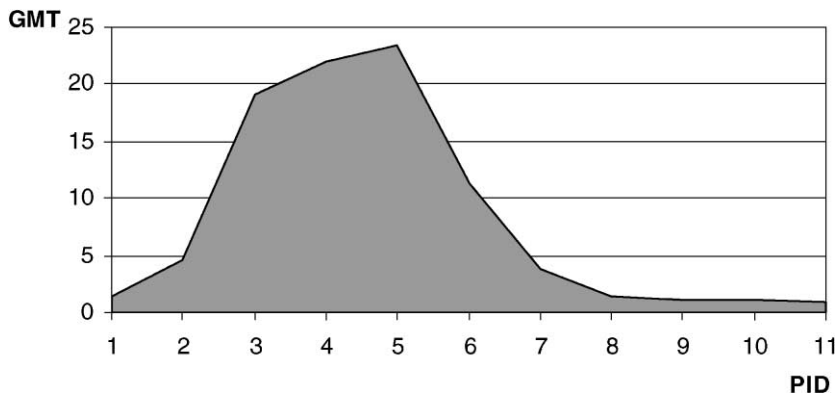


Fig. 1. GMT of the antigen detected by blocking ELISA in faeces from conventional pigs inoculated with the virulent isolate of the CV-777 strain of the PEDV.

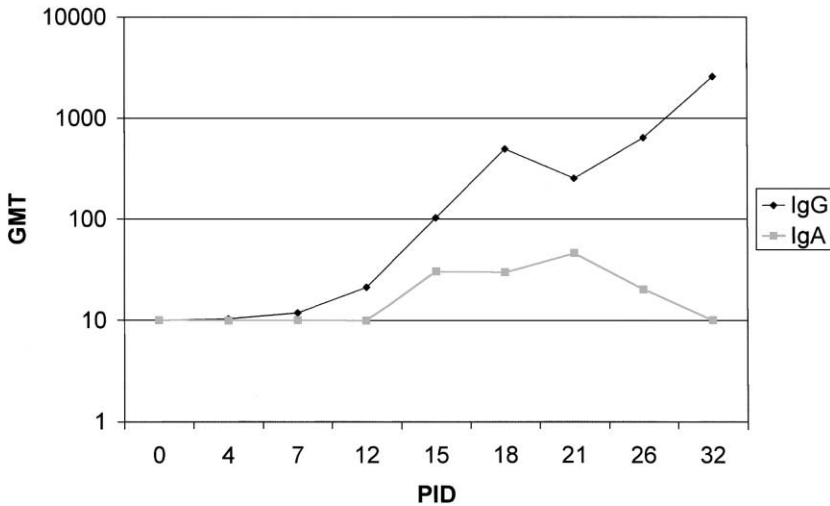


Fig. 2. Serum IgG and IgA GMT in conventional pigs inoculated with the virulent isolated of the CV-777 strain of the PEDV.

antibodies were firstly detected at PID 15, showing moderated values up to PID 28, in which the IgA level was again under detectable limits. The peak of IgA antibodies to PEDV occurred at PID 21 (GMT 46).

3.3. Standardisation of the ELISPOT

Optimal antigenic expression with the infected and fixed cell monolayers was obtained by inoculating 2.8×10^3 fluorescent focus-forming units of viral inoculum per well and fixing the plates after 12–15 h of incubation. These conditions guaranteed a citopatic effect between 80 and 90%, which gave a very high rate of fluorescence, but keeping the integrity of the monolayer.

Plates with the immunocaptured antigen yielded the best results when the semi-purified viral antigen diluted 1:25 was used (2.05 μg of protein per well) and the plates were incubated for 4 h at 37 °C or overnight at 4 °C.

Using infected and fixed cell monolayers unspecific spots were non-detected either in the plates with mock-infected cell monolayers or with MNC from naïve piglets and, therefore, every spot detected was considered a positive result. On the other hand, plates with the immunocaptured antigen showed some unspecific spots in the control wells and also when MNC from negative pigs were tested. Moreover, a higher number of spots were usually detected when the assay was performed over infected and fixed cell monolayers.

Consequently, due to the high specificity shown for the fixed PEDV infected cell plates and its higher sensitivity, we decided to use these plates for the rest of the experiments. In every assay mock-infected plates were included as negative control to assess the specificity.

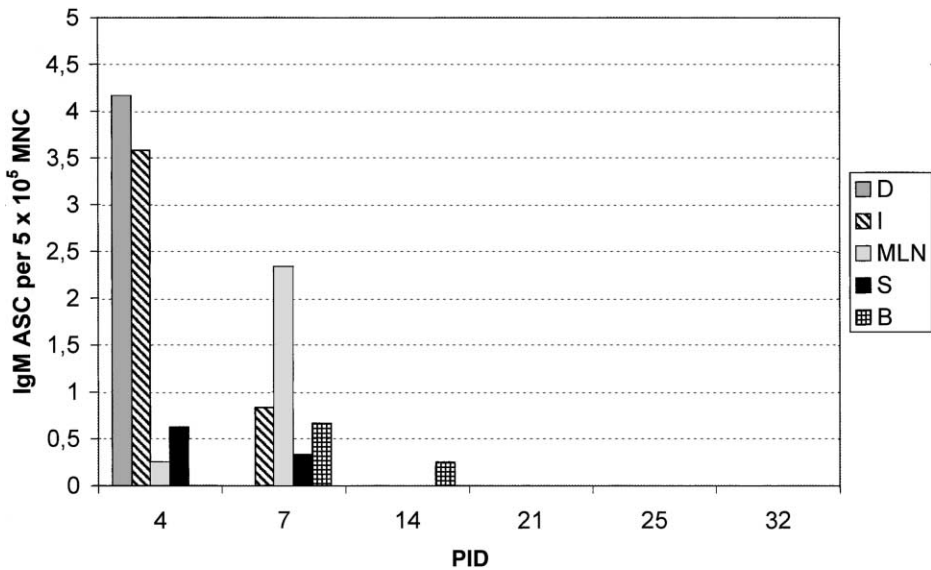


Fig. 3. Virus specific IgM ASC in duodenum (D) and ileum (I) lamina propria, mesenteric lymph nodes (MLN), spleen (S) and blood (B) of conventional pigs induced by oral inoculation with virulent PEDV. Pigs were sacrificed on PID 4, 7, 14, 21, 25 and 32.

3.4. Virus-specific ASC response

In order to evaluate the distribution of PEDV-specific ASC, MNC from mesenteric lymph nodes, lamina propria of duodenum and ileum, spleen and blood were recovered at various PID from PEDV exposed pigs and tested for antibody production by ELISPOT. Kinetics of those responses are summarised in Figs. 3–5 and Table 1.

No virus-specific ASC were detected by ELISPOT either with the control pigs or when the assay was performed with mock-infected cell plates.

PEDV-specific IgM ASC were the predominant response between PID 4 and PID 7. At PID 4 specific IgM ASC were found in all the tissues with the only exception in the blood, the strongest response corresponded to the intestinal lamina propria, where the number of IgM ASC was 6–7-fold greater than in mesenteric lymph nodes and spleen. At PID 7, the number of IgM ASC declined in the duodenum and the ileum, but not in mesenteric lymph nodes and systemic tissues (spleen and blood) and at PID 14 blood was the only tissue in which these cells were detected.

Although some IgA ASC were detected as early as PID 7 in duodenum lamina propria, the IgG and IgA ASC responses in the intestinal lymphoid tissues were evident from PID 21 on. IgG ASC were demonstrated in the three tissues at PID 21, being maximum in the duodenum (Fig. 4, 23.5 IgG ASC per 5×10^5 MNC). From this day and until the end of the experiment, the number of IgG ASC declined in the duodenum, but not in the mesenteric lymph nodes, where maximum was reached on PID 32. Although no significant statistical difference was demonstrated, the number of IgA ASC detected in the intestinal

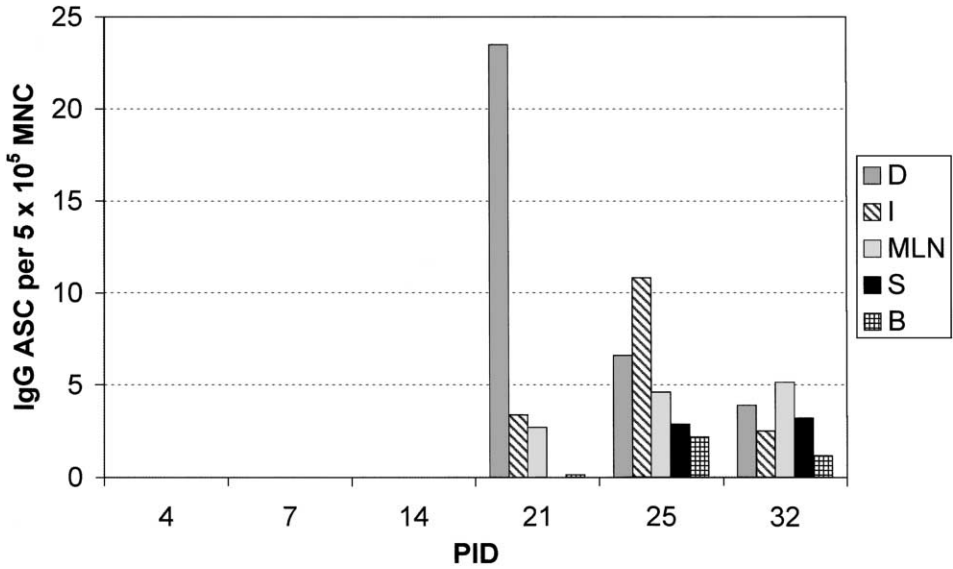


Fig. 4. Virus specific IgG ASC in duodenum (D) and ileum (I) lamina propria, mesenteric lymph nodes (MLN), spleen (S) and blood (B) of conventional pigs induced by oral inoculation with virulent PEDV. Pigs were sacrificed on PID 4, 7, 14, 21, 25 and 32.

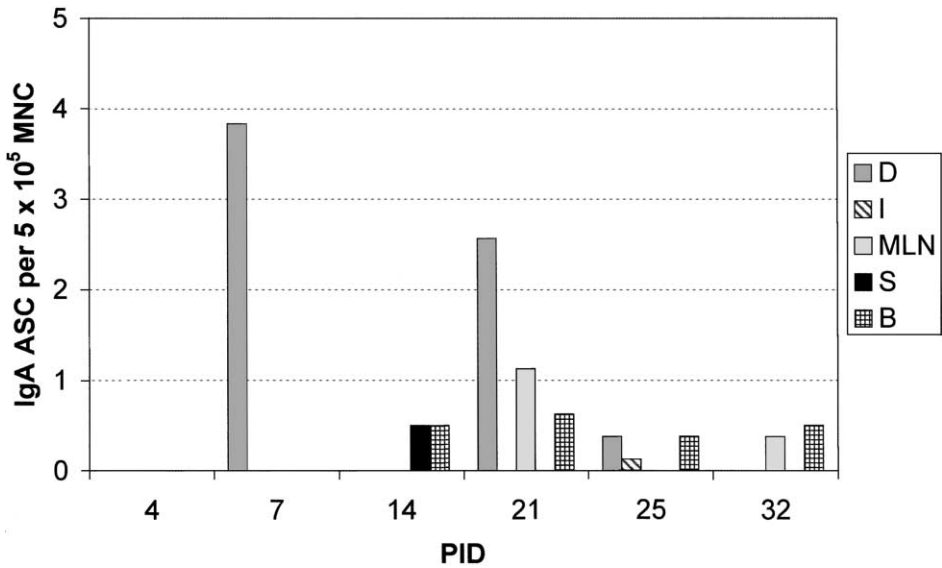


Fig. 5. Virus specific IgA ASC in duodenum (D) and ileum (I) lamina propria, mesenteric lymph nodes (MLN), spleen (S) and blood (B) of conventional pigs induced by oral inoculation with virulent PEDV. Pigs were sacrificed on PID 4, 7, 14, 21, 25 and 32.

Table 1

Numbers of isotype-specific ASC to PEDV (per 5×10^5 MNC) in duodenum and ileum lamina propria, mesenteric lymph nodes (MLN), spleen and blood from pigs experimentally exposed to the virulent isolate of the PEDV strain CV-777 and sacrificed on PID 4, 7, 14, 21, 25 and 32

PID	Duodenum			Ileum			MLN			Spleen			Blood		
	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG
4	4.2	0	0	3.6	0	0	0.25	0	0	0.6	0	0	0	0	0
7	0	3.8	0	0.8	0	0	2.3	0	0	0.3	0	0	0.6	0	0
14	0	0	0	0	0	0	0	0	0	0	0.5	0	0.25	0.5	0
21	0	2.6	23.5	0	0	3.4	0	1.1	2.7	0	0	0	0	0.6	0.1
25	0	0.4	6.6	0	0.12	10.8	0	0	4.6	0	0	2.9	0	0.4	2.2
32	0	0	3.4	0	0	2.5	0	0.4	5.1	0	0	3.2	0	0.5	1.2

lymphoid tissues by ELISPOT was, in general, lower than the number of IgG ASC. The highest IgA ASC response was also observed in the duodenum, where these cells were shown on PID 7, 21 and 25. The lowest response was obtained in ileum, in which IgA ASC were demonstrated only in a limited number at PID 25 (<1 IgA ASC per 5×10^5 MNC) (Fig. 5).

The number of IgG ASC in spleen and blood was lower than in the intestinal lymphoid tissues and these cells were evidenced later, between PID 25 and 32. A slow number of IgA ASC were detected in the spleen, but only at PID 14. However, in the blood, the presence of IgA ASC was proved from PID 14 until the end of the experiment on PID 32.

Due to the individual variability in the responses observed and mainly to the limited number of pigs used at each point in time, statistical comparison among tissues or days showed no significant differences.

3.5. Virus-specific ASC response after secondary *in vitro* stimulation

MNC were stimulated *in vitro* with different amounts of virus and over different periods. The best results were obtained when culturing the cells for 5 days with 4.6 μ l (238.6 ng of protein) of the semi-purified viral antigen per 5×10^5 MNC.

In vitro stimulation of the cells was only possible with the MNC from mesenteric lymph nodes, spleen and blood, but not with those from the intestinal lamina propria since they had an irregular survival due to their frequent contamination with the enteric microbial flora.

Results from the secondary immune response obtained after the *in vitro* stimulation are summarised in Table 2. Even though low numbers of IgM ASC were detected on PID 4, secondary antibody response was more prominent on PID 21 and 25, consisting of IgG and IgA ASC. The number of specific ASC increased from 38 to 1137 times after the secondary stimulation and in a similar way to the primary responses, IgG ASC were predominant in the systemic tissues (spleen and blood), whereas not in the MLN, in which the secondary IgA ASC response was higher at PID 21. *In vitro* stimulation of naïve MNC from the control group did not yield virus-specific ASC after 5 days of culture.

Table 2

Numbers of isotype-specific ASC to PEDV (per 5×10^5 MNC) after in vitro secondary stimulation of the MNC isolated from mesenteric lymph nodes (MLN), spleen and blood of conventional pigs experimentally exposed to the virulent isolate of the PEDV strain CV-777 on PID 4, 7, 14, 21, 25 and 32

PID	Mesenteric lymph nodes ^a			Spleen ^a			Blood ^a		
	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG
4	11 (44)	0.7 (-)	0 (-)	22 (36)	0 (-)	0 (-)	8 (-)	0 (-)	0 (-)
7	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
14	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0.8 (-)	0 (-)	0 (-)	0 (-)
21	0 (-)	44 (35)	38 (14)	0 (-)	220 (-)	586 (-)	0 (-)	29 (46)	267 (2136)
25	0 (-)	156 (-)	1137 (247)	0 (-)	7 (-)	97 (34)	0 (-)	49 (163)	1038 (478)

^a ASC after secondary in vitro stimulation/ASC after primary in vivo stimulation is given within the parenthesis.

4. Discussion

In the present work, we have made a characterisation of the humoral immune response occurring after the infection with PEDV. The study was carried out with conventional pigs, infected with a virulent, wild type isolate of the virus, with the aim of emulating the natural conditions in which the infection took place. Experimental models were 12-day-old, PEDV seronegative piglets. No older animals were used due to the high growth rhythm of porcines, which makes it very difficult to handle a large number of pigs for an extended period. However, these young animals, in spite of having a non-totally-mature-immune system, are immunocompetent from the birth and are capable of developing a complete immune response (Roth, 1999).

Virulent PEDV caused moderate to severe diarrhea in 30% of the inoculated piglets with an average duration of 1.7 days. The incidence and severity of the disease in our experiment was lower than previously described by other authors (Carvajal et al., 1995a; Debouck and Pensaert, 1984; Debouck et al., 1981) who found that almost 100% of the inoculated piglets, gnotobiotic and conventional, developed diarrhea over an average period of 7–10 days. This difference may be explained by the low dose of virus used in our experiment. This dose was experimentally determined in previous assays in order to guarantee infection but without causing severe disease, in order that immune response could be developed and detected in the piglets. However, viral antigen was detected in faeces of all the pigs for the average period of 5.4 days, starting between PID 1 and 3, as it has been described in similar experiments (Carvajal et al., 1995a).

Specific antibodies against PEDV were demonstrated by blocking ELISA in 100% of the inoculated pigs between PID 4 and 12. This result is closely related with previous studies which report seroconversion 1 week after PEDV infection (Carvajal et al., 1995a; Van Nieuwstadt and Zetstra, 1991). Although PEDV-IgG antibodies were always the predominant response in blood, in consistence with the pattern for distribution of the different immunoglobulin isotypes, an important IgA response was detected in blood from PID 15–25, simultaneous to the detection of IgA ASC in blood. These results indicate that even blood is not considered an important source of the typically mucosal-associated

immunoglobulin. Its detection is possible in the early stages after infection, probably in relation to the lymphocyte homing process (Corthesy and Kraehenbuhl, 1999; Kagnoff, 1996; Kantele et al., 1997; Salmi and Jalkanen, 1997).

In order to standardise ELISPOT, two different kinds of antigenated plates were compared, infected and fixed cell culture plates and semi-purified antigen immunocaptured plates. In spite of the fact that no statistical studies were carried out, ELISPOT performed with infected cell culture plates gave better results in terms of sensitivity and specificity. In the immunocaptured plates we used a Mab directed against S-protein of PEDV and, therefore, most of the viral antigen captured in the plate could be fragments of the spike more than the complete viral particle. On the other hand, viral inoculum for peritoneal immunisation of the animals was concentrated using ultracentrifugation and may then induce a high immune response against N and M protein which could be better detected with the infected and fixed cell plates.

Using the ELISPOT, we demonstrated the early development of a primary specific immune response against PEDV, formerly IgM ASC followed by IgG and IgA ASC responses. PEDV-specific IgM ASC were detected in all tissues between PID 4 and 7, with this response being higher and faster in duodenum and ileum lamina propria than in the other tissues, including mesenteric lymph nodes. The earlier presence of specific ASC in these tissues could be related to its anatomic proximity to the virus replication site in the enterocytes of the small intestine. The antigen could be delivered immediately to the lamina propria where it would stimulate the local immune response whereas its diffusion to the mesenteric lymph nodes would be more delayed (Corthesy and Kraehenbuhl, 1999; Kagnoff, 1996; Kraehenbuhl and Neutra, 1992; Neutra et al., 1996).

After this initial response, there was no important detection of PEDV-ASC in the intestine or mesenteric lymph nodes until PID 21. Maximum values of ASC were reached between PID 21 and 32, at the end of the experiment. Similar experiences with other porcine enteric viruses such as TGEV or rotavirus (Chen et al., 1995; Van Cott et al., 1993; Yuan et al., 1996) showed consistent IgA and IgG ASC responses as early as PID 12, with the maximum amounts of cells detected also being higher than those described in the report. This difference could be due to a lower sensitivity of the ELISPOT performed for PEDV which would not detect low numbers of specific-IgA and IgG ASC present in the first weeks after the infection. We also have to consider that experiences with rotavirus were carried out with gnotobiotic pigs in which the antigen-specific ASC/total ASC ratio is higher than in conventional animals. However, at least for TGEV, we cannot rule out the possibility of a higher antigenic stimulation as compared with PEDV.

Although duodenum registered the highest number of specific-IgG and IgA ASC of the three mucosal associated lymphoid tissues, we consider that this result is more related to the distribution and organisation of the intestinal lymphoid tissue than to real differences in the immune response between tissues. MNC purified from duodenal lamina propria belong to the diffuse lymphoid tissue, in which most of the cells, mainly the lymphocytes, are mature and active (Kraehenbuhl and Neutra, 1992; Pescovitz, 1999). On the other hand, MNC from ileum and mesenteric lymph nodes also include large numbers of naive cells from the germinal centres of the lymph nodes or the Peyer patches that were not removed from the ileum (Heel et al., 1997; Neutra et al., 1996; Pescovitz, 1999).

As a constant in our results, the number of IgG ASC in gut associated tissues was higher than the number of IgA ASC. This fact has also been described in previous reports (Van Cott et al., 1993; Yuan et al., 1996, 1998) studying immunity by ELISPOT in TGEV and rotavirus infections. Even though there is no clear explanation for this fact, the possibility of extraintestinal stimulation of the immune system has been proposed. However, PEDV has only been located in mesenteric lymph nodes apart from the intestinal mucosa (Debouck et al., 1981; Pensaert, 1999) and the possibility of systemic stimulation is very low. Further investigation is necessary to elucidate this question.

With regard to the response detected in the systemic lymph tissues and mainly in the blood, our results showed the presence of specific IgA ASC at PID 14, earlier than in mucosal associated lymphoid tissues. As mentioned previously, we explain this finding as a consequence of the lymphocyte homing, a process in which the lymphocytes migrate through the blood to the effector sites, such as the intestinal lamina propria, after completing its maturation in distal sites of the mucosal immune system (Corthesy and Kraehenbuhl, 1999; Kagnoff, 1996; Kantele et al., 1997; Salmi and Jalkanen, 1997). Thus, at least shortly after the infection, the PEDV specific ASC present in the blood could be considered more related to the response in mucosal associated lymph tissues than to the systemic response.

Secondary *in vitro* stimulation of the MNC from inoculated piglets was performed in order to determine the potential B-cell memory response after PEDV infection. No specific-ASC was evidenced after the secondary *in vitro* stimulation of MNC obtained from control animals with no previous contact with PEDV. This result confirms, as it has been described for other virus (Berthon et al., 1990; Van Cott et al., 1993; Yuan et al., 1996), that only previously *in vivo* stimulated cells are capable of developing a specific response when stimulated *in vitro* with PEDV antigen. Moreover, a limited number of IgM ASC were detected at PID 4, memory B cells appeared consistently at PID 21 in mesenteric lymph nodes, spleen and blood from PEDV infected pigs. Similar results have been found after oral inoculation of pigs with porcine rotavirus (Yuan et al., 1996) while in TGEV infected animals specific B memory cells were detected from PID 12 (Van Cott et al., 1993). Again, the number of PEDV-ASC per 5×10^5 MNC after secondary *in vitro* stimulation were lower than reported by Van Cott et al. (1993) in similar studies for TGEV. As we have suggested for the *in vivo* response, this difference could be attributed either to a lower sensitivity of the ELISPOT performed with PEDV or to differences in the immune response against each virus. In agreement with other authors (Berthon et al., 1990; Van Cott et al., 1993, 1994) and as described for the *in vivo* stimulated ASC, clear predominance of PEDV-specific IgG ASC was observed in systemic lymphoid tissues, blood and spleen, but not in the mesenteric lymph nodes where, at PID 21, IgA ASC outnumbered IgG ASC although reverse circumstances occurred at PID 25.

To sum up, a first attempt has been made to study the immune response developed in conventional piglets after infection with PEDV. This report shows the presence of specific-ASC from the different isotypes and in several locations of gut associated and systemic lymphoid tissues and also describes the IgA and IgG kinetics in serum. In addition, our study shows the presence of specific memory B cells from the third week after infection. Present results have important implications for future studies of immunity and protection against PEDV infection and consequently in the development and evaluation of future immunoprophylactic strategies to prevent and control the disease.

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