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Journal of Virological Methods 105 (2002) 37–47

Journal of Virological Methods

www.elsevier.com/locate/jviromet

Lymphoproliferative responses and protection in conventional piglets inoculated orally with virulent or attenuated porcine epidemic diarrhoea virus

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Received 13 September 2001; received in revised form 20 March 2002; accepted 21 March 2002

Abstract

Lymphocyte proliferative responses were evaluated in mucosal (mesenteric lymph nodes) and systemic (spleen and blood) lymphoid tissues of conventional piglets inoculated with the virulent or attenuated isolates of porcine epidemic diarrhoea virus (PEDV) strain CV-777 and challenged 21 days later with the virulent isolate of the same virus. A lymphoproliferative assay was developed in which mononuclear cells isolated from lymphoid tissues at different postinoculation and postchallenge days underwent a secondary in vitro stimulation with semipurified antigen obtained from PEDV-infected cell cultures. Vigorous lymphocyte proliferative responses were detected in the pigs inoculated with the virulent PEDV at postinoculation days 4–21, especially in the mesenteric lymph nodes and the blood; however, in the spleen this response was lower and less regular. The pigs inoculated with the attenuated virus showed a less intense response, the higher lymphocyte proliferation also corresponded to the mononuclear cells from mesenteric lymph nodes. Lymphocyte proliferation responses showed high correlations with protection against homologous challenge with virulent PEDV, and this correlation was higher in the gut associated lymphoid tissues (mesenteric lymph nodes). The cell proliferation response detected in blood mirrored that detected in the mesenteric lymph nodes, and showed also good correlation with protection. The results confirm that T-cell-helper function, assessed by lymphocyte proliferation responses, contributes to establishing a protective immune response against PEDV infections.

Keywords: Porcine epidemic diarrhoea virus; Lymphocyte proliferation; Porcine mucosal immunity; Protection

1. Introduction

Porcine epidemic diarrhoea is an enteric disease in pigs caused by a member of the Coronaviridae family, porcine epidemic diarrhoea virus (PEDV) (Cavanagh et al., 1994; Murphy et al., 1999). Because of the high prevalence and the severity of

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0166-0934/02/$ - see front matter
PII: S0166-0934(02)00063-0

clinical symptoms, porcine epidemic diarrhoea represents the most important viral infection of swine intestinal tract in Spain and in many other European and Asean countries (Carvajal et al., 1995b; Pensaert, 1999; Sueyoshi et al., 1995; Van Reeth and Pensaert, 1994), although not in the USA where the PEDV is not present but a high prevalence of transmissible gastroenteritis virus infections is still present (Saif, 1998; Saif and Wesley, 1999).

The disease is characterized by acute watery diarrhoea, depression and anorexia. Although morbidity is high and most of the animals in the herd can be affected within a week, mortality is usually low in adult animals (3%), which can recover in 7–10 days. However, when piglets less than 4–5 weeks are infected, mortality is usually 50% and can reach 90% in severe outbreaks, as usually happens in transmissible gastroenteritis. Besides the mortality, PEDV infection causes important economic loses due to the diminishing of the productive indexes (Pensaert, 1999).

As in many other viral infections of food animals, the lack of effective treatment makes immunity the main key for prevention and control of the disease. However, the development of candidate vaccines needs previous knowledge of immunological aspects related to the infection. Due to the enteric nature of the disease and the special configuration of the mucosal immune system, protection depends mostly on the local immune response (Corthesy and Kraehenbuhl, 1999; Kagnoff, 1996; Saif et al., 1994; Van Cott et al., 1994), and is the reason why these studies cannot be limited to blood but require organs containing gut associated lymphoid tissues, in which local immune response can be measured.

In spite of the fact that the importance of humoral immune response in gastroenteric viral infections of porcine is well recognized (Corthesy and Kraehenbuhl, 1999; Saif et al., 1994; Tô et al., 1998; Van Cott et al., 1994; Yuan et al., 1996), little is known about cell-mediated immunity, particularly in PEDV infections. However, it has been proposed that cellular immune response may play an important role in the protection and recovery from infection, besides the fact that production of antibodies is regulated by cytokines produced by activated T lymphocytes and other mononuclear cells (Corthesy and Kraehenbuhl, 1999; Kraehenbuhl and Neutra, 1992; Saif, 1999; Totterdell et al., 1988).

The aim of this study was to assess the cellular immune response following natural infection with PEDV and also after inoculation of an attenuated virus, and the contribution to the establishment of a protective response. Virus-specific lymphoproliferative responses of systemic tissues (spleen and blood) and mesenteric lymph nodes were studied in conventional piglets after primary inoculation with the virulent, wild type, strain CV-777 of PEDV or its cell culture attenuated form and after challenge, 3 weeks later, with a high dose of the virulent virus.

2. Material and methods

2.1. Cells and viruses

Vero cells were grown with Eagle's minimum essential medium (Gibco, Life Technologies) buffered with bicarbonate and supplemented with 5% (v/v) fetal 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).

The cell culture adapted strain of PEDV CV-777, attenuated by many passages, was propagated in Vero cells as described by Hofmann and Wyler (1989), infecting confluent monolayers of cells after removing the growth medium and adding the viral inoculum diluted in medium without fetal calf serum but containing 10 μl/ml of trypsin (Difco). A PEDV-infected cell lysate was used as attenuated PEDV inoculum.

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 and prepared in PBS for use as virulent PEDV inoculum. After oral inoculation, the animals were killed in the acute phase of diarrhoea, and the intestinal contents and the small intestine were collected at necropsy. 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. The richest fractions were pooled and stored at −70 °C.

2.2. Experimental design

A total of 62 conventional 11-day-old piglets, seronegative to PEDV and from a herd with no previous history of the disease were assigned to three different experimental groups which were maintained in isolation facilities to prevent virus circulation. Group 1 ($n = 29$) was inoculated with a low dose of the virulent isolate of PEDV strain CV-777 that was adjusted in a previous experiment to produce a high morbidity without causing severe disease in the animals. Pigs from group 2 ($n = 20$) were inoculated with 2.55×10^5 fluorescent focus-forming units per pig of the attenuated isolate of the same PEDV strain. The third group ($n = 13$) was mock-inoculated and served as control. Twenty-one days after inoculation, the three groups of pigs were challenged with the virulent PEDV, using a two times higher dose than that used to inoculate the group 1.

Animals were observed daily for clinical symptoms and rectal swabs were taken for 11 days after inoculation and for 9 days after challenge. Faecal scores were recorded as normal faeces, pasty faeces, semiliquid (moderate diarrhoea) and liquid (watery diarrhoea). At different postinoculation and postchallenge days (postinoculation days 4, 7 and 14, and postinoculation/postchallenge days 21/0, 25/4 and 33/12) subsets of each group of pigs ($n = 2–5$) were killed by injection of barbiturate overdose (Eutalender, Normon, Madrid, Spain). Hundred millilitre of blood were collected from cardiac cavities in 25% (v/v) acid citrate glucose. Spleen and mesenteric lymph nodes were also collected aseptically and placed in ice-cold wash medium (RPMI 1640 containing 10 mM HEPES and 200 mg of gentamicin and 20 mg ampicillin per ml). Three PEDV-seronegative, unexposed pigs served as negative control and were killed to obtain the background values for the lymphoproliferative assay.

2.3. Isolation of mononuclear cells

Mononuclear cells from blood and tissues were isolated as described previously (De Arriba et al., 2001a). Briefly, peripheral blood lymphocytes were obtained by density gradient centrifugation in Ficoll–Paque (Ficoll–Paque Research Grade, Pharmacia Biotech, Upsala, Sweden). Lymphocytes collected from the interface were washed twice in Hanks' balanced salt solution and suspended in 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 mg of ampicillin and 100 mg of gentamicin per ml (enriched medium).

Mononuclear cells from spleen and mesenteric lymph nodes were obtained by pressing the tissues through stainless steel screens (80 mesh) of a cell collector (Cellecter; E-C Apparatus Corp., FLA, USA). Cell suspensions were centrifuged, and the mononuclear cells were removed from the pellet by continuous and discontinuous gradient centrifugation in Percoll (Pharmacia Biotech.), washed twice with wash medium and resuspended in enriched medium. Viability of all mononuclear cells preparations was confirmed by the trypan blue exclusion test, in every case being $> 95\%$.

2.4. Lymphoproliferative assay

The lymphoproliferative assay for detection of PEDV-specific T cells was adapted from methods published previously (Brim et al., 1994, 1995; Ward et al., 1996). Semipurified PEDV antigen for in vitro stimulation of mononuclear cells cultures was obtained from lysates of PEDV-infected cell cultures that were concentrated 50 times by ultracentrifugation at $100{,}000 \times g$ for 2 h at 4 °C and then semipurified by ultracentrifugation through 20% sucrose under the same conditions. The most favourable concentration of antigen for optimal antigenic stimulation of the mononuclear cells was established by dose–response curves. A PEDV-control antigen was obtained giving the same treatment to mock-infected cultures. The T-cell mitogen phytohaemagglutinin (Gibco) was used as positive control at a final concentration of 10 μl/ml, following the manufacturer's instructions.

Optimal conditions of number of cells and duration of incubation were determined by preliminary studies. Mononuclear cells at a concentration of 5×10^5 cells per 100 μl and per well were placed in 96-well culture plates and stimulated in triplicate with the PEDV antigen or the control antigen or the phytohaemagglutinin. Cells were incubated for 72 h at 37 °C in 5% CO_2 and 18 h prior to harvest each well was labelled by pulsing 1 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech). Harvesting of cells was carried out on glass fiber filters (FilterMAT, Skatron Inc., Va, USA) and [^{3}H]thymidine incorporation was determined by liquid scintillation spectrophotometry.

The lymphocyte proliferative responses for each mononuclear cells sample assayed was expressed as the stimulation index (SI), calculated as SI = mean cpm of PEDV stimulated wells/mean cpm of control antigen stimulated wells, being cpm counts per minute.

2.5. ELISA for detection of antigen

Viral antigen was detected in faecal samples by the double antibody sandwich ELISA described by Carvajal et al. (1995a). This ELISA is based on the use of two monoclonal antibodies (Lelsytad CVI-66.31 and Lelystad CVI-66.49) directed specifically against the S protein of the virus. A blocking step with rabbit-anti PEDV hyperimmune serum was included to increase the specificity. Twofold serial dilutions of the samples were assayed starting at 1:2 and titres were expressed as the inverse of the lowest positive dilution. For calculation of the geometric mean titre (GMT), negative samples were given a titre of 1.

2.6. Statistical analyses

One-way analysis of variance followed by the paired Student's *t*-test was used to determine the nature of differences observed in virus shedding and lymphocyte proliferation responses among inoculated groups, tissues and days. Correlation between proliferation responses at challenge day and protection against the infection was established by Spearman's correlation coefficient (ρ). Significance was assessed at $P < 0.05$. For the analysis the SYSTAT for Windows v.5.03 (SYSTAT Inc.) and the spreadsheet Microsoft EXCEL v.7.0 (Microsoft Comp.) were used.

3. Results

3.1. Clinical signs and virus shedding

A summary of faecal virus shedding and clinical disease after inoculation and challenge is given in Table 1. After primary inoculation of pigs from group 1 with virulent PEDV, moderate to severe diarrhoea was observed in 33% of the animals and virus shedding in 100%. The onset of diarrhoea was observed between postinoculation days 2 and 4 and the average duration was 1.7 days. Virus shedding was detected in some of the pigs at postinoculation day 1 but most shed PEDV in faeces from postinoculation day 2 to 6. The average duration of the shedding period was 5.4 days. The GMT of viral antigen in faecal samples was measured using ELISA; it increased strongly from postinoculation day 2 and reached a peak at postinoculation day 5. Conversely, pigs from group 2 inoculated with the attenuated PEDV did not show typical signs of the disease and only one pig had moderate diarrhoea for 1 day and virus shedding was detected in only one sample at postinoculation day 5 and with a low titre. Differences between the GMT of antigen in the faeces of the two groups were significant statistically.

On the challenge day, at postinoculation day 21, pigs from group 1 were protected against infection and disease, none developed diarrhoea and not viral antigen was detected in the samples taken after challenge. Diarrhoea was not observed in pigs from group 2 after challenge either, but viral detection in rectal swab samples revealed that protection against the infection was only partial (25%), and the antigen was detected in 75% of the challenged animals (9 out of 12). In the control group moderate diarrhoea was seen in 46% of the pigs starting between postchallenge days 3 and 4 and with an average duration of 1.5 days. Viral antigen was detected in faeces of all of the challenged pigs from this group in which the

average duration of viral shedding (4.6 days) was significantly higher than in group 2 (2.6 days). The GMT of viral antigen detected in the control group was also significantly higher than in group 2.

3.2. PEDV-specific lymphocyte proliferative responses

In order to obtain the optimal secondary antigenic stimulation for the lymphoproliferative assay, two different doses of antigen were used, 25.5 ng of the semipurified antigen were added to each 5×10^5 mononuclear cells from spleen and blood, whereas in the mesenteric lymph nodes a minor dose, 12.8 ng, yielded the optimal stimulation of the cells. Mean cpm obtained after stimulation of each tissue with the different antigens and the mitogen are shown in Table 2. Background cpm, obtained after stimulation of each mononuclear cells culture with the control antigen, were low in mesenteric lymph nodes and blood (usually < 4000) but not in spleen, where these counts sometimes reached values close to 22,000.

After inoculation of group 1, a specific lymphocyte proliferation response was detected for the first time in the mesenteric lymph nodes at postinoculation day 4 (Fig. 1) and was maintained until challenge day (postinoculation day 21), when the maximum value of SI was found (18.82). SI in this tissue was significantly higher from postinoculation day 4 to 21 than that observed in unexposed pigs. The lymphoproliferative responses in mesenteric lymph nodes of pigs from group 2 increased significantly at postinoculation day 14 compared with their responses in previous days (Fig. 1) and SI reached its peak that day at 5.73. Values of the SI between postinoculation days 14 and 21 were significantly higher compared with responses for mononuclear cells from mesenteric lymph nodes in unexposed pigs. Group 1 SI was greater than group 2 at any PID, however statistical significance was only detected at postinoculation day 4.

Table 1
Summary of clinical signs and faecal virus shedding in pigs after inoculation with virulent (group 1) or attenuated (group 2) PEDV and after challenge with virulent PEDV

		Group 1	Group 2	Group 3
After inoculation				
Diarrhoea	Affected (%)	33	5	0
	Mean duration (days)	1.7	1	0
Virus shedding	Total (%)	100	5	0
	Onset (PID[a])	2	5	–
	Mean duration (days)	5.4	1	0
	Maximum GMT([c])	23.45 (a**, b**)	1.04	1
After challenge				
Diarrhoea	Affected (%)	0	0	46
	Mean duration (days)	0	0	1.5
Virus shedding	Total (%)	0	75	100
	Mean onset (PCD[b])	–	2	2.8
	Mean duration (days)	0	2.6 (c**)	4.6
	Maximum GMT	1 (a*, b*)	8.48	22.63

Group 3 was mock inoculated and served as control at challenge. Statistically significant differences among groups are denoted by the letters: 'a' when differences are between groups 1 and 2, 'b' when differences are between groups 1 and 3 and 'c' for differences between groups 2 and 3. Level of significance is defined by $*P \leq 0.5$, $**P \leq 0.01$.

[a] PID: postinoculation days.

[b] PCD: postchallenge days.

[c] GMT: geometric mean titre of antigen detected in faeces by ELISA.

Table 2
Mean counts per minute (cpm) and standard deviation (SD) obtained after stimulation of mononuclear cells purified from the mesenteric lymph nodes, spleen and blood from conventional pigs after inoculation with virulent (group 1) or attenuated (group 2) PEDV or mock-inoculation (group 3) and after challenge with virulent PEDV

PID/PCD		Mesenteric lymph nodes			Spleen			Blood		
		PHA	+Antigen	−Antigen	PHA	+Antigen	−Antigen	PHA	+Antigen	−Antigen
Group 1										
4	cpm	296,327	9894	3516	220,207	5582	5798	176,217	2698	3365
	SD	41,879	6985	3430	27,168	3615	2943	146,876	2147	5306
7	cpm	183,273	8894	870	72,395	5103	3147	111,903	11,223	3221
	SD	102,142	10,292	744	85,721	4249	2846	89,783	10,171	4570
14	cpm	276,632	4604	798	225,240	29,262	6192	177,598	30,399	1209
	SD	40,815	5275	466	40,027	10,014	4223	78,909	30,554	1151
21	cpm	253,626	17,031	685	420,930	47,401	15,065	207,769	40,785	1987
	SD	82,205	26,315	412	670,303	14,215	6197	78,627	14,088	2744
25/4	cpm	254,528	5216	1769	144,666	19,265	8761	169,954	2232	613
	SD	122,450	4137	2065	66,653	10,545	6496	116,665	1218	227
28/7	cpm	235,396	19,088	2331	111,489	49,502	12,213	97,342	21,894	1214
	SD	57,134	28,615	5009	122,765	65,581	8931	81,113	48,892	1049
33/12	cpm	227,316	90,627	14,805	181,428	45,797	15,428	247,312	80,924	2819
	SD	166,708	118,239	18,424	50,016	28,752	7,221	37,035	29,353	2235
Group 2										
4	cpm	180,910	2355	1815	190,566	6388	14,993	151,293	1085	1104
	SD	13,572	818	719	33,606	2428	5116	23,812	269	714
7	cpm	205,216	78,578	37,650	72,043	15,072	19,840	104,448	5,694	3140
	SD	28,792	46,364	7361	55,646	7665	7025	109,607	6,211	5023
14	cpm	69,212	19,339	7749	33,507	12,534	6472	231,848	10,162	1169
	SD	15,801	2479	6212	11,743	7479	1958	84,963	1719	478
21	cpm	218,616	8396	2718	132,831	38,779	22,027	39,591	2054	1024
	SD	53,430	1112	1015	86,796	28,081	11,851	8938	461	641
25/4	cpm	208,609	3937	1736	157,631	32,887	10,112	161,803	3195	905
	SD	32,096	3931	836	56,189	20,129	2428	54,129	2133	508
28/7	cpm	168,587	9071	5449	90,156	9500	5868	190,509	3576	1243
	SD	125,377	12,346	8558	100,169	10,007	6822	201,207	3626	1184
33/12	cpm	161,064	3225	1262	152,781	20,088	6359	195,160	4974	1060
	SD	73,553	2351	854	54,902	9759	3132	65,823	1989	866
Group 3										
25/4	cpm	146,209	1838	3172	102,547	1772	3995	111,207	2248	4016
	SD	75,416	1233	2956	58,162	1151	1862	20,815	1057	4333
28/7	cpm	200,475	3482	853	202,441	12,474	8661	246,291	1788	3426
	SD	237,592	3433	494	117,123	10,208	3216	61,526	271	6222
33/12	cpm	154,756	2533	633	174,091	12,752	6285	245,638	2149	450
	SD	46,384	2345	430	62,468	11,773	2387	118,582	1531	309
Background values										
0	cpm	183,232	25,995	14,851	284,154	11,805	3642	324,184	1966	772
	SD	61,111	20,849	10,670	82,662	17,049	5244	136,760	633	563

Mononuclear cells were stimulated with positive, negative antigen or the T-cell mitogen phytohaemagglutinin (PHA). Background values correspond to pigs with no previous contact to PEDV. PID, postinoculation day; PCD, postchallenge day.

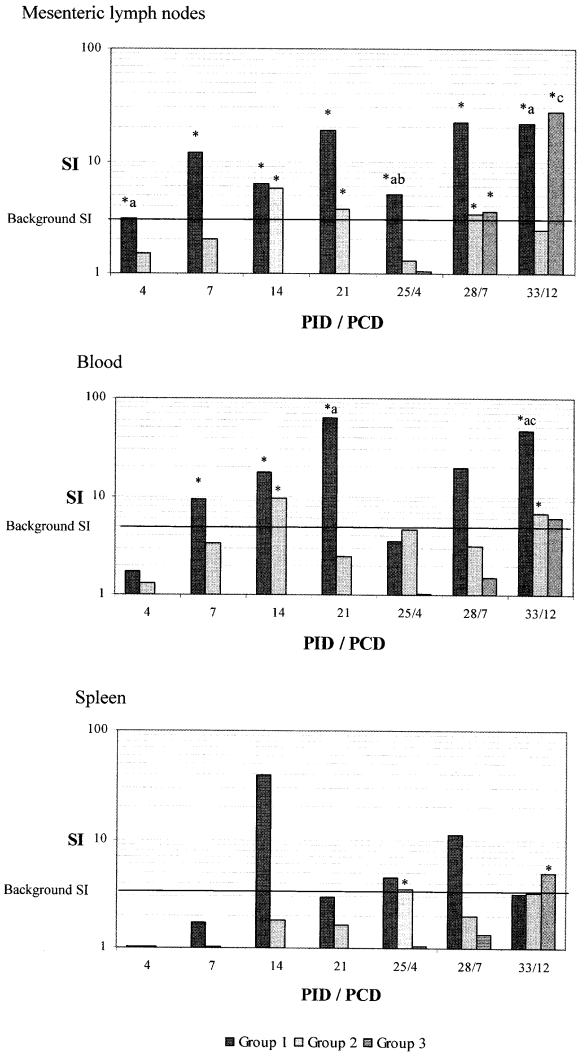

Fig. 1. Course of the virus-specific lymphocyte proliferative responses represented by SI for mononuclear cells from mesenteric lymph nodes, spleen and blood from pigs after inoculation with virulent (group 1) or attenuated (group 2) PEDV or mock-inoculation (group 3) and after challenge with virulent PEDV. The SI are the mean cpm of virus-specific stimulated wells versus mean cpm of control antigen stimulated wells, being cpm counts per minute. The mean value of the SI obtained from the group of unexposed pigs is represented by a line crossing the Y-axis. Statistically significant differences ($P \leq 0.05$) with values obtained in nonexposed pigs are noted as *. Differences between groups are nodded as: 'a' when differences are between groups 1 and 2, 'b' between groups 1 and 3 and 'c' for differences between groups 2 and 3.

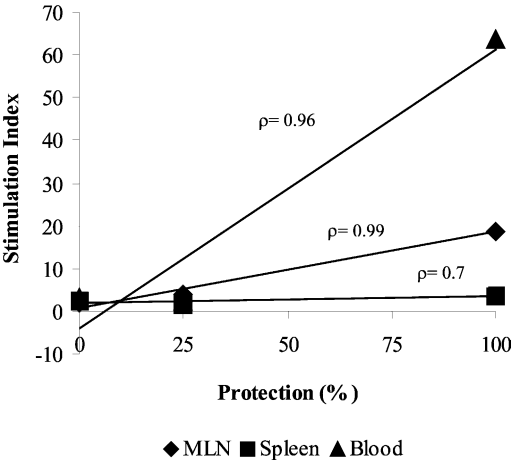

Fig. 2. Correlations between lymphocyte proliferative responses in mononuclear cells collected from mesenteric lymph nodes, blood and spleen from pigs inoculated with virulent or attenuated PEDV or mock-inoculated and protection against challenge 21 days later with virulent PEDV. Correlations were assessed by Spearman rank correlation test. Lymphocyte proliferative responses were expressed as mean cpm of PEDV stimulated wells versus mean cpm of control antigen stimulated wells, being cpm counts per minute.

Mononuclear cells purified from blood of group 1 showed a vigorous proliferative response after inoculation starting at postinoculation day 7 with significant increases over the following days. The SI obtained for this group in blood were significantly higher than values in blood of unexposed pigs between postinoculation days 7 and 21 and as in the mesenteric lymph nodes, the peak value occurred at postinoculation day 21 (Fig. 2). Virus-specific lymphoproliferative responses in blood from group 2 occurred at postinoculation day 14, the only day in which SI value was significantly higher than that in unexposed animals (SI = 9.58, $P < 0.001$). Likewise in mesenteric lymph nodes, the SI of group 2 were minor than the indexes of group 1, although the difference was statistical significant only at postinoculation day 21.

The magnitude of the virus-specific proliferation in the spleen of group 1 after inoculation was lower than in the other tissues, being also less regular. At postinoculation days 4 and 7, the response of this group was low and similar to the proliferation shown by unexposed pigs. In the following days there was an increase in the SI that

peaked at postinoculation day 14, however, the value of SI was not significantly higher than the background values at any postinoculation time. Group 2 did not show a virus-specific proliferative response in mononuclear cells from spleen after inoculation, with an SI similar to that in unexposed pigs.

After challenge at postinoculation day 21, the lymphoproliferative responses in mesenteric lymph nodes of group 1 underwent an important increase and even though at postchallenge day 4 (postinoculation day 25) the SI was lower than on challenge day (although not significantly), this value again reached its peak at postchallenge day 7 (postinoculation day 28) with a value of 29.25 (Fig. 1). In group 2 lymphocyte proliferation responses after challenge were low and only at postchallenge day 7 (postinoculation day 28) was the SI significantly higher than that in unexposed animals. Responses in group 3, the mock-inoculated control group, after challenge were similar to responses described in mesenteric lymph nodes of pigs from group 1 after inoculation with virulent PEDV, but showed a higher intensity. The SI in this group was significantly higher than in unexposed animals from postchallenge day 7 (postinoculation day 28) and reached 5 days later (at postchallenge day 12, postinoculation day 33) the highest value detected in the mesenteric lymph nodes of all the groups. When the SI of the three groups were compared, statistically significant differences were found at postchallenge day 4 (postinoculation day 25) between group 1 and groups 2 and 3 and at postchallenge day 12 (postinoculation day 33) the SI of groups 1 and 3 were significantly higher than index in group 2.

Lymphocyte proliferative responses in blood after challenge in group 1 were significantly lower at postchallenge day 4 (postinoculation day 25) compared to the challenge day, however, from postchallenge day 7 (postinoculation day 28) there were significant increases, reaching maximum value at postchallenge day 12 (postinoculation day 33) (Fig. 2), the only day that this value could be demonstrated significantly higher than that in unexposed pigs. Similarly to mesenteric lymph nodes, the response detected after challenge in blood from group 2 pigs was low, only the SI at postchallenge day 12 (postinoculation day 33) was significantly higher than unexposed animals index. In the control group, lymphoproliferative responses were low up to postchallenge day 12 (postinoculation day 33) and there was not statistical significance in the differences observed with regard to the unexposed animals. Comparisons between the SI in the blood after challenge in the different groups showed a higher response in group 1, although statistical significance was only shown at postchallenge day 12 (postinoculation day 33). The SI was lower in group 3 than in group 2, but not significantly.

In the spleen, responses after challenge of group 1 increased significantly at postchallenge day 7 (postinoculation day 28) with regard to the previous day, similar to the mesenteric lymph nodes. Group 2 response after challenge was maximum at postchallenge day 4 (postinoculation day 25), this being the only time in which the SI of mononuclear cells of spleen from this group was significantly higher than the SI of unexposed animals. The control group underwent for the first time a specific proliferation response at postchallenge day 12 (postinoculation day 33). The SI obtained in each group at each point in time were compared and no statistically significant differences were found.

3.3. Correlations

Correlations between lymphoproliferative responses detected in each tissue and group at the challenge day and protection against challenge, represented by the protection rate against infection, were established by the Spearman rank correlation test and are shown in Fig. 2. The magnitude of the response in all tissues examined at postinoculation day 21 correlated positively with protection against challenge, although statistical significance were not attained. The highest correlation was detected in the mesenteric lymph nodes ($\rho = 0.99$, $P = 0.08$).

4. Discussion

Protection in swine gastroenteric viral infections, as PED, has been related almost exclusively to the antibody immune responses. However, cell-mediated immunity must play an important role in

protecting and recovery from infection, besides the control function of the B cell-humoral responses carried out by T cell populations (Corthesy and Kraehenbuhl, 1999; Kraehenbuhl and Neutra, 1992; McGhee et al., 1992; Saif, 1999; Totterdell et al., 1988). Thus, without any B cell population deficiency in humans the lack of antibody and specific T-cell responses, resulting in rotavirus persistent infection with viral excretion in faeces for 15 months (Totterdell et al., 1988). Moreover, Welch et al. (1988), in pigs inoculated with transmissible gastroenteritis virus, related peaks of lymphoproliferative responses ending up with final virus shedding in faeces and the beginning of recovery from the disease.

In this study, an in vitro virus-specific proliferation assay was carried out as a method to estimate the cell-mediated immune response since this antigen-induced proliferation has been recognised as a property of CD4 + (T helper) cells in studies undertaken on pigs, mice and humans with rotavirus and coronavirus (Brim et al., 1994; Offit et al., 1992; Ward et al., 1996).

The specific proliferative response after inoculation of pigs with virulent PEDV was detected immediately in the mesenteric lymph nodes, the organs directly associated with the mucosal immune system. The maximum values were found around postinoculation day 21, just when a strong response of virus-specific antibody-secreting cells was detected in this organ and also in the duodenum and ileum lamina propria (De Arriba et al., 2001b). In pigs inoculated with the attenuated strain of PEDV this specific lymphoproliferative response was detected later, at postinoculation day 14 and it was lower that in pigs inoculated with the wild virus. This minor response of group 2 also corresponded to a low response of PEDV-specific antibody-secreting cells (De Arriba et al., 2001b). The difference observed between the lymphoproliferative responses of the two inoculated groups has also been described by other researchers (Brim et al., 1994, 1995; Ward et al., 1996) in other gastroenteric viruses of swine, describing that the lymphocyte proliferative responses induced by attenuated strains of transmissible gastroenteritis and rotavirus were significantly lower than that induced by the homologous virulent virus.

These results suggest that a protective antibody response to the virulent PEDV could be associated with previous development of a strong specific cell-mediated immune response. This consideration could be reinforced by the fact that antibody production by specialised B cells requires T cell help (Corthesy and Kraehenbuhl, 1999).

The virus-specific lymphocyte proliferative response in the systemic lymph tissues (blood and spleen) was observed later than in mesenteric lymph nodes, as well as being considerably lower in the spleen than in other tissues. This delay could be explained if it is considered that the PEDV-specific T cells located in blood and spleen originate in the inductive sites from the gut-associated lymphoid tissues, like the mesenteric lymph nodes, and its presence in systemic tissues is due to the homing process necessary for its maturation (Corthesy and Kraehenbuhl, 1999; Kagnoff, 1996; Kantele et al., 1997; Salmi and Jalkanen, 1997). The specific cell proliferation response in the blood was more similar to that observed in the mesenteric lymph nodes than in the spleen, especially in the group 1, in spite of both, blood and spleen, being linked to the systemic immune system. However, the blood, together with the lymphatic system, is the main vehicle for lymphocyte migration (Salmi and Jalkanen, 1997), and its lymphoid population may reflect primed T cells migrating to the gut for some time after an infection.

The lymphocyte proliferative responses at the challenge day showed high correlation with protection against challenge. Pigs from group 1 inoculated initially with the virulent PEDV, were 100% protected against infection 21 days later with a higher dose of the same virus whereas protection in group 2, inoculated with the attenuated PEDV, was just partial and only 25% of pigs were protected against infection with the virulent virus. The highest correlation was observed in mesenteric lymph nodes. This result again suggests that T-cell response, especially in the gut associated lymphoid tissues, contributes in an important way to the development of a protective immune response in PEDV infections.

The highly attenuated PEDV conferred partial protection against challenge with virulent virus in conventional pigs, this protection is related to the inoculated dose and increases when a higher dose is used (De Arriba et al., 2001b). Kweon et al. (1999) also described the induction of protective immunity by a attenuated strain of PEDV inoculated intramuscularly.

After the challenge, there was an increase in the lymphocyte proliferative response in pigs from group 1, however this increase was not reflected either by an enhancement of the virus-specific antibody secreting cell response or the GMT of PEDV-specific serum IgG and IgA (De Arriba et al., 2001b). Thus, Ward et al. (1996) also reported that pigs inoculated and challenged with virulent rotavirus strains showed after challenge lymphoproliferative responses similar or poorer than after inoculation. Although there is no clear explanation in this, the possibility remains that this secondary response after challenge could be related to the proliferation of cell clones involved in immune regulatory functions different to providing help for antibody production, such as T suppressor populations.

In this study the development of cell-mediated immunity occurred in systemic and lymphoid tissues after inoculation with virulent and attenuated strains of the PEDV. The results suggest that cell-mediated immune responses contribute significantly to the instauration of protective immune status against homologous virulent virus challenge. The lymphoproliferative responses both in gut associated lymphoid tissues and systemic tissues had a higher magnitude when virulent PEDV was used versus attenuated PEDV to inoculate pigs. However, higher doses and administration methods have to be assayed in order to develop vaccines.

Acknowledgements

We wish to thank Dr M.B. Pensaert for providing the wild type isolated of the PEDV strain CV-777 and Dr L.J. Saif and Dr L.A. Ward for laboratory training. We also wish to thank G.F. Bayón and B. Escudero for their excellent technical assistance. This work was funded by the Comisión Interministerial de Ciencia y Tecnología (CICYT) project No. AGF-960486. Salaries were provided by the Excelentisima Diputación Provincial de León.

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