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Immunogenicity and T cell recognition in swine of foot-and-mouth disease virus polymerase 3D

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Abstract

Immunization of domestic pigs with a vaccinia virus (VV) recombinant expressing foot-and-mouth disease virus (FMDV) 3D protein conferred partial protection against challenge with infectious virus. The severity reduction of the clinical symptoms developed by the challenged animals occurred in the absence of significant levels of anti-3D circulating antibodies. This observation suggested that the partial protection observed was mediated by the induction of a 3D-specific cellular immune response. To gain information on the T cell recognition of FMDV 3D protein, we conducted in vitro proliferative assays using lymphocytes from outbred pigs experimentally infected with FMDV and 90 overlapping peptides spanning the complete 3D sequence. The use of pools of two to three peptides allowed the identification of T cell epitopes that were efficiently recognized by lymphocytes from at least four of the five animals analyzed. This recognition was heterotypic because anti-peptide responses increased upon reinfection of animals with a FMDV isolate from a different serotype. The results obtained with individual peptides confirmed the antigenicity observed with peptide pools. Detection of cytokine mRNAs by RT-PCR in lymphocytes stimulated in vitro by individual 3D peptides revealed that IFN-γ mRNA was the most consistently induced, suggesting that the activated T cells belong to the Th 1 subset. These results indicate that 3D protein contains epitopes that can be efficiently recognized by porcine T lymphocytes from different infected animals, both upon primary and secondary (heterotypic) FMDV infection. These epitopes can extend the repertoire of viral T cell epitopes to be included in subunit and synthetic FMD vaccines.

Keywords: FMDV; 3D protein; Peptides

Introduction

Foot-and-mouth disease virus (FMDV) belongs to the genus aphthovirus of the family Picornaviridae and causes a highly contagious vesicular disease (FMD) of cloven-hoofed farm animals (reviewed in Pereira, 1981; Sobrino et al., 2001). FMD is considered the most important animal disease, and its devastating consequences have been dramatically illustrated by the epizootics that recently occurred in Taiwan and the United Kingdom (Knowles et al., 2001; Sobrino and Domingo, 2001). The FMDV particle contains a positive-strand RNA molecule of about 8500 nucleotides, enclosed within an icosahedral capsid comprising 60 copies each of four virus proteins VP1–4 (reviewed in Bachrach, 1977). The FMDV RNA encodes a unique polyprotein from which the different viral polypeptides are cleaved by viral proteases (Belsham, 1993) to render capsid proteins as well as nine different mature nonstructural proteins (NSP), the latter involved in functions that are relevant to the virus life cycle in infected cells (Mason et al., 2003; Porter, 1993). FMDV shows a high genetic and antigenic variability, reflected in the seven serotypes and the numerous variants described to date (Domingo et al., 1990).

FMD control is mainly implemented by using chemically inactivated whole virus vaccines (Barteling and Vreeswijk, 1991). Viral infection and immunization with conventional vaccines usually elicit high levels of circulating neutralizing antibodies, which correlate with protection against the

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homologous and antigenically related viruses (van Bekkum, 1969). However, chemically inactivated vaccines show several disadvantages, such as the requirement for a cold chain to preserve capsid stability, the need for periodic revaccination with virus strains antigenically similar to circulating viruses, and the risk of virus release during vaccine production (Barteling and Vreeswijk, 1991; Brown, 1992; Domingo et al., 1990). These limitations have led to the search for alternative, safe immunogens.

The main FMDV antigenic regions recognized by B lymphocytes have been shown to be in prominent structural motives exposed on the surface of the viral capsid (Acharya et al., 1989; reviewed in Brown, 1995; Mateu, 1995). A region in the G–H loop, around positions 140–160 of capsid protein VP1, has been identified as the main continuous antigenic site recognized by host B lymphocytes to produce neutralizing antibodies (Bittle et al., 1982; Pfaff et al., 1982). Attempts to use VP1 expressed in different systems have resulted in limited induction of neutralizing antibodies and protection (reviewed in Brown, 1992). Likewise, empty viral capsids are poorly produced in different expression systems and their expression in viral vectors or DNA vaccines has provided limited protection in natural hosts (Beard et al., 1999; Grubman et al., 1993). Immunization with adenovirus recombinants expressing P1 and the viral protease 3C conferred protection in pigs (Moraes et al., 2002). Using a different approach, immunization with synthetic peptides containing the antigenic loop G–H in VP1, either alone or in combination with the antigenic site at VP1 residues 200–213 (DiMarchi et al., 1986), resulted in induction of neutralizing antibodies but in limited protection in natural hosts (Taboga et al., 1997, reviewed in Brown, 1992; Sobrino et al., 1999).

The immune response against FMDV is T cell dependent (Collen, 1994). Peptides including the G–H loop and heterologous T helper epitopes have been reported to overcome genetic restriction for induction of an efficient antibody response in mice (Francis et al., 1987) and to confer protection to pigs (Wang et al., 2002). However, heterologous T helper epitopes would not prime for a specific secondary response upon viral infection. In recent years, several T cell epitopes recognized by cattle and swine lymphocytes have been identified in FMDV capsid proteins (Blanco et al., 2000; Collen et al., 1991; Rodríguez et al., 1994a, 1994b; van Lierop et al., 1995b; Zamorano et al., 1994). Inclusion of one of these T cell epitopes, identified in VP1 residues 21–40, in a tandem peptide with the B cell site A has been shown to overcome individual nonresponsiveness of cattle to peptide A (Collen et al., 1991). However, the available evidence indicates that major histocompatibility complex (MHC) polymorphism of host species significantly restricts the recognition of T cell epitopes identified in FMDV capsid proteins (García-Briones et al., 2000; Glass et al., 1991; van Lierop et al., 1995a, 1995b).

The contribution of NSP to protection against FMDV is poorly characterized. T cell epitopes relevant to the induction of a protective response have been described in the NSP of several viruses (Celis et al., 1990; Hacket et al., 1992), including FMDV (Blanco et al., 2001). An extension of the analysis of T cell epitopes to FMDV NSP offers the possibility of broadening the repertoire of viral epitopes recognized by host immune defenses. In addition, NSP exhibit a low degree of amino acid variation among different FMDV serotypes. This conservation permits identification of T cell epitopes that are recognized by immune lymphocytes in a heterotypic manner (Blanco et al., 2001), an important requirement for inclusion in a synthetic vaccine against this virus. 3D, the viral RNA polymerase, is efficiently recognized by bovine lymphocytes (Collen et al., 1998) and has been used, in combination with capsid precursor P1, in DNA vaccination strategies (Cedillo-Barón et al., 2001).

Vaccinia virus (VV) is a widely used model system to study the protective immune response, mediated by both CD4+ and CD8+ T cells, elicited by viral antigens (reviewed in Bennik and Yewdell, 1990). To address the contribution of 3D to the protective immune response to FMDV, we have analyzed the immune response and the protection against viral challenge induced in swine by VV recombinants expressing this protein. In addition, synthetic peptides corresponding to 3D protein have been used to

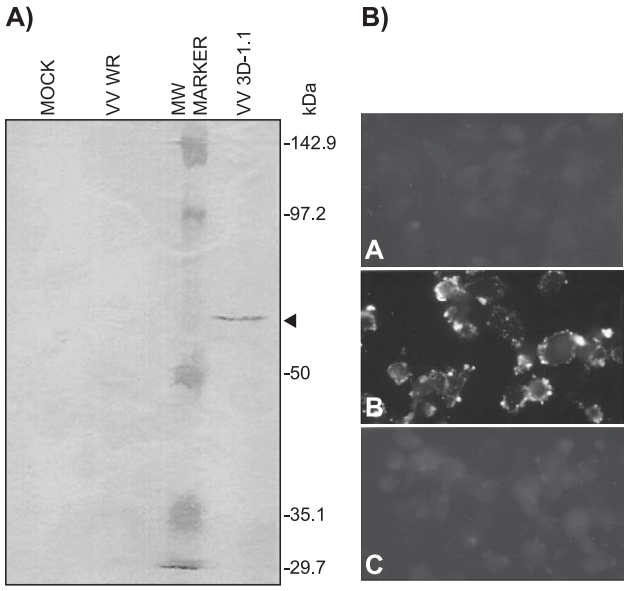

Fig. 1. Expression of 3D protein in cells infected with VV WR or recombinant VV 3D-1.1. (A) Bsc-1-uninfected cells or infected (at a m.o.i. = 1) with VV-3D recombinant clone 1.1.1., or VV-WR, were resolved on a 10% SDS-PAGE electrophoresis and transferred onto a nitrocellulose membrane. 3D protein was detected with a polyclonal antiserum against 3D protein, PE56 (Strebel et al., 1986), and revealed with horseradish-peroxidase-conjugated anti-rabbit donkey IgG. The band corresponding to protein 3D on the membrane is indicated by an arrowhead. (B) Bsc-1 cell monolayers either uninfected (A) or infected (at a m.o.i = 1) with VV-3D 1.1.1. (B), or VV WR (C), were fixed at 4.5 h pi with methanol for 10 min at −20 °C. Cells were incubated with polyclonal antiserum PE56 and developed with FITC-conjugated anti-rabbit antibody.

identify T cell epitopes in this protein consistently recognized by lymphocytes from infected animals.

Results

Construction of recombinant VV expressing FMDV 3D

We first studied the immune response elicited by the FMDV polymerase 3D expressed by a replicating vector. To this end, recombinant VV harboring a copy of 3D gene were obtained as described in Materials and methods. A recombinant clone, VV 3D-1.1, was selected to infect Bsc-1 cells that were analyzed for 3D protein expression. A product of the expected size (56 kDa) was detected by Western blotting in extracts of infected cells (Fig. 1A). Control cells, either uninfected or infected with VV WR, did not show 3D expression. The expression of this protein was also evaluated by indirect immunofluorescence. As shown in Fig. 1B, cells infected with VV 3D-1.1 exhibited a high intensity of 3D-specific fluorescence that appears in the periphery of infected cells. In FMDV infected cells, 3D expression is in the cytosol (data not shown). Thus, further experiments are required to study the possible association of 3D with membrane structures in the context of its expression in VV. Expression of 3D in infected cells was also confirmed by dot blot (data not shown).

Table 1
Pyrexia and clinical symptoms developed in pigs after FMDV challenge

Inoculum	Animal	Pyrexia[a]		Lesions[b]	
		Emergence	Duration	Snout[c]	Feet[d]
VV 3D	A1	3	3	1 (4)	1,2,0 (4)
	A2	3	4	1 (4)	1,1,1 (4)
	A3	3	3	none	0,1,1 (4)
VV WR	B1	2	5	3 (3)	3,2,3 (3)
	B2	1	7	3 (3)	2,3,2 (2)
E-MEM	C1	1	7	2 (3)	2,3,2 (2)
	C2	3	5	3 (3)	3,3,3 (2)

[a] Estimated as rectal temperature >39 °C. The day of emergence and the duration (in days) are indicated.
[b] The day of emergence of the lesions is indicated in parenthesis.
[c] Approximate size (diameter) of the secondary lesions in the snout: 1 (<5 mm), 2 (5–10 mm), 3 (>10 mm).
[d] The severity of the secondary lesions in the feet was estimated from 0 (none) to 3, based on the average size and number of the vesicles observed at the left fore, right hind, and left hind foot, respectively.

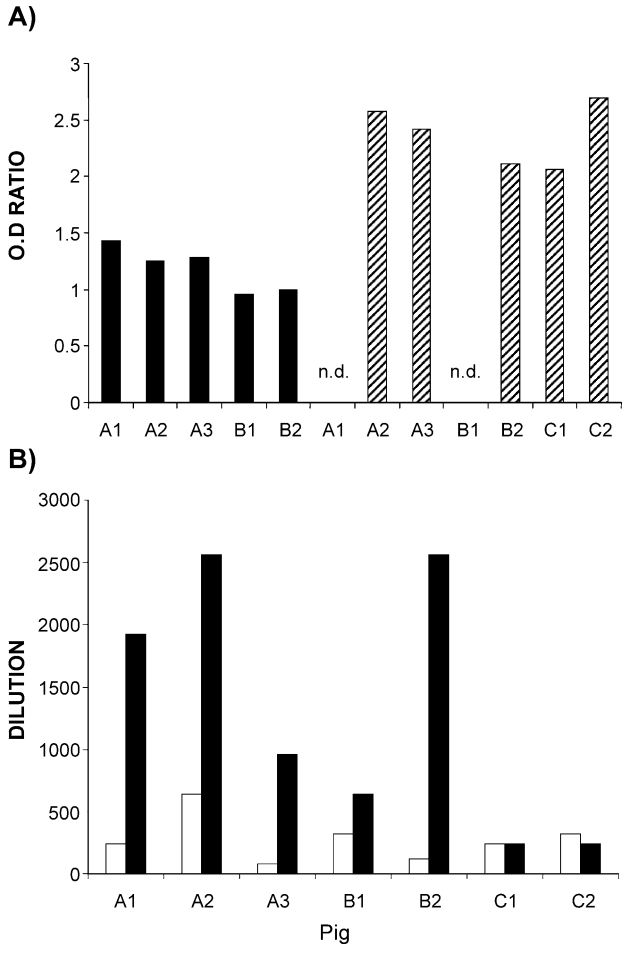

Fig. 2. Specific antibody responses in pigs inoculated with VV WR or VV 3D-1.1. (A) Ratio of ELISA titers (OD) against a FMDV 3D recombinant protein between serum collected at days 30 post re-inoculation (black bars) or 15 after viral challenge (stripped bars) and serum obtained before the first immunization (day 0). (B) ELISA titers against VV WR in sera from animals at day 0 (white bars) and at day 30 post re-inoculation (black bars). Data are expressed as the inverse of the serum dilution that gave 50% of the maximum OD obtained for each serum. n.d., not determined. The ELISAs used are detailed in Materials and methods.

Immune response elicited by recombinant VV 3D in pigs

To study the immune response and the protection induced by VV recombinants expressing 3D, three pigs (group A) were inoculated, oronasally and subcutaneously, with recombinant VV 3D-1.1, as detailed in Materials and methods. As controls, two (group B) and three (group C) animals were inoculated with VV WR or E-MEM, respectively. Animals of groups A and B received two equal doses of virus (10^9PFU) at days 1 and 30.

The induction of specific 3D antibodies in immunized pigs was estimated from serum samples obtained at days 0 (before infection), 30 post re-inoculation, and 15 days after challenge. As shown in Fig. 2A, the three animals inoculated with VV 3D-1.1 showed at day 30 post re-inoculation ELISA values (OD), relative to those observed at day 0, slightly higher than those found in pigs inoculated with VV WR. This difference was in the limit of significance and could be due to experimental variation. After challenge, all the animals showed similar OD ratios that were greater than those observed before challenge. The three pigs immunized with VV 3D-1.1 showed detectable antibody titers against VV at day 30 post re-inoculation as estimated by an ELISA using VV WR (Fig. 2B). In addition, no detectable neutralizing activity against the homologous FMDV C-S8 was

found in a parallel analysis of sera from day 30 post re-inoculation using a plaque reduction assay (data not shown).

Protection of immunized pigs against FMDV challenge

Forty-five days post re-inoculation, pigs from groups A, B, and C were challenged by intradermal injection of homologous FMDV C-S8. Clinical symptoms were monitored daily by measuring rectal temperatures and following the appearance of lesions in mouth and feet. The criteria considered as indicative of protection were the delay, reduction or absence of epithelial lesions and pyrexia (rectal temperature >39 °C). As shown in Table 1, a significant reduction in the clinical symptoms developed upon challenge was noticed in animals immunized with VV 3D (group A). Control animals (groups B and C) developed severe clinical signs: large vesicles in feet and snout, anorexia and lameness, and showed pyrexia from days 1 to 3 that lasted between 5 and 7 days post challenge. Conversely, pigs from group A showed a delay of 1–2 days in the onset of pyrexia and lesions (vesicles). In addition, only two of the three animals of this group

Table 2
Synthetic peptides used in this study[a]

Amino acid sequence	Peptide[b]		Amino acid sequence	Peptide[b]	
GLIVDTRDVEERVHV	1	(1–15)	AQYRNVWDVDYSAFD	47	(231–145)
TRDVEERVHVMRKTK	2	(6–20)	VWDVDYSAFDANHCS	48	(236–250)
ERVHVMRKTKLAPTV	3	(11–25)	YSAFDANHCSDAMNI	49	(141–255)
MRKTKLAPTVAHGVF	4	(16–30)	ANHCSDAMNIMFEEV	50	(246–260)
LAPTVAHGVFNPEFG	5	(21–35)	DAMNIMFEEVFRTEF	51	(251–265)
AHGVFNPEFGPAALS	6	(26–40)	MFEEVFRTEFGFHPN	52	(256–270)
NPEFGPAALSNKDPR	7	(31–45)	FRTEFGFHPNAEWYL	53	(261–275)
PAALSNKDPRLNEGV	8	(36–50)	GFHPNAEWYLKTLVN	54	(266–280)
NKDPRLNEGVVLDEV	9	(41–55)	AEWYLKTLVNTEHAY	55	(271–285)
LNEGVVLDEVIFSKH	10	(46–60)	KTLVNTEHAYENKRI	56	(276–290)
VLDEVIFSKHKGDTK	11	(51–65)	TEHAYENKRITVEGG	57	(281–295)
IFSKHRGDTKMSAED	12	(56–70)	ENKRITVEGGMPSGC	58	(286–300)
RGDTKMSAEDKALFR	13	(61–75)	TVEGGMPSGCSATSI	59	(291–305)*
MSAEDKALFRRCAAD	14	(66–80)	MPSGCSATSIINTIL	60	(296–310)
KALFRRCAADYASRL	15	(71–85)	SATSIINTILNNIYV	61	(301–315)
RCAADYASRLHSVLG	16	(76–90)	INTILNNIYVLYALR	62	(306–320)
YASRLHSVLGTANAP	17	(81–95)	NNIYVLYALRRAYEG	63	(311–325)
HSVLGTANAPLSIYE	18	(86–100)	LYALRRAYEGVELDT	64	(316–330)
TANAPLSIYEAIKGV	19	(91–105)	RAYEGVELDTYTMIS	65	(321–335)
LSIYEAIKGVDGLDA	20	(96–110)	VELDTYTMISYGDDI	66	(326–340)
AIKGVDGLDAMEPDT	21	(101–115)	YTMISYGDDIVVASD	67	(331–345)
DGLDAMEPDTAPGLP	22	(106–120)*	YGDDIVVASDYDLDF	68	(336–350)
MEPDTAPGLPWALQG	23	(111–125)	VVASDYDLDFEALKP	69	(341–355)
APGLPWALQGKRRGA	24	(116–130)	YDLDFEALKPHFKSL	70	(346–360)
WALQGKRRGALIDFE	25	(121–135)	EALKPHFKSLGQTYT	71	(351–365)
KRRGALIDFENGTVG	26	(126–140)	HFKSLGQTYTPADKS	72	(356–370)
LIDFENGTVGPEVEA	27	(131–145)	GQTYTPADKSDKGFV	73	(361–375)
NGTVGPEVEAALKLM	28	(136–150)	PADKSDKGFVLGHSI	74	(366–380)
PEVEAALKLMEKREY	29	(141–155)	DKGFVLGHSITDVTF	75	(371–385)
ALKLMEKREYKFACQ	30	(146–160)	LGHSITDVTFLKRHF	76	(376–390)
EKREYKFACQTFLKD	31	(151–165)	TDVTFLKRHFHMDYG	77	(381–395)
KFACQTFLKDEIRPM	32	(156–170)	LKRHFHMDYGTGFYK	78	(386–400)
TFLKDEIRPMEKVRA	33	(161–175)	HMDYGTGFYKPVMAS	79	(391–405)
EIRPMEKVRAGKTRI	34	(166–180)	TGFYKPVMASKTLEA	80	(396–410)
EKVRAGKTRIVDVLP	35	(171–185)	PVMASKTLEAILSFA	81	(401–415)
GKTRIVDVLPVEHIL	36	(176–190)	KTLEAILSFARRGTI	82	(406–420)
VDVLPVEHILYTRMM	37	(181–195)	ILSFARRGTIQEKLI	83	(411–425)
VEHILYTRMMIGRFC	38	(186–200)	RRGTIQEKLISVAGL	84	(416–430)
YTRMMIGRFCAQMHS	39	(191–205)	QEKLISVAGLAVHSG	85	(421–435)
IGRFCAQMHSNNGPQ	40	(196–210)	SVAGLAVHSGPDEYR	86	(426–440)
AQMHSNNGPQIGSAV	41	(201–215)	AVHSGPDEYRRLFEP	87	(431–445)
NNGPQIGSAVGCNPD	42	(206–220)	PDEYRRLFEPFQGLF	88	(436–450)
IGSAVGCNPDVDWQR	43	(211–225)	RLFEPFQGLFEIPSY	89	(441–455)
GCNPDVDWQRFGTHF	44	(216–230)	FQGLFEIPSYRSLYL	90	(446–460)
VDWQRFGTHFAQYRN	45	(221–235)	EIPSYRSLYLRWVNA	91	(451–465)
FGTHFAQYRNVWDVD	46	(226–240)	RSLYLRWVNAVCGDA	92	(456–470)

[a] Amino acid sequence (as in Toja et al., 1999) and numbering are indicated for each peptide.
[b] The yield of the chemical synthesis of peptides 22 and 59 (denoted by an asterisk) was low and did not allow its use in the proliferation experiments.

developed lesions at the snout and these lesions were of a small size (Table 1).

These results indicate that immunization of pigs with VV 3D-1.1 induced partial protection to viral challenge in the absence of induction of detectable neutralizing antibodies against FMDV. These results suggest that expression of 3D in pigs might elicit T cell responses that could play a role in the delay and reduction of the symptoms observed. Identification of T cell epitopes is an informative approach to understand the T immune mechanisms elicited by FMDV proteins (Blanco et al., 2000, 2001; van Lierop et al., 1995a, 1995b) and can allow identification of epitopic components relevant for the design of subunit vaccines. Thus, to gain insight on the contribution of 3D to the viral immune response, we conducted a new experiment to assess the antigenic specificity of the T cell recognition of 3D protein in infected pigs.

Identification of T cell epitopes recognized on 3D by pigs infected with FMDV

A new group of five pigs (D1–D5, group D) that was infected with FMDV C-S8 was used to study the antigenic specificity of the T cell response elicited by 3D protein. To this end, 92 overlapping (15-mer) peptides covering the amino acid sequence of 3D were synthesized (see Materials and methods and Table 2 for details) and used in lymphoproliferation assays. Due to the high number of peptides to be tested, assays were performed with pools of two to three overlapping peptides. These peptide pools were employed to stimulate in vitro lymphocytes obtained at days 0, 10, 21, 28, 35, 42, and 63 p.i. None of the peptide pools induced positive SI in PBMC collected before infection. Peptides inducing positive responses (SI $\geq$ 2.5) in at least four of the five animals studied are shown in Fig. 3, in which the higher SI observed at the different days p.i. analyzed are indicated. Responses against peptides and whole virus were detectable from day 21 p.i., mostly peaked at days 21 and 28 p.i, and this responsiveness was still observed at day 63 p.i. in animal D5 (data not shown). In general, lymphoproliferations were dose-dependent, and the highest SI were obtained with concentrations between 4 and 100 μg/ml (data not shown). In most of the pigs, the magnitude of the anti-FMDV response was higher than that induced by peptides. Even when individual variation was observed in the responses, groups of peptides were recognized by PBMC from at least four of the five pigs analyzed. This was the case for peptides (3D residues are indicated in brackets): 11,12 (51–70), 19,20,21 (91–115), 37,38 (181–200), 69,70 (341–360), and 77,78 (381–400) (Fig. 3).

The study of the T cells induced upon FMDV reinfection of pigs can allow the analysis of the epitope recognition during a secondary immune response, and is also an interesting approach for identification of T cell peptides conserved in viruses of different serotypes. To this end, animals from group D were inoculated at day 90 after the initial infection, with a heterologous type O FMDV (O-BFS). The five pigs developed an acute episode of the disease, and PBMC obtained at different days p.r.i. were used to perform lymphoproliferative assays, using 3D peptide pools as stimulator antigens. Positive responses were detected from day 10 p.r.i., earlier than was observed after the first infection. Responses were, in general, higher than those detected after the first infection, and several peptide pools induced responses higher than those elicited by FMDV C-S8 (data not shown). Lymphoproliferations were dose-dependent, and the highest values were obtained with peptide concentrations (4–100 μg/ml) similar to those found to be optimal after the first infection. The accumulated optimal SI for peptide pools inducing positive responses in four of the five pigs at day 10 p.r.i are shown in Fig. 4A. On average, individual variation in response to peptides was lower than that found after the first infection, although the level of responsiveness varied among animals

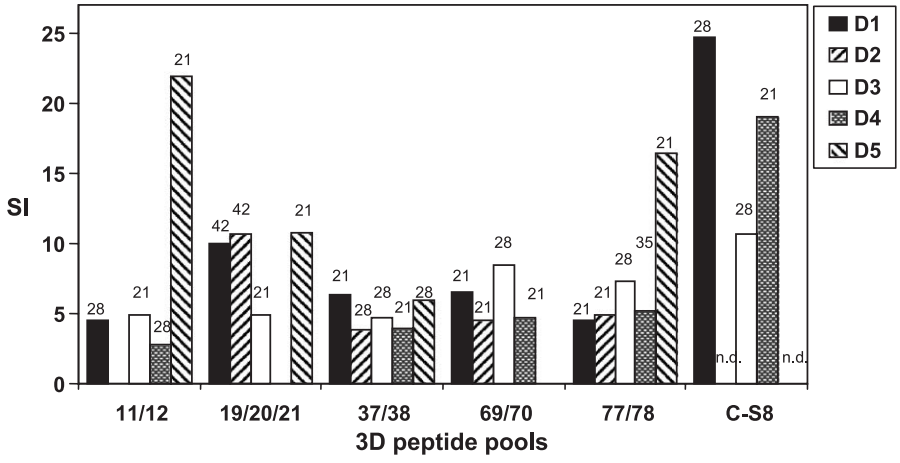

Fig. 3. Lymphoproliferations to 3D peptides by pigs infected with type C FMDV (C-S8). Peak responses to 3D peptide pools or FMDV C-S8 that induced positive SI ($\geq$ 2.5) in, at least, four of the five animals of group D. The day p.i. at which peak response was detected is indicated on the top of each bar. n.d., not done. The standard deviations of these values never exceeded 15% of the mean. The cpm in control cultures ranged from 500 and 2000.

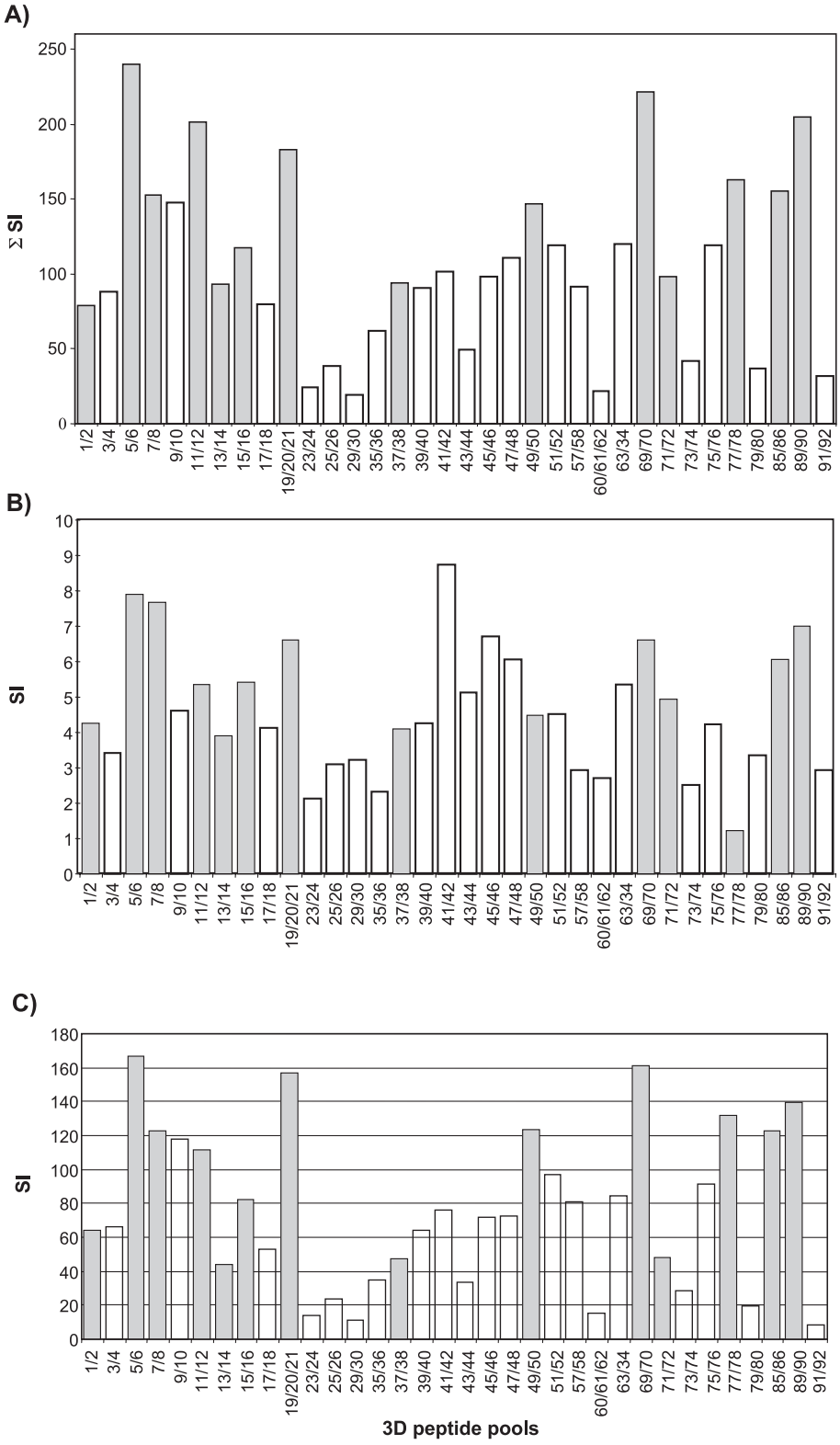

Fig. 4. Heterotypic lymphoproliferations to 3D peptides. Peak responses to peptide pools in animals of group D at day 10 p.r.i. with type O FMDV (O-BFS). Accumulated optimal SI from pigs D1, D2, D3, D4, and D5 (A). Optimal SI from pig D2 (B), and D3 (C). Peptides recognized by PBMC from at least four of the five reinfected animals are indicated by grey bars. The standard deviations of these values never exceeded 15% of the mean. The cpm in control cultures ranged from 300 to 2000.

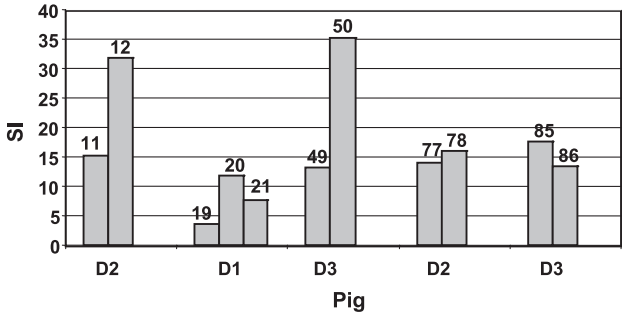

Fig. 5. Heterotypic lymphoproliferations to individual 3D peptides. Peak responses to individual peptides in animals of group D at different days p.r.i. with FMDV O-BFS. Peptide number is indicated on top of the bar. SI corresponded to day 51 p.r.i. The standard deviations of these values never exceeded 15% of the mean. The cpm in control cultures ranged from 300 to 2000.

(Figs. 4B, C). This pattern of response was maintained at days 21 and 35 p.r.i. (data not shown). The following 3D peptides were efficiently recognized by at least four of the five reinfected pigs: 1,2; 5–8; 11–16; 19–21; 37,38; 49,50; 69–72; 77,78; 85,86; and 89–90 (Fig. 4A), which included peptides identified as antigenic after the first infection (Fig. 3). When individual peptides from stimulator pools (11,12; 19,20,21; 49,50; 77,78; and 85,86) were included in proliferation assays, variations in the SI induced by individual peptides were observed between animals. However, in all cases, at least one of the overlapping peptides gave a high level response (Fig. 5).

As summarized in Fig. 6, the results obtained indicate that 3D protein regions contain T cell epitopes which are efficiently recognized by porcine T lymphocytes from different infected animals, both upon primary and secondary (heterotypic) FMDV infection.

Cytokine profiles induced in PBMC by different peptides

To gain information on the functional implications of the lymphoproliferative responses observed, antigenic peptides were used to determine the cytokine profiles that they induced upon in vitro incubation of PBMC from FMDV reinfected pigs. To this end, mRNA from the stimulated cells was extracted and amplified by RT-PCR using specific primers as described in Materials and methods. Table 3 summarizes the RT-PCR amplification of IFN-γ, IL-4, and IL-10 mRNAs in peptide-stimulated cultures of different pigs. For most of the peptides analyzed, positive amplification of IFN-γ mRNAs was detected. RT-PCR from PBMC cultures incubated in the absence of peptides did not render detectable amplification of IFN-γ, IL-4, and IL-10 mRNAs, although positive amplification was observed for G3PDH mRNA. These results suggest that the lymphoproliferations induced by 3D peptides are mediated by T cells and suggests that a Th1 response is produced when T cells from infected animals are stimulated with 3D FMDV peptides.

Table 3
Analysis of cytokine mRNA expression by RT-PCR

Peptide[a]	Pig[b]	G3PDH[c]	IFN-γ[d]	IL-4[d]	IL-10[d]
1	D2	+	+	–	–
11	D1	+	+	–	–
13	D1/D2	+	–/+	–	–
15	D2	+	+	–	–
20	D1/D2	+/+	+/+	+/+	–/–
72	D2	+	+	–	–
77	D2/D4	+/+	+/+	+/–	–
85	D2	+	+	–	–
89	D1	+	+	+	–

[a] Peptide used for PBMC stimulation.
[b] PBMC were obtained at day 35 p.r.i., except for pig D2 whose lymphocytes were collected at day 51 p.r.i.
[c] Positive amplification was obtained in PBMC cultured in the absence of peptide stimulation.
[d] Negative amplification was obtained in PBMC cultured in the absence of peptide stimulation.

Discussion

Partial protection in the absence of anti-FMDV neutralizing antibodies has been reported in cattle and pigs immu-

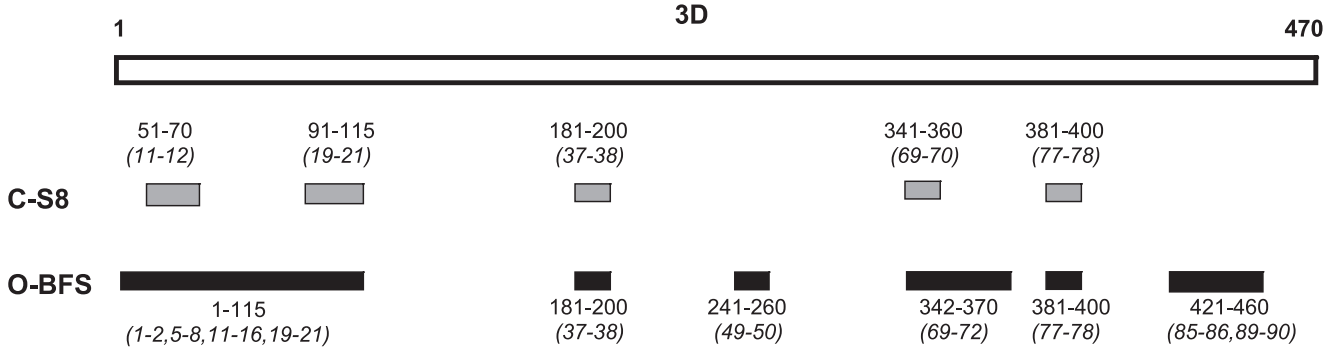

Fig. 6. Schematic representation of 3D peptides pools that were efficiently recognized by lymphocytes from at least four of the five pigs upon infection and reinfection with FMDV. Grey quadrangles denote sequences spanned by peptides recognized upon infection with FMDV C-S8. Black quadrangles denote sequence stretches containing peptides recognized upon reinfection with FMDV O-BFS. Amino acid positions and peptide numbering (as in Table 2) are indicated in normal and italic characters, respectively.

nized with VV and adenovirus expressing capsid proteins precursor P1 (Sanz-Parra et al., 1999a,1999b). These observations emphasize the role of the T cell response in the protection of pigs against FMDV infection (Childerstone et al., 1999; Collen, 1994). However, little is known on the role of FMDV specific T cells in protection mechanisms other than their contribution to induction of anti-FMDV antibodies, particularly on the activation and functional implications of FMDV CTL lymphocytes (Sobrino et al., 2002).

In this report, we have shown that inoculation of pigs with a VV recombinant expressing FMDV 3D protein (group A) results in a delay in the emergence and a lower severity of the lesions developed by immunized animals upon viral challenge, relative to those observed in control animals (groups B and C). This partial protection occurs in the absence of induction of detectable anti-FMDV neutralizing antibodies, providing new evidence in favor of the involvement of non-antibody-mediated responses in the induction of protection against FMDV. The immune mechanisms underlying the partial protection observed in animals immunized with VV 3D-1.1 remained unclear because the use of a recombinant 3D protein expressed as a fusion in *E. coli* did not allow detection of significant lymphoproliferative responses in infected animals (data not shown). This lack of responsiveness was likely to be due to the poor antigenicity of the 3D recombinant used (Blanco et al., 2001). Also, the difficulties for optimizing FMDV CTL assays with pig lymphocytes (Childerstone et al., 1999; Rodríguez et al., 1996) have impaired attempts to analyze the contribution of this response to the partial protections observed. However, these results emphasize the importance of the characterization of the T cell responses elicited by FMDV proteins.

Recognition of T cell epitopes by lymphocytes from different host species and individuals is restricted by the polymorphism of the MHC molecules, which are responsible for the presentation of foreign antigens by antigen presenting cells (Germain, 1999). Advances have been made in the last years in the analysis of the antigenic specificity of FMDV specific T helper cells, which are required for an efficient activation of specific B cells (reviewed in Collen, 1994; Sobrino et al., 2002).

One of the aims of these studies is the identification of FMDV T cell epitopes capable of inducing an effective response, although being widely recognized by MHC alleles frequent in natural populations of host species. Identification of such T cell epitopes is relevant for the improvement of new vaccines, particularly of those based on subunit vaccines (Rowlands, 1994; Sobrino et al., 1999). 3D polymerase is one of the FMDV proteins showing a lower degree of amino acid variation between isolates of different serotypes (Domingo et al., 1990). Therefore, 3D is an interesting candidate to contain conserved viral T cell epitopes, as suggested by its heterotypic recognition by bovine T cells (Collen, 1994) and the improvement of the immune response elicited by P1-based DNA vaccines when 3D protein was co-expressed (Cedillo-Barón, 2001).

To gain information of the T cell immunogenicity of FMDV 3D protein, we conducted an analysis of the antigenic specificity of the lymphocytes from a new group of pigs (group D) infected with FMDV C-S8. As reported for other FMDV proteins (Blanco et al., 2001; Rodríguez et al., 1994b), several peptides along 3D were efficiently recognized by lymphocytes from infected pigs and this recognition showed individual animal variation. Five peptide pools stimulated efficiently lymphocytes from four of the five animals tested (Fig. 3A) as summarized in Fig. 6. These peptides were consistently recognized by lymphocytes from animals reinfected with a type O FMDV. In addition, four of the five reinfected animals efficiently recognized eight new peptide pools (Fig. 3B) as indicated in Fig. 6. Consistently with induction of a secondary response, the proliferations observed were, in general, higher than those developed after the first infection. Proliferation to individual peptides confirmed that at least one of the peptides incorporated in the pools induced high proliferation levels in reinfected pigs (Fig. 5).

Although the number of animals used for this type of study was not sufficient for statistical demonstration, we think that the data are consistent with the identification of T cell epitopes in 3D able to stimulate porcine T cells following infection with viruses of different serotypes. This heterotypic recognition of FMDV peptides correlates well with the high level of 3D sequence conservation among different FMDV serotypes (99.2% homology between FMDV C-S8 and O-BFS) (Martínez-Salas et al., 1985). Heterotypic recognition of peptides corresponding to conserved FMDV proteins has been reported also for peptides in VP4 and 3ABC (Blanco et al., 2000, 2001).

Activation of a Th 1 response has been associated with efficient protection against FMDV (van Lierop et al., 1995a, 1995b). The cytokine profile in PBMC stimulated in vitro by 3D peptides revealed that IFN-γ mRNA synthesis was induced in most of the cases. These results suggest that T cells that become activated in response to 3D peptides phenotypically belong to the Th 1 subset. Further characterization of the responder cell populations is required to identify their phenotype, whether they belong to the CD4+ or CD8+ subset, and the contribution of the cytokines induced in FMDV immune response.

Taken together, the findings in this paper provide new evidence of the presence on 3D protein of antigenic regions efficiently recognized by porcine T lymphocytes. The reduced number of B and T cell epitopes included in subunit and synthetic FMDV vaccines is considered as one of their limitations for eliciting solid protective immune responses (Cedillo-Barón et al., 2001; Collen, 1994; Rowlands, 1994; Sobrino et al., 1999; van Lierop et al., 1995a, 1995b). The results described here can contribute to improve the repertoire of viral T cell epitopes to be included in the design of new FMDV vaccines.

Materials and methods

Recombinant vaccinia virus expressing 3D polypeptide

The recombinant VV expressing the 3D protein was constructed as follows: 3D gene sequence was amplified by PCR from plasmid pUC-3D carrying the whole 3D gene from FMDV C-S8 (J.C. Saiz, unpublished results), using the following primers (Isogen, Bioscience. The Netherlands): 3D1 (antisense), 5′-CCGAATTCTCTA-GAATGGGGTTGATCGGAT-3′; and 3D2 (sense), 3′-AAGCTAGCTCTAGATTATGCGCGTCCGCACAC-3′. These primers allowed amplification of the complete 3D gene sequence flanked by the recognition sequences of endonucleases *Eco*RI and *Nhe*I (at the 5′ and the 3′ end, respectively), as well as in-frame translational start and stop codons. The PCR product and plasmid pRB21 (Blasco and Moss, 1995) were digested with endonucleases *Eco*RI and *Nhe*I and ligated to obtain plasmid pRB21-3D. The integrity of 3D gene, including the initiation and stop codons, was confirmed by DNA sequencing. Plasmid pRB21-3D was used to produce recombinant VV using a plaque selection method (Blasco and Moss, 1992,1995). Briefly, subconfluent CV-1 monolayers were infected with the non-plaquing RB12a[4] vaccinia virus (Blasco and Moss, 1991,1995) and transfected with 10 μg of calcium phosphate-precipitated plasmid pRB21-3D. After 48 h of incubation, cells were detached from plastic and freeze-thawed three times to release the virus. Recombinant clones (plaque forming) were selected after three rounds of plaque purification on Bsc-1 cells. The presence of guanosine phosphoribotransferase (GPT) gene in the recombinants was confirmed as described (Blasco and Moss, 1995).

3D expression was tested by immunoblotting, indirect immunofluorescence (IIF), and dot-blotting of Bsc-1 cells infected with the different recombinant clones, using standard protocols. VV Western Reserve (VV WR) and recombinant vaccinia virus expressing 3D were grown and tittered by plaque assay in Bsc-1 cells.

Animal immunization, experimental infections, and virus challenge

To analyze the immunogenicity of VV 3D-1.1, 7 pigs (2-month-old Large White × Landrace), obtained from different litters, were inoculated by both oronasal and subcutaneous routes with 10^9 PFU each of VV 3D-1.1.1 (three animals, group A) or VV WR (two animals, group B). Two control animals were inoculated with medium (E-MEM) (group C). At day 30, animals were re-inoculated with the same regime. Forty-five days later (day 75 post inoculation), pigs were challenged intradermally, in the heel bulbs of the right forefoot, with 4.5×10^5 PFU of type C FMDV (C-S8). After challenge, rectal temperatures and clinical signs were monitored daily for 10 days.

To analyze the T cell response induced by 3D protein, five additional pigs (group D) were infected with FMDV C-S8, as described above. To study the specificity of the antigen recognition after a secondary response to FMDV, animals were reinfected with 10^4 PFU of a type O FMDV (O-BFS) at day 90 after infection with virus C-S8. In all cases, blood samples collected at different days post infection (p.i.) or post reinfection (p.r.i) were used to perform lymphoproliferative assays against different homologous FMDV C-S8 antigens.

Peptide synthesis

A total of 92 pentadecapeptides corresponding to the 3D sequence of FMDV C-S8 isolate (Toja et al., 1999) with a 10-residue overlap (sequences and numbering in Table 2) were synthesized by solid phase methods (Merrifield, 1963) using Fmoc chemistry as previously described (Mateu et al., 1996). After cleavage, all peptides were >80% pure by HPLC and had amino acid analyses and MALDI-TOF mass spectra consistent with the expected compositions.

Antibody determination

ELISA of anti-3D was performed using GST-3D fusion protein as antigen diluted (2.6 μg/ml) in carbonate buffer. After blocking with 3% BSA, 0.05% Tween 20, serum dilutions ranging from 1/10 to 1/400 were added and incubated for 12 h at 4 °C. All serum samples were preincubated with *E. coli* DH5-α strain extract, lysed by sonication. Bound antibodies were detected by an anti-swine immunoglobulin-horseradish peroxidase (Dako, Denmark). The reaction was developed with 0.4 mg/ml *o*-phenylenediamine (Sigma Co., St. Louis, MO) as chromogenic substrate. Results are expressed as the ratio of the OD at 492 nm determined for a 1/20 dilution of sera from immunized animals and sera obtained at day 0 (before immunization).

To evaluate the generation of anti-VV antibodies in animals from groups A, B, and C, 10^6 PFU/well of VV WR in carbonate buffer were used. The blocking step was done with 1% BSA, 0.05% Tween 20. Sera were preincubated 30 min at 37 °C with a Bsc-1 cell extract, and 2-fold dilutions (from 1/20 to 1/2500) were incubated overnight with the VV WR-coated plates at 4 °C. The secondary antibody used was an anti-swine immunoglobulin-horseradish peroxidase (Dako) and the reaction was developed with 0.4 mg/ml *o*-phenylenediamine (Sigma Co.) as chromogenic substrate.

Lymphoproliferation assays

Proliferation assays of swine lymphocytes were performed as described previously (Rodríguez et al., 1994b). Blood was collected in 5mM EDTA and used to obtain

Table 4
Synthetic oligonucleotides used for RT-PCR amplification of porcine cytokine mRNAs

Cytokine	Oligonucleotides	Product size (bp)	Mg^{2+} concentration (mM)
IFN-γ	aei 918 CTTACTGCCAGGCGCCCTTTTTTA aei 019 CTGATGGCTTTGCGCTGGATCT	389	1
IL-4	P 04F ACATCGTCAGTGCAAATAGAGC P 04R TTCAGCTTCAACACTTTGAG	438	1
IL-10	aei 035 TCTATTGCCTGATCTTCC aei 036 GAATGCTTCAGTTCTTCC	516	1
G3PDH	aei 021 AAGTTGTCATGGATGACCTTGGCCA aei 025 CATCACCATCTTCCAGGAGCGAG	298	0.5

peripheral blood mononuclear cells (PBMC). Assays were performed in 96-well round-bottomed microtiter plates (Nunc). Briefly, 2 to 3 × 10^5 PBMC per well were cultured, in triplicate, in complete RPMI supplemented with 10% FCS, in the presence of 5-fold serial dilutions of FMDV C-S8, ranging from 4 × 10^3 to 2 × 10^6 PFU, or synthetic peptides, ranging from 30 ng/ml to 100 μg/ml. Control cultures without viral antigens were included. Cells were incubated at 37 °C in 5% CO_2. After 4 days, cultures were pulsed with 0.5 μCi of methyl-[^{3}H]thymidine for 18 h. Cells were collected using a cell harvester and the incorporation of radioactivity into DNA was measured by liquid scintillation in a Microbeta counter (Pharmacia, Uppsala, Sweden). Results are expressed as stimulation index (SI; mean cpm of stimulated cultures/mean cpm of control cultures).

Detection of FMDV neutralizing activity

FMDV neutralizing activity in serum samples was analyzed using a modification of the plaque reduction assay, as previously described (Rodríguez et al., 1995). Briefly, about 100 PFU of FMDV C-S8 were incubated for 45 min, at room temperature, with serial dilutions of serum. The mixtures were employed to infect BHK-21 cell monolayers (about 2.4 × 10^6 cells), and the infection was allowed to proceed for 24 h in the presence of agar (0.6%) and E-MEM supplemented with 2.5% FBS. Monolayers were fixed and stained with acetic acid (10% v/v)-crystal violet (0.5% w/v), and the viral PFU scored. The PFU reduction was expressed as the percentage of PFU observed in the presence of sera from immunized animals relative to PFU in the presence of sera from day 0.

Cytokine detection

A total of 3 × 10^5 PBMC were incubated for 24 h with 100 μg/ml of each peptide. RT-PCR reactions were performed as previously described (Gómez del Moral et al., 1999; Sánchez et al., 1999). Briefly, total RNA was extracted by using 250 μl/3 × 10^5 cells of Ultraspec RNA isolation reagent (Biotecx Laboratories, Houston, TX) and used, following denaturing by heating 2 min at 65 °C, for cDNA synthesis in 5 μl of a reaction mixture containing: 1× Moloney murine leukemia RT buffer, 50 mM each deoxinucleoside triphosphate, 12.2 U of 12.5 U of Moloney murine leukemia virus reverse transcriptase (Epicenter, Madison, WI), 10 mM dithiothreitol, 0.5 mM oligo(dT), and 20 U of Rnasine (Promega). The reaction mixtures, adjusted to a final volume of 50 ml with RNase-free water, were incubated for 1 h at 37 °C.

For PCR amplification, 10 μl of cDNA was used in a total volume of 25 μl of a PCR mixture including 0.5–1 mM $MgCl_2$ (depending on the oligonucleotide pair employed) and 10 pmol each specific oligonucleotide pair. The reaction mixture was heated to 80 °C for 1 min to add 1 U of Dnazyme II DNA polymerase (Finnzymes Oy). PCR amplification was carried out with 40 cycles (denaturation at 94 °C for 45 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min). PCR products were electrophoretically resolved on 2% agarose gels containing ethidium bromide. Glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) amplification was used as an internal control for cellular RNA content and integrity. Positive amplification of each of the cytokine mRNAs assayed was observed in PBMC cultures incubated with lipopolysacharides (LPS) (1 μg/ml). The sequence of the primers used for amplification of different cytokine mRNAs, the expected size of amplified fragments, and the Mg^{2+} concentrations employed are shown in Table 4.

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